75

YEARS OF REPORTING ANALYTICAL SCIENCE 1915 - 1990

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

JOURNAL

MARCH/APRIL 1990
VOL. 73, NO. 2

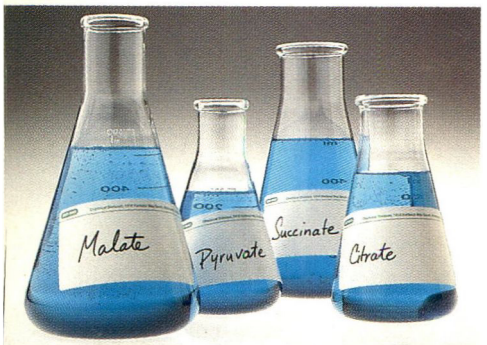
Since 1884
AOAC

JANCA 73(2) 193–338 (1990)
ISSN 0004-5756

Photo Courtesy Applikon Instruments

No sweat...just plain water

Why bother with complex gradient HPLC when you can separate all of these samples in plain DI water? All you need is an affordable, pretested, preplumbed HRLC® isocratic analyzer from Bio-Rad. Plus an Aminex® column especially tailored for your application. It's that easy!

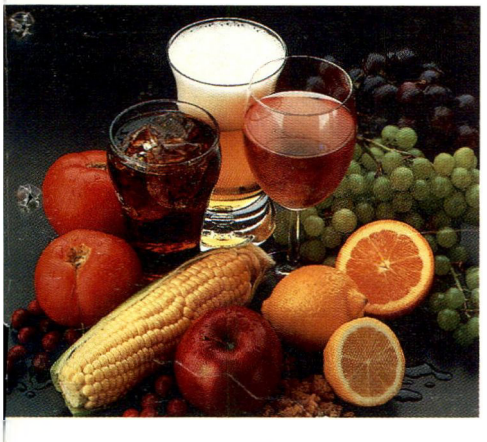


Biotechnology

Monitor ▪ substrate consumption ▪ byproducts of metabolism ▪ fermentation acids

Biomedical/biochemistry

Analyze ▪ citric acid cycle acids ▪ plasma organic acids ▪ sialic acid

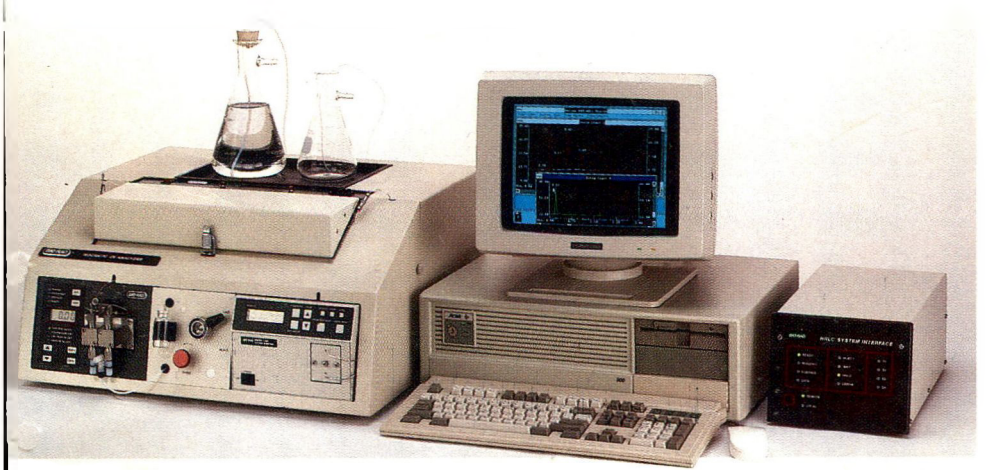


Fruits and vegetables

Monitor ▪ spoilage ▪ fruit juice adulteration ▪ grape juice quality ▪ tomato quality ▪ soluble vitamins

HRLC isocratic analyzers

▪ Simple, straightforward HPLC in DI water using unique Aminex column chemistries ▪ no elaborate sample prep ▪ data analysis and storage ▪ wide selection of columns to perform all of these analyses—and more



For more details on the simplest way to do all these jobs—and more—contact your Bio-Rad representative or call 1-800-4BIO-RAD.

BIO-RAD

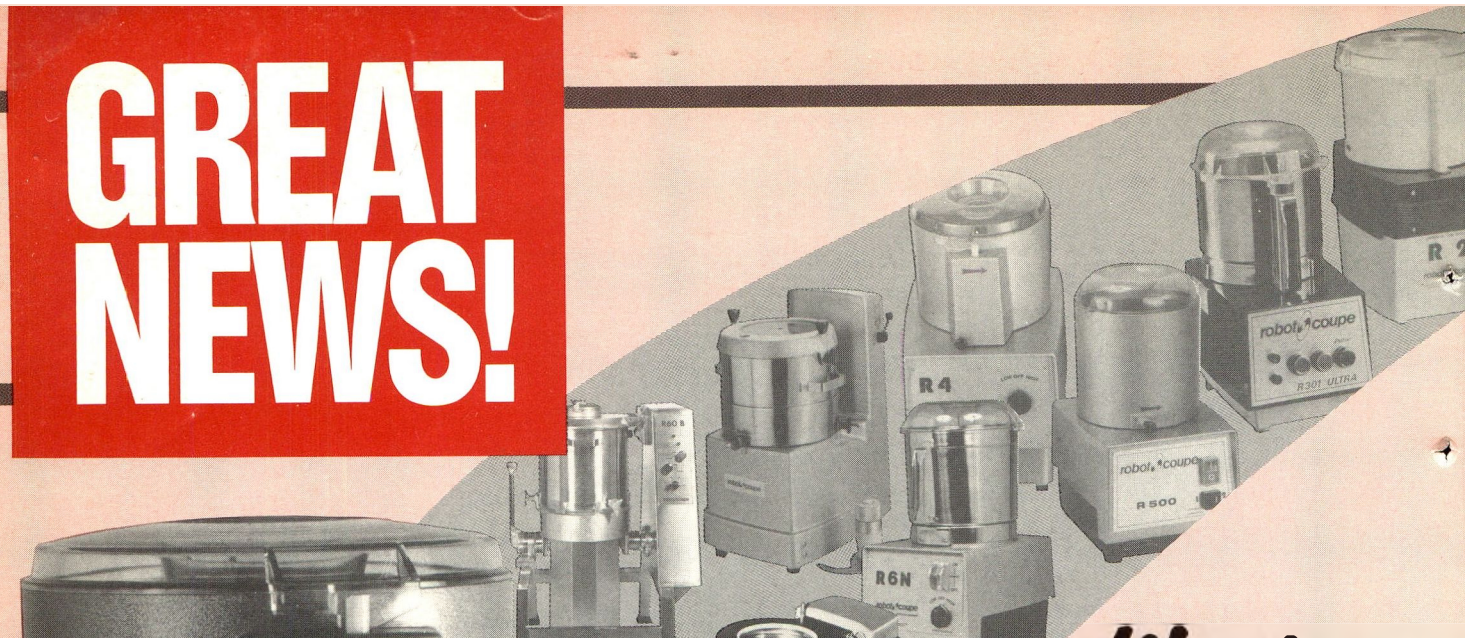
Chemical Division

1414 Harbour Way South
Richmond, CA 94804
(415) 232-7000
1-800-4-BIO-RAD

Also in Rockville Centre, NY; Hornsby, Australia; Vienna, Austria; Brussels, B[illegible]ium; Mississauga, Canada; Watford, England; Paris, F[illegible] Munich, Germany; Hong Kong; Milan, Italy; Tok[illegible] Utrecht, The Netherlands; and Glattbrugg, Sv[illegible]

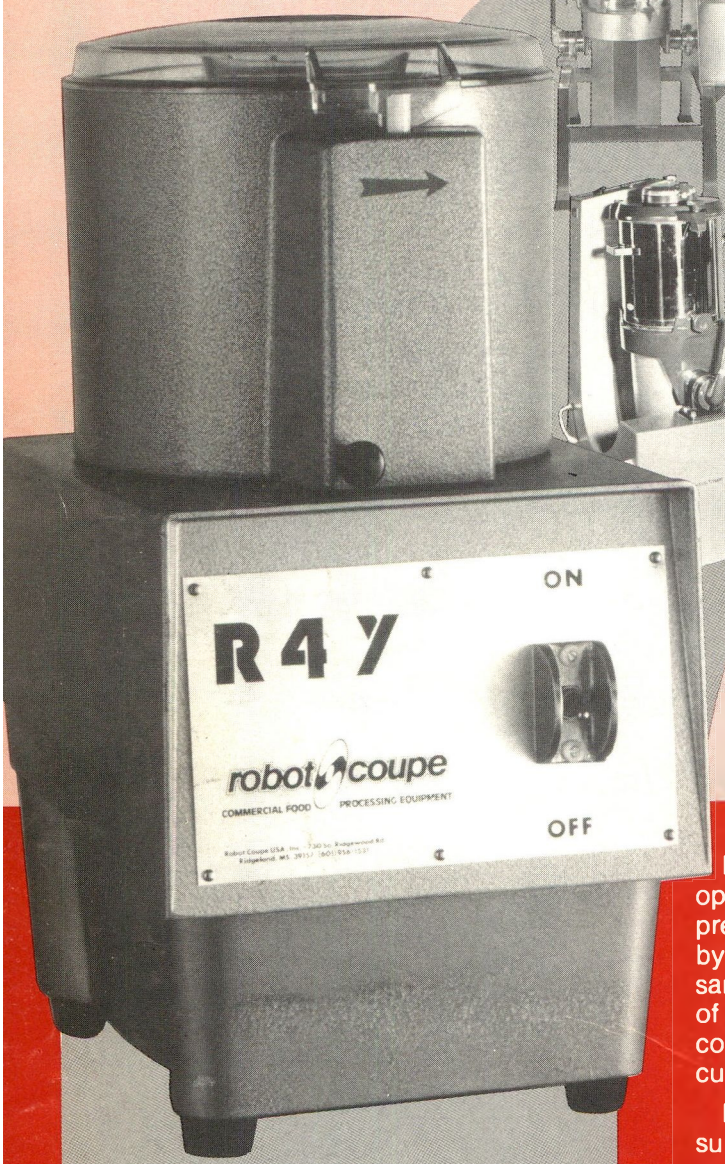
CIRCLE 98 ON READER SERVICE CARD

GREAT NEWS!



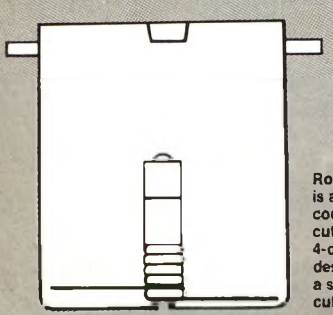
Meat Product Analysis is Going to Pot!

"Stock-Pot" That Is!



Meat analytical and research facilities now have the opportunity to select vertical cutter mixer equipment for preparing meat samples for analysis. Recently certified by USDA for use by their accredited laboratories, meat samples used for analysis are consistently representative of the whole production batch because of superior and consistent processing uniformly provided by the vertical cutter mixer.

Robot Coupe U.S.A., Inc. is a manufacturer and supplier of a full line of vertical cutter mixers with bowl capacities from 2 quarts up to 60 quarts all with the "stock pot" design. Write or call for full information:
1-800-824-1646.



Robot Coupe R4Y is a rugged, electric, counter-top vertical cutter mixer with 4-quart "stock-pot" design bowl and a stainless steel cutting blade.

robot coupe®

Robot Coupe U.S.A., Inc. • P.O. Box 16625
Jackson, Mississippi 39236-6625 • (601) 956-1531

CIRCLE 89 ON READER SERVICE CARD

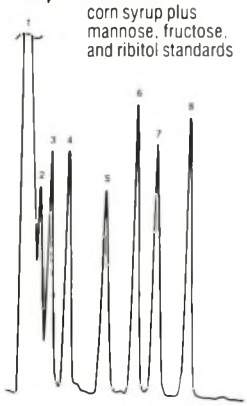
Just filter and go!

Biotechnologists discover the food industry's most reliable column technology.

The Aminex® line of HRLC® column chemistries from Bio-Rad has long been the most reliable way to analyze carbohydrates, organic bases, organic acids, and alcohols in food products. Now this superior Aminex technology has proven equally effective for many applications in the biomedical, pharmaceutical, and biotechnology fields. A simple isocratic separation is all that's needed to get results. Just filter the sample and go!

ANALYSIS OF OLIGOSACCHARIDES IN CORN SYRUP

Conditions
Detector: RI Monitor
Column: Aminex CSA
Sample: 2% 35 DE corn syrup plus mannose, fructose, and ribitol standards



Peaks:
1. Higher saccharides
2. Dp 4
3. Dp 3
4. Dp 2
5. Glucose
6. Mannose
7. Fructose
8. Ribitol

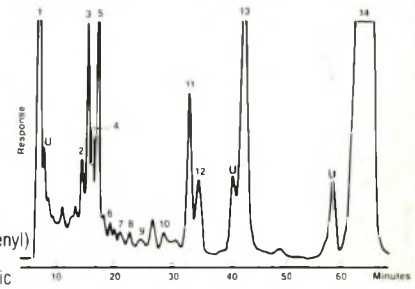
Food and beverage HRLC applications

♦ High fructose corn syrup ♦ Meat and fish spoilage analysis ♦ Sulfites in foods ♦ Apple juice adulteration ♦ Fusel alcohols ♦ Fermentation broths ♦ Oligosaccharides through DP11 ♦ Fermentable sugar analysis ♦ 35 DE corn syrup standard analysis ♦ –pentose, –tetrose, –triose, –maltose sugar analysis

ANALYSIS OF MICROBIAL FERMENTATION PRODUCTS

Conditions
Column: Aminex HPX-87H, 300 mm x 7.8 mm
Sample: *P. anaerobius* extract
Detector: UV Monitor @ 210 nm

Peaks:
1. Solvent Peak
2. Lactic
3. Fumaric
4. Formic
5. Acetic
6. Propionic
7. Isobutyric
8. Butyric
9. Isovaleric
10. Valeric
11. Media components
12. 4-methylvaleric
13. 3-(p-hydroxyphenyl) propionic
14. 3-phenylpropionic



Chromatogram courtesy of Guerrant, G.O., et al., *Journal of Clinical Microbiology*, **16** (2), 355 (1982)

Biochemical/biomedical HRLC applications

♦ Analyze plasma organic acids ♦ Monitor cell metabolites ♦ Measure acetylated amino sugars ♦ Detect serum lactate and pyruvate ♦ Follow citric acid cycle acids ♦ Determine fermentation metabolic byproducts ♦ Assess ketones, neutral metabolites in clinical patient studies

Booklets on HRLC food and beverage applications and biochemical and biomedical HRLC applications are yours for the asking. Call your Bio-Rad representative or contact:

BIO-RAD **Chemical Division**

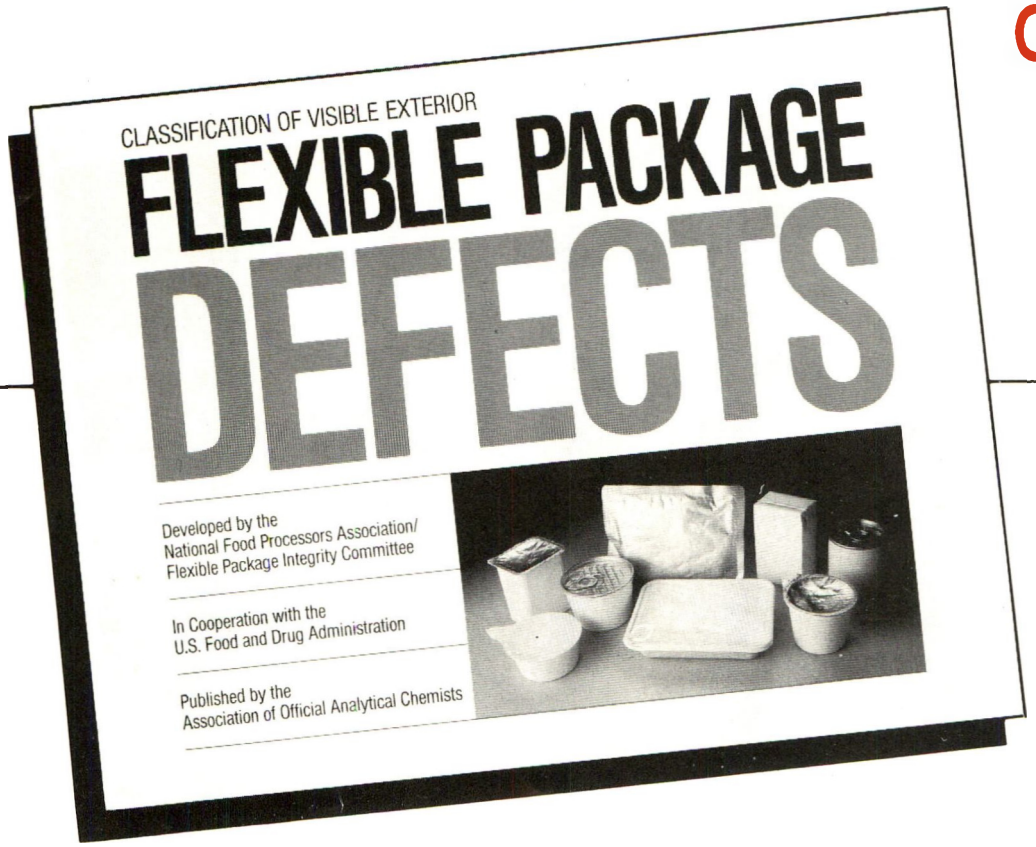
1414 Harbour Way South
Richmond, CA 94804
(415) 232-7000
800-843-1412

Also in Rockville Centre, NY; Hornsby, Australia; Vienna, Austria; Brussels, Belgium; Mississauga, Canada; Watford, England; Paris, France; Munich, Germany; Hong Kong; Milan, Italy; Tokyo, Japan; Utrecht, The Netherlands; and Glattbrugg, Switzerland.

CIRCLE 83 ON READER SERVICE CARD

It's the Chemistry that Counts.™

IDENTIFY POTENTIALLY DANGEROUS FOOD PACKAGE DEFECTS QUICKLY AND EASILY



Defective packages may leak and allow entrance of microorganisms that cause botulism and other forms of food poisoning. To avoid this risk, you need to be able to recognize harmful defects easily and quickly, before they reach the public.

To answer this need, AOAC is offering the poster,

Classification of Visible Exterior Flexible Package Defects

useful to all who develop, manufacture, regulate, distribute, or use these containers.

This easy-to-use poster
- Employs clear, full-color photos and a color code system to help you identify and classify defects
- Indicates what to look for in routine inspection
- Classifies potential hazard of defects as minor, major, or critical
- Addresses paperboard and flexible pouch packages and plastic packages with heat-sealed metal ends
- Unfolds from a pamphlet to a two-sided 24 by 36 inch chart

Prices (include handling and shipping):
$77.00 per package of 10 charts $25.00 per single chart

To order, please send check (U.S. funds on U.S. banks only) or credit card information (VISA or MasterCard number, expiration date, signature, and date) along with your name, address, and the number of posters you wish to order to:

AOAC, 2200 Wilson Blvd., Suite 400-J, Arlington, VA 22201-3301
(703) 522-3032, FAX (703) 522-5468
Credit card orders may be placed by phone or FAX.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS
JOURNAL

A Peer-Reviewed Journal Publishing Basic and Applied Research in the Analytical Sciences Related to Foods, Drugs, Agriculture, and the Environment

J A O A C

Table of Contents

MARCH/APRIL 1990 VOL. 73, NO. 2 JANCA 73(2)193–338 (1990)

 The *Journal of the Association of Official Analytical Chemists* (ISSN 0004-5756) is published bimonthly by the Association of Official Analytical Chemists, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. **Subscriptions** for Vol. 73 (1990) are: Members, $84.50 in U.S., $94.50 outside U.S.; Nonmembers, $125.00 in U.S., $145.00 outside U.S. Second class postage paid at Arlington, VA, and additional mailing offices. Abstracted or indexed in AGRICOLA, Analytical Abstracts, Biological Abstracts, Cambridge Scientific Abstracts, Chemical Abstracts, Current Advances in Ecological Sciences, Current Contents, and others. Printed in USA.
POSTMASTER: Send address changes to AOAC, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA.

New Products

Automatic Operation of SpeedVacs

A dryness controller reduces labor and enhances the concentrating and drying capabilities of existing SpeedVac systems. Using the dryness controller, Model SCD1020, an operator can set his/her SpeedVac to start and automatically end a run when the desired end-point dryness for samples has been achieved. Constant, precise control of the process ensures consistent results and protects bioactive samples from bumping and foaming. Savant Instruments, Inc.

Circle No. 318 on reader service card.

Advanced Purification Glass Columns

New sizes of biocompatible glass columns complement Waters' AP series of high performance columns. These AP-1 (10 mm id), AP-2 (20 mm id), and AP-5 (50 mm id) columns provide higher resolution and faster separation than open column methods, and are available in 4 column lengths enabling separation of microgram to gram quantities of biomolecules. Waters Chromatography Division of Millipore Corp.

Circle No. 319 on reader service card.

Magnetically Rotated Mixing System

A high speed, magnetically rotated packless mixing system, Autoclave 1.5-02 MagneDrive, provides a static torque of 60 in. pounds. The new MagneDrive is designed for blending or heat transfer of chemical reactions in research and pilot plant operations. Autoclave Engineers Group.

Circle No. 320 on reader service card.

HPCPC System

A high performance centrifugal partition chromatography (HPCPC) system uses a new liquid chromatographic technique with liquid-liquid partition, counter-current distribution to fractionate complex mixtures of chemical substances. It can be used to separate and purify a broad range of synthetic and naturally occurring chemical species; it offers distinct advantages for the isolation of polar substances and materials of biological origin, e.g., natural products, pharmaceutical biopolymers, antibodies, and genetically engineered molecules. Sanki Laboratories.

Circle No. 321 on reader service card.

Formaldehyde in Air Monitor

The new improved TGM 555 air monitor reliably and continuously monitors formaldehyde levels in the ppb range. Typical full scale range of the analyzer is 0–1 ppb, adjustable from 0–100 ppb full scale, detecting as low as 2 ppb, up to 0–10 ppm full scale. The unit using the pararosaniline procedure has no known interferences and has been shown to correlate with NIOSH chromotropic acid technique with ±3%. CEA Instruments, Inc.

Circle No. 322 on reader service card.

Environmental Standards for GC Analysis

A comprehensive line of environmental standards and kits includes purgeable, surrogate, and calibration check compounds for GC analysis of volatile, base-neutral/acid extractables and pesticides. The extensive volatile standards series provides a selection of single components and test mixtures, such as the purgeable halocarbon mixture for EPA method 601, a mixture of 28 components, and the companion EPA method 601 kit, with individual vials of each component, a sample of 2-chloroethylvinylether, plus the mixture. J&W Scientific.

Circle No. 323 on reader service card.

Universal, Low Cost LC Autosampler

The Model 738 autosampler can inject 1 μL to 1 mL from up to 96 different samples without any loss of sample and fits any liquid or ion chromatograph. Furthermore, the unit can make an umlimited number of injections from any single sample and requires no accessory gases for operation. Alcott Chromatography.

Circle No. 324 on reader service card.

Soil Safety Sampler

Designed for those interested in herbicide carryover, other chemical residue studies, soil structure investigations, or other studies where cross-contamination between samples

Essential References for Food Analysis from AOAC

☐ **FDA Bacteriological Analytical Manual, 6th Ed.**
1984. 448 pp. Looseleaf.
ISBN O-935584-29-3
Members: $41.85; Nonmembers: $46.50.

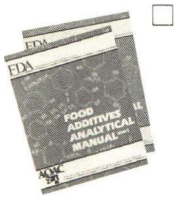
Provides regulatory and industry laboratories with methods for detection of microorganisms. Includes one FREE **Classification of Visible Can Defects** poster (see below).

☐ **Classification of Visible Can Defects-poster/ pamphlet**
1984. 24" x 36" chart. In color with photographs. Minimum order, 1 package of 10 charts, $40; 2nd package of 10, $30; 3rd package of 10, $25.

For rapid identification of can defects.

☐ **Principles of Food Analysis for Filth, Decomposition, and Foreign Matter–FDA Technical Bulletin No. 1**
1981. 286 pp. 2nd printing, 1985. Illustrated. Softbound. ISBN O-935584-33-1.
Members: $39.60; Nonmembers: $44.00.

Comprehensive laboratory manual/text on basic concepts of food sanitation analysis.

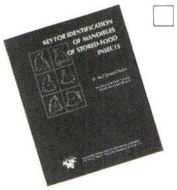


☐ **FDA Food Additives Analytical Manual (FAAM) Volumes I and II**
Volume I - 1983. 2nd printing, 1988. xv + 364 pp. Illustrated. Softbound.
ISBN O-935584-22-6. Volume II - 1987. xiv + 346 pp. Illustrated.
ISBN O-935584-34-X.
Members: $41.40 each volume;
Nonmembers: $46.00 each volume.

Each volume provides methodology for determining compliance with food additive regulations.

☐ **Training Manual for Analytical Entomology in the Food Industry–FDA Technical Bulletin No. 2**
1978. 184 pp. Looseleaf.
ISBN O-935584-11-0.
Members: $24.75; Nonmembers: $27.50.

With the aid of this text, organizations can set up their own in-house training.



☐ **Key for Identification of Mandibles of Stored-Food Insects**
1985. vi + 166 pp. Illustrated. 125 photographs. Softbound.
ISBN O-935584-32-3.
Members: $40.50; Nonmembers: $45.00.

Enables food sanitation analysts to identify species from all major stored-food insect pest groups.

☐ **Animal Drug Analytical Manual**
1985. xii + 352 pp. Illustrated. Looseleaf with binder. ISBN O-935584-30-7.
Members: $40.95; Nonmembers: $45.50.

Methods for determining drug residues in food producing animals and drugs in feeds.

ORDER THESE AOAC FOOD ANALYSIS PUBLICATIONS TODAY!

To order, please note quantity desired in the box beside each title; then complete and mail (or photocopy) this page and payment or credit card information to:

AOAC
Suite 400-J
2200 Wilson Boulevard
Arlington, VA 22201-3301 USA
(703) 522-3032, FAX (703) 522-5468

Total Book Order $ _____
All prices include handling and shipping.

☐ Check enclosed.
☐ Charge my ☐ VISA ☐ MasterCard.

Card Number_____Expires_____

Signature_____Date_____

US funds drawn on US banks only

Send to: (Please Print)

_____
Name

_____
Organization

_____
Street Address

_____
City

_____ _____
State (Country) Zip

Member No. VM_____is required to qualify for member price.

needs to be eliminated, this easy to use hand sampler withdraws soil cores ¾ inch in diameter and up to 12 inches in length. Soil cores are totally enclosed in 16 inch acetate liners that are easily inserted and removed from the soil tube. Oakfield Apparatus, Inc.
Circle No. 325 on reader service card.

Hydrogen Generator

A new hydrogen generator is available with many desirable features compared to older models. The Model 8200 hydrogen generator features a more reliable, higher capacity cell providing hydrogen flowrates of up to 250cc/min at 60 psi. A larger water reservoir allows for longer running times between refills, and several safety features have been added. Like the older models, only pure water needs to be added. This means no risks of handling highly caustic solutions. Alltech Associates, Inc.
Circle No. 326 on reader service card.

Sanitary, Automatic Washbottle

A new washbottle allows the user to direct a controlled forceful jet of washing or rinsing liquid in virtually any direction, and stops almost immediately as needed. The washbottle is easily operated by either hand, leaving one hand free for other needs. Because of its sanitary design, the spread of disease organisms is minimized. Guth Products Co.
Circle No. 327 on reader service card.

Lamps Specifically for Spectroscopy Applications

The series D800/900 Deuterium Lamps produce a continuous line free spectrum in the UV range between 160 and 360 nm. They are an ideal source of optical rays for spectral photometers, flourometers, refractometers, chromatographs, other photometric equipment, and atomic absorption spectroscopy. Lamp Technology Inc.
Circle No. 328 on reader service card.

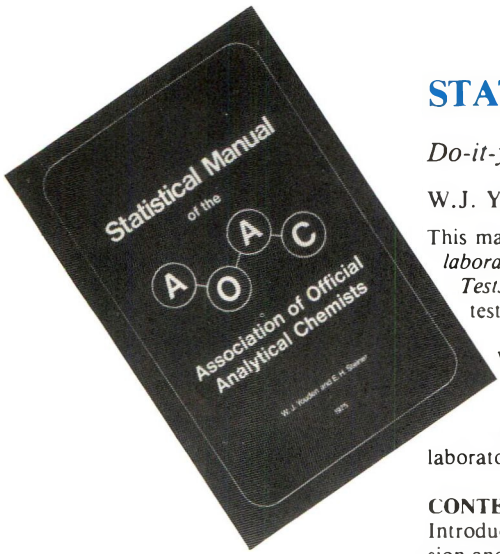
Refractive Index Detector

With an exceptionally wide RI range from 1.00 to 1.75, The Shodex SE-61 refractive index detector supports more applications than ever before. Detection limits of <1 μg/mL for sugars in water make its sensitivity unmatched among RI detectors. The signal-to-noise ratio is excellent. Drift is $<1.5 \times 10^{-7}$ RIU/h. Spectra-Physics.
Circle No. 329 on reader service card.

Motorized Pipet for 96-Well Plates

The eight-channel, 250 μL EDP-Plus M8 motorized electronic pipet is designed for use with 96-well plates. Five liquid measurement modes can be selected: pipet, multiple dispense, dilute, titrate, and measure. In addition, EDP-Plus M8 can mix liquids after dispensing, and can pick up and deliver up to 12 programmed volumes in each of three modes. Rainin Instrument Co.
Circle No. 330 on reader service card.

INDISPENSABLE STATISTICAL REFERENCE BOOKS for EVERY ANALYTICAL SCIENTIST



STATISTICAL MANUAL OF THE AOAC

Do-it-yourself statistical techniques for interlaboratory collaborative tests

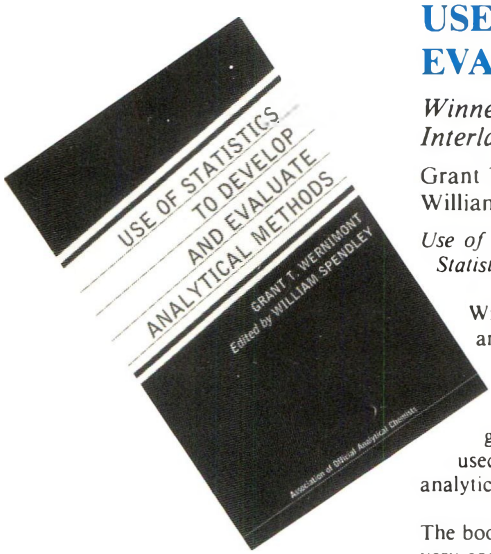
W.J. Youden and E.H. Steiner

This manual is a composite, with revisions, of two publications — *Statistical Techniques for Collaborative Tests,* by the late W.J. Youden, and *Planning and Analysis of Results of Collaborative Tests,* by E.H. Steiner — for use in statistical analysis regarding interlaboratory collaborative tests. The manual also presents guidelines for planning collaborative tests.

Written as a "do-it-yourself" manual for those with little or no experience with formal statistics, this publication presents simple and flexible statistical techniques, using examples related to familiar questions concerning the significance of apparent differences among results. It introduces the analysis of variance technique with examples of its use with interlaboratory tests.

CONTENTS
Introduction. Collaborative studies. Interpretation of collaborative test data. Measurement of precision and accuracy. Planning the collaborative test. Problems connected with collaborative tests. Application of collaborative results. Appendixes.

1975. 5th printing, 1987. 96 pages. With illustrations. Softbound. ISBN 0-935584-15-3.
Price—Members: $22.55 in U.S., $27.55 outside U.S. Nonmembers: $24.50 in U.S., $29.50 outside U.S.
Prices include handling and shipping.



USE OF STATISTICS TO DEVELOP AND EVALUATE ANALYTICAL METHODS

Winner of the American Statistical Association 1987 W.J. Youden Award in Interlaboratory Testing.

Grant T. Wernimont, Author
William Spendley, Editor

Use of Statistics is a natural extension of and a valuable addition to the classic Youden-Steiner, *Statistical Manual of the AOAC.*

With a knowledge of simple statistical procedures, the analytical investigator can use the designs and techniques described in this manual to determine and evaluate assignable causes of variability.

The book reviews the basic operations in the process of making measurements, offers suggestions for planning experimental work so that appropriate statistical methodologies can be used to interpret results, includes a number of experimental plans for developing and modifying analytical procedures, and discusses evaluation of data.

The book features scores of specific statistical analyses of real-life data, useful statistical tables, and very complete references.

CONTENTS
Introduction. The Measurement Process. Intralaboratory Development of an Analytical Process. Interlaboratory Evaluation of an Analytical Process. Appendixes: Tables. Statistical Computations. Glossary. Index.

1985. 2nd printing, 1987. xvi + 183 pages. 11 figures. 54 tables. Index. Glossary. Softbound. ISBN 0-935584-31-5.
Price—Members: $49.55 in U.S., $54.55 outside U.S. Nonmembers: $54.50 in U.S., $59.50 outside U.S.
Prices include handling and shipping.

To order, specify the book(s) you wish to purchase and send with your check or credit card information (VISA or MasterCard number, expiration date, signature, date) and your name and address to:
AOAC, 2200 Wilson Blvd., Ste. 400-J, Arlington, VA 22201-3301 USA
(703) 522-3032, FAX (703) 522-5468
(*U.S. funds on U.S. banks only*)

For Your Information

Meetings

April 2–6, 1990: AOAC Analytical Technology Week, Valley Forge, PA. Contact: AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

April 9–10, 1990: Mid-Canada AOAC Regional Section Meeting. Contact: Ezzat A. Ibrahim, Manitoba Agriculture, University of Manitoba, Feed Analysis Agriculture Service Co., Winnipeg, MB R3T 2X7, Canada, telephone 204/945-7675.

May 7–9, 1990: Northeast AOAC Regional Section Meeting. Contact: Jon Schermerhorn, New York State Dept of Agriculture and Markets, 1220 Washington, Albany, NY 12235, telephone 518/485-8098.

May 1990: Eastern Ontario-Quebec AOAC Regional Section Meeting. Contact: Mireille Gougeon, Institut Armand Frappier, 531 Boul des Prairies, Laval, PQ H2N 4Z3, Canada, telephone 514/687-5010.

May 10, 1990: New York-New Jersey AOAC Regional Section Meeting. Contact: Jeff Singer, Pall Corp., 30 Seacliff Ave, Glencove, NY 11542, telephone 516/671-4000.

June 4, 1990: Southeast AOAC Regional Section Meeting. Contact: M. Sher Ali, USDA, FSIS Eastern Laboratory, Russell Research Center: PO Box 6085, Athens, GA 30604, telephone 404/546-3571.

June 18–20, 1990: Midwest AOAC Regional Section Meeting. Contact: Max L. Foster, Kansas State Board of Agriculture, Division of Laboratories, 2524 W 6th St, Topeka, KS 66606, telephone 913/296-3301.

June 21–22, 1990: Pacific Northwest AOAC Regional Section Meeting. Contact: Steve Pope, Environmental Protection Agency, PO Box 549, Manchester, WA 98353, telephone 206/442-0370.

September 10–13, 1990: 104th AOAC Annual International Meeting and Exposition. The Clarion Hotel, New Orleans, LA. Contact: AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

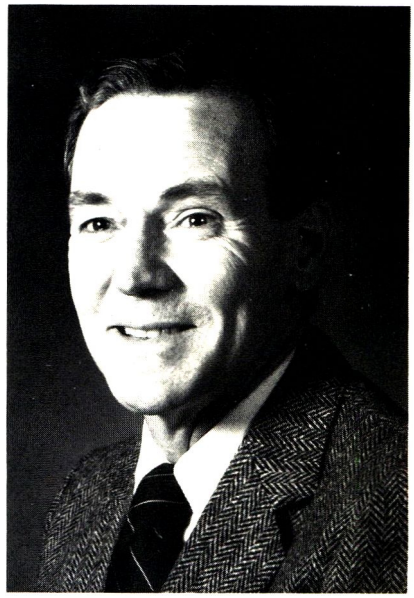
October 11–12, 1990: AOAC/Europe Regional Section Meeting, Brussels, Belgium. Contact: Ellen Jan de Vries, Duphar B.V., PO Box 900, NL 1380 DA Weesp, The Netherlands, telephone (31)-2940-79296.

Harvey W. Wiley Award to Landis W. Doner

Landis W. Doner, a research chemist in the Plant Science Research Unit at the Eastern Regional Research Center, U.S. Department of Agriculture, in Wyndmoor, PA, has been named to receive AOAC's 1990 Harvey W. Wiley Award, the most pregious recognition extended by AOAC for outstanding contributions to methodology. He will receive the Wiley Award at the opening session of the 104th AOAC Annual International Meeting and Exposition in New Orleans, LA, September 10–13, 1990.

Doner is internationally recognized for developing methods to combat food adulteration; analytical LC methods for sugars, vitamin C, and alkaloids; and HPTLC of sugars, and has developed new concepts and techniques of considerable interest to science. In the field of food adulteration, conventional analytical approaches are not sufficient for detecting the sophisticated adulterations that are practiced today. A high degree of creativity is required to extend existing theory and methodology and combat such economic frauds. Contributions to this and other areas of particular significance to analytical methodology and to AOAC garnered the Wiley Award for Doner.

For more than 20 years, Doner has been concerned wtih evidence that adulteration is widespread. His research in the 1970s concentrated on adulteration with products derived from corn or sugar cane and revealed that sugars in honey and apple and orange juices possess characteristic stable isotope ratios of carbon-13: carbon-12. Doner, in collaboration, established the uniformity in carbon-13:carbon-12 ratios of domestic and imported honey samples, providing the basis for using this ratio to detect the presence of HFCS in honey. As a result of a successful collaborative study, the SCIRA (stable carbon isotope ratio analysis)



method for honey adulteration was adopted by AOAC, and with Doner's leadership as AOAC Associate Referee in the area of isotope ratio analysis, SCIRA analyses have been extended to include detection of apple and orange juice adulteration.

Doner has also recently completed an AOAC collaborative study based on measurement of oxygen-18:oxygen-16 ratios in juice water as an indicator of adulteration. This is in response to unscrupulous, but scientifically astute processors, who have begun adulterating with beet invert syrups, which are not detectable on the basis of carbon isotope variations. This has been the major adulteration problem facing the orange juice industry. In addition, Doner has recently reported on a spectrometric determination of carbon-bound D/H ratios in orange sucrose, which, if applied, could result in significant decreases in orange juice adulteration with sugar beet derived syrups.

In work at the USDA Plant Science Research Unit, Doner is carrying out current research entitled "Reduction of Postharvest Microbial Spoilage: Role of Plant Extracellular Matrix in Resistance." The objectives of his work are to isolate and characterize cell well associated enzymes, hydroxyproline-rich glycoproteins, and polysaccharides, and to examine their interactions with pathogenic microorganisms. His special emphasis through independent and collab-

A Handbook on How to Set Up or Improve Laboratory Quality Assurance Programs

Quality Assurance Principles for Analytical Laboratories

by Frederick M. Garfield

former Assistant Administrator
U.S. Drug Enforcement Administration

This handbook provides essential information needed to design, document, implement, or improve a laboratory quality assurance program . . . A program that can enable you to document the credibility of your laboratory's analytical data.

The handbook also provides a rational and solidly based justification for commitment of resources to improved laboratory operation.

Drawing from published principles, practices, guidelines, and procedures, the book brings together the experiences of experts who have developed and implemented *successful* "QA" programs.

CONTENTS:

Chapters—

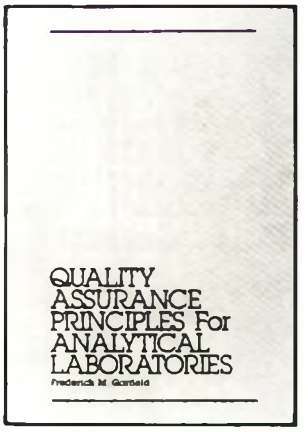
I. Administrative Considerations
II. Personnel Management
III. Management of Equipment and Supplies
IV. Records Maintenance
V. Sample Analysis
VI. Proficiency Testing
VII. Audit Procedures
VIII. Design and Safety of Facilities
IX. Laboratory Accreditation Programs and Good Laboratory Practices Regulations

Appendices—

A. Quality Assurance Publications and Programs
B. Forms Used by U.S. Federal Agencies
C. Instrument Performance Checks
D. Control Charts
E. FDA Audit Measure Procedures
F. Safety Publications
G. Glossary

1984. 224 pp. Softbound. ISBN 0-935584-26-9.

Price—Members: $43.25 in U.S., $48.25 outside U.S.
Nonmembers: $47.50 in U.S., $52.50 outside U.S.
Prices include handling and shipping.



Since 1884
AOAC

To obtain this book, send order and check or credit card information (VISA or MasterCard number, expiration date, signature, date) with your name and address to: AOAC, 2200 Wilson Blvd., Ste. 400-J, Arlington, VA 22201-3301 USA. (703) 522-3032, FAX (703) 522-5468. (U.S. funds on U.S. banks only).

orative research is in the structural and analytical areas.

Doner has also conducted research since the early 1980s resulting in greatly improved analytical method for sugars, vitamin C, and secondary plant metabolites, and developed an aminopropyl bonded phase HPTLC plate, which provides carbohydrate chemists with plates analogous to commercially available aminopropyl silica HPLC columns.

The author of 88 publications, Doner holds a B.S. degree from Winona State University, Winona, MN, and M.S. degree from North Dakota State University, and a Ph.D. degree from Purdue University. He is a member of AOAC, as well as the American Association for the Advancement of Science, and the American Chemical Society's Carbohydrate Division, Agricultural and Food Chemistry Division, and Philadelphia Section.

Help Wanted for "Methods of Air Sampling and Analysis"

The Intersociety Committee on Methods of Air Sampling and Analysis is soliciting members of AOAC who are interested in participating in preparing the fourth edition of the book "Methods of Air Sampling and Analysis." Proposed additional or updated topics include a new chapter on statistics, quality assurance, spiking, radioactive pollutants, indoor air pollutants, air toxics, asbestos, acid rain, impactors, continuous emission monitors, and visibility standards. Members of AOAC who are interested are invited to contact their representative and to indicate their areas of expertise.

Reference Materials Designed to Help Monitor Water Pollution

Environmental agencies, as well as others studying pollution in the nation's waterways, need materials containing an accurate composition of various compounds as a check to verify the reliability of laboratory instruments and methods. Now the National Institute of Standards and Technology has developed a bottled standard reference material (SRM) for this purpose. It contains marine sediment with a wide range of pollutant compounds of interest to environmental scientists. The sediment material, which has certified values for 11 polycyclic aromatic hydrocarbons (PAHs), was collected from the Chesapeake Bay area near Baltimore harbor. It is in dry powdered form, which can be reconstituted into wet form so that the compounds can be extracted by solvents for organic analysis. The material also contains noncertified values for other PAHs, polychlorinated biphenyls, and chlorinated pesticides. It is available from the Office of Standard Reference Materials, NIST, Room 204, Building 202, Gaithersburg, MD 20899. The SRM is among the more than 1,000 SRMs available in NIST's inventory. Each year about 44,000 units are sold to over 10,000 customers throughout the world.

Interim First Action Methods

The following methods have been reviewed by the appropriate General Referee, Committee Statistician, and Methods Committee, and have been approved interim official first action by the Chairman of the Official Methods Board: by the Committee on Pesticide Formulations and Disinfectants—(*1*) Gas chromatographic method for determination of *p,p'*-DDT in DDT technical and formulated products, submitted by D. L. Mount and F. C. Churchill (Centers for Disease Control, Center for Infectious Diseases, Division of Parasitic Diseases, Atlanta, GA); (*2*) Liquid chromatographic method for determination of technical deltamethrin and deltamethrin in pesticide formulations, CIPAC-AOAC method, submitted by A. R. Hanks (Purdue University, Dept of Biochemistry, West Lafayette, IN); by the Committee on Drugs and Related Topics—Liquid chromatographic method for determination of pentaerythritol tetranitrate in pharmaceuticals, submitted by M. Carlson (U.S. Food and Drug Administration, Minneapolis, MN); by the Committee on Foods I—Atomic absorption spectroscopic method for determination of polydimethylsiloxane residues in pineapple juice, submitted by R. D. Parker (Dow Corning Ltd, Barry, U.K.); by the Committee on Foods II—ASBC steam distillation method for determination of essential oil in hops and hop pellets, submitted by P. W. Gales (Anheuser-Busch, Inc., St. Louis, MO); by the Committee on Microbiology and Extraneous Materials—(*1*) Improved hydrophobic grid membrane filer (ISO-GRID) method for detection of *Salmonella* in foods, submitted by P. Entis (QA Laboratories, Ltd, Toronto, Ontario, Canada); (*2*) Presumptive identification by Vitek Gram-Negative Identification Card of *Salmonella*, *Escherichia coli*, and other *Enterobacteriaceae* isolated from foods, submitted by M. T. Knight, D. W. Wood, J. F. Black, G. Gosney, R. O. Rigney, and J. R. Agin (Q Laboratories, Inc., Cincinnati, OH) and C. K. Gravens and S. M. Farnham (Vitek Systems, Inc., Hazelwood, MO).

The methods will be recommended for adoption official first action at the 104th AOAC Annual International Meeting, September 10–13, 1990, at New Orleans, LA. Copies of the methods are available from AOAC Scientific Publications.

Harvey W. Wiley Awards Fund Contributors

The following members of AOAC have contributed to the Harvey W. Wiley Awards Fund: Otto Ackermann, Thomas G. Alexander, Canan D'Avela, Raymond J. Barber, Mark Billedeau, Leon Boyar, Juan Carlos Medina Bravo, Howard Casper, Charlie S. Chang, Forrest W. Cross, James W. Fitzgerald, Michael G. Goergen, Willie L. Hinze, Tetslya Kato, Paul G. King, Leo J. Lipinski, Jr, Janusz L. Lorenz, Stephen Ludvig, Frank G. Pilkiewicz, Forrest W. Quackenbush, Ulysses S. Rhea, Robert C. Rund, Peter M. Scott, Odette L. Shotwell, Bernard F. Taylor, John K. Taylor, Bradley L. Thompson, David C. Woollard, and Cas Woss.

The Harvey W. Wiley Award Fund was established in 1956 to honor Harvey W. Wiley, "Father of the Pure Food Laws" and a founder of AOAC. This fund supports the Harvey W. Wiley Award for the Development of Analytical Methods and the Harvey W. Wiley Scholarship Award. Contributions to sustain the Harvey W. Wiley Awards Fund will be appreciated and should be sent to AOAC.

New Sustaining Members

AOAC welcomes the following new private sustaining members: Kuwait

The JOURNAL OF THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

EDITORIAL PROFILE

The *Journal of the AOAC* is the official bimonthly journal of the Association of Official Analytical Chemists (AOAC). Published for analytical scientists who develop and use analytical methodology, it is used by its subscribers in their day-to-day work in the fields of food composition and contamination, feeds, pharmaceuticals, cosmetics, agricultural and household chemicals, water analysis, and environmental control. Users include analytical chemists, biologists, microbiologists, biochemists, toxicologists, spectroscopists, statisticians, forensic and other scientists in laboratory, administrative, and top management positions in industry, government, and universities in the United States and more than 90 other countries.

The *Journal of the AOAC* is a principal forum for exchange of information among methods researchers in the regulatory agencies and regulated industries.

The *Journal of the AOAC* has an average paid circulation of about 4,000 subscribers and a referenced readership of more than 25,000 purchasers and specifiers of laboratory equipment and supplies, testing apparatus, instrumentation, and related products and services. Subscribers order the *Journal* because it is professionally necessary to do so.

ADVERTISING RATES

Rate Card No. 14, Effective January 1988

ADVERTISING UNITS AND RATES

Size	1X	3X	6X
Full Page non-bleed, 7 × 10″ bleed, 8½ × 11¼″	$750	$700	$625
⅔ Page 4½ × 10″	$600	$560	$520
½ Page 7 × 5″	$500	$465	$420
⅓ Page 2¼ × 10″	$400	$375	$335

Color and Position Rates
are in addition to black and white space rates

2-Color Standard	$300
2-Color Matched	$400
4-Color Process	$575
Covers—2nd, 3rd, 4th; Full Page Only	$150
Bleed	No Charge

EARNED RATES, REBATES, AND SHORT RATES

Based on accumulated space used during 12-month periods from date of first insertion. Where earned rates and billed rates differ, advertisers will be rebated or short-rated, as applicable.

ISSUANCE AND CLOSING DATES

Journal of the AOAC is issued on the 15th of January, March, May, July, September, and November. **Closing date for receipt of insertion orders, copy, negatives, and camera-ready material is the first of the month preceding month of issue.**

CONDITIONS

Advertisers and advertising agencies assume full liability for all advertising content and assume full responsibility for any claims arising therefrom. All orders and instructions must be confirmed in writing. Publisher assumes no responsibility for printing errors or omissions.

The publisher shall not be bound by any condition other than those set forth in this ad or other advertising materials.

All advertising copy is subject to prior approval by the Editorial Board of the *Journal of the AOAC*.

AGENCY COMMISSION

15% to recognized agencies provided account is paid within 30 days of invoice date. No cash discount.

TERMS OF PAYMENT AND CANCELLATIONS

Payment is due within 30 days of invoice date. Space canceled after closing date will be billed to advertiser.

When copy changes are not received by deadline, publisher reserves right to repeat last previous copy, billing advertiser for space used.

MECHANICAL SPECIFICATIONS AND REQUIREMENTS

1. Trim size: 8¼ × 11″
2. Screens not to exceed 133 lines
3. The following reproduction materials are acceptable. Order of preference (all right reading):
   a. Film negatives—emulsion down
   b. Positive prints or mechanicals
   c. Scotch prints

   Other reproduction material can be converted and advertiser rebilled at publisher's cost.
4. The cost of repairing furnished ad materials will be charged to the advertiser.

INSERTION AND SHIPPING INSTRUCTIONS

Please send all advertising materials, contracts, and insertion orders to:

Marketing Manager, AOAC
Suite 400-J, 2200 Wilson Boulevard
Arlington, VA 22201-3301 USA
(703) 522-3032

Institute for Scientific Research, Safat, Kuwait, and Ohio Dept of Agriculture, Reynoldsburg, OH.

Fifteenth Edition of Official Methods of Analysis Published

The new 15th edition of *Official Methods of Analysis*, just published, comprises all the official methods of the Association.

For the first time, the methods have been published as a two volume set, and all have been assigned permanent reference numbers. New features are also representative of the increasing scope and technology of analytical chemistry, including methods on near infrared, ELISA, and DNA hybridization techniques.

While the number of AOAC official methods and the scope of their applications has multiplied dramatically in the past 100 years, the meaning and significance of the designation "AOAC official methods" has not changed. The term has consistently identified accurate, reproducible, and proven methods that have been validated through interlaboratory testing and found acceptable by AOAC. The 15th edition of *Official Methods of Analysis* continues the AOAC tradition as a recognized international source of methods that are definitive for their recommended uses and conditions.

The AOAC bylaws define official methods and outline the procedure for their adoption. Methods of analysis are adopted as official first action and official final action. No method is adopted "official first action" until it has been recommended by the appropriate General Referee and Methods Committee. A method is adopted "official final action" only after the adoption of such method as "official first action." In both cases, the method must be approved by vote of the membership at the annual business meeting. A two thirds majority of the voters present and voting is necessary for approval.

Both the first action and the final action methods are official and designate full acceptance by AOAC. Most first action methods are elevated to final action status after at least 2 years of official recognition. However, as stated in the Preface to the 8th edition, ". . . the distinction is more one of scientific courtesy than a difference in status. The same degree of performance is required by the Association for adoption of a method as first action as is required for its adoption as official. Since it is impossible for all chemists affected by a given method to participate in the collaborative study and since it is impossible to perform the study on all possible types and variations of samples that may be encountered in routine work, the Association, by custom, has provided the opportunity for those interested in the method to study it further before its final adoption." In instances where no feedback on a method occurs, it might remain in first action status. This does not affect the validity of the method, since it has passed all the requirements for official recognition, including vote by the membership."

A sampling or sample preparation or other type of procedure for which an interlaboratory study is impractical may be adopted as official when accumulated data or statistically planned studies indicate that the procedure is reliable. On recommendation of the General Referee and Methods Committee, these are adopted by the membership as "procedures."

An analytical method that has undergone interlaboratory study between annual meetings and has been approved by the appropriate General Referee and by the Method Committee, and Chairman of the Official Methods Board is designated "interim official first action" until the Association votes on full acceptance at the first subsequent annual meeting. This procedure serves to alert interested analysts and administrators to emerging methods that will be voted on at the next annual meeting.

The 15th edition of *Official Methods of Analysis* may be ordered now from AOAC for immediate delivery.

COMING IN THE NEXT ISSUE

SPECIAL REPORTS

- Evaluation of Protein Digestibility-Corrected Amino Acid Score Method for Assessing Protein Quality of Foods—*Ghulam Sarwar and Frank E. McDonough*
- Liquid Chromatographic Approaches to Antibiotic Residue Analysis—*William A. Moats*

METALS AND OTHER ELEMENTS

- Continuous Flow Vapor Generation for Inductively Coupled Argon Plasma Spectrometric Analysis. Part I: Selenium—*Mark L. Tracy and Gregory Möller*
- Influence of Automatic Dishwashings and Scrubbings on Release of Lead from Glazed Ceramicware—*John H. Gould, Susan C. Hight, George H. Alvarez, Catherine E. Nelson, and Stephen G. Capar*

and

75 YEARS OF REPORTING ANALYTICAL SCIENCE
1915–1990

Thanks to Reviewers

Each year at this time, we take the opportunity to convey our gratitude to the hundreds of individuals who voluntarily set aside valuable time to assist the *Journal of the AOAC* by reviewing submitted manuscripts. The reputation of a scientific journal rests on the quality of the science reported therein. Those of you listed below have helped immeasurably to assure the reputation of the *Journal of the AOAC* by insisting on high standards of experimental design, method execution, and data interpretation. Please accept our thanks for your contribution to fulfilling AOAC's mission.

R. G. Ackman
V. K. Agarwal
I. Ahmad
D. Anderson
R. Ashley
S. H. Ashoor
A. M. Au
R. Bailey
R. J. Baker
E. W. Balcer
R. Baldwin
J. Balsinger
R. Bandler
D. M. Barbano
F. Barbatsch
C. J. Barnes
R. W. Beaver
R. L. Beine
F. Belken
S. Bell
S. Bellman
G. A. Bennett
D. A. Benton
J. Benton-Jones
D. Bernard
M. Bicking
L. Bighley
P. D. Bland
S. I. Blittman
J. A. Boatman
J. L. Boese
K. W. Boyer
M. Brady
W. P. Brindly
M. Brooks
M. W. Brooks
K. Brunnemann
C. Brunner
W. Buchholz
W. Bucholz
V. Burse
L. C. Butler
A. D. Candlish
S. G. Capar
A. Caputi
W. F. Carey
G. Carignan
M. P. Carlson
E. E. Cary
A. J. Cessna
L. Chafetz
D. Chaput
M. J. Charles
A. Y. Chau
H. Cheng
V. Chew
T. C. Chiang
M. Chiba
F. S. Chu
D. Clark
W. P. Cochrane
R. J. Cole
M. R. Coleman
B. A. Colenutt
M. K. Conditt
R. F. Cook
E. D. Coppola
P. E. Corneliussen
J. E. Cowell
D. Craven
D. G. Cunningham
M. E. Cutrufelli
M. Czech
R. W. Dabeka
P. K. Dadgupta
B. A. Dawson
V. K. Dawson
A. Dekok
R. A. Depalma
A. V. Del Grosso
D. E. Dixon-Holland
J. W. Dolan
K. F. Donahue
L. W. Doner
A. L. Donoho
G. M. Doose
J. W. Dorner
H. Dorough
J. M. Dunham
J. Dziedzic
D. B. Easty
J. Eble
S. Eisenberg
R. G. Elkin
R. Eppler
D. Erney
A. A. Fatmi
E. Fedeli
M. Feeney
N. V. Fehringer
W. Fiddler
D. Firestone
D. L. Fish
J. Fisk
J. Flawrence
W. G. Fong
J. F. Frank
C. Franklin
N. Frawley
D. C. Fung
W. A. Garland
C. Garner
F. C. Garner
L. T. Gelbaum
J. Gilchrist
G. Gipson
U. Goff
J. Grant
R. Grappin
J. Gray
F. M. Gretch
H. Grill
P. A. Guarino
R. A. Guinivan
D. F. Gurka
J. Haas
C. C. Hach
H. C. Hamaker
A. Harding
E. E. Hargesheimer
M. Hasselberger
D. C. Havery
D. L. Heikes
C. J. Henry
J. Hershey
C. Hesseltine
R. Highet
D. A. Hinckley
C. A. Hitchcock
R. A. Hites
C. Ho
W. Holak
G. Hoogenboom
C. Hope
M. L. Hopper
L. Q. Huang
W. Hunter
S. Hurlbert
H. E. Hurst
W. Hurst
M. Ihnat
R. A. Isaac
A. V. Jain
W. G. Jennings
F. L. Joe
D. B. Johnson
J. L. Johnson
P. F. Kane
S. E. Katz
L. H. Keith
P. C. Kelly
S. Kevra
E. J. Kikta
H. J. Kim
D. Kindack
T. C. Knott
Y. Konishi
W. A. Korfmacher
S. Kornfeld
R. T. Krause
B. Krinitz
D. A. Krueger
I. Krull
F. Lancaster
W. O. Landen
R. D. Larson
R. R. Laski
D. R. Lauren
T. L. Lavy
M. W. Law
G. L. Lebel
M. Lebelle
J. A. Lebo
L. S. Lee
S. Lee
J. Lepore
S. Lesage
D. Leyden
V. Lopez-Avila
E. M. Lores
E. G. Lovering
W. Loy
R. G. Luchtefeld
R. C. Lundstrom
J. A. Maga
J. M. Magnuson
R. E. Majors
A. J. Malanoski
E. L. Malin
V. N. Mallet
H. Marks
P. Marquis
R. C. Massey
D. A. Masterorocco
J. D. Mattice
M. S. Maynard
F. D. McClure
F. McClure

S. McCormick
K. A. McCully
R. F. McFeeters
B. D. McGarvey
E. D. McGary
E. J. McGonigle
D. I. McGregor
G. McKay
V. J. Meinen
D. Millie
C. V. Mitchell
W. A. Moats
R. M. Montgomery
B. Mopper
M. A. Morgan
M. F. Morselli
D. J. Murray
E. Neidert
J. Neucere
G. A. Neville
W. Newsome
E. C. Nicolas
T. Nowicki
G. Nygard
D. D. Oehler
C. Olieman
F. I. Onuska
B. O. Page
S. W. Page
P. J. Palermo
C. H. Parfitt
D. L. Park
M. D. Parkins
O. W. Parks
W. Pask
J. T. Peeler
J. J. Pestka
D. R. Petrus
R. E. Phillips
R. J. Pierce
W. Pilnik
R. M. Pollman
I. Pomerantz
W. G. Potter
H. A. Price
R. L. Price
S. M. Prinsloo
L. Prosky
M. Rabinowitz
H. S. Ragheb
S. Ramesh
K. Ramsteiner
W. Ratnayaki
R. Reding
L. Reiman
K. Reimann
E. P. Reyes
H. L. Reynolds
B. D. Ripley
T. R. Romer
J. D. Rosen
F. Ross
L. Ross
P. F. Ross
S. C. Ross
G. Rottinghaus
N. W. Rowe
J. Rudling
R. C. Rund
G. E. Russell
A. M. Rustum
E. Sarnoff
G. Sarwar
L. D. Sawyer
W. S. Saxton
R. A. Scanlan
U. S. Schwertschlag
P. M. Scott
J. N. Seiber
N. P. Sen
P. Shea
E. Sheinin
A. J. Sheppard
J. Sherbon
J. Sherma
D. L. Shotwell
R. Shoup
D. H. Sieh
R. C. Sims
M. Singh
B. B. Sithole
P. T. Slack
R. V. Slates
J. E. Slayton
R. L. Smallidge
D. Smith
L. M. Smith
L. R. Smith
S. Solieau
T. D. Spittler
J. A. Springer
S. Spurlin
K. Stainbrecher
H. Stan
W. F. Staruskiewicz
W. Staruszkiewicz
B. Stavric
E. Steffen
K. Steinbrecher
F. H. Steinke
J. T. Stewart
D. B. Stonys
N. Strojny
J. Strom
R. D. Stubblefield
R. J. Stubbs
D. M. Sullivan
J. F. Suprock
R. A. Sweeney
K. Takatsuki
G. Takeoka
K. Tanaka
R. F. Taylor
M. H. Thomas
J. Thompson
C. Thorpe
S. Ting
D. F. Tompkins
V. Trost
W. J. Trotter
M. W. Trucksess
R. Tscherne
L. G. Tuinstra
J. F. Uthe
J. P. Van Buren
P. J. Van Soest
J. T. Vanderslice
T. L. Veum
A. B. Vilim
D. Wagner
C. R. Warner
J. D. Weber
M. M. Wekell
S. D. West
L. Weston
C. Wetherell
L. D. Wilken
T. D. Wilson
G. Wogan
B. L. Worobey
D. Wright
D. P. Wright
H. Wu
R. L. Yates
E. W. Zahnow
R. W. Zumwalt
L. C. Zygmunt

UPCOMING AOAC ANNUAL MEETINGS

104th AOAC Annual International Meeting in New Orleans, Louisiana
September 10–13, 1990
105th AOAC Annual International Meeting in Phoenix, Arizona
August 12–15, 1991

Books in Brief

The United States Pharmacopeia, 22nd revision, and The National Formulary, 17th edition. Published by The United States Pharmacopeial Convention, Inc., 12601 Twinbrook Pkwy, Rockville, MD 20852, 1990. 2,067 pp. Price: $250.00 (main volume and all 1990 supplements).

The 22nd revision of *The United States Pharmacopeia* and the 17th edition of *The National Formulary*, the official U.S. compendia of standards for drugs and the inactive ingredients in drug dosage forms, have been combined, for the third time, into a single volume. Together, they publish all the legally enforceable standards set by the United States Pharmacopeial Convention, Inc., (USP), on the strength, quality, purity, packaging, and labeling for drugs used in the United States. *The United States Pharmacopeia* includes nearly 3,000 monographs on drug substances and dosage forms; *The National Formulary* includes approximately 250 monographs on inactive agents or excipients known as pharmaceutic ingredients. Where an article is used as both a therapeutic agent and a pharmaceutic ingredient, it is included in *The United States Pharmacopeia*, with a cross-reference from *The National Formulary* to that USP monograph. A combined index facilitates reference to the contents of the respective compendia.

Standard Methods for the Examination of Water and Wastewater, 17th edition. Edited by Lenore S. Clesceri, Arnold E. Greenberg, and R. Rhodes Trussell. Published by American Public Health Association, 1015 15th St, NW, Washington, DC 20005, 1989, 1,644 pp. Price: $120.00 ($96.00 for APHA, AWWA, and WPCF members). ISBN 0-87553-161-X.

This internationally acclaimed reference includes more than 300 methods with step-by-step procedures for precise analysis of chemical constituents, sanitary quality, physical, and biological characteristics of water and wastewater. In all, more than 60 sections have been revised and 30 new methods added to reflect the latest technological advances and regulatory requirements. These major changes make the 17th edition of "Standard Methods" the most comprehensive, up-to-date compilation of water and wastewater analysis methods available anywhere.

Hazardous Waste Minimization Manual for Small Quantity Generators, 2nd edition. Published by the Center for Hazardous Materials Research, University of Pittsburgh, Applied Research Center, 320 William Pitt Way, Pittsburgh, PA 15238, 1989. 300 pp. Price: $40.00.

In understandable language, this manual is a valuable guide to business owners and managers on how to approach and implement a pollution prevention program and meet the waste minimization certification requirements of EPA. Industry can no longer afford to handle wastes after they are generated. Costs for disposal and treatment are soaring, requiring industry to explore new approaches and technologies to reduce hazardous wastes before they are generated. A special chapter of the manual targets waste reduction techniques for 11 specific industries. In addition, organizations and sources that can provide additional assistance in waste minimization are listed for each of the 50 states. This manual is an update of the first edition, which was awarded the 1988 Environmental Achievement Award for the best environmental product for the U.S. EPA Region III Center for Environmental Learning.

Cosmetic and Toiletry Formulations, 2nd edition. By Ernest W. Flick. Published by Noyes Data Corp., Mill Rd at Grand Ave, Park Ridge, NJ 07656, 1989. 964 pp. Price: $125.00. ISBN 0-8155-1218-X.

More than 1,800 cosmetic and toiletry formulations are described in the second edition of this well-received and useful book. The book is based on information obtained from industrial companies and their organizations. The data represent selections from manufacturers' descriptions, in their own words, made at no cost to, nor influence from, the makers or distributors of these materials. Only the most recent formulas have been included. It is believed that all of the tradenamed raw materials listed are currently available, which will be of utmost interest to readers concerned with raw material discontinuances.

Hazardous Waste Management Facilitates Directory: Treatment, Storage, Disposal, and Recycling. By U.S. Environmental Protection Agency, Versar, Inc., and Camp Dresser & McKee, Inc. Published by Noyes Data Corp., Mill Rd at Grand Ave, Park Ridge, NY 07656, 1989. 327 pp. Price: $64.00. ISBN 0-941459-02-0.

This book provides a listing of 1,045 commercial hazardous waste management facilities, along with information on the types of commercial services offered, e.g., treatment, storage, disposal, or recycling, and types of wastes managed. It is a compilation of recent data from EPA data bases. Facility name, address, contact person, and phone number are listed for each site as available. The purpose of this book is to assist in locating pertinent waste management facilities in specific geographic areas.

Algorithms for Chemists. By Jure Zupan. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1989. 290 pp. Price: $87.95. ISBN 0-471-92173-4.

This book describes and explains the many computational methods, and algorithms and their implementation, throughout the natural sciences in general, and chemistry in particular. The book is divided into three sections: data representation, data preprocessing, and data handling. It provides the chemist (or anyone interested in computerized data handling) with basic procedures encountered in the routine use of experimental data: from random number generation to two-dimensional pattern (image) processing. Among others, there are methods for handling chemical structures, different transformations, clustering, optimization, pattern recognition, cellular automata, and fundamentals of expert system design.

JAOAC

Instructions to Authors

Scope of Articles and Review Process

The *Journal of the AOAC* publishes articles that present, within the fields of interest of the Association: unpublished original research; new methods; further studies of previously published methods; background work leading to development of methods; compilations of authentic data of composition; monitoring data on pesticide, metal, and industrial chemical contaminants in food, tissues, and the environment; technical communications, cautionary notes, and comments on techniques, apparatus, and reagents; invited reviews and features. Emphasis is on research and development of precise, accurate, sensitive methods for analysis of foods, food additives, supplements and contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment. The usual review process is as follows: (*1*) AOAC editorial office transmits each submitted paper to appropriate subject matter editor, who solicits peer reviews; (*2*) editor returns paper to author for revision in response to reviewers' comments; editor accepts or rejects revision and returns paper to AOAC editorial office; (*3*) AOAC editorial staff edits accepted papers, returns them to authors for approval, and transmits approved manuscripts to typesetter; (*4*) typesetter sends page proofs to author for final approval.

General Information

Contributed manuscripts accepted for publication after peer review are subject to a charge of US$40 per printed page. Payment is not a condition of publication, however, and waivers are granted on receipt of a written request to the Managing Editor by an administrative officer of the author's institution.

Follow these instructions closely; doing so will save time and revision. For all questions of format and style not addressed in these instructions, consult recent issue of *Journal* or current edition of *Council of Biology Editors Style Manual*.

1. Write in clear, grammatical English.
2. To Managing Editor, AOAC, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA, submit typewritten original plus 3 photocopies (1 side only, white bond, 8½ × 11 in. [21½ × 28 cm]) of complete manuscript in order as follows—1. Title page; 2. Abstract; 3. Text (introduction, method or experimental, results and/or discussion, acknowledgments, references); 4. Figure captions; 5. Footnotes; 6. Tables with captions, one per page; 7. Figures.
3. Suggest in a covering letter the names of at least 4 qualified reviewers, i.e., individuals engaged in or versed in research of the type reported.
4. **DOUBLE SPACE all typed material.** Manuscripts not double spaced will be returned for retyping. Do not right justify or use proportional spacing; avoid hyphenation.
5. Use letter quality printer for word-processed manuscripts; manuscripts prepared on dot matrix printers of less than letter quality may be refused.

Format and Style

1. **Title page** (separate sheet, **double spaced**): Title of article, authors' names (full first, middle initial if any, full last), authors' addresses including mail codes.
2. **Abstract** (separate sheet, **double spaced**): ≤200 words. Provide specific information, not generalized statements.
3. **Text** (consecutive sheets, **double spaced**):

   Introduction. Include information on why work was done, previous work done, use of compound or process being studied.

   Method or Experimental. Consult recent issue of *Journal* for proper format. Separate special reagents/apparatus from details of procedure and list in sections with appropriate headings; list in generic and performance terms, avoid use of brand names. (Common reagents/apparatus or those which require no special treatment need not be listed separately.) Place detailed operations in separate sections with appropriate headings (e.g., Preparation of Sample, Extraction and Cleanup). Include necessary calculations; number of significant figures must reflect accuracy of method. Use metric units for measurements of quantities wherever possible. Write *Method* (recommendation for use of specific method) in imperative voice ("Add 10 mL . . . Heat to boiling . . . Wash flasks"); write *Experimental* (description of laboratory experiment) in passive or active voice ("Ten mL was added . . . We heated to boiling . . . Flasks were washed"). Note hazardous and/or carcinogenic chemicals.

   Results/Discussion. Cite tables and figures consecutively in text with Arabic numerals. *Do not* intersperse tables and figures in text.

   Acknowledgments. Give brief thanks (no social or academic titles) or acknowledge financial aid in this section.

   References. Submitted papers or unpublished oral presentations may not be listed as references; cite them in text as unpublished data or personal communications. Cite all references to previously published papers or papers in press in numerical order in text with number in parentheses on line (*not* superscript). List references numerically in "References" in *exactly* (arrangement, punctuation, capitalization, use of ampersand, etc.) styles of examples shown below or see recent issue of *Journal* for less often used types of entries. Follow *Chemical Abstracts* for abbreviations of journal titles.

   JOURNAL ARTICLE REFERENCE

   (1) Engstrom, G. W., Richard, J. L., & Cysewski, S. J. (1977) *J. Agric. Food Chem.* 25, 833–836

   BOOK CHAPTER REFERENCE

   (2) Hurn, B. A. L., & Chantler, S. M. (1980) in *Methods in Enzymology*, Vol. 70, H. VanVunakis & J. J. Langone (Eds), Academic Press, New York, NY, pp. 104–142

   BOOK REFERENCE

   (3) Siegel, S. (1956) *Nonparametric Statistics for the Behavioral Sciences*, McGraw-Hill Book Co., New York, NY

   OFFICIAL METHODS REFERENCE

   (4) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs 29.070–29.072

4. **Figure captions** (separate sheet(s), **double spaced**): Designate all illustrations, including schemes, as figures and include caption for every one. Identify curves (See **Figures**) and include all supplementary information in caption rather than on face of figure. Spell out word Figure.
5. **Footnotes** (separate sheet, **double spaced**): Avoid use of footnotes to text. Include "Received . . . Accepted . . ." line; location/date of presentation, if appropriate; present address(es) of author(s); identification of corresponding author, if not senior author; proprietary disclaimers; institution journal series numbers.
6. **Tables** (one per page, **double spaced**): Refer to recent issue of *Journal* for proper layout and style, especially use of horizontal lines. Do not draw in vertical lines. Include descriptive title sufficient that table stands alone without reference to text. Provide heading for *every* vertical column. Abbreviate freely; if necessary, explain in footnotes. Indicate footnotes by lower case superscript letters in alphabetical order. Do not use one-column tables; rather, incorporate data in text.
7. **Figures:** The *Journal* does not publish straight line calibration curves; state such information in text. Do not duplicate data in tables and figures. Submit original drawings or black/white glossy photographs with original manuscript; photocopies are acceptable only for review. Prepare drawings with black India ink or with drafting tape on white tracing or graph paper printed with nonreproducible green ink. Use a Leroy lettering set, press-on lettering, or similar device; use type at least 2 mm high to allow reduction to page or column size. Identify ordinate and abscissa and give value in *Journal* style (e.g., "Wavelength, nm," "Time, min"). Label curves with letters or numbers; avoid all other lettering/numbering on face of figure (see **Figure captions**). Identify each figure on back with number and authors' names.

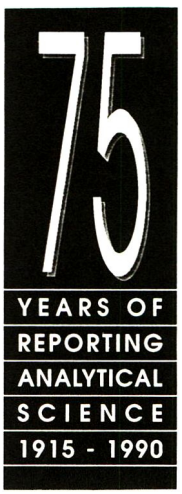
Miscellaneous

Abbreviation for liter is L; abbreviation for micron is μm. Do not italicize common Latin expressions such as et al. and in vitro; for nomenclature of spectrophotometry, gas chromatography, and liquid chromatography, follow practice of American Society for Testing and Materials (in particular, do not use "high performance," "high pressure," or the abbreviation "HP" with "liquid chromatography").

1/89

Cumulative Author Index

PAPERS THAT MADE A DIFFERENCE



The audience of the AOAC *Journal*, by its very nature, is concerned with the development, testing, and validation of analytical methods designed to verify the identity, purity, and, indeed, the suitability for use and distribution in commercial channels of a wide variety of materials from the food, drug, cosmetic, and allied industries.

As such, some of us who are "seasoned veterans" of analytical chemistry have witnessed the evolution of the concept of "analysis." We may recall 30 years ago when a senior member of the scientific staff of the Olin Chemical Corp. could call the use of a buret instrumental analysis. Today, we have hyphenated methodology, with a concomitant increase in sensitivity of at least 9 orders of magnitude.

Important ramifications have accompanied this increased sophistication, not the least of which, in my opinion, is our society's present day zero-risk mentality. It is possible, for example, to destroy the health of laboratory animals with large doses of Alar and then turn around and measure the compound in parts per million on the skin of apples collected from regional growers in the Northeast. The public, erring on the side of caution, called for the ban of the insecticide. An apple a day can still keep the doctor away but with the methodological capabilities of earlier times, we would never have had to worry about how many apples would cause us to visit the doctor.

Because of tragedies such as the thalidomide disaster in West Germany in the 1960s, U.S. regulatory agencies look much more critically at the gestation process in any product designed to come into direct contact with consumers, either internally or externally. Their attitude in regard to analytical methods has also gradually shifted toward increased analytical sophistication, particularly when monitoring residues of animal health products in comestibles. For example, sulfonamides were assayed colorimetrically at the low parts per million range in 1968 (1). Today, ELISA methods are designed to detect 1 ppb reliably (2). A trend away from tacit acceptance of methods supplied by industry and toward guidelines set by the regulatory bodies has developed.

Regulatory bodies have customarily subjected the analytical methods supplied to them as part of the application process to interlaboratory scrutiny. This usually has involved 2 or 3 field laboratories located in different parts of the country. This process has been described by a number of different organizations in various parts of the world, and most recently, this process was the subject of a paper in the AOAC *Journal* (3).

In his seminal publication on the subject, W. J. Youden codified the concept of interlaboratory investigation. Collaborative studies, as such investigations are popularly known, are designed to establish the reliability, ruggedness, and reproducibility (i.e., accuracy and precision) of a given procedure intended for broad application to pharmaceutical quality control and monitoring residues of a wide variety of materials.

Youden's paper was published in the AOAC *Journal* in 1963 under the simple title "Collaborative Test." This paper includes the criteria used to evaluate methods recommended for adoption by AOAC. A pioneering work in its field, Youden's discussion not only suggests statistical treatment of experimental data but also recognizes sources of experimental error and how to deal with them.

"The Collaborative Test" is reprinted here in its entirety. It appears quite appropriate for us to reread the paper inasmuch as the audience of the AOAC *Journal* is intimately involved with a variety of activities that have a deep impact on the public health and safety.

References

(1) Tishler, F., (1968) *J. Agric. Food Chem.* **16,** 50-53
(2) Dixon-Holland, D. E., & Katz, S. E. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 447-450
(3) Zaalberg, J. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 34-37

Joseph Westheimer
Journal Co-Editor for Drugs,
Cosmetics, and Forensic Materials

CLASSIC PAPER: REPRINTED FROM YOUDEN, W.J. (1963) *J. ASSOC. OFF. AGRIC. CHEM.* **46**, 55-62

SPECIAL SESSION ADDRESS

The Collaborative Test*

By W. J. YOUDEN (National Bureau of Standards, Washington, D.C.)

This paper discusses (a) the planning of collaborative tests, (b) a technique to establish that a procedure is ready for a collaborative test, and (c) the interpretation of the results of a collaborative test.

Introduction

The collaborative, or interlaboratory, test is an indispensable scrutiny of an analytical procedure to insure (a) that the description of the procedure is clear and complete and (b) that the procedure does give results that are in accord with any accuracy claims made for the procedure. A collaborative test should be a kind of final inspection. If the procedure has been properly studied before submitting it to a collaborative test, then the collaborative test has as its proper role the task of verifying any claims made for the procedure.

Planning a Collaborative Test

There are three matters to settle in planning a collaborative test. These are the number of collaborators, the number of materials sent to each collaborator, and the number of measurements made by each collaborator on each material. Inevitably certain compromises have to be made. A large number of collaborators is desirable because this will give confidence that analysts will not misinterpret the instructions and that the procedure has been tried under a wide range of environments. Increasing the number of materials provides evidence that the procedure is satisfactory over a wide range of amounts present and types of material. Repeat analyses on each material would provide information on the agreement of parallel analyses made under as nearly identical conditions as possible.

Increasing the number of materials and the number of analyses on each material adds considerably to the burden of work imposed on each collaborator. Often this has the unfortunate consequence of reducing the number of laboratories willing to participate as collaborators. Therefore it is important to hold to a minimum the work imposed on each collaborator. One only has to consider two extreme situations to see the importance of having an adequate number of collaborators. If you want to learn about a procedure, which would you rather have: Ten repeat analyses from one laboratory or a single analysis from each of ten laboratories? True, the information given by these alternatives is quite different, but the really useful information is given by the single results from the ten laboratories.

The best way to reduce the workload per laboratory is to reduce the number of repeat analyses made on each material (1). In spite of the long tradition to require at least duplicate determinations on each material, a strong case can be made for requiring just a single determination per material, unless repetitions are actually needed. There are several reasons behind this suggestion. First, the agreement of parallel determinations should be about as good in one laboratory as in another. After all, the equipment is specified and there is the presumption of qualified analysts. Certainly the laboratory environment will vary from laboratory to laboratory and the procedure may not be immune to these changes in environment. But *within* any one laboratory, parallel determinations will be exposed to the same environment and the agreement between the duplicates normally will not be impaired by reason of any local environmental peculiarity. For this reason it is not surprising that the *precision*, as revealed by repeat runs, is indistinguishably the same for all participating laboratories.

A second reason for not requiring repeat determinations is that rarely are enough data available to detect a two-fold difference in precision (standard deviation) between two

* Presented at the Referees' Meeting, Seventy-sixth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 16, 1962, at Washington, D.C.

CLASSIC PAPER: REPRINTED FROM YOUDEN, W.J. (1963) *J. ASSOC. OFF. AGRIC. CHEM.* **46**, 55-62

laboratories. Triplicate determinations on each of seven materials will give a four out of five chance of catching a two-fold difference in precision. It would take the equivalent of five repeat determinations on each of ten materials to have the same probability of detecting that one laboratory has a standard deviation 1.5 times that of another laboratory. Clearly this is a lot of extra work for each laboratory. On the other hand, the initiating laboratory should have ample records to establish the precision of the procedure. The precision, in any event, is usually of minor importance as compared with the larger error inevitably associated with the comparison of results from different laboratories.

One might also mention that many laboratories will not report a pair of duplicates that happen to show rather poor agreement. The temptation to run a third determination, or even another pair, is strong. The consequence of any such censoring of the data is to produce an estimate of the precision that is biased in the direction of making the precision appear to be better than it really is. Finally the precision can be estimated even if only single determinations are made, and such an estimate is immune from any replacements of the results first obtained. It is merely necessary that two materials, A and B, similar in composition and content, be included in the work. Let the results from n laboratories be as follows:

Material	*Laboratory Number*					*Av.*
	1	2	3	....	n	
A	a_1	a_2	a_3	....	a_n	$\bar{a}$
B	b_1	b_2	b_3	....	b_n	$\bar{b}$
Difference (A − B)	d_1	d_2	d_3	....	d_n	$\bar{d}$

Compute $s = \sqrt{\dfrac{\Sigma d^2 - n\bar{d}^2}{2(n-1)}}$ = standard deviation

You will observe that whatever local or systematic error a laboratory has drops out of the differences, $d_1, d_2, \ldots, d_n$. These differences should all be the same except for precision errors. So it is the variation among these differences that provides an estimate of the precision. The above formula is equivalent to deducting the mean difference, $\bar{d}$, from each of the n differences and calling the remainders d′. Thus $d_1 - \bar{d} = d_1'$. These remainders are squared and divided by $2(n-1)$, and the square root is taken.

$$s = \sqrt{\Sigma(d')^2/2(n-1)}$$

An estimate of the precision by this approach is more realistic in that it is protected against any selection of the data by replacement of repeat determinations that show larger than usual disagreement and the estimate is a consensus taken over all the participating laboratories.

We arrive, then, at the suggestion that the collaborative test include as many laboratories as possible, using as many materials as circumstances suggest, and that only single determinations be required. Some have raised the question that certain laboratories might run duplicates but report the averages as single determinations. A laboratory that does this is ill advised. First, the averages of two would give this laboratory an apparent standard deviation of only 0.707 that of laboratories running single determinations. But the data will not visibly reveal this if only because of the difficulty of showing small differences in precision. Rather less pleasing to such a laboratory is that this average reveals only the more clearly any systematic error the laboratory has in comparison with the consensus of all the laboratories. And it is on just this point that attention is going to be focused with the idea of asking such laboratories for explanations.

The Responsibility of the Initiating Laboratory

By no means an unusual occurrence is a collaborative test whose results obviously fall short of expectations based on data obtained by the initiating laboratory. The explanation is usually found in the fact that the initiating laboratory has a set of operations and equipment that is never varied. In fact, care is taken not to vary the routine in any particular. Naturally no light is shed on what may happen when the procedure on trial is used by a number of laboratories each of which establishes

CLASSIC PAPER: REPRINTED FROM YOUDEN, W.J. (1963) *J. ASSOC. OFF. AGRIC. CHEM.* **46**, 55-62

its own particular routine. Such things as the source and age of reagents and the concentrations of these reagents, the rate of heating, thermometer errors, humidity, and many other factors may be involved. One laboratory makes up a supply of nominally 1*M* acid and in fact achieves a concentration of 0.95. Another laboratory's solution may be 1.03*M*. Each laboratory gets good checks, of course, because it always uses the same solution, just as the initiating laboratory did.

The only protection against such sources of trouble which are disconcerting and difficult to discover is for the initiating laboratory deliberately to introduce minor reasonable variations in the procedure and observe what happens. These departures should be of the magnitude that a chemist might well expect to find among laboratories. At first this appears to throw much extra work on the initiating laboratory, but if the program is carefully laid out, a surprisingly small amount of work suffices.

We will suppose that as many as seven factors are selected for scrutiny. Perhaps the volume of solution is fixed at 100 and 110 ml; the time of waiting at some stage is tried at 30 and at 40 minutes. Different lots of reagent, slightly different concentrations, different times to bring solutions to boiling may also be tried. Now, if the procedure is "rugged" and therefore immune to modest (and inevitable) departures from some habitual routine, the results obtained should not be altered by these minor departures. If the results are altered, we should by all means know about it and warn the prospective user not to depart by more than some stated amount from the specified condition. Presumably most of these minor departures will show negligible effects, but if just one sensitive condition is spotted, we may save the very considerable effort that would have been expended in a disappointing collaborative test—particularly disappointing because it is all but impossible to track down the responsible conditions, since all the laboratories quite sincerely report that they followed the procedure.

What is needed is a scheme of attack that will conserve labor yet be sensitive enough to pick up fairly small effects if they should occur when some condition has been slightly altered. Negligible effects will be found for most changes. There is a program for making slight modifications in the procedure that has a very high efficiency in identifying those changes that do produce effects. The basic idea is not to study one alteration at a time but to introduce several changes at once, in such a manner that the effects of individual changes can be ascertained. Let A, B, C, D, E, F, and G denote the nominal values for seven different factors that might influence the result if their nominal values are slightly changed. Let their alternative values be denoted by the corresponding lower case letters a, b, c, d, e, f, and g. Now the conditions for running a determination will be completely specified by writing down these seven letters, each letter being either a capital or lower case. There are 2^7 or 128 different combinations that might be written out. Fortunately it is possible to

Table 1. Eight combinations of seven factors used to test the ruggedness of an analytical procedure

	Combination or Determination Number							
Factor Value	1	2	3	4	5	6	7	8
A or a	A	A	A	A	a	a	a	a
B or b	B	B	b	b	B	B	b	b
C or c	C	c	C	c	C	c	C	c
D or d	D	D	d	d	d	d	D	D
E or e	E	e	E	e	e	E	e	E
F or f	F	f	f	F	F	f	f	F
G or g	G	g	g	G	g	G	G	g
Observed result	s	t	u	v	w	x	y	z

CLASSIC PAPER: REPRINTED FROM YOUDEN, W.J. (1963) *J. ASSOC. OFF. AGRIC. CHEM.* **46**, 55–62

choose a subset of eight of these combinations that have an elegant balance between capital and lower case letters

The particular set of combinations is shown in Table 1. The table specifies the values for the seven factors to be used while running eight determinations. The results for the analyses are designated by the letters *s* through *z*. Let us see how to extricate the separate effects of the factor changes, even though four factors are always altered from the initial combination of all capitals. To find whether changing factor *A* to *a* had an effect, we compare the average $(s + t + u + v)/4$ with the average $(w + x + y + z)/4$. The table shows that determinations 1, 2, 3, and 4 were run with the factor at level *A* and determinations 5, 6, 7, and 8 with the factor at level *a*. Observe that this partition gives two groups of four determinations and that each group contains the other six factors twice at the capital level and twice at the lower case level. The effects of these factors, if present, consequently cancel out, leaving only the effect of changing *A* to *a*.

Inspection of Table 1 shows that whenever the eight determinations are split into two groups of four on the basis of one of the letters, all the other factors cancel out within each group. Every one of the factors is evaluated by all eight determinations. The effect of altering *G* to *g*, for example, is examined by comparing the average $(s + v + x + y)/4$ with the average of $(t + u + w + z)/4$. Suppose only six factors are explored. In that event, associate with *g* some meaningless operation such as solemnly picking up the beaker, looking at it intently, and setting it down again. Omit this meaningless operation for the determinations that involve *G*. (Be sure to look at the average difference between the G's and g's, because if they are large an explanation should be sought!)

Collect the seven differences for A − a, B − b, ... , G − g, and list them in order of size. If one or two factors are having an effect, their differences will be substantially larger than the group of differences associated with the other factors. Indeed, this ranking is a direct guide to the procedure's sensitivity to modest alterations in the factors. Obviously a useful procedure should not be affected by changes that will almost certainly be encountered between laboratories. If there is no outstanding difference, the most realistic measure of the analytical error is given by the seven differences obtained from the averages for capitals minus the averages for corresponding lower-case letters. Denote these seven differences by D_a, D_b, . . . , D_g. To estimate the standard deviation, square the differences and take the square root of 2/7 the sum of their squares. This estimate of the analytical error is realistic in that the sort of variation in operating conditions that will be encountered among several laboratories has been purposely created within the initiating laboratory. If the standard deviation so found is unsatisfactorily large, it is a foregone conclusion that the collaborative test will also give disappointing results. The collaborative test should never be undertaken until a procedure has been subjected to the abuse described above and satisfactory results obtained in spite of the abuse.

The schedule shown in Table 1 can be modified in various ways. An interesting variant is to replace the capitals with lower-case letters and vice versa. This creates eight new combinations. If all sixteen combinations are tried, smaller effects will be detected as well as possible mutual interferences of the factors. At this point a statistician will likely be of considerable assistance. There will be some who may see in this scheme a means of studying a procedure in its formative stage. Generally this is inadvisable, because substantial changes in the factors seldom act independently and a more complex schedule of factor values is appropriate. There are also schedules for eleven and fifteen factors which may be found useful (2–5).

If only those procedures that survive this planned introduction of minor modifications in the procedure were submitted to a collaborative test, then the latter would really take on the role of confirming that a good procedure has in fact been devised. Much disappointment would be avoided and sources of difficulty would be tracked down by this planned work within one laboratory. It should not be necessary to involve several laboratories in

CLASSIC PAPER: REPRINTED FROM YOUDEN, W.J. (1963) *J. ASSOC. OFF. AGRIC. CHEM.* **46**, 55-62

Table 2. Tabulation of results

	Material Number						
Laboratory No.	A	B	C	D	—	—	M
1	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
n	—	—	—	—	—	—	—

order to discover serious shortcomings in a procedure. Fewer collaborative tests would be needed and participation would be encouraged because the chance of a successful outcome would be very high.

The Interpretation of the Data

After the cooperating laboratories have made their reports, the results may be tabulated as shown in Table 2. Generally one would hope for a table with about forty or more entries, and every effort should be made to avoid missing entries.

It is useful to consider Table 2 as a whole and try to place the table in one of four categories. The hoped-for category is that the standard deviation as calculated for each column in the above table is acceptably small.

If $x_1, x_2, \ldots, x_n$ are the results tabulated in a column for any one material, the estimate for the standard deviation for that column is given by

$$s = \sqrt{(\Sigma x^2 - n\bar{x}^2)/(n-1)},$$

where $\bar{x}$ is the mean for the column. The standard deviation may, of course, vary with the amount present and it would be informative to prepare a graph plotting the standard deviation as ordinate against the amount present as abscissa. Some irregularity is to be expected, particularly if fewer than ten laboratories participate. A smooth curve should be drawn in with no attempt to follow the individual ups and downs. Values of the standard deviation read from this curve are very likely closer to the mark than the individual points. If the curve is approximately a straight line going near the origin, then the error is proportional to the amount present. Very often, in such an event, the error is expressed as per cent of the amount present and labeled the "Coefficient of Variation."

If the standard deviation when plotted against the amount present gives a series of points that show no trend, then the best fit is a horizontal line $Y = s^*$. That is, the standard deviation is the same over the range of amount present used in the work. The best value to use for s^* is not the average of the standard deviations found for the "M" columns. The squares of the standard deviations should be summed and divided by M, and the square root taken to get the best estimate of the standard deviation that will be appropriate for all the materials. This estimate of the standard deviation has $M(n-1)$ degrees of freedom. There should be at least 20 degrees of freedom to provide a reasonably good estimate of the standard deviation.

If the standard deviation as calculated for all, or most, of the columns is unacceptably large, the table of data may usually be classed in one of three categories. In order to determine the category, a convenient device is to prepare another table that better reveals certain features of the data, as follows: Scan the entries in the first column of Table 2 and assign the rank of 1 to the highest result, the rank of 2 to the next highest result and so on, until the rank of n is given to the lowest result in that column. Enter these ranks opposite the appropriate laboratories in the first column of the new table. If two laboratories are tied for fourth place, assign to each the rank of 4.5. If

CLASSIC PAPER: REPRINTED FROM YOUDEN, W.J. (1963) *J. ASSOC. OFF. AGRIC. CHEM.* **46**, 55-62

three are tied for second place, assign all three the rank of 3. This keeps the sum of the ranks, $n(n+1)/2$, the same for each column. Repeat this process for each column, and then sum the ranks assigned to the first laboratory and enter it as a laboratory score at the right of the row. Sum the ranks in each row. When the scores achieved by all the laboratories are added, the total should be $Mn(n+1)/2$, and this provides a convenient check on the work.

Should a laboratory turn in the highest result for each of "M" materials, its score would be M, the lowest possible. The highest possible score is nM and the average score is $M(n+1)/2$. The scores obtained by the n laboratories afford certain clues as to the reason why an unsatisfactory standard deviation was obtained from the reported results. The interpretation depends on the fact that for each combination of n laboratories and M materials, it is possible to compute a lower and an upper limiting score. Scores as low as or lower, or as large as or larger than these limiting scores are an indication of trouble. They mean that a laboratory with such an extreme score has a definite tendency to get persistently high or low results.

Now it is possible for the standard deviation to be unacceptably large and yet for no laboratory to turn up with an extremely low or high score. This would happen if the precision of the method is very poor. It may also happen if a laboratory tends to get high or low results for materials with low percentages and opposite results with materials of high percentages. If this happens with several laboratories, scores tend to cluster near the average score. Whatever the explanation, the evidence points to some defect in the procedure.

Another category arises when one or perhaps two laboratories have quite extreme scores. This laboratory (or both, if there are two) is the one chiefly responsible for the large standard deviations found for the individual columns. If the results from this laboratory are set aside, the standard deviation calculated by using the remaining laboratories may be acceptable. The basis for setting aside these results is that the limiting scores have been so chosen that only one collaborative test in twenty can be expected to include an extreme score by chance. An extreme score is, in consequence, a strong hint that the laboratory concerned has a pronounced bias, probably as a result of some deviation, unintentional or otherwise, from the procedure.

At this point it appears proper to query the laboratory with an extreme score to ascertain if the laboratory can offer any explanation for its results being consistently higher (or lower) than the results of the other participants.

In a very real sense a collaborative test reveals not only the performance of the procedure under test but also the performance of the laboratories doing the testing. The intent of this ranking device is to prevent a procedure from being unjustly rated poor when one or two laboratories are in fact responsible for

Table 3. Water-insoluble nitrogen results

	Results, %					Ranked Results					
Coll. No.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Coll. Score
7	4.59	1.46	5.64	2.19	27.32	9	5.5	6	4	3	27.5
8	4.94	1.52	5.68	2.28	26.44	1	1	3	2	10	17
9	4.80	1.40	5.62	2.12	26.89	3.5	8.5	7.5	6.5	8	34
10	4.73	1.46	5.65	2.09	27.17	5	5.5	5	8	4	27.5
11	4.72	1.51	5.62	2.12	27.00	6.5	2.5	7.5	6.5	6	29
12	4.80	1.51	5.80	3.29	27.48	3.5	2.5	1	1	1	9[a]
13	4.45	1.40	5.45	2.07	27.02	10	8.5	10	9	5	42.5
15	4.72	1.50	5.58	2.27	26.76	6.5	4	9	3	9	31.5
16	4.63	1.32	5.69	2.04	26.92	8	10	2	10	7	37
17	4.88	1.42	5.67	2.16	27.39	2	7	4	5	2	20

[a] Designates unusually low score.

CLASSIC PAPER: REPRINTED FROM YOUDEN, W.J. (1963) *J. ASSOC. OFF. AGRIC. CHEM.* **46**, 55-62

Table 4. Approximate 5% two-tail limits for ranking scores

No. of Labs.	Number of Materials												
	3	4	5	6	7	8	9	10	11	12	13	14	15
3		4	5	7	8	10	12	13	15	17	19	20	22
		12	15	17	20	22	24	27	29	31	33	36	38
4		4	6	8	10	12	14	16	18	20	22	24	26
		16	19	22	25	28	31	34	37	40	43	46	49
5		5	7	9	11	13	16	18	21	23	26	28	31
		19	23	27	31	35	38	42	45	49	52	56	59
6	3	5	7	10	12	15	18	21	23	26	29	32	35
	18	23	28	32	37	41	45	49	54	58	62	66	70
7	3	5	8	11	14	17	20	23	26	29	32	36	39
	21	27	32	37	42	47	52	57	62	67	72	76	81
8	3	6	9	12	15	18	22	25	29	32	36	39	43
	24	30	36	42	48	54	59	65	70	76	81	87	92
9	3	6	9	13	16	20	24	27	31	35	39	43	47
	27	34	41	47	54	60	66	73	79	85	91	97	103
10	4	7	10	14	17	21	26	30	34	38	43	47	51
	29	37	45	52	60	67	73	80	87	94	100	107	114
11	4	7	11	15	19	23	27	32	36	41	46	51	55
	32	41	49	57	65	73	81	88	96	103	110	117	125
12	4	7	11	15	20	24	29	34	39	44	49	54	59
	35	45	54	63	71	80	88	96	104	112	120	128	136
13	4	8	12	16	21	26	31	36	42	47	52	58	63
	38	48	58	68	77	86	95	104	112	121	130	138	147
14	4	8	12	17	22	27	33	38	44	50	56	61	67
	41	52	63	73	83	93	102	112	121	130	139	149	158
15	4	8	13	18	23	29	35	41	47	53	59	65	71
	44	56	67	78	89	99	109	119	129	139	149	159	169

the large scatter of the results.

Finally, the last category of unsatisfactory collaborative tests contains clearly unsatisfactory procedures. Sometimes the table of ranks shows little or no change in the assigned ranks as the eye moves from column to column in the table. In other words, a laboratory tends to hold its same rank for all materials. Usually there will be at least one very high and one very low score. What this tells is that each laboratory is doing the same thing very carefully every time. Some minor departure from a specified factor value, or even an arbitrarily chosen value for a factor because none was specified, is seriously influencing the analytical results. Obviously each laboratory is carefully following whatever routine it adopted. Now it is ridiculous to say that all the laboratories are inadequate. It makes better sense to conclude that there is a procedure so very vulnerable that it should never have been submitted to a collaborative test.

Illustrative Example of Ranking Technique

Table 3 shows a portion of a rather extensive collaborative test on nitrogen in fertilizers (6). The data for the water insoluble nitrogen are shown in the left half of Table 3 for ten of the participating laboratories. The right half of the table shows the ranks as-

CLASSIC PAPER: REPRINTED FROM YOUDEN, W.J. (1963) *J. ASSOC. OFF. AGRIC. CHEM.* **46**, 55–62

signed to the collaborators; the rank of one is given to the highest result and the rank of ten to the lowest result on each sample. It happens that the data are, in fact, averages of duplicates but this does not disturb the ranking technique. The result for Sample 4 by Collaborator 12 looks peculiar but even if the 3 is a misprint for 2 the ranking would not be altered.

The last column of the table shows the scores obtained for each collaborator by adding up the 5 ranks obtained with the 5 samples. The critical 5% probability scores for 10 laboratories and 5 samples are 10 and 45. Collaborator 12 runs persistently high and has a score of 9, which is in the critical region. The evidence indicates that Collaborator 12 has some individual manner of making the determination. Critical scores for as many as 15 collaborators and 15 samples are listed in Table 4 (5).

Discussion and Summary

This paper has considered several important aspects of collaborative test programs. The question of the distribution of the analytical effort is of prime importance. A broad basis for judgment requires enough laboratories and materials to be representative of the users and the materials likely to be submitted for analysis. In order to prevent unduly burdensome programs it is recommended that duplicates be eliminated and reliance placed on the initiating laboratory for information as to the precision of the procedure.

Another very important question concerns the need to make sure that the procedure is really ready for a collaborative test and that it will almost surely pass this final inspection. To that end an efficient and systematic way of disclosing possible weaknesses in the procedure has been presented in detail. The initiating laboratory should present evidence of the performance of the procedure when minor and seemingly inconsequential changes are made.

Finally a method has been described for evaluating unsatisfactory collaborative test results which should be valuable as a guide to determining the probable cause of the unsatisfactory results.

References

(1) Youden, W. J., *This Journal*, **45**, 169 (1962).
(2) Plackett, R. L., and Burman, J. P., *Biometrika*, **33**, 305 (1946).
(3) Yates, F., *Royal Statistical Society*, Supplement, **2**, 181 (1935).
(4) Youden, W. J., *Materials Research and Standards*, **1**, 862 (1961).
(5) Youden, W. J., *ibid.*, **2**, in press (1962).
(6) Davis, H. A., *This Journal*, **42**, 494 (1959).

ANTIBIOTICS IN FEEDS

Factors Influencing Optimization of Diffusion Assays for Antibiotics

MARIETTA SUE BRADY and STANLEY E. KATZ
Rutgers—The State University of New Jersey, Cook College, New Jersey Agricultural Experiment Station, Department of Biochemistry and Microbiology, New Brunswick, NJ 08903-0231

Agar nutrient content, cylinder charge volume, thickness (volume) of the agar layer, and incubation temperature were 4 factors varied to determine their effect(s) on the optimization of the cylinder-plate diffusion assay. Chlortetracycline was the pilot antibiotic and *Bacillus cereus* was used as the assay organism. Zones of inhibition were larger when the incubation temperature was lower than that which was commonly used and/or when the nutrient level was decreased; the zones were smaller when the incubation temperature was raised and/or when an increased nutrient level was used. The thickness (volume) of the assay layer played the most important role; the thinner the layer the less the effect the cylinder charge volume had on the zone diameter. The slopes of the response lines were minimally affected by cylinder charge volume. For a 7 mL assay layer per standard Petri plate, cylinder charge volumes ranging from 150 to 250 μL had little effect on zone diameter. The linearity of the response line was unaffected by assay layer thickness (volume), nutrient level, temperature of incubation, or cylinder charge volume. As long as the conditions for the assay were standardized, there were no discernible effects on recoveries or potencies.

Many of the official diffusion assays for antibiotics were developed between 20 and 40 years ago, before Youden (1) espoused the ruggedness principles. Accuracy, precision, and reproducibility of diffusion assays were relatively poor; the major consideration was to obtain reasonable linearity of the dose-response relationship so that a range of dilutions could be used for the estimation of the potency of the sample.

Kavanagh and Ragheb (2) summarized factors that influence accuracy and precision of diffusion assays, and suggested ways to minimize errors. Lees and Tootill (3) described 10 factors which affect zone size in plate assays. However, little consideration has been given to the interactions among these factors.

Cooper (4) stated that equilibrium conditions for diffusion through the agar exist as long as a reservoir of analyte remains in the cylinder after incubation. Ragheb (5) showed that variation in the charge volume of the cylinders could produce significant effects on the diameter of the zone of inhibition. He noted differences in apparent potency of as much as 50% for the same concentration using different charge volumes, even though a reservoir remained in the cylinder. Hasselberger (6) confirmed this effect.

Thus, a study of the interaction of 4 of the factors affecting zone diameter, slope of the response line, and recoveries has been made. Nutrient quality of media, thickness (volume) of assay agar layer, incubation temperature, and charge volume in cylinders were varied, and their interactions were examined to suggest a model to be used in the optimization of the cylinder-plate diffusion assay.

Received May 19, 1989. Accepted October 20, 1989.
Paper of the Journal Series, New Jersey Agricultural Experiment Station, Publication No. D-01112-02-89. Supported in part by state funds.

Experimental

Section numbers refer to *Official Methods of Analysis* (1990) 15th Ed. (7).

(a) *Antibiotic standard solution.*—Chlortetracycline, 100 μg/mL. Dissolve chlortetracycline hydrochloride standard in 10 mL 0.1N HCl; dilute to 100 mL with potassium phosphate buffer, pH 4.5. Solution is stable at least 2 weeks if stored at 4°C.

(b) *Dilution buffer.*—Phosphate buffer, pH 4.5, contains 13.6 g monopotassium phosphate, dissolved and diluted to 1 L with deionized water (**957.23B**).

(c) *Assay microorganism.*—Spores of *Bacillus cereus* ATCC 11778 are maintained in physiological saline at 4°C, after preparation as described by AOAC (**957.23D**).

(d) *Assay media.*—(*1*) Normal nutrient level: Dissolve 25.5 g Agar Medium D (**957.23C(d)**) in 1 L deionized water and boil 1 min with stirring. (*2*) 75% nutrient level: Dissolve 19.13 g Agar Medium D and 3.75 g plain agar in 1 L deionized water and prepare as above. (*3*) 125% nutrient level: Dissolve 25.5 g Agar Medium D, 1.5 g peptone gelysate, 0.75 g yeast extract, and 0.38 g beef extract in 1 L deionized water and prepare as above.

Cool each agar medium to 55–60°C before inoculating with 10 mL *B. cereus* spore suspension/L agar.

Preparation of Plates

After inoculated medium was mixed well, 7, 9, 12, or 15 mL agar medium was spread into standard (100 × 15 mm) Petri dishes. The medium in the plates was allowed to harden at room temperature, with lids ajar to facilitate escape of moisture.

Six cylinders were placed on each plate, using Shaw or equivalent dispenser, as described by AOAC (**957.23C(c)**).

Assay Design

The simplified plate diffusion design of Brady and Katz (8) was used, with 5 plates instead of 2. Cylinders were charged with 100, 150, 200, or 250 μL (±1%) of antibiotic standards using a Finn pipettor. The plates were incubated at 28, 30, or 32°C ± 0.1°C for 18–21 h.

Measurement of Potencies

Cylinders were removed and plates were washed gently with a stream of water to ensure the clearest measurement of the zones of inhibition. Diameters of the zones of inhibition were measured using a Fisher-Lilly zone reader. Potencies were determined using a calculator-generated least squares response line.

Experimental Design

The following 4 assay conditions were varied: Volume of analyte in the cylinders, thickness (volume) of the assay agar,

nutrient content of the assay agar, and incubation temperature.

Volume of analyte.—The 4 cylinder volumes used were 100, 150, 200, and 250 μL. On any individual plate, all cylinders were charged with the same volume of antibiotic standard.

Volume of assay agar.—Thickness of the agar was represented by the volume of agar spread in the plate. Volumes of 7, 9, 12, and 15 mL agar/plate were used.

Nutrient content of assay agar.—Agar Medium D was used at normal strength, at reduced strength (75% nutrient level), and at supplemented strength (125% nutrient level). In each case, the agar content of the medium was maintained at 1.5% to avoid altering the gelling properties of the media and diffusion of the antibiotic.

Incubation temperature.—The 3 incubation temperatures used for the plates were 28, 30, and 32°C ± 0.1°C. Incubation time was kept constant at 18–21 h.

For each combination of conditions, 5 plates constituted an assay. Each plate contained a standard curve of chlortetracycline at concentrations of 0.02, 0.04, 0.06, and 0.08 μg CTC/mL, and low and high "unknowns" of 0.03 and 0.06 μg CTC/mL. For each standard or unknown, the 5 zone diameter values were averaged, and the averages were used to calculate the standard response curve. Averages of the zone diameter values of the unknowns were used to determine percent recoveries.

Results and Discussion

The nutrient quality of the medium, volume (thickness) of the assay agar layer, incubation temperature, and charge volume in the cylinders were varied to determine their effects on the cylinder plate diffusion assay. To minimize any errors in the assay procedure, 5 plates per assay were used. Thus the effects of the variables studied were more easily discernible. Assay organism density was maintained as a constant. The inoculum was such that easily readable zones of inhibition were formed under the conditions of the study.

When each of the factors was varied and results were looked at separately, the effect on diameters of the zones of inhibition was predictable. Larger zones resulted from decreasing the nutrient level of the assay agar, the thickness of the agar layer, or the incubation temperature. Smaller zones resulted from increasing the nutrient level, agar thickness, or incubation temperature.

The most notable result was the interaction between the thickness of the assay agar layer and the charge volume in the cylinders. When the thinnest agar layer was used (7 mL/plate), the charge volume could be varied from 150 to 250 μL with little appreciable variation in zone diameters (Tables 1 and 2). Even with this thin layer, the 100 μL charge volume was insufficient to achieve consistency of response. The obvious deduction from this observation is that diffusion assay procedures that use assay layer volumes greater than 7 mL could be subject to greater variation of zone diameter for a given concentration of analyte if there is any inaccuracy or variation in charge volume. This is seen in the agar volumes of 12 or 15 mL, and seen to a lesser extent in the 9 mL assay volume, at normal nutrient levels.

At nutrient levels of 75 and 125% of normal, the minimal

Table 1. Average zone diameter (mm) as influenced by agar volume, nutrient level, and cylinder charge volume at 30°C incubation

Nutrient level:	100%				75%				125%			
CTC concn (μg/mL):	0.02	0.04	0.06	0.08	0.02	0.04	0.06	0.08	0.02	0.04	0.06	0.08
Agar volume (mL):	7				7				7			
μL/cylinder												
100	18.32	21.78	24.66	26.52	16.92	21.40	24.36	26.12	15.46	18.50	20.54	21.22
150	20.02	23.68	26.68	28.76	18.27	23.18	25.36	27.26	15.54	18.80	20.82	21.66
200	20.16	23.92	26.36	28.68	18.78	22.98	25.54	27.48	15.44	18.68	20.60	21.52
250	20.08	24.38	26.70	28.56	19.54	23.88	26.22	28.18	15.78	18.78	20.60	21.84
Agar volume (mL):	9				9				9			
μL/cylinder												
100	16.80	21.03	23.00	25.00	15.98	20.52	22.82	24.66	14.26	17.64	19.04	20.04
150	18.75	22.68	24.56	26.36	17.44	21.44	23.98	26.14	14.52	17.44	19.38	20.52
200	18.80	22.46	24.76	26.42	18.40	22.56	25.32	26.74	15.18	17.82	19.50	20.76
250	19.48	22.73	24.98	26.48	19.12	23.20	25.52	27.16	15.40	16.78	19.62	20.72
Agar volume (mL):	12				12				12			
μL/cylinder												
100	15.18	19.76	22.74	24.60	13.74	18.86	21.46	23.02	13.04	16.50	18.18	19.36
150	17.40	21.88	24.26	26.12	16.04	21.00	23.08	25.36	14.00	16.76	18.32	19.60
200	18.52	22.46	25.40	26.86	17.42	21.66	24.18	26.28	14.26	17.18	18.52	19.84
250	18.44	22.78	25.42	26.98	17.86	22.52	24.72	26.82	14.40	17.03	18.58	19.82
Agar volume (mL):	15				15				15			
μL/cylinder												
100	14.20	18.42	21.36	23.84	12.26	17.44	20.54	22.04	11.46	15.40	17.46	18.08
150	16.38	20.12	23.30	25.68	14.56	18.94	21.98	24.30	12.54	15.90	17.86	18.28
200	17.64	21.12	24.16	26.67	15.66	20.12	23.30	25.98	12.94	15.76	17.56	18.82
250	18.04	21.58	24.42	26.82	16.40	20.88	23.64	25.36	13.60	16.18	17.90	19.14

Table 2. Variation in zone diameter as a function of cylinder volume (150–250 μL) at 30°C as measured by coefficient of variation (%)

Nutrient level:	100%				75%				125%			
CTC concn (μg/mL):	0.02	0.04	0.06	0.08	0.02	0.04	0.06	0.08	0.02	0.04	0.06	0.08
Agar volume (mL)												
7	0.35	1.48	0.72	0.35	3.39	2.02	1.76	1.74	1.12	0.59	0.61	0.71
9	2.41	0.63	0.85	0.23	4.60	3.98	3.35	1.92	3.04	3.03	0.62	0.62
12	3.44	2.03	2.65	1.75	5.55	3.50	3.48	2.82	1.43	1.25	0.74	0.67
15	4.99	3.56	2.45	2.34	5.95	4.92	3.75	3.37	4.18	1.34	1.04	2.32

variation of zone diameter as a function of cylinder charge volume using a 7 mL assay agar volume is seen. These data suggest that at a given temperature of incubation, cylinder charge volumes of 200 μL or greater will have minimal influence on the size of the zone of inhibition, as long as the assay layer is as thin as possible. This result is not unexpected. The diffusion phenomenon consists of a horizontal diffusion vector and a vertical (downward) diffusion vector. The zone of inhibition is the resultant of these 2 vectors. As the assay agar thickness decreases, the downward diffusion diminishes and lateral diffusion predominates. When the agar layer is extremely thin, the charge volume in the cylinder should become inconsequential over a range of volumes. This was exactly what was noted. Assay agar volumes of 7 mL (9–12) and even 6 mL (13–16) have been used successfully.

Table 3 shows the relationship between the slope of the standard response curve, the charge volume in the cylinders, and the thickness (volume) and nutrient level of the agar layer. Increasing the charge volume in the cylinders shows a trend toward increasing the slope. Although there are trends toward decreasing the slope with increased agar nutrient levels and temperature of incubation, there are no dramatic changes in the slope of the assay response line when the charge volumes are 150 μL or greater. Any change in slope resulting from altering these factors is not enough to materially affect the sensitivity of the assay. Thus there is flexibility in the assay system without affecting the measurement of potency. Table 4 shows the recoveries of known concentrations at different thicknesses of the assay layer, temperatures of incubation, nutrient content, and charge volumes. As long as there was consistency in the assay conditions, there was very little effect on the ultimate measured potencies. This can be seen by comparison of average recoveries, when the cylinder charge volume is held constant while assay agar volume is varied, with recoveries when assay agar volume is held constant and cylinder charge volume is varied. The average recoveries and coefficients of variation are in close agreement (calculations not shown). The assay would be affected by these parameters only in the case of inability to read zone edges precisely or overlap of zones of inhibition.

Thus, the diffusion assay can be modified to meet special needs, rather than serving as an all-purpose assay that meets all uses somewhat less than adequately. For example, if fewer dilutions of an extract can be made by manipulating charge volume in the cylinders, nutrient content of the assay medium, or temperature of incubation, without altering conditions of similarity, these conditions should be manipulated. If a more sensitive response is necessary, such as is the case in

Table 3. Slopes of standard response curves as influenced by incubation temperature, agar volume, nutrient level, and cylinder charge volume

Incubation temp.:	28°C				30°C				32°C			
Charge (μL):	100	150	200	250	100	150	200	250	100	150	200	250
						Normal nutrient level						
Agar vol. (mL)												
7	16.1	13.4	13.4	13.5	13.6	14.5	13.9	12.8	11.2	10.8	10.2	11.0
9	15.3	13.8	14.4	14.6	13.4	12.5	12.6	12.1	12.4	9.8	11.6	10.8
12	16.9	15.6	13.7	14.3	15.7	14.4	14.1	14.3	13.3	12.6	11.2	11.9
15	16.0	16.3	15.2	14.3	15.7	15.4	14.9	14.4	16.4	13.7	11.6	13.1
						75% nutrient level						
Agar vol. (mL)												
7	13.4	13.3	12.5	10.8	15.4	14.9	14.4	14.2	12.9	13.0	11.9	12.5
9	14.7	13.4	12.2	12.0	14.4	14.3	14.1	13.4	13.8	12.9	12.2	12.6
12	15.6	15.4	14.4	13.9	15.5	15.2	14.6	14.7	14.6	13.6	12.8	12.8
15	17.0	15.5	14.8	13.4	16.5	16.1	16.9	15.0	15.2	14.0	13.4	12.8
						125% nutrient level						
Agar vol. (mL)												
7	11.9	11.6	10.9	11.1	9.8	10.5	10.3	10.0	10.2	9.8	9.1	8.7
9	12.0	11.2	11.9	11.4	9.5	10.1	9.3	8.8	11.9	10.3	10.2	9.2
12	12.6	11.8	11.4	11.1	10.5	9.2	9.1	9.0	11.9	10.4	9.5	9.4
15	13.2	12.9	12.7	11.3	11.4	9.9	9.8	9.1	11.2	10.6	10.2	8.8

Table 4. Recoveries (%) of standard solutions as influenced by incubation temperature, agar volume, nutrient level, and cylinder charge volume

Incubation temp.:	28°C					30°C					32°C				
Charge (μL):	100	150	200	250	(CV)	100	150	200	250	(CV)	100	150	200	250	(CV)
Normal nutrient level															
Agar volume (mL)															
7	103.6	102.5	100.3	98.9	2.09	100.0	98.9	98.0	98.6	0.85	101.7	100.9	100.0	101.8	0.82
9	98.9	100.1	99.1	102.2	1.51	104.2	99.8	97.6	98.6	2.90	103.1	104.9	102.6	102.5	1.08
12	104.1	103.1	103.6	103.1	0.46	99.3	99.6	100.2	103.6	1.97	103.9	98.3	97.0	100.1	3.00
15	100.9	98.1	101.0	100.2	1.34	98.6	99.1	100.3	97.7	1.10	102.6	98.0	99.1	104.0	2.81
CV, %	2.38	2.32	1.89	1.88		2.50	0.42	0.98	2.33		0.89	3.10	3.17	1.59	
75% nutrient level															
Agar volume (mL)															
7	97.1	99.4	96.4	100.3	1.88	100.1	99.2	100.2	101.6	0.99	99.8	104.5	99.2	101.2	2.34
9	98.6	102.9	99.3	102.4	2.14	99.1	97.8	100.5	98.5	1.16	100.9	98.8	102.8	98.7	1.94
12	104.1	99.4	99.9	99.8	0.88	100.3	100.2	98.1	99.1	1.04	102.8	100.5	99.0	99.5	1.68
15	98.5	99.3	100.9	98.9	1.05	102.7	100.7	98.9	101.1	1.54	96.2	98.8	100.5	97.2	1.92
CV, %	1.82	1.76	1.95	1.48		1.52	1.29	1.13	1.51		2.79	2.67	1.74	1.68	
125% nutrient level															
Agar volume (mL)															
7	100.1	98.6	102.2	99.4	1.54	99.0	98.8	101.0	98.6	1.12	102.0	103.7	100.9	104.9	1.72
9	100.6	96.4	101.2	98.1	2.25	103.8	101.9	99.9	98.4	2.33	102.0	103.7	103.2	102.6	0.71
12	100.9	100.3	101.4	98.1	1.45	103.1	103.4	99.5	99.2	2.22	105.7	106.0	100.9	103.2	2.29
15	103.7	104.9	106.4	99.1	3.04	103.1	103.5	102.0	101.8	0.57	100.7	103.1	105.5	107.7	2.90
CV, %	1.60	3.60	2.37	0.68		2.14	2.15	1.12	1.58		2.10	1.26	2.14	2.18	

residue analysis, the thinnest assay layer with the greatest cylinder charge volume at the lowest possible incubation temperature is certainly warranted. Similarly, if only small volumes of analyte are available, the analytical system can be adjusted to handle the situation. Tables 3 and 4 show that cylinder charge volumes as low as 100 μL give slopes that are very similar to those where charge volumes are 150 μL or greater, without impact on the final determination of potency. Since there is no impact on the final analytical result, such flexibility should be encouraged.

The diffusion assay offers a great deal of flexibility and adaptability to meet specific needs and should be utilized to its full potential. As long as the variables of the assay are consistent within the assay itself, there is no inherent loss of sensitivity, accuracy, or precision of the assay.

References

(1) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA

(2) Kavanagh, F., & Ragheb, H. S. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 943–950

(3) Lees, K. A., & Tootill, J. P. R. (1955) *Analyst* **80,** 95–111

(4) Cooper, K. E. (1972) in *Analytical Microbiology*, Vol. II, F. W. Kavanagh (Ed.), Academic Press, Inc., New York, NY, p. 14

(5) Ragheb, H. S. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 1071–1074

(6) Hasselberger, M. L. (1988) 102nd AOAC Annual International Meeting, Palm Beach, FL, August 29–September 1, 1988. Abstract No. 219

(7) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, secs **957.23B–D**

(8) Brady, M. S., & Katz, S. E. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 641–646

(9) Katz, J. M., & Katz, S. E. (1983) *J. Assoc. Off. Anal. Chem.* **66,** 635–639

(10) Katz, J. M., & Katz, S. E. (1983) *J. Assoc. Off. Anal. Chem.* **66,** 640–645

(11) Katz, J. M., & Katz, S. E. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 576–579

(12) Singer, C. J., & Katz, S. E. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 1037–1041

(13) Katz, S. E., & Fassbender, C. A. (1970) *J. Assoc. Off. Anal. Chem.* **53,** 968–972

(14) Katz, S. E., & Fassbender, C. A. (1972) *Bull. Environ. Contam. Toxicol.* **7,** 229–236

(15) Inglis, J. M., & Katz, S. E. (1978) *Appl. Environ. Microbiol.* **35,** 517–520

(16) Harpster, C. P., & Katz, S. E. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 1144–1148

COSMETICS

Methodology for Evaluation of Compatibilities of Cosmetic Perfumes and Plastic Containers

MYRIAM TAVERNA, ARLETTE E. BAILLET, and DANIELLE BAYLOCQ
Centre d'Etudes Pharmaceutiques, Laboratoire de Chimie Analytique, rue J. B. Clément, 92290 Chatenay-Malabry, France

The study was designed to investigate compatibility of plastic containers and perfumes. First, a gas chromatographic/mass spectrometric (GC/MS) system was developed for identification of fragrance components. Then a quantitative extraction and GC analysis was developed. Honeysuckle fragrance was added to a cosmetic emulsion at 0.5 mg/100 mg level. The cosmetic was stored for 2 months at 37°C in 6 different plastic containers and in a glass bottle as a test control. The cosmetic was extracted with dichloromethane and analyzed by GC. The results obtained after storage were compared with the original analysis and showed an important loss of some perfume components stored in different plastic containers. Sorption of these components was then studied by extracting components fixed by the plastic; some of the physicochemical factors involved in such interactions are identified.

Plastics are used extensively for packaging cosmetic products. The package has dual purposes—it must be both functional and attractive. The advantages of plastics for these purposes are numerous, especially their ability to produce attractive packs.

The basic interactions that can occur between the package and contents are migration of components of packages into contents, loss of ingredients of the cosmetic either into or through the package by adsorption or permeability, and atmospheric permeation. With the advent of plastic packaging, the problem of fragrance preservation has become more acute. Some papers have mentioned incompatibilities between some perfumes or essentials oils and plastics (1–3). In particular, the loss of perfume due to sorption by plastic may be important (3, 4); sorption loss can change the character and the intensity of a complex aroma (5). In fact, the aroma of the packaged product can be changed by preferential permeation of one or several components of the odorant vapor through the plastic film (6).

The aim of the present study was to develop an analytical method to quantitatively measure loss of fragrance components. Most researchers in this field use a sensory evaluation (5) or the permeation cell as methods to evaluate odor barrier properties of packaging films (6, 7). The proposed method uses an authentic model under the conditions occurring in practice. The perfume was added to an emulsion at low concentration. Six plastic containers, including those most used in the cosmetic market, were filled with the cosmetic. Combined gas chromatography/mass spectrometry was used to identify the volatile fragrance components.

After 2 months of storage in different plastics, the fragrance was extracted from the cosmetic product with dichloromethane and quantitatively analyzed by GC using internal (menthol) and external standardization. Reproducibility of the extraction was studied. We needed an extraction method that was not selective for only a few components of the perfume but was able to remove all the components without modifying the relative level of each.

Sorption of fragrance was also studied to determine some of the physicochemical factors involved in such interactions.

Received May 8, 1989. Accepted August 8, 1989.

Experimental

Reagents

(a) *Perfume.*—Dilution of honeysuckle fragrance (discharges and aromatic diffusions) was prepared at 10% in dichloromethane. Working solutions were prepared at concentrations of 0.5, 1.0, 2.0, and 4.0 g/100 mL.

(b) *Plastic containers.*—Polyamide (Selar PA 3426), polyethylene high density (H-700), styrene-acrylonitrile (Tyril 867E), polyvinylchloride (Dorlyl), polyethylene terephthalate (Arnite A06 100), and polypropylene (Hostalen PPU 1789).

(c) *Standard solutions.*—0.1 g of each perfume component and internal standard (menthol) was accurately weighed into a 100 mL volumetric flask, diluted to volume with dichloromethane, and mixed.

(d) *Internal standard solution.*—Menthol in dichloromethane at a concentration of 1 g/100 mL.

Apparatus

(a) *Gas chromatograph.*—Perkin Elmer, Model Sigma 3B equipped with flame ionization detector (FID) and 25 m × 0.22 mm id Carbowax 20 M capillary column (film thickness 0.25 μm). Peak areas were determined by electronic integration (Varian Model LCI 100). Operating conditions: nitrogen carrier gas, flow rate 0.4 mL/min, head pressure 14 psi. Split mode was used. Capillary column was programmed from 70 to 220°C at 3°/min with initial and final hold times of 5 and 10 min, respectively. Temperature of injector and detector was 250°C.

(b) *Gas chromatograph/mass spectrometer.*—Nermag R 10 10 C GC/MS interfaced to a Sidar data system. The R 10 10 C was working in the electron impact mode (70 eV) with a quadrupole filter (mass range 4–1000 amu). Capillary column of Carbowax 20 M, 50 m × 0.32 mm id, was used for gas chromatographic analysis. Column was programmed from 70 (5 min) to 220°C (10 min) at 3°/min. Nitrogen carrier gas, flow rate 2 mL/min. The split ratio was 1:60. Injector and interface temperatures were 250°C and 270°C, respectively.

Cosmetics Packaging

Perfume (0.5 g/100 g) was added to the final oil in water emulsion (which did not contain any perfume) with a Rayneri-type mixer. The emulsion contained 70% water and nonionic surfactants. The cosmetic was then packed in 6 differ-

ent plastic containers and in a glass bottle used as control. The filled products were stored at 37°C and ambient humidity in a Vismara-type oven for 2 months as described by Cannell (8).

Permeability Test

Periodic weight measurements of the cosmetic product were made during the storage, as a crude measure of the compatibility between the product and its different containers. Before weighing, containers were equilibrated to room temperature for 3 h.

Sample Extraction

After 2 months, the quantitative and qualitative composition of the perfume in the emulsions was analyzed by gas chromatography after liquid extraction as follows: A 2 g sample of cosmetic product was weighed into a glass tube, 1 mL internal standard solution was added, and the volume was diluted to 10 mL with dichloromethane. The mixture was heated 15 min in a 40°C water bath to break the emulsion, and stirred using a Vortex Ika Vibro-Fix followed by ultrasonic treatment.

The tube and contents were then frozen for 30 min, the supernate that contained fat excipients was removed, and the solution was analyzed by gas chromatography by injecting 2 μL.

Desorption of Perfumes from Plastics

A method for extracting perfume components from plastics was tested, and the most efficient extraction solvent was determined. Plastic containers were washed with water, dried, weighed, cut into small pieces (about 1 × 1 cm), and transferred to 100 mL glass-stopper flask. Twenty mL methanol was added, and each flask was placed in an ultrasonic bath for 30 min. Exactly 2 mL internal standard solution was added, and 2 μL aliquot of the mixture was injected into the gas chromatograph.

The methanol extraction was repeated until no more perfume was found in the extract solution.

Validation of Perfume Extraction from Cosmetic Emulsion

This extraction was carried out 6 consecutive times to study the possible variation in relative composition of the perfume components before and after extraction. The amount of each component of the perfume was determined by GC analysis and the resultant data were compared to those obtained from the same components after extraction of a perfume added in an emulsion at a known concentration (0.5 g/100 g).

Statistical tests were used to compare means obtained from the 2 samples.

The repeatability of the extraction method was also studied.

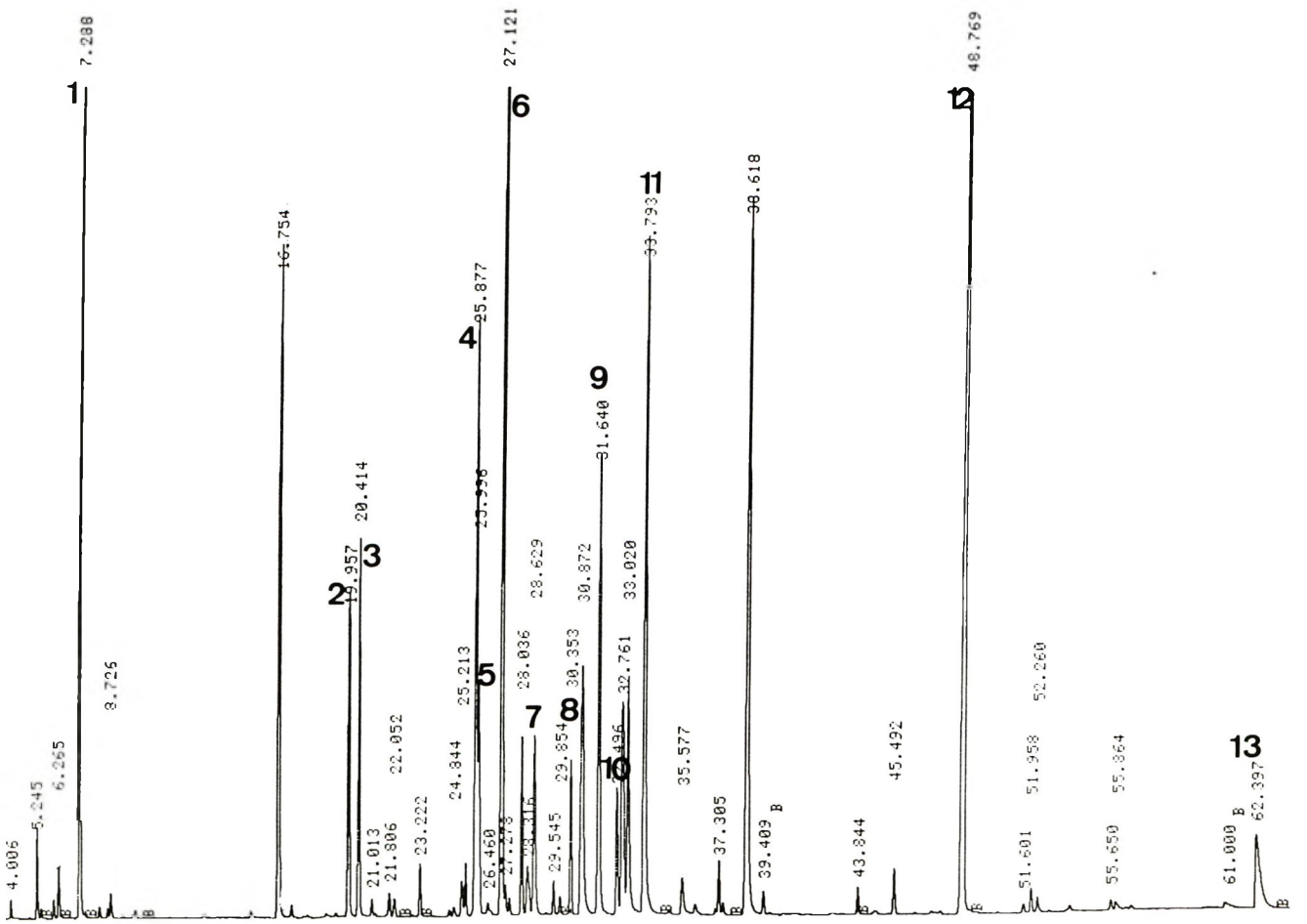


Figure 1. Chromatogram of honeysuckle perfume on capillary column of Carbowax 20M, 25 m × 0.22 id. Peak identification: 1, limonene; 2, linalool; 3, linalyl acetate; 4, α-terpineol; 5, terpinyl acetate; 6, benzyl acetate; 7, citronellol; 8,phenyl ethyl acetate; 9, geraniol; 10, benzyl alcohol; 11, phenyl ethyl alcohol; 12, hexyl cinnamic aldehyde; 13, benzyl salicylate.

Table 1. Concentration of some perfume components in a 1% perfume solution

Component	Mean[a] ± SD, mg/100 mL	RSD, %
Limonene	32.6 ± 2.6	8.0
Linalool	21.6 ± 0.4	1.8
Linalyl acetate	24.8 ± 0.8	3.2
α-Terpineol	37.1 ± 0.7	2.0
Benzyl acetate	98.6 ± 3.2	3.2
Citronellol	17.6 ± 0.6	3.6
Geraniol	39.6 ± 2.2	5.7
Benzyl alcohol	11.1 ± 0.8	7.3
Phenyl ethyl alcohol	58.1 ± 0.7	1.2
Hexyl cinnamic aldehyde	101.8 ± 5.6	5.5

[a] N = 6.

Table 2. Comparison of the level of perfume components extracted and in the perfume itself

	Recovered perfume[a]		Standard perfume	
Component	Mean ± SD[b]	RSD, %	Mean ± SD[b]	RSD, %
Limonene	11.72 ± 0.95	8.1	11.95 ± 0.96	8.0
Linalool	8.02 ± 0.39	4.8	7.92 ± 0.12	1.5
Linalyl acetate	9.55 ± 0.79	8.3	9.08 ± 0.25	2.7
α-Terpineol	14.05 ± 0.90	6.4	14.51 ± 0.40	3.0
Benzyl acetate	36.10 ± 0.85	2.4	36.15 ± 0.80	2.2
Phenyl ethyl alcohol	20.50 ± 1.36	6.6	21.30 ± 0.34	1.6

[a] Results are means of 5 separate extractions of the perfume added at a known concentration in the cosmetic.
[b] N = 5.

Determination

Peak area of each component and peak area of menthol standard solution were measured. Peak area of perfume components and peak area of menthol extracted from the cosmetic fragrance were measured. Concentration of each component in the fragrance (C_i, g/100 mL of perfume solution) was calculated:

$$C_i = (R_1/R_2) \times C_s$$

where R_1 and R_2 = ratios of peak area of component i to peak area of the internal standard for sample and standard preparation, respectively; and C_s = concentration of component in standard solution (g/100 mL).

Calculate relative percent of each component as follows:

$$C_i\% = C_i/\sum C_n$$

where $\sum C_n$ = sum of concentration of all components.

Quantitative analysis of perfume components in cosmetic preparation.—Calculate concentration of each component in cosmetic product (C_i', g/100 g cream):

$$C_i' = (R_1/R_2) \times C_s \times (V'/W') \times 100$$

where W′ = g sample of cosmetic product taken for extraction (2 g average); and V′ = mL sample extract (10.0 mL).

Calculate relative percent of each component in the cream as follows:

$$C_i'\% = C_i'/\sum C_n'$$

Sorption of perfume components by plastic containers.—Calculate concentration of perfume components sorbed by plastic (C_i'', g/100 g cream)

$$C_i'' = (R_1/R_2) \times C_s \times (V''/W'') \times 100$$

where V″ = mL sample extract (20 mL); and W″ = g cosmetic product stored in plastic container taken for analyses.

Calculate percentage of sorption for each component:

$$P = (C_i''/C_{ig}') \times 100$$

where C_{ig}' = concentration of component i in cream stored in glass.

Results and Discussion

The honeysuckle fragrance was analyzed by GC/MS. A mixture of fragrance (1 g/100 mL in methanol) and 0.1% internal standard (menthol) was analyzed by GC. A typical chromatogram is shown in Figure 1. Thirteen constituents of the perfume were identified; 6 were selected for the interaction studies (limonene, linalool, linalyl acetate, α-terpineol, benzyl acetate, and phenyl ethyl alcohol). Calibration graphs for the perfume components were constructed from 3 consecutive injections of the fragrance and were linear over the range of concentration used with regression coefficients ranging from 0.9994 to 0.9998 according to the component.

The fragrance solution was then quantitatively analyzed 6 times. The selected components were determined by using external and internal standards. The concentration was calculated and the repeatability coefficient of variation was determined (Table 1).

The repeatability of the assay was satisfactory; the coefficient of variation was 8% or less.

Validation of Extraction Method

To determine the relative percent of the components after extraction, 6 extractions were done on the same cosmetic product containing perfume at a known concentration and then compared to the perfume itself. A Shapiro Wilk test showed the distribution to be normal. A 2-sample comparison test using a Kruskall Wallis test showed the results to be insignificantly different (Table 2).

Weight Variation on Storage

The variation of the cosmetic weight was easily determined by periodic measurements during storage. A loss in weight of the cosmetic product was observed with each container except glass (Figure 2).

The loss in weight is generally the first test used to screen the suitability of plastics for a cosmetic, and depends on the water permeability of the container. Cosmetic products containing water packed in styrene-acrylonitrile (SAN) have lost up to 40% in weight after storage of 2 months at 37°C, probably due to loss of water (Figure 2). In fact, the dry appearance of the cream (which contained about 70% water) after 2 months corresponded to the loss in weight determined above.

The effect of the nature of plastics on permeability is well known (9). Thus, the copolymer styrene–acrylonitrile, which is a polar polymer, is the most permeable to water. On the other hand, cosmetics packed in high density polyethylene (PEHD) containers lost less than 3% weight. Crystallinity of the polymer seems to be involved in such phenomenon. Polyamide (PA) and styrene-acrylonitrile, which are both hydrophilic polymers, do not have the same barrier properties to water vapor. SAN, which is a highly branched copolymer,

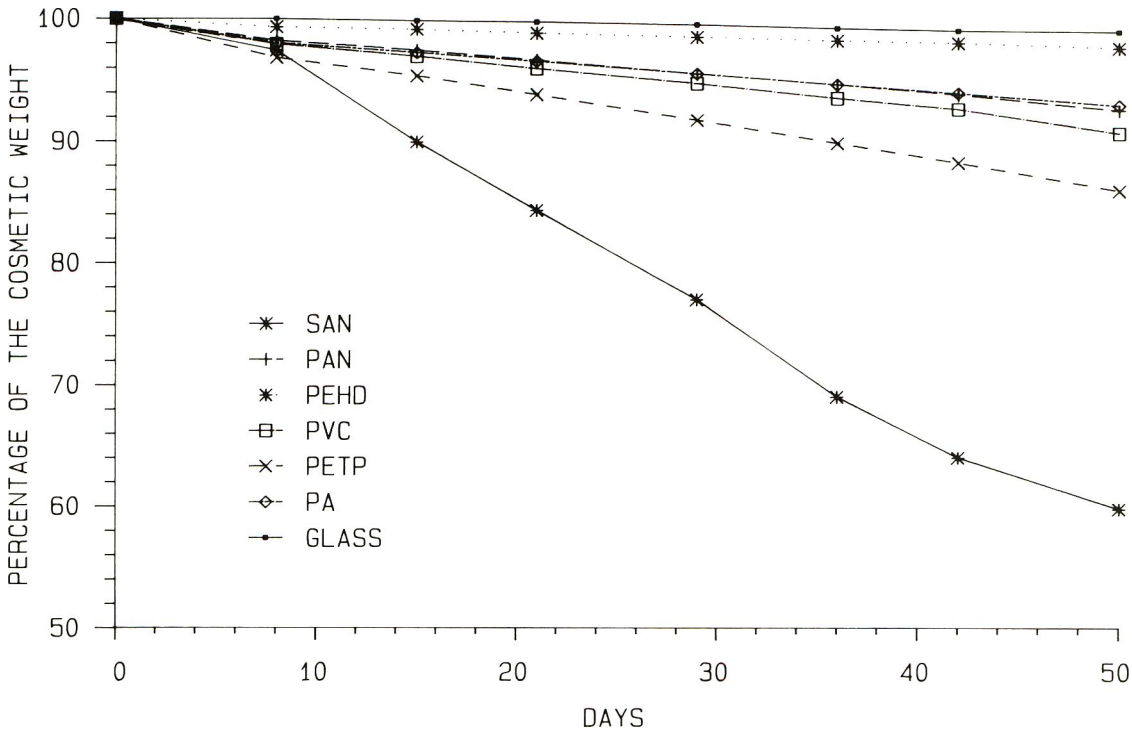


Figure 2. Variation in weight of cosmetic during 2 months of storage in various plastic containers and in glass: 1, glass; 2, PEHD; 3, PVC; 4, PA; 5, PAN; 6, PETP; 7, SAN.

has a low degree of crystallinity and is more permeable than PA to water.

Perfume Contents of Cosmetic after Storage

Perfume was extracted from cosmetics packed in different plastic containers and in a glass after 2 months. The concentration of selected components was determined and corrected according to the loss in weight previously observed, because the loss in weight would greatly affect the results (Table 3). Percent loss of each component was calculated with respect to the control. If the difference between control and sample was less than the RSD previously determined for each component (Table 2), the variation was not considered significant. Figure 3 illustrates the loss of perfume components from cream stored in different plastics.

A decrease of the level of each component was observed with PEHD and PVC and for every component except limonene for PA and PAN. This decrease was not the same for each perfume component. Barrier properties of plastic containers depend on the nature of the diffusing solute. Although some linalool was lost after storage in the polyamide container, no decrease in the limonene level was observed. PEHD was 3 times more permeable to benzyl than to linalyl acetate. Conversely, the significant increase in the concentration of limonene and linalyl acetate with SAN and of limonene, linalool, linalyl acetate, and terpineol with PETP was due to a concentration of these components not detected by weighing. According to the hydrophilic nature of these 2 plastic containers (9), we can suppose that some water from the cream was absorbed by the plastic container and led to a concentration of the constituents in the cosmetic product.

Component loss from the perfume is shown to be dependent on the film material. Generally, the loss is greater for cosmetics packed in PEHD containers. PEHD has a lower barrier performance compared to the other plastics, especially for benzyl acetate. Approximately 60% benzyl acetate was lost from the cosmetic packed in PEHD.

Perfume can be lost by sorption, permeation, or evaporation around a loose cap. Analysis performed on the plastic itself, after desorption, took into account only the sorption phenomenon.

Sorption of Perfume by Plastics

Desorption of perfumes sorbed by plastics as describe above showed that a migration of some perfume components took place (Table 4). But all components lost were not recovered in the plastic, and the absorptive process remained a minor phenomenon.

A similarity in structure and functional grouping between the diffusing molecule and the plastic seems to increase the

Table 3. Concentration of perfume components after 2 months of storage in various plastic containers and in glass (mg/1000 g cosmetic)

Component	Glass	PEHD	PA	PVC	PETP	PAN	SAN
Limonene	160.9	94.9	165.0	158.2	226.7	166.4	186.9
Linalool	151.7	109.1	131.4	133.2	192.1	129.1	143.6
Linalyl acetate	120.7	104.0	118.9	119.6	159.3	100.2	131.0
α-Terpineol	242.8	182.3	207.2	223.0	267.4	204.0	248.9
Benzyl acetate	515.3	172.4	402.8	425.5	524.5	402.8	482.8
Phenyl ethyl alcohol	371.9	250.1	265.1	293.3	362.9	265.4	298.1

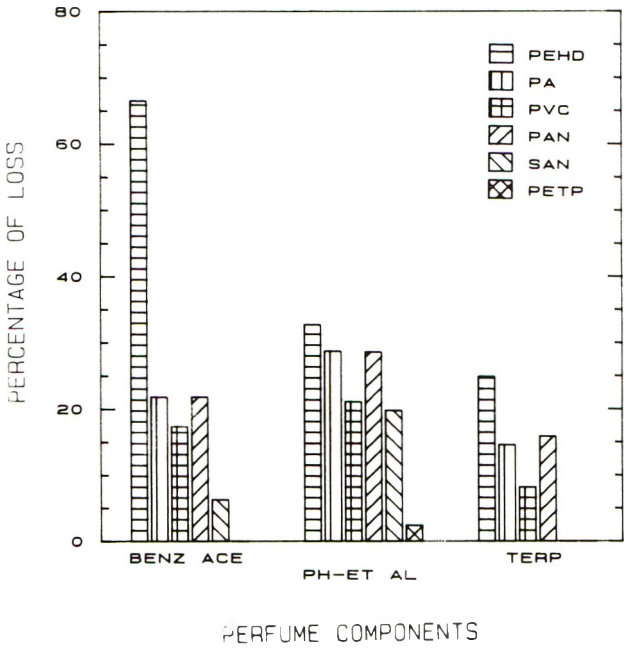


Figure 3a. Percentage loss of limonene, linalool, and linalyl acetate after 2 months of storage at 37°C in various plastic containers.

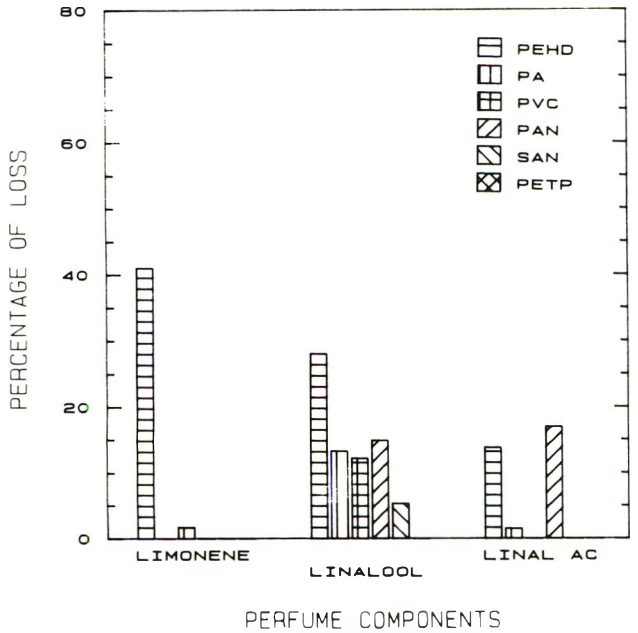


Figure 3b. Percentage loss of benzyl acetate, phenyl ethyl alcohol, and α-terpineol after 2 months of storage at 37°C in various plastic containers.

sorption phenomenon. Nylon has few polar sites and some compounds such as esters and alcohols (phenyl ethyl alcohol, benzyl acetate) can interact with these sites by hydrogen bonding. Limonene, which is a nonpolar compound, has no affinity for this plastic phase. These data were already supported by Eric (10) who studied the main parameters involved in chemical interactions between the plastic container and the product. Our results are consistent with Kwapong's

Table 4. Percentage of perfume components, originally present in the cream, sorbed by plastic containers after 2 months

Component	PEHD	PA	PVC	PETP	PAN	SAN
Limonene	6.5	0.0	0.9	0.0	0.0	6.9
Linalool	9.0	2.7	1.3	1.2	1.2	2.6
Linalyl acetate	2.4	1.6	2.1	0.0	2.7	0.0
α-Terpineol	2.8	3.6	0.0	0.0	0.5	0.0
Benzyl acetate	3.6	2.7	0.6	0.0	0.6	1.2
Phenyl ethyl alcohol	3.4	7.5	0.6	0.0	0.4	1.7

observations (5), which showed that a similarity of dipole moment between molecules and plastics increases diffusion through the polymer. PA has a greater affinity for components with a high dipole moment such as phenyl ethyl alcohol.

The proportion of crystalline zones in a polymer is an important parameter in determining the extent of sorption. Thus, a tight polymeric structure without amorphous zones is required for low permeability.

Conclusion

One of the objectives of this study was to develop a method for an objective evaluation of perfumes in cosmetic products that would accurately reflect the level of each component and eliminate the possibility of preferential loss of any component during the extraction procedure. We showed the validity of our method.

Then we proposed to determine the concentration of some components of the perfume after 2 months of storage in different plastics in order to investigate if an olfactory variation could occur under accelerated conditions.

The qualitative comparison between different molecular plastic systems showed the complexity of the physicochemical factors involved in interaction phenomenon. Some of these parameters were discussed. This procedure, using accelerated conditions of storage, can be very useful in the choice of suitable plastics for packaging of perfumed cosmetics. It is possible to predict the behavior of a perfume in a cosmetic stored in a given plastic container according to the perfume components, the nature of the plastic, and the type of cosmetic formulation.

References

(1) Introini, C. (1981) *Cosmet. Toiletr.* **96,** 37–53
(2) Nowak, G. A. (1977) *Parfüm. Kosmet.* **58** (4), 95–98
(3) Monte, W. C., & Landau-West, D. (1982) *J. Food Sci.* **47,** 1832–1835
(4) Becker, K., Koszinowski, J., & Piringer, O. (1987) *Parfüm. Kosmet.* **68** (5), 268–278
(5) Kwapong, G. O. Y., & Hotchkiss, J. H. (1987) *J. Food Sci.* **52,** 761–783
(6) Gilbert, S. G., et al. (1983) in *Instrumental Analysis of Foods*, Vol. 1, G. Charalambous & G. Inglit (Eds), Academic Press, New York, NY, p. 405
(7) Hatzidimitriu, E., Gilbert, S. G., & Loukakis, G. (1987) *J. Food Sci.* **52,** 472–474
(8) Cannell, J. S. (1984) presented at the Society of Cosmetic Chemists Symposium "Product Stability," Coventry, UK
(9) Botrel, J. (1982) in *Les Polymers, Chimie et Reglementation des Emballages*, Masson (Ed.), Paris, France
(10) Eric, B. (1972) *Am. Cosmet. Perfum.* **87** (7), 27–38

DECOMPOSITION

Outgrowth of Naturally Occurring *Clostridium botulinum* in Vacuum-Packaged Fresh Fish

TIMOTHY LILLY, JR, and DONALD A. KAUTTER
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

A total of 1074 test samples of commercial, domestic, vacuum-packaged fresh fish were studied to determine whether spoilage occurs before the products become toxic from naturally occurring *Clostridium botulinum* spores. The products were incubated for 12 days at 12°C (mild abuse). After incubation, they were tested for botulinal toxin and evaluated for organoleptic acceptability. Even when only marginally acceptable to laboratory personnel, none of the 1074 test samples were positive for *C. botulinum* toxin. Thus, the fish either contained no *C. botulinum* spores, or the spores were unable to grow out and produce toxin before spoilage made the product marginally unacceptable.

Temperature abuse of commercial vacuum-packaged or modified atmosphere-packaged fresh fish fillets can result in the rapid growth of *Clostridium botulinum* type E spores during storage (1-4). Because these organisms are nonproteolytic and can grow and produce toxin at very low temperatures (3.3°C), a *C. botulinum*-contaminated product may be toxic and still remain organoleptically acceptable with no noticeable signs of quality reduction or evidence of spoilage (5). This fact has caused concern about the possibility of growth of *C. botulinum* type E in fresh fish commercially vacuum-packaged or packaged in a modified atmosphere.

Earlier studies (6-9) have involved packages of fish fillets inoculated with spores of *C. botulinum* type E and held at different abusive temperatures. These studies indicated that under temperature abuse, most products packaged under vacuum or in a modified atmosphere became toxic before they became organoleptically unacceptable. Modified atmospheres alone do not provide the safety required for extended storage of fish with respect to bacterial outgrowth and toxin production by *C. botulinum* type E spores (6-9).

Although most consumers would reject a marginally acceptable fish product, i.e., one that is beginning to "turn," others might consume it despite its slight off-odor or loss of firm texture. Our objective, therefore, was to determine whether commercial vacuum-packaged fresh fish would become toxic from naturally occurring *C. botulinum* spores by the time of this "turning" point, and if toxicity would occur before spoilage made the product marginally unacceptable.

Experimental

Every other month for about 10 months, we received 100 vacuum-packaged fresh frozen raw fish that had been commercially produced throughout the United States and collected by Food and Drug Administration inspectors. Some packages contained individually wrapped, vacuum-packaged fish; others contained 10 fillets or steaks vacuum-packaged together in a plastic wrap. The frozen raw fresh fish products were stored in a 20°F (−6.7°C) walk-in refrigerator until analysis. Fish species and number of samples studied were pollock (50), haddock (40), cod (40), salmon (40), turbot (20), catfish (20), trout (10), ocean perch (50), flounder (40), cusk (40), hake (30), mahi (20), halibut (20), monk (30), dab (24), sole (30), pompano (20), tuna (20), blackfish (20), red snapper (50), tile bass (20), swordfish (20), shark (30), Gulf redfish (20), grouper (20), white fish (10), and farm-grown trout (340).

The vacuum-packaged fish were thawed and incubated for 12 days at 12°C (mild abuse) in a refrigerator. The temperature was monitored by a Model 615 continuous temperature recording thermometer (Kernco Instruments, Co., El Paso, TX). Vacuum-packaged fish fillets inoculated with a 0.1 mL suspension of approximately 35 *C. botulinum* type E spores/mL served as controls. In some instances, however, the packages were institutional-sized, each containing 10 or more vacuum-packaged fish. If the package had been inoculated, all 10 or more fish could have become toxic and the sample rendered useless for experimentation. In these instances, 2 tubes of inoculated trypticase-peptone-glucose-yeast extract broth served as controls.

After 12 days of incubation, acceptability of the fish was determined by sensory analysis. Laboratory personnel evaluated each fish product for appearance, odor, and texture and the results were recorded. After evaluation, each fish test sample was put into a Waring blender along with just enough gel phosphate buffer to blend into a slurry. After blending, the samples were centrifuged in the cold, trypsinized, and injected intraperitoneally into each of 2 mice. If the extracts were presumptively positive for toxin, as evidenced by mouse death, toxin neutralization tests were performed by procedures outlined in the *FDA Bacteriological Analytical Manual* (10).

Results and Discussion

At the time of analysis, the majority of the fish were only marginally acceptable (i.e., they had developed a "fishy" off-odor, and in most instances the flesh had started to lose its firm texture). None of the 1074 vacuum-packaged fresh fish products were positive for *C. botulinum* toxin. The controls in all of the studies were positive for *C. botulinum* type E toxin. Statistically these results show a 95% confidence level that the true contamination rate of *C. botulinum* type E spores in the fish tested is between 0 and 0.34%.

In the past, numerous studies have demonstrated that distribution of *C. botulinum* spores in marine and fresh water environments is ubiquitous and that the distribution of spores in fish fillets or other fish portions is sporadic, varying markedly within different areas of the environment. The absence of botulinal toxin in the uninoculated fish used in this study may indicate that the specific fillets used in these experiments were not contaminated with *C. botulinum* or that the number of spores present was not sufficient to produce discernible levels of botulinal toxin during the 12-day storage period at 12°C.

Results of these studies indicate that 1074 commercial

Received June 15, 1989. Accepted August 3, 1989.

vacuum-packaged fresh fish either did not contain the spores of *C. botulinum*, or, if the spores were present, they were unable to grow out and produce toxin before the product was marginally unacceptable. Under very strict temperature control and without temperature abuse, vacuum packaging and/or modified-atmosphere packaging may be safely applied to extend the shelf-life of fresh fish, provided that proper refrigeration temperature (38°F or 3.3°C) is absolutely maintained during distribution, storage, and retailing, and in the home.

REFERENCES

(1) Clingman, C. D., & Hooper, A. G. (1986) *Dairy Food Sanit.* **6,** 194-197
(2) Genigeorgis, C. A. (1985) *Int. J. Food Microbiol.* **1,** 237-251
(3) Hintlian, C. B., & Hotchkiss, J. H. (1986) *Food Technol.* **40**(12), 70-76
(4) Insalata, N. F., Fredericks, G. J., Berman, J. H., & Borker, E. (1967) *Food Technol.* **21**(3), 78-80
(5) Kautter, D. A. (1964) *J. Food Sci.* **29,** 843-849
(6) Llobrera, A. T. (1983) Ph.D. Dissertation, Texas A & M University, College Station, TX
(7) Post, L. S., Lee, D., Solberg, M., Furgang, D., Specchio, J., & Graham, C. (1985) *J. Food Sci.* **50,** 990-996
(8) Stier, R. F., Bell, L., Ito, K. A., Shafer, B. D., Brown, L. A., Seeger, M. L., Allen, B. H., Porcuna, M. N., & Lerke, P. A. (1981) *J. Food Sci.* **46,** 1639-1642
(9) Wilhelm, K. A. (1982) *Mar. Fish. Rev.* **44,** 17-20
(10) Kautter, D. A., Lynt, R. K., & Solomon, H. M. (1984) in *FDA Bacteriological Analytical Manual*, 6th Ed., AOAC, Arlington, VA, pp. 18.01-18.10

FEED ADDITIVES

Diastatic Activity of Forage Additive Products Containing Malt Flour

INTEAZ ALLI, ANTONIO DILOLLO, SELIM KERMASHA, CHRISTINE THIFFAULT, and FRANCEAN DUMAIS
Department of Food Science and Agricultural Chemistry, Macdonald College of McGill University, 21,111 Lakeshore Rd, Ste Anne de Bellevue, Quebec H9X 1C0, Canada

A method has been developed for the measurement of diastatic activity of forage additive products which contain malt flour as active ingredient. The procedure involves extraction of the enzyme activity with calcium chloride solution, incubation of the extract with excess soluble starch, and measurement of the unhydrolyzed starch by the starch-iodine reaction. The conditions established for optimum enzyme activity were 1% calcium chloride solution for enzyme extraction, and enzyme-starch reaction pH and temperature of pH 5.5 and 40°C, respectively. With the combination of these experimental conditions, the diastatic activity, expressed as g starch hydrolyzed per g material per h (SKB) (a) of malt diastase was 75.9 SKB units, (b) of malt flour was 32.8 SKB units, and (c) of 2 forage additive products were 0.205 SKB unit and 0.044 SKB unit.

The use of enzymes and microorganisms in commercial forage additive products is now widespread. These products serve as fermentation aids and are considered to promote favorable conditions for the preservation of ensiled forage (1). The presence of malt flour containing diastase enzymes in a forage additive product was shown to enhance the liberation of fermentable sugars from an ensiled forage (2). The observed increase in the content of soluble sugars was, in all likelihood, the result of the diastatic activity on starch in the forage by starch-degrading enzymes in the malt flour present in the forage additive.

A survey of the published literature indicates that considerable work has been done on the measurement of diastatic activity of cereals and cereal products. The classical Wohlgemuth (3) procedure has served as the basis for several of the methods, such as the *Food Chemicals Codex* method (4), the AOAC method (5), and the AACC method (6) which are currently used for measuring α-amylase activity. Standstedt et al. (7) acknowledged that the Wohlgemuth method measures the activities of combined α- and β- amylase; for the measurement of α-amylase alone, it is necessary to eliminate variability due to β-amylase activity. However, these workers suggested that, for most practical situations, measurement of total dextrinizing power resulting from combined activities of both α- and -β-amylase may be all that is required.

Although α-amylase is considered to be primarily dextrinizing in its activity, β-amylase also has been shown to reduce the dextrinization time of starch hydrolysis (8). In fact, the starch-degrading properties of enzymes in malt flour are due not only to α- and β-amylase, but could be affected by at least the following 4 additional enzymes: phosphorylase, amylopectin 6-glucanohydrolase, oligo-1,6-glucosidase (limit dextrinase), and α-glucosidase (9, 10). Consequently, measurement of diastatic activity or starch degradation based on the original Wohlgemuth method could in fact, be a measurement of the combined activity of these 6 enzymes.

Received April 4, 1989. Accepted September 27, 1989.

The objective of the present work was to measure the total diastatic activity or the starch degradation due to all enzymes present in a commercial forage additive product containing malt flour. The approach taken was to measure by colorimetry (11-13) the decrease in the intensity of the blue starch-iodine complex as an indicator of starch degradation (3). Since the aim was to measure total diastatic activity and not specifically α-amylase activity, no attempt was made to remove the influence of β-amylase as is done in some standardized methods. The AOAC method for diastase activity of malt does not attempt to eliminate the action of β-amylase; in this respect the approach used in the procedure described in this paper is similar to that of the AOAC method for diastase activity. However, the AOAC method determines activity by measurement of the reducing power of the enzyme-substrate medium after 30 min of reaction. Yoo et al. (14) has suggested that α-amylase activities determined by the starch-iodine reaction method were approximately 4-6 times higher than those from the reducing value method.

Experimental

Materials

(a) *Forage additives.*—Two commercial products containing malt flour as active ingredient and malt flour were obtained from manufacturers of the products.

(b) *Malt diastase.*—Purchased from Fisher Scientific, Montreal, Quebec.

Reagents

(a) *Starch solution.*—Add 1.2 g Lintner potato starch (Sigma Chemical Co.) to 15 mL water with stirring. Add this suspension slowly, with stirring, to 120 mL boiling water. Boil gently for 2 min and then cool under running tap water. Dilute to 200 mL with water.

(b) *Stock iodine solution.*—Prepare according to procedure described in *Food Chemicals Codex* method. Dissolve 11.0 g potassium iodide and 5.5 g iodine in ca 200 mL water. Dilute to 250 mL with water and mix. Store in dark bottle.

(c) *Dilute iodine solution.*—Dissolve 20 g potassium iodide in 300 mL water and add 2 mL stock iodine solution. Quantitatively transfer to 500 mL volumetric flask, dilute to volume with water, and mix. Prepare fresh solution daily.

(d) *Acetate buffer solution.*—Dissolve 120 mL glacial acetic acid and 164 g anhydrous sodium acetate in water and dilute to 1 L. Adjust to desired pH with dilute HCl or dilute NaOH.

Procedure

(a) *Preparation of enzyme extract [according to procedure of Perten (12)].*—Weigh appropriate quantity (0.5-5.0 g) of material to be assayed for diastatic activity. Add 100 mL $CaCl_2$ solution of desired concentration and incubate 1 h

at 30°C; mix well at 15 min intervals. Filter mixture and transfer suitable volume of filtrate to 100 mL volumetric flask. Add 10 mL acetate buffer of desired pH and dilute to 100 mL with water.

(b) *Colorimetric reaction [according to procedure of Perten (12)].*—Transfer 30 mL prepared enzyme extract to 125 mL Erlenmeyer flask. Place this extract and starch substrate solution, separately, in shaker water bath to attain desired temperature. Add 10 mL starch substrate solution to enzyme extract and return to water bath. At 5, 10, 15, and 20 min intervals, transfer 2 mL enzyme-substrate reaction mixture to 125 mL Erlenmeyer flask containing 50 mL acetic acid solution (10%) and 10 mL dilute iodine solution. Mix and read absorbance immediately at 575 nm.

Add 2 mL calcium chloride solution (1%), 50 mL acetic acid solution (10%), and 10 mL dilute iodine solution to 250 mL Erlenmeyer flask. Mix and use this solution to adjust spectrophotometer to zero at 575 nm.

Add 2 mL soluble starch solution to 6 mL calcium chloride solution (1%). Mix and then pipet 2 mL of the above solution into 125 mL Erlenmeyer flask containing 50 mL acetic acid solution and 10 mL dilute iodine solution. Read absorbance at 575 nm. This represents substrate control (12).

Calculation of Diastatic Activity

The period of half-life ($t_{1/2}$) procedure of Hagberg (13) was used to calculate diastatic activity. The period of half-life ($t_{1/2}$) was calculated from the formula:

$$t_{1/2} = 0.30103\ t/(\log E_0 - \log E_t)$$

where t = time of reaction (min); E_0 = absorbance at time 0; and E_t = absorbance after reaction time t. Calculated $t_{1/2}$ value is based on mean of absorbance readings from 3 reaction times as suggested by Hagberg. From calculated $t_{1/2}$ value, diastatic activity expressed as H units (12, 13) was calculated using the following equation:

$$\text{Diastatic activity (H units)} = [W_{starch}(g) \times 60\ \text{min}]/[W_{sample}(g) \times t_{1/2}]$$

where W_{starch} = weight of starch and W_{sample} = weight of sample in hydrolyzing mixture (12, 13). H units obtained by this procedure were converted to SKB units by using the relation of 1 H unit × 0.42 = 1 SKB unit (13).

Results and Discussion

The effect of pH, temperature, and calcium chloride concentration on diastatic activity was investigated to determine the optimum conditions for activity of the starch degrading enzymes. Kneen et al. (15) demonstrated that the stability of α-amylase and β-amylase was influenced by these 3 factors. Temperature was the most important factor with regard to β-amylase; for α-amylase, pH was more important than temperature. In addition, calcium concentration had a stabilizing effect on α-amylase but a destabilizing effect on β-amylase.

Table 1. Effect of pH on diastase activity

pH	Diastase activity, g/g/h[a]		
	Malt diastase	Malt flour	Forage additive A
4.80	59.6 (±5.63)	—	0.044 (±0.0036)
5.15	72.4 (±4.13)	8.5 (±0.22)	0.063 (±0.0047)
5.50	72.7 (±3.13)	17.0 (±0.53)	0.077 (±0.0017)
6.00	66.8 (±3.48)	15.8 (±0.26)	0.069 (±0.0083)
6.50	45.5 (±1.58)	10.9 (±0.06)	0.037 (±0.0020)

[a] Results are means (standard deviations) of triplicate determinations.

Table 2. Effect of temperature on diastase activity

Temperature	Diastase activity, g/g/h[a]		
	Malt diastase	Malt flour	Forage additive A
20°C	13.4 (±0.72)	4.8 (±0.28)	0.019 (±0.0033)
30°C	57.8 (±2.51)	12.4 (±1.17)	0.055 (±0.0039)
40°C	113.7 (±3.83)	22.6 (±0.08)	0.145 (±0.0187)
50°C	42.8 (±1.81)	13.9 (±0.18)	0.075 (±0.0051)

[a] Results are means (standard deviations) of triplicate determinations.

Effect of pH

Table 1 shows the effect of pH of the reaction medium on the diastase activity of commercial malt diastase, malt flour, and a forage additive product. The pH of maximum activity for the 3 materials was 5.5. However, comparison of the means of diastatic activity using the t-test (16) indicated that the activity of malt diastase at pH 5.5 was not significantly ($P < 0.05$) different from that at pH 5.15 or pH 6.0. For malt flour, the activity at pH 5.5 was significantly higher ($P < 0.05$) than that at pH 5.15 and pH 6.0. For the forage additive, the activity at pH 5.5 was significantly higher ($P < 0.05$) than that at pH 5.15 but not significantly different ($P < 0.05$) than that at pH 6.0. These results suggest that the optimum reaction pH was 5.5. Hoskam (8) demonstrated that conversion of erythrodextrin by α-amylase was greatest between pH 4.5 and 5.6. Several methods for measurement of α-amylase activity for cereals and cereal products [FCC method, AOAC method, and AACC method] are based on the procedure of Standstedt et al. (7) which utilizes an acetate buffer of pH 4.8. The FCC and AOAC procedures for diastase activity for malt flour use an acetate buffer of pH 4.6. Greenwood and Milne (17) summarized that the pH value of higher-plant, bacterial, and fungal α-amylase and β-amylase all lie between pH 5.0 and 6.0.

Effect of Temperature

Table 2 shows the effect of temperature on diastase activity of malt diastase, malt flour, and a forage additive. The temperature for maximum diastase activity of the 3 materials was 40°C. Comparison of the means of diastatic activity using the t-test indicated that the activity obtained at 40°C was significantly higher ($P < 0.05$) than that obtained at 20°C, 30°C, or 50°C. Kneen et al. (15) working with temperatures above 50°C reported that temperature was the predominant factor influencing β-amylase stability. These workers concluded that β-amylase stability was dependent on both temperature and pH. Several standardized methods, such as the FCC method, the AOAC method, and the AACC method, use a reaction temperature of 30°C for α-amylase activity. However, Hagberg (13) suggested that α-amylase activity can be determined more readily at temperatures higher than 30°C. For diastase activity, an incubation temperature of 20°C is used (FCC and AOAC). Yoo et al. (14) demonstrated that the activity of *Bacillus licheniformis* α-amylase increases at temperatures between 25°C and 65°C. Greenwood and Milne (17) reported that purified α-amylase lost activity rapidly above 50°C although this deactivation was reduced in the presence of excess calcium ions.

Table 3. Effect of calcium chloride concentration on diastase activity of malt diastase

Calcium chloride concn, %	Diastatic activity, g/g/h[a]
0.0	14.4 (±0.62)
0.1	27.0 (±0.69)
0.2	32.1 (±1.47)
0.5	43.1 (±9.29)
1.0	45.9 (±0.25)
5.0	37.8 (±1.34)

[a] Results are means (standard deviations) of triplicate determinations.

Table 4. Diastase activity of malt diastase, malt flour, 2 commercial forage additive products

Source	Diastase activity, g/g/h[a]
Malt diastase	75.9 (±9.53)
Malt flour	32.8 (±2.55)
Forage additive A	0.205 (±0.0212)
Forage additive B	0.044 (±0.0093)

[a] Results are means (standard deviations) of 4 replicate determinations.

Effect of Calcium Chloride Concentration

Table 3 shows the effect of calcium chloride concentration on diastase activity of malt diastase. The calcium solution was used for extraction of the enzymes from the malt diastase. The results show that maximum activity was obtained with the enzyme extract prepared by extracting with a 1.0% calcium chloride solution. However, the activity of extract from 0.5% $CaCl_2$ solution was not significantly different ($P > 0.5$) than that from 1.0% $CaCl_2$. Calcium is considered as a co-factor for α-amylase activity while β-amylase requires no co-factor for activity (16). Several standardized methods (e.g., FCC method, AOAC method, and AACC method), for α-amylase activity utilize 0.5% sodium chloride solution. For diastase activity, these methods also use 0.5% NaCl solution with the exception of the AACC method which utilize water for preparation of the enzyme extract. Kneen et al. (15) reported that conditions for the maximum retention of α-amylase activity included the presence of calcium. In the procedure of Perten (12) and Hagberg (13), 0.2% calcium chloride solution is used for extraction of α-amylase.

Diastase Activity Using Optimum Conditions

Table 4 shows the activities of malt diastase, malt flour, and 2 forage additive products using the combination of optimum conditions of calcium chloride concentration (1%) for enzyme extraction, of pH (5.5) and of temperature (40°C) of enzyme-substrate reaction. The diastase activities of malt four and diastase were 32.8 g/g/h (SKB units) and 75.9 SKB units, respectively. Perten (12) reported SKB values of 23 and 46 for 2 samples of malt and SKB values of 120 and 480 for 2 enzyme preparations. The activity of the 2 forage additive products were 0.205 and 0.044 SKB unit. The

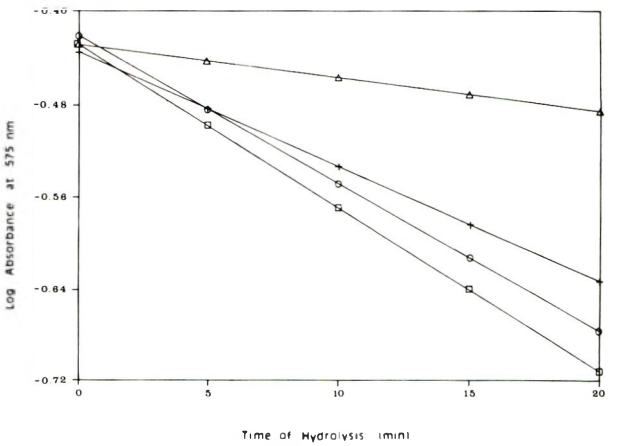


Figure 1. Plot of log absorbance vs reaction time for malt diastase (+ - - - +), malt flour (□ - - - □), and 2 commercial forage additive products containing malt four, additive A (○ - - - ○), and additive B (△ - - - △).

plots of log absorbance vs reaction time for the data from which the activities were obtained, are shown in Figure 1. For the 4 materials which were investigated (Table 4), absorbance vs reaction time plots are similar to those obtained for the conversion erythrodextrin (13) or limit dextrin (12). These results suggest that by using optimum experimental conditions the original Wohlgemuth method can be modified and used to measure diastase activity of malt-containing forage additives.

REFERENCES

(1) Bolsen, R. K. (1978) in *Fermentation of Silage*, M. E. McCullough (Ed), National Feed Ingredient Association, West Des Moines, IA, pp. 181–200
(2) Alli, I., & Baker, B. E. (1982) *J. Dairy Sci.* **65,** 1472–1478
(3) Wohlgemuth, J. (1908) *Biochem. Z.* **9,** 1–9
(4) *Food Chemicals Codex* (1981) 3rd Ed., National Academy Press, Washington, DC, pp. 479–485
(5) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Washington, DC
(6) *Approved Methods of the American Association of Cereal Chemists* (1983) 8th Ed., American Association of Cereal Chemists, Inc., St. Paul, MN
(7) Standstedt, R. M., Kneen, E., & Blesk, M. J. (1939) *Cereal Chem.* **16,** 712–723
(8) Hoskam, E. G. (1947) *Biochim. Biophys. Acta* **1,** 419–427
(9) Briggs, D. E. (1964) *Enzymologia* **26,** 355–363
(10) Meredith, W. O. S. (1965) *Proc. Am. Soc. Brew. Chem.* 5–10
(11) Möttönen, K. (1969) *J. Sci. Food Agric.* **20,** 279–288
(12) Perten, H. (1966) *Cereal Chem.* **43,** 336–342
(13) Hagberg, S. (1961) *Cereal Chem.* **38,** 241–250
(14) Yoo, J. J., Hong, J., & Hatch, R. T. (1987) *Biotech. Bioengin.* **30,** 147–151
(15) Kneen, E., Standstedt, R. M., & Hollenbeck, C. M. (1943) *Cereal Chem.* **20,** 399–423
(16) Steel, R. G. D., & Torrie, J. H. (1960) in *Principles and Procedures of Statistics*, McGraw Hill Book Co. Inc., New York, NY, pp. 67–78
(17) Greenwood, C. T., & Milne, E. A. (1968) *Adv. Carbohydrate Chem.* **23,** 281–366

FOOD ADDITIVES

Determination of Sulfite in Foods and Beverages by Ion Exclusion Chromatography with Electrochemical Detection: Collaborative Study

HIE-JOON KIM
U.S. Army Natick Research, Development and Engineering Center, Food Engineering Directorate, Natick, MA 01760

Collaborators: V. Chu; K. R. Conca; B. Frost; J. R. Heuser; T. Huang; Y.-K. Kim; S. Kupina; D. J. Mayers; J. H. Nguyen

A liquid chromatographic (LC) method for determination of total sulfite in foods and beverages by alkali extraction followed by ion exclusion chromatographic separation and electrochemical detection (IEC-EC) was collaboratively studied by 9 laboratories. Blind duplicate samples of starch, diluted lemon juice, wine cooler, dehydrated seafood, and instant mashed potatoes were analyzed without spiking and with added sulfite at 2 levels. The initial sulfite levels varied from 0 to 384 ppm SO_2, and the levels added varied from 10 to 400 ppm. The initial sulfite levels determined by the IEC-EC method and the Monier-Williams method were in good agreement. Recovery of added sulfite by the IEC-EC method was generally higher than that by the Monier-Williams method. Within-laboratory repeatability (RSD_r) for the IEC-EC method varied from 4.4 to 26.0%, and overall reproducibility (RSD_R) varied from 8.5 to 39.3%. The collaborators found the method to be fast, sensitive, and easy to use, which makes it a useful alternative to the Monier-Williams method. The method has been adopted official first action.

Effective January 9, 1987, the U.S. Food and Drug Administration (FDA) requires that the presence of sulfite at or above 10 ppm be declared on the label of food products (1). The Bureau of Alcohol, Tobacco, and Firearms published a similar labeling regulation for alcoholic beverages (2). Recently, the U.S. Environmental Protection Agency (EPA) established a tolerance limit of 10 ppm for sulfur dioxide in or on grapes (3).

The current official method for sulfite analysis is the Monier-Williams method (4). In this method, sulfite is released from the food as sulfur dioxide by a lengthy (1.75 h) distillation in the presence of a strong acid. The sulfur dioxide is oxidized in a trap to sulfuric acid, which is determined either by titration with alkali or gravimetrically. Results by the Monier-Williams method are subject to uncertainties at the 10 ppm level. FDA modified the Monier-Williams method to improve the sensitivity (by using more dilute titrant) and to minimize the potential interference (by decreasing the condenser temperature) (1). The analysis time of about 2 h still makes the Monier-Williams method unsuitable for rapid screening.

Received for publication August 18, 1989.

This report was presented at the 102nd AOAC Annual International Meeting, August 29-September 1, 1988, at Palm Beach, FL.

The recommendation was approved interim official first action by the General Referee, the Committee on Foods II, and the Chairman of the Official Methods Board. The method was adopted official first action at the 103rd AOAC Annual International Meeting, September 25-28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73**, January/February issue.

A number of alternative methods have been developed recently. These new methods come under 2 categories. Under one category, acid distillation is used as in the Monier-Williams method to separate sulfite from the food matrix, but the alkali titration is replaced by a more selective determinative step. The sulfur dioxide collected in the trap is either separated by ion exchange chromatography and detected by conductivity measurements (5-7) or determined by more selective detection techniques such as redox titration (8), coulometric titration (9), or polarography (10, 11) without further separation.

Under another category, a direct alkali extraction method was extensively investigated in an effort to eliminate the acid distillation step. The reversibly bound sulfite is released more efficiently from the foods by alkali than by acid (12, 13). Nevertheless, separation of sulfite from the alkali extract has been a challenging problem. Fortunately, several novel separation techniques facilitated the selective determination of sulfite in the alkali extract of the foods. Examples include flow injection analysis (14), ion exclusion chromatography with electrochemical detection (15, 16), headspace techniques (17, 18), and a reverse-phase ion pairing liquid chromatographic (LC) method with spectrophotometric detection (19).

Among these various methods, the differential pulse polarographic method was adopted official first action (20), and the FDA-optimized Monier-Williams method (21) and the flow injection method (14) have received interim official first action approval by AOAC. Recently, Holak and Specchio (22) improved the differential pulse polarographic method by replacing acid distillation with alkali treatment followed by acidification and nitrogen purging. The present paper reports the collaborative study results for the determination of total sulfite by the IEC-EC method. A comparison with results by the Monier-Williams method is included and explanations for the difference are suggested.

Collaborative Study

The 9 participants were equipped with an LC system with an anion exclusion column and an electrochemical detector. Five participants used an anion exclusion column supplied by Wescan Instruments, Inc. (2051 Waukegan Rd, Deerfield, IL); two by Waters Chromatography Division (34 Maple St, Milford, MA); one each by Bio-Rad Laboratories (1414 Harbour Way South, Richmond, CA) and Brownlee Labs, Inc. (2045 Martin Ave, Santa Clara, CA). Four participants used electrochemical detectors supplied by Wescan; two each by Bioanalytical Systems, Inc. (2701 Kent Ave, West Lafayette, IN) and Waters; and one by Bio-Rad. All participants used a Polytron homogenizer (Brinkmann Instruments, Cantiague Rd, Westbury, NY) or an equivalent.

Sample Preparation

Five food and beverage samples with the initial sulfite level ranging from 0 to 384 ppm were used. These were corn starch (initial level, 0 ppm), diluted lemon juice (21 ppm), wine cooler (22 ppm), dehydrated seafood (35 ppm), and instant mashed potatoes (384 ppm). Corn starch, lemon juice, and wine cooler were purchased from local stores. The participants were asked to dilute the lemon juice 10-fold before use. Dehydrated seafood was prepared by dipping a surimi-type seafood section in an 80 mg sodium sulfite/L solution and freeze-drying. The dehydrated seafood was pulverized to fine powder to achieve sample homogeneity. Instant mashed potatoes were obtained from a commercial supplier.

Spike Preparation

Vials containing accurately weighed amounts of sodium sulfite (amount not revealed to participants) and excess sodium chloride were provided. The participants were instructed to dissolve the contents of the vial in a given volume of buffer and make dilutions according to the instructions. Duplicate samples and spiking mixtures were sent to the participants. Spiking levels of 10 and 30 μg/g were used for starch, diluted lemon juice, and wine cooler; 40 and 80 μg/g for dehydrated seafood; and 80 and 400 μg/g for instant mashed potatoes.

Instructions to Collaborators

Detailed instructions were sent to the participants. They were asked to practice the procedure before performing the study.

Monier-Williams Method

The Associate Referee determined the amount of sulfite in the samples with and without the spike in duplicate by the modified Monier-Williams method (1) except that cold tap water was used for cooling the condenser.

Statistical Analysis

Statistical analysis was applied to each sample at each spiking level according to the AOAC statistical manual (23).

990.31 Sulfites in Foods and Beverages

Ion Exclusion Chromatographic Method

First Action 1990

(Applicable to determination of SO_2 at $\geq$10 ppm. Not applicable to dark colored foods or ingredients where SO_2 is strongly bound, e.g., caramel color. Method does not detect naturally occurring sulfite.)

Method Performance:

$s_r = 4.7$; $s_R = 8.9$; $RSD_r = 12.3\%$; $RSD_R = 21.2\%$

A. Principle

SO_2 is released by direct alkali extraction. Diluted portions of liquid samples or diluted filtrates of solid samples are injected into LC or ion exclusion chromatographic system equipped with anion exclusion column and electrochemical detector.

B. Apparatus

(a) *Ion exclusion chromatograph.*—Any liquid chromatographic or ion chromatographic system equipped with anion exclusion column (sulfonated polystyrene/divinylbenzene) and electrochemical (amperometric) detector can be used. Equilibrate anion exclusion column (preferably high speed column, 4.6 × 100 mm) and electrochemical detector set at +0.6 V on platinum working electrode vs Ag/AgCl reference electrode with 20mM H_2SO_4 eluant. Adjust attenuation on integrator or chart recorder so that signal from 0.60 ppm SO_2 solution yields ca $\frac{1}{2}$ full scale deflection.

(b) *Homogenizer.*—Polytron or equivalent.

C. Reagents

(a) *Buffer.*—pH 9. Prepare 20mM Na_2HPO_4, 10mM D-mannitol solution in deionized water and degas.

(b) *Sulfuric acid solution.*—20mM. Add 1.07 mL concentrated H_2SO_4 to water in 1 L volumetric flask. Dilute to volume with water and degas.

(c) *Sodium sulfite standard.*—Determine purity of Na_2SO_3 as follows: Accurately weigh ca 250 mg Na_2SO_3 into exactly 50 mL 0.1N I solution in glass flask. After 5 min at room temperature, add 1 mL HCl and titrate excess I with 0.1N $Na_2S_2O_3$ using 1% aqueous starch solution as indicator (1 mL 0.1N I consumed = 6.302 mg Na_2SO_3).

(d) *Sulfite standard solutions.*—(*1*) *Stock solution.*—Prepare 1000 ppm SO_2 solution by dissolving 196.9 mg Na_2SO_3 in 100 mL pH 9 buffer, (a). Prepare stock solution daily. (*2*) *Working solution.*—Dilute stock solution to 0.60 ppm with same pH 9 buffer. Working solution must be freshly prepared from stock solution every 2 h.

D. Determination

Dilute liquid sample with pH 9 buffer so that height of sulfite peak from sample is similar to that of 0.60 ppm standard within 50%. For solid samples, homogenize 0.2–1.0 g sample in 10–100-fold excess pH 9 buffer for 1 min with homogenizer and filter (0.2–0.45 μm). Dilute filtrate as necessary, comparing signal intensity with that of 0.60 ppm working standard solution. For acidic samples such as lemon juice, if pH of diluted sample is <8, adjust to pH between 8 and 9 with dilute NaOH solution or perform extraction with 100mM Na_2HPO_4, 10 mM D-mannitol solution.

Inject 0.60 ppm standard solution, and then inject prepared, diluted test sample.

Extraction, filtration, dilution, and injection should be done within 10 min because sulfite concentration in extract tends to decrease gradually.

Errors due to gradual decrease in detector sensitivity during multiple sample injections can be minimized by injecting standard solution alternately with sample injections. Cleaning electrode at beginning of each chromatographic run may alleviate decrease in sensitivity. To clean electrode, apply −1.0 V for several min followed by +1.8 V for several more min and then equilibrate at +0.6 V. Alternatively, short train of electrode cleaning voltages can be applied automatically after each injection.

E. Calculations

Calculate ppm SO_2 in sample as follows:

$$SO_2, \text{ ppm} = 0.60 \times (PH/PH') \times \text{dilution factor}$$

where PH and PH' = peak height from sample and standard, respectively, and dilution factor takes into account initial dilution for extraction and any subsequent dilution.

Ref.: JAOAC 73, March/April issue (1990)

Table 1. Collaborative results for recovery by IEC-EC method of sulfite added to starch at 10 and 30 ppm levels (blind duplicate samples)

	Added, 0 ppm	Added, 10 ppm			Added, 30 ppm		
Coll.	Found	Found	Rec.	Rec., %	Found	Rec.	Rec., %
1	0	8.9	8.9	89	33.6	33.6	112
	0	9.6	9.6	96	33.2	33.2	111
2	0	6.7	6.7	67	22.5	22.5	75
	0	8.0	8.0	80	22.6	22.6	75
3	0	6.4	6.4	64	24.4	24.4	81
	0	7.1	7.1	71	26.3	26.3	88
4	0	7.6	7.6	76	27.3	27.3	91
	0	6.7	6.7	67	25.4	25.4	85
5	0	7.1	7.1	71	27.9	27.9	93
	0	6.9	6.9	69	28.1	28.1	94
6	0	7.2	7.2	72	27.0	27.0	90
	0	5.5	5.5	55	21.6	21.6	72
7	15.4[a]	23.8	8.4	84	44.9	29.5	98
	21.2[a]	28.8	7.6	76	44.9	23.7	79
8	0	8.8	8.8	88	31.0	31.0	103
	0	10.0	10.0	100	32.8	32.8	109
9	0	16.8	16.8[a]	168	24.6	24.6	82
	0	21.1	21.1[a]	211	30.1	30.1	100

[a] Data rejected by Dixon test.

CAS-7446-09-5 (sulfur dioxide)

Results and Discussion

Nine participants reported the sulfite concentration as ppm SO_2 from blind duplicate analyses of 5 food and beverage samples without spike and with added sulfite at 2 levels. Peak height was used for calculation. One set of data was rejected before the statistical analysis because an old standard sulfite solution was used (diluted lemon juice, 30 μg/g spike, Collaborator 1). The reported data are summarized in Tables 1–5.

The results of the statistical analysis are summarized in Table 6. The relative standard deviation for repeatability (RSD_r) for the initial sulfite content of the 4 sulfite-containing samples varied from 4.4 to 13.2% (average 8.1%). The relative standard deviation for reproducibility (RSD_R) varied from 8.5 to 25.8% (average 15.3%) for the same samples.

For recovery studies carried out at pH 9, RSD_r varied from 5.4 to 26.0% (average 14.0%) and RSD_R varied from 13.0 to 39.3% (average 23.6%). The larger values of RSD_r and RSD_R in the recovery results reflect the fact that the variations due to experimental errors are compounded when the

Table 2. Collaborative results for recovery by IEC-EC method of sulfite added to diluted lemon juice at 10 and 30 ppm levels (blind duplicate samples)

	Added, 0 ppm	Added, 10 ppm			Added, 30 ppm		
Coll.	Found	Found	Rec.	Rec., %	Found	Rec.	Rec., %
1	29.7[a]	40.3	10.6	106	89.7	60.0[b]	200
	29.5[a]	41.0	11.5	115	88.4	58.9[b]	196
2	19.7	31.2	11.5	115	59.7	40.0	133
	20.3	34.9	14.6	146	46.2	25.9	86
3	21.8	51.7	29.9[c]	299	82.0	60.2[a]	201
	21.9	30.5	8.6[c]	86	50.9	29.0[a]	97
4	23.3	30.9	7.6	76	46.1	22.8	76
	22.9	32.7	9.8	98	51.0	28.1	94
5	21.6	32.1	10.5	105	55.4	33.8	113
	20.3	32.2	11.9	119	52.1	31.8	106
6	24.6[c]	35.2	10.6	106	54.8	30.2	101
	14.8[c]	21.8	7.0	70	36.2	21.4	71
7	12.4[a]	21.5	9.1	91	43.5	31.1	104
	11.9[a]	21.3	9.4	94	50.2	38.3	128
8	16.5	28.6	12.1	121	43.8	27.3	91
	22.3	35.0	12.7	127	52.3	30.0	100
9	23.6	36.4	12.8	128	53.9	30.3	101
	19.6	37.6	18.0	180	53.1	33.5	112

[a] Data rejected by Dixon test.
[b] Data rejected because old standard was used.
[c] Data rejected by variance range test.

Table 3. Collaborative results for recovery by IEC-EC method of sulfite added to wine cooler at 10 and 30 ppm levels (blind duplicate samples)

	Added, 0 ppm	Added, 10 ppm			Added, 30 ppm		
Coll.	Found	Found	Rec.	Rec., %	Found	Rec.	Rec., %
1	15.3[a]	25.0	9.7	97	54.8	39.5	132
	9.0[a]	26.3	17.3	173	46.4	37.4	125
2	18.8	27.8	9.0	90	52.2	33.4[a]	111
	15.8	19.7	3.9	39	35.9	20.1[a]	67
3	20.2	34.7	14.5	145	48.8	28.6	95
	20.6	36.2	15.6	156	49.4	28.8	96
4	21.9	32.2	10.3	103	52.9	31.0	103
	21.0	32.2	11.2	112	52.8	31.8	106
5	23.1	30.5	7.4	74	54.6	31.5	105
	21.5	30.4	8.9	89	52.1	30.6	102
6	23.4	32.5	9.1	91	56.3	32.9	110
	23.4	33.9	10.5	105	56.6	33.2	111
7	17.7	26.8	9.1	91	48.1	30.4	101
	17.4	26.4	9.0	90	51.6	34.2	114
8	23.2	44.5	21.3	213	64.9	41.7	139
	22.2	42.4	20.2	202	59.9	37.7	126
9	28.4	39.2	10.8	108	55.4	27.0	90
	29.4	43.9	14.5	145	60.2	30.8	103

[a] Data rejected by variance range test.

difference of 2 sets of data is taken. The Horwitz boundary of the historically expected reproducibility is 11.3–22.6% at 10 ppm and 8.3–16.5% at 80 ppm (24). Of 15 RSD_R values in Table 6, 5 were significantly above the boundary (wine cooler, 10 ppm spike; dehydrated seafood, all three; instant mashed potatoes, 400 ppm spike). These somewhat high variations are believed to be due to the high reactivity of the sulfite. Comparable variations were observed in the differential pulse polarographic analysis (11) and the Monier-Williams analysis (21) for total sulfite in foods.

Source of Error

Since sulfite reacts with various components of the foods reversibly and irreversibly, accurate determination of total sulfite is a difficult task. The concentration of the extracted sulfite in the alkali buffer tends to decrease gradually due to oxidation and recombination with the food constituents. The oxidative loss can be minimized with mannitol. When the food is homogenized, certain chemical reactions could take place to produce compounds that are reactive toward sulfite. Enzymatic browning reaction is a good example. In such cases, the sulfite content could be underestimated unless the extract is injected without delay.

Errors can also result if the sample and the standard solution used for calculation are not injected sequentially because the detector sensitivity may change over time. Detector sensi-

Table 4. Collaborative results for recovery by IEC-EC method of sulfite added to dehydrated seafood at 40 and 80 ppm levels (blind duplicate samples)

	Added, 0 ppm	Added, 40 ppm			Added, 80 ppm		
Coll.	Found	Found	Rec.	Rec., %	Found	Rec.	Rec., %
1	39.5	69.1	29.6	74	95.2	55.7	70
	44.8	59.2	14.4	36	98.5	53.7	67
2	37.4	52.7	15.3	38	80.7	43.3	54
	39.1	51.4	12.3	31	77.8	38.7	48
3	50.4	92.2	41.8	105	111.6	61.2[a]	77
	36.7	88.8	52.1	130	122.9	86.2[a]	108
4	38.0	65.4	27.4	69	100.2	62.2	78
	50.8	79.1	28.3	71	117.9	67.1	84
5	39.5	73.7	34.2	86	118.7	79.2	99
	38.5	74.5	36.0	90	117.3	78.8	99
6	23.3	49.8	26.5	66	83.5	60.2	75
	22.4	60.8	38.4	96	95.4	73.0	91
7	24.4	40.1	15.7	39	60.4	36.0	45
	26.6	41.4	14.5	36	59.8	33.2	42
8	24.7	57.5	32.8	82	112.8	88.1	110
	25.6	62.6	37.0	93	112.6	87.0	109
9	37.4	68.3	30.9	77	113.2	75.8	95
	37.8	68.5	30.7	77	117.0	79.2	99

[a] Data rejected by variance range test.

Table 5. Collaborative results for recovery by IEC-EC method of sulfite added to instant mashed potatoes at 80 and 400 ppm levels (blind duplicate samples)

	Added, 0 ppm	Added, 80 ppm			Added, 400 ppm		
Coll.	Found	Found	Rec.	Rec., %	Found	Rec.	Rec., %
1	383	528	145[a]	181	1038	655	164
	406	460	54[a]	68	746	340	85
2	327	411	84	105	861	534	134
	336	422	86	108	558	222	56
3	426	520	94[b]	118	938	512	128
	390	609	219[b]	274	773	383	96
4	402	484	82	103	888	486	122
	436	532	96	120	890	454	114
5	406	504	98	123	821	415	104
	402	495	93	116	820	418	105
6	380	466	86	108	767	387	97
	361	469	108	135	717	356	89
7	345	420	75	94	682	337	84
	395	453	58	73	770	375	94
8	341	456	115	144	797	456	114
	362	467	105	131	797	435	109
9	394	510	116	145	805	411	103
	415	512	97	121	826	411	103

[a] Data rejected by variance range test.
[b] Data rejected by Dixon test.

tivity depends on the condition of the working electrode and could decrease as much as 40% over an 8 h period if the surface of the working electrode is contaminated by a continuous injection of concentrated samples. This problem can be alleviated by injecting dilute samples as well as by injecting standard solution alternately with the sample. The electrode can be cleaned occasionally by applying − 1.0 V for several minutes followed by + 1.8 V for another several minutes before re-equilibrating at + 0.6 V. Alternatively, a short train of electrode cleaning voltages can be applied automatically after each injection (25).

The observed variations (see recovery results in Tables 1–5) are probably due to a combination of these possible errors. These experimental errors can be minimized by training and experience.

Comparison with Monier-Williams Method

Comparison of the IEC-EC method with the Monier-Williams method has been investigated in enzymatic and nonenzymatic browning reaction systems, foods containing naturally occurring sulfite, and carbohydrate-type food ingredient that yields a false positive response by the Monier-Williams method (26). Good overall correlation, particularly for foods containing less than 10 ppm SO_2, was also demonstrated (27).

In the present study, the spiking experiments were performed at pH 9 by the collaborators. The recovery results are summarized in Table 7. The Associate Referee repeated the recovery study with the same sample matrixes and the same spiking levels under conditions that allow maximum interaction between sulfite and the sample. To do that, the food and beverage samples were spiked at pH 2, instead of pH 9, with respective amounts of sulfite by mixing 1.0 g sample with 2.0 mL spiking solution. After 5 min, the sulfite was extracted with the pH 9 buffer and determined by the IEC-EC method. The results are included in Table 7. Recoveries at pH 2 were generally lower than those obtained at pH 9 but are comparable with those by the Monier-Williams method. Overall, the data in Table 7 show that the results by the IEC-EC method and the Monier-Williams method are in good agreement even though several discrepancies were noted. Explanations of the discrepancies are suggested in the following.

In the case of starch, 8 participants reported zero sulfite. Collaborator 7 reported 15.4 and 21.2 ppm for the duplicate samples. These results by Collaborator 7 appear to be due to insufficient separation of a food component on the anion exclusion column and were rejected by the Dixon test (23). When starch was subjected to the Monier-Williams distillation, severe darkening was observed. The 7.5 ppm SO_2 obtained by the Monier-Williams method probably represents volatile organic acids produced by the caramelization reaction of the starch. A similar false positive was observed with erythorbic acid (26) and grapes (28). When the starch is distilled with added sulfite, there could be a reaction between sulfite and the intermediate products and melanoidins of the caramelization reaction, leading to irreversible binding of the sulfite. This irreversible binding could explain the low recovery of added sulfite from starch by the Monier-Williams method.

The higher initial sulfite level in wine cooler observed by the Monier-Williams method might be due to carbonate, which is carried to the trap as carbon dioxide and redissolved, even though it is expected to be expelled eventually by the nitrogen gas. Consistently higher results were obtained from instant mashed potatoes by the IEC-EC method than by the Monier-Williams method (16). A similar observation was made by other investigators (18, 22). Darkening of the sample was also observed during the acid distillation of instant mashed potatoes. Therefore, it appears that the discrepancy is due to the irreversible binding of sulfite, present at high concentrations in instant mashed potatoes, to the caramelization reaction products. It should be noted that no such discrepancy was observed from the protein-rich dehydrated seafood. Thus, it appears that the Monier-Williams method

Table 6. Summary of statistical analysis of collaborative study results for determination of total sulfite by IEC-EC method

Spike ppm	$\sum n_L$	$\bar{X}$[a]	s_r	RSD_r, %	s_R	RSD_R, %
Starch						
0	16	0	0	0	0	0
10	16	7.7	0.7	9.5	1.3	16.3
30	18	27.3	2.4	8.8	3.9	14.1
Diluted lemon juice						
0	12	21.2	2.1	9.8	2.1	9.8
10	16	11.2	1.9	17.0	2.7	24.1
30	14	30.3	5.2	17.2	5.2	17.2
Wine cooler						
0	16	21.8	1.0	4.4	3.7	17.1
10	18	11.8	2.4	20.4	4.6	39.3
30	16	32.9	1.8	5.4	4.3	13.0
Dehydrated seafood						
0	18	35.4	4.7	13.2	9.1	25.8
40	18	28.8	5.3	18.5	11.2	38.9
80	18	63.2	3.8	6.0	18.9	29.9
Instant mashed potatoes						
0	18	384	19.5	5.1	32.5	8.5
80	14	92.8	10.2	11.0	16.3	17.5
400	18	422	109.8	26.0	109.8	26.0

[a] Mean initial sulfite level for unspiked samples. Mean recovery of added sulfite for spiked samples.

Table 7. Comparison of SO_2 recovery data by IEC-EC and Monier-Williams methods[a]

SO_2 added, ppm	IEC-EC pH 9[b] ppm	IEC-EC pH 9[b] %	IEC-EC pH 2[c] ppm	IEC-EC pH 2[c] %	Monier-Williams[d] ppm	Monier-Williams[d] %
Starch						
0	0	—	—	—	7.5	—
10	7.7	77	5.7	57	3.1	31
30	27.3	91	22.5	75	11.9	40
Diluted lemon juice						
0	21.2	—	—	—	17.8	—
10	11.2	112	8.0	80	8.8	88
30	30.3	101	23.5	78	23.6	79
Wine cooler						
0	21.8	—	—	—	28.1	—
10	11.8	118	7.8	78	8.7	87
30	32.9	110	32.2	107	25.4	85
Dehydrated seafood						
0	35.4	—	—	—	38.9	—
40	28.8	72	20.9	52	27.9	70
80	63.2	79	36.1	45	47.1	59
Instant mashed potatoes						
0	384	—	—	—	278	—
80	93	116	59	74	68	85
400	422	106	380	95	332	83

[a] Initial sulfite level for unspiked samples. Recovery of added sulfite for spiked samples.
[b] $\bar{X}$ from Table 6.
[c] Average of duplicate spiking experiments at pH 2 by the Associate Referee.
[d] Average of duplicate determinations by the Associate Referee.

may be subject to negative bias as well as positive interference depending on the nature of the sample. In the alkali extraction method, such a negative bias does not occur because no heat is involved.

Overall, the recovery of added sulfite was higher by the IEC-EC method than by the Monier-Williams method. The average recovery among different samples and spiking levels varied from 72 to 118% by the IEC-EC method at pH 9, from 45 to 107% by the IEC-EC method at pH 2, and from 31 to 88% by the Monier-Williams method.

It should be noted that the alkali extraction used in the IEC-EC method does not effectively release sulfite bound to certain pigments such as the nonenzymatic browning reaction products (26). Therefore, lower results could be obtained by the IEC-EC method than by the Monier-Williams method from such foods and food ingredients as caramel color. The IEC-EC method does not detect naturally occurring sulfite in Allium and Brassica vegetables (26). This limitation could be an advantage since FDA would take the natural sulfite levels in these vegetables into account in the enforcement of the labeling regulation (1).

Collaborator Comments

All participants agreed that the IEC-EC method is fast and sensitive. It was commonly observed that injection of a solution containing 10 ppb SO_2 yielded a signal with a signal-to-noise ratio of 5. Therefore, they felt that 0.01 ppm is the detection limit for sulfite in liquid samples or in the alkali extract.

Most participants also noted that there is little interference. The absence of interference is due to the combination of 2 selective processes, namely, anion exclusion chromatographic separation and amperometric detection. One collaborator observed interference in starch. Eight other collaborators observed no sulfite in starch. A proper selection of the column might overcome such a problem.

It was also pointed out that sulfite concentration in the extract tends to decrease gradually. This problem could be overcome by an immediate injection (within 10 min) after extraction and filtration. A gradual decrease in detector sensitivity during multiple sample injections was noted as a drawback of electrochemical detection. The errors due to a change in detector sensitivity could be minimized by injecting a standard sulfite solution adjacent to the sample injection. This problem can also be overcome by cleaning the electrode at the beginning of each chromatographic run (25).

Recommendation

The method is recommended for use for most foods and beverages at all sulfite levels. Exceptions are darkly colored foods or ingredients such as caramel color where sulfite is strongly bound and released only by acid distillation. The method does not detect naturally occurring sulfite.

It is recommended that the method be adopted official first action.

Acknowledgments

The collaborative efforts of the following participants are greatly appreciated:

V. Chu, Bio-Rad Laboratories, Richmond, CA

K. R. Conca, U.S. Army Natick RD&E Center, Natick, MA

B. Frost, Waters Chromatography Division, Milford MA

J. R. Heuser, National Food Processors Association, Washington, DC

T. Huang, Bioanalytical Systems, Inc., West Lafayette, IN

Y.-K. Kim, Kim & Associates, Wayland, MA

S. Kupina, Heublein Wines, Madera, CA

D. J. Mayers, Michigan Dept of Agriculture

J. H. Nguyen, Wescan Instruments, Inc., San Jose, CA

The author acknowledges the support and valuable comments from Irwin Taub, Chief, Technology Acquisition Division, and Tom Yang, Chief, Advanced Foods Branch, Technology Acquisition Division, Food Engineering Directorate, U.S. Army Natick Research, Development and Engineering Center.

REFERENCES

(1) *Fed. Regist.* (1986) **51,** 25012–25020
(2) *Fed. Regist.* (1986) **51,** 34706–34709
(3) *Fed. Regist.* (1989) **54,** 20125–20126
(4) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **20.123–20.125**; (1990) 15th Ed., **962.16**
(5) Sullivan, D. M., & Smith, R. L. (1985) *Food Technol.* **39**(7), 45–53
(6) Anderson, C., Warner, C. R., Daniels, D. H., & Padgett, K. L. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 14–19
(7) Cooper, P. L., Marshall, M. R., Gregory, J. F., III, & Otwell, W. S. (1986) *J. Food Sci.* **51,** 924–928
(8) DeVries, J. W., Ge, H., Ebert, F. J., Magnuson, J. M., & Ogawa, M. K. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 827–830
(9) Greyson, J., & Zeller, S. (1987) *Am. Lab.*, July, pp. 44–51
(10) Stonys, D. B. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 114–117
(11) Holak, W., & Patel, B. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 572–578
(12) Monier-Williams, G. W. (1927) *Analyst* **52,** 343–344
(13) Wedzicha, B. L. (1984) *Chemistry of Sulphur Dioxide in Foods*, Elsevier Applied Science Publisher, New York, NY, p. 45
(14) Sullivan, J. J., Hollingworth, T. A., Wekell, M. M., Newton, R. T., & Larose, J. E. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 542–546
(15) Kim, H.-J., & Kim, Y.-K. (1986) *J. Food Sci.* 51, 1360–1361
(16) Kim, H.-J., Park, G. Y., & Kim, Y.-K. (1987) *Food Technol.* **41**(1), 85–91
(17) Hamano, T., Mitsuhashi, Y., Matsuki, Y., Ikuzawa, M., Fujita, K., Izumi, T., Adachi, T., Nonogi, H., Fuke, T., Suzuki, H., Toyoda, M., Ito, Y., & Iwaida, M. (1979) *Z. Lebensm. Unters. Forsch.* **168,** 195–199
(18) Lawrence, J. F., & Chadha, R. K. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 930–933
(19) Warner, C. R., Daniels, D. H., Pratt, D. E., Joe, F. L., Jr, Fazio, T., & Diachenko, G. W. (1987) *Food Addit. Contam.* **4,** 437–445
(20) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, **987.04**; "Changes in Official Methods of Analysis" (1987) *J. Assoc. Off. Anal. Chem.* **70,** 387–388, secs **20.C01–20.C08**
(21) Hillery, B. R., Elkins, E. R., Warner, C. R., Daniels, D., & Fazio, T. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 470–475
(22) Holak, W., & Specchio, J. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 476–480
(23) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
(24) Horwitz, W. (1982) *Anal. Chem.* **54,** 67A–76A
(25) Nguyen, J. H., Kim, H.-J., & Gjerde, D. T. (1988) *Am. Lab.*, May, pp. 122–126
(26) Kim, H.-J. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 266–272
(27) Kim, H.-J., Kim. Y.-K., & Smith, M. (1988) *Food Technol.* **42**(11), 113–116
(28) Kim, H.-J., Conca, K. R., & Richardson, M. J., *J. Assoc. Off. Anal. Chem.*, submitted for publication

Determination of Free (pH 2.2) Sulfite in Wines by Flow Injection Analysis: Collaborative Study

JOHN J. SULLIVAN,[1] THOMAS A. HOLLINGWORTH, MARLEEN M. WEKELL,[2] VICTOR A. MEO, and ALI ETEMAD-MOGHADAM
U.S. Food and Drug Administration, Seafood Products Research Center, Bothell, WA 98041-3012
JOHN G. PHILLIPS
U.S. Department of Agriculture, Agricultural Research Center, Philadelphia, PA 19118
BARRY H. GUMP
California State University, Department of Enology, Fresno, CA 93740

Collaborators: S. Chew; J. Farley; K. Faul; L. Habeger; J. Moller; U. Sics; S. Stieg; D. Stonys

A method for the determination of free sulfite in wine by flow injection analysis (FIA) is described. The method involves liberation of sulfur dioxide from the wine at pH 2.2, with detection by decolorization of a malachite green solution. The method was collaboratively studied, and the results indicated an average reproducibility of 12% for white wine samples (average level 12.1 ppm SO_2) and 26% for red wine samples (average level 3.1 ppm). When the FIA method was compared to an aeration/oxidation method, the results indicated a high degree of correlation between the 2 methods. The FIA method has been adopted by AOAC official first action.

When present in foods and beverages, sulfiting agents can undergo a number of reactions, including oxidation and binding to other constituents. "Sulfites" are therefore present both as various combined forms and as free sulfite anion, depending on pH and the level of other constituents present (1). In wines, much of the sulfite combines with acetaldehyde to form the hydroxy-sulfonate adduct, with only a small proportion present as free sulfite. Since the level of free sulfite is an important parameter in wine making and in the processing of sulfited foods, it is important to have rapid, accurate analytical methods available to differentiate free from combined sulfite.

The determination of that portion of the total sulfite present that is free is very difficult because sample handling and treatments can liberate weakly bound forms of sulfite. Bolin et al. (2) noted that sulfite was bound in dried apples by a variety of mechanisms, some of which were susceptible to weak acid hydrolysis and some susceptible to weak base hydrolysis. They found that any sample treatment, including the conditions specified to liberate free sulfite in wines, measured both free sulfite anion and that portion of the bound sulfite that is labile under weak acid conditions. It is apparent that a very delicate equilibrium exists between free sulfite anion and the various forms of bound sulfite, and the method actually defines what the free sulfite level will be. This makes it very difficult to compare different free sulfite methods and to accurately define a true free sulfite level in a particular sample. Nevertheless, methods for the determination of free sulfite are useful since the relative levels of free sulfite can be determined for related samples, with the precaution that the sample treatment conditions be rigorously reproduced from sample to sample.

A number of investigators have reported methods of analysis for free sulfite in foods and beverages. The sample work-up procedures vary, but all include the addition of strong acid (3–5) and strong base (6). In addition, a procedure has been described that uses no sample work-up (2). The most accurate free sulfite determinations are made on an undisturbed sample, because addition of acid, base, or any other extractant can alter the relative proportions of free and bound sulfite (2, 7).

Flow injection analysis (FIA) can be used to measure total sulfite levels in foods and beverages (8, 9), and a procedure has been described for free sulfites in wines (10). Based on our work on the measurement of total sulfites by FIA (8), a method was developed to measure free sulfite in wines. This includes the free sulfite anion and sulfite that is liberated rapidly at room temperature at about pH 2.2. These conditions are much less rigorous than those conditions normally used to measure free sulfite (7) in wine, which include pH <1 and reaction times of several minutes. After development of the FIA method, it was subjected to an interlaboratory collaborative study. The results of the study are presented here.

Collaborative Study

Wine samples purchased from a local supermarket represented a variety of both red and white wines. The wines were dispensed into 25 mL glass ampules, which were filled to allow for a minimum of headspace. The ampules were heat-sealed with a propane torch and stored at 4°C.

Two ampules of each wine were submitted to the collaborators as blind duplicates. The collaborators were asked to store the samples at 4°C until the time of analysis. Once the FIA instrument was set up and was operating well, as indicated by a reproducible and linear standard curve, collaborators were to proceed with analysis of the wine samples. The ampules were to be removed from the refrigerator, and the contents mixed well and allowed to come to room temperature. For the determination, the neck of the ampule was removed and the sample was injected into the FIA instrument without delay. The collaborators were asked to operate the FIA system and process the data as described below.

Received for publication August 18, 1989.
This report was presented at the 102nd AOAC Annual International Meeting, August 29–September 1, 1988, at Palm Beach, FL.
The recommendation was approved interim official first action by the General Referee, the Committee on Foods II, and the Chairman of the Official Methods Board. The method was adopted official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73**, January/February issue.
[1] Present address: Varian Associates, Inc., 2700 Mitchell Dr, Walnut Creek, CA 94598.
[2] Address correspondence to this author.

990.30 Sulfite (Free) in Wines

Flow Injection Analysis Method

First Action 1990

(Applicable to determination of free sulfite and bound sulfite that is labile at pH 2.2.)

Method Performance:

$s_r = 0.55$; $s_R = 1.18$; $RSD_r = 8.77\%$; $RSD_R = 19.27\%$

A. Principle

Injected sample aliquot is mixed with concentrated citric acid solution (pH about 2), which forms SO_2 gas from free sulfite and from portion of bound sulfite that is labile under these conditions. SO_2 gas diffuses across Teflon membrane in gas-diffusion cell into flowing stream of malachite green solution. Malachite green is decolorized in proportion to amount of SO_2 gas that diffuses across membrane. Degree of decolorization is measured spectrophotometrically.

B. Apparatus

Flow injection analyzer.—See **990.29B(a)**. Construct FIA system as shown in Fig. **990.29**, *except* use 15 μL sample valve, and use citric acid reagent, **C(b)**, as donor reagent in place of both H_2SO_4 and NaOH reagents.

C. Reagents

(**a**) *Malachite green solutions.*—See **990.29C(a)**(*1*) and (*2*).

(**b**) *Citric acid reagent.*—0.5M. Dissolve 192 g citric acid in ca 1700 mL H_2O, add 60 mL absolute ethanol, and dilute to 2 L with water.

(**c**) *Phosphate buffer reagent.*—0.3M. See **990.29C(c)**.

(**d**) *FIA rinsing reagent.*—0.04M NaOH. Dissolve 1.6 g NaOH in 1 L H_2O.

(**e**) *Ethanol solution.*—1%. Dilute 10 mL absolute ethanol, nondenatured, to 1 L with water.

(**f**) *Sulfite standard solutions.*—All standard solutions must be freshly prepared daily.

(*1*) *Stock solution.*—500 ppm as SO_2. Dissolve 98.4 mg Na_2SO_3 in 90 mL 1% ethanol solution, (**e**), and dilute to 100 mL.

(*2*) *Intermediate solution.*—50 ppm as SO_2. Dilute stock solution with 1% ethanol solution to obtain 50.0 ppm solution.

(*3*) *Working solutions.*—0–40 ppm as SO_2. Dilute intermediate solution with 1% ethanol solution to obtain standards of 40, 30, 20, 10, 5, 3, and 0 ppm.

D. Determination

(**a**) *System start-up.*—Begin pumping citric acid donor reagent, **C(b)**, and 2 recipient reagents, **C(a)**(*2*) and **C(c)**, through pump tubes specified in Fig. **990.29**, and carry out start-up procedure described in **990.29E(a)**.

(**b**) *Sample analysis.* Repeatedly inject 15 μL of 40 ppm sulfite working standard solution, **C(f)**(*3*), until peak height is stable and recorder deflection is 60–90% full scale. It may be necessary to adjust recorder span to achieve desired peak height.

Five injections of 40 ppm standard should yield series of 5 peaks with CV for peak heights ≤5%. If this precision is not achieved, determine and correct source of problem.

Once system has stabilized, inject 15 μL portions of sulfite working standard solutions, **C(f)**(*3*), injecting each standard once.

No sample pretreatment is required. Thoroughly mix undiluted wine and inject 15 μL portion directly into FIA system. To prevent loss of free SO_2, protect sample from atmosphere until just before injection. Inject all samples in duplicate. After about 10 sample injections, repeat series of standards.

For wines with free SO_2 levels <3 ppm, increase sensitivity either by injecting >15 μL portion or by decreasing full scale response on detector. Prepare standards in range of samples (e.g., 2 ppm, 1 ppm) and repeat determination of low level samples using standards of 0–3 ppm for calibration.

After all samples have been analyzed, thoroughly rinse FIA manifold by pumping water through all 4 lines for 15 min, followed by FIA rinsing reagent, **C(d)**, for 15 min. Manifold can be stored with this reagent. Release tension on pump rollers to prolong tubing life.

(**c**) *Data reduction.*—Determine peak height for each sample and standard injection to ±0.5 mm. If blank injection produced peak, subtract this peak height from all samples and standards. Average peak heights of each injection for individual standard concentrations and plot peak height vs ppm SO_2.

Determine SO_2 concentration in wine sample directly from standard curve.

Ref.: JAOAC **73**, March/April issue (1990).

CAS-7446-09-5 (sulfur dioxide)

Results and Discussion

To preserve the delicate balance between the free and bound forms of sulfite, efforts were made to minimize sample pretreatment or alteration of the physical conditions in the wine. Within the FIA manifold, the only physical changes the wine undergoes are a drop in pH from the in situ level to about pH 2.2 and a dilution into the donor reagent stream. The wine is exposed to those conditions for only about 20 s. This pH is near the optimum for aldehyde-sulfite complexes (*1*), and yet is sufficiently low to provide excellent response in the FIA system. Under these conditions, no response is observed in the FIA instrument for either the formaldehyde or acetaldehyde addition products of sulfite. Standards are utilized for free sulfite with only ethanol added (to inhibit oxidation of sulfite), and 100% of the sulfite is present in these standards as free sulfite. Configuration of the FIA system in this manner ensures that a response is observed for only free sulfite and that portion of the bound sulfite that is labile under these mild conditions.

To determine how accurately the FIA system was measuring free sulfite, we compared results obtained using the FIA method with those for the aeration/oxidation method (*4*). Results of these comparative studies (Table 1) indicated a fairly good correlation between the 2 methods for red wine and a somewhat poorer correlation for white wine, with FIA values slightly higher. The reason for these discrepancies are likely related to differences in sample handling required for the 2 methods. Differences in the physical conditions to which the sample is exposed could lead to measuring a differing proportion of the loosely bound sulfite.

In addition, we tried to measure accuracy by determining the recovery of free sulfite from spiked wine samples. Unfortunately, recoveries were low (about 20%) because addition of sulfite shifted the equilibrium of free vs bound in favor of the bound form. These studies indicated that no good measure of the accuracy of the FIA method would be available

Table 1. Comparison between the FIA and aeration/oxidation (A/O) methods for determination of free sulfite (ppm SO_2) in wine

Sample	FIA[a]	A/O
White 1	14.1	7.7[b]
White 2	38.7	22.8[b]
White 3	19.1	10.2[b]
Red 1	4.2	4.8[b]
Red 2	7.9	8.7[b]
Red 3	10.7	9.5[b]
Red (536)	0.6	2.2[c]
Red (906)	1.2	3.0[c]

[a] Average of duplicate determinations in the Associate Referee's laboratory.
[b] Average from A/O collaborative study.
[c] Average of duplicate determinations.

and like all free sulfite methods, sample handling conditions define the level of free sulfite found. However, even with these considerations in mind, the FIA method has a number of advantages that make it useful. Treatment conditions are tightly controlled and reproducible (a feature inherent to FIA), so laboratory-to-laboratory and day-to-day fluctuations in results should be low. In addition, FIA methods are well suited to both quality control and research situations. By flow injection analysis, one analyst can analyze about 100 samples per day. Considering these advantages, we determined that an interlaboratory collaborative study was warranted.

Collaborative Study

The collaborative study on free sulfite by FIA was conducted as part of a larger study on total sulfite by FIA (11). The only modifications in the FIA system to enable analysis of free sulfite were to change the sample loop and to replace the donor reagents with the citric acid solution used for free sulfites. The collaborators reported little difficulty with the free sulfite analyses except that some of the samples had very low levels, which involved measuring heights of small peaks.

The study samples were configured to provide 6 sets of blind duplicates randomly numbered and representing both white and red wines. Samples were submitted to 9 laboratories; 8 collaborators returned results. Since the proportion of free to bound sulfite was expected to change over time, all collaborators were requested to analyze the samples over a specified 7 day period. Four of the 8 laboratories observed this time frame and 3 more completed the analyses within the next 7 days. However, one laboratory delayed analysis until 41 days after the receipt of samples. Since free sulfite levels could decrease substantially in wine over this time period, data from that laboratory were eliminated from further consideration.

The results of the collaborative study (Table 2) illustrate the excellent precision of FIA for the determination of free sulfite. At free sulfite levels over 5 ppm, the average reproducibility was 12% (RSD_R) and at levels below 5 ppm it was 26%. As expected, the white wines contained somewhat higher levels of free sulfites than did the red wines. No data were eliminated from the set on the basis of outlier tests (Dixon, Grubbs), and correlation was excellent between the results obtained by the Associate Referee and the collaborators (Table 2).

Table 2. Results of collaborative study on determination of free sulfite (ppm SO_2) in wine by FIA method[a]

	White wine			Red wine		
Coll.	N	O	S	P	Q	T
1	20.0	10.1	7.3	2.2	4.0	3.6
	18.9	10.1	6.9	2.2	3.8	3.2
2	19.7	11.4	6.7	1.9	5.6	3.7
	20.5	11.2	7.0	1.8	5.3	3.7
3	17.1	11.8	6.0	.6	3.3	2.2
	19.1	11.7	7.1	1.0	3.9	2.6
4	18.8	10.7	6.9	1.8	5.3	3.5
	18.5	10.5	6.0	1.7	3.9	3.0
5	21.2	13	6.9	1.2	3.0	2.5
	21.5	12.4	6.8	1.1	3.2	2.5
6	20.2	10.7	7.1	2.2	4.6	3.8
	19.7	12	8.2	2.7	7.3	3.2
7	16.7	8.6	5.0	2.4	4.1	2.9
	15.3	7.8	5.0	2.3	4.3	2.9
Mean	19.1	10.9	6.6	1.8	4.4	3.1
Repeatability						
s_r	0.8	0.4	0.5	0.2	0.8	0.3
RSD_r, %	4.0	4.1	7.4	10.1	18.7	8.3
Reproducibility						
s_R	1.8	1.5	0.9	0.6	1.2	0.5
RSD_R, %	9.4	13.5	13.4	35.8	26.5	17.0
Assoc. Ref. (mean ppm)	20.4	10.5	6.6	2.0	4.3	3.3

[a] Data represent results of paired blind duplicates.

A number of collaborative studies have been conducted on free sulfite methods. A study on a polarographic method for free sulfite in foods and beverages resulted in RSD_r and RSD_R values of 27 and 48%, respectively (5). In an earlier study on free sulfite in wine by the Ripper method, RSD_r and RSD_R values were 18 and 26%, respectively (3). For both of these studies, the majority of samples exceeded 5 ppm apparent free sulfite; levels at which flow injection analysis resulted in RSD_r and RSD_R values of 5 and 12%, respectively. It is likely that the poorer precision in earlier free sulfite methods was caused by the extensive sample handling steps involved and the lower pH specified, which resulted in measurement of bound sulfite that is labile under weak acid conditions.

Conclusions

The FIA method for the determination of free sulfite in wine is a precise and accurate method for that proportion of sulfite liberated from wine at pH 2.2. The fact that this includes both the free sulfite anion and some proportion of the weakly bound sulfite should not detract from utility of the method for rapid quality control or for research. Although RSD_r and RSD_R values for the red wine samples averaged a somewhat high 12 and 26%, respectively, the cause for this can be traced to the extremely low levels of free sulfite in these samples (average 3.1 ppm). The FIA method can be altered to increase sensitivity, which would improve precision for these low-level samples.

Recommendation

It is recommended that the FIA method for the determination of free sulfite in wine be adopted official first action. In addition, it is suggested that a precautionary note be included to state that the method measures free sulfite anion in wine

and any combined sulfite that is labile under the mild exposure conditions used in the FIA manifold.

Acknowledgments

The authors thank the following collaborators:

Shirley Chew, Food and Drug Administration (FDA), San Francisco, CA

Joyce Farley, FDA, Seattle, WA

Kent Faul, FDA, Denver, CO

Larry Habeger, FDA, Minneapolis, MN

Jurgen Moller, Tekator, Hoganas, Sweden

Uldis Sics, FDA, Kansas City, MO

Scott Stieg, Lachat, Oconomowoc, MI

Dalia Stonys, FDA, Detroit, MI

REFERENCES

(1) Wedzicha, B. L. (1984) *Chemistry of Sulphur Dioxide in Foods*, Elsevier Applied Science, Essex, England

(2) Bolin, H. R., Steele, R. J., & Davis, E. G. (1985) *J. Food Sci.* **50,** 1649–1651

(3) Vahl, J. M., & Converse, J. E. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 194–199

(4) Amerine, M. A., & Ough, C. S. (1980) *Methods of Analysis of Musts and Wines*, John Wiley and Sons, New York, NY

(5) Holak, W., & Patel, B. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 572–578

(6) Anderson, C., Warner, C. R., Daniels, D. H., & Padgett, K. L. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 14–19

(7) Ough, C. S. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 5–7

(8) Sullivan, J. J., Hollingworth, T. A., Wekell, M. M., Newton, R. T., & LaRose, J. E. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 542–546

(9) Moller, J., & Winter, B. (1985) *Fres. Z. Anal. Chem.* **320,** 451–456

(10) Application Note, No. ASN 61-21/83, Tecator AB, Hoganas, Sweden

(11) Sullivan, J. J., Hollingworth, T. A., Wekell, M. M., Meo, V. A., Saba, H. H., Etemad-Moghadam, A., Eklund, C., Phillips, J. G., & Gump, B. H. (1990) *J. Assoc. Off. Anal. Chem.* **73,** 35–42

Determination of Ten *N*-Nitrosoamino Acids in Cured Meat Products

JOHN W. PENSABENE and WALTER FIDDLER

U.S. Department of Agriculture, Eastern Regional Research Center, 600 E Mermaid Ln, Philadelphia, PA 19118

A rapid, sensitive, and accurate solid-phase extraction method was developed for the measurement of 10 *N*-nitrosoamino acids (NAAs) in cured meat products. In the procedure, the comminuted meat was mixed with sulfamic acid and Celite, and then added to a glass column containing anhydrous sodium sulfate. The column was washed with pentane, and the NAAs were eluted with ethyl acetate. The eluate was concentrated, then derivatized with diazomethane followed by acetic anhydride–pyridine reagent. The NAA methyl esters and their acylated hydroxy derivatives were separated by gas chromatography on a DB-5 fused silica capillary column and quantitated with a thermal energy analyzer, a chemiluminescence detector specific for nitric oxide derived from the thermal denitrosation of nitrosamines. Recovery of 10 of the NAAs exceeded 75% at the 10 ppb level. The method is applicable to a wide range of cured meat products.

Of all the *N*-nitrosoamino acids (NAAs; Figures 1 and 2), *N*-nitrosoproline (NPRO) and, to a lesser extent, *N*-nitrososarcosine (NSAR) have been the most widely detected in cured meats (1–3), beer and malts (4, 5), fish (6), and tobacco products (7). More recently, *N*-nitrosothiazolidine-4-carboxylic acid (NTHZC) and its 2-hydroxymethyl derivative (NHMTHZC) have been confirmed in meats (3, 8–10), and 3-(*N*-nitroso-*N*-methylamino) propionic acid (NMAPA) and 4-(*N*-nitroso-*N*-methylamino) butyric acid have been found in tobacco and tobacco products (11).

With the exception of NSAR (12, 13), most NAAs have been reported to be noncarcinogenic. They can, however, be converted to carcinogenic *N*-nitrosamines by decarboxylation at the high temperatures that may be encountered during cooking or in burning tobacco. For instance, NPRO forms *N*-nitrosopyrrolidine (14), NSAR forms *N*-nitrosodimethylamine (1), *N*-nitrosohydroxyproline (NHPRO) forms *N*-nitrosohydroxypyrrolidine (15), and NTHZC forms *N*-nitrosothiazolidine (9).

Two NAAs, NPRO and NTHZC, have been proposed as indicators of in vivo nitrosation in human and animal studies by their measurement in urine, because they both appear to be excreted without undergoing metabolic transformation. Other than the potential of NAAs to form aliphatic and alicyclic volatile nitrosamines, NAAs are thought to make up a large portion of the apparent total nonvolatile nitrosamine content of various food products subjected to nitrite treatment or nitrogen oxide exposure (10).

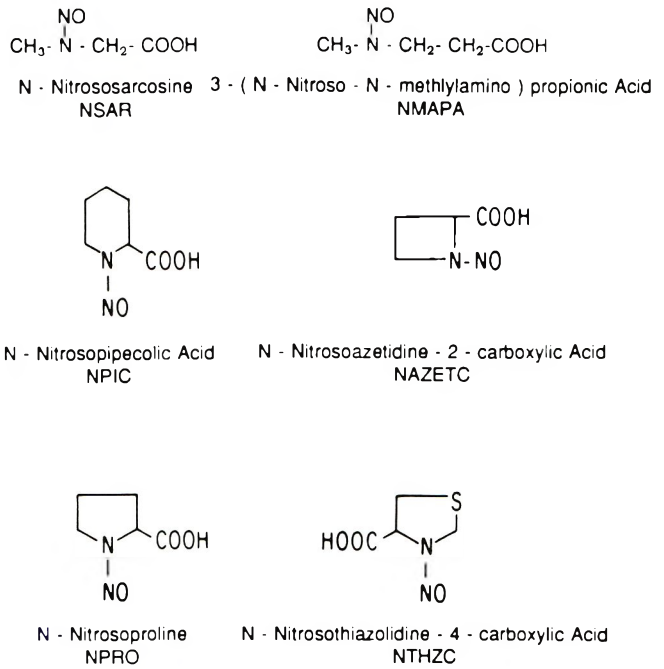
While many procedures have been published for the determination of individual NAAs in various foods and biologically derived samples, to date, all of these methods have been limited to only a few NAAs that can be analyzed at one time. In addition, these methods require lengthy sample preparation and changes to achieve NAA isolation without significant interference even with a specific detector like the thermal energy analyzer (TEA). Detection of nitrosohydroxy-containing amino acids has been especially difficult because of poor chromatographic (GC) separation and peak broadening if the hydroxyl group is not derivatized.

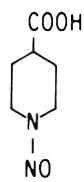
Despite the use of liquid chromatography (LC) with TEA by several researchers, NAA analysis has not been carried out extensively because of the inability to use the requisite reverse-phase solvents with this detector. Therefore, direct analysis of NAAs by LC-TEA has been limited to nonionizable neutral compounds like *N*-nitrosodiethanolamine

Presented in part at the 101st Annual International Meeting of AOAC, San Francisco, CA, Sept. 14–17, 1987.

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

Received May 29, 1989. Accepted September 11, 1989.





N - Nitrosoisonipecotic Acid
NINA

Figure 1. *N*-Nitrosoamino acids.

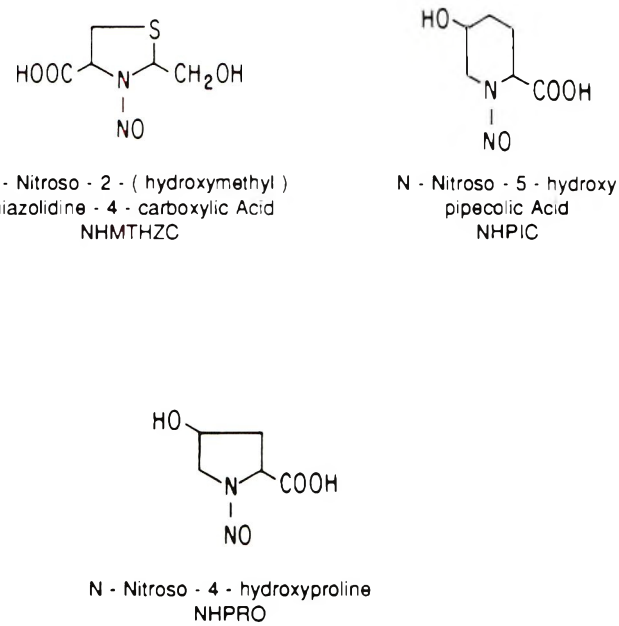


Figure 2. *N*-Nitrosohydroxyamino acids.

(16) and a few NAAs where the ionization is suppressed by the solvents used in the mobile phase (17).

In the present paper, we describe a solid-phase extraction GC-TEA method where a combination of 10 NAAs can be isolated, separated, and detected. A detailed description of this new method and the results obtained from the analysis of selected cured meat samples that demonstrate the applicability of the method are reported herein.

METHOD

Reagents

(a) *Celite 545.*—Not acid-washed (Fisher Scientific Co.).

(b) *Ethyl acetate, n-pentane, pyridine, ethyl ether, and dichloromethane (DCM).*—Distilled-in-glass solvents (Burdick and Jackson Laboratories).

(c) *Sulfamic acid.*—1% in 1N sulfuric acid.

(d) *Diazomethane.*—15 mg/mL. Prepare from *N*-methyl-*N*-nitroso-*p*-toluene-sulfonamide, following directions on the bottle (Aldrich Chemical Co.); store in −20°C freezer until used.

(e) *Acetic anhydride-pyridine reagent.*—Prepare 20 mL reagent by mixing 15 mL acetic anhydride and 5 mL pyridine (prepared fresh monthly).

(f) *N-Nitrosopipecolic acid (NPIC) internal standard solution.*—0.10 μg NPIC/mL in DCM.

(g) *N-Nitrosoamino acids.*—NSAR, NMAPA, *N*-nitrosoazetidine-4-carboxylic acid (NAZETC), NPRO, NPIC, NTHZC, *N*-nitrosoisonipecotic acid (NINA), NHPRO, *N*-nitrosohydroxypipecolic acid (NHPIC), and NHMTHZC (structures are shown in Figures 1 and 2) were synthesized from their corresponding amino acids and sodium nitrite under acidic conditions, and purified by either vacuum distillation or recrystallization according to general procedure described previously (18). Structures were verified by GC-MS as their methyl esters.

(h) *Methyl esters of the N-nitrosoamino acids.*—GC working standard, each 0.10 μg/mL in DCM except for 0.25 μg/mL of NHMTHZC. Esters were prepared separately, then combined to make the standard.

(i) *Silica and Florisil Sep-Pak cartridges.*—Waters Associates.

(j) *Cured meat samples.*—Purchased from local suppliers and ground twice before analysis.

(k) *Other reagents.*—Purchased from local suppliers and used without further purification.

Apparatus

Usual laboratory equipment and the following items:

(a) *Mortar and pestle.*—Glass, 473 mL (16 oz, A. H. Thomas, Co.).

(b) *Chromatographic column.*—Glass, 350 × 32 mm id with 60 × 6 mm id drip tip.

(c) *Rotary evaporator.*—Buchi (Brinkmann Instrument Co.).

(d) *Evapo-mix.*—Buchler Instrument Co.

(e) *Tamping rod.*—Glass, 450 mm long, with 12 mm diameter disk on the end, prepared by glassblower.

(f) *Gas chromatograph-thermal energy analyzer (GC-TEA).*—Varian Aerograph gas chromatograph Model 2700, or equivalent, interfaced with thermal energy analyzer Model 502. Operating conditions: 30 m × 0.527 mm DB-5 fused silica capillary column (J & W Scientific), helium carrier gas at 25 mL/min, column oven programmed from 80 to 200°C at 4°/min; injector port, 200°C; TEA furnace, 485°C; TEA vacuum, 1.0 mm; liquid nitrogen cold trap.

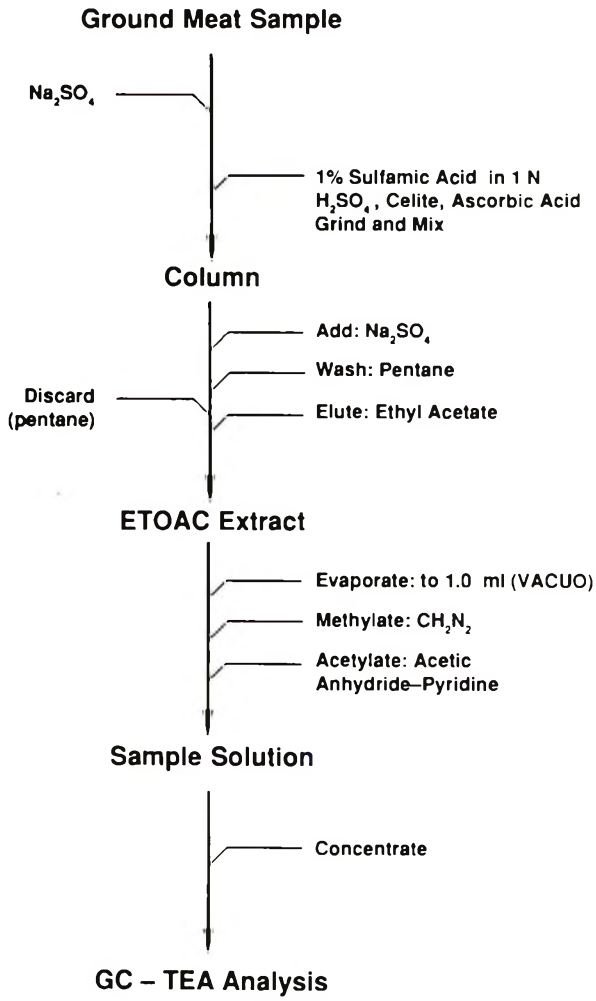


Figure 3. Schematic of *N*-nitrosoamino acid method.

Procedure

(a) *Sample analysis.*—(Flow diagram of method is shown in Figure 3.) Add 30 g anhydrous, granular sodium sulfate to glass column containing glass wool plug at bottom. Accurately weigh 10.0 ± 0.1 g ground meat sample into mortar. Add 1.0 mL NPIC internal standard solution to sample, using 1.0 mL transfer pipet, 0.5 g ascorbic acid, and then 1 mL of 1% sulfamic acid solution. Mix sample with pestle, wait 1 min, add 25 g Celite to mortar, and grind entire mixture gently at first and then with moderate pressure for 1 min.

Quantitatively transfer dry powder mixture to chromatographic column and tamp with tamping rod to achieve overall height of ca 100 mm. Add 15 g anhydrous sodium sulfate to top of column. Rinse mortar and pestle with 25 mL pentane and add rinse to column, immediately followed by 225 mL of the same solvent. Collect eluate in 250 mL graduated cylinder. When level of pentane in column descends to top of sodium sulfate, add 200 mL ethyl acetate. After 225 mL eluate has been collected, change receivers.

Collect remaining eluate in 300 mL round-bottom flask, and reduce solvent on vacuum rotary evaporator to 1 mL (40°C water bath). Quantitatively transfer residues using 1–2 mL ethyl acetate, to 16 × 145 mm test tube. Reduce volume to ca 0.2 mL on Evapo-mix at 40°C or use stream of nitrogen), and then add 3 mL ether solution containing diazomethane while test tube is heated and shaken on Evapo-mix at 40°C for 20 min. Reduce volume to 0.5 mL. Add 0.25 mL acetic anhydride–pyridine reagent, and then heat and shake at 40°C for an additional 45 min. Quntitatively transfer solution to 4 mL concentrator tube, rinsing with DCM, and concentrate solution to 1.0 mL in 80°C water bath using micro-Snyder column.

If further cleanup of sample is necessary due to interfering GC peaks or residue in sample, Silica or Florisil Sep-Pak cartridge can be used with equivalent results. For Silica, dilute sample to 4 mL with pentane–DCM solvent mixture (50 + 50), inject onto Silica cartridge, and collect eluate in 4 mL concentrator tube. Wash cartridge with additional 2 mL solvent mixture. Concentrate sample in water bath to 1.0 mL. For Florisil, concentrate sample to <0.5 mL, and then dilute to 4 mL with pentane. Inject sample mixture onto Florisil cartridge, discarding eluant. Inject 4 mL of DCM–ether solution (70 + 30) onto cartridge and collect eluate in 4 mL concentrator tube. Concentrate sample in water bath to 1.0 mL.

(b) *Nitrosoamino acid determination.*—Inject 5.0 μL of derivatized NAA standard at lowest GC-TEA attenuation that yields peaks suitable for quantitation (3 × signal/noise). Repeat standard injection to ensure reproducibility of retention time and response. Inject 5.0 μL sample solution and measure peak heights. Presence of solvents other than DCM will not significantly change response to nitric oxide generated from nitrosoamino acids. Volatile nitrosamines, if present, will elute with initial pentane wash from column.

(c) *Statistical analysis.*—Statistical analyses were carried out according to the methods of Snedecor and Cochran (19).

Results and Discussion

In developing an analytical procedure for the determination of NAAs, emphasis was placed on deriving a rapid and reliable method for simultaneously determining a number of NAAs that are likely to be present in complex food substrates. The majority of published methods use some form of extraction, solvent partitioning, and lengthy centrifugation of the sample prior to quantitation of the NAAs directly by LC or after derivatization prior to GC. These methods are inherently slow, they are often plagued by emulsion problems due to fat in the samples, and they are limited in the number and type of NAAs that can be simultaneously separated on a chromatographic column. We have previously developed methods for volatile *N*-nitrosamines in foods based on solid-phase extraction techniques (20, 21). These methods have proved to be so versatile that we were able to use the same extraction principle for the isolation of both simple and hydroxylated NAAs from cured meat samples. Dull et al. (22) recently analyzed urine samples for NPRO using a variation of this solid-phase extraction procedure.

To ensure destruction of residual nitrite, and to prevent artifactual NAA formation, sulfamic acid was added to the sample matrix. This, in addition to ascorbic acid, a proven nitrosation inhibitor, helped ensure against this possibility. The sulfamic acid was also used as an acidulant to help facilitate NAA extraction.

The isolation of the NAAs from the food matrix was not the major obstacle encountered in developing this procedure. Instead, the selection of the best technique for the optimum derivatization of the NAAs and selection of a GC column that could simultaneously separate both nonhydroxylated and hydroxylated NAAs required the most effort.

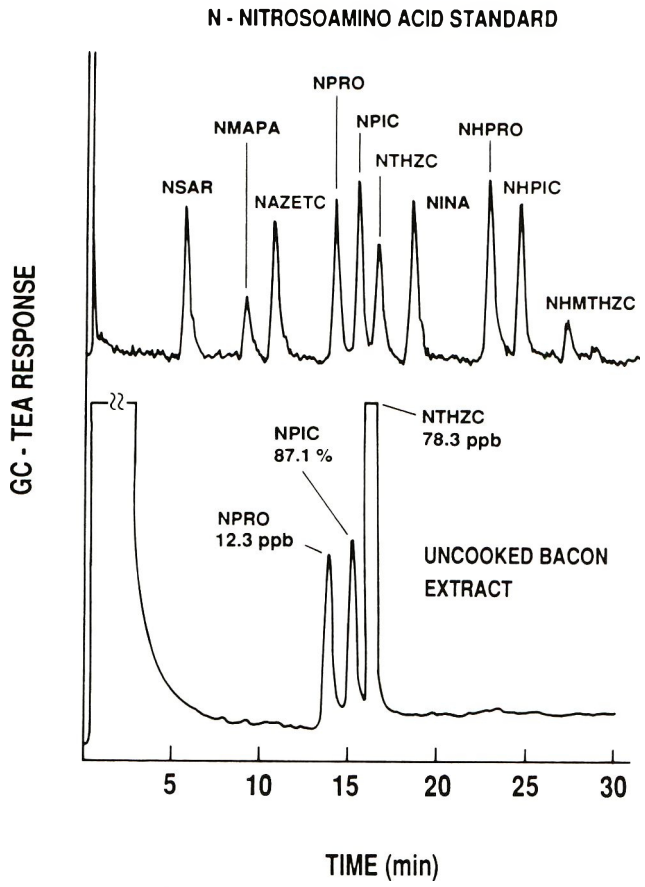


Figure 4. GC-TEA chromatogram of *N*-nitrosoamino acid standard and sample.

There are many methods used for derivatizing NAAs; however, the most frequently used involve some form of methylation (23) or silylation (24). Preparing volatile GC derivatives of the simple NAAs works well using either of these 2 common methods, whereas complete derivatization of the hydroxylated NAAs has always been less than favorable (25). It has been reported that the GC analysis of the methyl ester of NHPRO exhibits a low TEA response due to peak broadening caused by nonspecific adsorption and the free hydroxyl group (25). We found this to be true not only with NHPRO but also with NHPIC and NHMTHZC.

Ohshima and Kawabata (26) studied this problem and found that acylation with acetic anhydride–pyridine was best for derivatizing 6 hydroxylated nitrosamines. In a mixture of both simple and hydroxylated NAAs, we also found that methylation followed by acylation proved to be the most reliable method for preparing volatile GC derivatives. Conversion of the carboxyl groups of the 10 NAA standards at the 10 ppb level to their methyl esters with diazomethane immediately followed by acylation of the 3 hydroxy groups (Figure 2) with acetic anhydride–pyridine was always greater than 95%. If the samples were derivatized with acetic anhydride–pyridine only or acylated and then methylated, the overall conversion varied but was consistently less than 70%.

For separation and subsequent quantitation of the NAAs by GC-TEA, most published methods are quite similar. Sen and Kubacki (27) published a review of methods for determining derivatized, nonvolatile *N*-nitroso compounds in foods and included particular GC columns used by various researchers. The main criteria for successful determination of NAAs must be that the column can resolve the NAAs and that no non-nitrosamine peaks interfere with the quantitation of these compounds.

Our investigation of GC columns showed that packed columns, regardless of the mobile phase, were inadequate for separating mixtures of derivatized NAAs. For example, 5% Silar 10 CP on 100–120 mesh Supelcoport was good for the simple NAA derivatives, but was unsuitable for all the hydroxy NAA derivatives. OV-225 was usable for the hydroxy NAA derivatives, but, when they were combined with the other NAA derivatives, the long retention times of NHPRO, NHPIC, and NHMTHZC caused unacceptable peak broadening. We found that a DB-5 capillary column was able to adequately separate the 10 NAA derivatives with good resolution of the peaks (Figure 4, standard). Minimum detectable levels were NSAR, NAZETC, NPRO, NTHZC, NINA, NHPRO, NHPIC, 2 ppb; NMAPA, ppb 5; NHMTHZC, 10 ppb. The only compound that gave a doublet on this column was NHMTHZC; no other NAA compound gave an indication of its syn and anti conformers. Sen et al. (28) recently reported that this NHMTHZC could be decarboxylated during cooking to form 2-(hydroxymethyl)-*N*-nitrosothiazolidine in fried bacon.

The recovery of the 10 NAAs added to nitrite-free pork belly at the 10 ppb level was determined to evaluate the efficiency of this method. The results were NSAR, 83.9 ± 8.7%; NMAPA, 77.0 ± 11.7%; NAZETC, 76.7 ± 8.9%; NPRO 86.7 ± 6.5%; NPIC, 85.1 ± 3.5%; NTHZC, 87.8 ± 5.2%; NINA, 86.0 ± 7.0%; NHPRO, 79.1 ± 3.2%; NHPIC, 84.6 ± 3.8%; NHMTHZC, 80.9 ± 6.4%. Statistical analysis of the results showed that the NPIC internal standard was significantly correlated ($P < 0.05$) with NSAR, NAZETC, NPRO, NTHZC, and NHPRO.

A ruggedness test of the method for the determination of 10 ppb of each NAA showed that packing the extraction column too tightly (less than 100 mm) could cause NAA values to vary significantly. We have observed this same phenomenon when applying the solid-phase extraction method for determining *N*-nitrosopyrrolidine in fried bacon and *N*-nitrosodimethylamine in minced fish and surimi-meat frankfurters (20, 21). The method was also checked for artifactual nitrosoamino acid formation by spiking cured pork belly samples with 25 ppb thiazolidine carboxylic acid prior to analysis, and then checking for NTHZC after sample workup; none was detected.

Six cured meat samples were analyzed in duplicate to determine the within-laboratory repeatability of the method. NSAR ranged from 3.5 to 6.5 ppb (corrected), NPRO from 3.2 to 17.2 ppb (corrected), and NTHZC from 15.0 to 517.8 ppb (corrected); no other NAAs were detected in these samples. The recovery of the NPIC internal standard (10 ppb) ranged from 72.2 to 98.0% with a mean of 85.1%. The analysis of variance showed that the variable within-laboratory repeatability standard deviation for the 3 NAAs found was 1.1 ppb for NSAR, 1.6 ppb for NPRO, 3.9 ppb for NTHZC, and 5.1% for recovery of NPIC internal standard.

To check the applicability of this method, 15 samples from 7 different cured meat products (cure-pumped and dry-cured bacon, pork side meat, frankfurter, ham, pepperoni, and lebanon bologna) were analyzed for NAAs. NTHZC was the only compound found in all samples, ranging from 27.9 to 3661 ppb. This is consistent with our previous finding of this nitrosamine in a wide variety of smoked products (8). NPRO

(ND to 156.9 ppb) and NSAR (ND to 5.6 ppb) were also detected in these samples. Recovery of the NPIC internal standard averaged 86.3%. These results indicate that the present method can be used to analyze meat products for NAAs. Sample chromatograms of a standard and a cured meat product extract are shown in Figure 4.

We have developed a method for the simultaneous isolation and quantitation of 10 NAAs that is accurate, precise, and specific. The method is easy to perform and can be applied to varying types of cured meat products.

Acknowledgments

The authors thank Robert A. Gates for the statistical analysis of the data and Mark A. Hayes of J & W Scientific, Inc., for use of the DB-5 capillary column.

REFERENCES

(1) Janzowski, C., Eisenbrand, G., & Preussmann, R. (1978) *Food Cosmet. Toxicol.* **16,** 343–348
(2) Pensabene, J. W., Feinberg, J. I., Piotrowski, E. G., & Fiddler, W. (1979) *J. Food Sci.* **44,** 1700–1702
(3) Tricker, A. R., Perkins, M. J., Massey, R. C., Bishop, C., Key, P. E., & McWeeny, D. J. (1984) *Food Addit. Contam.* **1,** 245–252
(4) Pollock, J. R. A. (1981) *J. Inst. Brew.* **87,** 356–359
(5) Sen, N. P., Tessier, L., & Seaman, S. W. (1983) *J. Agric. Food Chem.* **31,** 1033–1036
(6) Sen, N. P., Tessier, L., Seaman, S. W., & Baddoo, P. A. (1985) *J. Agric. Food Chem.* **33,** 264–266
(7) Brunnemann, K. D., Scott, J. C., & Hoffmann, D. (1983) *J. Agric. Food Chem.* **31,** 905–909
(8) Pensabene, J. W., & Fiddler, W. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 1077–1080
(9) Sen, N. P., Seaman, S. W., & Baddoo, P. A. (1985) *Food Technol.* **39,** 84–88
(10) Massey, R. C., Crews, C., Dennis, M. J., McWeeny, D. J., Startin, J. R., & Knowles, M. E. (1985) *Anal. Chim. Acta* **174,** 327–330
(11) Ohshima, H., Nair, J., Bourgade, M.-C., Friesen, M., Garren, L., & Bartsch, H. (1985) *Cancer Lett.* **26,** 153–162
(12) Wogan, G. N., Pagilialunga, S., Archer, M. C., & Tannenbaum, S. R. (1975) *Cancer Res.* **35,** 1981–1984
(13) Preussmann, R., & Stewart, B. W. (1984) *N-Nitroso Compounds in Chemical Carcinogens*, ACS Monograph 182, ACS, Washington, DC, pp. 643–828
(14) Pensabene, J. W., Fiddler, W., Gates, R. A., Fagan, J. C., & Wasserman, A. E. (1974) *J. Food Sci.* **39,** 314–316
(15) Lee, J. S., Bills, D. D., Scanlan, R. A., & Libby, L. M. (1977) *J. Agric. Food Chem.* **25,** 422–423
(16) Rosenberg, I. E., Gross, J., & Spears, T. (1980) *J. Soc. Cosmet. Chem.* **31,** 237–252
(17) McWeeny, D. J. (1983) *Food Chem.* **11,** 273–287
(18) Pensabene, J. W., Fiddler, W., Dooley, C. J., Doerr, R. C., & Wasserman, A. E. (1972) *J. Agric. Food Chem.* **20,** 274–277
(19) Snedecor, G. W., & Cochran, W. G. (1979) *Statistical Analysis*, 6th Ed., Iowa State University Press, Ames, IA
(20) Pensabene, J. W., Miller, A. J., Greenfield, E. L., & Fiddler, W. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 151–156
(21) Pensabene, J. W., & Fiddler, W. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 839–843
(22) Dull, B. J., Hotchkiss, J. H., & Vecchio, A. J. (1986) *Food Chem. Toxicol.* **24,** 843–845
(23) Sen, N. P., Donaldson, B. A., Seaman, S., Iyengar, J. R., & Miles, W. F. (1978) *Environmental Aspects of N-Nitroso Compounds*, E. A. Walker, M. Castegnaro, R. E. Lyle, & L. Griciute (Eds), IARC Scientific Publication No. 19, IARC, Lyon, pp. 373–393
(24) Eisenbrand, G., Janzowski, C., & Preussmann, R. (1975) *J. Chromatogr.* **115,** 602–606
(25) Ishibashi, T., Kawabata, T., & Tanabe, H. (1980) *J. Chromatogr.* **195,** 416–420
(26) Ohshima, H., & Kawabata, T. (1979) *J. Chromatogr.* **169,** 279–286
(27) Sen, N. P., & Kubacki, S. J. (1987) *Food Addit. Contam.* **4,** 357–383
(28) Sen, N. P., Seaman, S. W., Baddoo, P. A., & Weber, D. (1989) *J. Agric. Food Chem.* **37,** 717–721

METHOD PERFORMANCE

Diagnostic Data Evaluation. Part I. Collaborative Studies: How To Do It

ANTHONY J. MALANOSKI
U.S. Department of Agriculture, Food Safety and Inspection Service, Washington, DC 20250

A generic protocol is described to add diagnostic data evaluation to collaborative study evaluations. The protocol would enable the individual performing the evaluation to determine more specifically the causes for unsuccessful studies. The protocol also formalizes the techniques for establishing performance standards for both the analysts and the method.

The evaluation of collaborative studies is usually limited to the simple determination of whether or not the resulting data meet the requirements that have been established for a successful study. The minimum diagnostic analysis that is performed is primarily of a statistical nature. For example, outliers can be and have been eliminated on the basis of statistical tests such as the Dixon (1), Grubbs (2, 3), or Youden ranking (4). A complete diagnostic evaluation of collaborative studies provides additional information on causes for a less than successful collaborative study. The techniques that will be described for the evaluation of collaborative studies are applicable to the evaluation of other data sets.

One important criterion must be observed for the diagnostic data evaluation to be meaningful. The evaluation must be performed by a chemist who is familiar with the analytical procedure and statistics, or jointly by a chemist and a statistician. Final decisions as to the acceptability of any of the data are the primary responsibility of the chemist; acceptability is based on the knowledge of the chemist about the chemical processes of the method. It is a professional judgment that cannot be assigned to any individual who does not have knowledge of the chemistry and the acceptable variability associated with each individual process. A statistician seldom has the opportunity to become familiar with each separate process that is included in the entire method.

Preliminary standards of performance for the analytical method should be established before any evaluation is initiated. The performance standards are best established by a chemist with training in statistics, or jointly by the chemist and a statistician. Data acceptability, in general, would then be based on the preliminary standards, and on the chemist's and statistician's knowledge, experience, and professional judgment.

The statistics that will be used for the diagnostic data evaluation of the collaborative study will be limited to descriptive statistics. Descriptive statistics are a tool used by chemists to describe or characterize a method with quantitative descriptors. It enables the chemist to establish standards of performance for a method which the chemist then uses to determine when the analyst is competent to perform the analysis or when additional training should be suggested.

The search for objective means to characterize and measure the performance of an analytical method resulted in a review of some of Youden's early publications (4–7). Youden's statement that the collaborative test could also be used to evaluate the individual analyst's performance set the dual purpose of establishing standards of performance for a method and for analysts. The primary emphasis of AOAC has been method evaluation. With the interest in analytical quality control (QC) and quality assurance (QA) programs, the evaluation of analysts and laboratories has become more important than method evaluation. The technique used for diagnostic data evaluation provides a means of defining the performance of the individual analyst.

In the statistical evaluation of collaborative studies, the standard deviation or variance that is determined for repeatability is the average (pooled) of the standard deviations or variances of the analysts, independent of analyst bias. Many collaborative studies do not have an adequate number of observations from each analyst to determine repeatability for each analyst. Increasing the number of data points for each participant is necessary and has been a practice of the Chemistry Division of the Food Safety and Inspection Service for method or analyst validations (Validation process, FSIS Good Regulatory Analytical Laboratory Practice, in preparation). The number of results must be increased to provide sufficient data points to also determine individual analyst performance, as proposed previously by the author (8).

With the emphasis on QC and QA programs and analytical productivity in the recent years it has become necessary to establish standards of performance for the analysts as well as for the method. When standards of performance are established for the method and are used in the laboratory, single sample analysis can be justified (Single, duplicate, replicate analysis, FSIS Good Regulatory Analytical Laboratory Practice, in preparation). Ideally, the standards of performance should be established from a collaborative study (8). The collaborative study protocol should, therefore, evaluate the performance of the method and of the analyst(s).

The basic purposes of evaluation of collaborative studies (and other studies) by chemists are (*1*) to provide estimates of the repeatability and reproducibility of an analytical method; (*2*) to validate the preliminary performance standards of the method and of the analysts; (*3*) to determine that the analyst performance standards meet established performance standards; (*4*) to establish product standards; (*5*) to determine that the established product standards are met.

The requirements for these different purposes may not be identical but they are similar enough that the same techniques for data evaluation are used. First and foremost it must be understood that the chemist is concerned only with describing what occurred during the course of the analytical process that will provide information to make professional decisions about the acceptability of the results. Chemists are not concerned with making inferences about processes or products for which the analyses are performed. It is the statistician's responsibility to make the inferences required about the product or the process. The primary responsibility of the regulatory analytical chemist is to provide the best analytical results possible with the method and to describe or

Received February 3, 1987. Resubmitted November 9, 1988. Accepted February 20, 1989.

characterize what has been done so scientists and managers who are not familiar with the specific method can make professional decisions about the acceptability of the data.

The prerequisites to perform data evaluation are (*1*) a working knowledge of descriptive statistics, as provided by a basic course in statistics; (*2*) a knowledge of the chemical processes of the analytical method and the instruments used; (*3*) a scientific calculator with a statistical function which uses the value (n − 1) in the denominator to determine the variance or standard deviation. With the availability of the personal computers and appropriate software, the diagnostic data evaluation can be performed more efficiently.

Several assumptions concerning the samples and the competence of the analysts must be made and are the foundation of all data evaluations: (*1*) the samples submitted for analysis are homogeneous and are representative of the product; (*2*) all analysts are competent to perform the analysis; (*3*) the average of all reported results for the sample is the best estimate of the quantity of the analyte in the product.

These assumptions must be considered valid until the evaluation process raises questions about either sample homogeneity or the analyst's competence to perform the analysis. If the initial data evaluation is successful, then all assumptions were valid. It is when the study is less than successful that it is necessary to perform the diagnostic evaluation to identify the causes of the failure. For natural products, one of the major causes of failure is sample nonhomogeneity. The second factor in the failure of method studies is that participating analysts may not have had sufficient training and experience with the method. The 2 factors confound the problem of identifying the root cause of the failure. Diagnostic data analysis as proposed in this manuscript is an attempt to resolve this problem.

Descriptive Statistics

The basic descriptive statistics used in the evaluation process are listed below:

(*1*) The averages (x) for:
- a. the sample ($\bar{x}$),
- b. the analyst's normalized results ($\bar{x}_a$) (described later).

(*2*) The standard deviations (s) for:
- a. the method repeatability (s_o),
- b. the method reproducibility (s_x),
- c. the analyst's repeatability (s_a).

(*3*) The relative standard deviations (RSD) for:
- a. the method repeatability RSD (RSD_o),
- b. the method reproducibility RSD (RSD_x),
- c. analyst's repeatability RSD (RSD_a).

(*4*) The critical differences (r) ($s*2*2^{0.5} = 2.83$):
- a. the method repeatability (r_o) for:
  - i. initial values [$2.83*s_o$ (s constant) or $2.83*\bar{x}*RSD_o$ (RSD constant)],
  - ii. normalized differences [$2.83*s_o$],
  - iii. normalized ratios [$2.83*RSD_o$].
- b. the method reproducibility (R) for:
  - i. initial values [$2.83*s_x$ (s constant) or $2.83*\bar{x}*RSD_x$ (RSD constant)],
  - ii. normalized differences [$2.83*s_x$],
  - iii. normalized ratios [$2.83*RSD_x$].
- c. the analyst's repeatability (r_a) for:
  - i. normalized differences [$2.83*s_a$],
  - ii. normalized ratios [$2.83*RSD_a$].

(*5*) The critical difference (r_{o99}) (99% confidence interval: $s*2.59*2^{0.5} = 3.65$):
- a. the method repeatability for:
  - i. initial results [$3.65*s_o$ (s constant) or $3.65*\bar{x}*RSD_o$ (RSD constant)],
  - ii. normalized differences [$3.65*s_o$],
  - iii. normalized ratios [$3.65*RSD_o$].
- b. the method reproducibility (R_{99}) for:
  - i. initial results [$3.65*s_x$ (s constant) or $3.65*\bar{x}RSD_x$ (RSD constant)],
  - ii. normalized differences [$3.65*s_x$],
  - iii. normalized ratios [$3.65*RSD_x$].
- c. the analyst's repeatability (r_{a99}) for:
  - i. normalized differences [$3.65*s_a$],
  - ii. normalized ratios [$3.65*RSD_a$].

The critical differences are and have been used to determine the need for additional analyses whenever analytical values are available from 2 laboratories or when duplicate analyses were performed. Whenever a critical difference is exceeded, additional analyses of the sample or product are indicated. The use of the standard deviation or relative standard deviation of the individual analyst has been possible only since it became possible to determine them for the individual. The repeatability statistics for the "method" are used only when the statistic for analyst's repeatability is not available.

The nature of the chemical process determines which of the above statistics are applicable and should be used in the evaluation process. In particular, the standard deviation and the relative standard deviation are statistics that should seldom be used to evaluate the same process. If all reactions are stoichiometrically complete, all transfers are quantitatively complete, and recoveries of the analyte are constant, then the standard deviation can be considered to be constant over some range that can be determined. If, however, the reactions are not complete and/or unfavorable partitions occur, then the coefficient of variation will be constant over a range that can be determined.

Performance Standards

The need for clearly defined method performance standards and the techniques for establishing them was noted in 1982 (9). It is with established performance standards that critical differences can be used to identify suspect results. It is possible to identify initial performance standards with quantitative descriptors from the initial method evaluation by the chemist who develops the method. That initial method characterization should have a sufficient quantity of data to provide the applicable analytical range of the analysis, the average recovery (where the recovery is applicable), and the s_a or RSD_a values for a single analyst. An error analysis can be performed to verify the first estimate for s_a or RSD_a (Error analysis in method evaluation, FSIS Good Regulatory Analytical Laboratory Practice, in preparation). When insufficient data are available, these estimates can be used: the first approximation for s_o or RSD_o should be 1.2 times the s_a or RSD_a value, and the first approximation for s_x or RSD_x should be 1.5 times the s_o or RSD_o value.

The standards that should be determined for a method and should be included in the written description are as follows:

(*1*) The applicable range for the analytical method.

(*2*) The standard deviations for:

a. method repeatability (s_o),
b. method reproducibility (s_x),
c. individual analyst repeatability (s_a).

(*3*) The relative standard deviation for:
a. method repeatability (RSD_o),
b. method reproducibility (RSD_x),
c. analyst repeatability (RSD_a).

(*4*) Averages
a. recovery with the acceptable range; determined with the formula, $s_x*2/n^{0.5}$,
b. normalized average for either s or RSD as appropriate.

(*5*) Correlation coefficient (wherever 3 or more data points at different levels are available, linear regression should be performed; number of data points used in the regression should be listed).

The tests that may be used to evaluate the data for outliers are the Grubbs test, the Dixon test, and the Youden ranking test. The 3 tests are to be used only when it is not possible to determine or estimate the critical differences for the method from s_a, s_o, and s_x. The introduction of standards of performance for the method and for the analyst eliminate the need to submit the data to statistical outlier tests. The critical differences are used for the determination of "performance outliers" on the basis of Good Regulatory Analytical Laboratory Practices. Whenever an analyst's blind duplicate results or whenever 2 laboratories' results exceed the appropriate critical difference, the samples should be reanalyzed and the causes of the excessive difference should be determined. The normalized average is used in place of the Youden ranking test to determine analyst's bias. It is possible and appropriate to eliminate the statistical outlier tests because performance standards are or will be established and are used.

One factor not mentioned previously that must be emphasized is that analyses for regulatory purposes must be performed by analyst(s) who have demonstrated their competence to perform that analysis. For regulatory purposes, the acceptable "average analysts" are only those in the higher percentile of the acceptable laboratories (the acceptable percentile is determined by the regulatory agency). With Good Laboratory Practices, diagnostic data evaluation identifies the analyst who may require additional training. The present protocol for diagnostic data evaluation was not written with that restriction. It can be used to identify analysts who may require additional training and may provide the opportunity to salvage a collaborative study.

Data sets and the evaluation of those data sets are part of a hierarchy which begins with the evaluation of the data from a single analyst or laboratory. The purpose of the data set from one laboratory is either an estimate of repeatability as a preliminary performance standard or to demonstrate the analyst's readiness to perform analysis. The second category, to evaluate factors for QC/QA purposes, also consists of data from a single analyst or laboratory. Standards of performance for the method have been established and are available. The third category of data for evaluation is a 3-or-more-laboratory method validation study which is used to determine method performance characteristics and to establish analyst performance standards. The fourth category in the data evaluation hierarchy is the evaluation of a method collaborative study. The only difference between the 2 types of method studies is the number of laboratories that participate. The final data sets that are subjected to statistical analysis are check sample data that are used for analyst or laboratory QC/QA purposes.

Data Evaluation

The operational sequence for data evaluation is as follows:

(*1*) List the performance standards for the method.

a. If the purpose of study is to establish performance standards, then list the standards that are the goal for the method.

b. If standards of performance have been established for the method and the evaluation is done for QC/QA purposes, list the performance standards.

(*2*) List the data in a format that would permit the data to be easily scanned and manipulated for statistical analysis, either manually or by computer.

(*3*) Determine $\bar{x}$, s_x, and RSD_x for each sample, following the procedures of Youden or Steiner (5). Or, alternately, use Wernimont (9).

(*4*) Compare the s_x or the RSD_x values to the proposed or established performance standards of the method.

a. If the performance standards or the proposed standards of the method are met, then no additional diagnostic analysis may be necessary.

b. If the performance standards are not met, additional diagnostic analysis may be necessary. The critical differences for analyst duplicates and between-laboratories results must be reviewed to assure that each sample was homogeneous and that each analyst had achieved the acceptable level of competence.

(*5*) If it is applicable, determine the least square linear regression equations for each analyst. Compare the correlation coefficients, the slopes, and the intercepts for consistency and to determine if established performance standards are met. Inconsistencies should be identified so that when considered with the other diagnostic information they could be excluded from statistical analysis.

(*6*) Determine the differences of the extreme values for each sample and compare them to the critical difference values, R and R_{99}.

a. If the difference of the extreme sample values exceeds the R_{99} value, subtract the sample average from each of the suspect results. Exclude the sample result with the higher absolute difference. Recalculate $\bar{x}$, s_x, and RSD_x values for the samples. Compare the recalculated values to the standards.

b. If the s_x or the RSD_x performance standard has not been met, calculate the difference value of the extreme sample values of the remaining data.

c. If the difference value exceeds the critical value of R or R_{99}, the sample may not be homogeneous or an analyst may not be performing adequately. Do not include the data in any pooled evaluation for the estimation of the applicable range or pooled standard deviation or the coefficient of variation until the data have been tested for analyst repeatability.

(*7*) Normalize the data.

a. If s is constant, subtract the sample average from each result.

b. If RSD is constant, divide each result by the sample average.

(*8*) Delete and do not include in the statistical evaluation any individual normalized value that exceeds $3s_x$ (s constant) or exceeds the range of $1.000 \pm 3RSD$ (RSD constant). This may be considered an arbitrary rule, but any individual value

that is more than 3 standard deviations from the average value should not be considered a valid result (analysis should be repeated after the sample is remixed).

(*9*) Determine $\bar{x}_a$, s_a, and RSD_a.

(*10*) Compare s_a and RSD_a values to the established or proposed standards for s_a and RSD_a. If either is equal to or less than the performance standards, no additional diagnostic evaluation is necessary.

(*11*) If the performance standards are not met, determine the difference of the extreme values of the normalized data for the analysts who have not met the s_a or RSD_a standards and compare them to the r_a and r_{a99} critical difference values.

a. If s is constant, exclude the higher absolute value of any difference that exceeds the r_{a99} critical difference. Determine $\bar{x}_a$, s_a, and RSD_a.

b. If RSD is constant, subtract 1.000 from each value of any pair that exceeds the r_{a99} critical difference. Exclude the higher absolute value and determine $\bar{x}_a$, s_a, and RSD_a.

(*12*) If the performance standards are met with the exclusion of one value from an analyst, no additional diagnostic analysis is necessary. If the performance standards are not met, then the data from the analysts who have not met the standards must be examined more intensely. In the same manner, the reported values for each sample are examined if the standards for reproducibility have not been met.

(*13*) Examine the normalized data for each analyst who did not meet the s_a or RSD_a standards, to determine if any additional differences between the remaining extreme values are equal to or less than the critical difference values of r_a or r_{a99} critical differences. List all differences that exceed the r_a or r_{a99} differences. Subtract the values from 0 (s constant) or subtract 1.000 (RSD constant) from each pair of values. Identify the value that has the higher absolute value for consideration for exclusion.

(*14*) Examine the normalized data for any sample that did not meet the standards for s_x or RSD_x. List any differences between extreme sample results that are equal to or greater than the R and R_{99} critical difference. Subtract 0 (s constant) or 1.000 (RSD constant) from the individual values. Identify the analytical value that has the highest absolute difference for consideration for exclusion.

(*15*) Tabulate the values for the samples and the analysts that were the cause for the critical differences to be exceeded. In addition to the values identified in sections 13 and 14, add any value identified in the earlier analysis.

(*16*) The process of identifying data that when excluded from the statistical analysis would cause the performance standards to be met is a matter of trial and error and professional judgment.

Usually, only 2 sources of excessive variation can be identified. The major cause of variation is that due to the individual analyst which can be evaluated by means of either s_a or RSD_a. The second is homogeneity which can be evaluated by means of the critical differences, r_a and R. The basic purpose of diagnostic evaluation is to identify and delete from the statistical analysis the minimum quantity of data that can be identified as a cause of failure to meet performance standards. The deleted data, if the opportunity had been available, would have been subjected to reanalysis according to Good Regulatory Analytical Laboratory Practices. One of the exceptions to the exclusion of the minimum amount of data is the exclusion of all data for an analyst if more than 20% of the analyst's data is suspect. This rule is based on the percentage of data that can be excluded from a successful AOAC collaborative study and on the maintenance of scientific integrity of the study. When more than 20% of the data are excluded and the remainder of the data are included, the charge can be made that only selected data are being included. The second exception would be the exclusion of all data for a sample if sample nonhomogeneity is suspected.

a. Delete and do not include in the further statistical evaluation individual values that cause a critical difference greater than r_{a99}.

b. Delete and do not include in the further statistical evaluation any values that cause a critical difference greater than R_{99}.

c. Delete all data for a sample or analyst if 3 or more data points in the sample or analyst's data set exceed R or r_a.

d. Delete the data from any analyst with an $\bar{x}_a$ that is not in the 95% confidence range. The range for the difference normalized average is $0 \pm s_x*2/n^{0.5}$. The range for the ratio normalized average is $1 \pm 2\ RSD_x/n^{0.5}$.

(*17*) Tabulate the remaining data and determine (if sufficient data remain) the $\bar{x}$, s_x, s_o, RSD_x, and RSD_o according to Youden or Steiner (5). Normalize the acceptable data with the sample averages of the acceptable data and determine $\bar{x}_a$, s_a, and RSD_a. All performance standards should be met at this time; if they are not, a review of the method, the proposed performance standards, and the quality controls for the analysis is required to determine the causes for the failure.

(*18*) Determine the standards of performance of the method. The performance standards are based on s_a, s_o, and s_x when s is constant or on RSD_a, RSD_o, and RSD_x when RSD is constant.

a. s_a or RSD_a: Square the s_a or RSD_a values for each analyst. Determine the average and the standard deviation for the squared values. The square root of the sum of the average and twice the standard deviation is the s_a or the RSD_a of the performance standard.

b. s_o or RSD_o: Square the s_o or the RSD_o value of each of the samples of the study. Determine the average and the standard deviation for the squared values. The square root of the sum of the average and twice the standard deviation is the s_o or the RSD_o of the performance standard.

c. s_x or RSD_x: Square the s_x or the RSD_x values of each of the samples. Determine the average and the standard deviation of the squared values. The square root of the sum of the average and twice the standard deviation becomes the s_x or the RSD_x of the performance standard.

(*19*) The Grubbs, Dixon, and Youden ranking tests are to be used primarily to evaluate data for the exclusion of statistical outliers in the determination of quality control standards and factors.

Summary

The diagnostic techniques described enable the chemist with the aid of a statistical calculator to evaluate collaborative studies with a minimum assistance from a statistician. If a personal computer is available with appropriate software, the analysis can be accomplished even more efficiently. More important, the techniques provide the means for identifying a less than successful study. When performance standards are used, statistical tests for outliers are not necessary. The standard deviations or the relative standard deviations from the performance standards, either known or proposed, are the basis for determining outlying results.

The techniques of the previous sections are used and will be discussed in the additional parts of the Diagnostic Data Evaluation series: Part II. Collaborative Study Evaluation: Coefficient of Variation Considered To Be A Constant; Part III. Collaborative Study Evaluation: Standard Deviation Considered To Be A Constant; Part IV. Evaluation of A Check Sample Series To Determine Analyst Performance; Part V. Ruggedness "Stress" Test Evaluation; Part VI. Data Evaluation To Establish Product Standards.

REFERENCES

(1) Dixon, W. J. (1953) *Biometrics* **19,** 74–89
(2) Grubbs, F. E. (1969) *Technometrics* **11,** 1–21
(3) Grubbs, F. E. (1972) *Technometrics* **14,** 847–848
(4) Youden, W. J. (1961) *Materials, Research and Standards*, pp. 862–867
(5) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
(6) Youden, W. J. (1962) *J. Assoc. Off. Anal. Chem.* **45,** 169–173
(7) Youden, W. J. (1967) *J. Assoc. Off. Anal. Chem.* **50,** 1007–1013
(8) Malanoski, A. J. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 1333–1338
(9) Wernimont, G. T. (1985) *Use of Statistics to Develop and Evaluate Analytical Methods*, W. Spendley (Ed.), AOAC, Arlington, VA

Diagnostic Data Evaluation. Part II. Collaborative Study Evaluation: Coefficient of Variation Considered To Be A Constant

ANTHONY J. MALANOSKI
U.S. Department of Agriculture, Food Safety and Inspection Service, Washington, DC 20250

The principles described in Part I were used to determine standards of performance for the method and for analysts from a collaborative study of the determination of nitrosopyrrolidine (NPYR). Performance standards for the method were 10.4% RSD_a, 9.2% for RSD_o, and 14.2% for RSD_x.

The usual assumption that has been a standard in the collaborative study evaluation process of the Association of Official Analytical Chemists is that the chemists who participate in the studies are competent and can perform the analysis satisfactorily. This assumption is stated by Youden (1) and may have been to some extent misinterpreted by many of his successors. The purpose of the collaborative study was to determine the performance of the method as conducted by the "average analyst." However, no provisions were ever made for determining an average analyst or the standards of performance to be used to judge the competence of analysts.

For regulatory analytical purposes, "average analysts" should be those analysts contained in the 90th or 95th percentile of all laboratories. The analysts who are not in the acceptable range should not perform regulatory analysis until the causes of the higher variance or bias are corrected by additional training. The normalization evaluation process that is the basis of the present series of reports is to provide an objective measure of "bias" (bias as used in this evaluation process is not the difference from the "true value" but the difference from the average of all participating analysts/laboratories) of both analyst repeatability and laboratory reproducibility. With the emphasis on quality control and quality assurance of the last few years, this objective evaluation is increasingly needed.

The evaluation process should be more than a statistical evaluation of the data. A diagnostic analysis should be added to identify analysts who have not demonstrated the competence to perform the analysis. Two basic premises must be made and accepted in all analytical work. The first is that the analyst will perform all tasks of the method in the same manner each and every time the analysis is performed. The second is that over the range of interest the function of the method is continuous, that is, the standard deviation or the relative standard deviation (as appropriate) can be considered to be constant for concentrations of the analyte over the range of interest. With these assumptions, it is possible to establish and describe performance standards by means of standard, simple descriptive statistics. It cannot be overemphasized that performance standards are established using the professional judgment of the chemist. Descriptive statistics are only the tool used to provide the basis for making the decision on the applicable standard.

The data that will be used in the example evaluation are taken from a nitrosamine collaborative study (2). Only the data for *N*-nitrosopyrrolidine (NPYR) will be used. To establish performance standards, it is not necessary but it is useful to perform an error analysis of the method to obtain an estimate of the repeatability for the performance that may be obtained (Error analysis in method evaluation, FSIS Good Regulatory Analytical Laboratory Practice, in preparation). If the error analysis is not done, then it is possible that the standards that would be established may not truly represent the method. This is particularly true when the empirical formula developed by Horwitz (3) is applied to residue methods. For the mineral oil vacuum distillation method used for the NPYR collaborative study, the relative standard deviation (RSD_x) of 30% would have been acceptable because the level of the study was in the part per billion range. When the error analysis was performed, the estimate for the RSD_a was 10% and the RSD_x was 15%.

Performance Standards

The proposed performance standards are:

$$RSD_a = 10\%$$
$$RSD_x = 15\%$$
$$R = (\bar{X}_1 - \bar{X}_2)/n^{0.5}*2*2^{0.5}$$
$$R_{99} = (\bar{X}_1 - \bar{X}_2)/n^{0.5}*2.58*2^{0.5}$$

Received February 3, 1987. Resubmitted November 9, 1988. Accepted February 20, 1989.

Table 1. *N*-Nitrosopyrrolidine (ppb) collaborative study data

	Samples								
Anal.	1	2	3	4	5	6	7	8	9
1	4.08[a]	5.90[a]	9.66	12.8	14.0	18.9	9.68	17.4	13.2
	3.92[a]	8.25[a]	9.67	12.8	14.1	18.2	10.5	18.0	14.1
					15.9		11.2	17.4	
					16.0			16.2	
2	3.0	7.4	8.6	10.4	15.0	18.0	9.86	21.2	10.1[a]
	3.11	7.6	9.5	12.1	13.3	18.8	8.5	18.0	16.6
					12.8		9.2	17.2	
					13.8			15.8	
3	2.67[a]	8.01	9.75	12.3	15.2	18.7	8.80	21.7	16.3
	2.68[a]	7.66	9.11	12.0	14.8	18.1	9.55	21.6	15.9
					14.1		10.60	23.0	
					13.7			20.2	
4	3.32	7.33	8.87	12.1	14.0	17.8	10.7	18.0	17.3
	2.86	7.37	9.38	11.8	14.0	17.8	10.6	18.1	15.6
					14.4		10.5	17.7	
					15.2			19.1	
5	3.28	8.37	10.5	12.4	15.1	19.4	10.7	20.7	18.2
	3.75	7.85	10.0	13.4	15.6	19.2	10.4	22.1	16.6
					17.5		11.2	22.3	
					16.1			21.2	
6	3.2	5.6	7.7	11.0	12.2	14.7	10.4	15.8	15.5
	3.0	7.1	7.5	11.0	14.0	16.8	9.7	14.7	12.6
					11.6		8.7	14.5	
					12.5			14.3	
7	3.30	7.66	8.90	10.6	15.3	17.2	10.5	13.2[a]	17.9
	—	8.21	8.73	12.8	13.0	23.2[a]	—	18.7	17.2
					15.8		11.4	16.0	
					14.9			21.4	
8	3.47	6.66	9.40	10.8	11.3	16.2	9.82	15.8	18.0
	3.12	7.08	8.19	12.5	14.3	15.3	9.01	15.5	14.5
					12.3		10.30	15.3	
					14.6			15.8	
9[b]	2.34	5.66	6.11	9.31	9.90	15.3	11.10	17.8	10.8
	2.35	4.47	6.43	7.53	8.14	11.4	7.22	13.8	12.2
					14.8		6.80	14.3	
					11.5			12.9	
All data:									
$\bar{x}$	3.144	7.156	8.778	11.536	13.909	17.5	9.882	17.686	15.444
s_x	0.49	1.08	1.19	1.45	2.86	2.45	1.17	2.87	2.49
RSD_x	15.6	15.1	13.6	12.6	13.4	14.0	11.8	16.2	16.4
Data for analyst 9 excluded:									
$\bar{x}$	3.219	7.464	9.091	11.925	14.263	17.67	10.079	18.216	15.967
s_x	0.247	0.693	0.816	0.906	1.405	1.398	0.826	2.644	1.733
RSD_x	7.7	9.3	9.0	7.6	9.9	7.9	8.2	14.5	10.9

[a] Data not included in the statistical analysis.
[b] All data for analyst 9 excluded from the statistical analysis.

$$r_a = (\bar{x}_1 - \bar{x}_2)/n^{0.5}*2*2^{0.5}$$
$$r_{a99} = (\bar{x}_1 - \bar{x}_2)/n^{0.5}*2.58*2^{0.5}$$
$$\bar{x}_a = 1 \pm RSD_x*2/n^{0.5}$$
$$\bar{x}_{a99} = 1 \pm RSD_x*2.58/n^{0.5}$$

where $\bar{x}$ = the average of the replicate analytical values for the same sample; $\bar{x}_a$ = the average of analytical values for the same analyst; n is the number of replicates or values in the determination of the average; and * = the symbol for multiplication.

Experimental

The fortification levels for the study for samples 1–6 were 0.00, 4.89, 6.84, 9.78, 12.7, and 16.6 ppb, respectively. Samples 7, 8, and 9 were bacon from normal production and did not require fortification. The experimental work is described in ref. 1.

Data Evaluation

The raw data are listed in Table 1. The averages, standard deviations, and relative standard deviations were determained for each sample with a hard-wired scientific calculator. The RSD_x values ranged from 12.6 to 16.4% and, by visual observation, were sufficiently homogeneous to determine a pooled RSD_x. The diagnostic evaluation was performed to determine the RSD_o, the RSD_a, and the pooled RSD_x to establish the performance standards.

Diagnostic Evaluation

To determine the linearity of the analytical determinations

Table 2. Normalized NPYR collaborative study data

Anal.	Sample 1	2	3	4	5	6	7	8	9	Analyst statistics
1	1.298	0.824	1.100	1.110	1.007	1.080	0.980	0.984	0.872	$\bar{x}$ = 1.055
	1.247	1.153	1.102	1.110	1.014	1.040	1.062	1.018	0.931	s = 0.113
					1.143		1.133	0.984		RSD = 10.7
					1.150		0.916			
2	0.954	1.034	0.980	0.902	1.078	1.028	0.998	1.199	0.667[a]	$\bar{x}$ = 0.988
	0.989	1.062	1.082	1.049	0.956	1.074	0.860	1.018	1.096	s = 0.105
					0.920		0.931	0.973		RSD = 10.6
					0.992		0.893			
3	0.849	1.119	1.111	1.056	1.093	1.069	0.891	1.227	1.076	$\bar{x}$ = 1.069
	0.852	1.070	1.038	1.040	1.064	1.034	0.966	1.221	1.050	s = 0.110
					1.014		1.073	1.300		RSD = 10.3
					0.985			1.142		
4	1.056	1.024	1.010	1.049	1.007	1.017	1.083	1.018	1.142	$\bar{x}$ = 1.037
	0.910	1.030	1.065	1.023	1.007	1.017	1.073	1.023	1.030	s = 0.044
					1.035		1.062	1.001		RSD = 4.3
					1.093			1.080		
5	1.043	1.170	1.196	1.075	1.086	1.108	1.083	1.170	1.202	$\bar{x}$ = 1.146
	1.193	1.097	1.139	1.162	1.122	1.097	1.052	1.250	1.096	s = 0.064
					1.258		1.113	1.261		RSD = 5.6
					1.158			1.199		
6	1.018	0.782	0.877	0.954	0.877	0.840	1.052	0.893	1.024	$\bar{x}$ = 0.910
	0.954	0.992	0.854	0.954	1.007	0.960	0.982	0.831	0.832	s = 0.079
					0.834		0.880	0.820		RSD = 8
					0.899			0.808		
7	1.050	1.070	1.014	0.919	1.100	0.983	1.062	0.746	1.182	$\bar{x}$ = 1.063
	—	1.147	0.994	1.110	0.935	1.326[a]	—	1.057	1.136	s = 0.125
					1.136		1.154	0.905		RSD = 11.8
					1.071			1.210		
8	1.104	0.931	1.071	0.936	0.812	0.926	0.994	0.893	1.189	$\bar{x}$ = 0.970
	0.992	1.073	0.933	1.084	1.028	0.874	0.912	0.876	0.957	s = 0.950
					0.884		1.042	0.865		RSD = 9.8
					1.050			0.893		
9[b]	0.744	0.791	0.696	0.807	0.712	0.874	1.123	1.006	0.713	$\bar{x}$ = 0.778
	0.747	0.625	0.732	0.653	0.584	0.651	0.731	0.780	0.806	s = 0.133
					1.064		0.688	0.808		RSD = 17.1
					0.827			0.729		
All data:										
n	17	18	18	18	36	18	26	36	18	
$\bar{x}$	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
s_x	0.157	0.151	0.136	0.126	0.134	0.140	0.118	0.162	0.164	
RSD_x	15.7	15.1	13.6	12.6	13.4	14.0	11.8	16.2	16.4	
Data for analyst 9 excluded:										
n	15	16	16	16	32	15	23	32	15	
$\bar{x}$	1.034	1.036	1.035	1.034	1.026	1.010	1.020	1.021	1.054	
s_x	0.133	0.110	0.093	0.079	0.101	0.080	0.084	0.155	0.1145	
RSD_x	12.8	10.6	9.0	7.6	9.9	7.9	8.2	15.2	10.9	

[a] Data excluded from statistical analysis.
[b] Data of analyst 9 excluded from the statistical analysis.

for each analyst, the least square linear regression was performed on the fortified sample results. For determination of the regression equation, x was the analytical value and y was the fortification level. It was also necessary to calculate the regression equation to predict the concentration of NPYR present in the bacon that was fortified. The intercept is the best estimate of the NPYR concentraton in the bacon that was fortified. It has a negative value because the prediction is made for the concentration of the NPYR that was added. The equations and the correlation coefficients are:

Analyst	*Linear regression equation*	*Correlation coefficient*
1	y = 1.0758x − 3.543	0.9857
2	y = 1.1022x − 3.004	0.9879
3	y = 1.0744x − 3.111	0.9959
4	y = 1.1174x − 3.397	0.9978
5	y = 1.0194x − 3.484	0.9935
6	y = 1.2458x − 3.199	0.9825
7	y = 0.9043x − 1.331	0.9730
8	y = 1.2510x − 3.957	0.9730
9	y = 1.2068x − 0.8612	0.9143

Analysts 7 and 9 have correlation coefficients and intercepts that appear to be different from the values for the other analysts. The diagnostic evaluation is necessary to determine if some or all of the data should be excluded from the statistical analysis.

Table 3. Extreme differences between analyses for the same sample for normalized NPYR data

	Sample								
Anal.	1	2	3	4	5	6	7	8	9
1	0.051	0.329[a]	0.002	0.000	0.143	0.040	0.153	0.102	0.059
2	0.035	0.028	0.102	0.107	0.158	0.054	0.138	0.306[a]	0.429[b]
3	0.003	0.049	0.073	0.026	0.079	0.035	0.182	0.158	0.026
4	0.146	0.014	0.055	0.022	0.086	0.000	0.011	0.022	0.112
5	0.150	0.073	0.057	0.087	0.172	0.011	0.081	0.029	0.106
6	0.064	0.110	0.023	0.000	0.130	0.120	0.172	0.085	0.192
7	—	0.077	0.020	0.191	0.201	0.343	0.092	0.464[b]	0.046
8	0.112	0.142	0.138	0.142	0.238	0.052	0.130	0.017	0.232
9	0.003	0.166	0.036	0.152	0.480[b]	0.223	0.435[b]	0.277	0.093

Critical differences:

$r_a = 0.10*2*2^{0.5} = 0.28$

Analyst 1, sample 2
Analyst 2, sample 8

$r_{a99} = 0.10*2.58*2^{0.5} = 0.36$

Analyst 2, sample 9
Analyst 7, sample 8
Analyst 9, sample 5
Analyst 9, sample 7

[a] Critical difference exceeded for r_a.
[b] Critical difference exceeded for r_{a99}.

Since the RSD is constant, the data was normalized by dividing the individual analytical values by the sample average. The normalized values, the averages, the standard deviations, and the relative standard deviations for each sample are listed in Table 2. The statistics were determined as a quality control check on the normalization. The average for each sample should be 1.000 and the RSD_x values should be the same as the values for the raw data. The statistics ($\bar{x}_a$, s_a, and RSD_a) are also listed in Table 2. Analysts 1, 2, 3, 7, and 9 have RSD_a values that are larger than the proposed performance standard. For analyst 9, the RSD_a and $\bar{x}_a$ of 17.1% and 0.778, when combined with the linear regression equation intercept, support the exclusion of all data on the basis that the analyst did not have sufficient experience with the method and did not demonstrate analytical competence in the analysis. All of the data from that analyst were excluded from the subsequent statistical evaluation.

The diagnostic data evaluation was continued with an examination of the critical differences to determine the cause for the differences for analyst 7 in the linear regression equation. With replicate sample analysis, 3 critical differences can be used to identify possible performance outliers. Two are based on r_a and the third is based on R. The first critical difference (r_a) is the difference between the 2 repli-

Table 4. Sample averages of normalized NPYR data

	Samples								
Anal.	1	2	3	4	5	6	7	8	9
1	1.273	0.988	1.101	1.110	1.078	1.060	1.058	0.976	0.901
2	0.972	1.048	1.031	0.976	0.986	1.051	0.930	1.021	0.881
3	0.850	1.094	1.074	1.053	1.039	1.051	0.977	1.222	1.149
4	0.983	1.027	1.037	1.036	1.035	1.017	1.073	1.030	1.086
5	1.118	1.133	1.167	1.118	1.156	1.102	1.089	1.222	1.149
6	0.986	0.887	0.865	0.954	0.904	0.900	0.971	0.838	0.928
7	—	1.108	1.004	1.014	1.060	1.154	1.108	0.979	1.159
8	1.048	1.002	1.002	1.010	0.943	0.900	0.983	0.881	1.073

Critical differences between samples for the same analyst:

$r_a = 0.10*2*2^{0.5}/2^{0.5} = 0.200$

$r_{a99} = 0.10*2*2^{0.5}/2^{0.5} = 0.258$
Analyst 1 samples 1–9 0.372
Analyst 3 samples 8–1 0.372

Critical differences between analysts for the same sample:

For sample 1
$R_{99} = 0.15*2*2^{0.5}/2^{0.5} = 0.390$
Analysts 1–3 0.423

For sample 8
$R_{99} = 0.15*2*2^{0.5}/4^{0.5} = 0.28$
Analysts 3–6 0.384
Analysts 5–6 0.384
Analysts 3–8 0.341
Analysts 5–8 0.341
Analysts 3–1 0.246
Analysts 5–1 0.246
Analysts 3–7 0.241
Analysts 5–7 0.241

Table 5. Compilation of performance outliers

	Sample					
Anal.	1	2	6	7	9	8
1	1.273[ab]	0.824 1.153 (r_a = 0.329)			0.901[a]	0.976
2					0.667 1.096 (r_{a99} = 0.429)	1.021
3	0.850[ab]					1.222[a]
4						1.030
5						1.222
6						0.838
7			0.983 1.326 (r_a = 0.343)	0.746 1.210 (r_{a99} = 0.464)		0.979
8						0.881

[a] 0.372 exceeds the r_{a99} of 0.258.
[b] 0.423 exceeds the R_{99} of 0.390.

cates or the difference between the extreme values if there are more than 2 replicate values. The differences are listed in Table 3. The differences that exceed the critical differences are also listed. The second critical difference (r_a) is the difference between the extreme values for the average value for each sample for each analyst. The averages of the normalized analytical results are listed in Table 4. The third critical difference (R) is the difference between the extreme values of the averages of the analysts. These differences are calculated from Table 4. The differences that exceed the critical difference are identified and listed with Table 4.

Table 5 is the compilation of the normalized sample values that exceed the performance standard for critical differences by any of the 3 methods. The determination of performance outliers is made after a review of the compiled data and, when necessary, with an additional review of Table 4 to determine if there are any additional differences between results that exceed the critical difference for either analyst or sample.

Two samples, sample 9 for analyst 2 and sample 6 for analyst 7, have normalized differences of 0.429 (1.096 − 0.667) and 0.343 (1.326 − 0.983), respectively, that exceed the critical difference r_{a99} value of 0.36. The result that is the performance outlier of a pair is the sample result that has the larger absolute difference from 1.000. The sample results represented by the normalized values of 0.667 and 1.326 are excluded as performance outliers and are excluded from any subsequent statistical evaluation.

Two analysts have differences between samples that exceed the critical difference of r_{a99} of 0.258 for the sample averages. They are analyst 1 with a difference of 0.372 between samples 1 and 9 for the average values of 1.273 and 0.901, and analyst 3 with a difference of 0.372 between samples 1 and 8 for the average values of 0.850 and 1.222. The average values of 1.273 and 1.222 have the larger absolute difference from the normalized sample average and the analytical results that are included in the average must be considered for exclusion as performance outliers. The between-sample difference of 0.285 between samples 1 and 2 exceeds the r_{a99}; therefore, the results for sample 1 of analyst 1 are excluded as performance outliers. One value for sample 2 of analyst 1 was also identified as a performance outlier. With the identification of 3 of 4 results at the lowest level of the study as outliers, all results of analyst 1 at the lowest levels were excluded on the basis that the analyst had not achieved the necessary proficiency for the analysis of NPYR at these levels.

For analyst 3, the largest difference from the normalized sample average is 1.227 for sample 8. However, there are also differences between sample 1 and other samples that exceed the r_a of 0.20 (samples 1 and 2, 1 and 3, and 1 and 9). The results that are identified as the outlier values are those for sample 1. The values are excluded because the analyst had not achieved the required proficiency at the lowest level of the study.

Sample 8 for analyst 3 was not excluded because of the large number of differences between analysts for the sample that exceeded the R and r_{99} values. The causes for the greater variability for this sample may be due either to sample nonhomogeneity, to the method not being applicable at this level, or to the analysts not having the proficiency to recover NPYR quantitatively at this level.

Table 6 is the normalized data after the exclusion of all performance outliers. The statistical evaluation was performed with and without data for sample 8.

To determine the concentration of NPYR in the control bacon sample, the least square linear regression equations were calculated after the exclusion of the performance outliers. The revised equations are:

Analyst	*Linear regression equation*	*Correlation coefficient*
1	y = 1.0668x − 3.427	0.9764
2	y = 1.1022x − 3.004	0.9879
3	y = 1.1068x − 3.566	0.9924
4	y = 1.1117x − 3.397	0.9978
5	y = 1.0194x − 3.484	0.9935
6	y = 1.2458x − 3.199	0.9825
7	y = 1.0954x − 3.249	0.9753
8	y = 1.2510x − 3.957	0.9730

The intercept values range from 3.004 to 3.957 ppb NPYR; for practical reasons, the average of the intercept values can be considered to be the best estimate of the NPYR in the bacon that was fortified. The average value is 3.41 ppb NPYR. It was necessary to determine the best estimate of the

Table 6. Normalized NPYR collaborative study data with performance outliers omitted

	Analyst							
Sample	1	2	3	4	5	6	7	8
1		0.932		1.031	1.019	0.994	1.025	1.078
		0.966		0.888	1.165	0.932		0.969
2		0.991	1.073	0.982	1.121	0.750	1.026	0.892
		1.018	1.026	0.987	1.052	0.951	1.100	1.029
3	1.063	0.946	1.073	0.976	1.155	0.847	0.979	1.034
	1.064	1.045	1.002	1.032	1.100	0.825	0.960	0.901
4	1.073	0.872	1.031	1.015	1.039	0.922	0.889	0.906
	1.073	1.015	1.006	0.990	1.124	0.922	1.073	1.048
5	0.982	1.052	1.066	0.982	1.059	0.855	1.073	0.792
	0.989	0.933	1.038	0.982	1.094	0.982	0.912	1.003
	1.115	0.898	0.989	1.010	1.227	0.813	1.108	0.852
	1.122	0.968	0.961	1.066	1.129	0.876	1.045	1.034
6	1.069	1.018	1.058	1.007	1.097	0.832	0.973	0.916
	1.030	1.064	1.024	1.007	1.086	0.950		0.866
7	0.960	0.978	0.873	1.062	1.062	1.032	1.042	0.974
	1.042	0.843	0.948	1.052	1.032	0.962		0.894
	1.111	0.913	1.052	1.042	1.111	0.863	1.131	1.022
8	0.955	1.164	1.191	0.988	1.136	0.867		0.867
	0.988	1.038	1.186	0.944	1.213	0.807	1.027	0.851
	0.955	0.944	1.263	0.972	1.224	0.796	0.787	0.840
	0.889	0.867	1.109	1.049	1.164	0.785	1.175	0.867
9	0.827		1.021	1.084	1.140	0.971	1.121	1.127
	0.883	1.040	0.996	0.977	1.040	0.789	1.077	0.908
All data:								
n	19	22	21	23	23	23	19	23
$\bar{x}_a$	1.010	0.978	1.047	1.008	1.113	0.884	1.032	0.943
s_a	0.084	0.077	0.087	0.042	0.061	0.079	0.083	0.089
RSD_a	8.3	7.8	8.3	4.2	5.5	8.9	8.1	9.1
Data for sample 8 excluded:								
n	15	18	17	19	19	19	16	19
$\bar{x}_a$	1.027	0.972	1.014	1.009	1.098	0.898	1.033	0.961
s_a	0.085	0.064	0.052	0.044	0.053	0.078	0.073	0.088
RSD_a	8.3	6.6	5.1	4.4	4.9	8.6	7.1	9.1

NPYR in the bacon that was fortified to determine the recovery of the NPYR added to the fortified samples. The 2 formulas to determine recovery are:

Formula 1: Recovery, % = $(x_i - B)\ 100\%/F$
Formula 2: Recovery, % = $x_i\ 100\%/(F + B)$

where x_i = the individual analytical value; B = the average value for NPYR in the control sample; and F = the fortification level. Formula 2 provides the best estimate of NPYR recovery for each analyst and sample because the level of the NPYR in the control sample is significant.

The data were analyzed according to Steiner (1) and the recoveries, standard deviations, and relative standard deviations are listed in Table 7. The RSD results are equal to or lower than the proposed standard of performance.

Table 8 is a compilation of all analyst average recoveries, standard deviations, and relative standard deviations for the fortified samples determined by the 2 recovery determination formulas. The pooled method recovery, s_o, and RSD_o for all analysts are calculated with and without the data of analyst 9.

When the first formula is used, the underlying assumption is that the recovery of NPYR in the control is constant in all

Table 7. Tabulated statistics for NPYR collaborative study (performance outliers excluded)

Sample	$\bar{x}$	Red[a]	s_o	s_L[b]	s_x	RSD_o	RSD_L[b]	RSD_x
1	3.21	94.1	0.246	0.086	0.261	7.7	2.7	8.1
2	7.46	89.9	0.536	0.457	0.705	7.2	6.1	9.4
3	9.09	88.7	0.451	0.703	0.836	5.0	7.7	9.2
4	11.92	90.4	0.859	0.299	0.909	7.2	2.5	7.6
5	14.26	88.5	1.030	0.970	1.490	7.2	6.8	10.0
6	17.71	88.5	0.694	1.319	1.490	3.4	7.4	8.4
7	10.00	—	0.675	0.461	0.817	6.8	4.6	8.2
8	18.21	—	1.300	2.372	2.704	7.1	13.0	14.8
9	15.92	—	1.403	1.156	1.818	7.0	7.3	11.4

[a] The recovery was determined with Formula 2: % R = $(x_i\ 100\%)/(F + B)$.
[b] Laboratory bias = s_L and RSD_L.

Table 8. Compilation of the recoveries ($\bar{x}_a$), s_a, and RSD_a for the fortified samples by 2 recovery formulas

	Formula 1				Formula 2			
Anal.	n	$\bar{x}_a$	s_a	RSD_a	n	$\bar{x}_a$	s_a	RSD_a
1	12	90.65	13.65	15.1	14	96.1	11.6	12.1
2	12	85.3	7.11	8.3	14	88.0	5.1	5.8
3	12	90.8	4.4	4.9	14	89.0	6.0	6.7
4	12	87.3	3.4	3.9	14	89.5	3.4	3.8
5	12	100.2	5.9	5.9	14	99.2	5.1	5.1
6	12	71.8	10.2	14.2	14	80.0	7.5	9.4
7	11	91.5	12.7	13.9	13	92.7	9.5	10.3
8	12	80.0	10.2	12.8	14	85.5	8.8	10.3
9	12	53.4	17.3	32.4	14	65.6	10.6	16.2
	n	$\bar{x}$	s_o	RSD_o	n	$\bar{x}$	s_o	RSD_o
Pooled	107	83.44	10.39	12.5	125	87.29	7.96	9.1
Exclude analyst 9:								
Pooled	95	87.2	9.16	10.5	111	90.0	7.57	8.4

Table 9. Tabulated analyst normalized averages ($\bar{x}_a$) and confidence intervals for the average

Anal.	n	$\bar{x}_a$	95% Confidence interval	99% Confidence interval
1	19	1.010	0.936–1.064	0.917–1.083
	15	1.027	0.928–1.072	0.907–1.093
2	22	0.978	0.940–1.060	0.923–1.077
	18	0.972	0.934–1.066	0.915–1.085
3	21	1.047	0.939–1.061	0.921–1.079
	17	1.014	0.932–1.068	0.912–1.088
4	23	1.008	0.942–1.058	0.925–1.075
	19	1.004	0.936–1.064	0.917–1.083
5	23	1.113[a]	0.942–1.058	0.925–1.075
	19	1.098[a]	0.936–1.064	0.917–1.083
6	23	0.884[a]	0.942–1.058	0.925–1.075
	19	0.898[a]	0.936–1.064	0.917–1.083
7	19	1.032	0.936–1.064	0.917–1.083
	16	1.033	0.930–1.070	0.910–1.090
8	23	0.943	0.942–1.058	0.925–1.075
	19	0.961	0.936–1.064	0.917–1.083

[a] Data considered for exclusion, but not excluded for practical purposes because results for NPYR are reported to single decimal place for values below 10 ppb and to whole integers above 10 ppb.

determinations. This is not consistent with the chemistry of the process. Use of the second formula assumes that the recovery of NPYR in the control sample is a variable and that when losses occur the same percentage loss occurs in both the fortified NPYR and the control NPYR. Recoveries determined with Formula 2 are 3.85% higher for all analysts and 2.8% higher after the exclusion of analyst 9. The pooled s_o and RSD_o are also lower with Formula 2. For the determination of recoveries where the analyte is present at concentrations almost equal to the lowest level studied, Formula 2 is recommended for use.

The normalized data of Table 6 were reviewed for analyst bias. The 95% confidence range for acceptable $\bar{x}_a$ is $1 \pm 0.0283RSD_x/n^{0.5}$. The 99% confidence range for $\bar{x}_a$ is $1 \pm 0.365RSD_x/n^{0.5}$. Table 9 tabulates $\bar{x}_a$ and the acceptable ranges for those normalized averages. Analyst 5 has a high bias at the 99% confidence level, while analyst 6 has a low bias also at the 99% confidence level. The most probable cause of bias is an error in the NPYR standard of 3–5% in each laboratory, with one being high and the second being low.

The standards of performance for RSD_a, RSD_o, and RSD_x were calculated with the following procedure: (*1*) Square the individual appropriate RSD. (*2*) Determine the average and the standard deviation. (*3*) The square root of the sum of the average and twice the standard deviation is the performance standard.

Conclusions

The standards of performance for the method are:

Applicable range:	3.0–20.8 ppb
RSD_a:	10.4%
RSD_o:	9.2%
RSD_x:	14.2%
r:	$2*2^{0.5}*RSD_a*(x_1 + x_2)/2 = 0.147*(x_1 + x_2)$
R:	$2*2^{0.5}*RSD_x*(X_1 + X_2)/2 = 0.201*(X_1 + X_2)$

where x_1 = analyst's first result; x_2 = analyst's second result; X_1 = result from the first laboratory; X_2 = result from the second laboratory.

References

(1) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA
(2) Greenfield, E. L., Smith, W. J., & Malanoski, A. J. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 1319–1332
(3) Horwitz, W., Kamps, L. R., & Boyer, K. W. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 1344–1354

MICROBIOLOGICAL METHODS

Dry Rehydratable Film for Enumeration of Total Aerobic Bacteria in Foods: Collaborative Study

MICHAEL S. CURIALE and THERESE SONS
Silliker Laboratories, 1304 Halsted St, Chicago Heights, IL 60411
J. SUE McALLISTER and BARBARA HALSEY
3M Microbiology Products, 3M Center 270-3N-04, St. Paul, MN 55144
TERRANCE L. FOX
3M Riker, 3M Center 270-3A-01, St. Paul, MN 55144

Collaborators: T. Baldy; M. Brodsky; E. Föllmi; A. Ghafoor; B. Halsey; A. Izat; M. Laws; P. Mah; J. S. McAllister; J. McGregor; P. McGrew; C. Okolo; D. Richter; L. Roth; S. Sandoval; D. Shafer; S. Sharp; T. Sons; K. Swanson

A rehydratable dry-film plating procedure for aerobic plate counts has been compared to the standard agar plate method (966.23B and C, 15th ed.; 46.014–46.015, 14th ed.) in a collaborative study by 12 laboratories. Each laboratory analyzed the normal microflora of 3 samples in duplicate for 6 products. The aerobic plate counts ranged from 1.0×10^3 to 1.0×10^8 cfu/g. The products were flour, nuts, frozen raw shrimp, spice, frozen raw ground turkey, and frozen and refrigerated vegetables. Repeatability standard deviations of the 2 methods did not differ significantly for 13 of 18 test samples. For 1 shrimp and 2 turkey samples, the dry-film method had lower repeatability variances ($P < 0.05$) and for 1 spice sample the agar method had lower repeatability variances ($P < 0.05$). Relative standard deviations of repeatability were between 1.7 and 15.5% for the dry-film method and 1.2 and 16.0% for the agar method. Relative standard deviations of reproducibility ranged from 2.4 to 23.4% for the dry-film method and 2.3 to 18.8% for the agar method. The dry rehydratable film method has been adopted official first action for determination of the aerobic plate count.

The rehydratable dry-film plating method has been evaluated in 2 previous collaborative studies, first, for use with raw and pasteurized milk (1) and then for all dairy foods (2). Those methods have been adopted by AOAC, **986.33,** 15th ed. (**46.B05–46.B07,** 14th ed.) and **989.10,** 15th ed. (**46.E01–46.E04,** 14th ed.), respectively (3). The efficacy of the dry-film method has been well documented for these applications (4–11).

The dry-film plating method has been shown to be useful for the accurate determination of aerobic plate counts for a broad variety of food types in independent studies. It has been compared to the standard agar method for determining the aerobic counts in poultry (11–13); ground beef (11, 14); vegetables (15); and raw ground pork, pecans, thyme, and wheat flour (11). Correlations between the 2 plate media have also been reported for black pepper, corn meal, paprika, peanut butter, pecans, thyme, flour, blue crab, frozen egg, and potatoes (McAllister, Halsey, and Fox, personal communication). It was concluded on the successes of these studies that an interlaboratory collaborative study be undertaken with a representative set of samples to satisfy AOAC guidelines (16).

Received for publication August 2, 1989.
This report was presented at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO.
The recommendation was approved interim official first action by the General Referee, the Committee on Microbiology and Extraneous Materials, and the Chairman of the Official Methods Board. The method was adopted official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* 73, January/February issue.

Collaborative Study

Test Products

Six food types were chosen for this study. Flour (white, wheat, rye), nut halves, raw shrimp, spices (pepper, thyme, onion powder), ground turkey, and vegetables (refrigerated mushrooms, frozen carrots, frozen peas) were selected to provide sufficient variation to represent most food types. In addition, these foods provide a challenge to both test methods because they include a variety of factors that can interfere with enumeration. Interference can be caused by particulates that obscure colonies, by gel or agar liquefying organisms, by spreading organisms, or by high levels of mold.

Preparation of Test Samples

Three lots of each food type were obtained at local retail markets. Each contained a level of aerobic bacteria typical for that product type. The aerobic counts of the different lots were different for all food types except nuts. Each sample lot was thoroughly mixed by placing it in a large plastic bag and shaking the bag. Then, for each collaborator, duplicate samples of about 75 g were placed in 8 oz Whirl-Pak bags.

Distribution of Samples to Collaborators

The collaborators received 6 samples consisting of 3 blind duplicate pairs for each food. Samples were shipped by overnight delivery 1 week prior to the scheduled date of analysis. Collaborators checked the condition of the samples on arrival and then stored them at the appropriate temperature until the scheduled date of analysis.

Microbiological Analysis of Test Samples

All collaborators analyzed each set of samples on the same day. Collaborators prepared sample homogenates according to method **966.23B,** 15th ed. (**46.014,** 14th ed.). Decimal dilutions of 10^{-2} through 10^{-6} were used for all foods except nuts, which were diluted 10^{-1} through 10^{-5}. All samples

Table 1. Samples analyzed by laboratories in collaborative study on enumeration of total aerobic bacteria in foods by dry rehydratable film method

Lab.	Flour	Nuts	Shrimp	Spice	Turkey	Vegetables
1	Y[a]	Y	Y	Y	Y	Y
2	Y	Y	Y	Y	Y	Y
3	Y	Y	Y	Y	Y	Y
4	N[b]	N[b]	Y	N[b]	Y	Y
5	Y	Y	Y	Y	Y	Y
6	Y	Y	Y	Y	Y	Y
7	Y	Y	Y	Y	Y	Y
8	Y	N[c]	Y	Y	Y	Y
9	Y	N[c]	Y	Y	Y	Y
10	Y	Y	Y	Y	Y	Y
11	—[d]	—	Y	—	Y	Y
12	Y	Y	—	Y	—	—

[a] Y-collaborator participated in analysis of these samples.
[b] Collaborator received samples, but did not receive revised instructions prior to analysis of samples. Data not used.
[c] Collaborator included mold count with bacteria count for agar plates. Data not used.
[d] Collaborator did not analyze these samples.

were plated in duplicate on dry-film plates according to the proposed procedure and with plate count agar according to **966.23C,** 15th ed. (**46.015,** 14th ed.).

Data Analysis

Colony counts were recorded on data sheets that were provided and then returned to the Associate Referee for analysis. For calculating and reporting the aerobic plate counts by the 2 methods, the procedure in ref. 17 was used as a guide. The counting range of 30–300 colonies per plate was used as recommended by **966.23C.**

Colony counts were converted to $\log_{10}$ counts/mL for statistical analysis. It was assumed that the log counts would be normally distributed and of homogeneous variance. The paired *t*-test was applied to mean log differences to determine any significant differences in recovery of organisms between methods. The components of variance were calculated using standard statistical techniques (18). Repeatability and reproducibility estimates were calculated according to AOAC procedures (19). Correlated repeatability variances were compared by a Pitman-Morgan style analysis (20). In all statistical tests, a resulting value of $P < 0.05$ indicated a significant difference. Outliers detected by the Cochran test were excluded from the analysis (16). Most of these outliers were obvious by simple examination of the results. Data were also excluded in some instances because of technical errors. These are described in more detail under each product and in Table 1.

990.12 Aerobic Plate Count in Foods
Dry Rehydratable Film Method
First Action 1990

Method Performance:

Flour
$s_r = 0.225$; $s_R = 0.246$; $RSD_r = 5.3\%$; $RSD_R = 5.8\%$
Nuts
$s_r = 0.272$; $s_R = 0.674$; $RSD_r = 7.4\%$; $RSD_R = 18.4\%$
Shrimp
$s_r = 0.540$; $s_R = 0.615$; $RSD_r = 9.8\%$; $RSD_R = 11.1\%$
Spice
$s_r = 0.274$; $s_R = 0.303$; $RSD_r = 6.0\%$; $RSD_R = 6.6\%$
Turkey
$s_r = 0.278$; $s_R = 0.348$; $RSD_r = 5.3\%$; $RSD_R = 6.6\%$
Vegetables
$s_r = 3.10$; $s_R = 0.454$; $RSD_r = 6.3\%$; $RSD_R = 9.2\%$

A. Principle

See **989.10A.**

Table 2. Collaborative results for aerobic plate counts of flour samples ($\log_{10}$ cfu/g) by dry-film (Petrifilm) aerobic count (PSM) and standard methods agar (SMA) methods[a]

	White				Rye				Wheat			
	Sample 2		Sample 4		Sample 1		Sample 5		Sample 3		Sample 6	
Coll.	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA
1	3.279	3.114	3.602	3.362	4.301	4.230	3.982	3.968	5.114	4.959	5.146	5.079
2	3.556	3.643	3.447	3.591	4.114	4.146	4.000	4.176	5.204	5.279	5.114	5.279
3	3.322	3.279	3.580	3.491	4.342	4.279	4.342	4.462	4.851	4.806	5.176	5.041
5	3.176	2.954	3.519	3.255	3.806	3.857	4.079	4.079	5.114	4.079	5.114	5.041
6	3.301	3.531	3.602	3.301	4.204	3.991	4.000	3.903	5.519	5.255	5.041	5.000
7	3.531	3.491	2.398	2.778	4.041	4.146	4.279	4.362	5.301	4.623	5.146	5.380
8	3.820	3.806	3.568	3.568	4.204	4.079	4.477	4.415	5.681	5.531	5.380	5.431
9	3.415	3.544	3.462	3.613	4.114	4.204	3.799	3.934	5.146	5.176	5.079	5.255
10	3.078	3.415	3.556	3.663	3.826	3.892	3.778	3.881	5.146	5.204	5.000	5.146
12	3.519	3.398	3.342	3.415	3.959	4.079	4.079	4.176	4.748	5.000	5.000	5.041
Mean	3.400	3.418	3.408	3.404	4.091	4.090	4.082	4.136	5.182	4.991	5.120	5.170

[a] Samples were distributed as blind duplicates.

Table 3. Collaborative results for aerobic plate counts of nut samples (log_{10} cfu/g) by dry-film (Petrifilm) aerobic count (PSM) and standard methods agar (SMA) methods[a]

	Lot 1				Lot 2				Lot 3			
	Sample 1		Sample 4		Sample 2		Sample 5		Sample 3		Sample 6	
Coll.	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA
1	3.643	3.114	3.380	2.898	3.672	2.724	3.415	3.079	3.633	3.079	3.934	3.491
2	3.462	3.462	3.556	3.362	3.362	3.322	3.813	4.255	3.114	3.342	3.415	3.398
3	3.505	4.146	3.771	4.041	3.919	4.491	3.633	4.114	3.431	4.146	3.519	4.462
5	3.756	4.079	3.255	3.690	4.114	4.477	3.763	4.322	3.230	3.653	4.477	4.491
6	4.845	4.462	4.845	4.491	4.643	4.362	4.491	4.230	5.663	5.447	5.845	5.531
7	3.415	3.778	3.462	3.973	3.301	4.491	3.447	3.934	3.398	3.964	3.623	3.591
10	3.255	3.544	2.914	3.000	3.505	3.176	3.362	3.699	3.176	3.602	2.959	3.477
12	2.964	3.672	2.954	3.716	2.732	3.978	3.176	3.973	3.806	3.851	3.146	3.643
Mean	3.606	3.782	3.517	3.646	3.656	3.878	3.638	3.951	3.682	3.886	3.865	4.011

[a] Samples were distributed as blind duplicates.

B. Apparatus

See **989.10B(a)** and (c)–(e).

C. Reagent

Dilution water.—To prepare stock solution, dissolve 34 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with 1N NaOH (ca 175 mL), and dilute to 1 L with water. To prepare buffered water for dilutions, dilute 1.25 mL stock solution to 1 L with boiled and cooled water. Autoclave 15 min at 121°.

D. Sample Preparation

See **966.23B.**

E. Determination

Place dry-film aerobic count plate on flat surface. Lift top film and inoculate 1 mL sample onto center of film base. Carefully place top film down on inoculum. Distribute sample over prescribed growth area with downward pressure in center of plastic spreader device (recessed side down). Leave plate undisturbed 1 min to permit gel to solidify. Incubate plates 48 ± 3 h at 35 ± 1°.

In incubator, place plates in horizontal position, clear side up, in stacks not exceeding 20 units. Count plates promptly after incubation period. If impossible to count at once after required incubation, store plates at 0–4.4° for not >24 h. Avoid this as a routine practice.

Use standard colony counter for counting purposes. Magnifier-illuminator may also be used to facilitate counting. Colonies stain in various shades of red. Count all colonies in countable range (30–300 colonies).

To compute bacterial count, multiply total number of colonies per plate (or average number of colonies per plate if counting duplicate plates of same dilution) by reciprocal of dilution used. When counting colonies on duplicate plates of consecutive dilutions, compute mean number of colonies for each dilution before determining average bacterial count. Estimated counts can be made on plates with >300 colonies and should be reported as estimated counts. In making such counts, circular growth area can be considered to contain ca twenty 1 cm squares. To isolate colonies for further identification, lift top film and pick colony from gel.

Ref.: JAOAC **73,** March/April issue (1990).

Results and Discussion

Twelve laboratories participated in the study. Ten laboratories analyzed all 6 product types. One laboratory analyzed the flour, nuts, and spice samples only; another laboratory analyzed the turkey, shrimp, and vegetable samples only (Table 1). The plate counts for the individual samples are presented in Tables 2–7. Repeatability and reproducibility analyses are presented in Table 8.

Flour

White, rye, and wheat flour samples with low, medium, and high aerobic counts, respectively, were analyzed (Table 2). Collaborator 10 reported the presence of spreader colonies on agar plates for sample 6. Collaborator 11 reported the presence of liquefying bacteria on the dry-film for samples 1, 2, 3, and 5 and spreader colonies on the agar plates for samples 1, 3, 5, and 6. Collaborator 5 noted that white flour particles might be mistaken for colonies on agar plates at the 10^{-1} and 10^{-2} dilutions. All reported data were included in the analysis.

The mean log dry-film and agar plate counts were in agreement for the 3 lots of flour. Mean counts were higher by the agar method for white and rye flour. The repeatability standard deviations (s_r) of the dry-film and agar methods were not significantly different for the white, rye, or wheat flour samples (Table 8). The repeatability relative standard deviations (RSD_r) ranged from 3.2 to 9.3% for the dry-film method and 3.5 to 6.2% for the agar method. The reproducibility standard deviations (s_R) of the methods appeared comparable (Table 8). The reproducibility relative standard deviation percentages (RSD_R) were between 4.1 and 9.3% for the dry-film method and 4.4 and 7.3% for the agar method.

Nut Halves

Three different lots of walnut halves were analyzed (Table 3). All lots had the same level of aerobic bacteria. Many of the collaborators indicated that the agar plates were difficult to evaluate because of proliferation of mold. The mold did not appear to grow on the dry-film plates, which indicates an advantage of the dry-film method. A frequent practice is to add cycloheximide to plate count agar to inhibit mold growth. It was not recommended in this study because the standard method does not have this provision. Collaborators 8 and 9 indicated that the agar plate counts were a total of the mold and bacterial colonies. Consequently, the results from these laboratories were excluded from the analysis.

The mean dry-film count was lower than the agar count for each of the 3 lots of nuts. These differences, however, were not significant. Repeatability of the 2 methods was not signif-

Table 4. Collaborative results for aerobic plate counts of shrimp samples (log_{10} cfu/g) by dry-film (Petrifilm) aerobic count (PSM) and standard methods agar (SMA) methods[a]

	Lot 1				Lot 2				Lot 3			
	Sample 1		Sample 6		Sample 3		Sample 4		Sample 2		Sample 5	
Coll.	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA
1	4.176	4.279	4.531	4.431	5.322	5.556	5.041	5.398	6.756	5.968	7.176	6.613
2	4.322	4.505	4.919	4.716	4.447	5.079	5.041	5.362	6.690	7.380	6.398	7.255
3	5.672	5.690	5.623	5.255	5.491	5.724	6.114	6.301	7.255	6.851	7.146	6.881
4	4.114	2.740	4.398	3.833	4.041	3.279	4.491	4.415	6.279	6.342	6.322	5.431
5	4.301	4.380	4.663	4.681	4.491	4.792	5.763	5.716	7.146	7.633	7.255	7.477
6	4.845	4.519	4.681	4.716	5.146	4.944	7.491	7.380	6.041[b]	7.362[b]	8.114[b]	7.643[b]
7	4.544	4.342	4.949	4.477	4.602	4.643	5.987	6.491	7.505	7.255	7.398	7.398
8	5.041	5.041	5.806	5.771	4.982	7.806	6.114	6.279	8.146[c]	8.000[c]	—[c]	—[c]
9	4.531	4.556	4.875	4.954	4.505	4.602	4.690	4.991	6.255	6.230	6.342	6.792
10	4.799	4.556	5.041	6.146	4.863	5.079	6.204	6.204	7.568	7.531	7.663	7.477
11	4.114	3.924	5.041	4.806	5.398	5.556	4.342	4.477	5.763	5.806	6.748	6.875
Mean	4.587	4.412	4.957	4.890	4.845	5.188	5.571	5.729	6.880	6.778	6.939	6.911

[a] Samples were distributed as blind duplicates.
[b] Outlier; data for sample 2 and sample 5 (SMA) also excluded from analysis.
[c] Results for sample 5 reported as too numerous to count. All data for lot 3 excluded from analysis.

icantly different (Table 8). For the dry-film procedure, RSD_r values ranged from 5.1 to 10.1% and for the agar method, from 5.0 to 8.5%. The reproducibility variance for the 2 methods was higher for the nut samples than any of the other samples in the study (Table 8). The RSD_R values were between 13.6 and 23.4% for the dry-film method and between 13.2 and 18.6% for the agar method.

Shrimp

Three different lots of peeled, deveined whole shrimp were analyzed (Table 4). Lots 1, 2, and 3 contained low, medium, and high levels of aerobic bacteria, respectively. None of the collaborators experienced difficulty with the analyses. A high count, determined to be an outlier by the Cochran test, was obtained by laboratory 6 on sample 5 by the dry-film method. Laboratory 8 reported "too numerous to count" results for sample 5 by both plating procedures. Consequently, the results from laboratories 6 and 8 for lot 3 were excluded from the statistical analysis.

Relative to the agar method, slightly higher mean counts were obtained by the dry-film method for lots 1 and 2. Differences between the 2 methods for each of the 3 lots were not significant. The repeatability of the agar method was significantly higher than it was for the dry-film method for lot 1 ($P < 0.05$), but not for the other 2 lots (Table 8). The repeatability and reproducibility standard deviations (Table 8) for the dry-film method were lower than those obtained for the standard agar method for all 3 test lots. RSD_r values ranged from 3.9 to 15.5% for the dry-film method and from 5.7 to 16.0% for the agar method. RSD_R values ranged from 8.2 to 15.7% for the dry-film and from 9.8 to 18.8% for the agar method.

Spice

Three types of spice—pepper, thyme, and onion powder—were analyzed (Table 5). The spices contained low, medium, and high levels of aerobic bacteria, respectively. Most laboratories reported the presence of liquefying bacteria and spreader colonies on plates for a majority of the samples. Data from Laboratory 8 were excluded from the analysis because of an apparent dilution error. Laboratory 9 reported particle interference by the dry-film procedure for pepper

Table 5. Collaborative results for aerobic plate counts of spice samples (log_{10} cfu/g) by dry-film (Petrifilm) aerobic count (PSM) and standard methods agar (SMA) methods[a]

	Pepper				Thyme				Onion powder			
	Sample 3		Sample 5		Sample 2		Sample 6		Sample 1		Sample 4	
Coll.	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA
1	3.447	3.505	4.491	4.415	4.778	4.431	4.740	4.279	5.544	5.447	5.531	5.447
2	3.505	3.732	4.431	4.580	4.380	4.491	4.380	4.580	5.544	5.663	5.602	5.740
3	3.477	3.740	3.623	3.771	4.114	4.079	4.857	4.176	5.491	5.431	5.415	5.415
5	3.580	3.633	3.892	3.903	4.643	4.591	4.477	4.602	5.447	5.398	5.204	5.398
6	3.699	3.556	3.778	3.914	4.748	4.431	4.556	4.491	5.462	5.491	5.785	5.544
7	3.792	3.940	4.544	4.568	4.556	4.681	4.643	4.591	5.477	5.708	5.771	5.519
8	3.799	3.806	3.114	3.041	4.778	4.820	4.973	5.041	—[b]	—[b]	5.742[b]	5.643[b]
9	3.792	3.556	4.000	4.000	4.724	4.996	4.978	4.929	5.556	5.653	5.613	5.531
10	3.146	4.114	3.415	4.041	4.079	4.146	4.041	4.230	5.544	5.653	5.491	5.763
12	3.415	3.462	3.699	3.602	4.519	4.279	4.322	4.230	5.531	5.663	5.663	5.580
Mean	3.565	3.705	3.899	3.984	4.532	4.495	4.597	4.515	5.511	5.567	5.564	5.549

[a] Samples were distributed as blind duplicates.
[b] No counts reported for sample 1; all data for onion powder excluded from analysis.

Table 6. Collaborative results for aerobic plate counts of turkey samples (log_{10} cfu/g) by dry-film (Petrifilm) aerobic count (PSM) and standard methods agar (SMA) methods[a]

	Lot 1				Lot 2				Lot 3			
	Sample 1		Sample 5		Sample 2		Sample 3		Sample 4		Sample 6	
Coll.	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA
1	3.987	4.114	4.041	4.176	5.204	5.322	5.279	5.255	5.740	5.908	5.748	5.949
2	3.996	4.114	3.973	4.204	5.041	5.176	5.415	5.362	6.398	6.681	5.491	5.663
3	4.996	5.447	4.820	5.255	6.602	6.531	6.491	6.462	7.690[b]	7.978[b]	—[b]	6.968[b]
4	4.041	3.505	3.944	3.663	5.255	5.230	5.415	5.362	6.362	6.041	5.633	5.591
5	4.230	4.255	4.204	4.279	5.462	5.580	5.431	5.415	6.447	6.602	6.398	6.505
6	4.279	4.301	4.255	4.255	5.398	5.322	5.447	5.204	6.380	6.342	5.602	5.491
7	4.204	4.079	4.079	3.991	5.114	5.176	5.301	5.204	6.505	6.447	6.230	6.447
8	4.301	4.447	4.415	4.079	5.322	5.431	5.690	5.771	6.255	6.255	7.505	7.398
9	4.041	4.079	3.886	4.114	5.279	5.462	5.079	5.204	5.929	6.398	5.398	6.146
10	4.176	4.255	4.114	4.255	6.041	6.230	5.792	5.690	6.792	7.176	6.380	6.519
11	4.114	3.591	4.041	3.785	5.301	5.041	4.869	3.863	5.973	5.940	5.591	5.892
Mean	4.215	4.199	4.161	4.187	5.456	5.500	5.474	5.345	6.278	6.379	5.998	6.160

[a] Samples were distributed as blind duplicates.
[b] Laboratory accident was reported for sample 6 (PSM); all data for lot 3 excluded from analysis.

and thyme samples. These results appeared similar by examination to those reported by the other collaborators and were not outliers by the outlier tests; they were included in the analysis.

Mean log counts for the onion powder and pepper were lower by the dry-film method than by the agar method. For thyme samples, the mean dry-film counts were higher. None of these differences were significant. The repeatability standard deviations of the dry-film method were higher than those for the agar method for all 3 spices (Table 8). Only the result for thyme was significant ($P < 0.01$). Reproducibility standard deviations of the 2 methods were similar (Table 8). The RSD_r values were between 2.3 and 10.9% for the dry-film method and between 1.2 and 10.0% for the agar method. RSD_R values were between 2.4 and 10.9% for the dry-film method and between 2.3 and 10.0% for the agar method.

Turkey

Three lots of ground turkey meat were analyzed in the study (Table 6). Lots 1, 2, and 3 contained low, medium, and high levels of aerobic bacteria, respectively. The collaborators did not report significant difficulties with the analyses. Laboratory 3 did not report counts for the dry-film plates for sample 6, so all data for lot 3 by that laboratory were excluded from the analysis.

Mean log counts obtained by the dry-film for lots 1 and 2 were lower and for lot 3 they were higher than by the agar method. Only the difference for lot 3 was significant ($P = 0.023$). Significantly, repeatability standard deviations (Table 8) were higher for the agar method compared to the dry-film method for lot 1 ($P < 0.05$) and lot 2 ($P < 0.01$). For lot 3, the difference was not significant. The RSD_r values ranged from 1.7 to 7.5% for the dry-film method and from 2.6 to 6.9% for the agar method. For reproducibility (Table 8), RSD_R values were between 6.6 and 8.4% for dry-film and between 7.9 and 10.9% for agar.

Vegetables

Three different vegetables were analyzed (Table 7). No difficulties were reported. The carrots, peas, and mushrooms

Table 7. Collaborative results for aerobic plate counts of vegetables samples (log_{10} cfu/g) by dry-film (Petrifilm) aerobic count (PSM) and standard methods agar (SMA) methods[a]

	Carrots				Peas				Mushrooms			
	Sample 1		Sample 3		Sample 2		Sample 6		Sample 4		Sample 5	
Coll.	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA
1	3.771	4.041	3.462	3.505	3.799	3.732	3.740	3.908	7.380	7.531	8.000	8.415
2	3.301	3.398	3.462	3.580	4.114	3.996	3.964	4.079	7.748	7.898	7.255	7.633
3	4.204	4.362	4.462	4.079	4.708	4.681	4.491	4.978	7.146	8.301	7.580	8.301
4	3.531	3.380	3.447	3.146	3.748	3.716	3.863	3.881	6.716	6.690	6.653	6.591
5	3.477	3.380	4.146	4.114	3.886	3.978	3.591	3.778	8.114	8.114	6.978	7.398
6	3.301	3.079	3.699	3.556	3.954	3.903	3.954	3.954	7.580	7.415	8.342	8.079
7	3.204	3.230	3.230	3.380	3.851	4.041	3.447	3.505	8.415	8.230	8.041	8.000
8	6.643[b]	6.792[b]	3.602[b]	3.613[b]	4.079	4.079	3.940	3.940	—[c]	—[c]	—[c]	—[c]
9	3.477	3.491	3.602	3.431	3.851	3.881	3.763	3.903	6.869	7.362	6.114	6.041
10	3.114	3.114	3.924	3.792	3.732	3.756	3.690	3.820	6.568	6.820	6.968	6.914
11	2.929	2.954	3.568	3.398	3.903	3.919	3.898	3.732	7.919	7.964	7.519	7.447
Mean	3.431	3.443	3.700	3.598	3.966	3.971	3.849	3.953	7.446	7.632	7.345	7.482

[a] Samples were distributed as blind duplicates.
[b] Outlier; data for carrot samples 3 also excluded from analysis.
[c] Result reported as too numerous to count; data excluded from analysis.

Table 8. Statistical analysis of collaborative results for aerobic plate counts in foods

Product	Type	Contamination level	s_r		RSD_r, %		s_R		RSD_R, %	
			PSM	SMA	PSM	SMA	PSM	SMA	PSM	SMA
Flour	white	low	0.316	0.210	9.3	6.2	0.316	0.250	9.3	7.3
	rye	medium	0.154	0.142	3.8	3.4	0.203	0.182	5.0	4.4
	wheat	high	0.165	0.288	3.2	5.7	0.209	0.318	4.1	6.3
Nuts	lot 1	—[a]	0.180	0.186	5.1	5.0	0.583	0.492	16.4	13.2
	lot 2	—[a]	0.214	0.332	5.9	8.5	0.496	0.571	13.6	14.6
	lot 3	—[a]	0.380	0.272	10.1	6.9	0.881	0.735	23.4	18.6
Shrimp	lot 1	low	0.338	0.504*	7.1	10.8	0.487	0.716	10.2	15.4
	lot 2	medium	0.806	0.875	15.5	16.0	0.816	1.025	15.7	18.8
	lot 3	high	0.267	0.392	3.9	5.7	0.564	0.671	8.2	9.8
Spice	pepper	low	0.406	0.384	10.9	10.0	0.406	0.384	10.9	10.0
	thyme	medium	0.196**	0.076	4.3	1.7	0.282	0.293	6.2	6.5
	on. pwd.	high	0.125	0.066	2.3	1.2	0.131	0.126	2.4	2.3
Turkey	lot 1	low	0.070	0.108*	1.7	2.6	0.275	0.457	6.6	10.9
	lot 2	medium	0.171	0.299**	3.1	5.5	0.440	0.549	8.1	10.1
	lot 3	high	0.458	0.435	7.5	6.9	0.518	0.493	8.4	7.9
Vegetables	carrots	low	0.307	0.309	8.6	8.8	0.386	0.382	10.8	10.9
	peas	medium	0.129	0.157	3.3	4.0	0.277	0.321	7.1	8.1
	mushrooms	high	0.431	0.441	5.8	5.8	0.649	0.675	8.8	8.9

* Significantly higher repeatability ($P < 0.05$).
** Significantly higher repeatability ($P < 0.01$).
[a] Aerobic counts for different lots were not significantly different.

contained low, medium, and high levels of aerobic bacteria. The results for the carrot samples from laboratory 8 were discarded as outliers by the Cochran test. The same laboratory reported all dilutions by both plating methods for the mushroom samples as "too numerous to count." These data, therefore, were not available for analysis. Laboratory 9 reported that the mushroom samples froze during shipping. Data for these samples were not excluded from analysis because the counts were consistent with those reported by the other laboratories.

Differences of the mean log counts for the vegetable samples of the 2 methods were not significantly different. Counts were higher by the dry-film method for carrots as well as by the agar method for mushrooms and peas. Repeatability and reproducibility standard deviations and relative standard deviations of the 2 methods were similar (Table 8). RSD_r values were between 3.3 and 8.6% for dry-film and between 4.0 and 8.8% for agar. RSD_R values were between 7.1 and 10.8 for dry-film and between 8.1 and 10.9% for agar.

Summary

In this study, 6 food products (flour, nut halves, shrimp, spice, raw turkey, and refrigerated and frozen vegetables), selected as representative foods, were analyzed by 12 laboratories for aerobic bacteria by both the proposed dry-film method and the standard agar method. Each laboratory analyzed 3 samples in blind duplicate from each food product. The repeatabilities were not significantly different for the majority of the samples, and the results indicated equivalence of the dry-film method and the agar method.

Recommendation

On the basis of these results, the dry rehydratable film method has been adopted official first action for the enumeration of total aerobic bacteria in food.

Acknowledgments

The authors thank the following individuals for their time and assistance:

Thomas Baldy, General Mills, Minneapolis, MN

M. Brodsky and E. Föllmi, Ontario Ministry of Health, Weston, Ontario, Canada

Amy Izat, University of Arkansas, Fayetteville, AR

Mildred Laws, Holly Farms, Wildsboro, NC

Penny Mah and Lawrence Roth, Alberta Food Laboratory, Edmonton, Alberta, Canada

John McGregor, P. McGrew, S. Sandoval, and A. Ghafoor, Louisiana State University, Baton Rouge, LA

Christian Okolo, Conagra Frozen Food Co., Batesville, AR

Dawn Richter, Ore-Ida Foods, Ontario, OR

Dawn Shafer and Katie Swanson, Pillsbury, Minneapolis, MN

Sandra Sharp, National Marine Fisheries Service, Pascagula, MS

References

(1) Ginn, R. E., Packard, V. S., & Fox, T. L. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 527–531
(2) Curiale, M. S., Fahey, P., Fox, T. L., & McAllister, J. S. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 312–318
(3) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA; (1984) 14th Ed. and its supplements, AOAC, Arlington, VA
(4) Ginn, R. E., Packard, V. S., & Fox, T. L. (1984) *J. Food Prot.* **47,** 753–756
(5) McAllister, J. S., Ramos, M. S., & Fox, T. L. (1987) *Dairy Food Sanitation* **7,** 632–635
(6) Senyk, G. F., Kozlowski, S. M., Noar, P. S., Shipe, W. F., & Bandler, D. K. (1987) *J. Dairy Sci.* **70,** 1152–1158
(7) Hughs, D., & Sutherland, P. S. (1987) *Aust. J. Dairy Technol.* **42,** 59–61
(8) Sato, T., Nakana, K., Sato, S., & Kurita, T. (1987) *Tohoku J. Vet. Clin.* **10,** 38–45

(9) Yashida, K., Mizuochi, S., Hartua, M., Simizu, Y., & Morichi, T. (1987). *Food Hygene* **28,** 483–486
(10) Ottaviani, F., & Arvati, G. (1988) *Il Latte* **13,** 299–303
(11) Chain, V. (1988) Master's thesis, Kansas State University
(12) Bailey, J. S., & Cox, N. A. (1987) *J. Food Prot.* **50,** 643–644
(13) McAllister, J. S., Stadtherr, M. P., & Fox, T. L. (1988) *J. Food Prot.* **51,** 658–659
(14) Smith, L. B., Fox, T. L., & Busta, F. F. (1985) *J. Food Prot.* **48,** 1044–1045
(15) Denis, C., Normandie, A., & Bardet, M. (1987) *Infos-Ctifl* **37,** 19–22
(16) "Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis" (1988) *J. Assoc. Off. Anal. Chem.* **71,** 161–173
(17) Messer, J. W., Peeler, J. T., & Gilchrist, J. E. (1984) in FDA *Bacteriological Analytical Manual,* AOAC, Arlington, VA, 4.01–4.10
(18) Neter, J., & Wasserman, W. (1974) *Applied Linear Statistical Models,* Richard D. Irwin, Inc., Homewood, IL
(19) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC,* AOAC, Arlington, VA
(20) Snedecor, G. W., & Cochran, W. G. (1980) *Statistical Methods,* 7th Ed., Iowa State University Press, Ames, IA

Colorimetric Deoxyribonucleic Acid Hybridization Assay for Rapid Screening of *Salmonella* in Foods: Collaborative Study

MICHAEL S. CURIALE and MARY JOAN KLATT
Silliker Laboratories, 1304 Halsted St, Chicago Heights, IL 60411
MARK A. MOZOLA
GENE-TRAK Systems, 31 New York Ave, Framingham, MA 01701

Collaborators: T. Arista; B. Bennett; M. Bidlack; M. Brodsky; F. Chan; B. Ciebin; M. Cirigliano; F. Dever; P. Elliot; J. Fechner; E. Föllmi; D. Ford; J. Fukuoka; J. Gardner; M. Ghassali; L. Larkin; C. Llabrès; R. McKenna; D. Nolan; N. Ramos; D. Reed; C. Roderick; B. Rose; M. Schulke; V. Smith; T. Sons; A. Thomas; S. Thompson; J. Troller; T. Wagner; J. Walters; S. Weathersby

A collaborative study was performed in 11 laboratories to validate a colorimetric DNA hybridization (DNAH) method for rapid detection of *Salmonella* in foods. The method was compared to the standard culture method for detection of *Salmonella* in nonfat dry milk, milk chocolate, soy isolate, dried whole egg, ground black pepper, and raw ground turkey. Samples inoculated with high (0.4–2 cells/g) and low (0.04–0.2 cells/g) levels of *Salmonella* and uninoculated control samples were included in each food group analyzed. There was no significant difference in the proportion of samples positive by DNAH and culture procedure for any of the 6 foods. The colorimetric DNA hybridization assay screening method has been adopted official first action as a rapid screening method for detection of *Salmonella* in all foods.

The official culture method for the detection of *Salmonella* in food products requires at least 4 days to obtain negative results (1, 2). Deoxyribonucleic acid hybridization (DNAH) technology is a proven method for the rapid screening of foods and food ingredients for *Salmonella* (3–6). The DNAH method, including cultural enrichment steps and assay, requires only 48 h for completion. DNA probes that are specific for *Salmonella* species are used. Detection of *Salmonella* by this assay requires hybridization of a ^{32}P-labeled DNA probe to regions of chromosomal DNA that are unique to *Salmonella*. The method has been evaluated through precollaborative comparative and collaborative studies to compare the efficacy of the method with that of the standard or existing official cultural methods (6, 7). These evaluations of method performance indicated that DNAH was as sensitive as the culture method, and was applicable to a variety of foods. The DNAH method for the detection of *Salmonella* in foods was adopted official first action by AOAC in 1987 (8), and received final action status in 1989 (9).

Use of the AOAC DNAH method (**987.10,** 15th Ed.; **46.C07-46.C16,** 14th Ed. [8]) has been limited to sites that handle radioisotopes. To overcome this limitation, a second generation nucleic acid hybridization assay for the detection of *Salmonella* in foods has been developed by GENE-TRAK Systems, Framingham, MA. The assay utilizes an enzymatic detection method and a colorimetric end point. Experience with the colorimetric DNA hybridization assay indicated that it could be easily and reliably performed by laboratory technicians with typical microbiology laboratory skills.

A collaborative study was performed to evaluate the efficacy of the *Salmonella* colorimetric DNA hybridization method compared to the conventional culture procedures of AOAC and BAM. The study was designed to meet AOAC requirements for collaborative studies (10).

Collaborative Study

The food types included were the same as those used in previous collaborative studies for rapid *Salmonella* methods (7, 11). The foods (raw ground turkey, finely ground black pepper, soy flour, dry whole egg, milk chocolate, and nonfat dry milk) represent a variety of product types, require different pre-enrichment conditions, and each has been implicated as a potential source of *Salmonella*. Samples (about 75 g each) of each product were prepared at Silliker Laboratories, Inc., Chicago Heights, IL, and distributed to the 11 collaborators.

Received for publication August 2, 1989.
This report was presented at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO.
The recommendation was approved interim official first action by the General Referee, the Committee on Microbiology and Extraneous Materials, and the Chairman of the Official Methods Board. The method was adopted official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73,** January/February issue.

Table 1. Test products, test organisms, and inoculation levels used in collaborative study of colorimetric DNA hybridization assay

Product	Salmonella organisms	Inoculation level	Most probable number/g
Nonfat dry milk	*Salmonella bovis-morbificans*	low	0.023
	Salmonella cubana	high	0.93
Chocolate	*Salmonella senftenberg*	low	0.12
	Salmonella anatum	high	0.75
Soy flour	*Salmonella typhimurium*	low	1.1
	Salmonella blukwa	high	11.0
Dried whole egg	*Salmonella arizonae*	low	0.093
	Salmonella infantis	high	2.4
Pepper	*Salmonella rubislaw*	low	0.24
	Salmonella abaetuba	high	4.6
Turkey	naturally contaminated, *Salmonella* sp. (B:G complex)[a,b]	low	0.04
	naturally contaminated, *Salmonella* sp. (B:G complex)	high	0.09

[a] Species not determined; group (O) and Spicer-Edwards (H) reactions presented in parentheses.
[b] Organism reported for turkey was identified by the principal laboratory.

Collaborators received a set of 12 samples for each food. Two samples of each food type were uninoculated controls. Five samples were inoculated with low levels (1–5 cells/25 g) of *Salmonella*, and 5 samples were inoculated with high levels (10–50 cells/25 g). A combination of 2 *Salmonella* species was inoculated into each product except turkey which was naturally contaminated (Table 1).

Test samples were shipped via an overnight delivery service. One set of test samples was sent to each participating collaborator on the Wednesday preceding the Monday that analyses were to be initiated. Collaborators were instructed to analyze each sample by both the standard cultural procedure and by the DNAH method. The *Salmonella* levels in each product were determined by the Most Probable Number (MPN) method using standard culture methods on one set of samples on the day analyses were initiated. This determination was performed by the Associate Referee (MSC). Prior to initiation of the study, materials necessary for performance of the DNAH assay not normally available in a laboratory routinely performing *Salmonella* analyses were shipped to each collaborator by GENE-TRAK Systems. Results obtained by collaborators were submitted on supplied data forms to the Associate Referee for review, compilation, and data analysis.

Preparation and Shipment of Samples

Methods for preparation of collaborative samples and distribution to collaborators have been described previously (11).

Sample Analysis

Each sample was analyzed by both the BAM/AOAC culture and DNAH methods. Pre-enrichment, selective enrichment, isolation, and confirmation of isolates for the BAM/AOAC analyses were performed as described (1, 2). For all products except turkey, the following steps were used for the DNAH method. Sample pre-enrichments prepared for the BAM/AOAC methods were used for the DNAH procedure. After 22–26 h incubation, a separate set of tetrathionate brilliant green and selenite cystine selective enrichments were inoculated from each pre-enrichment, i.e., a set independent of that used for the BAM/AOAC method. Selective enrichments were incubated for 6 h at 35°C, mixed with a vortex mixer, and 1 mL was transferred from each into separate Gram negative (GN) broths (10 mL/tube). The selective enrichments were returned to 35°C for an additional 12–18 h, to be used for the confirmation of assay positive samples. The inoculated GN broths were incubated at 35°C for 12–18 h prior to assay. After incubation, 0.25 mL from each GN broth was combined in a clean 12 × 75 mm glass test tube. The remaining GN broth and selective enrichment cultures were refrigerated. Positive DNAH assay results were confirmed culturally by streaking the corresponding refrigerated GN broths and selective enrichment cultures to xylose lysine desoxycholate agar (XLD), Hektoen enteric agar (HE), and bismuth sulfite agar (BS) as described for confirmation in the culture procedure. All subsequent steps in identification of suspicious colonies were performed by the same methods used for the standard culture procedure (1, 2).

Direct selective enrichment of raw poultry as indicated by BAM (2) was used for the culture method. Because of the evidence indicating improved recovery of *Salmonella* from raw meats with the use of pre-enrichment (11), raw poultry samples to be evaluated by DNAH assay were pre-enriched as described for cooked meats (1, 2). After 22–26 h incubation at 35°C, one set of selective enrichments was inoculated from each pre-enrichment. The selective enrichments were incubated for 16–18 h at 35°C. Following incubation, selective enrichments were mixed and 1 mL was transferred from each selective enrichment into separate GN broths (10 mL/tube). The selective enrichments were returned to 35°C for an additional 6 h. The inoculated GN broths were incubated at 35°C for 6 h prior to assay. All other steps involving the analyses of raw turkey were the same as previously described for other foods.

Analysis of Data

Collaborators were instructed to send all results to the Associate Referee. Data for each food type were collated and numbers of false negative results by the DNAH and BAM/AOAC methods were tallied. False negative rates were calculated by 2 methods. According to Fleiss (12), the false negative rate is the number of false negative results divided by the total number of negative samples. Zweig and Robertson (13) define the rate as the number of false negative results divided by the total number of positive samples.

990.13 *Salmonella* in Foods

Colorimetric Deoxyribonucleic Acid Hybridization Screening Method

First Action 1990

Method is test procedure for presence of *Salmonella* in all foods. Because certain percentage of false positive reactions may be encountered, positive assays must be confirmed by standard culture methods (see *Confirmation of Positive DNA Hybridization Results*). *Note:* DNA probes used in this assay are nonreactive with subgenus V salmonellae.

A. Principle

Detection of *Salmonella* ribosomal RNA (rRNA) uses specific DNA probes. Following pre-enrichment, selective enrichment, and post-enrichment of test samples, bacteria are lysed, and labeled *Salmonella*-specific DNA probes are added for solution phase hybridization. If *Salmonella* rRNA is present in test sample, fluorescein-labeled detector probe and polydeoxyadenylic acid (poly dA)-tailed capture probe will hybridize to target rRNA sequences. Polydeoxythymidylic acid (poly dT)-coated solid phase (plastic dipstick) is then introduced into hybridization solution. Base pairing between poly dA and poly dT facilitates capture of probe:target hybrid nucleic acid molecules onto solid support. Unbound probe is removed by washing, and dipsticks are incubated in horseradish peroxidase–antifluorescein conjugate solution. Conjugate binds to fluorescein label present on hybridized detector probe. Unbound conjugate is washed away, and dipsticks are incubated in substrate–chromogen solution. Reaction of horseradish peroxidase with substrate converts chromogen to blue compound. Reaction is stopped with acid, which changes color of chromogen to yellow. Absorbance at 450 nm is measured. Absorbance in excess of threshold value indicates presence of *Salmonella* in test samples.

B. Method Performance

Results	*Percent*	*95% Confidence Range (Approx.)*
Agreement[1]	97.6	94.8–100
False neg. rate (DNAH)[2]	4.8	2.0–7.6
False neg. rate (culture)[3]	5.4	2.6–8.2
Agreement[4]	97.6	96.5–98.7
False neg. rate (DNAH)[5]	1.5	0.5–2.5
False neg. rate (culture)[6]	1.7	0.6–2.8

[1] This rate reflects number of samples read identically between AOAC/BAM (*Bacteriological Analytical Manual*, 1984, 6th ed., AOAC, Arlington, VA) culture method and DNAH method. Variance calculated according to cluster sampling method.

[2] This rate reflects number of samples found to be positive by AOAC/BAM culture method and negative by DNAH method divided by total number of negative samples.

[3] This rate reflects number of samples found to be positive by DNAH method and negative by AOAC/BAM culture method divided by total number of negative samples.

[4] This rate reflects number of samples read identically between AOAC/BAM culture method and DNAH method. Variance calculated assuming a binomial distribution.

[5] This rate reflects number of samples found to be positive by AOAC/BAM culture method and negative by DNAH method divided by total number of positive samples.

[6] This rate reflects number of samples found to be positive by DNAH method and negative by AOAC/BAM culture method divided by total number of positive samples.

Of 11 laboratories, 4 had complete agreement between DNAH and culture methods; 10 laboratories showed agreement on 98.4% of the samples.

C. Apparatus

Items (**a**)–(**d**) are available from GENE-TRAK Systems (31 New York Ave, Framingham, MA 01701).

(**a**) *Photometer.*—To measure *A* at 450 nm. Blank and sample wells to accommodate 12 × 75 mm test tubes containing solution volume of 1 mL.

(**b**) *Test tube racks.*—3 are needed. Plastic, heat-resistant (65°), to accommodate at least fifty 12 × 75 mm test tubes. Minimum of 5 wells per row with 18 mm spacing between wells (measured between centers of wells).

(**c**) *Dipstick holders.*—Plastic device to hold 5 dipsticks in row with 18 mm spacing between dipsticks (center to center).

(**d**) *Wash basins.*—4 are needed. Metal, heat-resistant (65°), 10 × 10 × 9 cm containers, with covers.

(**e**) *Test tubes.*—Glass, 12 × 75 mm.

(**f**) *Heating water bath.*—Capable of maintaining 65 ± 1.0°. Able to accommodate 1 test tube rack and 1 wash basin and water level of 5 cm.

(**g**) *Repeater pipet.*—Optional. With syringe-barrel tips, capable of accurately delivering aliquots of 0.1, 0.25, and 0.75 mL.

D. Reagents

Items (**a**)–(**m**) are available as Colorimetric GENE-TRAK® *Salmonella* Assay (DNA Hybridization Test for Detection of *Salmonella*) from GENE-TRAK Systems. Store *Salmonella* probe solution, enzyme conjugate 100× concentrate, substrate solution, chromogen solution, positive control solution, and negative control solution at 2–8°. Store all other solutions and dipsticks at room temperature (<30°). Sufficient for 100 determinations.

(**a**) *Lysis solution.*—1 bottle (10 mL). Contains 0.75N NaOH. (*Caution:* Avoid contact with skin; if contact occurs, wash skin thoroughly with water.)

(**b**) *Neutralization solution.*—1 bottle (10 mL). Contains 2.0M Tris, pH 7.5.

(**c**) *Salmonella probe solution.*—1 bottle (10 mL). Contains fluorescein-labeled, *Salmonella*-specific, synthetic oligonucleotide DNA probe and poly-deoxyadenylic acid-tailed, *Salmonella*-specific, synthetic oligonucleotide DNA probe in 0.1M Tris, pH 7.5; 1mM Na_2 ethylenediamine tetraacetate; 0.1% bovine serum albumin; 0.01% NP-40 (nonionic detergent); and 0.1% Na azide.

(**d**) *Wash solution 20× concentrate.*—2 bottles (250 mL each). Contains 1.0M Tris, pH 7.5; 0.4M Na_2 ethylenediamine tetraacetate; 3.0M NaCl, and 0.2% Tween-20.

(**e**) *Enzyme conjugate 100× concentrate.*—1 vial (1 mL). Contains horseradish peroxidase–antifluorescein polyclonal antibody conjugate.

(**f**) *Substrate solution.*—1 bottle (56 mL). Contains urea peroxide.

(**g**) *Chromogen solution.*—1 bottle (28 mL). Contains tetramethylbenzidine.

(**h**) *Stop solution.*—1 bottle (25 mL). Contains 4.0N H_2SO_4. (*Caution:* Avoid contact with skin; if contact occurs, wash skin thoroughly with water.)

(**i**) *Dipsticks.*—2 containers (50 each). Polystyrene dipsticks coated with poly-deoxythymidylic acid.

(j) *Positive control solution.*—1 bottle (5 mL). Contains formaldehyde-inactivated *Salmonella typhimurium.*

(k) *Negative control solution.*—1 bottle (5 mL). Contains formaldehyde-inactivated *Citrobacter freundii.*

(l) *Package insert.*

(m) *Data sheets.*

(n) *GN broth.*—20 g tryptose, 1 g dextrose, 2 g D-mannitol, 5 g Na citrate, 0.5 g Na desoxycholate, 4 g K_2HPO_4, 1.5 g KH_2PO_4, and 5 g NaCl. Dissolve ingredients in 1 L H_2O. Dispense 10 mL portions into 16 × 125 mm test tubes. Cap tubes loosely and autoclave 15 min at 121°. Final pH should be 7.0 ± 0.2 at 25°.

(o) *Diagnostic reagents.*—Necessary for culture confirmation of positive DNA hybridization assays. See **967.25B.**

E. General Instructions

Components in kit are intended for use as integral unit. Do not mix components from different kit lot numbers.

Include 1 positive control and 1 negative control with each group of test samples.

Do not touch fin portion of dipstick with fingers; hold by handle only. Do not reuse dipsticks or wash solution.

Use separate pipets or tips for each sample and kit reagent to avoid cross-contamination. Exercise care not to contaminate substrate-chromogen mixture with enzyme conjugate.

Return reagents requiring refrigeration to 2–8° storage immediately after use. Refer to storage requirements on individual reagent bottle labels.

Treat all materials in contact with bacterial cultures as biohazardous material and decontaminate by appropriate methods.

Caution: Some reagents in kit contain 0.1% Na azide. Disposal of these reagents into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential hazards.

Components and procedures of this test kit have been standardized for use in GENE-TRAK assay. Use of components or procedures other than those supplied or recommended by GENE-TRAK Systems may yield unsatisfactory results, and should be tested before use.

F. Sample Preparation

(a) *Pre-enrichment.*—Pre-enrich sample in nonselective medium to initiate growth of salmonellae. Procedure will vary with product type and must be performed as indicated in **967.26A** or in *Bacteriological Analytical Manual* (1984) 6th ed., AOAC, Arlington, VA, Chapter 7, sec. C, with the following exception:

Raw meats and raw milk products: Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (ca 20 000 rpm). Cap jar securely and let stand 60 min at room temperature. Mix contents well by shaking and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 using sterile 1N NaOH or HCl; cap jar securely and mix contents well before determining final pH. Aseptically transfer contents to sterile, wide-mouth, screw-cap 500 mL jar. Loosen jar cap ¼ turn and incubate 24 ± 2 h at 35°.

(b) *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment culture to tube containing 10 mL selenite cystine broth (prewarmed to 35°) and 1 mL to tube containing 10 mL tetrathionate broth (prewarmed to 35°) as in **967.26B(a).** Incubate 6 h at 35° with the following exception:

Raw meats and raw milk products: Incubate selenite cystine and tetrathionate broths for 18 ± 2 h at 35°.

(c) *Post-enrichment.*—Remove selective enrichment cultures from incubation and mix by hand or with vortex mixer. Transfer 1 mL tetrathionate culture to tube containing 10 mL GN broth (prewarmed to 35°). Transfer 1 mL selenite cystine culture to separate tube containing 10 mL GN broth (prewarmed to 35°). Incubate GN broths 12–18 h at 35° with the exception of raw meats and raw milk products (see below). Return tetrathionate and selenite cystine tubes to 35° for incubation up to total of 24 ± 2 h.

Raw meats and raw milk products: Incubate GN broths 6 h at 35°. Return tetrathionate and selenite cystine tubes to 35° for incubation up to total of 24 ± 2 h.

G. DNA Hybridization Assay

(*1*) Label sufficient number of 12 × 75 mm glass test tubes. Include tubes for one positive control and one negative control per assay. Place tubes in rack in rows of five.

(*2*) Remove sample GN broths from 35° incubation. Vortex-mix or otherwise mix each culture. For each sample, pipet 0.25 mL from each of the 2 GN broths (one derived from tetrathionate, one from selenite cystine) into single tube. Record sample numbers on data sheet.

(*3*) Mix positive and negative control solutions. Pipet 0.5 mL positive control solution into positive control tube. Pipet 0.5 mL negative control solution into negative control tube. Return controls to 2–8° storage.

(*4*) Add 0.10 mL lysis solution to each tube. Shake racks of tubes by hand for 5 s. Incubate tubes 5 min at room temperature.

(*5*) Add 0.1 mL neutralization solution to each tube. Shake rack of tubes by hand for 5 s. Cover tubes with aluminum foil. Place rack of tubes in 65° water bath and incubate 15 min.

(*6*) Remove foil. Add 0.10 mL *Salmonella* probe solution to each tube. Remove rack from water bath and shake by hand for 5 s. Cover tubes with foil, and return to 65° water bath. Incubate 15 min at 65°. Return probe solution to 2–8° storage.

(*7*) For each 25 tests performed, prepare 1.3 L 1× wash solution. Add 65 mL 20× wash solution to 1235 mL distilled or deionized H_2O.

(*8*) Prepare wash basin containing 300 mL 1× wash solution. Cover basin and place in 65° water bath until needed. Prepare second wash basin (300 mL) and keep at room temperature.

(*9*) Place appropriate number of dipsticks into dipstick holders. Rinse dipsticks 2–3 min in 1× wash solution at room temperature. Remove excess solution by blotting to absorbent paper (touch tip of fin portion of dipstick to paper).

(*10*) Remove foil from sample tubes and place dipsticks into tubes. Incubate dipsticks in tubes in 65° water bath for 1 h.

(*11*) Set up second rack of 12 × 75 mm tubes and label appropriately.

(*12*) Prepare sufficient 1× enzyme conjugate by mixing 100× enzyme conjugate concentrate and 1× wash solution. Dispense 0.75 mL 1× enzyme conjugate into each empty tube. Return remaining 100× enzyme conjugate concentrate to 2–8° storage.

(*13*) Remove dipsticks from tubes. Wash dipsticks sequentially with gentle shaking for 1 min each, first in 65° wash solution, then in room temperature wash solution.

Table 2. Collaborative results for detection of *Salmonella* in nonfat dry milk by BAM/AOAC and DNAH assay methods

	BAM/AOAC												DNAH - Assay[a]												DNAH - Confirmed[b]											
Collaborator	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
2	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
4	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
5	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
6	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-
7	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
8	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
9	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
10	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
11	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-

[a] Samples positive by DNAH assay before culture confirmation.
[b] Samples positive by DNAH assay and confirmed positive by isolation of Salmonella from culture broth.
[c] Samples 1, 2, 3, 4, and 5 were inoculated at a high level; samples 8, 9, 10, 11, and 12 were inoculated at a low level; and samples 6 and 7 were uninoculated control samples.

(*14*) Blot dipsticks on absorbent paper. Place dipsticks into second set of tubes containing enzyme conjugate. Incubate 20 min at room temperature.

(*15*) Set up third rack of tubes and label appropriately. Include additional tube for blank.

(*16*) Prepare mixture of substrate-chromogen consisting of 2 parts substrate to 1 part chromogen solution. Dispense 0.75 mL substrate-chromogen into each empty tube. Return remaining substrate solution and chromogen solution to 2-8° storage.

(*17*) Prepare 2 basins each containing 300 mL fresh 1× wash solution at room temperature.

(*18*) Remove dipsticks from enzyme conjugate tubes. Wash dipsticks sequentially with gentle shaking for 1 min each in fresh room temperature wash solution.

(*19*) Blot dipsticks on absorbent paper. Place dipsticks into third set of tubes containing substrate-chromogen. Incubate 20 min at room temperature.

(*20*) Remove dipsticks from tubes and discard. Add 0.25 mL stop solution to each tube containing substrate-chromogen, including blank. Shake rack of tubes by hand to mix contents.

(*21*) Measure absorbance at 450 nm. To read each tube, place reference tube in reference well on left side of photometer and sample tube in sample well on right side. Absorbance will be displayed digitally. Wait for reading to stabilize before recording result for each tube on data sheet.

(a) Determine negative control absorbance value by placing tube labeled "Blank" in reference well on left side of photometer. Place negative control tube in sample well on right side of photometer.

(b) Determine positive control absorbance value by placing tube labeled "Blank" in reference well on left side of photometer. Place positive control tube in sample well on right side of photometer.

(c) Determine test sample absorbance value by placing negative control tube in reference well on left side of photometer and test sample tube in sample well on right side.

H. Data Analysis

(1) *A* for negative control should be ≤0.15 (read against blank).

(2) *A* for positive control should be ≥1.00 (read against blank). If these results are not obtained, assay should be repeated.

Negative criterion.—Test sample is considered negative (nonreactive for presence of *Salmonella*) if *A* is ≤0.10 (read against negative control).

Positive criterion.—Test sample is considered positive (reactive for presence of *Salmonella*) if *A* is >0.10 (read against negative control).

I. Confirmation of Positive DNA Hybridization Results

Samples found positive by DNA hybridization assay must be confirmed by standard culture methods. Except in rare cases, confirmation can be achieved from GN broths alone. However, tetrathionate broth and selenite cystine broth cultures should be retained (2-8°) for later evaluation in cases where confirmation from GN broths is not obtained. For confirmation, streak cultures to HE, XLD, and BS plates as described in **967.26B** and identify typical and suspicious colonies as in **967.26C, 967.27,** and **967.28.**

Ref.: JAOAC **73,** March/April issue (1990).

Results

Data submitted by collaborators are summarized in Tables 2-7. Table 8 presents a comparison of BAM/AOAC and DNAH assay and confirmed DNAH assay data for all foods, including statistical analysis.

Nonfat Dry Milk

Salmonella MPNs determined on nonfat dry milk samples on the day collaborative samples were initiated indicated levels of 0.93/g and 0.023/g for the high and low level inoculated samples, respectively (Table 1). Eleven laboratories analyzed the nonfat dry milk samples; however, laboratory 3 encountered difficulties with the analysis and did not report complete results. The 10 reporting laboratories found all of the high level samples and 48 of 50 low level samples positive for *Salmonella* by both BAM/AOAC and DNAH assay (Table 2). Each of these positive assays was confirmed through isolation of *Salmonella* from the DNAH assay associated culture broths. Laboratories 6 and 11 each reported

Table 3. Collaborative results for detection of *Salmonella* in milk chocolate by BAM/AOAC and DNAH assay methods

	BAM/AOAC												DNAH - Assay[a]												DNAH - Confirmed[b]											
Collaborator	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12
1	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
2	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
3	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
4	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
5	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
6	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
7	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
8	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
9	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+

[a] Samples positive by DNAH assay before culture confirmation.
[b] Samples positive by DNAH assay and confirmed positive by isolation of Salmonella from culture broth.
[c] Samples 3, 4, 5, 6, and 7 were inoculated at a high level; samples 8, 9, 10, 11, and 12 were inoculated at a low level; and samples 1 and 2 were uninoculated control samples.

sample 12 as positive by the culture method, but negative by DNAH assay. These results were interpreted as false negative assays by the DNAH method. Collaborator 2 found one uninoculated control sample positive by DNAH assay but negative by BAM/AOAC. This sample was not confirmed by culture and was considered a false positive reaction by DNAH assay.

Milk Chocolate

Salmonella MPNs determined on milk chocolate samples on the day collaborative samples were initiated were 0.75/g and 0.12/g for the high and low level inoculated samples, respectively (Table 1). Eleven collaborators analyzed the milk chocolate samples and results for nine are presented in Table 3. The 9 laboratories reported each of the 45 low level and 45 high level inoculated samples positive by both BAM/AOAC and DNAH assay. Each of the positive DNAH assays was confirmed by isolation of *Salmonella* from the culture broths.

Collaborator 6 found one uninoculated control sample that was negative by BAM/AOAC, to be positive by DNAH assay. This sample was not confirmed by culture and was interpreted as a false positive reaction by DNAH assay.

Data from 2 laboratories (Nos. 10 and 11) were eliminated from the analysis because the DNAH negative control A_{450} readings were above 0.15, the highest acceptable level for this control. Laboratory 11 noted that following addition of probe, samples were incubated for 60 min instead of the recommended 15 min. This protocol deviation may have caused the high reading in this laboratory. Both laboratories completed the analysis of the samples, adjusting for the high negative control, and correctly identified all positive and negative samples by both the DNAH and culture methods.

Soy Isolate

Salmonella MPNs determined on soy isolate samples on the day collaborative samples were initiated were 1.1/g and 11.0/g for the low and high level inoculated samples, respectively (Table 1). Eleven collaborators analyzed the soy isolate samples. Ten laboratories found all inoculated high and low level samples positive by both BAM/AOAC and DNAH assay methods (Table 4). Each of the positive assays was

Table 4. Collaborative results for detection of *Salmonella* in soy isolate by BAM/AOAC and DNAH assay methods

	BAM/AOAC												DNAH - Assay[a]												DNAH - Confirmed[b]											
Collaborator	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
2	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
3	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
4	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
5	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
6	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
7	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
8	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-
9	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
10	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
11	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-

[a] Samples positive by DNAH assay before culture confirmation.
[b] Samples positive by DNAH assay and confirmed positive by isolation of Salmonella from culture broth.
[c] Samples 1, 2, 3, 4, and 5 were inoculated at a low level; samples 6, 7, 8, 9, and 10 were inoculated at a high level; and samples 11 and 12 were uninoculated control samples.

Table 5. Collaborative results for detection of *Salmonella* in dried whole egg by BAM/AOAC and DNAH assay methods

	BAM/AOAC												DNAH - Assay[a]												DNAH - Confirmed[b]											
Collaborator	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12
1	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
2	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
4	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
6	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
7	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
9	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
10	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
11	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-

[a] Samples positive by DNAH assay before culture confirmation.
[b] Samples positive by DNAH assay and confirmed positive by isolation of Salmonella from culture broth.
[c] Samples 2, 3, 4, 5, and 6 were inoculated at a high level; samples 7, 8, 9, 10, and 11 were inoculated at a low level; and samples 1 and 12 were uninoculated control samples.

confirmed by culture. In one laboratory (No. 8), one low level inoculated sample was negative by DNAH assay but positive by BAM/AOAC. This sample was interpreted as a false negative for the DNAH assay method. All 11 laboratories found all 22 uninoculated control samples negative by both BAM/AOAC and DNAH assay methods.

Dried Whole Egg

Salmonella MPNs determined for dried whole egg were 0.093/g and 2.4/g for the low and high level inoculated samples, respectively, on the day collaborative samples were initiated (Table 1). Eleven laboratories analyzed the egg samples but data from 3 laboratories (Nos. 3, 5, 8) were eliminated due to invalid assay results (Table 5). The remaining 8 laboratories found all inoculated high and low level samples positive by both BAM/AOAC and DNAH assay methods. All positive DNAH assays were confirmed by culture. All 16 uninoculated control samples were negative by both methods.

The results from laboratory 3 were excluded because the negative control for the DNAH assay was above the 0.15 A_{450} limit. The collaborator completed the analyses, adjusting for the negative control result, and observed complete agreement between the DNAH and culture methods. Results for laboratories 5 and 8 were excluded because positive control results were below 1.00 A_{450} as specified for the assay. In both cases, the collaborators proceeded with the analyses. Laboratory 5 reported all inoculated samples as positive and all uninoculated samples negative by both methods. The A_{450} readings for all but the positive control appeared consistent with those reported by laboratories with acceptable positive control results. Laboratory 8 reported 9 of 10 inoculated samples positive by the hybridization assay and 10 of 10 positive by culture. The A_{450} readings for these samples were lower than those reported by other laboratories, suggesting that signal generation was low for all samples and not just the positive control.

Pepper

Salmonella MPNs determined for pepper were 0.24/g and 4.6/g for the low and high level inoculated samples, respectively (Table 1). Eleven laboratories analyzed the pepper, but data from laboratory 11 were eliminated due to invalid assay results. Nine of the remaining 10 collaborators found all of the inoculated high and low level samples positive by both methods; all positive DNAH assays were confirmed by culture. The remaining data indicated that one collaborator (No. 9) found one high level inoculated sample negative by

Table 6. Collaborative results for detection of *Salmonella* in pepper by BAM/AOAC and DNAH assay methods

	BAM/AOAC												DNAH - Assay[a]												DNAH - Confirmed[b]											
Collaborator	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
2	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
3	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
4	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
5	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
6	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
7	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
8	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
9	-	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	+
10	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+

[a] Samples positive by DNAH assay before culture confirmation.
[b] Samples positive by DNAH assay and confirmed positive by isolation of Salmonella from culture broth.
[c] Samples 1, 2, 3, 4, and 5, were inoculated at a high level; samples 8, 9, 10, 11, and 12 were inoculated at a low level; and samples 6 and 7 were uninoculated control samples.

Table 7. Collaborative results for detection of *Salmonella* in turkey by BAM/AOAC and DNAH assay methods

	BAM/AOAC												DNAH - Assay[a]												DNAH - Confirmed[b]											
Collaborator	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12	1[c]	2	3	4	5	6	7	8	9	10	11	12
1	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-	+	+	-	+	+
2	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+
3	-	-	-	+	-	-	-	+	+	+	+	+	-	-	-	+	-	-	-	+	+	+	+	+	-	-	-	+	-	-	-	+	+	+	+	+
4	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+
5	-	-	-	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+
6	-	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+
7	-	-	-	-	+	-	-	+	+	-	+	+	-	-	-	+	+	+	-	+	+	-	+	+	-	-	-	+	+	+	-	+	+	-	+	+
8	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+
9	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+
11	-	-	-	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+

[a] Samples positive by DNAH assay before culture confirmation.
[b] Samples positive by DNAH assay and confirmed positive by isolation of Salmonella from culture broth.
[c] Samples 3, 4, 5, 6, and 7 were at a high level; samples 8, 9, 10, 11, and 12 were at a low level; and samples 1 and 2 were uninoculated control samples.

both the BAM/AOAC and DNAH assay methods (Table 6). All 22 uninoculated control samples were negative by both methods.

Results from laboratory 11 were excluded because the negative control reading exceeded the 0.15 OD_{450} limit. This collaborator continued the analyses, adjusting for the high reading, and observed perfect correlation between the DNAH and culture methods.

Turkey

Salmonella MPNs for the turkey samples were 0.04/g and 0.09/g, respectively (Table 1). Ten collaborators analyzed the turkey samples. Two of 50 high level samples and 43 of 50 low level samples were positive by both methods, and all positive DNAH assays for these 45 samples were confirmed. Six high level and 5 low level samples were negative by BAM/AOAC but positive by DNAH assay (Table 7). Four of the high level and all 5 of the low level samples were confirmed by isolation of *Salmonella* from the DNAH assay associated culture broths. These 9 samples were interpreted as false negatives by the BAM/AOAC culture procedure. The 2 unconfirmed high level samples were interpreted as false positives for the DNAH assay, but may alternatively represent failures of the culture confirmation procedures. One low level and 4 high level samples that were positive by BAM/AOAC were negative by DNAH assay. These samples were interpreted as false negatives by DNAH assay. Thirty-eight high level, 1 low level, and 20 uninoculated control samples were negative by both methods.

Overall, the recovery of *Salmonella* from the low level sample was better than from the high level samples for both the culture and the DNAH methods. The reason for this is not clear. It is most likely that the MPN determination for the low level sample, 0.04/g, was an underestimation of the true count. This may occur when the distribution of *Salmonella* in the portion used for MPN analysis is not typical of the distribution in the batch.

Discussion

Eleven laboratories participated in the collaborative study. Ten of the laboratories analyzed all foods and 1 collaborator (No. 10) analyzed all but the turkey samples, which had not arrived on time because of problems with the delivery service. One collaborator (No. 3) failed to submit data for the nonfat dry milk samples. Data from 1 laboratory (No. 11) that analyzed pepper, 2 laboratories (Nos. 10 and 11) that analyzed milk chocolate, and 3 laboratories (Nos. 3, 5, and 8) that analyzed dried whole egg were eliminated due to invalid assay results. All remaining data were available for analysis.

Agreement was perfect between BAM/AOAC and DNAH assay methods for dried whole egg and pepper food groups. Of 696 samples analyzed, there were 8 false negatives by the DNAH assay method, 2 for nonfat dry milk (Table 2, sample 12, collaborators 6 and 11), 1 for soy isolate (Table 4, sample 1, collaborator 8), and 5 for turkey (Table 7, sample 3, collaborator 6; sample 4, collaborators 5 and 11; and samples 5 and 10, collaborator 1). There were 4 false positives by DNAH assay, 1 for nonfat dry milk (Table 2, sample 6, collaborator 2); 1 for milk chocolate (Table 3, sample 1, collaborator 6); and 2 for turkey (Table 7, sample 4, collaborator 1 and sample 5, collaborator 8). There were 9 false negatives by the BAM/AOAC procedure, all were found in turkey (Table 7, samples 4 and 6, collaborator 7; sample 7, collaborators 6 and 11; sample 9, collaborator 4; and samples 8, 9, 11, and 12, collaborator 1). Of the 696 samples analyzed, 521 were positive and 154 were negative by both DNAH assay and BAM/AOAC, indicating 97.6% agreement. The false negative rates were calculated by the method of Fleiss (12) and by the method of Zweig and Robertson (13). The rate as defined by Fleiss is the number of false negative assays divided by the total number of negative samples. Zweig and Robertson define the rate as the number of false negative assays divided by the total number of positive samples or one minus sensitivity. The Fleiss definition is recommended by AOAC (F. McClure, FDA, Washington, DC, personal communication), while the Zweig and Robertson definition has been used for the evaluation of results of AOAC collaborative studies on other rapid methods for *Salmonella* (**986.35B,** 15th Ed. [**46.B22,** 14th Ed.]; **987.10B** [**46.C08**]; **987.11B** [**46.C18**]; **989.12B** [**46.E10**]; **989.13B** [**46.E17**]; **989.14** [**46.E24**]; **989.15B** [**46.E33**]) (1). Results for that calculation procedure are presented here to allow comparison with previous collaborative studies. Likewise, the method for computing the confidence interval has

Table 8. Performance of DNAH and BAM/AOAC methods for detection of *Salmonella* in foods and comparison of 2 calculation procedures for false negative rate and confidence interval

Product	Level, MPN/g	DNAH method				BAM/AOAC method			
		FN rate,[a] %	FN 95% CI, %	FN rate,[b] %	FN 95% CI, %	FN rate,[a] %	FN 95% CI, %	FN rate,[b] %	FN 95% CI, %
Nonfat dry milk	0.023	9.1	8.3–9.6	4.0	0.0–9.4	0.0	—[c]	0.0	—[c]
	0.93	0.0	—[c]	0.0	—[c]	0.0	—[c]	0.0	—[c]
Milk chocolate	0.12	0.0	—[c]	0.0	—[c]	0.0	—[c]	0.0	—[c]
	0.75	0.0	—[c]	0.0	—[c]	0.0	—[c]	0.0	—[c]
Soy isolate	1.1	4.3	3.8–4.8	1.8	0.0–5.3	0.0	—[c]	0.0	—[c]
	11	0.0	—[c]	0.0	—[c]	0.0	—[c]	0.0	—[c]
Dried whole egg	0.093	0.0	—[c]	0.0	—[c]	0.0	—[c]	0.0	—[c]
	2.4	0.0	—[c]	0.0	—[c]	0.0	—[c]	0.0	—[c]
Ground black pepper	0.24	0.0	—[c]	0.0	—[c]	0.0	—[c]	0.0	—[c]
	4.6	0.0	0.0–0.5	0.0	—[c]	0.0	0.0–0.5	0.0	—[c]
Turkey	0.04	6.3	0.0–21.1	40.0	9.6–70.4	6.3	0.0–14.2	40.0	9.6–70.4
	0.09	4.5	3.8–5.3	2.0	0.0–6.0	19.2	16.8–21.7	10.2	1.7–18.7
Total		4.8	2.0–7.6	1.5	0.5–2.5	5.4	2.6–8.2	1.7	0.6–2.8

[a] False negative (FN) rate was calculated according to Fleiss (12) as number of false negative results divided by number of negative responses by method per inoculation level and uninoculated control samples. Confidence interval (CI) was calculated according to ratio method of Cochran (14).

[b] False negative (FN) rate was calculated according to Zweig and Robertson (13) as number of false negative results divided by number of positive samples. Confidence interval (CI) was calculated by binomial method.

[c] Standard error equaled zero.

been revised. The results for both methods are presented in Table 8.

A total of 365 isolates of *Salmonella* have been analyzed using the colorimetric DNAH assay (S. Wilson et al., GENE-TRAK Systems, Framingham, MA, manuscript submitted for publication). The assay was nonreactive with 7 isolates, *S. balboa*, *S. bongor*, *S. brookfield*, *S. malawi*, *S. mareqrosso*, *S. camdeni*, and *S.* spp. CDC 2269 1308-83. All of these belong to subgenus V (five) of the Kauffman scheme for biochemical differentiation (15). Reported isolations of subgenus V salmonellae are rare and these serovars have not been encountered in domestic or imported food samples analyzed by the U.S. Food and Drug Administration during the period 1974–1985 (16).

Recommendation

Based on the findings of this collaborative study, the colorimetric GENE-TRAK *Salmonella* Assay method is recommended for adoption official first action as a rapid screening method for the detection of *Salmonella* in all foods, with the known limitation that it is nonreactive with subgenus V salmonellae.

Acknowledgments

The authors thank the following scientists and laboratories that participated in this study:

T. Arista, N. Ramos, and C. Roderick, U.S. Food and Drug Administration, Dallas, TX

B. Bennett, C. Llabrès, and B. Rose, U.S. Dept of Agriculture, Food Safety and Inspection Service, Beltsville, MD

M. Bidlack, J. Gardner, and L. Larkin, Ross Laboratories, Columbus, OH

M. Brodsky, F. Chan, B. Ciebin, E. Föllmi, and A. Thomas, Ontario Ministry of Health, Weston, Ontario, Canada

M. Cirigliano, M. Ghassali, and R. McKenna, Thomas J. Lipton, Inc., Englewood Cliffs, NJ

F. Dever and P. Elliot, Campbell Soup Co., Camden, NJ

J. Fechner, M. Schulke, V. Smith, and T. Wagner, Kraft, Inc., Glenview, IL

D. Ford, T. Sons, and S. Weathersby, Silliker Laboratories, Chicago Heights, IL

J. Fukuoka, Michelson Laboratories, Commerce, CA

D. Nolan and J. Troller, The Procter and Gamble Co., Cincinnati, OH

D. Reed, S. Thompson, and J. Walters, Hershey Foods Corp., Hershey, PA

References

(1) *Official Methods of Analysis* (1990) 15th Ed. and (1984) 14th Ed., AOAC, Arlington, VA

(2) FDA *Bacteriological Analytical Manual* (1984) 6th Ed., AOAC, Arlington, VA

(3) Fitts, R., Diamond, M., Hamilton, C., & Neri, M. (1983) *Appl. Environ. Microbiol.* **46,** 1146–1151

(4) Fitts, R. (1985) *Food Technol.* **39**(3), 95–102

(5) Curiale, M. S., Flowers, R. S., Mozola, M. A., & Smith, A. E. (1986) in *DNA Probes: Applications in Genetic and Infectious Disease and Cancer*, L. S. Lerman (Ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 143–148

(6) Flowers, R. S., Mozola, M. A., Curiale, M. S., Gabis, D. A., & Silliker, J. H. (1987) *J. Food Sci.* **52,** 781–785

(7) Flowers, R. S., Klatt, M. J., Mozola, M. A. Curiale, M. S., Gabis, D. A., & Silliker, J. H. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 521–529

(8) "Changes in Official Methods of Analysis" (1987) *J. Assoc. Off. Anal. Chem.* **70,** 394–396, secs **46.C07–46.C16**

(9) "Changes in Official Methods of Analysis" (1989) *J. Assoc. Off. Anal. Chem.* **72,** 196

(10) "Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis" (1988) *J. Assoc. Off. Anal. Chem.* **71,** 161–172

(11) Flowers, R. S., Ekner, K., Gabis, D. A., Robison, B. J., Mattingly, J. A., & Silliker, J. H. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 786–798

(12) Fleiss, J. L. (1981) *Statistical Methods for Rates and Proportions*, 2nd Ed., John Wiley & Sons, New York, NY, pp. 4–8

(13) Zweig, M. H., & Robertson, M. H. (1987) in *Immunoassay: A Practical Guide*, D. W. Chan & R. T. Perlstein (Eds), pp. 97–127

(14) Cochran, W. G. (1963) *Sampling Techniques*, 2nd Ed., John Wiley & Sons, New York, NY, pp. 64–67

(15) *Bergey's Manual of Systematic Bacteriology* (1984) Vol. 1, Williams and Wilkins, Baltimore, MD, pp. 427–458

(16) Wagner, D. E., & McLaughlin, S. (1986) *J. Food Prot.* **49,** 734–738

MYCOTOXINS

Improved Spectrophotometric Determination of Cyclopiazonic Acid in Poultry Feed and Corn

IVAN CHANG-YEN and KESHORE BIDASEE
Department of Chemistry, University of the West Indies, St. Augustine, Trinidad

An improved visible spectrophotometric method has been developed for cyclopiazonic acid in poultry feed and corn. The method is based on the reaction of cyclopiazonic acid with Ehrlich reagent and detection at 580 nm. Reaction conditions were optimized with respect to reaction and measurement times and acid and Ehrlich reagent concentrations. Calibration curves were linear from 1 to 20 μg cyclopiazonic acid in 3 mL Ehrlich reagent, with a lower detection limit of 0.08 mg/kg for 50 g samples of poultry feed and corn. Recoveries from 50 g samples of poultry feed spiked with cyclopiazonic ranging from 0.16 to 1.20 mg/kg averaged 93.8%. Moldy corn and poultry feed samples analyzed by this method contained between 1 and 4 mg/kg cyclopiazonic acid.

The mycotoxin cyclopiazonic acid (CPA), which was first isolated from cultures of *Penicillium cyclopium* Westling (1), has since been shown to be produced by many species of *Aspergillus* and *Penicillium* on a range of sample types (2–5). When 54 strains of *A. flavus* isolated from various sources were grown on autoclaved agricultural products, 26% of them produced CPA and aflatoxins, 76% produced CPA alone, and 7% produced aflatoxins only (2). CPA has been shown to be toxic to a variety of animals (6–8) and has been associated with Kodua poisoning in humans (9). The recent discovery that a significant percentage of CPA from contaminated food ingested by chickens could accumulate in the flesh (10) has serious implications for human health.

Previous methods for the quantitation of CPA involved thin-layer chromatography (TLC) with Ehrlich reagent (*p*-dimethylaminobenzaldehyde in acid solution) for visualization, with visual (3, 5) or densitometric (11) determination; or ligand exchange liquid chromatography (LC) with ultraviolet detection (10, 12).

A colorimetric method for estimation of CPA also has been described (13) involving TLC cleanup prior to reaction with Ehrlich reagent. However no detection limit was given, the problem of rapid color fading was not defined, and the method is disadvantageous in the time-consuming cleanup procedure. The present study seeks to significantly improve on this relatively inexpensive procedure for accurate and precise determination of CPA in poultry feeds and corn.

METHOD

Apparatus

(a) *Wrist action shaker.*—Gallenkamp SGL-705-010X.

(b) *Rotary evaporator.*—Buchi Model SB.

(c) *Chromatographic columns.*—25 × 0.8 cm id, each plugged at lower end with glass wool and packed with 3.75 g silica gel (Merck, 70–230 mesh) in chloroform.

(d) *Erlenmeyer flasks.*—250 and 500 mL, with ground-glass stoppers.

(e) *Filter paper.*—Whatman No. 541, 7.5 cm diameter.

(f) *Buchner flask.*—500 mL, fitted with Buchner funnel, 7.5 cm diameter.

(g) *Separatory funnels.*—500 mL.

(h) *Round-bottom flasks.*—100, 250, and 500 mL, with ground-glass stoppers.

(i) *Spectrophotometer.*—Perkin Elmer Model 552 with stoppered 4 mL, 1 cm pathlength glass cuvets.

(j) *Syringe.*—Glass, 500 μL, SGE 500A-FN.

Reagents

(a) *Solvents.*—Chloroform, acetone, methanol, distilled water, all glass-distilled in our laboratory before use.

(b) *Sodium sulfate.*—Anhydrous, analytical grade.

(c) *Cyclopiazonic acid.*—(Sigma Chemical Co. C1530). Prepare 100 μg/mL solution in methanol and confirm purity by measuring absorbance at 282 nm, and by thin-layer chromatography. For spectrophotometric analysis, prepare stock solution of 400 μg CPA/mL in acetone and dilute in acetone to produce standards ranging from 5 to 100 μg/mL. Prepare fresh diluted standards weekly and keep in darkened cupboard when not in use.

(d) *p-Dimethylaminobenzaldehyde.*—(Aldrich 12,647-7). Dissolve 4 g in 100 mL 5.5M aqueous HCl. Prepare fresh immediately prior to use and keep tightly stoppered to exclude air when not in use.

(e) *Other reagents.*—1M aqueous NaOH, 2M HCl, saturated NaCl solution.

Sample Extraction

Weigh representative 50 g subsamples of finely divided sample into 500 mL Erlenmeyer flasks. To each add 200 mL chloroform–methanol (85 + 15) solvent and shake 30 min on wrist-action shaker. Let settle 2–3 min, decant supernatant liquid as completely as possible, and suction-filter through Whatman No. 541 filter into 500 mL Buchner flask. Repeat the extraction of solid residue with similar volume of fresh solvent, suction-filter entire slurry, and combine with previous filtrate.

Transfer filtrate to separatory funnel, add 50 mL 1M NaOH, and shake vigorously for 2 min to back-extract CPA into aqueous layer. Let layers separate, drain lower chloroform layer into Erlenmeyer flask, and collect upper aqueous layer in second separatory funnel. Repeat back-extraction twice again and combine aqueous extracts. In final back-extraction, add 25 mL methanol and 25 mL saturated NaCl solution in addition to 1M NaOH to minimize emulsion formation.

Acidify combined aqueous extracts with 2M HCl and extract 3 times with 25 mL volumes of chloroform, collecting lower chloroform layers in 250 mL Erlenmeyer flask. To combined chloroform extracts, add 5 g anhydrous Na_2SO_4

Received March 23, 1989. Accepted August 11, 1989.

Table 1. Recovery of CPA added to feed samples

CPA added, mg/kg	Recovery,[a] %
0.16	79.1 ± 10.3
0.32	100.3 ± 0.0
0.60	87.1 ± 4.8
0.80	97.2 ± 1.4
1.20	105.1 ± 2.2
Mean recovery (n = 5)	93.8%
SD (n = 10)	3.9

[a] Results are means of duplicate analyses ± absolute deviations about the means.

Table 2. Analysis of moldy feed and corn samples

Sample		CPA concn, mg/kg
Poultry Feeds[a]	1	2.05
	2	1.00
	3	1.35
	4	2.00
Corn[b]	1	1.90
	2	2.85
	3	4.00

[a] Feeds held at 80–85% R.H., 22–30°C for 3 weeks.
[b] Samples received in visibly molded state.

with swirling to absorb moisture and decant into 250 mL round-bottom flask. Rinse flask and sodium sulfate with additional 25 mL solvent and add rinse to original extract. Evaporate to dryness on rotary evaporator in 50°C water bath.

Purification of Extracts

Redissolve dried extracts in 0.5 mL chloroform and transfer with Pasteur pipet to chromatographic column packed with silica gel in chloroform. Rinse flask with two 0.5 mL volumes of chloroform and add washings to the column. Elute column with 30 mL each of chloroform, chloroform–acetone (1 + 1), and acetone, and discard these eluates. Elute cyclopiazonic acid with 50 mL acetone–methanol (3 + 1) into 100 mL round-bottom flask. Evaporate solvent to dryness on rotary evaporator, stopper flask tightly, and maintain extract in dried state until analysis. Redissolve each extract in exactly 1 mL acetone just before spectrophotometric determination.

Spectrophotometric Analysis

Into 4 mL glass cuvet, pipet 3 mL Ehrlich reagent. Add by syringe an aliquot (0.2 mL) of CPA standard or sample in acetone. Stopper tightly and invert several times. Wipe faces of cuvet clean and place in sample compartment of spectrophotometer. Measure absorbance at 580 nm against blank of 0.2 mL acetone in 3 mL Ehrlich reagent within 2 min after initial inversion of the cuvet. If sample solution absorbance exceeds that of highest standard, dilute sample solution with acetone and repeat procedure with diluted solution. Plot calibration curve of absorbance vs corresponding μg CPA used, or calculate linear regression equation, and use to determine amount of CPA in 0.2 mL aliquot of sample extract.

Hence, calculate CPA concentration in original 50 g sample from the equation:

$$\text{CPA, mg/kg} = (C/50) \times (1.0/0.2)$$

where C = μg CPA in 0.2 mL sample extract from total volume of 1.0 mL.

Recovery Studies on CPA

Grind poultry feed to pass 1 mm mesh. Weigh duplicate 50 g aliquots in 500 mL Erlenmeyer flasks and spike with various amounts of CPA, equivalent to 0.16–1.20 mg/kg in spiked samples. Carry out extraction and cleanup procedures described to produce 1 mL extracts in acetone. Carry out spectrophotometric analysis as described in the previous section and calculate percent recovery of method at each level of fortification (Table 1).

Analysis of Poultry Feed and Corn Samples

Samples of poultry feed obtained from local manufacturers were stored in 50 g aliquots in sterilized paper bags at 80–85% relative humidity and 20–30°C ambient temperature for 3 weeks to determine the effects of such storage conditions on CPA production. Corn samples were obtained in a visibly molded state from a feed manufacturer, ground to pass 1 mm sieve, and analyzed immediately by the procedure described (Table 2).

Results and Discussion

The use of sodium hydroxide as the initial back-extraction reagent for CPA from chloroform–methanol extracts was shown to be effective for meat samples (10), and the present method shows this to be equally effective for poultry feed and corn. The use of methanol and sodium chloride in the final back-extraction step was necessary to minimize emulsion formation and loss of CPA. The column purification step was also necessary to minimize background absorbance caused by coextractives from samples.

Timed measurements of absorbance of CPA-Ehrlich reagent solutions showed that absorbance values reached maximum values after 1 min, remained constant for an additional 1 min, and then decreased slowly but appreciably thereafter. All readings for samples and standards were therefore taken within 2 min of reaction.

The absorption maximum of a 0.2 mL solution containing 10 μg CPA in acetone and 3 mL Ehrlich reagent prepared according to Lansden (11) was determined to be 580 nm with a shoulder at 545 nm (Figure 1). By systematically varying in turn the hydrochloric acid concentration (4M to 8M) and *p*-dimethylaminobenzaldehyde concentration (0.01 to 0.06 g/mL) and monitoring the reaction at 580 nm over extended periods of time, we determined the following optimal conditions: hydrochloric acid, 5.5M; *p*-dimethylaminobenzaldehyde, 0.04 g/mL; time for stable absorbance to be reached, 1 min; length of time constant absorbance maintained, 1 min.

Acetone proved to be a better solvent than methanol (13) for redissolving the purified extracts for spectrophotometric analysis. The calibration curves of absorbance vs μg CPA concentration were linear from 1 to 20 μg CPA in 3 mL Ehrlich reagent, and may be described by the equation: $y = 0.042x + 0.037$, $r = 0.999$, where y = absorbance value at 590 nm, x = μg CPA, and r = correlation coefficient.

The overall procedure has a lower detection limit for CPA in poultry feed and corn of 0.08 mg/kg, this being equivalent to an absorbance value twice that of uncontaminated samples. Since the absorbances of uncontaminated corn and poultry feeds were similar, and since the poultry feeds con-

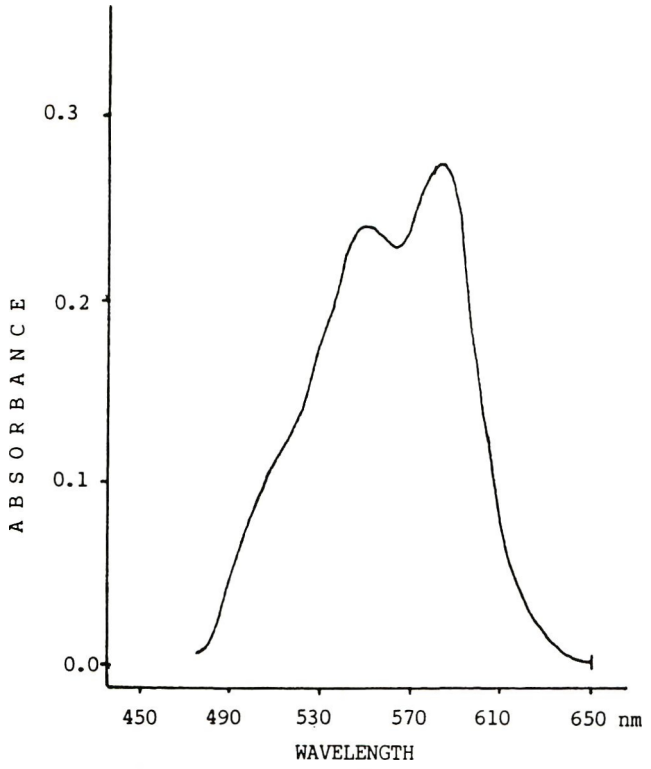


Figure 1. Visible spectrum of CPA-Ehrlich reagent reaction.

tained a high percentage of corn, it was thought unnecessary to carry out similar recovery studies on corn. The mean recovery (93.8%) obtained by the present method compares very well with that (85%) of a previous method for corn (11), and the lower limit of detection is one-half that of the TLC procedure (11).

Also in the present procedure, initiation of the reaction between CPA and Ehrlich reagent in a cuvet allows for more exact timing of absorbance measurements than that of the previous colorimetric method (13). This effectively overcomes the problems associated with rapid fading of color of the reaction product and increases accuracy and precision of measurement. Redissolution of purified sample extracts in acetone just before spectrophotometric determination also minimizes errors due to solvent evaporation and volume change.

The poultry feed analyzed in this study was deliberately molded in controlled humidity chambers under conditions similar to those experienced annually during the local wet season. During this period the sale of visibly molded feeds is not uncommon. Molded aliquots of feed and corn were also analyzed by an established method (14) and found to contain aflatoxins near to or below the detection limits of the method, established under our laboratory conditions as (mg/kg): 0.1 B_1, 0.5 G_1, 0.4 B_2, and 1.0 G_2. These levels are in direct contrast to the CPA levels in aliquots of similarly molded feed samples, ranging from 1.00 to 2.05 mg/kg, and in corn from 1.90 to 4.00 mg/kg. The corn samples, however, were obtained in an already visibly molded state from a local feed manufacturer, shortly before the corn was incorporated into animal feed formulations.

A previous study of bulk animal feeds and feed components in Trinidad (15) was unable to detect aflatoxin contamination, even on samples that were heavily molded. Our microbiological studies on the moldy feed and corn used in the present study have confirmed the presence of several species of *Aspergillus*, notably *A. flavus* Link (identified by the Commonwealth Mycological Institute, Kew, U.K.) as well as *Penicillium*, *Cladosporium*, *Rhizopus*, *Mucor*, and *Fusarium* species. The *A. flavus* Link isolates from corn and feed samples were subcultured on boiled brown rice and sterilized poultry feed, respectively, for 2 weeks. Analysis of the cultures showed these fungal isolates to be strong CPA but minimal aflatoxin producers, as observed in the moldy samples.

It is also possible that other fungal isolates could have contributed to the CPA levels found. Crystals of CPA isolated chromatographically from the *A. flavus* cultures were analyzed by TLC and IR and UV spectroscopy, respectively. The sample spectra and R_f values closely matched those of reference data generated under similar analytical conditions (16). The acetone extracts of moldy corn and feed samples were also analyzed by TLC using CPA standards for comparison and Ehrlich reagent spray, to confirm the presence of CPA in samples.

Since local legislation at present only defines maximum permitted levels of aflatoxins and a few other mycotoxins, but not CPA, the presence of such CPA-producing fungi on feeds and corn has serious implications for human and animal health. A large scale survey of animal feeds, feed compounds and foods is being undertaken to fully assess the extent of CPA contamination.

Acknowledgments

The authors are grateful to R. Best and A. Chen for provision of samples for the study, and the Commonwealth Mycological Institute, Kew, U.K., for identification of the fungal isolates.

REFERENCES

(1) Holzapfel, C. W. (1968) *Tetrahedron* **24,** 2101–2119
(2) Gallagher, R. T., Richard, J. L., Stahr, H. M., & Cole, R. J. (1978) *Mycopathologia* **66,** 31–36
(3) Lansden, J. A., & Davidson, J. I. (1983) *Appl. Environ. Microbiol.* **45,** 766–769
(4) Cole, R. J. (1984) in *Mycotoxins: Production, Isolation, Separation, and Purification*, V. Betina (Ed.), Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 405–414
(5) Trucksess, M. W., Mislivec, P. B., Young, K., Bruce, V. R., & Page, S. W. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 123–126
(6) Purchase, I. F. H. (1971) *Toxicol. Appl. Pharmacol.* **18,** 114–123
(7) Dorner, J. W., Cole, R. J., Lomax, L. G., Gosser, H. S., & Diener, U. L. (1983) *Appl. Environ. Microbiol.* **46,** 698–703
(8) Lomax, L. G., Cole, R. J., & Dorner, J. W. (1984) *Vet. Pathol.* **21,** 418–424
(9) Rao, B. L., & Husain, A. (1985) *Mycopathologia* **89,** 177–180
(10) Norred, W. P., Cole, R. J., Dorner, J. W., & Lansden, J. A. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 121–123
(11) Lansden, J. A. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 964–966
(12) Lansden, J. A. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 728–730
(13) Rathinavelu, A., & Shanmugasundaram, L. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 38–40
(14) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, Secs **26.031, 26.049, 26.050**
(15) Chang-Yen, I., & Felmine, J. (1987) *Trop. Agric. (Trin.)* **64,** 283–286
(16) Cole, R. J., & Cox, R. H. (1981) in *Handbook of Fungal Metabolites*, Academic Press, New York, NY, pp. 497–500

Liquid Chromatographic Method for Determination of Aflatoxins B_1, B_2, G_1, and G_2 in Corn and Peanut Products: Collaborative Study

DOUGLAS L. PARK,[1] STANLEY NESHEIM, MARY W. TRUCKSESS, MICHAEL E. STACK, and RICHARD F. NEWELL[2]

Food and Drug Administration, Division of Contaminants Chemistry, Washington, DC 20204

Collaborators: K. Ainsworth; N. Bezodis; K. T. Faughnan; K. Kurz; L. H. Marion; M. Navarre; D. R. Newkirk; G. R. Nixon; R. Schneider; W. Steiner; P. Steyn; E. Tarter; D. W. Trinder; R. Wong; P. H. Yu

A collaborative study of a liquid chromatographic method for the determination of aflatoxins B_1, B_2, G_1, and G_2 was conducted in laboratories located in the United States, Canada, South Africa, and Switzerland. Twenty-one artificially contaminated raw peanuts, peanut butter, and corn samples containing varying amounts of aflatoxins B_1, B_2, G_1, and G_2 were distributed to participating laboratories. The test portion was extracted with methanol–0.1N HCl (4 + 1), filtered, defatted with hexane, and then partitioned with methylene chloride. The concentrated extract was passed through a silica gel column. Aflatoxins B_1 and G_1 were derivatized with trifluoroacetic acid, and the individual aflatoxins were determined by reverse-phase liquid chromatography with fluorescence detection. Statistical analysis of the data was performed to determine or confirm outliers, and to compute repeatability and reproducibility of the method. For corn, relative standard deviations for repeatability (RSD_r) for aflatoxin B_1 ranged from 27.2 to 8.3% for contamination levels from 5 through 50 ng/g. For raw peanuts and peanut butter, RSD_r values for aflatoxin B_1 were 35.0 to 41.2% and 11.2 to 19.1%, respectively, for contamination levels from 5 through 25 ng/g. RSD_r values for aflatoxins B_2, G_1, and G_2 were similar. Relative standard deviations for reproducibility (RSD_R) for aflatoxin B_1 ranged from 15.8 to 38.4%, 24.4 to 33.4%, and 43.9 to 54.0% for corn, peanut butter, and raw peanuts, respectively. The method has been adopted official first action for the determination of aflatoxins B_1, B_2, G_1, and G_2 in peanut butter and corn at concentrations ≥13 ng total aflatoxins/g.

A review of the scientific literature noted over 20 reports of liquid chromatographic (LC) methods with potential applications to aflatoxin control programs (1). Most of these methods use either a normal-phase column with a silica gel-packed flow cell for fluorescence detection (2), trifluoroacetic acid (TFA) derivatization of the toxins and a reverse-phase column with fluorescence detection (3), or post-column derivatization with iodine in water (4–7). Two methods, a normal-phase LC method and a reverse-phase method, were previously studied collaboratively but neither gave acceptable results (8). Based on performance of the TFA derivatization method with the Smalley Check Sample Program (J. McKinney, Ranchers Cotton Oil, personal communication, 1985) and intensive practical application in Canada and the United States, this method (3) was selected for collaborative study under the sponsorship of AOAC and the International Union of Pure and Applied Chemistry (IUPAC).

Received for publication April 17, 1989.

This report was presented at the 102nd AOAC Annual International Meeting, August 29–September 1, 1988, at Palm Beach, FL.

The recommendation was approved interim official first action by the General Referee, the Committee on Foods I, and the Chairman of the Official Methods Board. The method was adopted official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73**, January/February issue.

This study was carried out under the joint sponsorship of AOAC and the Commission on Food Chemistry of the International Union of Pure and Applied Chemistry (IUPAC).

[1] Present address: University of Arizona, Department of Nutrition and Food Science, Tucson, AZ 85721.

[2] Division of Mathematics.

Collaborative Study

Ten laboratories were each furnished with 21 coded test portions of raw peanuts, peanut butter, and corn artificially contaminated with aflatoxins B_1, B_2, G_1, and G_2. Test portions were prepared by obtaining sufficient quantities of corn, raw peanuts, and peanut butter as free as possible from aflatoxin contamination and adding various amounts of aflatoxins B_1, B_2, G_1, and G_2. Contamination levels ranged from 13 to 65 ng total aflatoxins/g for raw peanuts and peanut butter, and from 13 to 130 ng/g for corn. The nonspiked product served as a negative control. Collaborators were supplied with practice test portions, reference standards, method directions, and test portions of each commodity as blind duplicates at all contamination levels.

990.33 Aflatoxins in Corn and Peanut Butter

Liquid Chromatographic Method

First Action 1990

AOAC–IUPAC Method

(Applicable to determination of aflatoxins B_1, B_2, G_1, and G_2 at ≥13 ng total aflatoxins/g or 5 ng aflatoxin B_1/g in corn and peanut butter)

Method Performance:

(*Note:* The average aflatoxin concentrations found were used as the denominator to calculate the RSD values.)

Corn, 5 ng aflatoxin B_1/g
$s_r = 0.92$; $s_R = 1.10$; $RSD_r = 27.2\%$; $RSD_R = 32.3\%$

Corn, 10 ng aflatoxin B_1/g
$s_r = 0.78$; $s_R = 0.96$; $RSD_r = 12.8\%$; $RSD_R = 15.8\%$

Corn, 50 ng aflatoxin B_1/g
$s_r = 2.87$; $s_R = 13.29$; $RSD_r = 8.3\%$; $RSD_R = 38.4\%$

Peanut butter, 5 ng aflatoxin B_1/g
$s_r = 0.63$; $s_R = 1.37$; $RSD_r = 11.2\%$; $RSD_R = 24.4\%$

Peanut butter, 10 ng aflatoxin B_1/g
$s_r = 1.19$; $s_R = 2.48$; $RSD_r = 13.1\%$; $RSD_R = 27.2\%$

Peanut butter, 25 ng aflatoxin B_1/g
$s_r = 3.86$; $s_R = 6.75$; $RSD_r = 19.1\%$; $RSD_R = 33.4\%$

A. Principle

Aflatoxins are extracted and purified, derivatized with trifluoroacetic acid (aflatoxins B_1 and G_1 to B_{2a} and G_{2a}, respectively), separated by reverse-phase liquid chromatography, and detected by fluorescence. Method can measure

0.1 ng of aflatoxins B_1, B_2, G_1, and G_2. Detection limit is ca 0.3 ng/g for each aflatoxin.

B. Apparatus

Equipment specified is not restrictive; other suitable equipment can be substituted.

(a) *Liquid chromatograph.*—Varian Model 5000 with Model 7125 Rheodyne septumless injector, or equivalent; Fluorichrom fluorescence detector (Varian); 7-54 and 7-60 excitation filters (360 nm), 3-73 and 4-76 glass emission filters (440 nm), fitted with Varian flowcell; integrator or recorder, 0.5 cm/min chart speed. Flow rate 1.0 mL/min. Set up detector, preferably with tungsten source, using low lamp, high gain, attenuation 20, or adjust range to give minimum half-scale deflection with 1.25 ng aflatoxin B_{2a} or G_{2a}. For optimum performance, detector should be left on continuously.

(b) *LC column.*—15 cm × 4.6 mm id, Supelcosil LC-18 No. 5-8230, 5 μm, or equivalent. (*Note:* New LC columns or those that have been stored in methanol for extended periods require conditioning with concentrated standards in order to achieve optimum resolution and sensitivity for aflatoxins B_{2a} and G_{2a}. Make 5 consecutive injections of a derivatized standard containing G_{2a} and B_{2a} at 0.5 μg/2.0 mL.)

(c) *Cleanup column.*—20 cm × 1 cm id, with Teflon stopcock and coarse frit bed support (glass wool bed support not recommended); detachable glass solvent reservoir with 24/40 fitting.

(d) *Adjustable pipets.*—10-100 μL and 100-1000 μL with disposable tips (Eppendorf).

(e) *Filter tube.*—Glass, 15 cm × 2.5 cm id, with coarse frit bed support (glass wool not recommended).

C. Reagents

(a) *Solvents.*—Distilled-in-glass grade methanol, hexane, methylene chloride, benzene, acetone, acetonitrile. Anhydrous ethyl ether (Mallinckrodt No. 0848 or equivalent) stored in metallic container. Glass-bottled ether may form peroxides soon after opening which degrade aflatoxins.

(b) *LC elution solvent.*—H_2O-CH_3CN-methanol (700 + 170 + 170). Adjust ratio of water to obtain baseline resolution of aflatoxins B_{2a} and G_{2a}.

(c) *Silica gel for column chromatography.*—E. Merck Silica Gel 60, 0.63-0.2 mm (No. 7734). Activate by drying 4 h at 100°. Cool to room temperature. Weigh desired quantity (100 g) into glass-stopper container. Add 1 mL H_2O in small increments; agitate silica gel between additions. Shake or tumble mechanically 4-6 h. If mechanical tumbler or shaker is not available, shake manually 5 min/h over 8 h. Let stand 16 h. Perform suitability test as in **D**.

(d) *Trifluoroacetic acid (TFA).*—Eastman Kodak No. 6287 (assay by titration ≥98.5%), or equivalent. Transfer 1 or 2 mL TFA to 1 dram vial with Teflon-lined cap. Keep in freezer when not in use. Discard if discoloration appears.

(e) *Sodium sulfate.*—Anhydrous, coarse granular (BDH No. ES0760-40 [EM Science No. SX0760-3], or equivalent). Sift out fines to obtain 20-40 mesh range. Heat 2-3 h at 600° to remove organic impurities.

(f) *Aflatoxin standard solutions.*—(*1*) *Aflatoxin stock solutions.*—10 μg/mL. Prepare individual stock solutions in benzene-CH_3CN (98 + 2) and determine concentration of each by measuring UV absorbance as in **971.22A** and **B**.

(*2*) *Working standard solutions.*—Use Eppendorf pipet to transfer appropriate quantity of stock solutions to each 4 dram vial to obtain following amounts of aflatoxins in each vial, respectively:

Vial	B_1 and G_1, ng	B_2 and G_2, ng
1	250	125
2	500	250
3	1000	500
4	2000	1000

Evaporate solutions to dryness under gentle stream of nitrogen (drying may be facilitated by warming to 40°). Using Eppendorf pipet, add 200 μL hexane and 50 μL TFA to each vial, cap, and vortex-mix 30 s. Let solutions stand 5 min; then, add 10.0 mL H_2O-CH_3CN (9 + 1) and vortex-mix 30 s. Let layers separate 5-10 min, or centrifuge at 1000 rpm for 30 s. Final concentration of aflatoxins is as follows:

Vial	B_1 and G_1, μg/10.05 mL	B_2 and G_2, μg/10.05 mL
1	0.25	0.125
2	0.5	0.25
3	1.0	0.50
4	2.0	1.00

D. Silica Gel Suitability Test

Prepare 2.0 g silica gel column as in **G**. Transfer appropriate quantity of aflatoxin stock solutions to 50 mL beaker to contain 500 ng each of aflatoxins B_1 and G_1 and 250 ng each of aflatoxins B_2 and G_2. Evaporate mixture to dryness under gentle stream of nitrogen. Add 2-3 mL CH_2Cl_2 and swirl 10 s. Quantitatively transfer to silica gel column with 2 ca 1 mL portions of CH_2Cl_2, using wash bottle. Proceed as in **G**, using the 2 wash solvents and eluting with CH_2Cl_2-acetone (9 + 1). Derivatize as in **H** and perform LC analysis. Compare chromatogram with that of derivatized standard. Peak heights (areas) should be nearly identical to those for working standard, i.e., B_1, G_1 = 0.5 μg/10.05 mL; B_2, G_2 = 0.25 μg/10.05 mL. Recovery should be ≥90%.

E. Extraction

Transfer 50 g corn (prepared as in **972.26A**) or 50 g peanut butter (prepared as in **968.22C**) to 1 L blender jar, add 200 mL methanol followed by 50 mL 0.1N HCl, and blend 3 min at high speed. Filter through 24 cm Whatman No. 1 paper, or equivalent. (*Note:* Finely ground corn meal may require centrifugation at 1000 rpm for 10 min.) Filtrate may not be completely clear. Collect 50 mL filtrate.

F. Partition

Transfer 50 mL filtrate to 250 mL separatory funnel. Add 50 mL 10% NaCl solution, swirl, add 50 mL hexane, and shake gently ca 30 s. Let phases separate and drain lower aqueous layer into another 250 mL separatory funnel. Discard hexane layer. Add 25 mL CH_2Cl_2 and shake moderately 30 s. If emulsion occurs, break up with clean Pasteur pipet. Let phases separate and drain lower CH_2Cl_2 layer through 4 cm coarse granular, anhydrous Na_2SO_4 in glass filter tube. Collect eluate in 250 mL beaker. Repeat partition with 2 additional 25 mL portions of CH_2Cl_2, and vigorously shake and drain as above. Collect eluate in the 250 mL beaker.

(*Note:* Good stopping point if necessary.) Evaporate eluate on steam bath under gentle stream of nitrogen to 2–3 mL (1–2 mm layer of eluate covers bottom of beaker).

G. Column Chromatography

Slurry 2.0 g silica gel with ca 10 mL ether–hexane (3 + 1) in 30 mL beaker, pour slurry into cleanup column, and wash beaker with additional 5 mL ether–hexane solvent to effect transfer. Keep stopcock closed and let silica gel settle without tamping. Wash sides of column with 2–3 mL ether-hexane (3 + 1), using wash bottle. After gel settles, open stopcock, and, while column drains, add ca 1 cm anhydrous granular Na_2SO_4. Transfer extract from partition, **F,** to column. Wash lip of beaker with 0.5 mL CH_2Cl_2, using wash bottle, and collect wash in column. Wash beaker with ca 2 mL CH_2Cl_2 and add wash to column. Do not use more than 5–6 mL CH_2Cl_2 to transfer extract to column.

With stopcock fully open, add 25 mL benzene–acetic acid (9 + 1), and then add 30 mL ether–hexane (3 + 1) to column, draining each wash to top of Na_2SO_4. Discard washes. Elute aflatoxin with 100 mL CH_2Cl_2–acetone (90 + 10) and collect eluate in 250 mL beaker. Evaporate solvent on steam bath under gentle stream of nitrogen to ca 6 mL. Quantitatively transfer to 3 dram vial, using 2–3 mL CH_2Cl_2 as wash. (*Note:* Good stopping point if necessary.)

Evaporate eluate almost to dryness on steam bath in an aluminum block under gentle stream of nitrogen. Evaporate remaining 200 μL just to dryness under gentle stream of nitrogen by holding vial in palm of hand and slowly rotating vial.

H. Derivatization

Add 200 μL hexane to column extract, **G.** Then, add 50 μL TFA (using Eppendorf pipet), cap vial, and vortex-mix vigorously for 30 s (*exactly*). This procedure must be followed closely to ensure consistent reaction yields. Let mixture stand 5 min. Using Eppendorf pipet, add 1.950 mL H_2O–CH_3CN (9 + 1). Vortex-mix vigorously for 30 s (*exactly*), and let layers separate 10 min or centrifuge at 1000 rpm for 30 s. Concentration is 10 g/2 mL aqueous CH_3CN.

I. LC Determination

Using instrument parameters previously described, **B,** successively inject 25 μL of derivatized standard solutions. Prepare standard curve to check linearity of responses. Inject 25 μL TFA-treated sample solution (lower aqueous phase). If sample peaks are outside linear range, dilute aliquot of TFA-treated sample solution to suitable volume with H_2O–CH_3CN (9 + 1), re-mix on vortex mixer, and inject another 25 μL portion. Calculate individual aflatoxin concentrations as follows. Use responses of standard containing 500 ng B_1 and G_1, and 250 ng B_2 and G_2 for calculations.

$$\text{Aflatoxins, ng/g} = (P/P') \times C \times (2/10) \times 1000 \times D$$

where P and P' = peak areas (integrator counts) or heights for sample and standard, respectively, per 25 μL injection; C = concentration of individual aflatoxins in standard solution (0.5 or 0.25 μg/10.05 mL); and D = dilution factor if 2 mL solution for injection is diluted.

Ref.: JAOAC **73,** March/April issue (1990).

Table 1. Collaborative study results for determination of aflatoxin by liquid chromatography[a]

Lab.	Aflatoxin added	Corn 0	13	13	26	26	130	130
1	B_1	0.2	3.0	3.9	5.4	8.0	31.0	40.0
	B_2	0.0	1.0	1.3	2.0	2.5	11.0	13.0
	G_1	0.1	1.9	3.7	2.5	8.6	6.6	40.0
	G_2	0.0	0.6	1.3	1.1	2.6	2.9	13.0
	Total	0.3	6.5	10.2	11.0	21.7	51.5	106.0
2	B_1	0.1	4.1	3.7	5.7	5.6	9.1	9.8
	B_2	0.0	1.5	1.2	2.8	2.8	6.0	6.2
	G_1	0.0	3.6	3.2	4.8	4.7	7.3	7.5
	G_2	0.0	1.5	1.1	2.5	2.6	4.6	4.6
	Total	0.1	10.7	9.2	15.8	15.7	27.0	28.1
3	B_1	0.0	2.9	16.3	5.8	5.7	32.6	29.9
	B_2	0.0	1.2	7.2	2.4	2.7	14.4	12.8
	G_1	0.0	3.6	17.6	7.0	7.3	36.7	32.6
	G_2	0.0	0.9	5.2	1.9	2.0	10.1	8.6
	Total	0.0	8.7	46.3	17.1	17.7	93.7	83.9
4	B_1	0.0	7.0	0.0	7.0	6.0	13.0	34.0
	B_2	0.0	3.0	0.0	3.0	3.0	15.0	15.0
	G_1	0.0	8.0	0.0	8.0	7.0	21.0	37.0
	G_2	0.0	3.0	0.0	3.0	3.0	14.0	13.0
	Total	0.0	21.0	0.0	21.0	19.0	63.0	99.0
5	B_1	0.0	3.2	2.9	5.3	6.5	33.0	32.3
	B_2	0.0	1.4	1.3	2.3	2.9	14.5	14.5
	G_1	0.0	3.0	3.4	5.6	7.0	34.1	35.4
	G_2	0.0	1.0	1.1	1.8	2.3	11.4	11.4
	Total	0.0	8.6	8.7	15.0	18.7	93.0	93.6
6	B_1	0.0	3.9	3.9	6.8	7.2	36.7	38.8
	B_2	0.0	1.3	1.4	2.5	2.6	12.8	13.0
	G_1	0.0	4.7	4.5	7.7	7.8	40.0	44.9
	G_2	0.0	1.4	1.4	2.5	2.5	13.1	14.2
	Total	0.0	11.3	11.2	19.5	20.1	102.6	110.9
7	B_1	0.0	3.1	5.2	9.7	10.3	55.5	58.0
	B_2	0.0	0.6	1.0	2.1	2.4	18.1	14.1
	G_1	0.0	0.9	2.9	5.4	5.7	18.1	31.9
	G_2	0.0	0.1	0.7	1.4	1.5	10.4	9.1
	Total	0.0	4.7	9.8	18.6	19.9	102.1	113.1
8	B_1	0.2	3.5	5.0	6.4	6.6	29.1	34.5
	B_2	0.0	1.3	1.9	2.4	2.4	11.5	12.9
	G_1	0.2	3.9	4.6	6.9	7.3	30.5	34.5
	G_2	0.0	1.6	2.1	2.8	2.6	12.5	14.1
	Total	0.4	10.2	13.5	18.5	18.8	83.6	95.9
9	B_1	0.3	1.6	1.7	4.1	21.7	4.1	58.0
	B_2	0.0	0.4	0.2	1.4	7.7	1.5	14.1
	G_1	0.3	2.1	2.0	5.4	28.7	5.4	31.9
	G_2	0.1	0.4	0.3	1.6	8.9	1.8	9.1
	Total	0.7	4.5	4.2	12.5	67.0	12.8	113.1
10	B_1	0.3	1.5	3.9	4.8	4.3	40.4	42.5
	B_2	0.2	0.9	1.3	1.8	1.5	13.3	13.4
	G_1	0.3	0.6	3.9	1.9	1.7	35.3	40.2
	G_2	0.2	0.4	1.1	0.7	0.7	11.5	13.0
	Total	1.0	3.4	10.2	9.2	8.2	100.5	109.1

[a] Results (ng/g) are single determinations on blind duplicate test portions at each concentration (except nonspiked sample).

Table 2. Collaborative study results for determination of aflatoxin by liquid chromatography[a]

Lab.	Aflatoxin added	Peanut butter 0	13	13	26	26	65	65
1	B_1	2.5	6.3	6.1	12.0	12.0	22.0	24.0
	B_2	0.5	2.0	1.9	3.9	3.6	7.1	7.1
	G_1	0.4	4.1	3.9	7.6	8.2	12.0	16.0
	G_2	1.3	2.9	2.6	3.7	2.8	4.7	5.2
	Total	4.7	15.3	14.5	27.2	26.6	45.8	52.3
2	B_1	2.5	5.4	5.7	4.5	6.3	7.9	7.8
	B_2	0.0	1.8	1.9	2.6	3.6	5.0	4.9
	G_1	1.1	4.2	4.3	3.2	4.6	6.5	6.3
	G_2	0.0	1.7	1.9	1.7	2.7	3.4	3.5
	Total	3.6	13.1	13.8	12.0	17.2	22.8	22.5
3	B_1	2.2	6.3	5.8	10.4	10.5	18.8	20.6
	B_2	0.0	2.0	1.9	3.8	3.7	8.4	8.3
	G_1	0.0	4.9	4.5	9.0	8.7	19.5	21.6
	G_2	0.0	1.3	1.3	2.6	2.4	6.1	5.9
	Total	2.2	14.5	13.4	25.8	25.3	52.9	56.4
4	B_1	0.0	5.0	0.0	10.0	7.0	28.0	22.0
	B_2	0.0	2.0	0.0	4.0	4.0	12.0	9.0
	G_1	0.0	4.0	0.0	8.0	8.0	26.0	21.0
	G_2	0.0	2.0	0.0	4.0	3.0	11.0	8.0
	Total	0.0	13.0	0.0	26.0	22.0	77.0	60.0
5	B_1	1.8	5.7	5.4	6.3	8.4	21.1	20.1
	B_2	0.0	2.0	2.0	2.4	3.4	8.4	8.0
	G_1	0.9	4.7	4.6	3.8	7.4	20.4	18.7
	G_2	0.0	1.7	1.6	1.4	2.6	7.4	6.3
	Total	2.7	14.1	13.6	13.9	21.8	57.3	53.1
6	B_1	2.8	6.9	6.5	11.5	11.4	23.8	24.4
	B_2	0.4	1.9	1.9	3.5	3.5	7.8	8.2
	G_1	0.9	5.4	5.7	9.5	10.2	23.7	24.8
	G_2	0.1	1.9	2.2	3.5	3.5	8.4	9.0
	Total	4.2	16.1	16.3	28.0	28.6	63.7	66.4
7	B_1	0.5	7.2	7.1	11.1	10.7	28.1	28.0
	B_2	0.0	1.6	1.7	2.7	2.8	7.4	7.5
	G_1	1.7	6.1	6.2	9.2	7.0	20.5	20.6
	G_2	0.1	1.2	1.3	2.1	2.0	5.1	5.2
	Total	2.3	16.1	16.3	25.1	22.5	61.1	61.3
8	B_1	2.0	5.3	4.9	7.5	9.3	10.7	19.5
	B_2	0.3	1.8	1.8	1.3	3.1	7.2	7.1
	G_1	0.5	3.9	3.2	5.8	7.5	9.1	15.0
	G_2	0.1	1.5	1.7	1.4	2.9	7.6	7.2
	Total	3.0	12.5	11.6	16.0	22.8	34.6	48.7
9	B_1	1.4	3.2	2.0	5.0	6.9	19.1	9.4
	B_2	1.2	1.0	0.6	1.3	2.1	6.6	2.8
	G_1	6.9	3.3	2.3	5.9	7.3	30.5	12.0
	G_2	0.0	1.0	0.7	1.8	2.1	8.8	3.4
	Total	9.5	8.5	5.6	14.0	18.4	65.0	27.6
10	B_1	2.6	4.5	6.7	11.5	9.4	29.4	20.2
	B_2	0.3	1.4	2.2	3.4	2.9	8.3	6.4
	G_1	0.7	2.7	5.5	9.4	7.3	28.6	17.0
	G_2	0.3	1.5	1.7	2.9	2.3	9.9	5.5
	Total	3.9	10.1	16.1	27.2	21.9	76.2	49.1

[a] Results (ng/g) are single determinations on blind duplicate test portions at each concentration (except nonspiked sample).

Table 3. Collaborative study results for determination of aflatoxin by liquid chromatography[a]

Lab.	Aflatoxin added	Raw peanuts 0	13	13	26	26	65	65
1	B_1	0.4	1.5	3.1	3.2	0.7	16.0	4.4
	B_2	0.0	0.7	1.1	1.4	0.4	5.6	2.2
	G_1	0.2	0.3	2.6	0.3	0.2	18.0	0.4
	G_2	0.0	0.0	1.1	0.2	0.0	6.3	0.1
	Total	0.6	2.5	7.9	5.1	1.3	45.9	7.1
2	B_1	0.0	4.1	3.7	5.6	0.1	7.6	7.6
	B_2	0.0	1.5	1.3	2.7	0.0	4.7	4.9
	G_1	0.2	3.6	3.4	4.7	0.1	6.1	6.2
	G_2	0.0	1.4	1.3	2.5	0.0	3.7	3.7
	Total	0.2	10.6	9.7	15.5	0.2	22.1	22.4
3	B_1	0.0	2.8	3.7	6.4	7.0	19.0	3.5
	B_2	0.0	1.4	1.5	2.7	2.8	7.7	1.3
	G_1	0.0	3.1	4.0	8.0	7.6	21.8	3.9
	G_2	0.0	0.9	1.0	1.9	1.8	5.2	1.0
	Total	0.0	8.2	10.1	19.0	19.2	53.6	9.7
4	B_1	0.0	3.0	7.0	7.0	4.0	20.0	11.0
	B_2	0.0	2.0	3.0	4.0	3.0	10.0	8.0
	G_1	0.0	4.0	7.0	8.0	5.0	21.0	14.0
	G_2	0.0	2.0	3.0	3.0	3.0	9.0	7.0
	Total	0.0	11.0	20.0	22.0	15.0	60.0	40.0
5	B_1	0.0	2.4	3.3	5.7	—[b]	15.1	17.3
	B_2	0.0	1.3	1.4	2.6	—[b]	7.3	7.3
	G_1	0.0	3.0	4.1	6.0	—[b]	17.7	20.1
	G_2	0.0	1.0	1.2	2.0	—[b]	5.8	6.3
	Total	0.0	7.7	10.0	16.3	—[b]	45.9	51.0
6	B_1	0.0	3.6	4.1	7.1	7.6	19.6	18.3
	B_2	0.0	1.2	1.4	2.5	2.7	6.9	6.3
	G_1	0.4	4.0	4.8	8.3	9.4	22.2	20.8
	G_2	0.0	1.2	1.4	2.6	2.9	7.2	6.5
	Total	0.4	10.0	11.7	20.5	22.6	55.9	51.9
7	B_1	0.0	5.0	3.4	—[b]	8.9	18.6	30.9
	B_2	0.0	1.1	0.8	—[b]	2.4	4.0	7.2
	G_1	0.0	2.4	3.3	—[b]	5.9	9.3	17.8
	G_2	0.0	0.6	0.7	—[b]	1.6	1.8	4.7
	Total	0.0	9.1	8.2	—[b]	18.8	33.7	60.6
8	B_1	0.7	2.8	2.7	6.0	5.7	15.0	12.9
	B_2	0.2	1.1	1.0	2.4	2.2	6.0	5.8
	G_1	0.5	2.3	3.0	5.8	4.7	11.4	12.8
	G_2	0.2	1.2	1.3	2.8	2.6	6.9	6.2
	Total	1.5	7.4	8.0	17.0	15.2	39.3	37.6
9	B_1	—[c]	1.0[c]	0.5[c]	0.5[c]	1.2[c]	4.0[c]	1.7[c]
	B_2	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]
	G_1	—[c]	0.5[c]	0.2[c]	0.7[c]	0.4[c]	1.2[c]	0.4[c]
	G_2	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]	—[c]
	Total	—[c]	1.5[c]	0.7[c]	1.2[c]	1.6[c]	5.2[c]	2.1[c]
10	B_1	0.4	4.6	3.0	6.9	6.0	23.8	16.3
	B_2	0.3	1.5	1.0	2.2	2.2	7.5	5.7
	G_1	0.4	4.9	2.1	5.1	2.8	26.2	12.5
	G_2	0.3	1.4	0.7	1.9	1.1	8.1	4.5
	Total	1.4	12.4	6.8	16.1	12.1	65.6	39.0

[a] Results (ng/g) are single determinations on blind duplicate test portions at each concentration (except nonspiked sample).
[b] Sample lost.
[c] Operator unable to set instrument parameters properly; data not used in statistical analysis.

Table 4. Statistical analysis of collaborative data for LC determination of aflatoxins B_1, B_2, G_1, and G_2

No. of labs	Aflatoxin	Added, ng/g	Av.[a] rec., %	s_r	s_R	RSD_r, %[b]	RSD_R, %[b]	Outliers
				Corn				
10	B_1	5	80	3.47	3.47	86.7	86.7	0
8[c]		5	67	0.92	1.10	27.2	32.3	2
10	B_2	1.5	98	1.54	1.54	102.9	102.9	0
8[c]		1.5	75	0.22	0.43	20.4	38.9	2
10	G_1	5	78	3.72	3.72	95.5	95.5	0
8[c]		5	61	1.07	1.24	35.7	41.3	2
10	G_2	1.5	83	1.15	1.15	95.7	95.7	0
	Total	13	71					
10	B_1	10	76	3.98	3.98	56.0	56.0	0
8[c]		10	64	0.78	0.96	12.8	15.8	2
10	B_2	3	71	1.44	1.44	53.5	53.5	0
9[d]		3	65	0.22	0.41	9.0	17.0	1
10	G_1	10	75	5.37	5.44	76.7	77.7	0
8		10	64	0.46	2.00	7.7	33.4	2
10	G_2	3	64	1.67	1.69	69.7	70.3	0
8[c]		3	56	0.38	0.76	6.8	35.8	2
	Total	26	62					
10	B_1	50	66	13.17	15.26	16.9	40.2	0
8[c]		50	69	2.87	13.29	8.3	38.4	2
10	B_2	15	82	3.01	3.78	24.5	30.7	0
9[c]		15	85	1.17	2.99	9.1	23.2	1
10	G_1	50	58	10.80	12.82	37.9	45.0	0
10[d]	G_2	15	67	2.88	3.87	28.5	38.3	0
	Total	130	70					
				Peanut butter				
10	B_1	5	104	1.27	1.81	23.9	34.1	0
9[d]		5	112	0.63	1.37	11.2	24.4	1
10	B_2	1.5	111	0.80	0.86	29.5	32.7	0
8[c]		1.5	123	0.20	0.20	11.1	11.1	2
10[c]	G_1	5	82	1.14	1.47	27.2	34.9	0
10[c]	G_2	1.5	105	0.47	0.64	29.5	40.3	1
9[d]		1.5	110	0.14	0.53	9.0	33.0	1
	Total	13	107					
10	B_1	10	96	1.19	2.48	13.1	27.2	0
10	B_2	3	80	0.56	0.83	18.0	26.8	0
10	G_1	10	78	1.23	1.93	16.6	26.1	0
10	G_2	3	69	0.60	0.76	23.0	29.1	0
	Total	26	81					
10	B_1	25	81	3.86	6.75	19.1	33.4	0
10	B_2	7.5	65	1.18	1.86	15.9	25.2	0
10	G_1	25	74	5.31	6.90	28.7	37.3	0
10	G_2	7.5	87	1.78	2.20	26.2	33.4	0
	Total	65	77					
				Raw peanuts				
10	B_1	5	66	1.16	1.45	35	43.9	0
9	B_2	1.5	89	0.29	0.50	22.1	38.4	0
8[d]		1.5	80	0.19	0.25	16.1	20.5	1
10	G_1	5	62	1.14	1.64	36.6	52.9	0
9	G_2	1.5	79	0.40	0.64	33.2	52.9	0
	Total	13	72					
10	B_1	10	55	1.74	2.75	33.1	52.9	0
9	B_2	3	61	0.82	0.98	35.8	42.5	0
10	G_1	10	50	1.52	3.18	32.6	67.7	0
9	G_2	3	49	0.72	1.08	38.1	57.1	0
8[d]		3	52	0.26	0.99	13.4	52.0	1
	Total	26	55					

Table 4. Continued

No. of labs	Aflatoxin	Added, ng/g	Av.[a] rec., %	s_r	s_R	RSD_r, %[b]	RSD_R, %[b]	Outliers
10	B_1	25	57	5.81	7.61	41.2	54.0	0
9	B_2	7.5	80	1.97	2.09	32.8	34.9	0
10	G_1	25	54	6.89	8.12	52.2	61.5	0
9	G_2	7.5	69	2.14	2.42	41.2	46.6	0
	Total	65	65					

[a] Average natural aflatoxin B_1 contamination levels found by collaborators for corn, peanut butter, and raw peanuts were 0.11, 1.8, and 0.17 ng/g, respectively; calculated recovery values do not take into account this source of aflatoxins. Outliers excluded for total recoveries.
[b] Average aflatoxin concentration found was used as denominator in calculating RSD values.
[c] Outlier results from 2 laboratories removed.
[d] Outlier results from 1 laboratory removed.

Results and Discussion

Results reported by each participating laboratory are presented in Tables 1–3. Laboratory 9 initially had difficulty in setting up instrument parameters, which resulted in erroneous results for the raw peanuts; consequently, those results were excluded from the study. Because of a leaking injector, values for one of the raw peanut test portions from laboratory 5 were not reported. Laboratory 7 lost one test portion during cleanup. Statistical analysis of the data was performed to determine or confirm outliers, and to compute repeatability (RSD_r) and reproducibility (RSD_R) relative standard deviations (9). Table 4 summarizes the statistical analysis; the Dixon, Cochran, and Grubbs tests were used to determine or confirm outliers. Overall agreement was good between results reported by participating laboratories.

All collaborators returned chromatograms that showed excellent resolution of the individual toxins. Average recovery values at all levels were 67.8, 88.1, and 63.8% for corn, peanut butter, and raw peanuts, respectively. Comparative recovery values for the 3 commodities at the levels tested are presented in Table 4. Some positive results were observed with the nonspiked ("0") test portions; for peanut butter, the average for aflatoxin B_1 was 1.8 ng/g. The raw peanut and corn blanks averaged 0.17 and 0.11 ng/g, respectively. This natural contamination phenomenon probably accounts for the high recoveries observed in samples of peanut butter containing low levels of aflatoxins. Between-laboratory variability (reproducibility, RSD_R) was good for corn and peanut butter samples (for aflatoxin B_1, 15.8–38.4% and 24.4–33.4%, respectively). For raw peanuts, however, RSD_R values were high, 43.9–54.0%. Additional study is recommended for this commodity. The method performed well at all contamination levels tested, 13 ng total aflatoxins/g to 65 ng/g for peanut butter, and 13 to 130 ng/g for corn, which compared favorably (RSD) with the AOAC method for aflatoxins in cottonseed products (**26.052–26.060,** 14th Ed.; **980.20,** 15th Ed. [10]) (11). Recovery values observed in the present study were lower, however.

Collaborators' Comments

General comments about the method confirmed that it was straightforward, and limited difficulties were encountered during the analyses of these products. Two laboratories reported some emulsion and turbidity problems with corn samples. One laboratory reported the formation of a gel after vortex-mixing the water–acetonitrile mixture of the raw peanut samples. Another laboratory stressed that although good results were obtained, the method was very time consuming. As an alternative to the specified excitation (360 nm) and emission (440 nm) filters for the detection system, 365 and 450 nm, respectively, were successfully used by 2 laboratories.

Recommendation

On the basis of the results obtained in the AOAC–IUPAC collaborative study, the Associate Referee recommends that the liquid chromatographic method be adopted official first action for the determination of aflatoxins B_1, B_2, G_1, and G_2 in peanut butter and corn at concentrations $\geq$13 ng total aflatoxins/g.

Acknowledgments

The authors thank the following collaborating laboratories for their participation in the joint AOAC–IUPAC study:

AOAC Collaborators:

Keith Ainsworth, Cargill, Inc., Wayzata, MN

Nancy Bezodis, Nabisco Brands Ltd, Toronto, Ontario, Canada

Kevin T. Faughnan, Best Foods Research and Engineering Center, Union, NJ

Michele Navarre and Louise Hebert Marion, Health and Welfare Canada, Health Protection Branch, Longueuil, Quebec, Canada.

David R. Newkirk, Food and Drug Administration, Center for Veterinary Medicine, Beltsville, MD

G. R. Nixon, R. Wong, and K. Kurz, Health and Welfare Canada, Health Protection Branch, Burnaby, British Columbia, Canada

E. Tarter, Health and Welfare Canada, Health Protection Branch, Scarborough, Ontario, Canada

P. H. Yu, Diversified Research Laboratory, Toronto, Ontario, Canada

IUPAC Collaborators:

R. Schneider and W. Steiner, Kantonales Laboratorium Zurich, Zurich, Switzerland

P. Steyn and D. W. Trinder, Council for Scientific and Industrial Research, National Food Research Institute, Pretoria, South Africa

References

(1) Scott, P. M. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 242–248
(2) Panalaks, T., & Scott, P. M. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 583–589
(3) Tarter, E. J., Hanchay, J. P., & Scott, P. M. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 597–600
(4) Thorpe, C. W., Ware, G. M., & Pohland, A. E. (1982) Proc. V

Int. IUPAC Symp. Mycotoxins Phycotoxins, Sept. 1-3, 1982, Vienna, Austria, pp. 52-55
(5) Tuinstra, L. G. M. T., & Haasnoot, W. (1983) *J. Chromatogr.* **282,** 457-462
(6) Sheppard, M. J., & Gilbert, J. (1984) *Food Addit. Contam.* **1,** 325-335
(7) Gilbert, J., & Sheppard, M. J. (1985) *Food Addit. Contam.* **2,** 171-183
(8) Campbell, A. D., Francis, O. J., Jr, Beebe, R. A., & Stoloff, L. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 312-316
(9) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC,* AOAC, Arlington, VA, pp. 21-26
(10) *Official Methods of Analysis* (1984) 14th Ed. and (1990) 15th Ed., AOAC, Arlington, VA
(11) Pons, W. A., Jr, Lee, L. S., & Stoloff, L. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 899-906

Rapid Screening Method for Deoxynivalenol in Agricultural Commodities by Fluorescent Minicolumn

WILLIAM C. GORDON and LINDA J. GORDON
Rialdon Diagnostics, PO Box 1995, College Station, TX 77841

A rapid screening procedure based on the selective adsorption of deoxynivalenol (DON) from extracts of wheat and corn has been developed. DON is extracted from the sample with acetonitrile–water (85 + 15) and partially purified on a preparative minicolumn. Solvent is evaporated and the residue is dissolved in toluene–acetone (95 + 5) and chromatographed on a novel detector minicolumn which selectively adsorbs DON. A blue fluorescence is produced when the column is heated 5 min at 100°C. The procedure is capable of detecting DON at ≥ 500 ng/g. Forty-three wheat samples, contaminated with DON at 60–6300 ng/g, were assayed by gas chromatography-mass spectroscopy (GC-MS) of the heptafluorobutyryl derivative of DON and by the selective adsorption procedure. Comparison of results showed 91% agreement between data from the 2 methods. Selective adsorption assays were positive for all samples that were ≥ 500 ng/g by GC-MS (no false negatives) and were negative for 85% of samples < 500 ng/g (4/27 false positives). These four samples contained > 200 ng/g by GC-MS. Samples of wheat (64), corn (23), soybeans (8), and sorghum (6) were extracted and extracts were assayed by thin-layer chromatography and the selective adsorption procedure. Selective adsorption assays agreed with TLC results.

Deoxynivalenol (DON), also known as vomitoxin, is one of a group of closely related secondary fungal metabolites called trichothecenes. This mycotoxin is produced by some species of *Fusarium* which, under certain climatic conditions, invade crops such as wheat and corn in the field and in storage. Grain contaminated with DON has been implicated in decreased feed consumption, decreased weight gain, vomiting, feed refusal, and diarrhea in swine and other livestock (1-5), and skin irritation, hemorrhaging, and immunosuppression in laboratory animals (6, 7). DON is considered a potential carcinogen (8), and has been associated with esophageal cancer in humans (9).

Regulatory limits or levels of concern for the presence of DON in wheat and other grain products destined for human and animal consumption have been established in Canada, the United States, and the Soviet Union (10, 11). The limits, ranging from 500 to 4000 ppb (ng/g), reflect concentrations associated with observed health effects, although documentation in this area is limited. DON occurs in food and feed concurrently with other *Fusarium* toxins, notably nivalenol and zearalenone (12, 13). Combined and synergistic toxicity upon consumption, therefore, is possible. The detection of DON may have considerable value as a marker for *Fusarium* contamination.

Various chromatographic methods for the analysis of DON have been reported (14, 15) and 2 methods have been adopted official by AOAC (16) for the quantitation of DON in wheat. Effective monitoring of the feed and food supply by industries and regulatory agencies, however, would be significantly enhanced by methods that are more rapid and less technically involved yet can accurately and reliably screen for DON at levels at which health effects begin to occur. Lower costs and faster assays associated with screening methods could result in routine testing of more commodities, thus generating a more complete profile of DON occurrence. Confirmation and sensitive quantitation could be accomplished by the more involved AOAC methods. Methods based on thin-layer chromatography (TLC) and liquid chromatography (LC) technology have been described that are simpler or more rapid than the AOAC methods (17-21), and commercial immunoassays have recently become available for DON screening.

We now report an improved rapid screening method based on selective adsorption (SA) principles, in a minicolumn format, that uses a novel procedure for the binding and conversion of DON to allow fluorescent detection. Procedures are described for the SA screen that have reduced manipulations and time requirements over the TLC and LC methods, have improved stability over the immunochemical methods, and provide reproducible and accurate detection of DON at 500 ppb and above. The preparative minicolumn can be used to prepare duplicate extracts of positive samples for confirmation and quantitation by an official AOAC method.

METHOD

Safety Note

DON should be handled with caution and treated as a toxic substance.

Received June 12, 1989. Accepted October 10, 1989.

Apparatus

(a) *Selective adsorption (SA) DON screening system* (Figure 1).—Screening system includes: (*1*) *Preparative (P) minicolumn*, prepacked with a mixture of pretreated charcoal, alumina, aluminosilicates and silicas to adsorb non-trichothecene mycotoxins and other naturally occurring compounds that would interfere with the measurement of DON by the detector (D) minicolumn. (*2*) *Detector (D) minicolumn*, prepacked with silicas and a selective mineral as a detection band that binds and chemically converts certain compounds (e.g., DON) to fluorescent species. (*3*) *Syringe flow-aide*, supplies positive pressure to facilitate rapid processing of the sample through each minicolumn. (*4*) *Test tube collar*, provides the proper positioning of each minicolumn with test tubes. (*5*) *UV viewer*, a longwave (365 nm) UV lamp designed for determining fluorescence of DON on D minicolumn.

The screening system is available as the SAM-DON™ Assay for Deoxynivalenol (Rialdon Diagnostics, PO Box 1995, College Station, TX 77841).

(b) *Thin-layer chromatography (TLC) system.*—20 μL capillary tubes, developing tank with lid, silica gel TL plate, longwave (365 nm) UV lamp (UVP, Inc., PO Box 1501, San Gabriel, CA 91778), spray bottle, oven (to 140°C).

(c) *Solvent evaporation apparatus.*—Heat-block or water bath (to 100°C), compressed nitrogen, gas-stream ports.

Reagents

(a) *Solvents.*—Acetone, acetonitrile, chloroform, methanol, toluene, all ACS grade, used as received; distilled water.

(b) *Standard solutions.*—Type A trichothecenes: contain 200 μg/mL each of T-2 toxin and diacetoxyscirpenol (DAS) in methanol (Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178). Type B trichothecenes: contain 20 μg/mL each of deoxynivalenol (DON), nivalenol (NIV), and fusarenon X (FX) in methanol (Romer Labs, PO Box 2095, Washington, MO 63090). Others: aflatoxin B_1 (1 μg/mL), zearalenone (100 μg/mL), ochratoxin A (20 μg/mL), and citrinin (100 μg/mL) (Sigma Chemical Co.).

(c) *Extraction solvent.*—Mix 850 mL acetonitrile with 150 mL water.

(d) *$AlCl_3$ spray solution.*—Dissolve 20 g $AlCl_3.6H_2O$ in 100 mL methanol.

Sample Extraction—SA and TLC

Prepare finely ground sample (e.g., to pass 20 mesh screen) and mix thoroughly. Place 10 g representative sample in beaker and add 40 mL extraction solvent. Stir sample intermittently (5–10 times) over 15 min period and let particles settle (1–2 min). Use 2 mL supernatant liquid for SA and another 2 mL for TLC.

Preparative (P) Minicolumn Cleanup

Fit test tube collar onto 16 × 125 mm glass test tube, and place P minicolumn into collar. Add 2 mL sample extract to P minicolumn. Fit syringe flow-aide on top of minicolumn and use positive pressure to push sample through minicolumn. When all sample extract has entered column matrix, remove syringe and add 0.5 mL extraction solvent to minicolumn. Use syringe to push all liquid through P minicolumn. Remove collar and evaporate all solvent by heating to 100°C, using heat block or water bath. Evaporation is enhanced by focusing a stream of nitrogen onto the extract. Evaporate to complete dryness.

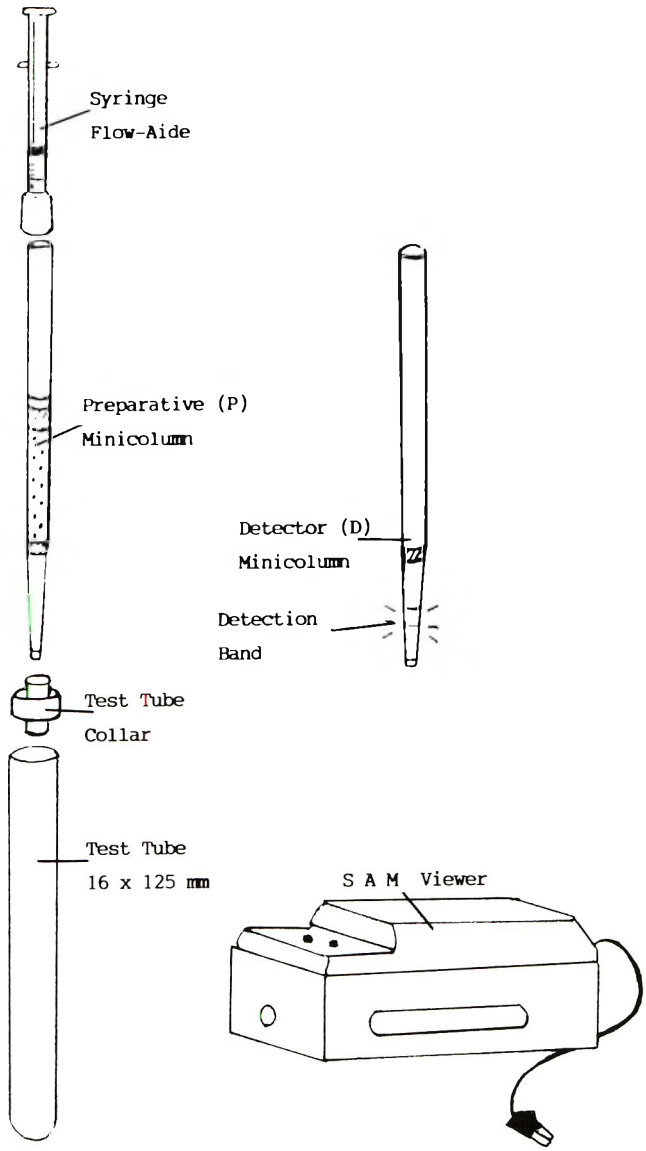


Figure 1. Selective adsorption screening system for deoxynivalenol.

DON Detection by D minicolumn and TLC

Add 0.5 mL toluene–acetone (95 + 5) to cooled extract residue and dissolve by agitation. Fit test tube collar onto 16 × 125 mm test tube and place D minicolumn into collar (see Figure 1). Transfer entire dissolved residue by pipet to D minicolumn and let liquid drip through no faster than 1 drop/s. Use syringe flow-aide for control and to evacuate all residual liquid.

Place D minicolumn in beaker and heat 5 min in 100°C oven. Insert cooled D minicolumn into UV viewer, or position within 2 cm from UV lamp (365 nm wavelength) in darkened area. Readily detectable blue fluorescence at detection band (Figure 1) indicates presence of DON in sample at 500 ppb or greater.

Negative and positive sample should be run to familiarize analyst with expected color development. The 500 ppb concentration is represented by 2 mL of a 125 ng/mL DON solution in acetonitrile–water (85 + 15), processed through

Table 1. Recovery of selected mycotoxins from SA preparative (P) minicolumns

Mycotoxin	Amount,[a] μg	Recovery from P minicolumn Range,[b] μg	%
Deoxynivalenol[c]	2	1.8–2.0	90–100
Nivalenol[c]	2	1.6–2.0	80–100
Fusarenon X[c]	2	1.8–2.0	90–100
T-2 toxin[d]	2	1.8–2.0	90–100
Diacetylscirpenol[d]	2	1.8–2.0	90–100
Zearalenone	20	0	0
Aflatoxin B_1	1	0	0
Ochratoxin A	10	0	0
Citrinin	20	0	0

[a] Mycotoxin spiked into 2 mL acetonitrile–water (85 + 15), applied to P minicolumn, and eluted with 5 mL same solvent.
[b] Triplicate samples. Recovery quantitated by TLC analysis.
[c] Type B trichothecene.
[d] Type A trichothecene.

same procedure as a sample extract. Fluorescence signal generated on D minicolumn may be used as reading guide for analysis of other samples.

For confirmation by TLC, process duplicate 2 mL sample from initial sample extract through same P minicolumn by same procedure used to prepare sample for SA analysis. Add 0.5 mL toluene–acetone (95 + 5) to cooled extract residue and dissolve by agitation. Transfer solution to small vial (1–2 mL) and evaporate to dryness using nitrogen stream.

Add 50 μL $CHCl_3$–acetone (9 + 1) to vial, redissolve residue with aid of 20 μL capillary tube, and spot entire amount onto TLC plate, using multiple applications. Develop plate in toluene–acetone (1 + 1) until solvent is ca 1 cm from top of plate. Dry plate, spray with $AlCl_3$ solution, and heat 10 min at 140°C. Examine plate under longwave UV light for blue fluorescent spots at same R_f as DON standard. Compare intensity of DON in extract with that of 2 mL of 125 ng/mL DON standard, which represents 500 ppb (ng/g) sample, carried through same procedure.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The AOAC first action method, **986.18,** was used for GC analysis, except that mass spectrometry single ion monitoring was used to detect the heptafluorobutyrate derivative of DON. The GC-MS analysis was performed by an independent laboratory on wheat samples collected during the 1987 wheat harvest. The ground samples were divided and representative subsamples were provided for SA and TLC analysis.

Results and Discussion

Extraction efficiency was compared between the described method and the use of a wrist shaker for 30 min, using 4 wheat samples in the 2–6 ppm (μg/g) range. Extracts were analyzed by the described TLC method. No difference between methods was detected. While extraction efficiency may differ with different commodities (22), the more rapid procedure was used for corn, sorghum, and soybean samples as well as for wheat.

The SA preparative (P) and detector (D) minicolumns were characterized regarding specificity, sensitivity, capacity, and other features relative to designing a procedure to provide an accurate, reproducible, and rapid assay for screening at the 500 ppb DON level, the lowest level recommended by regulatory concerns (10, 11). The reproducibility

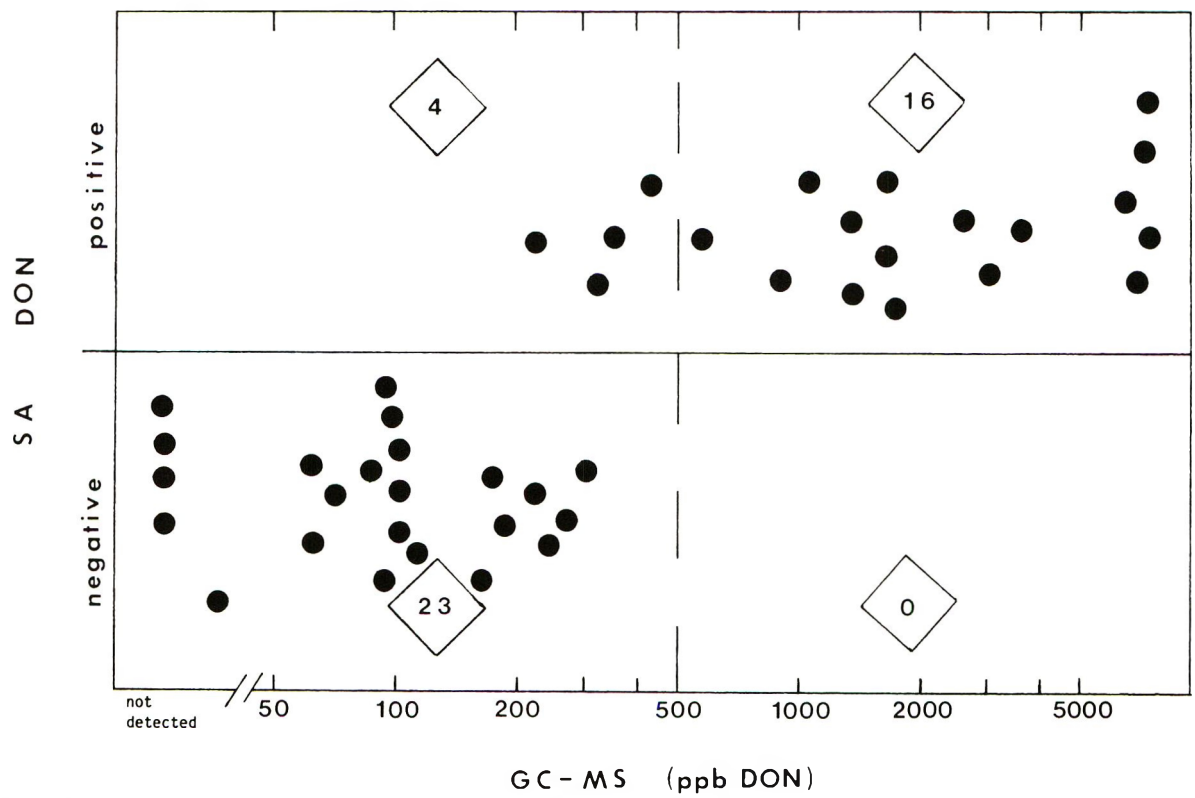


Figure 2. Correlation of SA with GC-MS data for analysis of DON in naturally contaminated wheat samples. Samples were screened for DON as positive or negative at 500 ppb level by SA and quantitated by GC-MS on replicate samples.

Table 2. Comparison of SA with TLC data from analysis of DON in wheat, corn, soybean, and sorghum samples

Sample type	No. of samples	SA results[a]	TLC results[a] Positive	TLC results[a] Negative
Wheat	29	+	28	1[b]
	35	−	0	35
Corn	3	+	3	0
	20	−	0	20
Soybeans	0	+	0	0
	8	−	0	8
Sorghum	0	+	0	0
	6	−	0	6
Total	101:32	+	31	1
	69	−	0	69

[a] Positive (+) = ≥ 500 ppb (ng/mL); negative (−) = < 500 ppb.
[b] Positive for nivalenol by TLC.

of analysis at the selected screening level was assessed by assaying 10 replicates of 3 standard solutions spiked at 100, 125, and 150 ng/mL DON to represent 400, 500, and 600 ppb samples, respectively. The resulting signals were compared with a previously assayed 500 ppb sample. The number of replicates ≥ 500 ppb were 2/10 for 400 ppb, 8/10 for 500 ppb, and 10/10 for 600 ppb. These results were produced by a single analyst using a single lot of each minicolumn.

The specificity of the P minicolumn for the recovery of selected mycotoxins is shown in Table 1. Greater than 90% of both type A and type B trichothecenes were recovered from the P minicolumn; no detectable aflatoxin B1, ochratoxin A, zearalenone, or citrinin was recovered. The type B trichothecenes produced a bright blue fluorescence under UV light at the detection band of the D minicolumn. This signal was enhanced 5- to 10-fold by heating the minicolumn for 5 min at 100°C. Type A trichothecenes produced no fluorescence, with or without heating.

The SA method was compared in a blind fashion with a GC-MS method performed by an independent laboratory in the evaluation of 43 wheat samples including white winter, red spring, amber durum, Canada feed, Canada prairie spring, and soft white spring. The results, shown in Figure 2, indicate that 100% of the samples (16/16) measuring above 500 ppb by GC-MS were positive by SA at the 500 ppb screening level. Of samples measuring below 500 ppb by GC-MS, 85% (23/27) were negative by SA. Four samples positive by SA were assayed at 400, 320, 290, and 200 ppb by GC-MS. These apparent discrepant measurements could be due to discrepant DON standards, sample or assay variation, or a combination of these factors.

The SA results were confirmed by TLC using the same sample extract and P minicolumn. The TLC method, thus, had the same level of concordance with the GC-MS method as did SA. These results demonstrate the accuracy of the SA method in screening wheat samples for DON at the ≥ 500 ppb level, and also demonstrate the efficacy of the coupled TLC application to confirm the screening results.

The ability of the SA procedure to sufficiently remove interfering compounds present in extracts of corn, soybeans, and sorghum as well as wheat was determined using TLC as verification. The data in Table 2 show that 31 of 101 samples were positive for DON above 500 ppb by TLC analysis and that 100% of these were positive by SA. The positive wheat samples are those shown in Figure 1 ranging from 550 to 6300 ppb by GC-MS analysis. Sixty-nine samples were below 500 ppb by TLC and 68 were negative by SA. The overall agreement was 99%. One sample from *Fusarium*-contaminated wheat was positive by SA and was shown to be DON-negative but nivalenol-positive by TLC. The SA assay detects nivalenol, which is a type B trichothecene.

The SA DON assay procedure showed accurate analysis of DON with no false negatives and few false positives compared with GC-MS and TLC as reference methods on samples tested. The SA method is simple, rapid, and solvent-efficient and can be coupled conveniently and effectively to a rapid TLC confirmation method by virtue of reuse of the SA preparative column. The preparative (P) and detector (D) minicolumns promise a stable and reliable screening assay for DON in selected agricultural commodities. The assay procedures described are tailored for DON screening although other type B trichothecenes may be detected. Further investigations are required to determine the analytical ability of these selective adsorption techniques to differentiate or combine the detection and measurement of this important group of toxins.

Acknowledgments

We acknowledge the scientific contributions of Timothy D. Phillips and Beverly A. Clement, Texas A&M University, regarding the detection column of the described assay, which is included in a pending patent with them, the authors, and Norman C. Heidelbaugh, Texas A&M University, as co-inventors. We thank Thomas Nowinski, Canadian Grain Commission, for access to wheat samples and for analytical results included in Figure 2.

References

(1) Forsyth, D. M., Yoshizawa, T., Morooka, N., & Truite, J. (1977) *Appl. Environ. Microbiol.* **34,** 547–552
(2) Friend, D. W., Trenholm, H. L., Fiser, P. S., Thompson, B. K., & Hartin, K. E. (1983) *Can. J. Anim. Sci.* **66,** 765–772
(3) Trenholm, H. L., Cochrane, W. P., Cohen, H., Elliot, J. I., Farnsworth, E. R., Friend, D. W., Hamilton, R. M. G., Standish, J. F., & Thompson, B. K. (1983) *J. Assoc. Off. Anal. Chem.* **66,** 92–97
(4) Ueno, Y. (1983) *Dev. Food Sci.* **4,** 135–146
(5) Cote, L. M., Reynolds, J. D., Vesonder, R. F., Buck, W. B., Swanson, S. P., Coffey, R. C., & Brown, D. C. (1984) *J. Am. Vet. Med.* **184,** 189–192
(6) Forsell. J. H., Witt, M. F., Tai, J.-H., Jensen, R., & Pestka, J. J. (1986) *Food Chem. Toxicol.* **24,** 213–219
(7) Pestka, J. J., Tai, J.-H., Witt, M. F., Dixon, D. E., & Forsell, J. H. (1987) *Food Chem. Toxicol.* **25,** 297–308
(8) Shoental, R. (1985) in *Advances in Cancer Research*, Vol. 45, J. Klein & S. Weinhouse (Eds), Academic Press, London, UK, pp. 217–290
(9) Marasas, W. F., van Rensburg, S. J., & Mirocha, C. J. (1979) *J. Agric. Food Chem.* **27,** 1108–1110
(10) *Food Chem. News* (1982) **24,** 23
(11) van Egmond, H. P. (1987) "Current limits and regulation on mycotoxins," MYC 87/9.2, Joint FAO/WHO/UNEP Second International Conference on Mycotoxins, Bangkok, Thailand, Sept. 28–Oct. 3, 1987, Food and Agriculture Organization of the United Nations, Rome, Italy
(12) Tanaka, T., Hasegawa, A., Yamamoto, S., Lee, U.-S., Sugiura, Y., & Ueno, Y. (1988) *J. Agric. Food Chem.* **36,** 979–983
(13) Jelinek, C. F., Pohland, A. E., & Wood, G. E. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 223–230
(14) Scott, P. M. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 876–883
(15) Scott, P. M. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 75–80
(16) *Official Methods of Analysis* (1990) 15th Ed., AOAC, Arlington, VA, secs. **986.17** and **986.18**

(17) Eppley, R. M., Trucksess, M. W., Nesheim, S., Thorpe, C. W., Wood, G. E., & Pohland, A. E. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 43-45
(18) Chang, H. L., DeVries, J. W., Larson, P. A., & Patel, H. H. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 52-54
(19) Shannon, G. M., Peterson, R. E., & Shotwell, O. L. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 1126-1128
(20) Sylvia, V. L., Phillips, T. D., Clement, B. A., Green, J. L., Kubena, L. F., & Heidelbaugh, N. D. (1986) *J. Chromatogr.* **362,** 79-85
(21) Trucksess, M. W., Flood, M. T., Mossoba, M. M., & Page, S. W. (1987) *J. Agric. Food Chem.* **35,** 445-448
(22) Trenholm, H. L., Warner, R. M., & Prelusky, D. B. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 645-649

Criteria for Determining Purity of *Fusarium* Mycotoxins

GLENN A. BENNETT and ODETTE L. SHOTWELL
U.S. Department of Agriculture, Agricultural Research Service, Northern Regional Research Center, Peoria, IL 61604

Physical and chemical properties that may be used to determine the purity of several *Fusarium* mycotoxins have been investigated. A combination of analytical procedures, which include high performance thin-layer chromatography (HPTLC), liquid chromatography (LC), gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), ultraviolet spectrometry (UV), and nuclear magnetic resonance (NMR) spectrometry have been used to examine mycotoxin standards obtained from commercial sources and from laboratory fermentations. Results of this investigation indicate that commercially available standards are greater than 90% pure, but the label weight of purchased reference standards in individual containers should be verified. Mycotoxin standards, determined to be greater than 98% pure by HPTLC, LC, and GC/MS, were examined by UV spectrometry and the coefficients of extinction were determined. An interlaboratory study, involving 5 collaborators who determined coefficients of extinction (in methanol) for identical samples, gave the following results: α-zearalenol (λ236 = 28 538 ± 558); β-zearalenol (λ238 = 24 963 ± 747); deoxynivalenol (λ219 = 6395 ± 349, lot 1), (6020 ± 228, lot 2); and T-2 toxin (λ202 = 3681 ± 255). UV maxima and coefficients of extinction are also reported for HT-2 toxin (λ202 = 1959), diacetoxyscirpenol (λ203 = 2487), neosolaniol (λ203 = 2644), nivalenol (λ220 = 5142), and fusarenon-X (λ217 = 5997).

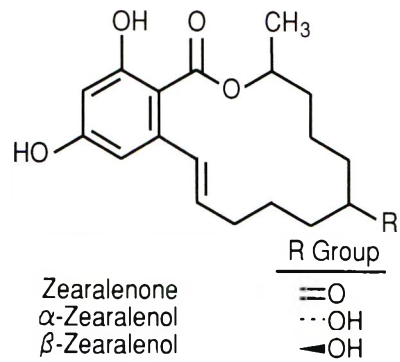
Trichothecene mycotoxins (Figure 1), such as T-2 toxin, deoxynivalenol, nivalenol, and the estrogenic mycotoxin, zearalenone, are produced by a number of *Fusarium* species found around the world. Contamination of cereals and feeds with trichothecene mycotoxins occasionally causes intoxication in humans and in farm animals (1-3). Zearalenone contamination of animal feeds appears to be a worldwide problem (4). Analytical procedures for detection and quantitation of most *Fusarium* mycotoxins at parts per billion levels now exist (5), and the validity and accuracy of such procedures are directly dependent on reference standards used in the assays. Since reference standards are generally used as dilute solutions (ng or μg/mL), impurities would not be detectable at these concentrations. Although the problem of purity of trichothecene standards has been recognized (6), mycotoxin literature lacks nondestructive procedures to determine purity of several of the scarce and expensive trichothecenes. Extensive physicochemical data for characterizing and identifying some 15 mycotoxins (including T-2 toxin and zearalenone) have been published (7). Our investigation is directed toward the development of a nondestructive procedure that can be used to determine purity of purchased or laboratory-prepared reference standards.

Received July 27, 1989. Accepted October 30, 1989.
The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

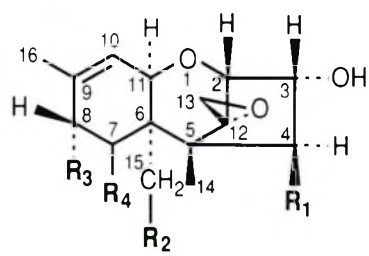
METHODS

Apparatus

(a) *Spectrophotometry.*—Perkin-Elmer Model Lambda 4B (Perkin-Elmer Corp., Norwalk, CT 06859).



R Group	
Zearalenone	=O
α-Zearalenol	···OH
β-Zearalenol	◄OH



	R_1	R_2	R_3	R_4
T-2	OAc	OAc	ISV	H
HT-2	OH	OAc	ISV	H
DAS	OAc	OAc	H	H
Neosolaniol	OAc	OAc	OH	H
DON	H	OH	=O	OH
NIV	OH	OH	=O	OH
Fusarenon-X	OAc	OH	=O	OH

Figure 1. Structures of mycotoxins used in this study.

(b) *High performance thin-layer chromatographic plates.*—Precoated plates, silica gel, 10 × 10 cm (E. Merck, Darmstadt, FRG).

(c) *Liquid chromatograph.*—Spectra-Physics Model 8100 equipped with reverse-phase column (150 × 4.6 mm) Zorbax ODS or C_8 (Dupont Instrument, Wilmington, DE 19898).

(d) *Detectors.*—Kratos Model 980 fluorescence detector, variable wavelength (Kratos, Ramsey, NJ 07446) and LDC III UV detector, variable wavelength (Milton Roy, Riviera Beach, FL 33419).

(e) *Gas chromatography.*—Spectra-Physics Model 7100 chromatograph equipped with on-column injector and flame ionization detector (FID). Fused-silica capillary column (12 m × 0.53 mm id) with DB-5 phase at 0.25 μm film thickness.

(f) *Mass spectroscopy.*—Finnigan Model 4535/TSQ GC/MS equipped with a 15 m DB-1 capillary column (0.23 mm id).

(g) *Nuclear magnetic resonance.*—Bruker Model 300 MHz. ^{1}H NMR obtained on 1 mg samples in deuterio chloroform ($CDCl_3$).

Reagents

(a) *Solvents.*—Methanol, acetonitrile, chloroform (LC grade); water (LC quality, Barnstead NANO pure II system); ethanol, acetone, carbon tetrachloride (reagent grade).

(b) *LC mobile phase.*—Acetonitrile–water (25 + 75 and 40 + 60), degas with purified helium.

(c) *HPTLC solvents.*—Chloroform–acetone (60 + 40) for trichothecenes, chloroform–ethanol (93 + 7) for zearalenone and zearalenols.

(d) *Spray reagents.*—*For trichothecenes.*—Reagent A: Dissolve 1 g 4-(*p*-nitrobenzyl)pyridine in 100 mL chloroform–carbon tetrachloride (40 + 60). Reagent B: Mix 5 mL tetraethylenepentamine (Aldrich Chemical Co., Milwaukee, WI 53233) with 50 mL chloroform–carbon tetrachloride (40 + 60). Reagent B is stable for 1 week in refrigerator. *For zearalenone and zearalenols.*—Dissolve 20 g aluminum chloride in 100 mL ethanol.

(e) *Derivatization reagents.*—Trimethylsilylimidizole–bis(trimethylsilyl)acetamide–trimethylchlorosilane (3 + 3 + 2 v/v/v) (Tri-Sil TBT) and *N*-heptafluorobutyrylimidazole (HFBI) (Pierce Chemical Co., Rockford, IL 61105).

(f) *Mycotoxin standards.*—T-2 toxin, HT-2, neosolaniol, diacetoxyscirpenol (Sigma Chemical Co., St. Louis, MO 63178); zearalenone, α-zearalenol, β-zearalenol (International Minerals and Chemicals, Terre Haute, IN 47808); deoxynivalenol, nivalenol, fusarenon-X (Wako Chemicals, USA, Dallas, TX 75234 and Myco Lab, Chesterfield, MO 63017).

High Performance Thin-Layer Chromatography

Apply zearalenone, α-zearalenol, and β-zearalenol (25 μg each toxin) in acetonitrile to HPTLC plate with a 10 μL syringe. Develop plates in chloroform–ethanol (97 + 3) in unlined glass tanks until solvent front is 3/4 distance to top of plate. After air drying, observe plates under long-wave and short-wave UV; spray with aluminum chloride (20% in ethanol) and re-examine under long-wave UV for fluorescent zones other than those produced by primary standard. Apply 25 μg trichothecenes (T-2 toxin, HT-2 toxin, nivalenol, fusarenon-X, neosolaniol, and deoxynivalenol) to HPTLC plates and develop in chloroform–acetone (60 + 40). Air dry and spray plate lightly, but completely, with 4-(*p*-nitrobenzyl)pyridine (Reagent A) and heat 30 min at 150°C. Let plate cool and spray with tetraethylenepentamine solution (Reagent B). Trichothecenes appear as blue zone on white background (8).

Liquid Chromatography

Prepare working solutions of each toxin by accurately dispensing 100 μL stock solution (1 μg/μL) into amber vials with Teflon-lined screw caps. Evaporate solvent under gentle stream of nitrogen, and dissolve residue in mobile phase to give following concentrations: zearalenone, α-zearalenol, and β-zearalenol (25 μg/mL). Inject 20 μL onto reverse-phase (C_8 or C_{18}) column, elute at 1.0 mL/min, and monitor eluant at 236 nm. This provides for 100-fold increase over level that can be easily quantitated by LC with UV detector. Dilute stock solutions of trichothecenes (1 μg/μL) to working solutions containing 0.250 μg/μL of T-2, HT-2, diacetoxyscirpenol, and neosolaniol, and 0.125 μg/μL of deoxynivalenol, nivalenol, and fusarenon-X. These concentrations provide 100 times minimum levels that can be quantitated by LC with UV detector at 210 nm for T-2, HT-2, diacetoxyscirpenol, and neosolaniol, and at 219 nm for deoxynivalenol, nivalenol, and fusarenon-X.

Gas Chromatography and Gas Chromatography/Mass Spectroscopy

Prepare TMS derivatives as described by Gilbert et al. (9) so that final concentration of derivatized standard is 1 μg/μL. Inject (on column) 1–2 μL standard onto capillary column and temperature-program oven from 150–250°C at 5°/min. Adjust sensitivity of FID detector so that major peak is 90–100% full scale. Record detector signal on recording integrator to determine presence and relative percentage of TMS contaminant (if any) that may be present. Use same TMS preparations (1 μg/μL) to examine toxins by GC/MS. Record positive CI spectra (full scan) with isobutane as reagent gas.

Nuclear Magnetic Resonance Analysis

Determine ^{1}H NMR spectra on 1 mg samples of toxin standard dissolved in $CDCl_3$ as described by Beremand et al. (10).

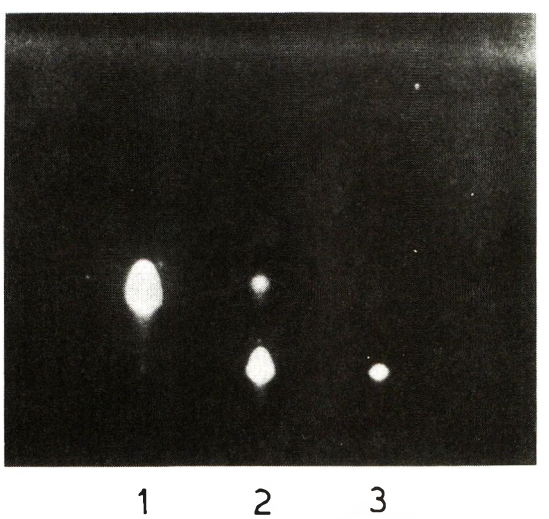


Figure 2. HPTLC detection of zearalenone (1), α-zearalenol (2), and β-zearalenol (3), showing impurities detected with aluminum chloride reagent.

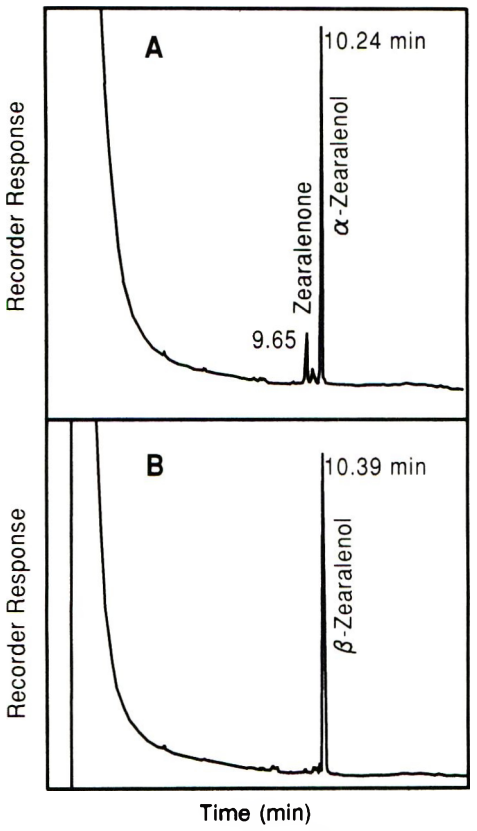


Figure 3. Capillary gas chromatogram (0.53 mm) of impure α-zearalenol (A) and β-zearalenol (B) standards (TMS) showing impurities at 9.65 min (zearalenone) and 9.92 min (unknown).

UV Spectra and Coefficients of Extinction

Prepare zearalenone, α-zearalenol, and β-zearalenol standards at 10 μg/mL methanol. Prepare trichothecene standards at 100 μg/mL methanol for T-2 toxin, diacetoxyscirpenol, and neosolaniol; at 50 μg/mL for HT-2; and at 25 μg/mL for deoxynivalenol, fusarenon-X, and nivalenol. Scan solvent from 400 to 190 nm to determine suitability for use. Rinse cuvet with working solution of each toxin prior to obtaining UV spectra from 400 to 190 nm. Calculate coefficient of extinction for each toxin at UV maximum as follows:

$$\sum_{\lambda} = [\text{mw} \times 1000 \times \text{absorbance (OD)}]/\text{concn } (\mu\text{g/mL})$$

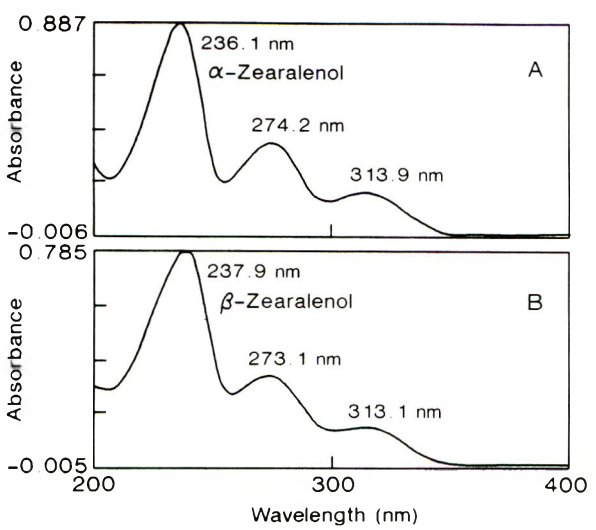


Figure 4. UV spectra of pure α-zearalenol (A) and β-zearalenol (B) in methanol (10 μg/mL).

Interlaboratory Study on Standards

Samples of mycotoxin standards that were determined to be pure (>98%) by LC, HPTLC, and GC/MS were supplied to 5 different collaborators. The following samples were provided as dry films in amber vials: deoxynivalenol (125 μg from 2 different commercial sources; T-2 toxin (500 μg); α-zearalenol (50 μg); and β-zearalenol (50 μg). Collaborators were instructed to dissolve each standard in 5.00 mL Spectroquality methanol and determine the UV spectra from 400 to 190 nm. Coefficients of extinction at UV maxima were calculated and reported with other data on instrument make and model, scan rate, wavelength accuracy, cuvet volume, and path length. Collaborators were also requested to determine the coefficient of extinction for T-2 toxin in acetonitrile at 25 μg/mL.

Results and Discussion

Figure 2 shows impurities in α-zearalenol (2) and β-zearalenol (3) reference standards that can be detected on HPTLC plates sprayed with 20% aluminum chloride. The α-zearalenol contains approximately 5% zearalenone, which is also present, but at lower levels, in the β-zearalenol. Pure reference standards (10 and 25 μg each) that have been properly stored produced only one zone on HPTLC plates. The aluminum chloride spray does not detect all organic impurities (which can be detected by charring after sulfuric acid spray), but does detect degradation or conversion products more commonly found in reference standards. Figures 3A and 3B are GC chromatograms of the impure α- and β-zearalenol standards (1.4 μg of TMS derivative). Impurities were detected at 9.65 min (zearalenone) and 9.92 min (unknown). Chromatograms of standards showing one zone on HPTLC plates also showed only one peak for each standard. A UV detector, although less sensitive and less specific than the fluorescence detector, was used because it indicates more readily the presence of impurities. Similar purity for the α- and β-zearalenol standards was determined by LC. Confirmation of purity was established by GC/MS of the TMS derivative of each standard.

These reference samples were used in the interlaboratory study to evaluate the reliability of using coefficients of extinction to determine purity of purchased or laboratory-prepared reference standards. The UV spectra of zearalenone, α-zearalenol, and β-zearalenol show similar adsorption maxima (236, 274, and 316 nm); however, the coefficients of extinction are characteristically different. Figure 4 shows the UV spectra of pure α-zearalenol (A) and β-zearalenol (B) at 10 μg/mL in methanol. Values for the coefficients of extinction of selected, pure *Fusarium* toxins were determined in 5 different laboratories. Type of instrument and scan speed (30–600 nm/min) did not have a significant effect on values obtained (Table 1). Standard deviations for UV maxima were less than 1 nm, and CV_R (reproducibility) values were less than 0.45%. Coefficients of extinction at UV maximum are shown in Table 2. The value for α-zearalenol (λ236 = 28 538 ± 803) is less than the established value for zearalenone (λ236 = 29 512 ± 376), which has been used to determine the purity of reference standards of this toxin (11). The coefficient of extinction for β-zearalenol (λ238 = 24 963 ± 1041) is, in turn, less than for the α-isomer and exhibits a UV

Table 1. Spectrophotometers, scan rates, and UV maxima reported for interlaboratory study of selected *Fusarium* mycotoxins

Coll.	Instrument	Scan rate, nm/min	UV maxima, nm[a]				
			α-Zearalenol	β-Zearalenol	DON, Lot 1	DON, Lot 2	T-2
1	Cary 219	30	235.4	237.5	218.3	218.3	—[b]
2	Gilford	240	236.5	239.0	220.0	220.0	201.5
3	Beckman DU-7	600	234.5	237.0	218.0	218.0	202.5
4	Shimadzu 160	480	236.2	238.2	219.7	219.8	202.2
5	Beckman DU-7	120	234.8	237.4	218.7	219.3	202.5
Average			235.5	237.8	218.9	219.1	202.2
SD			0.86	0.79	0.87	0.89	0.50
CV_R, %			0.37	0.33	0.40	0.41	0.20

[a] Accuracy of spectrophotometers is 0.5 nm.
[b] Instrument could not measure maximum at 202 nm.

maximum of 238 nm. Although limited, this interlaboratory study indicates that these values can be used to determine the purity of α-zearalenol and β-zearalenol standards. CV_R values of extinction coefficients obtained in the interlaboratory study were 2.815 and 4.174 for α- and β-zearalenol, respectively. Storage of these standards in methanol, protected from light and refrigerated (4°C), has been successful for 18 months.

Purity of trichothecene standards were evaluated by similar procedures. HPTLC of T-2, HT-2, diacetoxyscirpenol, and neosolaniol (25 μg each) indicated that these standards were pure. However, further examination by LC, UV, and GC/MS indicated that the HT-2 was contaminated with several minor components (Figure 5). The UV spectrum indicates an impurity at 220 nm, and LC shows minor components, detectable at 210 nm, which elute prior to HT-2

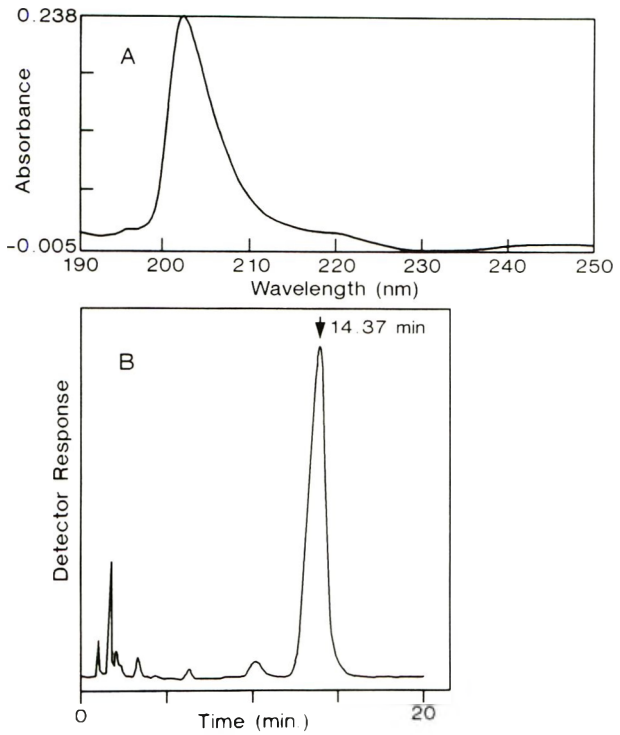


Figure 5. UV spectrum of HT-2 standard in methanol, showing indication of impurity at 220 nm. Lower pattern shows a liquid chromatogram of this standard with impurities detectable at 210 nm.

(14.37 min) with an acetonitrile–water (25 + 75) solvent system. We were unable to obtain HT-2 in crystalline form, and manipulation of the clear, amorphous gel presented problems in preparation of reference solutions. Other type A trichothecenes studied were pure as determined by LC, UV, and GC/MS examination. Neosolaniol, T-2, and diacetoxyscirpenol produced similar spectra, and the coefficients of extinction (at 203 nm) for these toxins were determined to be 2644, 3822, and 2487, respectively. Coefficient of extinction for this T-2 reference standard was determined to be 3681 ± 362 by 4 other analysts (Table 2). NMR analysis of 2 T-2 standards from different sources indicated only minor differences in purities (Figure 6). No impurities were detected in these samples by GC/MS.

Type B trichothecenes included in this study were deoxynivalenol, nivalenol, and fusarenon-X. Figure 7 shows the UV spectra of nivalenol and deoxynivalenol standards determined to be pure by LC, HPTLC, and GC/MS. The respective coefficients of extinction were calculated to be λ220 = 5142 and λ219 = 5913 (Table 3). Samples of deoxynivalenol standards from 2 different sources were also examined by 5 collaborators; the average values and standard deviations were 6395 ± 475 and 6020 ± 301. CV_R values were 7.432 and 5.005%, respectively. Fusarenon-X, estimated to be 94% pure by GC and LC analyses, contained small amounts of nivalenol and an unknown. UV examination of this standard gave a coefficient of extinction of λ217 = 5997 (Table 3).

Although the purity of most reference standards is about 95%, we encountered considerable variation in the weights of toxins in individual vials from commercial sources. A sample of T-2 (worst case encountered) was labeled to contain 5 mg; the vial actually contained 3.500. Caution must be exercised in determining actual amounts of toxin received. Whenever small amounts (1–5 mg) of standards are purchased and accurate weights cannot be determined, we recommend that the concentration of the toxin be determined by comparing the coefficient of extinction, in methanol, to an established value. The reference standard can then be stored as a dry film or in an appropriate solvent; some trichothecenes are unstable in methanol (12). In this laboratory, we have determined that the coefficients of extinction for the trichothecenes are 2.5× higher in acetonitrile than in methanol. However, the UV maximum is shifted to 194–196 nm, and collaborating laboratories were unable to measure UV maxima at this wavelength.

We have studied purity of several of the major trichothe-

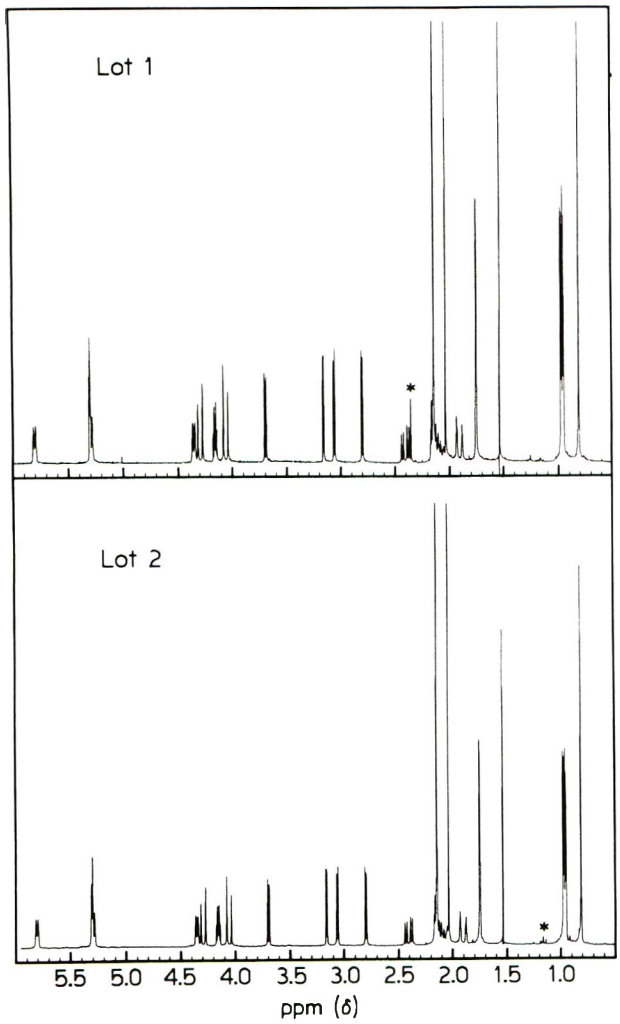


Figure 6. NMR spectra of two commercial lots of T-2 toxin. Minor differences in spectra noted at 1.1–1.2 ppm and 2.3–2.4 ppm.

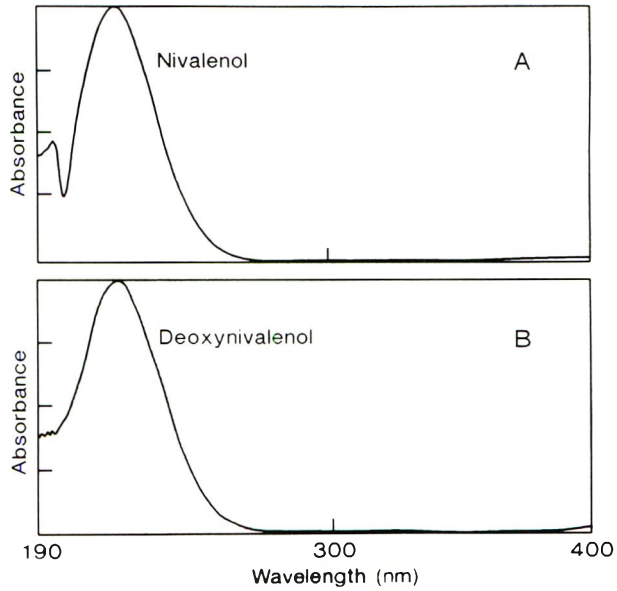


Figure 7. UV spectra of nivalenol (A) and deoxynivalenol (B) in methanol (25 μg/mL) showing UV maxima at 220 and 219 nm.

Table 3. Coefficient of extinction values for trichothecene reference standards in methanol

Trichothecene	Wavelength, nm	Coefficient of extinction in methanol
HT-2[a]	203	1959
Diacetoxyscirpenol	203	2487
Neosolaniol	203	2644
Nivalenol	220	5142
Fusarenon-X[a]	217	5997
DON	219	5913
T-2 toxin	203	3822

[a] Reference standard contained impurities.

cene mycotoxins and α- and β-zearalenol by a variety of methods. Standards determined to be pure were then examined by UV and the coefficients of extinction were determined. The ministudy to determine the coefficients of extinction for selected standards demonstrate that the method is reliable and gives reproducibility CVs of less than 10%. Such nondestructive determinations are needed to conserve scarce and expensive standards, to ensure accurate analyses whenever surveys are conducted to determine occurrence of these toxins (13), and to establish accurate and realistic guidelines for the control of the toxins (14).

Table 2. Results of study to determine reproducibility of extinction coefficients of selected *Fusarium* mycotoxins

Coll.	α-Zearalenol, 236 nm	β-Zearalenol, 238 nm	DON, Lot 1, 219 nm	DON, Lot 2, 219 nm	T-2, 202 nm
1	29 190	26 130	7067	6467	—[a]
2	27 142	23 358	5808	5676	3534
3	28 900	24 700	6110	5870	3650
4	28 685	25 099	6550	6147	4191
5	28 771	25 526	6441	5940	3348
Average	28 538	24 963	6395	6020	3681
SD	803	1041	475	301	362
CV_R, %	2.815	4.174	7.432	5.005	9.840

[a] Instrument would not measure UV maximum at 202 nm.

Acknowledgments

The authors express their appreciation to David Weisleder for NMR spectra, Ron Plattner for MS spectra of trichothecenes, and Terry Lolling for technical assistance. We also thank the following for participating in the interlaboratory study to determine UV spectra and coefficients of extinction for mycotoxin standards: H. L. Trenholm, Animal Research Center, Agriculture Canada, Ottawa; R. M. Eppley, Food and Drug Administration, Washington, DC; P. M. Scott, Health and Welfare Canada, Ottawa; W. Hagler, North Carolina State University, Raleigh, NC; and L. Clark, International Minerals and Chemical Corporation, Terre Haute, IN.

REFERENCES

(1) Ueno, Y. (1986) *Rev. Environ. Toxicol.* **2**, 303–341
(2) Joffee, A. Z. (1986) "Effects of Fusariotoxins in Humans," in *Fusarium Species: Their Biology and Toxicology*, A. Z. Joffee (Ed.), Wiley, Chichester, England, pp. 225–292

(3) Ueno, Y. (1983) *Trichothecenes—Chemical, Biological, and Toxicological Aspects*, Elsevier, New York, NY
(4) Bennett, G. A., & Shotwell, O. L. (1979) *J. Am. Oil Chem. Soc.* **56,** 812-819
(5) Shotwell, O. L. (1986) "Chemical Survey Methods for Mycotoxins," in *Modern Methods in the Analysis and Structure Elucidation of Mycotoxins*, R. J. Cole (Ed.), Academic Press, Inc., New York, NY, pp. 70-80
(6) Eppley, R. M. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 892-893
(7) Pohland, A. E., Schuller, P. L., Steyn, P. S., & Van Egmond, H. P. (1982) *Pure Appl. Chem.* **54,** 2219-2284
(8) Takitani, S., Asabe, Y., Kato, T., Suzuki, M., & Ueno, Y. (1979) *J. Chromatogr.* **172,** 335-342
(9) Gilbert, J., Startin, J. R., & Crews, C. (1985) *J. Chromatogr.* **319,** 376-381
(10) Beremand, M. N., VanMiddlesworth, F., Taylor, S., Plattner, R. D., & Weisleder, D. (1988) *Appl. Environ. Microbiol.* **54,** 2759-2766
(11) Pathre, S. V., Mirocha, C. J., & Fentoss, S. W. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1268-1272
(12) Wei, R.-D., & Chu, F. S. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 202-203
(13) Tanaka, T., Hasegawa, A., Yamamoto, S., Lee, U. S., Sugiura, Y., & Ueno, Y. (1988) *J. Agric. Food Chem.* **36,** 979-983
(14) Van Egmond, H. P. (1987) "Current Limits and Regulations on Mycotoxins," in *Joint FAO/WHO/UNEP Second International Conference on Mycotoxins*, Bangkok, Thailand, Sept. 28-Oct. 3, 1987

PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Gas Chromatographic–Electron Capture Detection Method for Determination of 29 Organochlorine Pesticides in Finished Drinking Water: Collaborative Study

VIORICA LOPEZ-AVILA
Acurex Corp., 485 Clyde Ave, Mountain View, CA 94039
RAYMOND WESSELMAN
U.S. Environmental Protection Agency, 26 W Martin Luther King Dr, Cincinnati, OH 45268
KENNETH EDGELL
Bionetics Corp., 16 Triangle Park Dr, Cincinnati, OH 45246

Collaborators: F. DeRoos; N. S. Dodhiwala; D. Erickson; E. E. Hargesheimer; D. Lane; B. Lee; A. Liu; J. Malik; C. Nulton; W. Smalligan; B. Woods

A joint U.S. Environmental Protection Agency/AOAC interlaboratory method validation study was conducted on EPA Method 508, Determination of Chlorinated Pesticides in Water by Gas Chromatography with an Electron Capture Detector, to determine the mean recovery and precision for analyses of 29 pesticides in reagent water and finished drinking water. The study design was based on Youden's nonreplicate plan for collaborative tests of analytical methods. The waters were spiked with 29 pesticides at 6 concentration levels, as 3 Youden pairs. Eleven volunteer laboratories extracted the spiked test waters with methylene chloride, performed a solvent exchange with methyl *tert*-butyl ether, and analyzed an aliquot of each extract by gas chromatography with electron capture detection. Results were analyzed using an EPA computer program, Interlaboratory Method Validation Study (IMVS), which measured recovery and precision for each of the 29 pesticides and compared the performance of the method between water types. Method 508 was judged acceptable for all analytes tested. Only 3 analytes (α-chlordane, 4,4′-DDE, and methoxychlor) exhibited practical significant matrix effects. The method has been adopted official first action.

The Office of Drinking Water (ODW) of the U.S. Environmental Protection Agency (EPA) gathers water quality data to provide information on water resources and drinking waters to monitor safe drinking water quality, establish maximum contaminant limits, assure compliance with existing regulations, and develop further regulations under the Safe Drinking Water Act (1986) and its amendments. The success of these activities depends on the reliability of the data provided by drinking water laboratories. Therefore, it is important to establish the precision and bias of the analytical methods. These are best measured in interlaboratory method validation studies.

The Environmental Monitoring Systems Laboratory, Cincinnati, OH (EMSL-Cincinnati), develops or selects analytical methods and provides quality assurance (QA) support for Agency programs that involve water and wastewater regulations. In EMSL-Cincinnati, the responsibility for providing QA support is assigned to the Quality Assurance Research Division (QARD). The QARD program is designed to provide the QA support needed to establish the reliability and legal defensibility of water and wastewater data collected by the Agency, the state regulating authorities, the private sector, and commercial laboratories that perform compliance analyses. One of QARD's activities is to conduct interlaboratory method validation studies to evaluate analytical methods selected for the Agency's operating programs, such as the Office of Drinking Water.

In the past, EMSL-Cincinnati obtained contract laboratories to participate in interlaboratory studies. This had advantages in that EMSL-Cincinnati could require and control exact instrumentation, quality control, scheduling, and completeness of data packages. However, the increasing cost of conducting interlaboratory studies caused EMSL-Cincinnati to consider means of reducing study costs. One option was an alternative source of laboratories. Concurrently, AOAC expressed interest in obtaining validated methods for its purposes through joint studies with EPA. With its solid association with the state and private laboratories and a strong group of associate and general referees, the 2 organizations agreed that voluntary participation could be accomplished on methods of common interest.

In July and August 1988, the AOAC Associate Referee provided copies of the method and a description of the study requirements to over 30 laboratories. A prestudy performance evaluation sample was sent in September 1988 to those laboratories who were interested in participating in the study, to allow them to gain experience with the method and to provide an initial evaluation of performance. Data were returned by 18 laboratories in October 1988. Study samples were mailed in November 1988, and data were returned from 11 laboratories in January 1989.

The objective of the study was to characterize multilaboratory performance of Method 508 in terms of recovery, overall and single-analyst precision, and the effect of water type on

Received for publication August 2, 1989.
This report was presented at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO.
The recommendation was approved interim official first action by the General Referee, the Committee on Environmental Quality, and the Chairman of the Official Methods Board. The method was adopted official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* 73, January/February issue.
The study was conducted by The Bionetics Corp. (Contract No. 68-03-3254) under the direction of the Quality Assurance Research Division, Environmental Monitoring Systems Laboratory, Cincinnati, OH. The 11 participating laboratories were obtained through AOAC. Analytical work was completed in January 1989.
The information contained in this document, funded in part by the U.S. Environmental Protection Agency (USEPA) under a contract with The Bionetics Corp., Cincinnati, OH, Contract No. 68-03-3254, has been subjected to USEPA and AOAC peer and administrative review and has been approved for publication as an EPA document. Mention of trade names or commercial products does not constitute endorsement or recommendation for use by USEPA or AOAC.

recovery and precision for 29 pesticides. The study was conducted under the direction of QARD, EMSL-Cincinnati, with the cooperation of 11 participating laboratories obtained by AOAC. As primary contractor to QARD, Bionetics Corp. was responsible for the preparation and distribution of samples, user instructions, and report forms, and for screening the returned data for gross errors. The raw data were evaluated statistically by QARD using a computer program, Interlaboratory Method Validation Studies (IMVS) (1).

Collaborative Study

The study design was based on Youden's nonreplicate design for collaborative evaluation of precision and recovery for analytical methods (2). Two similar, yet different, samples were prepared at each of 3 levels over the selected range of concentrations, such that the concentrations of the pair varied between 5 and 20% of the mean of the pairs. Each laboratory was directed to analyze each extract and report 1 value for each analyte at each concentration level. Analyses of the spiked reagent water evaluated the proficiency of the method on a sample free of interferences; analyses of the finished drinking water (provided by each participating laboratory) were intended to reveal the effects of matrix interferences on the method.

Spiking solutions, calibration standards, and quality control samples were prepared and heat-sealed in ampoules, each containing approximately 1.5 mL solution. Prior to distribution, ampoule solutions were analyzed against standards freshly prepared from neat materials. At the completion of the study, the ampoules were analyzed again to verify the stability of the solutions sent to the collaborators, over the time period of the study.

Each of the 11 participating laboratories was sent 24 sample ampoules (6 concentrations for each of 2 waters for 2 groups of target compounds), 4 calibration standard concentrates, 2 internal standard concentrates, 2 surrogate standard concentrates, 4 quality control samples with acceptance limits, copies of Method 508, report forms, and a questionnaire. The laboratories were instructed to analyze the samples in strict accord with the written method and to complete the analyses and report forms within 60 days from receipt of samples.

Treatment of Data

The returned data were grouped by water type and arranged in 6 subsets defined by the 6 concentration levels used in the study and evaluated using the EPA Interlaboratory Method Validation Study computer program (1). Youden's laboratory ranking procedure was used to reject laboratories that had a consistently high or low bias in their submitted data. This test was applied to each analyte data set, for each water type, at the 5% level of significance. Next, missing, "less than," zero, and "nondetect" data were rejected. Finally, Thompson's test for individual outliers (3), suggested in the ASTM Standard Practice D2777-86, was applied to the data using a 5% significance level. If an individual data point was rejected based on this test, it was removed from the subset, and the test was repeated using the remaining data in the subset. Only one repeat of Thompson's test was allowed for each subset.

Besides providing summary statistics for mean recovery, overall method precision, and single-analyst precision, the IMVS program calculated the relationships between mean recovery and true concentration and between precision and mean recovery. These relationships were calculated in the form of linear regression equations using the weighted least squares technique. These regression equations can be used to estimate the mean recovery, overall precision, and single-analyst precision at any value within the concentration range studied. The IMVS also determined statistically significant matrix effects between the matrix waters for each of the 29 analytes.

990.06 Organochlorine Pesticides in Water

Gas Chromatographic Method

First Action 1990

(Applicable to determination of 29 organochlorine pesticides in finished drinking water)

Method Performance:

Pesticide	*Reagent Water*				*Finished Drinking Water*			
	s_r	s_R	RSD_r, %	RSD_R, %	s_r	s_R	RSD_r, %	RSD_R, %
Aldrin	6.2	13.2	6.7	14.3	4.9	15.3	5.8	18.2
α-BHC	6.3	14.1	6.2	13.8	8.9	18.8	9.4	19.9
β-BHC	4.1	15.7	4.2	15.9	13.2	21.8	14.1	23.3
γ-BHC	5.4	15.8	5.4	15.7	6.5	13.8	6.9	14.7
δ-BHC	5.0	12.0	5.2	12.4	7.0	12.9	7.6	14.0
α-Chlordane	6.3	14.0	6.2	13.8	6.1	14.4	7.0	16.4
γ-Chlordane	4.7	12.4	5.0	13.1	6.3	12.1	7.2	13.9
Chlorobenzilate	6.8	18.4	6.8	18.3	12.7	22.5	14.4	25.5
Chloroneb	9.5	16.2	10.0	17.0	8.8	16.9	9.9	19.0
Chlorothalonil	9.4	23.4	9.8	24.4	9.3	16.9	10.1	18.3
DCPA	5.1	16.5	5.0	16.3	12.4	20.8	13.3	22.2
4,4′-DDD	8.4	14.7	8.6	15.1	9.4	13.6	10.3	14.8
4,4′-DDE	9.2	16.3	9.4	16.6	6.8	16.8	8.0	19.9
4,4′-DDT	4.9	13.7	5.0	14.0	9.3	14.4	10.8	16.7
Dieldrin	8.7	15.3	9.0	15.8	5.8	12.4	6.5	14.0
Endosulfan I	6.8	13.4	7.0	13.9	6.6	10.9	7.3	12.1
Endosulfan II	5.9	12.0	6.0	12.3	5.8	11.0	6.4	12.2
Endosulfan sulfate	11.8	16.8	11.9	16.9	12.7	23.4	13.2	24.3
Endrin	10.6	13.5	10.5	13.6	5.6	11.0	6.3	12.3

Pesticide	Reagent Water				Finished Drinking Water			
	s_r	s_R	RSD_r, %	RSD_R, %	s_r	s_R	RSD_r, %	RSD_R, %
Endrin aldehyde	9.7	11.8	10.2	12.4	6.7	8.9	7.6	10.2
Etridiazole	4.9	15.4	5.1	15.9	6.9	22.2	7.5	24.0
Hexachlorobenzene	4.4	11.7	5.1	13.7	1.8	10.0	2.1	11.8
Heptachlor	6.9	12.0	7.0	12.3	7.3	9.3	7.4	9.4
Heptachlor epoxide	4.9	12.2	5.1	12.7	6.5	8.3	6.8	8.7
Methoxychlor	11.2	19.8	10.6	18.8	13.2	26.5	14.0	28.1
cis-Permethrin	7.5	13.9	7.9	14.6	9.5	14.5	11.3	17.3
trans-Permethrin	14.6	26.1	15.4	27.6	14.9	33.6	18.1	40.7
Propachlor	5.5	13.3	5.6	13.4	8.5	15.5	9.0	16.5
Trifluralin	6.0	13.2	6.7	14.8	5.8	12.9	6.8	15.1

A. Principle

Measured volume of sample (1 L) is extracted with CH_2Cl_2 by shaking in separatory funnel or mechanical tumbling in bottle. CH_2Cl_2 extract is separated, dried with anhydrous Na_2SO_4, solvent-exchanged with methyl *tert*-butyl ether (MTBE), and concentrated to 5 mL. Pesticides are separated and measured by capillary column gas chromatography with electron-capture detection. Estimated method detection limits range from 0.0015 μg/L for α- and γ-chlordane to 5 μg/L for chlorobenzilate; values for 24 compounds range between 0.025 and 0.5 μg/L.

B. Apparatus

(a) *Separatory funnel.*—2000 mL, with TFE-fluorocarbon stopcock, and ground-glass or TFE-fluorocarbon stopper.

(b) *Tumbler bottle.*—1.7 L (Wheaton Roller Culture Vessel), with TFE-fluorocarbon lined screw cap. Cut liners to fit screw cap from sheets (Pierce Catalog No. 012736) and extract overnight with methanol before use.

(c) *Kuderna-Danish (K-D) apparatus.*—(*1*) *Concentrator tube.*—10 or 25 mL, graduated (Kontes K-570050-1025 or K-570050-2525, or equivalent). Check calibration of concentrator tube at volumes used in method. Use ground-glass stoppers to prevent evaporation of extracts. (*2*) *Evaporation flask.*—500 mL (Kontes K-570001-0500, or equivalent). Attach to concentrator tube with springs. (*3*) *Snyder columns.*—3-ball macro (Kontes K-503000-0121, or equivalent); 2-ball micro (Kontes K-569001-0219, or equivalent).

(d) *Vials.*—Glass, 5–10 mL capacity, with TFE-fluorocarbon lined screw caps.

(e) *Separatory funnel shaker.*—Capable of holding 2 L separatory funnels and shaking them with rocking motion to thoroughly mix funnel contents (Eberbach Co., Ann Arbor, MI).

(f) *Tumbler.*—Capable of holding and tumbling bottles, (b), end-over-end at 30 turns/min (Associated Design and Manufacturing Co., Alexandria, VA).

(g) *Boiling stones.*—Carborundum, No. 12 granules (Arthur H. Thomas Co., No. 1590-033). Heat 30 min at 400° before use. Cool and store in desiccator.

(h) *Water bath.*—Heated, capable of control ±2°. Use bath in hood.

(i) *Balance.*—Analytical, capable of accurately weighing to nearest 0.0001 g.

(j) *Gas chromatograph.*—Temperature-programmable system suitable for use with capillary columns, including syringes, analytical columns, gases, detector, and strip chart recorder. Data system is recommended for measuring peak areas. Primary column: 30 m × 0.25 mm id DB-5 fused-silica capillary column, 0.25 μm film thickness (J&W Scientific, Inc.). Confirmation column: 30 m × 0.25 mm id DB-1701 fused silica capillary column, 0.25 μm film thickness (J&W Scientific, Inc.). Operating conditions: injection volume 2 μL splitless with 45 s delay; He carrier gas at 30 cm/s linear velocity; injector 250°; detector 320°; oven programmed from 60 to 300° at 4°/min; electron-capture detector.

C. Reagents

(a) *Standard solutions.*—Use standards of test compounds with purity >96% to prepare stock solutions at 1 mg/mL in MTBE. Commercially prepared stock standards may be used at any concentration if they are certified by manufacturer or independent source. These stock standards may be available from U.S. Environmental Protection Agency Toxic and Hazardous Materials Repository, Research Triangle Park, NC. Store solutions at room temperature and protect from light. Replace stock standard solutions after 2 months, or sooner if comparison with laboratory control standards indicates degradation.

(b) *Internal standard solution.*—Prepare pentachloronitrobenzene (purity 98%) stock solution at 0.1 mg/mL in MTBE. Add 5 μL stock solution to 5 mL sample extract to give final internal standard concentration of 0.1 μg pentachloronitrobenzene/mL.

(c) *Surrogate solution.*—Prepare 4,4′-dichlorobiphenyl (purity 96%) stock solution at 0.5 mg/mL in MTBE. Add 50 μL stock solution to 1 L sample prior to extraction to produce surrogate concentration of 25 μg 4,4′-dichlorobiphenyl/L in sample and, assuming quantitative recovery, 5.0 μg/mL in extract.

(d) *Instrument performance solution.*—Prepare individual stock standard solutions containing chlorothalonil, chlorpyrifos, DCPA, and δ-BHC at 0.10 mg/mL in MTBE. For assessing instrument performance, combine 50 μL chlorothalonil stock solution, 2 μL chlorpyrifos stock solution, 50 μL DCPA stock solution, and 40 μL δ-BHC stock solution in 100 mL volumetric flask and dilute to volume with MTBE.

(e) *Solvents.*—Acetone, methylene chloride, and CH_3COCH_3, CH_2Cl_2, and MTBE. Distilled-in-glass quality, or equivalent.

(f) *Phosphate buffer.*—pH 7. Mix 29.6 mL 0.1N HCl and 50 mL 0.1M dipotassium hydrogen phosphate.

(g) *Sodium sulfate.*—Granular, anhydrous. ACS grade. Heat in shallow tray for ≥4 h at 450° to remove interfering organic substances.

(h) *Sodium chloride.*—Crystals. ACS grade. Heat in shallow tray for ≥4 h at 450° to remove interfering organic substances.

(i) *Reagent water.*—Water reasonably free of contami-

Table 990.06A Gas Chromatographic Conditions, Chemical Abstracts Service Registry Numbers, Peak Identification Codes, Relative Retention Times, and Estimated Method Detection Limits for 29 Organochlorine Pesticides

Analyte	CAS No.	Peak No.[a]	Relative Retention Time[b]		Estd MDL, μg/L
			Primary[c]	Confirmation[d]	
Aldrin	309-00-2	A7	1.18	1.12	0.075
α-BHC	319-84-6	A3	0.93	0.97	0.025
β-BHC	319-85-7	B4	0.98	1.18	0.010
γ-BHC	319-86-8	B5	1.03	1.22	0.010
δ-BHC	58-89-9	A5	0.99	1.04	0.015
α-Chlordane	5103-71-9	B9	1.31	1.31	0.0015
γ-Chlordane	5103-74-2	B8	1.28	1.29	0.0015
Chlorobenzilate	501-15-6	B11	1.41	1.42	5.0
Chloroneb	2675-77-6	A1	0.75	0.77	0.50
Chlorothalonil	2921-88-2	A6	1.04	1.17	0.025
DCPA	1897-45-6	B7	1.21	1.21	0.025
4,4′-DDD	72-54-8	B12	1.42	1.38	0.0025
4,4′-DDE	72-55-9	B10	1.35	1.32	0.010
4,4′-DDT	50-29-3	A16	1.48	1.48	0.060
Dieldrin	60-57-1	A11	1.35	1.35	0.020
Endosulfan I	959-98-8	A10	1.30	1.28	0.015
Endosulfan II	33213-65-9	A14	1.40	1.45	0.015
Endosulfan sulfate	1031-07-8	B13	1.47	—[e]	0.015
Endrin	72-20-8	A12	1.38	1.38	0.025
Endrin aldehyde	7421-93-4	A15	1.43	1.52	0.024
Etridiazole	2593-15-9	B1	0.69	0.67	0.025
Heptachlor	76-44-8	B6	1.11	1.08	0.010
Heptachlor epoxide	1024-57-3	A9	1.24	1.24	0.015
Hexachlorobenzene	118-74-1	B3	0.94	—[e]	0.0077
Methoxychlor	72-43-5	B14	1.57	1.58	0.050
cis-Permethrin	52645-53-1	A17	1.72	—[e]	0.50
trans-Permethrin	52645-53-1	B15	1.73	—[e]	0.50
Propachlor	1918-16-7	A2	0.85	0.91	0.50
Trifluralin	1582-09-8	B2	0.93	—[e]	0.025

[a] Identification of chromatographic peaks shown in Figs **990.06A** and **990.06B**. Letters indicate which spiking mixture (A or B) contains the analyte.
[b] Retention time relative to internal standard = 1.00.
[c,d] See **B(j)** for column description and operating conditions.
[e] Data not available.

nation that would prevent determination of any analyte of interest.

(**j**) *Preservative.*—Mercuric chloride solution. 10 mg $HgCl_2$ (ACS grade)/mL reagent water, (**i**).

(**k**) *Sodium thiosulfate.*—$Na_2S_2O_3$. Granular, anhydrous. ACS grade.

D. Preparation of Sample Bottles

Add 1 mL preservative, **C(j)**, to glass sample bottle. If residual chlorine is expected to be present in samples, add 80 mg $Na_2S_2O_3$, **C(k)**, to sample bottle before collection.

E. Sample Collection

Collect 1 L grab samples in glass bottles by conventional sampling practices. Since bottles contain preservative and $Na_2S_2O_3$, do not prerinse bottles with sample before collection. Add sample to bottle containing preservative, seal sample bottle, and shake vigorously 1 min. Refrigerate samples at 4° from time of collection until extracted. Protect from light. Extract samples within 7 days of sample collection.

F. Sample Preparation

(**a**) *Automated extraction method.*—Add preservative, **C(j)**, to any samples not previously preserved. Mark water meniscus on side of sample bottle for later determination of sample volume. Add 50 μL surrogate stock solution, **C(c)**, to sample. If mechanical separatory funnel shaker is used, pour entire sample into 2 L separatory funnel. If mechanical tumbler is used, pour entire sample into tumbler bottle. Adjust sample to pH 7 by adding 50 mL phosphate buffer, **C(f)**. Check pH and add H_2SO_4 or NaOH if necessary.

Add 100 g NaCl to sample, seal, and shake to dissolve salt. Add 300 mL CH_2Cl_2 to sample bottle, seal, and shake 30 s to rinse inner walls. Transfer solvent to sample contained in separatory funnel or tumbler bottle, seal, and shake 10 s, venting periodically. Repeat shaking and venting until pressure release is not observed during venting. Reseal and place sample container in appropriate mechanical mixing device (separatory funnel shaker or tumbler). Shake or tumble sample for 1 h.

After extraction, pour contents of tumbler bottle into 2 L separatory funnel. Let organic layer separate from water phase for ≥10 min. If emulsion interface between layers is more than one-third volume of solvent layer, complete phase separation mechanically. Collect CH_2Cl_2 extract in 500 mL erlenmeyer flask containing ca 5 g anhydrous Na_2SO_4. Swirl flask to dry extract; let flask sit 15 min. Determine original sample volume by refilling sample bottle to mark and trans-

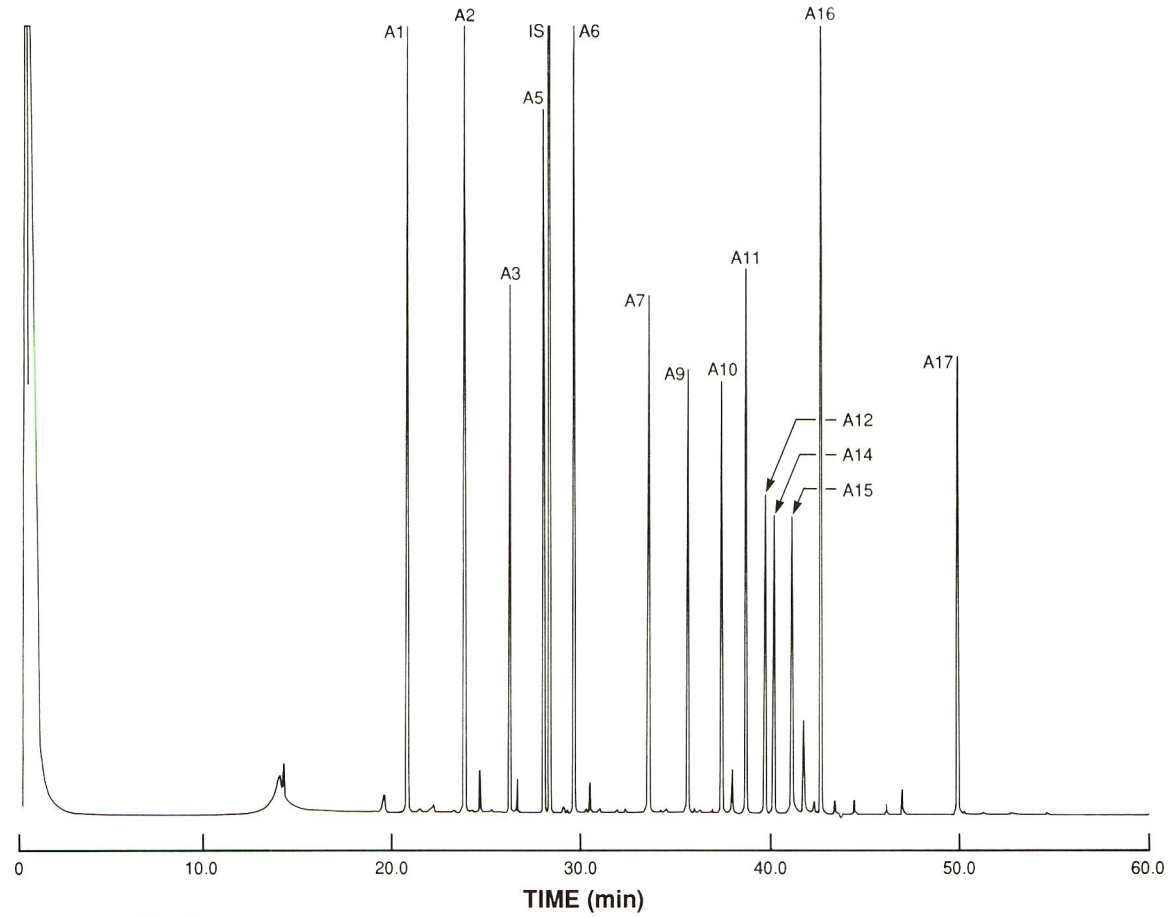


FIG. 990.06A—GC/ECD chromatogram of Group A compounds analyzed on 30 m × 0.25 mm id DB-5 fused-silica capillary column (0.25 μm film). See B(j) for operating conditions and Table 990.06A for peak identification. IS = internal standard.

ferring water to 1000 mL graduated cylinder. Record sample volume to nearest 5 mL.

(b) *Manual extraction method.*—Add preservative, C(j), to samples not previously preserved. Mark water meniscus on side of sample bottle for later determination of sample volume. Add 50 μL surrogate stock solution, C(c), to sample. Pour entire sample into 2 L separatory funnel. Adjust sample to pH 7 by adding 50 mL phosphate buffer, C(f). Check pH and add H_2SO_4 or NaOH if necessary. Add 100 g NaCl to sample, seal, and shake to dissolve salt. Add 60 mL CH_2Cl_2 to sample bottle, seal, and shake bottle 30 s to rinse inner walls.

Transfer solvent to separatory funnel and extract sample by vigorously shaking funnel for 2 min with periodic venting to release excess pressure. Let organic layer separate from water phase for ≥10 min. If emulsion interface between layers is more than one-third volume of solvent layer, complete phase separation mechanically. Collect CH_2Cl_2 extract in 500 mL erlenmeyer flask containing ca 5 g anhydrous Na_2SO_4. Add second 60 mL portion of CH_2Cl_2 to sample bottle and repeat extraction procedure a second time, combining extracts in erlenmeyer flask. Perform third extraction in same manner. Swirl flask to dry extract; let flask sit for 15 min. Determine original sample volume by refilling sample bottle to mark and transferring water to 1000 mL graduated cylinder. Record sample volume to nearest 5 mL.

G. Extract Concentration

Assemble K-D concentrator by attaching 25 mL concentrator tube to 500 mL evaporation flask. Decant CH_2Cl_2 extract into concentrator. Rinse remaining Na_2SO_4 with two 25 mL portions of CH_2Cl_2 and decant rinses into concentrator.

Add 1 or 2 clean boiling stones to evaporation flask and attach macro-Snyder column. Prewet column by adding ca 1 mL CH_2Cl_2 to top. Place K-D apparatus on 65–75° water bath so that concentrator tube is partially immersed in hot water, and entire lower, rounded surface of flask is bathed with hot vapor. Adjust vertical position of apparatus and water temperature as required to complete concentration in 15–20 min. At proper rate of distillation, balls of column will actively chatter, but chambers will not flood. When apparent volume of liquid reaches 2 mL, remove K-D apparatus and let it drain and cool ≥10 min.

Remove Snyder column and rinse flask and its lower joint with 1–2 mL MTBE, collecting rinse in concentrator tube. Add 5–10 mL MTBE and fresh boiling stone. Attach micro-Snyder column to concentrator tube and prewet column by adding ca 0.5 mL MTBE to top. Place micro K-D apparatus on water bath so that concentrator tube is partially immersed in hot water. Adjust vertical position of apparatus and water temperature as required to complete concentration in 5–10 min. When apparent volume of liquid reaches 2 mL, remove

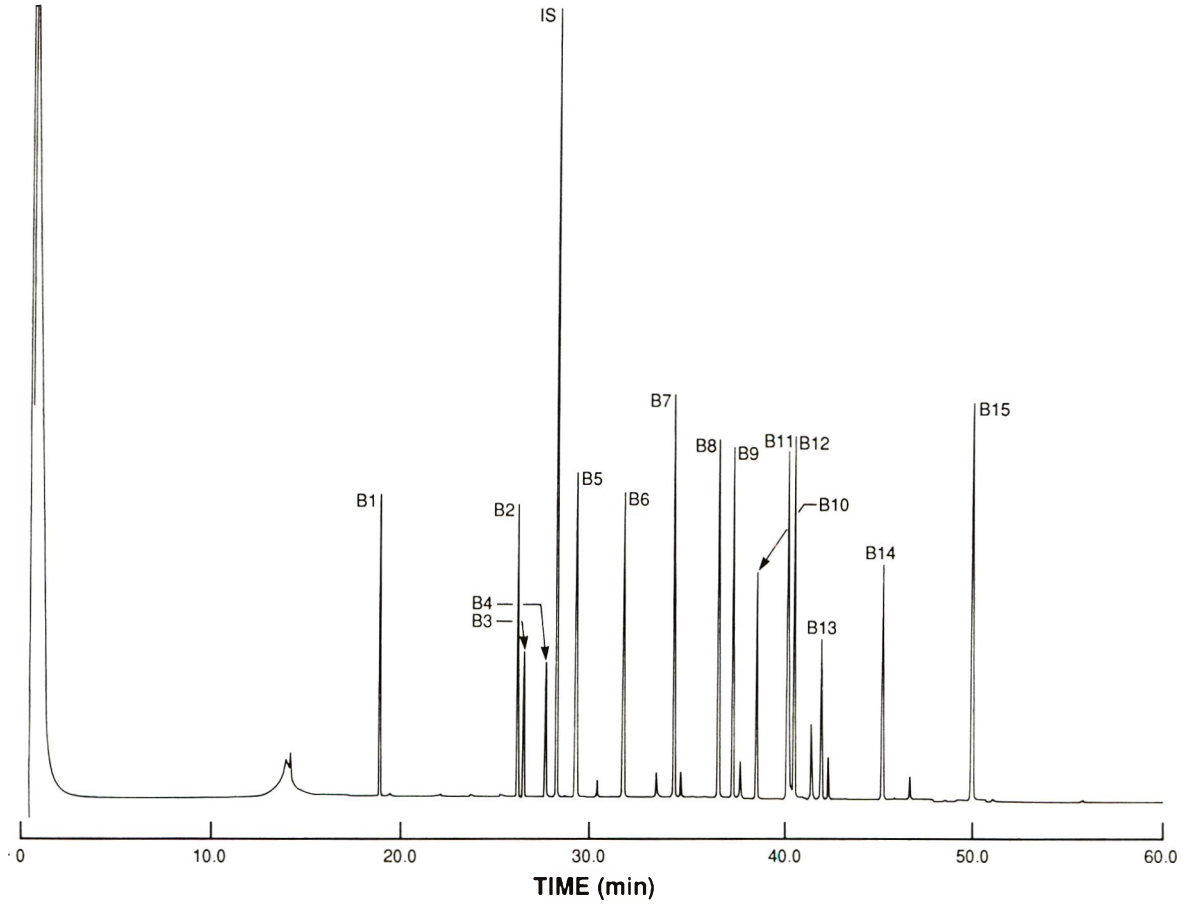


FIG. 990.06B—GC/ECD chromatogram of Group B compounds analyzed on 30 m × 0.25 mm Id DB-5 fused-silica capillary column (0.25 μm film). See B(j) for operating conditions and Table 990.06A for peak identification. IS = internal standard.

apparatus from bath and let it drain and cool. Add 5–10 mL MTBE and boiling stone and reconcentrate to 2 mL. Remove micro K-D apparatus from bath and let it drain and cool. Remove micro-Snyder column, and rinse walls of concentrator tube while adjusting volume to 5.0 mL with MTBE.

Add 5 μL internal standard stock solution, **C(b)**, to sample extract, seal, and shake to distribute internal standard. Transfer extract to appropriate-size TFE-fluorocarbon-sealed, screw-cap vial and store at 4° until analysis. A 14-day maximum extract storage time is recommended.

H. Calibration of Gas Chromatograph with Electron Capture Detector

Table **990.06A** summarizes recommended operating conditions for gas chromatography and retention times observed using this method. Examples of separations using these conditions are shown in Figs **990.06A** and **990.06B**. Initially, perform 5-level calibration within linear range of detector, using internal standard and relative response factors. If response factor (RF) value over working range is constant (≤10% RSD), average RF can be used for calculations. Verify calibration curve daily using 1 or 2 calibration standards. If response of any analyte varies >20% from average relative response factor for initial calibration, analysis of single-level standard must be repeated with fresh standard. Alternatively, new calibration curve must be prepared.

I. Quality Control

Minimum quality control requirements for this method include (*1*) initial demonstration of method performance, (*2*) analysis of surrogate standard in each sample (acceptable recovery is 70–130%), (*3*) monitoring of internal standard area counts in each sample (area of internal standard should be within 30% of area in calibration standard), (*4*) analysis of method blanks as continuing check on sample contamination, (*5*) analysis of spiked samples as continuing check on method recovery, and (*6*) analysis of instrument QC standard to ensure acceptable instrument performance (Table **990.06B**).

Table 990.06B Instrument Quality Control Standard

Test	Analyte	Concn, μg/L	Requirements
Sensitivity	chlorpyrifos	0.002	detection of analyte; S/N >3
Chromatographic performance	DCPA	0.05	PSF between 0.80 and 1.15 PGF between 0.80 and 1.15
Column performance	chlorothalonil	0.05	R > 0.50
	δ-BHC	0.04	

Calculate peak symmetry factor (PSF), peak Gaussian factor (PGF), and resolution between the 2 peaks (R) as follows:

$$PSF = w(1/2)/[0.5 \times W(1/2)]$$

where $w(1/2)$ = width of peak front at half height, assuming peak is split at its highest point; and $W(1/2)$ = peak width at half height.

$$PGF = [1.83 \times W(1/2)]/W(1/10)$$

where $W(1/2)$ = peak width at half height, and $W(1/10)$ = peak width at tenth height.

$$R = t/W$$

where t = difference in elution times between the 2 peaks, and W = average peak width of the 2 peaks at the baseline.

Demonstrate initial method performance by extracting four 1 L samples of reagent water, C(i), spiked at concentration ca 10 times estimated method detection limit. Calculate average percent recovery and standard deviation of percent recovery. For acceptable performance, relative standard deviation should be <20% and analyte mean recoveries should be within 70–130%.

Ref.: JAOAC **73**, March/April issue (1990).

Results and Discussion

Rejection of Outliers

For the entire study, the IMVS computer program rejected 12.8% of the 3828 data points submitted. The percentage of rejected data was 5.4 for reagent water and 7.4 for finished drinking water (Table 1) of the 132 data points/analyte. Trifluralin had the largest number of outliers, 32, while aldrin had no outliers. Seven compounds had less than 10 outliers, 11 compounds had between 11 and 20 outliers, and 11 compounds had between 21 and 32 outliers.

The number of data rejected for each laboratory is presented in Table 2. The laboratory ranking test accounted for 84.5% of all rejected data. Of the 11 laboratories submitting data, laboratory 18 had the highest number of data rejected, 30.7% of their total submitted data, which seemed excessive compared with the remaining laboratories. Their laboratory notebook stated an increased column flow rate (no rates given) prior to the analyses of the sample extracts. As a result, hexachlorobenzene and trifluralin were no longer resolved and coeluted, making quantitation impossible. Laboratory 18 reported no data for these 2 compounds, which represents 24 data points of the 107 total rejected data points.

Four of the 6 laboratories exhibiting the highest number of rejected data used thick film capillary columns ranging from 0.52 to 2.65 μm, instead of the recommended 0.25 μm film column. The remaining laboratories used the 0.25 μm film thickness column.

An initial review of the regression equations revealed higher variability in the chlorothalonil reagent water data set than in the finished drinking water data set. Examination of the data showed the retention of 2 extremely low data points. In our judgment, these 2 data points biased the chlorothalonil reagent water data set, causing it to be inconsistent with the other chlorothalonil data sets. As a result, the data points were removed and the summary statistics were recalculated, including the revision of the linear regression equations. The revised chlorothalonil reagent water equations are presented in Table 3.

Table 1. Total number of rejected data by compound by water type and in total

Compound	Reagent water[a]	Finished drinking water[a]	Total rejected data[b]
Aldrin	0	0	0
α-BHC	7	0	7
β-BHC	3	0	3
γ-BHC	1	7	8
δ-BHC	15	15	30
α-Chlordane	14	8	22
γ-Chlordane	2	13	15
Chlorobenzilate	14	12	26
Chloroneb	3	12	15
Chlorothalonil	8	0	8
DCPA	2	6	8
4,4′-DDD	3	13	16
4,4′-DDE	6	10	16
4,4′-DDT	12	18	30
Dieldrin	1	14	15
Endosulfan I	7	12	19
Endosulfan II	13	6	19
Endosulfan sulfate	8	6	14
Endrin	13	12	25
Endrin aldehyde	12	12	24
Etridiazole	2	13	15
Heptachlor	9	16	25
Heptachlor epoxide	2	14	16
Hexachlorobenzene	6	15	21
Methoxychlor	7	6	13
cis-Permethrin	14	7	21
trans-Permethrin	8	13	21
Propachlor	0	6	6
Trifluralin	13	19	32
Total	205 (5.4%)[c]	285 (7.4%)	490 (12.8%)
Average	7.1	9.8	16.9

[a] Total submitted data by water type was 66 (6 concentrations × 11 laboratories).
[b] Total submitted data by compound was 132 (6 concentrations × 11 laboratories × 2 water types).
[c] Total submitted data for the study was 3828.

Table 2. Total number of rejected data by laboratory by outlier test and in total

Laboratory code	Laboratory ranking test[a]	Thompson's outlier test[a]	Total rejected data[b]
01	48	1	49
02	48	6	54
03	42	14	56
04	12	8	20
06	18	1	19
07	0	23	23
09	0	0	0
15	66	2	68
17	12	3	15
18	90	17	107
19	78	1	79
Total	414 (84.5%)	76 (15.5%)	490
Average			44.5

[a] Level of significance 0.05.
[b] Total submitted data by laboratory was 348 (6 concentrations × 29 compounds × 2 water types).

Table 3. Regression equations for method precision and mean recovery for reagent water[a]

Compound	Concn range, μg/L	Single-analyst precision, s_r	Overall precision, s_R	Mean recovery, X
Aldrin	0.03–1.38	0.061X + 0.004	0.130X + 0.009	0.909C + 0.007
α-BHC	0.02–1.00	0.059X + 0.001	0.127X + 0.005	1.015C + 0.004
β-BHC	0.02–1.00	0.034X + 0.004	0.148X + 0.005	0.975C + 0.006
γ-BHC	0.03–1.51	0.047X + 0.005	0.147X + 0.007	0.998C + 0.006
δ-BHC	0.02–1.01	0.050X + 0.001	0.119X + 0.002	0.958C + 0.005
α-Chlordane	0.03–1.50	0.062X + 0.000	0.138X + 0.000	1.008C + 0.003
γ-Chlordane	0.03–1.51	0.048X + 0.002	0.129X + 0.001	0.936C + 0.005
Chlorobenzilate	1.00–50.00	0.067X + 0.022	0.178X + 0.117	0.993C + 0.263
Chloroneb	1.00–50.08	0.111X − 0.016	0.159X + 0.275	0.942C + 0.280
Chlorothalonil	0.05–2.51	0.096X + 0.001	0.233X + 0.001	0.955C + 0.001
DCPA	0.05–2.51	0.047X + 0.002	0.161X + 0.002	0.998C + 0.013
4,4′-DDD	0.05–2.50	0.087X − 0.001	0.150X + 0.000	0.970C + 0.006
4,4′-DDE	0.02–1.00	0.093X + 0.001	0.166X + 0.000	0.982C + 0.000
4,4′-DDT	0.12–6.01	0.044X + 0.017	0.140X + 0.002	0.976C + 0.006
Dieldrin	0.04–2.01	0.089X + 0.000	0.150X + 0.009	0.962C + 0.009
Endosulfan I	0.03–1.51	0.070X + 0.000	0.127X + 0.009	0.957C + 0.006
Endosulfan II	0.03–1.49	0.059X + 0.001	0.120X + 0.002	0.974C + 0.003
Endosulfan sulfate	0.03–1.51	0.115X + 0.003	0.158X + 0.007	0.988C + 0.004
Endrin	0.03–1.50	0.108X − 0.002	0.134X + 0.002	0.991C + 0.002
Endrin aldehyde	0.05–2.49	0.105X − 0.004	0.121X + 0.003	0.940C + 0.007
Etridiazole	0.05–2.48	0.049X + 0.002	0.149X + 0.010	0.960C + 0.007
Heptachlor	0.02–1.00	0.068X + 0.001	0.100X + 0.011	0.961C + 0.009
Heptachlor epoxide	0.03–1.50	0.049X + 0.002	0.122X + 0.005	0.950C + 0.006
Hexachlorobenzene	0.01–0.50	0.049X + 0.000	0.124X + 0.003	0.841C + 0.003
Methoxychlor	0.10–5.01	0.108X − 0.004	0.190X − 0.003	1.044C + 0.016
cis-Permethrin	1.00–50.08	0.077X + 0.034	0.138X + 0.204	0.938C + 0.314
trans-Permethrin	1.00–50.12	0.096X − 0.001	0.233X + 0.001	0.955C + 0.001
Propachlor	1.00–50.08	0.052X + 0.098	0.119X + 0.370	0.978C + 0.317
Trifluralin	0.05–2.51	0.064X + 0.003	0.144X + 0.004	0.888C + 0.004

[a] X = mean recovery; C = analyte true concentration.

Mean Recovery

Mean recoveries were calculated for each of the 29 pesticides by inserting a midpoint concentration value in the range studied into the regression equations presented in Tables 3 and 4. The results, given in Table 5, show excellent recoveries from reagent water, ranging from 85.4% for hexachlorobenzene to 105.2% for methoxychlor, with an average recovery of 97.1%. In finished drinking water, the recoveries of the 29 compounds were slightly lower but judged acceptable, ranging from 82.5% for *trans*-permethrin to 99.0% for heptachlor, with an average of 90.1%. The small standard deviations about the pooled mean recoveries suggest that the method worked well in either water matrix.

For purposes of discussion and interpretation, compounds with calculated recoveries outside 2 standard deviations from the pooled mean recovery were reviewed further. In reagent water, the 2 standard deviation range was 89.3–104.9%. Only hexachlorobenzene (85.4%) fell outside this range. A review of the hexachlorobenzene data set showed no unusual data. It was concluded that the statistics reported for hexachlorobenzene were acceptable and representative of the method. In finished drinking water, the 2 standard deviation range was 81.6–98.6%. Heptachlor (99.0%) was the only pesticide to exceed this range, but the value was not considered excessive enough to warrant further data review.

Precision

The overall standard deviation (S_R) is the precision associated with measurements generated by a group of laboratories, while the single-analyst standard deviation (S_r) is the precision associated with performance in an individual laboratory. The overall and single-analyst precisions were calculated using the midpoint value of the range tested in the precision regression equations presented in Tables 3 and 4. The overall (RSD_R) and single-analyst precisions (RSD_r), expressed as relative standard deviations, are presented in Table 6.

The pooled mean overall precision for the 29 pesticides in reagent water, expressed as RSD_R, was 15.5%, ranging from 12.3% for endosulfan II and heptachlor to 27.6% for *trans*-permethrin. In finished drinking water, the pooled mean overall precision was 17.7%, ranging from 8.7% for heptachlor epoxide to 40.7% for *trans*-permethrin. The overall precisions of the finished drinking water data were slightly more variable than for the reagent water data as might be expected because of the differences in the sources and treatment of the waters tested in the participating laboratories.

For purposes of discussion and interpretation, compounds whose overall precision, as RSD_R, exceeded 25% (approximately 1.5 times the average overall precision) were subjected to further data review. In reagent water, *trans*-permethrin (27.6%) was the only compound that exceeded this limit. A review of the *trans*-permethrin data revealed an anomalous data point, which was dissimilar to the other data in the set. However, when this data point was removed and the statistics recalculated, only a slight improvement in the summary statistics resulted. Removal of this data point, therefore, was not believed to be justified. The statistical summaries for *trans*-permethrin are acceptable as presented and represent the

Table 4. Regression equations for method precision and mean recovery for finished drinking water[a]

Compound	Concn range, μg/L	Single-analyst precision, s_r	Overall precision, s_R	Mean recovery, X
Aldrin	0.03–1.49	0.048X + 0.008	0.175X + 0.005	0.826C + 0.008
α-BHC	0.02–1.00	0.094X − 0.000	0.198X + 0.000	0.940C + 0.003
β-BHC	0.02–1.00	0.142X − 0.001	0.227X + 0.003	0.923C + 0.005
γ-BHC	0.03–1.51	0.070X − 0.001	0.138X + 0.006	0.938C + 0.002
δ-BHC	0.02–1.01	0.066X + 0.005	0.133X + 0.004	0.905C + 0.007
α-Chlordane	0.03–1.50	0.070X + 0.000	0.164X + 0.000	0.870C + 0.005
γ-Chlordane	0.03–1.51	0.072X + 0.000	0.138X + 0.001	0.865C + 0.005
Chlorobenzilate	1.00–50.00	0.146X − 0.042	0.243X + 0.292	0.874C + 0.207
Chloroneb	1.00–50.08	0.100X − 0.024	0.185X + 0.110	0.883C + 0.218
Chlorothalonil	0.05–2.51	0.100X + 0.001	0.180X + 0.004	0.920C + 0.000
DCPA	0.05–2.51	0.136X − 0.003	0.224X − 0.003	0.920C + 0.015
4,4′-DDD	0.05–2.50	0.102X + 0.001	0.146X + 0.002	0.908C + 0.008
4,4′-DDE	0.02–1.00	0.081X − 0.001	0.203X − 0.002	0.842C + 0.002
4,4′-DDT	0.12–6.01	0.110X − 0.005	0.162X + 0.012	0.858C + 0.009
Dieldrin	0.04–2.01	0.065X − 0.000	0.140X − 0.000	0.882C + 0.006
Endosulfan I	0.03–1.51	0.072X + 0.001	0.117X + 0.003	0.898C + 0.004
Endosulfan II	0.03–1.49	0.064X − 0.000	0.119X + 0.002	0.901C + 0.002
Endosulfan sulfate	0.03–1.51	0.132X − 0.000	0.233X + 0.007	0.948C + 0.009
Endrin	0.03–1.50	0.062X + 0.001	0.120X + 0.002	0.893C + 0.001
Endrin aldehyde	0.05–2.49	0.076X − 0.001	0.097X + 0.005	0.874C + 0.003
Etridiazole	0.05–2.48	0.074X + 0.001	0.240X − 0.000	0.916C + 0.009
Heptachlor	0.02–1.00	0.072X + 0.001	0.075X + 0.009	0.980C + 0.005
Heptachlor epoxide	0.03–1.50	0.066X + 0.001	0.084X + 0.004	0.944C + 0.006
Hexachlorobenzene	0.01–0.50	0.013X + 0.002	0.097X + 0.005	0.833C + 0.004
Methoxychlor	0.10–5.01	0.142X − 0.004	0.285X − 0.007	0.936C + 0.017
cis-Permethrin	1.00–50.08	0.112X + 0.012	0.161X + 0.292	0.833C + 0.200
trans-Permethrin	1.00–50.12	0.184X − 0.087	0.410X − 0.063	0.814C + 0.287
Propachlor	1.00–50.08	0.087X + 0.061	0.158X + 0.185	0.925C + 0.353
Trifluralin	0.05–2.51	0.066X + 0.002	0.147X + 0.004	0.847C + 0.006

[a] X = mean recovery; C = analyte true concentration.

method. In finished drinking water, chlorobenzilate (25.5%), methoxychlor (28.1%), and *trans*-permethrin (40.7%) exceeded the 25% limit. The methoxychlor data sets show the same variability across all concentration levels. The overall precision statistics for methoxychlor were judged acceptable and indicate a higher variability in the recoveries from finished drinking water as opposed to reagent water. Examination of the chlorobenzilate and *trans*-permethrin data sets reveals the retention of several anomalous data points within each data set. However, the variability of the data sets was large enough that removal of these data points would not have improved the overall precision statistics to any degree. As a result, their removal was not justified. The higher variability for *trans*-permethrin may have resulted from it being the last compound to elute from the chromatographic column, at approximately 63 min. Peak geometry is not as sharp for late eluting compounds and results in higher variability in peak area integrations.

The pooled mean single-analyst precision, expressed as RSD_r, of the 29 pesticides in reagent water was 7.5%, and ranged from 4.2% for β-BHC to 15.4% for *trans*-permethrin. In finished drinking water, the pooled mean single-analyst precision was 9.1%, and ranged from 2.1% for hexachlorobenzene to 18.1% for *trans*-permethrin, slightly higher in finished drinking water than in reagent water. Precision statistics generated in other method validation studies have shown average overall precision approximately twice the average single-analyst precision. This same relationship was observed here with the reagent water overall/single-analyst ratio of 15.5/7.5 = 2.07 and the finished drinking water ratio of 17.7/9.1 = 1.94.

For purposes of discussion and interpretation, compounds whose single-analyst precision, expressed as RSD_r, were greater than 13% (approximately 1.5 times the average single-analyst precision) were subjected to further review. In a few cases, single-analyst precisions exceeded 13% but were less than 14.5%; however, these instances were not considered of major concern and will not be discussed. Only *trans*-permethrin exceeded this limit in reagent water (15.4%) and finished drinking water (18.1%). As stated previously, the removal of a few errant data points from the *trans*-permethrin data set did not improve the single-analyst precision substantially.

Effect of Water Type

The data across water types were subjected to an analysis of variance test using the IMVS computer program to determine the effect of water type on recovery and precision. Only 4 compounds (α-chlordane, DCPA, 4,4′-DDE, and methoxychlor) had statistically significant matrix effects due to water type but this does not necessarily mean that the effect is of practical significance. Practical significance was determined by a review of the retained data and judging whether the statistically significant matrix effects were influenced by the retention of one or more errant data points. If no anomalies were observed, the statistically significant matrix effects were considered to be of practical significance.

A review of the DCPA finished drinking water data set

Table 5. Estimated recoveries calculated from regression equations by compound and by water type, as percent of true concentration[a]

Compound	Concn, µg/L[b]	Recovery, %		Compound	Concn, µg/L[b]	Recovery, %	
		Reagent water	Finished drinking water			Reagent water	Finished drinking water
Aldrin	0.7	92.0	83.8	Endosulfan II	0.7	97.9	90.4
α-BHC	0.5	102.3	94.6	Endosulfan sulfate	0.7	99.3	96.1
β-BHC	0.5	98.7	93.4	Endrin	0.7	99.4	89.5
γ-BHC	0.7	100.6	94.2	Endrin aldehyde	1.0	94.8	87.7
δ-BHC	0.5	96.9	92.0	Etridiazole	1.0	96.7	92.5
α-Chlordane	0.7	101.3	87.6	Heptachlor	0.5	97.9	99.0
γ-Chlordane	0.7	94.3	87.2	Heptachlor epoxide	1.0	95.7	95.0
Chlorobenzilate	25.0	100.4	88.3	Hexachlorobenzene	0.25	85.4	84.9
Chloroneb	25.0	95.3	89.2	Methoxychlor	2.0	105.2	94.4
Chlorothalonil	1.0	95.7	92.1	*cis*-Permethrin	25.0	95.1	84.1
DCPA	1.0	101.0	93.5	*trans*-Permethrin	25.0	94.5	82.5
4,4′-DDD	1.0	97.5	91.6	Propachlor	25.0	99.1	93.9
4,4′-DDE	0.5	98.2	84.6	Trifluralin	1.0	89.2	85.4
4,4′-DDT	3.0	97.9	86.1				
Dieldrin	1.0	97.1	88.8	Average		97.1	90.1
Endosulfan I	0.7	96.6	90.3	Std dev.		3.9	4.2

[a] Weighted linear regression equations for Method 508.
[b] Midpoint value in concentration range studied.

Table 6. Statistical parameters calculated from regression equations for Method 508 by compound and by water type[a]

Compound	Concn, µg/L[b]	Reagent water		Finished drinking water		Reagent water		Finished drinking water	
		s_r	s_R	s_r	s_R	RSD_r, %	RSD_R, %	RSD_r, %	RSD_R, %
Aldrin	0.7	6.2	13.2	4.9	15.3	6.7	14.3	5.8	18.2
α-BHC	0.5	6.3	14.1	8.9	18.8	6.2	13.8	9.4	19.9
β-BHC	0.5	4.1	15.7	13.2	21.8	4.2	15.9	14.1	23.3
γ-BHC	0.7	5.4	15.8	6.5	13.8	5.4	15.7	6.9	14.7
δ-BHC	0.5	5.0	12.0	7.0	12.9	5.2	12.4	7.6	14.0
α-Chlordane	0.7	6.3	14.0	6.1	14.4	6.2	13.8	7.0	16.4
γ-Chlordane	0.7	4.7	12.4	6.3	12.1	5.0	13.1	7.2	13.9
Chlorobenzilate	25.0	6.8	18.4	12.7	22.5	6.8	18.3	14.4	25.5
Chloroneb	25.0	9.5	16.2	8.8	16.9	10.0	17.0	9.9	19.0
Chlorothalonil	1.0	9.4	23.4	9.3	16.9	9.8	24.4	10.1	18.3
DCPA	1.0	5.1	16.5	12.4	20.8	5.0	16.3	13.3	22.2
4,4′-DDD	1.0	8.4	14.7	9.4	13.6	8.6	15.1	10.3	14.8
4,4′-DDE	0.5	9.2	16.3	6.8	16.8	9.4	16.6	8.0	19.9
4,4′-DDT	3.0	4.9	13.7	9.3	14.4	5.0	14.0	10.8	16.7
Dieldrin	1.0	8.7	15.3	5.8	12.4	9.0	15.8	6.5	14.0
Endosulfan I	0.7	6.8	13.4	6.6	10.9	7.0	13.9	7.3	12.1
Endosulfan II	0.7	5.9	12.0	5.8	11.0	6.0	12.3	6.4	12.2
Endosulfan sulfate	0.7	11.8	16.8	12.7	23.4	11.9	16.9	13.2	24.3
Endrin	0.7	10.6	13.5	5.6	11.0	10.5	13.6	6.3	12.3
Endrin aldehyde	1.0	9.7	11.8	6.7	8.9	10.2	12.4	7.6	10.2
Etridiazole	1.0	4.9	15.4	6.9	22.2	5.1	15.9	7.5	24.0
Heptachlor	0.5	6.9	12.0	7.3	9.3	7.0	12.3	7.4	9.4
Heptachlor epoxide	1.0	4.9	12.2	6.5	8.3	5.1	12.7	6.8	8.7
Hexachlorobenzene	0.25	4.4	11.7	1.8	10.0	5.1	13.7	2.1	11.8
Methoxychlor	2.0	11.2	19.8	13.2	26.5	10.6	18.8	14.0	28.1
cis-Permethrin	25.0	7.5	13.9	9.5	14.5	7.9	14.6	11.3	17.3
trans-Permethrin	25.0	14.6	26.1	14.9	33.6	15.4	27.6	18.1	40.7
Propachlor	25.0	5.5	13.3	8.5	15.5	5.6	13.4	9.0	16.5
Trifluralin	1.0	6.0	13.2	5.8	12.9	6.7	14.8	6.8	15.1
Average						7.5	15.5	9.1	17.7
Std dev.						2.6	3.4	3.4	6.7

[a] s_r and s_R = standard deviations for repeatability and reproducibility, respectively. RSD_r and RSD_R = corresponding relative standard deviations.
[b] Midpoint value in concentration range studied.

revealed the retention of an anomalous data point in the data set. Therefore, the statistically significant matrix effect for DCPA was judged to be of no practical significance. The statistically significant matrix effects for α-chlordane, 4,4′-DDE, and methoxychlor were considered to be of practical significance. Each of these compounds had lower recoveries and poorer overall precision in finished drinking water at all concentration levels compared with reagent water. The reagent water recoveries were approximately 10% higher, with better precision, than the comparable finished drinking water samples.

Conclusions and Recommendations

The objective of this study was to characterize the performance of Method 508 in terms of recovery, overall precision, single-analyst precision, and the effect of water type on recovery and precision. Through use of the IMVS computer program, statistical analyses of 3828 analytical values provided estimates of recovery and precision for each of the 29 pesticides, which are expressed as regression equations in Tables 3 and 4. These equations may be used to predict the recovery and precision of the 29 chlorinated pesticides over the concentration range tested.

The summary statistics from this study show that analytical results for 29 chlorinated pesticides were acceptable in reagent water and in finished drinking waters. The overall and single-analyst precisions were similar in the 2 waters. Recoveries of the 29 pesticides were slightly lower in the finished drinking waters than in reagent water.

The low number of rejected data in this study demonstrates the general applicability of the method. Practical, significant matrix effects were found for α-chlordane, 4,4′-DDE, and methoxychlor in the finished drinking waters.

On the basis of this interlaboratory method validation study, it is recommended that the method be adopted official first action.

Acknowledgments

The following collaborators participated in this study:

Fred DeRoos, Twin City Testing Corp., St. Paul, MN

Nikhil S. Dodhiwala, Acurex Corp., Mountain View, CA

Dennis Erickson, Enviro-Test Laboratories, Edmonton, Alberta, Canada

Erika E. Hargesheimer, City of Calgary, Glenmore Waterworks Laboratory, Calgary, Alberta, Canada

Dottie Lane, Western Research Institute, Laramie, WY

Bill Lee, Canada Centre for Inland Waters, Burlington, Ontario, Canada

Ann Liu, California Water Service, San Jose, CA

J. Malik, Monsanto Agricultural Co., St. Louis, MO

Carter Nulton, Weston Analytical Laboratory, Lionville, PA

W. Smalligan, Gerber Products Co., Fremont, MI

Bob Woods, Analytical Technologies, Inc., Phoenix, AZ

References

(1) Outler, E. C., & McCreery, J. H. (1982) *Interlaboratory Method Validation Study: Program Documentation*, Battelle Columbus Laboratories, Columbus, OH

(2) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA, p. 88

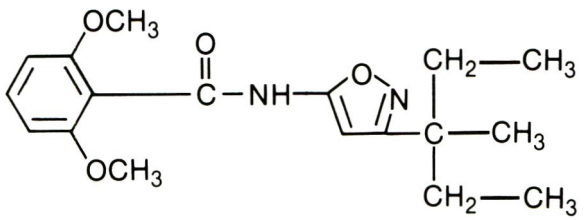
(3) Thompson, W. R. (1935) *Ann. Math. Stat.* **6**, 214

Liquid Chromatographic Determination of the Herbicide Isoxaben and Its Soil Metabolite in Soil and Soil-Turf Samples

BONNIE S. RUTHERFORD
Lilly Research Laboratories, Division of Eli Lilly and Co., Agricultural Analytical Chemistry, Greenfield, IN 46140-0708

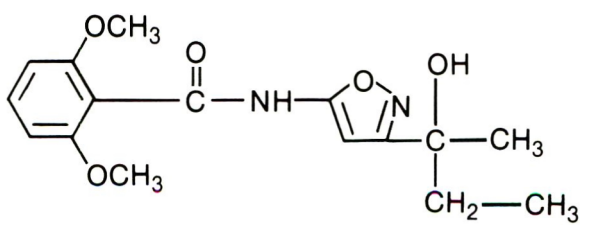
A method is described for the determination of residues of isoxaben and its principal soil metabolite in soil and soil-turf samples. Both compounds are extracted from samples by refluxing with methanol–water. An aliquot of the extract is partitioned into dichloromethane and purified by alumina column chromatography. Separate fractions containing isoxaben and metabolite are collected and subjected to liquid chromatography at conditions that are optimized for each compound. The detection limit for both compounds is 0.005 ppm. Residue identities are confirmed by chromatography on a different LC system.

Isoxaben, N-[3-(1-ethyl-1-methylpropyl)-5-isoxazolyl]-2,6-dimethoxybenzamide (I), is being marketed in Europe as a



I

preemergence herbicide for control of annual broadleaf weeds in cereal crops (1). It has recently been registered in the United States for use in the non-crop specialty area, where it acts as a selective preemergence surface-applied herbicide to control annual broadleaf weeds in established turf, ornamentals, non-bearing trees and vines, ground covers, and other non-cropland uses (2). Soil metabolism studies conducted with ^{14}C-isoxaben in the laboratory and under field conditions have shown that isoxaben and its metabolite, N-[3-(1-hydroxy-1-methylpropyl)-5-isoxazolyl]-2,6-dimethoxybenzamide (II), are the major residues occurring in



II

soils treated with isoxaben (D. P. Rainey and L. K. Graper, 1984–85, Lilly Research Laboratories, unpublished data). Four additional degradation products were detected in much lower amounts (less than 5 percent of the applied isoxaben). The major metabolite (II) reached levels of approximately 20% of the applied isoxaben during the laboratory soil study and 9% during the field study. Therefore, a method was developed for the determination of residues of isoxaben and metabolite in soil and soil-turf samples in order to study the dissipation of isoxaben under actual use conditions.

Received May 29, 1989. Accepted September 13, 1989.

Experimental

Apparatus

(a) *Liquid chromatograph.*—LC pump equipped with a UV detector capable of operation at 0.01 AUFS at 254 nm and a constant volume injector. Conditions for assay: 250 × 4.6 mm id column packed with Spherisorb ODS II, 5 μm particle size; mobile phase 70 + 30 (v/v) methanol–water for isoxaben or 60 + 40 (v/v) methanol–water for metabolite; flow rate 1 mL/min, injection volume 50–70 μL. Under these conditions, the retention time of isoxaben is 7 min and 5.5 min for the metabolite. Conditions for confirmatory assay: 250 × 4.6 mm id Zorbax CN column, 5 μm particle size; mobile phase 45 + 55 acetonitrile–water for isoxaben or 35 + 65 acetonitrile–water for metabolite; flow rate 1 mL/min, injection volume 50–70 μL. Under these conditions, the retention time of isoxaben is 8.5 min, and 6.5 min for the metabolite.

(b) *Grinder.*—Homoloid Model F-3493-00-171, or equivalent, with screen having a hole size of 0.45 cm.

(c) *Soil mixer.*—Hobart Model C-100, or equivalent.

(d) *Reflux apparatus.*—Water-cooled tubes with heating mantles.

(e) *Rotary vacuum evaporators.*—Rinco, or equivalent, with 45°C water bath.

(f) *Filter paper.*—Prefolded, Schleicher & Schuell No. 588, or equivalent.

(g) *Chromatography columns.*—25 cm × 14 mm id glass, equipped with stopcocks and 250 mL solvent reservoirs.

Reagents

(a) *Solvents.*—Methanol, dichloromethane, ethyl acetate, acetonitrile (all LC grade).

(b) *Solutions.*—Methanol–water (80 + 20, v/v); sodium chloride, 5% aqueous; dichloromethane–ethyl acetate (80 + 20, v/v); dichloromethane–methanol (99 + 1, v/v), (98 + 2, v/v), (97 + 3, v/v).

(c) *Sodium sulfate.*—Anhydrous, methanol-washed.

(d) *Alumina.*—Alcoa F-20 (containing ca 4% water as determined by loss-on-drying technique).

(e) *Liquid nitrogen.*

(f) *Standard solutions.*—Prepare separate solutions for isoxaben and metabolite. (*1*) *Stock standard solution.*—50 μg/mL in methanol. (*2*) *Fortification standard solution.*—1.25 μg/mL in methanol.

(g) *Standard curve solutions.*—Prepare at least 3 solutions over the range of 0.125–5.0 μg/mL in appropriate mobile phase solution.

Sample Storage and Preparation

Refrigerate soil or soil-grass samples until prepared for assay. Thoroughly grind and mix samples as described previously (3). If sample analysis will be delayed, storage stability

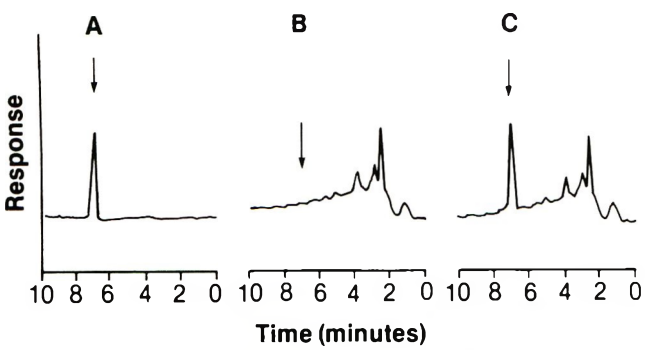


Figure 1. Chromatograms demonstrating determination of isoxaben in soil: A, isoxaben standard, 31 ng; B, control soil sample; C, control soil sample fortified with 0.025 ppm isoxaben, equivalent to 98% recovery.

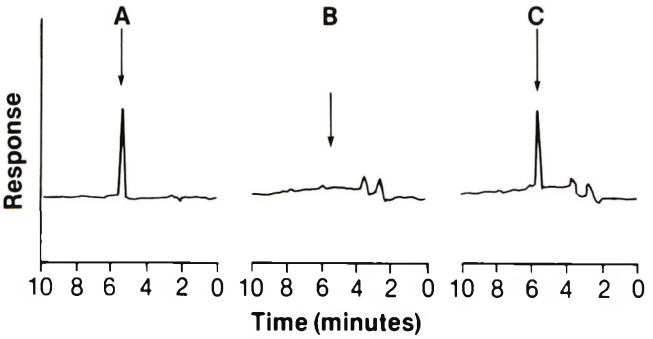


Figure 2. Chromatograms demonstrating determination of metabolite (II) in soil: A, metabolite standard, 31 ng; B, control soil sample; C, control soil sample fortified with 0.025 ppm metabolite, equivalent to 88% recovery.

samples should be prepared at a concentration of 0.025 ppm by fortifying 50 g control soil with 1.25 μg each of isoxaben and metabolite. Refrigerate all samples until analyzed.

Extraction Procedure

Weigh 50 g prepared soil or soil-turf mixture into 500 mL boiling flask. Add 200 mL methanol-water (80 + 20) and attach water-cooled condenser to flask. Heat to boiling and reflux sample 1 h. Cool sample to room temperature. Filter portion of sample and collect 100 mL aliquot.

Liquid-Liquid Partition

Transfer filtered aliquot to 250 mL separatory funnel containing 50 mL of 5% sodium chloride solution. Extract aqueous solution with three 70 mL portions of dichloromethane, draining each extract through funnel containing layer of sodium sulfate into 250 mL or larger boiling flask. *Note*: Do not let any water into boiling flask. Rinse sodium sulfate with 20 mL dichloromethane. Evaporate combined dichloromethane extracts to dryness on rotary vacuum evaporator.

Purification

Prepare alumina column by placing plug of glass wool in bottom of glass chromatography column and adding 13 mL (dry volume) alumina. Tap sides of column gently to settle alumina. Cover alumina with 1.5 cm layer of sodium sulfate. Wash column with 30 mL dichloromethane, draining solvent to top of column.

Transfer sample residue to column using two 5 mL portions of dichloromethane and letting each portion pass into absorbent layer. Rinse boiling flask with 25 mL dichloromethane and add rinse to column, washing down sides of column and draining to top of absorbent. Wash column with 50 mL dichloromethane–ethyl acetate (80+20), followed by 25 mL dichloromethane–methanol (99+1). Discard all column effluent to this point.

Add 50 mL dichloromethane–methanol (99+1) and collect eluate in 125 mL boiling flask. This fraction contains isoxaben.

Wash the column with 20 mL dichloromethane–methanol (98+2). Discard eluate. Add 75 mL dichloromethane–methanol (97+3) and collect eluate in 125 mL boiling flask. This fraction contains metabolite II. *Note*: Solvent volumes given should be checked with each batch of alumina to determine column profile for isoxaben and metabolite.

Evaporate collected fractions by rotary-vacuum evaporation. Dissolve each residue in 1.0 mL (or other appropriate volume) of methanol–water in same proportions as mobile phase for each compound.

Liquid Chromatography Procedure

Inject standard curve and sample solutions into appropriate LC system for isoxaben or metabolite. Measure the peak height of each isoxaben or metabolite peak. Dilute sample solutions as needed with mobile phase so that responses are within range of standard curve. Repeat procedure on alternative LC system for confirmatory assay.

Calculation

Determine concentration of each sample solution in μg/mL from appropriate standard curve:

$$\text{Isoxaben (or metabolite), ppm} = (A \times C \times E \times 100)/(B \times D)$$

where A = residue concentration calculated from standard curve, μg/mL; B = sample weight, g; C = aliquot factor; D = % recovery from fortified control samples; and E = final sample volume, mL. (Aliquot factor accounts for portions of sample which are not carried through entire procedure. For this method, aliquot factor is 2.)

Results and Discussion

Assay by gas chromatography was not possible because both isoxaben and its metabolite are subject to thermal degradation. Since both compounds exhibit ultraviolet absorption maxima at 254 nm, liquid chromatography was deemed the method of choice for detection of the compounds. Soil and soil-turf samples required alumina column cleanup prior to UV detection. Polarity differences between the analytes dictated different mobile phase compositions for the 2 compounds. Since the compounds were easily separated on the alumina column, it was advantageous to collect them individually and quantitate them on separate LC systems, rather than trying to quantitate both compounds by gradient elution LC.

Typical LC chromatograms are depicted in Figures 1 and 2 for standards, control samples, and fortified control samples of isoxaben and metabolite, respectively. Recovery data were obtained at several fortification levels to ensure that the method would perform satisfactorily over a wide range of residue levels. Table 1 contains recovery data from soil and soil-turf samples fortified with isoxaben. Table 2 contains

Table 1. Isoxaben recovery from fortified control samples

Soil type	Source (state)	Fortification level, ppm	N	Recovery, % Mean	SD
Clay loam	IN	0.005	8	91.5	2.9
		0.050	8	95.5	2.7
Sandy clay loam	TX	0.10	3	90.3	6.7
Sandy loam	(greenhouse)	0.10	3	92.1	3.4
	TX	0.025	9	102	10.0
	GA	1.0	2	101	0.0
	TX	1.0	2	99.4	0.9
Silt loam	IN	0.1	12	83.7	6.4
Loam	IN	0.025	9	101	6.6
Sand	FL	0.025	11	107	5.0
Clay loam + turf	IL	1.0	2	98.4	0.9
Sand + turf	FL	0.025	22	99.8	7.9

recovery data for the metabolite. Overall recovery for isoxaben from several soil types was 96.8%. Fortification levels examined ranged from 0.005 to 1.0 ppm. Recoveries for metabolite were more variable, but the overall recovery was 87.4% for fortification levels of 0.005 to 1.0 ppm.

Both compounds have demonstrated linearity of response from 0.125 to 5.0 μg/mL in their respective LC systems. The practical limit of detection (LOD) for both compounds is 0.005 ppm in soil and soil-turf samples. This is equivalent to approximately 9 ng injected on the column at the conditions of the method. This amount results in a peak height which is equivalent to 3 times baseline noise in most control samples.

The most critical step in the method is the alumina column cleanup procedure. The column elution profile must be determined with standards whenever a new batch of alumina is received or a new analyst attempts the procedure. The proper fractions to collect for optimal recovery of the 2 compounds must be determined before actual samples are attempted. The sample residue must be completely dry before transfer to the column with dichloromethane. The mixed-solvent ratios and volumes must be accurately measured to ensure good recovery of the compounds.

Isoxaben and metabolite solutions are quite stable under normal use conditions. The stock solutions were stable for 8 months when refrigerated. Fortification and standard curve solutions were stable for 4 months at room temperature when kept tightly stoppered. Stability for longer periods of time was not evaluated.

Table 2. Metabolite recovery from fortified control samples

Soil type	Source (state)	Fortification level, ppm	N	Recovery, % Mean	SD
Clay loam	IN	0.005	9	105	9.2
		0.025	9	96.2	2.2
Sandy loam	TX	0.025	13	70.7	15.3
		1.0	2	90.3	0.9
	GA	1.0	2	91.9	1.8
Loam	CA	0.005	3	94.9	6.0
		0.025	3	99.5	1.1
	IN	0.025	10	74.9	14.4
Sand	FL[a]	0.005	3	76.7	9.7
	FL[a]	0.025	3	102	2.1
	FL[b]	0.025	13	72.2	9.7
Clay loam + turf	IL	1.0	2	92.0	0.0
Sand + turf	FL[b]	0.025	25	70.3	13.5

[a] FL = Boynton Beach, Florida.
[b] FL = Gulf Breeze, Florida.

Residues suspected of being isoxaben or metabolite may be confirmed using the alternative LC systems by comparison of sample peak retention times to the retention times of reference standards injected concurrently. Both column type and mobile phase solvents differ from the quantitation system, so residues may be confirmed in this manner.

Acknowledgments

I gratefully acknowledge the technical assistance provided by F. L. Powers, L. G. Turner, and J. J. Ulmer.

REFERENCES

(1) Huggenberger, F., Jennings, E. A., Ryan, P. J., & Burow, K. W. (1982) *Proc. Br. Crop Prot. Conf.-Weeds* **1**, 47–52
(2) Colbert, F. O., & Ford, D. H. (1987) *Proc. West. Soc. Weed Sci.* **40**, 155–163
(3) West, S. D., & Rutherford, B. S. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 572–575

Polymeric Film Dialysis in Organic Solvent Media for Cleanup of Organic Contaminants

JAMES N. HUCKINS, MARK W. TUBERGEN, JON A. LEBO, ROBERT W. GALE, and TED R. SCHWARTZ
U.S. Fish and Wildlife Service, National Fisheries Contaminant Research Center, Rte 2, 4200 New Haven Rd, Columbia, MO 65201

Dialytic enrichment, using a nonpolar polymeric film or membrane, is proposed as a new approach for the separation of organic contaminants from fish lipid. Nonpolar organochlorine analytes diffuse from the fish lipid through a polyethylene membrane into cyclopentane. Separations of 48 h or less in duration afforded excellent recoveries for all analytes tested and removed 93% of the fish oil. Kinetics of membrane diffusion and possible controlling factors are elucidated for selected analytes and model compounds. Potential advantages of this technique over conventional lipid-removal methods such as gel permeation chromatography include simplicity, large sample capacity, reduced solvent requirements, and amenability to interfacing in-line with other enrichment modules.

In 1980, Byrne and Aylott (1) patented a device consisting of a nonpolar organic solvent separated from water by semipermeable membranes (regenerated cellulose, vinyl chloride, polyvinylidene fluoride, or polytetrafluoroethylene) that concentrated organic molecules from water. This represented a new application of dialysis in conjunction with the liquid-liquid extraction of nonpolar organic contaminants. Miere et al. (2) appear to have been the first investigators to use polyethylene film for dialysis of nonpolar organic contaminants from water into organic solvents. Implicit in this work was the suggestion that nonporous (3), synthetic polymeric films, including low density polyethylene and polypropylene, could serve as semipermeable membranes for dialysis of organic molecules from aqueous environments into relatively nonpolar organic solvents. Heindorf and Zabik (unpublished data, Michigan State University, 1989) extended this concept to solvent-filled bags of polymeric films, designed for field monitoring of organic aqueous contaminants.

We know of no report in the literature on the use of low density polyethylene film or other similar hydrophobic films for dialysis of organic contaminants (less than about 550 daltons) from fish extracts into organic solvent. The largest mass fraction of lipids in extracts of fish is generally composed of triglycerides (4) with molecular weights of at least 600 daltons. Consequently, size exclusion or gel permeation chromatography (GPC) is often chosen as the initial cleanup method for organic analytes smaller than 550 daltons (5). The use of polyethylene or polypropylene films as semipermeable membranes for dialysis separations of analytes from lipids appears to offer several advantages over existing methods: (*1*) polymeric film dialysis is easy to implement—no costly hardware such as an automated gel permeation chromatograph is required; (*2*) these films are stable in a variety of organic solvents, unlike most dialysis or ultrafiltration membranes; (*3*) the method can be scaled up or down for cleanup of a wide range of sample masses; (*4*) the amount of solvent required for bulk cleanup is markedly reduced, e.g., 5 g fish oil can be processed with 200–500 mL cyclopentane; whereas, the same amount of oil would require 3 L mobile phase if size exclusion chromatography were used (5); (*5*) surface area/volume ratios and film thicknesses can be manipulated to adjust the times to equilibrium or completion of separations; (*6*) analysts have the flexibility to use reactive, adsorptive, and other chromatographic methods in sequence without interim evaporation steps because nonpolar hydrocarbon solvents such as cyclopentane can be used for dialysis; and (*7*) the method can be automated.

We report the use of polyethylene film for dialytic separations in organic solvent media of fish extracts spiked with a number of organochlorine compounds. The choices of dialyzing solvent, kinetics of residue transport through polyethylene film, implications of varying the surface area/volume ratios, and problems encountered are discussed. Future analytical applications of this technology and approaches to validation are also proposed.

The purpose of the present paper is to suggest a potentially useful new approach for the separation of lipids from organic contaminants, not to validate a method, nor to propose this new approach as a complete enrichment procedure by itself. Although we did not attempt to establish optimally low quantitation limits for the analytes used, we did demonstrate the utility of the cleanup technique for relatively large lipid masses containing contaminants at environmentally relevant concentrations.

Experimental

Materials

(a) *Solvents.*—All solvents used were pesticide grade, or equivalent.

(b) *Adsorbents.*—Silica gel 60 (70–230 mesh, from E. Merck) was washed with CH_2Cl_2 and stored at 130°C until used. Sulfuric acid–silica gel was made by combining silica gel (treated as described above) with concentrated H_2SO_4 (20 + 1 w/w) and shaking the mixture until a homogeneous powder resulted.

(c) *Fish oil.*—A large grass carp (*Ctenopharyngodon idella*), obtained from a control pond at the National Fisheries Contaminant Research Center, was frozen, sawed into small pieces, ground with a meat grinder, and then blended with anhydrous Na_2SO_4 that had been baked at 475°C. The dry mixture of fish and sodium sulfate was packed into large glass columns and extracted with CH_2Cl_2. The solvent was removed by rotary evaporation, and the lipid was stored at −20°C.

(d) *Analytes.*—Mirex, *p,p'*-DDT, and *trans*-nonachlor were obtained from EPA Chemical Repository. Four analytes were from Ultra Scientific, Inc.: octachloronaphthalene; octachlorodibenzofuran; 2,4,6-trichlorobiphenyl (IUPAC congener 30); and 2,2',3,3',4,5,6,6'-octachlorobiphenyl (IUPAC congener 199). Perdeuterated 3,3',4,4'-tetrachlorobiphenyl (IUPAC congener 77) was obtained from MSD Isotopes (perdeuterated compound was used because of an existing supply at our laboratory).

Received July 17, 1989. Accepted October 10, 1989.

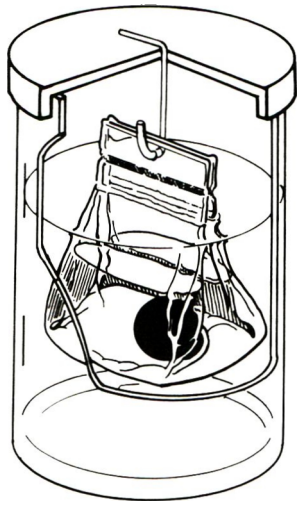


Figure 1. Cutaway representation of dialysis chamber, solvent, and polyethylene bag arrangement. Chamber is cylindrical vessel of amber glass (12 cm high × 7.5 cm id) with aluminum foil cover. Polyethylene bag, prepared as described in text, contains lipid solution and glass weight and is suspended from a wire hook. Bag, shown near end of second 24 h dialysis period, is somewhat turgid.

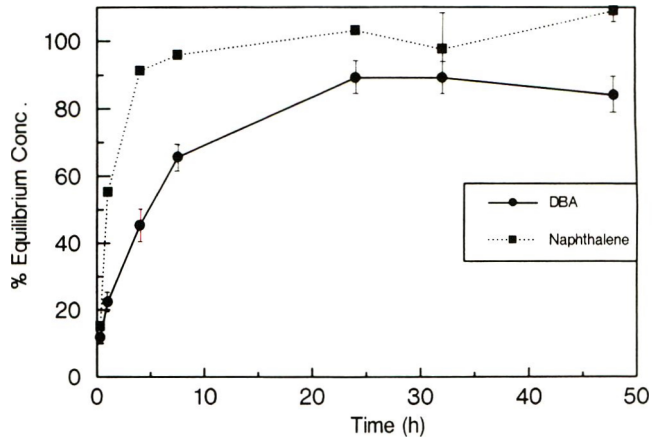


Figure 2. Rates of transport of naphthalene and dibenz[a,h]anthracene (DBA) through polyethylene film at constant temperature. Each data point represents 3 replicates. Error bars signify standard errors (most of the error bars are obscured by symbols). Percentages of equilibrium concentrations (y axis) for analytes were calculated on the premise that equilibrium partition coefficients of analytes between lipid and dialysate phases were 1.0. Analyte concentrations measured in dialysates were corrected for diminishing volumes of total liquid and divided by the appropriate maximum (equilibrium) concentrations, resulting in the plotted percentage data.

(e) *Radiolabeled analytes.*—^{14}C-3,3′,4,4′-tetrachlorobiphenyl (TCB), ^{14}C-2,2′,5,5′-TCB (IUPAC congener 52), and ^{14}C-mirex were obtained from Pathfinder Laboratories Inc.; ^{14}C-naphthalene from ICN Laboratories; and ^{14}C-dibenz[a,h]anthracene from Amersham Corp. Tritium-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a gift from Alan Poland of the University of Wisconsin, had recently been purified by chromatography on columns of dispersed carbon and alumina (6). All radiolabeled compounds except TCDD were examined for purity by silica gel thin-layer chromatography, followed by autoradiography.

Equipment

(a) *Gas chromatography.*—Varian 3700 gas chromatograph (GC) equipped with ^{63}Ni electron capture detector (ECD) was used for analysis of nonradiolabeled compounds. GC column was DB1 bonded phase fused silica capillary (30 m × 0.25 mm id) with retention gap of 60 cm × 0.32 mm id.

(b) *Polyethylene bags.*—Enclosures were made from a single lot of "layflat" tubing (2 in. [5.1 cm] wide and 0.002 in. [51 μm] thick) obtained from Cope Plastics, Inc. Polyethylene was rinsed with pentane and air-dried. Bags were then formed by heat-sealing ends of tubing segments (heat sealer from Clamco Corp., operated at 270°C).

(c) *Liquid scintillation counter.*—Beckman Model 3801, Beckman Instruments.

Procedure

An array of environmentally prevalent organochlorine contaminants was spiked into 5 g portions of carp lipid at concentrations of 40 ng/g each. Each 5 g lipid sample was also spiked with ca 1 ng (ca 0.09 μCi) of ^{3}H-labeled TCDD. Grass carp consisted of 20% lipid by weight; spike concentrations listed above are expressed relative to 25 g samples of fish (whole body wet weight) rather than to lipid weights.

The following procedure was used for preparing lipid-filled bags. Lengths (18 cm) of polyethylene tubing were sealed at one end, glass spheres were added for weight, and lipid solutions (5.0 g spiked lipid diluted to 12 mL with cyclopentane) were added by pipet. Most air was voided, and the bags were immediately sealed at the other end, bent into a U-shape, and placed in dialysis chambers with 250 mL portions of cyclopentane (Figure 1). The bags were configured in a U-shape to reduce heat-seal contact with solvent and to increase the surface area of polyethylene film exposed to the solvent. (*Caution*: Exposure of thermal seals to hydrocarbon solvent may lead to their eventual failure.) All experiments were conducted at about 25°C.

After first 24 h dialysis period, cyclopentane in the dialysis chambers was drawn off and replaced with fresh cyclopentane. When another 24 h had elapsed, the second portions of cyclopentane dialysates were drawn off and combined with the first. After rotary evaporation to volumes of ca 10 mL, the concentrated dialysate solutions were further enriched. Glass columns (2 cm id) with open reservoirs were packed with 3 cm segments of silica gel atop 5 cm segments of H_2SO_4–silica gel atop 6 cm segments of silica gel. Sodium sulfate was placed at the top, and these adsorbents were presaturated with the eluant, CH_2Cl_2–cyclopentane (10 + 90 v/v). The concentrated dialysates were applied to tops of columns, and the organochlorine compounds were eluted with 100 mL eluant. The eluates were concentrated to 5.0 mL volumes, and 1.0 mL of each was used for radiometric determination of TCDD recovery. The remaining portions of the eluates were evaluated by capillary GC/ECD.

Design of the kinetics experiments was similar to that used in the previously described recovery study. Three replicate dialysis experiments were performed for each of 5 analytes selected. The 2 major differences were that cyclopentane was not removed (except for minute samplings of dialysates) or replaced during 48 h dialysis period, and that residues in the dialysates outside the bags were measured directly by liquid scintillation counting without chromatographic cleanup. Af-

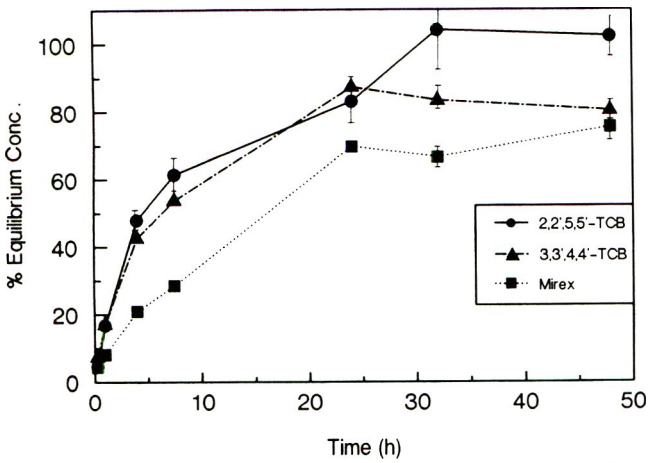


Figure 3. Rates of transport of selected organochlorines through polyethylene film at constant temperature. Each data point represents 3 replicates. Use of error bars and percentages of equilibrium concentrations is defined in Figure 2. TCB is tetrachlorobiphenyl.

ter 48 h, radioactive residues were also measured in the solvent–lipid mixtures inside bags and in membrane walls. Dialysates were sampled at 0.2, 1, 4, 8, 24, 32, and 48 h (Figures 2 and 3). The values plotted in Figures 2 and 3 were corrected for solvent losses from chambers, which were caused by evaporation, osmosis of solvent into the bags, and removal of dialysis solvent during sampling. For descriptive purposes, linear statistics were applied to replicate analyte ratio (percent) data plotted in Figures 2 and 3.

Results

Recoveries were good for all organochlorine compounds studied. Table 1 summarizes the results of GC/ECD and radiometric analyses of enriched dialysates. Figure 4 is a representative chromatogram from the GC/ECD analyses. Several non-interfering extraneous peaks were present in the chromatograms, e.g., peak a in Figure 4 appeared to represent a polyethylene leachate. However, subsequent experiments showed that presoaking membranes 48 h in cyclopentane reduced the mass of polyethylene leachate by about 90%.

Dialysis experiments were also performed with bags containing unspiked lipid to determine the efficiency of the lipid removal process. After two 24 h dialysis periods, the dialysates contained 350 ± 20 mg (n = 6) of lipid, indicating that 93% of the 5.0 g carp lipid remained in the bags. Additional experiments showed that 95.4% of the lipid was retained in the bags after a single 24 h dialysis period. At the end of the two 24 h dialysis periods, the bags contained 50 ± 2 mL (n = 6) of total liquid; the increase was due to the osmotic transport of cyclopentane into the lipid.

Results of experiments (constant temperature) on the dialysis kinetics of selected organochlorines and model polycyclic aromatic hydrocarbons (PAHs) indicate that molecular size or molecular weight affects transport rates through the polyethylene film (Figures 2 and 3). This observation follows theories of organic compound diffusion through polymeric films (3). The role of molecular size in mediating diffusion rates is illustrated in Figure 2 by the marked difference in the transport rates of naphthalene and dibenz[a,h]anthracene. Dialytic recovery of naphthalene (128 daltons, 2 rings)

Table 1. Percent recoveries of organochlorines by dialysis from spiked (40 ng/g) lipid. Dialysates were cleaned up by chromatography on columns of silica gel/H_2SO_4–silica gel before gas chromatography

Compound	Mean[a]	SD	CV, %
Congener 30	92	1.6	1.7
trans-Nonachlor	88	2.4	2.8
Congener 77	88	3.7	4.2
p,p'-DDT	101	2.2	2.2
Congener 199	98	2.1	2.1
Mirex	95	2.5	2.6
Octachloronaphthalene	90	1.8	2.0
Octachlorodibenzofuran	88	6.1	6.9
^{3}H-TCDD	99[b]	0.9	1.0

[a] Mean of 6 replicates.
[b] Radiometric recoveries.

reached 90% in 4 h; whereas, 24 h was required to obtain the same recovery of dibenz[a,h]anthracene (278 daltons, 5 rings). Despite the ability of TCB isomer 77 to assume a coplanar configuration, differences between the diffusion rates of TCB isomers 77 and 52 did not appear to be significant (Figure 2). The diffusion rate of mirex (546 daltons; a fully chlorinated modified cubane structure) was lowest, as expected (Figure 3), and steady state may not have been reached in the 48 h study period. Steady state was assumed when 2 or more sequential mean values (including associated standard deviations) of analyte concentration ratios in the dialysate did not appear to be significantly different. Although mirex was obviously below the actual size exclusion limit of our lot of polyethylene film (saturated with cyclopentane), its relatively slow diffusion rate through the polymer required additional time for dialysis, and resulted in increased lipid carryover. This suggests that mirex approximates an operational size limit for this particular method.

Comparison of results from the kinetics studies with those from analyte recovery studies suggests that substitutions of fresh solvent for dialysates after 24 h did little to improve recoveries of any of the test compounds except mirex. Losses of test compounds to the polyethylene film were negligible because <2% of each radiolabeled analyte used in the kinetics studies was present in the polymer matrix at termination (48 h).

Discussion

The potential of polyethylene tubing for dialysis separations of contaminants from biogenic material was demonstrated. This simple technique appears to be particularly useful for removing nonpolar organic analytes from bulk lipids. The dialysis time (48 h) used in this work was not optimal for the organochlorines tested; kinetics experiments indicated that shorter solvent exposure (i.e., 24 h) resulted in better lipid discrimination (95.4%) with little or no reduction in analyte recovery. Further reductions in dialysis time can obviously be achieved by increasing the surface area/volume ratio of the tubing: a thin film of lipid extract in flattened layflat polyethylene tubing has much greater surface area than in tubing with a turgid, cylindrical configuration.

The choice of solvent used in dialysis separations greatly affects transport rates of analytes through polyethylene film. Hildebrand solubility parameters for a number of organic solvents and polyethylene (Heindorf and Zabik, unpublished

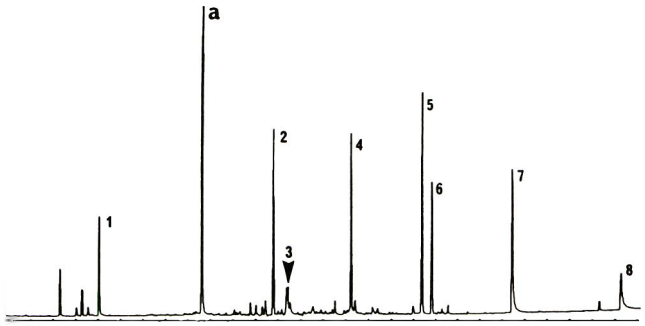


Figure 4. ECD chromatogram of typical dialysate from lipid spiked at concentrations of 40 ng/g with each of the following compounds: (*1*) congener 30; (*2*) *trans*-nonachlor; (*3*) congener 77; (*4*) *p,p'*-DDT; (*5*) congener 199; (*6*) mirex; (*7*) octachloronaphthalene; and (*8*) octachlorodibenzofuran. ^{3}H-TCDD was too dilute in dialysates to appear in the chromatograms. Peak a is caused by leachate from the polyethylene bag.

data, Michigan State University, 1989) suggest that hydrocarbon solvents such as hexane or pentane should interact favorably with polyethylene. Favorable interactions between a polymer and a solvent consist of increased polymer liquidity (or reduced rigidity) and polymer matrix swelling. These types of interactions are necessary, in most cases, for adequate analyte transport rates because diffusion rates are reduced by structural rigidity (crystallinity) and by the related compactness of linear polyethylene chains. Cyclopentane proved to be the best solvent for dialytic enrichment of organics during preliminary solvent evaluation tests. This conclusion was based on the criterion of minimization of time to equilibrated distribution of analytes between the 2 phases. The molecular planarity and compactness of cyclopentane appear to be responsible for its superior performance.

There is great potential for further developments in the use of this simple separation approach. Other polymeric films (polyvinyl chloride, polyimides, and polyethylene–vinyl acetate copolymer) compatible with selected organic solvents may be available, which should permit dialysis of more polar organics yet have similar size exclusion properties to polyethylene (3). The use of nonpolar dialyzing solvents such as cyclopentane should permit direct in-line interfacing of dialysis chambers (without the evaporation steps used in this work) with enrichment modules containing adsorbents such as potassium silicate, H_2SO_4–silica gel, alumina, silica gel, and carbon.

Analyses of reference samples, such as cod liver oil with known quantities of polynuclear aromatic hydrocarbons and organochlorines, are necessary for method validation. In addition, direct comparisons to existing bulk lipid removal methods such as gel permeation chromatography are needed.

Acknowledgments

We thank J. D. Petty for his encouragement, suggestions, and literature review, G. Gibson for her technical expertise, L. Sappington for plotting the data, and T. La Point for his support and advise on plots.

References

(1) Byrne, G. A., & Aylott, R. I. (1980) British Patent 1566253, Concentrator for Removing Organic Materials from Aqueous Systems

(2) Miere, J. P., Mappes, G. W., Tucker, E. S., & Dietrich, M. W. (1977) in *Identification and Analysis of Organic Pollutants in Water*, L. H. Keith (Ed), Ann Arbor Science Publishers, Inc., Ann Arbor, MI, pp. 113–133

(3) Hwang, S.-T., & Kammermeyer, K. (1975) *Membranes in Separations, Techniques of Chemistry*, Vol. 7, John Wiley & Sons, New York, NY

(4) Henderson, J. R., & Tocher, D. R. (1987) *Prog. Lipid Res.* **26,** 281–347

(5) Stalling, D. L., Tindle, R. C., & Johnson, J. L. (1972) *Anal. Chem.* **44,** 1768–1773

(6) Smith, L. M., Stalling, D. L., & Johnson, J. L. (1984) *Anal. Chem.* **56,** 830–842

Gas Chromatographic Method for Determination of Chlorpyrifos and Its Metabolite 3,5,6-Trichloro-2-Pyridinol (TCP) in Dates

CHARLES R. MOURER, GREGORY L. HALL, WILLIAM E. WHITEHEAD, and TAKAYUKI SHIBAMOTO
University of California at Davis, Department of Environmental Toxicology, Davis, CA 95616

A method is described for the determination of the insecticide chlorpyrifos and its metabolite TCP in green, unprocessed, and processed dates with the seeds incorporated. After extraction, chloropyrifos is cleaned up using Florisil and analyzed using a gas chromatograph (GC) equipped with a nitrogen/phosphorus detector. TCP is derivatized using bis-(trimethylsilyl)-acetamide (BSA) to form the TCP-derivative and analyzed by a gas chromatograph equipped with a Hall electrolytic conductivity detector. Recoveries of chlorpyrifos from all fortified dates (0.05 and 0.1 ppm) ranged from 86 to 110% with an average of 94.5%. Recoveries of TCP from all fortified dates (0.1 and 0.2 ppm) ranged from 79 to 99% with an average of 86%. Limits of detection for chlorpyrifos and TCP in green, unprocessed, and processed dates were 0.02 and 0.05 ppm, respectively.

Chlorpyrifos (*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is an insecticide used for control of various pests on field, nut, and vegetable crops. It was first introduced in 1965 by Dow Chemical Co.

Existing methods for rice (1) and sugar beets (2) deal exclusively with the parent chlorpyrifos. Other methods for corn (3) and wheat (4) analyze for the parent chlorpyrifos and its oxygen analog, but not the metabolite TCP (3,5,6-trichloro-2-pyridinol). Several multi-residue methods exist (5–7), but they do not incorporate the metabolite or deal with the difficult matrix of dates. Methods for chlorpyrifos and TCP in hay and oil of peppermint (8), peaches (9), and lettuce (10) all use electron-capture detection and analyze TCP as a derivative. A selective detector can minimize background interferences and improve the limit of detection of a method.

The present paper represents an adaptation of previously reported methods, plus innovations in cleanup and extraction techniques and the use of specific detectors to permit the determination of chlorpyrifos and its metabolite TCP in green, unprocessed, and processed dates.

METHOD

Apparatus

(a) *Food cutter.*—Model 8141 Hobart food cutter.

(b) *Blender.*—Tissumizer with medium grind probe (Tekmar, PO Box 37202, Cincinnati, OH 45222).

(c) *Gas chromatograph.*—Varian Vista Model 402 controlled Vista Model 6000/6500 GC equipped with dual detectors. (*1*) For chlorpyrifos analysis: Nitrogen/phosphorus detector; 167 cm × 2 mm id glass column packed with 5% OV-101 on 60–80 mesh Gas Chrom Q; parameters: helium carrier gas 35 mL/min, hydrogen 5 mL/min, and air 175 mL/min; injector, column oven, and detector 220°C, 190°C, and 250°C, respectively.

(*2*) For TCP-derivative analysis: Tracor Hall conductivity detector (Model 700A) (specific for chlorine); 198 cm × 2 mm id glass column packed with 1.5% SP 2250/1.95% SP 2401 on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA 16823); parameters: helium carrier gas 20 mL/min, hydrogen 50 mL/min; injector, column oven, detector base, and combustion chamber 220°C, 130°C, 280°C, and 900°C, respectively. Electrolyte for Hall cell, *n*-propanol (ChromAR grade, Mallinckrodt, Inc.), 0.5 mL/min.

(d) *Nitrogen evaporator.*—Meyer N-Evap analytical evaporator (Organomation Associates, Inc., PO Box 5 TPK STA., Shrewsbury, MA 01545).

Reagents

(a) *Solvents.*—Resi-analyzed grade acetone, benzene, ethyl acetate, and hexane (J. T. Baker Chemical Co.; VWR Scientific, Inc., 3745 Bayshore Blvd, Brisbane, CA 94005) and ACS grade ethyl ether, anhydrous (Fisher Scientific, Co., 2170 Martin Ave, Santa Clara, CA 95050).

(b) *Silylation reagent.*—Bis(trimethylsilyl) acetamide (BSA) (Aldrich Chemical Co., PO Box 2060, Milwaukee, WI 53201).

(c) *Florisil.*—Pesticide grade, 80–100 mesh, dried in 105°C oven until use (Floridin Co., Berkeley Springs, WV).

(d) *Analytical standards.*—99% pure chlorpyrifos and 3,5,6-trichloro-2-pyridinol (Dow Chemical Co., Midland, MI).

Preparation of Sample

Remove growing stems from ca 1000 g dates and chop in food cutter with dry ice for 10 min until powdery. After dry ice has dissipated, weigh 10 g ground dates into 250 mL Erlenmeyer flask with 7 g Celite 545. (Fortifications were done at this point.) Add 150 mL acetone and 15 mL water. Blend samples in Tissumizer 2 min at high speed. Rinse blender shaft with 10 mL acetone. Let stand 1 min and then decant acetone-water mixture through 5.5 cm Whatman No. 1 filter paper in Buchner funnel under light vacuum. Wash filter cake 3 times with 10 mL acetone before transferring filtrate to 500 mL round-bottom flask.

Separation of Chlorpyrifos and TCP

Evaporate acetone filtrate on rotary evaporator (40 ± 2°C) until only watery liquid remains (no acetone present). Decant liquid into 125 mL separatory funnel, rinse boiling flask twice with 30 mL hexane, and transfer rinses to separatory funnel. Rinse boiling flask with 25 mL 0.1M $NaHCO_3$ and transfer rinse to separatory funnel. Slowly shake for good mixing, and then let stand to separate.

Drain bottom $NaHCO_3$ layer into second 125 mL separatory funnel and add 25 mL 0.1M $NaHCO_3$ to hexane in first separatory funnel; slowly shake and let stand. Drain bottom $NaHCO_3$ layer into the second separatory funnel and transfer top hexane layer to 250 mL separatory funnel. Wash combined $NaHCO_3$ layers twice with 30 mL benzene, and add each benzene wash to hexane in the 250 mL separatory funnel. Back-wash hexane-benzene solution with 25 mL

Received June 4, 1989. Accepted September 16, 1989.

Table 1. Chlorpyrifos recovery studies

Sample	Replicate	Level, ppm	Found, ppm	Rec., %
		Green dates		
Control	—	—	<0.001	—
AR-1	1	0.05	0.043	86
	2	0.05	0.044	88
	3	0.05	0.046	92
	Av.	—	—	92
AR-2	1	0.10	0.090	90
	2	0.10	0.092	92
	3	0.10	0.095	95
	Av.	—	—	92
		Unprocessed dates		
Control	—	—	<0.001	—
AR-1	1	0.05	0.050	100
	2	0.05	0.046	92
	3	0.05	0.049	98
	Av.	—	—	97
AR-2	1	0.10	0.088	88
	2	0.10	0.092	92
	3	0.10	0.096	96
	Av.	—	—	92
		Processed dates		
Control	—	—	<0.001	—
AR-1	1	0.05	0.047	97
	2	0.05	0.050	100
	3	0.05	0.055	110
	Av.	—	—	102
AR-2	1	0.10	0.095	95
	2	0.10	0.93	93
	3	0.095	95	
	Av.	—	—	95

Table 2. Trichloropyridinol recovery studies

Sample	Replicate	Level, ppm	Found, ppm	Rec., %
		Green dates		
Control	—	—	<0.010	—
AR-1	1	0.10	0.087	87
	2	0.10	0.088	88
	3	0.10	0.083	83
	Av.	—	—	86
AR-2	1	0.20	0.171	85
	2	0.20	0.187	93
	3	0.20	0.161	81
	Av.	—	—	86
		Unprocessed dates		
Control	—	—	<0.010	—
AR-1	1	0.10	0.087	87
	2	0.10	0.99	99
	3	0.10	0.80	80
	—	—	—	88
AR-2	1	0.20	0.160	80
	2	0.20	0.173	87
	3	0.20	0.157	79
	Av.	—	—	82
		Processed dates		
Control	—	—	<0.010	—
AR-1	1	0.10	0.090	90
	2	0.10	0.083	83
	3	0.10	0.80	84
	Av.	—	—	84
AR-2	1	0.20	0.187	93
	2	0.20	0.173	87
	3	0.20	0.176	88
	Av.	—	—	89

0.1M $NaHCO_3$. After layers have separated, transfer bottom $NaHCO_3$ layer to the second 125 mL separatory funnel containing previous $NaHCO_3$ washes.

Filter hexane–benzene mixture through anhydrous sodium sulfate and 12.5 cm Whatman No. 1 filter paper into 250 mL round-bottom flask. Rinse separatory funnel with 25 mL benzene and also pour rinse through sodium sulfate filter.

Hexane–benzene mixture in round-bottom flask contains chlorpyrifos, ready for Florisil cleanup; $NaHCO_3$ solution contains TCP.

TCP Extraction and Derivatization

Add 2 mL concentrated hydrochloric acid to $NaHCO_3$ layer. (*Caution*: Pressure in separatory funnel from evolving CO_2 requires slow shaking with frequent venting, until no gas forms.) Add 40 mL benzene, shake, let stand, and drain bottom $NaHCO_3$ layer into 125 mL separatory funnel. Drain top benzene layer through anhydrous sodium sulfate in Whatman No. 1 filter paper (15.0 cm) into 250 mL round-bottom flask. Repeat extraction of $NaHCO_3$ twice with 40 mL benzene. Combine benzene washes in the round-bottom flask. Rinse sodium sulfate/filter with 25 mL benzene. Evaporate benzene to dryness on rotary evaporator. Transfer residue to 15 mL graduated centrifuge tube, using hexane, and concentrate solvent on nitrogen evaporator to 1 mL. Add 20 μL BSA derivatizing agent, mix on vortex mixer, and let stand 5 min. Adjust volume with hexane to facilitate analysis of TCP by Hall detector.

Florisil Cleanup for Chlorpyrifos

Evaporate hexane–benzene solution from "separation of chlorpyrifos and TCP" step to dryness on rotary evaporator and add 5 mL hexane; evaporate again to dryness and add 5 mL hexane. Pack cleanup column (12 mm id × 100 mm with 175 mL solvent reservoir) with glass wool plug, 1 cm anhydrous sodium sulfate, and 5 cm Florisil and top with 1 cm anhydrous sodium sulfate. Tap sides of column to pack column uniformly. Pre-wet cleanup column with 15 mL hexane and then add sample, using disposable pipet. Wash round-bottom flask twice with 5 mL of hexane, each time adding wash to column just as last of solvent sinks into Florisil; discard eluate. Add 50 mL 5% ethyl ether in hexane to column and collect eluate in 100 mL round-bottom flask. Evaporate solvent on rotary evaporator to near dryness, transfer sample to 15 mL graduated centrifuge tube with hexane, and adjust volume to facilitate analysis. Analyze for chlorpyrifos, using nitrogen/phosphorus detector.

Gas Chromatographic Analysis

Determine chlorpyrifos residues using nitrogen/phosphorus detector by comparing peak heights from known amounts of samples injected to standard curve. Prepare chlorpyrifos standard curves by injecting 3 μL each of the 25 pg/μL, 50 pg/μL, and 100 pg/μL chlorpyrifos standards. Plot peak heights (mm) vs known amounts of chlorpyrifos (pg) represented in each injection. Adjust volumes of samples so majority of sample peak heights are mid-scale when 3 μL sample is

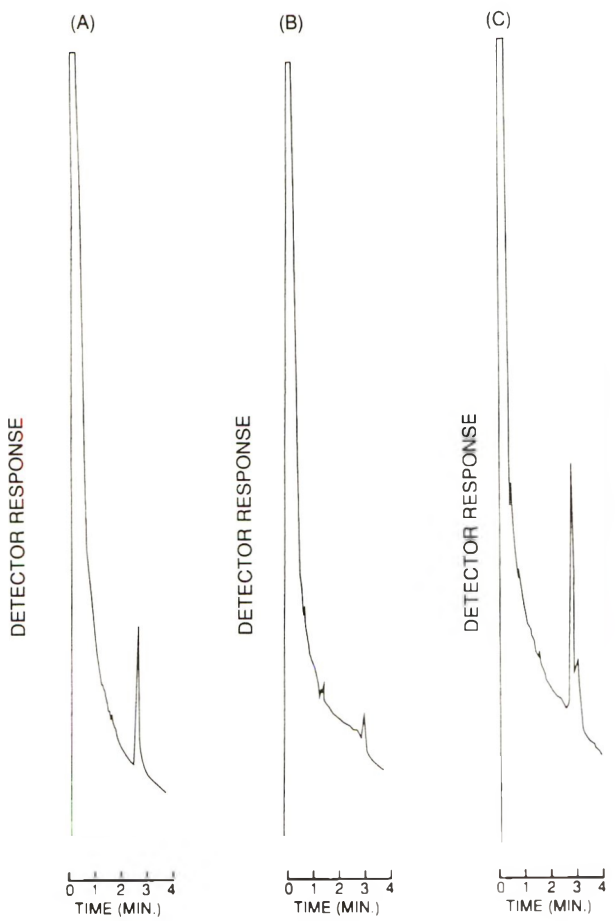


Figure 1. Typical N/P detector chromatograms: A, 75 pg chlorpyrifos standard; B, 3.0 mg date control; C, 3.0 mg date fortified with 0.05 ppm chlorpyrifos.

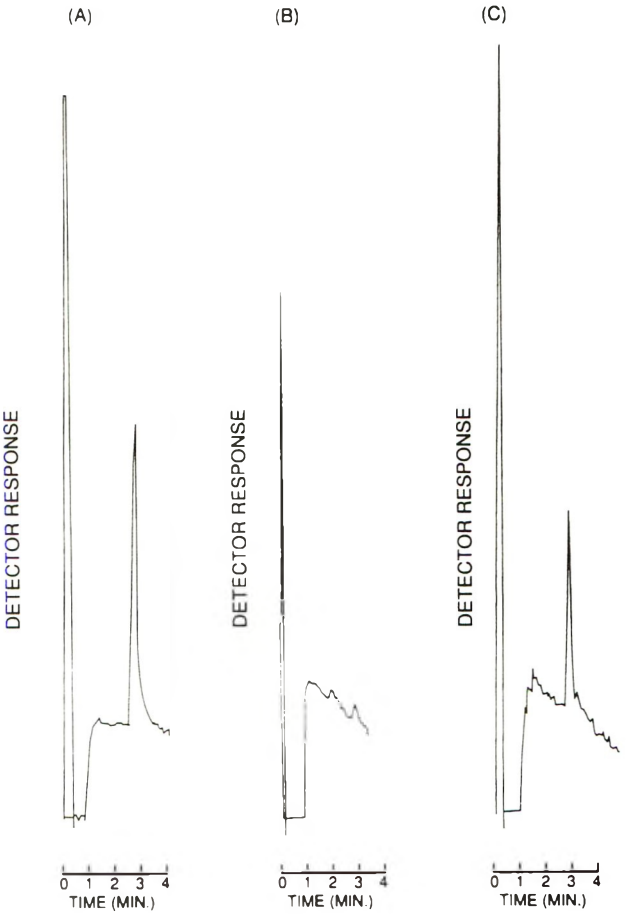


Figure 2. Typical Hall detector chromatograms: A, 2 ng TCP-derivative standard; B, 10.0 mg control dates; C, 15.0 mg dates fortified with 0.10 ppm TCP.

injected. Peak heights of injected samples can be converted directly from standard curve to amount of chlorpyrifos (pg) represented in the injection. These samples are very stable and can be stored before analyzing if necessary.

To determine TCP residues, first derivatize TCP using bis(trimethylsilyl) acetamide (BSA) to form TCP-derivative. Freshly prepare standard solutions used to make standard curves with each group of samples to be analyzed. Use peak heights obtained from chromatograms of samples injected to determine amount of TCP represented, by comparing peak heights found to those of standard curve plotting amount of TCP-derivative vs peak height.

The Hall conductivity detector is used for specificity for chlorine and because it can be vented to allow excess BSA and early chromatographing compounds from going through the detector. Chromatograms from these samples are clean of interferences and easy to interpret. The TCP-derivative is somewhat unstable and should be analyzed within a few hours after reacting TCP with BSA for the best results.

Fortification

Recoveries of chlorpyrifos and its metabolite TCP were determined in triplicate at fortification levels of 0.05 and 0.10 ppm for the parent compound and 0.10 and 0.20 ppm for the metabolite. Fortifications of green, unprocessed, and processed dates were made directly on each type of date after homogenation and weighing of the 10 g aliquot, but prior to adding the extracting solvent. Fortified samples were then carried through the procedure as outlined. The fortification solution was 5 ng/μL of chlorpyrifos and 10 ng/μL of TCP in ethyl acetate; 100 and 200 μL volumes of the fortification solution were added for a fortification of 0.05 ppm chlorpyrifos and 0.10 ppm TCP or 0.10 ppm chlorpyrifos and 0.20 ppm TCP, respectively. The level of fortification used was determined by the requirements of the U.S. Department of Agriculture, Agricultural Research Service, Interregional Research Project Number 4 (IR-4) program to validate the methodology for dates.

Results and Discussion

Recovery data for chlorpyrifos and TCP at 2 levels of fortification for each of the 3 stages of date production are presented in Tables 1 and 2, respectively. Table 1 shows recoveries for chlorpyrifos in each of the 3 stages of date production with a range of 86–110% and an average of 96% for 0.05 ppm fortification, and a range of 88–96% with an average of 93% for 0.10 ppm fortification. Table 2 shows recoveries for TCP in each of the 3 stages of date production with a range of 80–99% and an average of 86% for 0.10 ppm fortification and a range of 79–93% with an average of 86% for 0.20 ppm fortification. Overall recoveries are 95% for chlorpyrifos and 86% for TCP. Evaluation of the recoveries presented in Tables 1 and 2 shows no significant differences in recoveries among the 3 stages of date production.

Recovery investigations were also carried out by means of

separate fortifications of parent and TCP at a level of 1000 ppm each to study the possibility of compound degradation or conversion as a result of the extraction, partitioning, and cleanup procedure; none was observed.

Typical gas chromatograms for chlorpyrifos analytical standard, control dates, and dates fortified at 0.05 ppm chlorpyrifos are shown in Figure 1. Under the previously stated GC conditions, chlorpyrifos has a retention time of 3.14 min. By conservatively estimating a 25 pg chlorpyrifos minimum detectable peak (10 mm and noise of 1 mm) and 15 mg amount of injection (3 μL extract from a total volume of 2.0 mL), 0.002 ppm could be attained, if lower limits of detection were required.

Typical gas chromatograms of analytical standard of TCP-derivative, control dates, and dates fortified at 0.10 ppm TCP are shown in Figure 2. A retention time of 3.18 min for TCP-derivative was attained using the GC conditions stated previously. By conservatively estimating TCP-derivative minimum detectable peak at 0.3 ng (10 mm peak and noise of 2 mm) and 30 mg amount of injection (3 μL date extract from a total volume of 1.0 mL), it is clear that the 0.05 ppm limit of detection required for the method could be greatly exceeded.

A combined hexane and benzene extraction was required to separate chlorpyrifos and TCP. This was due to the formation of emulsions with green date samples when benzene extraction was used alone. The combination of hexane washes eliminated the emulsions and extracted some chlorpyrifos, and the additional washes with benzene removed the remaining chlorpyrifos from the sodium bicarbonate solution.

The methodology described is specific for the separation and determination of chlorpyrifos and TCP residues in dates, but should be adaptable to any watery or sugar-containing crop.

Acknowledgments

The authors gratefully acknowledge M. M. Barnes and Richard Warner of the University of California at Riverside for providing the control dates used in the recovery studies; Richard Farquharson of Dow Chemical Co. for providing the opportunity to develop the methodology for the study; and Dow Chemical Co. of Midland, Michigan, for furnishing the analytical standard required for this investigation.

REFERENCES

(1) Winterlin, W. L., Moilanen, K., & Burgoyne, W. E. (1968) *Down Earth* **24,** 34-47
(2) Wetters, J. H. (1973) *Dow Chemical*, ACR **73.5,** 1-19
(3) Bowman, M. C., & Beroza, M. (1967) *J. Agric. Food Chem.* **16,** 651-653
(4) Struble, D. L., & McDonald, S. (1973) *J. Econ. Entomol.* **66,** 769-772
(5) *Pesticide Analytical Manual* (1979) Vol. I, Food and Drug Administration, Washington, DC
(6) Luke, M. A., Froberg, J. E., Doose, G. M., & Masumoto, H. T. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1187-1195
(7) Di Muccio, A., Cicero, A. M., Camoni, I., Pontecorvo, D., & Dommarco, R. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 106-108
(8) Inman, R. D., Kiigemagi, V., & Deinzer, M. L. (1981) *J. Agric. Food Chem.* **29,** 321-323
(9) Guinivan, R. A., Thompson, N. P., & Bardalaye, P. C. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 210-214
(10) Braun, H. E. (1974) *J. Assoc. Off. Anal. Chem.* **57,** 182-188

Liquid Chromatographic Determination of 5-(Methylamino)-2-Phenyl-4-[3-(Trifluoromethyl)phenyl]-3-(2*H*)-Furanone in Soil

THOMAS C. MUELLER and PHILIP A. BANKS
University of Georgia, Agronomy Department, Athens, GA 30605
PARSHALL B. BUSH
University of Georgia, Cooperative Extension Service, Athens, GA 30605
WILLIAM C. STEEN
U.S. Environmental Protection Agency, College Station Rd, Athens, GA 30605

A rapid, sensitive method is described for the determination of 5-(methylamino)-2-phenyl-4-[3-(trifluoromethyl)phenyl]-3-(2*H*)-furanone (RE-40885) concentrations in 3 soil types. The method consists of extraction of soil samples with methanol, filtration, liquid chromatographic separation of methanol-soluble components using a C_{18} column, and UV detection at 275 nm. Recoveries were 94, 96, and 94% from the Greenville, Cecil, and Dothan soils, respectively. Average relative standard deviation was 8.0% in the Greenville soil. The qualitative limit of detection was 20 ppb and the limit of quantitation was 40 ppb in 25 g soil samples.

A new herbicide, 5-(methylamino)-2-phenyl-4-[3-(trifluoromethyl)phenyl]-3-(2*H*)-furanone (RE-40885), synthesized by Chevron Chemical Co. (Richmond, CA, USA), is being investigated for use in peanuts, cotton, cereals, and grain sorghum (1, 2). Probable field application rates are 0.56–0.84 kg/ha (unpublished data). Due to the newness of the herbicide, methods for its analysis in soil samples have not been described. In this report, methodology developed for the determination of RE-40885 at ppb to ppm levels in 3 soil types using an external standard liquid chromatographic method with UV detection will be described.

Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—Model SCL-6A control unit, SIL-6A auto-injector, LC-6A solvent delivery system, and SPD-6A variable wavelength detector set at 275 nm (all from Shimadzu, Kyoto, Japan). Model 3390A reporting integrator (Hewlett-Packard Co., Analytical Group, Palo Alto, CA 94303).

(b) *Analytical column.*—25 cm × 4.6 mm id 5 μm LC-C_{18}, in-line 1 cm × 1.5 mm pellicular C_{18} guard column (Alltech, Chicago, IL, USA).

(c) *Solvents.*—LC grade (J. T. Baker Inc., Phillipsburg, NJ 08865).

(d) *Mobile phase.*—Methanol–water (72 + 28 v/v) at flow rate of 1.0 mL/min.

(e) *Analytical standards.*—RE-40885 (Chevron, Richmond, CA, USA); atrazine (Ciba-Geigy, Raleigh, NC, USA); benefin and trifluralin (Elanco, Greenfield, IN, USA); alachlor (Monsanto, St. Louis, MO, USA); and chlorothalonil (Fermenta, Mentor, OH, USA).

Soil Selection

Soil was collected from 3 areas to represent major soil associations in the southeastern United States. Soil 1 is a Greenville sandy clay loam, soil 2 is a Cecil loam (typical of the eroded piedmont area), and soil 3 is a Dothan loamy sand (typical of large areas of the coastal plain) (Table 1). Soil samples were air-dried and passed through a 10 mesh (2 mm) sieve before analysis. All samples were collected from areas with no known previous exposure to RE-40885.

Received August 7, 1989. Accepted October 16, 1989.

Mention of trade names or commercial products does not constitute endorsement by the U.S. Environmental Protection Agency.

Extraction

The UV spectra of RE-40885 was obtained using a scanning spectrophotometer (Perkin-Elmer 559A, Oak Brook, IL). The purity of the RE-40885 analytical material was established using gas chromatography/mass spectrometry (GC/MS) (Hewlett-Packard 5840-5985B). Various combinations of acetonitrile–water, methanol–water, and acetonitrile–2-propanol were evaluated for herbicide retention time and capacity factor (k′) (3). The accuracy of the analysis was examined by injection of a series of standards containing 5–700 ng/mL RE-40885 in methanol. This range corresponded to herbicide soil concentrations of 10–1400 ppb, using the described methodology.

Analytical herbicide (in methanol) was added to triplicate flasks containing 25 g of soil 1 to achieve RE-40885 concentrations of 40, 50, 80, 100, 120, 160, 200, 250, 500, and 1000 ng/g soil. After addition of the herbicide, the methanol was removed under a gentle air stream. The soil/herbicide mixture was mixed thoroughly with a wrist-action shaker for 60 min and allowed to equilibrate statically for 24 h. The soil was extracted with 50 mL methanol by shaking for 60 min on a wrist-action shaker. The soil extracts were then filtered through 2 Whatman No. 1 filter papers (Whatman, Clinton, NJ, USA) to remove particulates, and 50 μL aliquots were injected and compared to RE-40885 standards. Soils 2 and 3 were similarly spiked with 200 and 1000 ng/g soil.

Twenty-five g of each soil was spiked with RE-40885 (0.4 μg/g), atrazine (3.57 μg/g), benefin (2.20 μg/g), trifluralin (1.90 μg/g), alachlor (3.10 μg/g), and chlorothalonil (1.10 μg/g). Triplicate flasks for each soil were analyzed with the described method.

Results and Discussion

The UV spectra of RE-40885 showed an absorbance maximum at 275 nm. GC/MS analysis of RE-40885 (analytical grade) indicated a purity of > 99%. Injection of 50 μL of RE-40885 standard produced a peak with a retention time of 2.7–22.6 min, depending on the mobile phase (Table 2). On the basis of k′ values, we selected a mixture of methanol–water (72 + 28) which resulted in a retention time of approximately 7 min (Figure 1a). A standard curve plot was linear in the 10–1400 ppb range (soil basis), with an $r^2 = 0.99$ (data not shown.) The analytical standards (in methanol) and the methanol soil extracts were stable over time, and showed no change in detector response when stored at 0°C for 20 and 2 weeks, respectively. Soil samples containing RE-40885 residues were stable for 12 months when stored at −15°C.

Recoveries for soil 1 ranged from 77 to 110% of applied

Table 1. Soil characteristics

Soil	Series	Sand, %	Silt, %	Clay, %	Organic matter,[a] %	pH[b]
1	Greenville	51	24	25	2.1	6.4
2	Cecil	50	37	13	1.4	5.2
3	Dothan	83	11	6	0.5	7.0

[a] Determined by Wakley-Black dichromate method.
[b] Determined by 1:1 soil:water suspension technique.

Table 2. Solvent systems for RE-40885 analysis

Mobile phase	Solvent ratio, v/v	Retention time, min	k'[a]
Acetonitrile–water	80 + 20	3.30	0.65
Acetonitrile–water	60 + 40	6.99	2.50
Methanol–water	80 + 20	5.11	1.55
Methanol–water	72 + 28	7.00	2.50
Methanol–water	70 + 30	9.42	3.71
Methanol–water	60 + 40	22.60	10.30
Acetonitrile–2-propanol	80 + 20	2.82	0.41
Acetonitrile–2-propanol	60 + 40	2.73	0.36

[a] Capacity factor; t_0 for system = 2.00 min.

Table 3. Determination of RE-40885 recovery from soil 1 with methanol extraction, methanol–water (72 + 28) mobile phase.

Spiked concn, ng/g	Detected concn,[a] ng/g	RSD, %
0	0	0
40	43.0	23.1
50	41.4	24.1
80	76.5	12.0
100	97.6	1.1
120	107.0	3.7
160	155.8	5.1
200	220.7	3.9
250	193.0	6.7
500	451.0	4.6
1000	927.0	4.0

[a] Mean values obtained from 3 flasks. Average recovery = 94%.

herbicide, with the average recovery being 94% (Table 3). The precision of the analytical procedure over the range of 40–1000 ppb was acceptable, with the average relative standard deviation over this range being 8.0%. This analytical range corresponds to soil concentrations of approximately 400–600 from a RE-40885 field application. Our limit of detection for quantitation was defined as 40 ppb; where the signal-to-noise ratio was 3 (Figure 1c). However, qualitatively, the limit of detection of RE-40885 may be extended as low as 20 ppb. Results for soils 2 and 3 were similar to soil 1.

Herbicides, including RE-40885, often are applied in combination with other pesticides. The pesticides examined for detection interference represent major classes of pesticides used in crops where RE-40885 has potential for use. These commonly used chemicals did not elute in the retention window of RE-40885, and did not interfere with the method. Pesticides that eluted and their retention times in minutes were: atrazine (6.25), chlorothalonil (10.05), alachlor (17.5), and trifluralin (18.28). Benefin did not cause a detector response with this method.

Acknowledgments

The authors thank Rick Arrendale for obtaining and interpreting the GC/MS spectra.

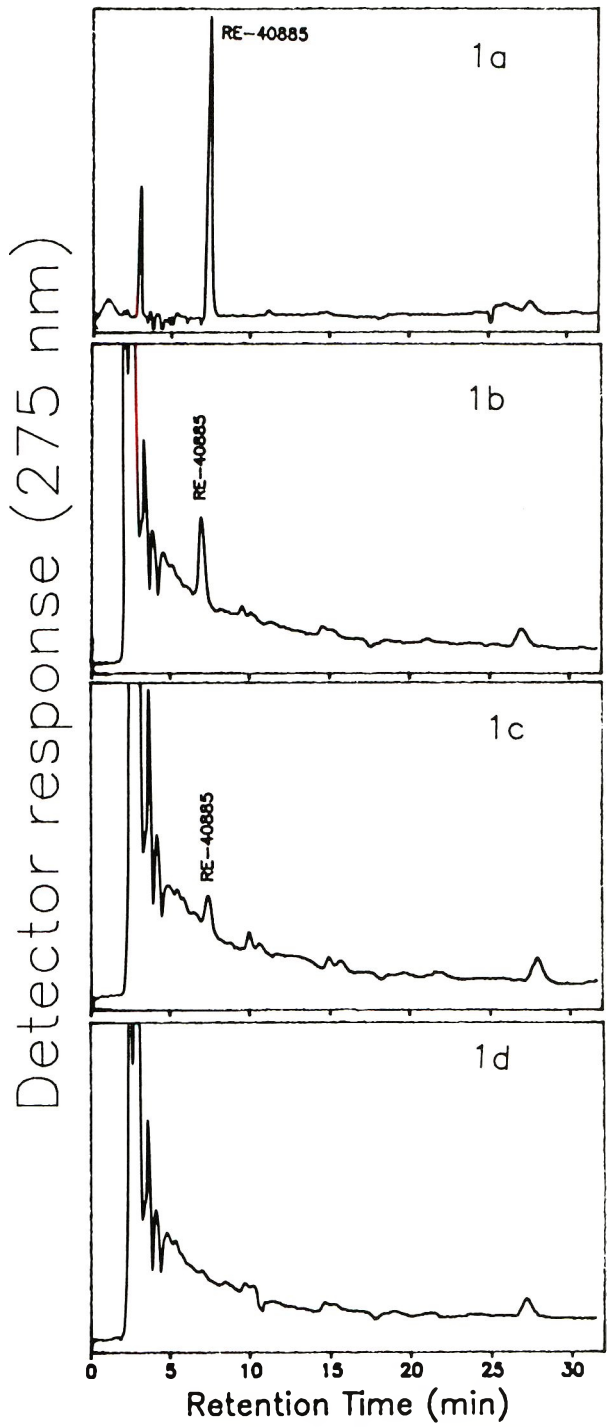


Figure 1. Liquid chromatograms of analytical standard and methanol extracts of soil spiked to achieve various RE-40885 concentrations: a, 200 ppb RE-40885 analytical standard; b, Soil 1 extract containing 120 ppb RE-40885; c, Soil 1 extract containing 40 ppb RE-40885; d, Soil 1 extract containing no RE-40885.

References

(1) Ward, C. E., Lo, W. C., Pomider, P. B., Tisdell, F. E., Ho, A. W., Chiu, C., Tuck, D. M., Bernardo, C. R., Fong, P. J., Omid, A., & Butea, K. A. (1987) in *Synthesis and Chemistry of Agrochemicals*, D. R. Baker (Ed.), American Chemical Society, Series 335, Washington, DC, pp. 66–73

(2) Rogers, D. D., Kirby, B. W., Hulbert, J. C., Bledsoe, M. E., & Hill, L. V. (1987) *Brit. Crop Prot. Conf.-Weeds* **8**, 69–75

(3) Snyder, L. R., & Kirkland, J. J. (1979) *Introduction to Modern Liquid Chromatography*, John Wiley and Sons, New York, NY, p. 74.

Relative Retention Time Diagram as a Useful Tool for Gas Chromatographic Analysis and Electron-Capture Detection of Pesticides

MASAMI OMURA, KUMIKO HASHIMOTO, KUNIO OHTA, TOMOYUKI IIO, SHIGEKAZU UEDA, KEIKO ANDO, and HIKARU HIRAIDE
Hamamatsu City Health Center, 2-11-2 Kamoe Hamamatsu, Shizuoka 432, Japan
NAOHIDE KINAE
University of Shizuoka, School of Food and Nutritional Sciences, 395 Yada, Shizuoka 422, Japan

To establish efficient operating conditions for gas chromatographic (GC) analysis of pesticides that are detected by electron-capture detector (ECD), separation degrees of 40 pesticides and their relative retention times (RRT) vs aldrin were determined. Eleven liquid phases, categorized according to the McReynolds constant (MC), were used: OV-1, OV-3, DC-550, and OV-17 as non- or low polar liquid phases (MC $\leqq$ 1000), OV-22, QF-1, and XE-60 as medium polar liquid phases (1000 < MC $\leqq$ 2000), PEG-20M, DEGA, and DEGS as high polar liquid phases (2000 < MC), and a mixture of DC-200 and EPON1009. An RRT diagram was prepared by plotting the RRT of each pesticide on the horizontal axis and the MC values on the vertical axis. The RRT diagram could be used to describe the properties of pesticides—their behavior on each liquid phase and the precise operational conditions for qualitative GC analysis. The non- or low polar liquid phases were best suited for GC analysis of organochlorine pesticides having low polarity. Their detection limits ranged from 0.01 to 1.0 ng.

Unknown peaks frequently appear on gas chromatograms of residual pesticides contained in environmental samples such as foods, water and sediments. Most investigators note the difficulty in identifying and determining the content of the peaks because of insufficient information in the literature.

We have tried to develop a simple and efficient method for qualitative analysis of well known pesticides in Japan. In the previous paper (1), we proposed an application of the relative retention time (RRT) diagram for GC analysis of organophosphorus pesticides.

A number of reports deal with RRTs of residual pesticides that are determined by gas chromatography and electron-capture (ECD) or flame photometric detectors (FPD). Some researchers use a packed column or a capillary column under isothermal or temperature-programmed operating conditions (1–12). Thompson et al. (2) observed a good correlation between the column temperature and the RRTs of 95 pesticides. Saxton (12) tried to identify several pesticides by using the emergence temperature indices.

On the basis of these results, we proposed the RRT diagram as a compass for a relation between the RRTs of pesticides and the polarity of liquid phases (1). In the present paper, we have attempted to prepare the RRT diagram for 40 ECD-sensitive pesticides by using 11 different liquid phases, and to generalize the diagram theory. We also examine the application of the diagram to establishing operating conditions in GC analysis.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph.*—GC-163, Hitachi, Tokyo, Japan, with ^{63}Ni electron-capture detector (ECD). Peak heights and peak areas were calculated with a data processor (Chromatopac D-2000, Hitachi, Tokyo, Japan).

(b) *Pesticides.*—The 40 pesticides studied are listed in Table 1 according to the ISO name (International Standards Organization) (13). Twenty-six of the pesticides are regulated by the Japanese Food Sanitation Laws. All pesticide standards and solvents used were analytical grade (purchased from Wako Pure Chemical Co., Osaka, Japan, or Gaskuro Kogyo Co., Tokyo, Japan). A standard solution of each pesticides was prepared by dissolving it in acetone and/or hexane at 1000 μg/mL and then diluting to a predetermined working level (generally 25–75% full scale deflection). Aldrin was used as a reference compound for comparison with the data in other reports.

(c) *Chromatographic columns.*—The length and the inside diameter of silanized columns were standardized to 2 m and 3 mm, respectively. Gas Chrom Q (80–100 mesh, Gaskuro Kogyo Co.) coated with 2% liquid phase was used for the column material. The nitrogen carrier gas was regulated to ca 30–50 mL/min, to maintain the retention time of aldrin at 5 min. The detector and column oven temperatures were 350°C and ca 165–200 °C, respectively.

(d) *Liquid phases.*—On the basis of the McReynolds constant (0 $\leqq$ MC < about 4500) (14), 3 classifications were chosen for the RRT diagram. From the 11 liquid phases, OV-1, OV-3, DC-550, and OV-17 were selected as non- or low polar liquid phases with MC < 1000. The OV-22, QF-1, and XE-60 were selected as medium polar liquid phases (1000 < MC $\leqq$ 2000). The PEG-20M, DEGA, and DEGS were utilized as high polar liquid phases (2000 < MC). A mixed phase of DC-200 and EPON 1009, showing an ill-defined MC score, which was generally used for pesticide analysis, was also examined. All liquid phases used are shown in Table 2.

The retention time of aldrin as a guideline substance in each liquid phase was adjusted to exactly 5 min. A gas chromatograph was operated by means of isothermal analysis to maintain the thermal stability of ECD and to determine the accuracy of retention times during operation.

Procedure

In this experiment, the general sensitivity and separation degrees were determined for the RRT diagram. First, the sensitivity of the electron-capture detector to each pesticide was measured. Since the response of the detector to a pesticide depends on the functional groups in the molecule, the amount of pesticide yielding 10% full scale deflection was determined under favorable operating conditions.

The detection limits (ng) were classified into the following 3 types: Class A (A $\leqq$ 0.1 ng), Class B (0.1 < B $\leqq$ 1.0), Class C (1.0 < C).

Next, the general qualities of 11 liquid phases were evalu-

Received May 17, 1989. Accepted September 28, 1989.

Table 1. ECD-sensitive pesticides used

No.[a]	Name[b]	Groups				MW	Other names
		Cl	P	S	NO_2		
1	Aldrin	6				365	
2	α-BHC	6				291	
3	β-BHC	6				291	
4	γ-BHC	6				291	
5	δ-BHC	6				291	
6	Captafol	4		1		349	Difolatan
7	Captan	3		1		301	Orthocide
8	α-Chlorfenvinphos	3	1			360	α-CVP, Vinyphate
9	β-Chlorfenvinphos	3	1			360	β-CVP, Vinyphate
10	Chlornitrofen	3			1	319	CNP, MO
11	Chlorobenzilate	2				325	Akar
12	Chlorothalonil	4				266	TPN, Daconil
13	Chlorpyrifos	3	1	1		351	Dursban
14	Chlorpyrifos-methyl	3	1	1		323	Reldan, Dawreldan
15	Cyanofenphos		1	1		303	CYP, Surecide
16	*o,p'*-DDD	4				320	
17	*p,p'*-DDD	4				320	
18	*o,p'*-DDE	4				318	
19	*p,p'*-DDE	4				318	
20	*o,p'*-DDT	5				354	
21	*p,p'*-DDT	5				354	
22	Dichlobenil	2				172	DBN, Casoron
23	Dichlorvos	2	1			221	DDVP, Vapona
24	Dicofol	5				370	Kelthane
25	Dieldrin	6				381	
26	α-Endosulfan	6		1		407	Malix, α-Benzoepin
27	β-Endosulfan	6		1		407	Malix, β-Benzoepin
28	Endrin	6				381	
29	EPN		1	1	1	323	
30	Fenitrothion		1	1	1	277	MEP, Sumithion
31	Tetrachlorophthalide	4				272	Fthalide, Rabcide
32	Heptachlor	7				373	
33	Parathion		1	1	1	291	
34	Parathion-methyl		1	1	1	263	
35	Pentachlorophenol	5				266	PCP
36	Phosalone	1	1	2		368	Rubitox
37	Prothiophos	2	1	2		345	Tokuthion
38	Quintozene	5			1	295	PCNB
39	Tetradifon	4		1		356	Tedion
40	Thiobencarb	1		1		258	Benthiocarb, Saturn

[a] Used for identification in tables and figures.
[b] International Standards Organization.

Table 2. Liquid phases examined and their McReynolds constants

No.	Liquid phase	McReynolds constant	Analytical temp., °C
1	OV-1	222	170
2	OV-3	423	190
3	DC-200 + EPON1009	—[a]	185
4	DC-550	620	200
5	OV-17	884	195
6	OV-22	1075	185
7	QF-1	1500	165
8	XE-60	1785	165
9	PEG-20M	2308	195
10	DEGA	2764	180
11	DEGS	3543	183

[a] Unknown.

ated. The relative value (A/H: area to height) of each pesticide peak was readily obtained from the peak area and peak height in the liquid phase. The separation degrees were classified into the following 4 types as seen in Table 3 by A/H values, peak shape, and tailing: 1 is assigned to pesticides showing an A/H value less than 60 and a sharp symmetrical peak; 2 = pesticides showing an A/H value from 60 to 160 and a moderate tailing peak; 3 = the pesticides showing an A/H value from 160 to 700, low sensitivity, and marked tailing peak; × = the pesticides showing an A/H value over 700 and no noticeable peak.

Table 4 shows the RRTs of the 11 liquid phases re-listed in sequence on the basis of the RRTs on a DC-550 column. From these results, an RRT diagram was prepared by plotting RRTs on the horizontal axis and MC values on the vertical axis to examine the behaviors of 40 pesticides.

Table 3. Detection limits (DL)[a] and separation degrees (SD)[b] of ECD-sensitive pesticides on 11 liquid phases

No.	Name	OV-1		OV-3		DC200 + EPON-1009		DC-550		OV-17		OV-22		QF-1		XE-60		PEG-20M		DEGA		DEGS	
		DL	SD	DL	SD	DL	SD	DL	SD	DL	SD	DL	SD	DL	SD	DL	SD	DL	SD	DL	SD	DL	SD
1	Aldrin	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1
2	α-BHC	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	B	1	A	1	A	1
3	β-BHC	A	1	A	1	A	1	A	1	A	1	A	1	A	1	B	2	B	1	B	2	B	2
4	τ-BHC	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	B	1	B	1	A	1
5	δ-BHC	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	2	B	1	—	X	B	2
6	Captafol	B	2	B	2	—	X	C	2	B	3	C	3	C	3	C	3	—	X	—	X	—	X
7	Captan	A	1	A	1	B	1	B	1	B	1	B	1	B	2	B	2	—	X	—	X	—	X
8	α-Chlorfenvinphos	B	2	B	1	B	1	B	1	B	2	A	1	C	3	B	2	B	2	B	1	B	2
9	β-Chlorfenvinphos	B	2	B	1	B	1	B	1	B	2	A	1	B	3	B	2	B	2	B	2	B	2
10	Chlornitrofen	B	2	B	2	B	2	B	2	B	2	B	2	B	3	B	3	B	3	C	3	B	3
11	Chlorobenzilate	C	2	C	2	B	1	C	1	C	2	C	2	C	3	C	2	C	3	C	3	C	3
12	Chlorothalonil	A	1	A	1	A	1	A	1	A	1	A	1	A	2	A	2	—	X	—	X	—	X
13	Chlorpyrifos	A	1	A	1	A	1	A	1	A	1	A	1	B	1	B	1	B	1	B	1	A	1
14	Chlorpyrifos-methyl	A	1	A	1	A	1	A	1	A	1	A	1	B	1	B	1	B	1	B	1	A	2
15	Cyanofenphos	B	2	B	2	B	2	B	2	B	3	B	2	B	3	B	3	C	3	C	3	B	3
16	o,p'-DDD	A	1	B	1	A	1	B	1	B	2	A	1	B	2	B	2	B	2	B	2	B	2
17	p,p'-DDD	A	1	B	2	A	1	B	1	B	2	A	2	B	2	B	2	C	3	C	3	B	3
18	o,p'-DDE	A	1	A	1	A	1	B	1	A	1	A	1	A	1	A	1	B	2	B	1	B	2
19	p,p'-DDE	A	1	A	1	A	1	A	1	A	2	A	1	A	2	A	2	A	2	B	2	A	2
20	o,p'-DDT	A	1	A	2	A	1	B	1	A	2	A	2	A	2	B	2	B	3	C	2	B	2
21	p,p'-DDT	A	2	A	2	A	1	B	2	B	2	A	2	B	2	B	2	B	3	C	3	B	3
22	Dichlobenil	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1
23	Dichlorvos	B	1	B	1	B	1	B	1	B	1	B	1	B	1	B	1	—	X	B	1	B	1
24	Dicofol	B	1	B	2	B	1	B	3	B	3	B	3	B	3	C	2	B	2	B	1	B	2
25	Dieldrin	A	1	A	1	A	1	A	1	A	2	A	1	A	2	A	2	A	2	B	2	A	2
26	α-Endosulfan	A	1	A	1	A	1	A	1	A	1	A	1	A	2	A	1	A	2	B	1	A	2
27	β-Endosulfan	A	1	A	1	A	1	A	1	A	2	A	2	B	2	B	2	B	2	B	2	B	3
28	Endrin	A	1	A	1	B	3	B	1	A	2	A	2	B	2	A	2	B	2	B	2	B	2
29	EPN	B	2	B	2	B	2	B	2	B	3	B	3	C	3	B	3	—	X	C	3	C	X
30	Fenitrothion	A	1	A	1	A	1	A	1	A	1	A	1	B	2	B	2	—	X	B	2	B	2
31	Tetrachlorophthalide	A	1	A	2	A	1	A	1	A	2	A	1	B	2	B	2	B	2	B	2	B	3
32	Heptachlor	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1
33	Parathion	A	1	A	1	A	1	A	1	A	1	A	1	B	2	B	2	B	2	B	2	B	2
34	Parathion-methyl	A	1	A	1	A	1	A	1	A	1	A	1	B	2	B	2	—	X	B	2	B	2
35	Pentachlorophenol	—	X	—	X	B	1	—	X	—	X	—	X	—	X	—	X	—	X	C	3	C	3
36	Phosalone	B	2	B	3	B	2	C	2	B	3	B	3	C	3	C	3	—	X	—	X	—	X
37	Prothiophos	A	1	A	1	A	1	A	1	A	2	A	1	A	2	B	2	B	2	B	1	A	2
38	Quintozene	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1
39	Tetradifon	B	2	B	3	B	3	B	2	B	3	B	3	B	3	C	3	C	3	C	3	C	X
40	Thiobencarb	C	1	C	1	C	1	C	1	C	1	C	1	C	1	C	1	C	1	C	1	C	1

[a] Detection limit amount of pesticide giving approximately 10% full scale recorder response. Class A: A ≦ 0.1 (ng); class B: 0.1 < B ≦ 1.0; class C: 1.0 < C; — = could not be determined.

[b] Levels of separation: 1 = very good peak with 0 < A/H (= peak area/peak height) ≦ 60; 2 = good peak with 60 < A/H ≦ 160; 3 = poor and tailing peak with 160 < A/H ≦ 700; X = could not be determined.

Results and Discussion

Sensitivity of ECD to Pesticides and Their Separation Degrees

Takeuchi and Tuge (15) selected 226 stationary phases from 700 commercial liquid phases and investigated their suitability to GC analysis of pesticides; 12 of them were preferable. Koda (16) and Omura et al. (17) discussed the relation between inorganic and organic character of liquid phases (I/O value) and their MC values by the organic conceptual diagram. From their work, we selected 11 typical liquid phases on the basis of use frequency and the polarities.

Table 3 shows the ECD response to pesticides by peak height in each liquid phase. The minimum detection limit of aldrin as a reference compound ranged from 0.01 ng on OV-1 and OV-22 to 0.04 ng on DEGA. In non- or low polar liquid phases, level A(≦ 0.1 ng) was attached to most pesticides. Their minimum detection limits ranged from 0.01 to 1.0 ng in all liquid phases.

The values shown in this table do not equal the minimum detection limits (MDL) which are measured under the most favorable GC conditions for each compound, but they do follow the MDL. In this experiment, each compound is calculated compared to standard aldrin with a fixed retention time.

On the other hand, when the MDL was greater than 10 in

Table 4. Relative retention times of ECD-sensitive pesticides on 11 liquid phases

No.	Name	OV-1	OV-3	DC200 + EPON1009	DC-550[a]	OV-17	OV-22	QF-1	XE-60	PEG-20M	DEGA	DEGS
23	Dichlorvos	0.08	0.06	0.10	0.06	0.07	0.06	0.27	0.13	—[b]	0.15	0.20
22	Dichlobenil	0.08	0.11	0.11	0.11	0.12	0.12	0.31	0.25	0.27	0.28	0.36
2	α-BHC	0.33	0.39	0.40	0.43	0.45	0.45	0.59	0.86	0.79	1.16	1.32
4	γ-BHC	0.42	0.50	0.51	0.57	0.61	0.63	0.79	1.28	0.28	1.85	2.14
38	Quintozene	0.45	0.53	0.50	0.57	0.61	0.58	0.86	0.75	0.63	0.85	0.88
3	β-BHC	0.37	0.53	0.56	0.65	0.74	0.72	0.98	4.18	2.69	6.56	7.10
5	δ-BHC	0.44	0.64	0.65	0.78	0.90	0.92	1.11	4.17	0.51	—	6.30
32	Heptachlor	0.78	0.79	0.79	0.80	0.80	0.78	0.85	0.91	0.92	0.98	1.00
12	Chlorothalonil	0.51	0.73	0.70	0.85	1.03	0.96	2.84	3.09	—	—	—
14	Chlorpyrifos-methyl	0.70	0.80	0.76	0.91	1.03	1.10	1.40	1.54	1.73	1.99	2.25
34	Parathion-methyl	0.70	0.84	0.89	0.98	1.17	1.24	3.46	3.56	—	4.84	6.47
1	Aldrin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
40	Thiobencarb	0.97	1.06	1.06	1.17	1.33	1.44	1.70	1.90	1.93	2.32	2.40
30	Fenitrothion	0.86	1.01	1.02	1.18	1.42	1.54	3.97	4.04	—	5.00	6.43
13	Chlorpyrifos	1.04	1.14	1.03	1.25	1.38	1.46	1.83	1.87	1.74	1.94	2.03
24	Dicofol	1.07	1.26	1.23	1.31	1.56	1.56	2.41	2.53	2.98	3.71	4.03
33	Parathion	1.04	1.18	1.16	1.33	1.51	1.58	4.72	4.32	3.64	4.61	5.59
8	α-Chlorfenvinphos	1.65	1.49	1.67	1.61	1.85	2.00	4.85	3.71	3.04	3.60	4.09
31	Tetrachlorophthalide	1.09	1.49	1.58	1.73	2.24	2.25	4.60	5.44	4.79	7.73	9.19
9	β-Chlorfenvinphos	1.69	1.63	1.84	1.84	2.15	2.37	5.25	4.32	3.65	4.41	5.14
26	α-Endosulfan	1.67	1.75	1.77	1.86	1.97	2.03	2.79	2.70	2.55	3.36	3.68
18	*o,p'*-DDE	1.66	1.74	1.65	1.86	2.06	2.24	1.80	2.47	2.97	3.21	3.14
7	Captan	1.25	1.58	1.81	1.99	2.54	2.99	5.06	7.16	—	—	—
25	Dieldrin	2.01	2.12	2.14	2.27	2.48	2.60	3.48	3.88	3.67	4.46	4.88
37	Prothiophos	2.05	2.14	1.96	2.33	2.55	2.70	3.18	3.58	3.18	3.34	3.23
19	*p,p'*-DDE	2.13	2.22	2.09	2.33	2.56	2.74	2.47	3.34	3.88	4.41	4.17
16	*o,p'*-DDD	2.18	2.38	2.25	2.64	3.05	3.37	3.03	5.46	5.74	7.60	7.59
28	Endrin	2.27	2.47	3.35	2.75	3.09	3.35	4.15	4.38	3.99	5.13	5.54
11	Chlorobenzilate	2.88	2.89	2.98	3.18	3.57	3.87	5.43	7.93	10.73	13.57	14.34
27	β-Endosulfan	2.35	2.78	2.93	3.25	3.81	4.09	5.47	8.93	8.10	11.69	13.39
20	o,p'-DDT	2.94	3.02	2.86	3.28	3.73	4.16	3.22	4.62	5.58	7.44	5.94
17	*p,p'*-DDD	2.79	3.13	2.98	3.46	4.01	4.39	4.66	9.47	3.72	13.77	13.66
10	Chlornitrofen	3.38	3.80	3.91	4.24	5.10	5.36	9.59	10.57	11.87	16.53	17.68
21	*p,p'*-DDT	3.81	3.98	3.79	4.32	4.91	5.44	5.08	8.52	4.05	13.64	13.17
15	Cyanofenphos	3.67	4.28	3.97	5.16	6.72	7.76	15.57	19.28	18.11	24.47	29.75
6	Captafol	3.87	4.82	—	6.23	8.23	10.10	13.98	28.49	—	—	—
29	EPN	5.69	6.44	6.68	7.77	10.04	11.70	21.60	27.59	—	36.52	43.88
39	Tetradifon	6.75	7.74	8.54	9.15	11.82	13.41	25.25	30.67	29.82	45.44	48.27
36	Phosalone	7.52	8.69	8.99	10.50	13.53	15.48	30.78	46.08	—	—	—
35	Pentachlorophenol	—	—	0.83	—	—	—	—	—	—	14.04	19.37

[a] Listed in order of relative retention time on DC-550. Absolute retention time for aldrin was adjusted to approximately 5 min on each liquid phase.
[b] Could not be determined.

high polar liquid phases such as PEG-20M, DEGA, and DEGS, these phases often failed to separate the pesticides from each other. In general, an increase of the polarity of the liquid phase resulted in a decrease in the detection limit.

The separation degrees for 11 liquid phases were shown in Table 3. Most pesticides containing aldrin and chlorpyrifos were level 1, indicating a sharp symmetrical peak. Several compounds containing chlornitrofen and cyanofenphos were level 2, indicating slight tailing. Several compounds containing phosalone and tetradifon were level 3, indicating greater tailing. A few compounds containing pentachlorophenol and captafol were marked by ×, indicating no noticeable peak.

Most organochlorine pesticides have lower polarity compared with organophosphorus pesticides. In the previous report (1), we found that 3 liquid phases, OV-17, QF-1, and XE-60, having medium polarity (MC: 884–1785), were suitable for analysis of organophosphorus pesticides. In this experiment, non- or low polar liquid phases containing OV-1, OV-3, DC-550, OV-17, OV-22, QF-1, and XE-60 having MC < 1800 were suitable for analysis of ECD-sensitive pesticides. The use of a liquid phase having MC < 1000 was preferable. The separation degrees of the high polar liquid phases such as PEG-20M, DEGA, and DEGS evaluated unfavorably compared to those of non- or low polar liquid phases. But these high polar liquid phases would be suitable for analysis of high polar pesticides. On the principle of compatibility (16, 18), we inferred that the non- or low polar pesticides match the non- or low polar liquid phases and that the high polar pesticides match the high polar liquid phases.

Some broad peaks generally appeared on the chromatograms, eluting later than aldrin. Some pesticides marked by dashes in Tables 3 and 4 did not elute from the columns. This behavior may result from thermal decomposition of the pesticides in the column and/or in the injection port, and their

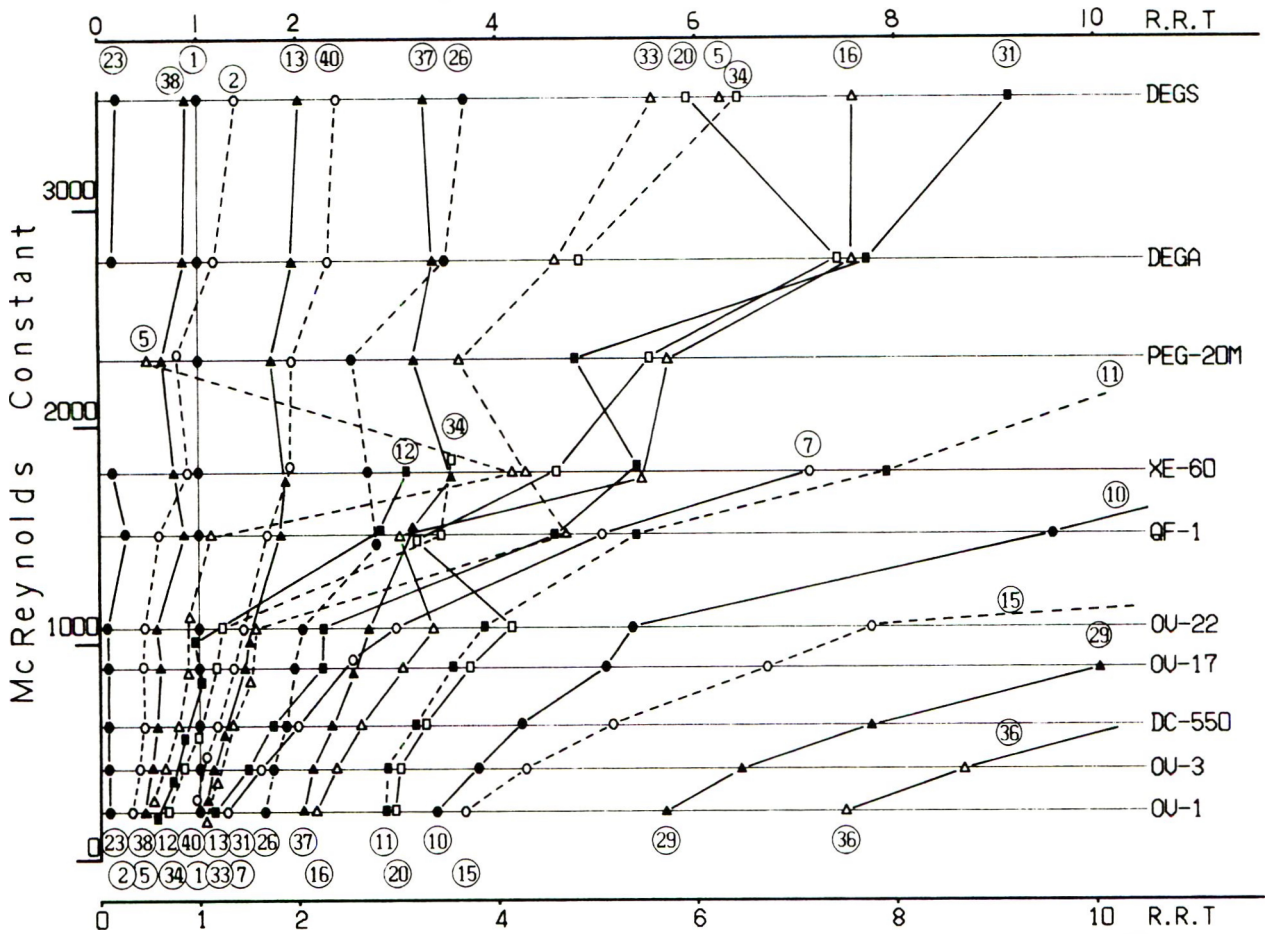


Figure 1. Relative retention time diagram-1 of ECD-sensitive pesticides: 1, aldrin; 2, α-BHC; 5, δ-BHC; 7, captan; 10, chlornitrofen; 11, chlorobenzilate; 12, chlorothalonil; 13, chlorpyrifos; 15, cyanofenphos; 16, o,p'-DDD; 20, o,p'-DDT; 23, dichlorvos; 26, α-endosulfan; 29, EPN; 31, tetrachlorophthalide; 33, parathion; 34, parathion-methyl; 36, phosalone; 37, prothiophos; 38, quintozene; 40, tiobencarb.

sorption with the liquid phase. In addition, relatively large molecular weight pesticides or high polar pesticides could not be analyzed in the high polar columns because of the limited working temperature of the liquid phases.

RRT Examination

The retention time of aldrin (5 min) is represented as 1 RRT as shown in Figures 1 and 2. The RRTs of other pesticides in the 11 liquid phases were then obtained with respect to aldrin. Separations were good for all pesticides except pentachlorophenol in DC-550 liquid phase. So, the RRTs shown in Table 4 were sorted in the column of DC-550. In the column filled with nonpolar liquid phase, the RRTs distribution of most pesticides falls within the 10-fold unit of that of aldrin.

RRTs in the non- or low polar liquid phases were analogous to each other. As the polarity of liquid phases increase, the RRTs of pesticides increase and peak tailing becomes more pronounced. The relatively large molecular weight pesticides and the compounds with polar radicals in the molecules had large RRTs in high polar liquid phases. Thus, the results were considered to reflect a correlation between the polarity of liquid phase and the physical property of the pesticide.

The MC values of OV-101, DC-200, and SE-30 were very similar with that of OV-1; therefore, the RRTs of these liquid phases were nearly the same.

According to recent reports (9, 10, 12), chlorpyrifos is also used as a reference compound for RRT examination. Many GC detectors are sensitive to chlorpyrifos; therefore, the data obtained in this study can be converted to a chlorpyrifos base.

Plinsloo et al. (7, 8) evaluated the separation efficiency of 50 organophosphorus pesticides and 70 ECD-sensitive pesticides based on peak symmetrical factors in 9 liquid phases. They recommended that 4% Reoplex-400 was suitable for analysis of organophosphorus pesticides, and a mixed column filled with 1.5% OV-17 and 1.25% QF-1 was suitable for ECD-sensitive pesticides. These results are similar to our data.

Preparation and Application of RRT Diagram

In GC analysis, we often need to know the relation between compounds and liquid phases. The RRT findings from our experiment could be represented graphically and show a correlation among pesticides, RRTs, and liquid phases.

The preparation method of the RRT diagram of 40 pesticides based on the RRTs is shown in Figures 1 and 2. (Two figures were used because the 40 pesticides were too many to plot in a single diagram.) The set MC (14) indicates the polarity of liquid phase on the vertical axis. The next set of

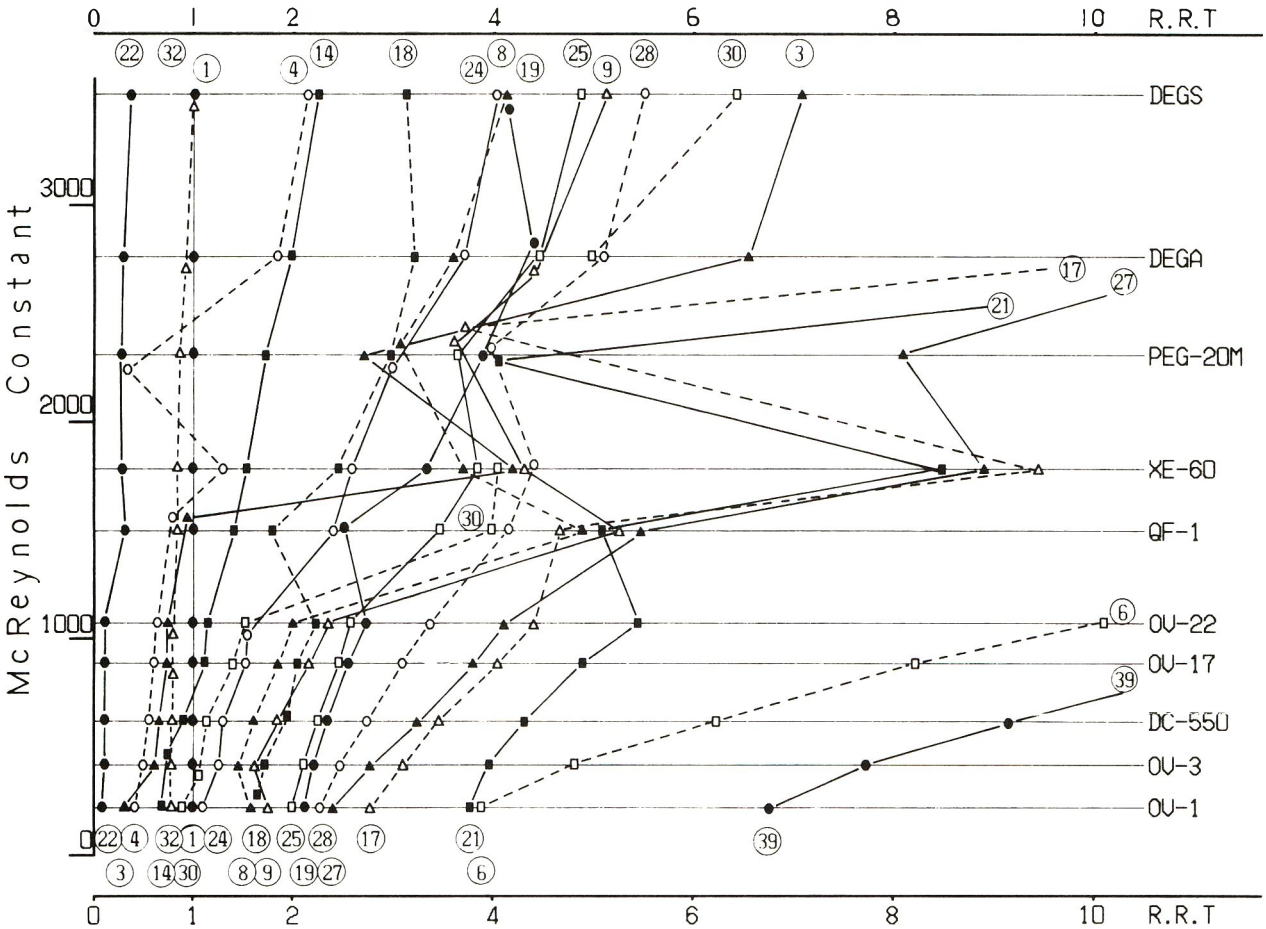


Figure 2. Relative retention time diagram-2 of ECD-sensitive pesticides: 1, aldrin; 3, β-BHC; 4, γ-BHC; 6, captafol; 8, α-chlorfenvinphos; 9, β-chlorfenvinphos; 14, chlorpyrifos-methyl; 17, *p,p'*-DDD; 18, *o,p'*-DDE; 19, *p,p'*-DDE; 21, *p,p'*-DDT; 22, dichlobenil; 24, dicofol; 25, dieldrin; 27, β-endosulfan; 28, endrin; 30, fenitrothion; 32, heptachlor; 39, tetradifon.

RRTs is plotted on the horizontal axis. Then, 10 horizontal liquid phase lines are drawn from nonpolar OV-1 to high polar DEGS. The RRTs of 40 pesticides are plotted on the liquid phase lines, and the RRT points of each pesticide are connected on the liquid phase lines to draw a zigzag curve.

By using this diagram, it is possible to demonstrate the identical behavior patterns among the liquid phases for given pesticides. These patterns can be broadly classified into 3 groups: Group A containing heptachlor has similar polarity, chemical composition, and molecular weight as those of aldrin, and therefore its RRT behavior curve is almost parallel to that of aldrin. In group B containing dicofol, the slope tends to deviate from that of aldrin due to an increase of the polarity of the liquid phase. In group C, containing chlorobenzilate and EPN, their RRTs tend to become extremely large in polar liquid phases.

In general, it is necessary to use 2 or more liquid phases for identification of chemical compounds in GC analysis and sometimes to use a combination of liquid phases whose elution sequence is reversed in order to get the most reliable results. In our study of RRT diagrams, identification is improved on liquid phases with MC values 500 units apart or greater. Such liquid phase combinations would be a very useful means for identifying and separating residual pesticides.

As a result of our experiments, we provide the following 2 examples for the RRT diagram, for known compounds and for unknown compounds.

For known compounds.—If the MC values of 2 or more liquid phases have already been set on the RRT diagram and the MC value of a new liquid phase is known, one can set the MC value of the new liquid phase somewhere between those of 2 liquid phases. Doing so allows you to obtain an approximate RRT of the new liquid phase because it intersects with the predetermined RRT curve of the 2 known liquid phases. From this intersection, it is also possible to estimate GC conditions of the objective compound.

For unknown compounds.—First, the RRTs of the unknown compound must be determined from some of the 10 liquid phases. A plot of the obtained RRTs on the RRT diagram produces a zigzag curve of the unknown compound. One can then compare it with the RRT behavior of the other compounds. By this comparison, it is possible to estimate approximate properties of the unknown compound such as polarity.

The RRT diagram provides a convenient visual impression of the relation among the separations, RRTs of each compound, and liquid phases. The diagram is not only useful for screening the compound in GC analysis but also may be used to establish the operating conditions for GC-MS analysis that are essential to identify the compound. Furthermore, the RRT diagram is useful for GC analysts to graphically and

visually compare the relations among objective materials, liquid phases, and RRTs. If an improved RRT diagram using chlorpyrifos as a reference compound is completed, it would allow us to have a much clearer understanding of each compound.

REFERENCES

(1) Omura, M., Hashimoto, K., Ohta, K., Shinzi, K., Ando, K., Shimizu, Y., & Hiraide, H. (1988) *Eisei Kagaku* (in Japanese) **34,** 282–290
(2) Thompson, J. F., Mann, J. B., Apodaca, A. O., & Kantor, E. J. (1975) *J. Assoc. Off. Anal. Chem.* **58,** 1037–1050
(3) Kawamura, Y., Takeda, M., & Uchiyama, M. (1978) *J. Food Hyg. Soc.* (in Japanese) **19,** 511–517
(4) Ambrus, A., Visi, E., Zakar, F., Hargitai, E., Szabo, L., & Papa, A. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 749–768
(5) Matsumoto, M. (1984) *J. Food Hyg. Soc.* (in Japanese) **25,** 410–417
(6) Tsunoda, N. (1986) *Eisei Kagaku* (in Japanese) **32,** 91–100
(7) Prinsloo, S. M., & De Beer, P. R. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 1100–1108
(8) Prinsloo, S. M., & De Beer, P. R. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 878–888
(9) *Pesticide Analytical Manual*, Vol. 1, U.S. Food and Drug Administration, Washington, DC
(10) Fehringer, N. V., & Walters, M. S. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 91–95
(11) Suprock, J. F., & Vinopal, J. H. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 1014–1017
(12) Saxton, W. L. (1987) *J. Chromatogr.* **393,** 175–194
(13) Tomisawa, T., & Kamizi, M. (1982) *Saishin Nouyaku Data Book* (in Japanese), Soft Science, Tokyo, Japan
(14) McReynolds, W. O. (1970) *J. Chromatogr. Sci.* **8,** 685–695
(15) Takeuchi, T., & Tuge, S. (1978) *Gaschromatography Saikin No Shinpo* (in Japanese), Kagaku no Ryoiki Zoukan No. 120, Nankodo, Tokyo, Japan, p. 47–61
(16) Koda, Y. (1984) *Yukigainenzu, Kiso To Ouyo-* (in Japanese), Sankyo Syuppan, Tokyo, Japan, p. 23, 76
(17) Omura, M., Sato, S., & Koda, Y. (1979) presented at *ACS/CSJ Chemical Congress*, Honolulu, Hawaii, Abstr. 139
(18) Nipponkagakukai. (1975) *Sin Zikken Kagaku Kouza, Kihonsosa* [1] (in Japanese), Maruzen, Tokyo, Japan, p. 223, 253

Extraction of Gasoline Constituents from Soil

SUSAN G. DONALDSON
University of Nevada-Reno, Department of Range, Wildlife and Forestry, 1000 Valley Rd, Reno, NV 89512
GLENN C. MILLER[1]
University of Nevada-Reno, Department of Biochemistry, Reno, NV 89557
W. W. MILLER
University of Nevada-Reno, Department of Range, Wildlife and Forestry, 1000 Valley Rd, Reno, NV 89512

The recovery of gasoline constituents from soil by using a sonication/extraction method was evaluated. Ten characteristic compounds were studied: benzene, toluene, *n*-heptane, *m*-xylene, nonane, *n*-propylbenzene, 1,2,4-trimethylbenzene, *n*-butylbenzene, 1,2,4,5-tetramethylbenzene, and dodecane. Modifications of extraction method EPA 3550 were tested using flame ionization gas chromatography with a 15 m × 0.53 mm id fused-silica capillary column. For dry soils, reducing total solvent extraction volume from 400 to 250 mL resulted in equivalent recoveries and shorter analysis times. Recovery of the gasoline constituents from wet soils was significantly lower than from dry soils for all methods studied. Recoveries were also dependent on the relative constituent volatility. Higher molecular weight compounds were recovered at greater than 80% of the initial amount applied from dry soils; while recoveries of benzene and heptane were generally less than 5%. Using the most efficient extraction procedure, recovery of unleaded gasoline from a dry, spiked soil was 43.2% when expressed on a total petroleum hydrocarbon basis, and recovery from a wet, spiked soil was 21.8%.

Since the 1940s, it has been a common practice to bury fuel storage tanks underground to guard against fire hazards and explosions. As these underground gasoline storage tanks near the end of their effective lifetime, leakage of hydrocarbons into soils is becoming a common problem. A portion of these spills remains trapped in the soil, immobilized by capillarity and adsorption. This fraction can present a long-term threat to groundwater, as relatively cleaner recharge water may desorb or solubilize gasoline components. Remediation requires a reliable and rapid means of evaluating the total gasoline content of contaminated soils.

Several techniques can be used to extract hydrophobic organic compounds from soils. One method is based on EPA Method 3550 (1), a sonication/extraction procedure, followed by gas chromatographic analysis using flame ionization detection. Johnsen and Starr (2) compared the sonication procedure with blender, roller, and Soxhlet extraction for pesticide-spiked soils. Sonication of the samples increased the recoveries of several compounds, although recoveries by Soxhlet extraction were comparable. Dunnivant and Elzerman (3) also found the sonication method to be equal to or more efficient than Soxhlet for extraction of PCBs (polychlorinated biphenyls) from sediments. Furthermore, sonication resulted in reduced sample preparation time and decreased volumes of solvents and sample, and allowed substitution of less expensive glassware.

Method 3550 was originally intended for use when extracting nonvolatile and semivolatile organics from solids such as soils. The need for a rapid, inexpensive screening method for use in guiding excavations of contaminated sites has resulted in variations of Method 3550 being applied to quantification of fuel contamination in soils. Such contamination often consists of unknown amounts of both gasoline and heavier weight diesel fuel; total petroleum hydrocarbons is then estimated by applying the adaptation of Method 3550 for low

[1] Address correspondence to this author.
Received May 17, 1989. Accepted September 30, 1989.

level concentrations. The method variations most commonly used include reductions in solvent volume, use of a tank style sonicator rather than a horn-type sonicator, and gravity filtration without the use of a Buchner funnel or centrifugation.

The purpose of the present investigation was to determine the analytical recovery of a mixture of 10 hydrocarbons representing a range of gasoline constituents from both dry and wet spiked soil. Soil samples were extracted using various modifications of EPA Method 3550, and recovery efficiencies were determined for each constituent. The effects of changing sonicator type and filtration apparatus on the efficiency of Method 3550 were not investigated.

METHODS

Experimental Design

Individual recoveries of each of 10 hydrocarbons (grand mix) added to air-dry and wet soils at concentrations of 200 mg/kg were determined using modifications of EPA Method 3550. Compounds examined were benzene, *n*-heptane, toluene, *m*-xylene, nonane, *n*-propylbenzene, 1,2,4-trimethylbenzene, *n*-butylbenzene, 1,2,4,5-tetramethylbenzene, and dodecane. These compounds are representative of the C_6–C_{12} ingredients commonly found in gasoline mixtures (4) and were selected, in part, because each could be resolved from the mixture by gas chromatography.

Decreasing the hot water bath temperature as a method of increasing recoveries was investigated by concentrating spiked samples of a mixture of acetone–methylene chloride (1 + 1) from 10 to 1 mL, using a micro-Snyder column. The change in recovery resulting from differing initial solvent volumes was determined by concentrating spiked samples of the solvent mixture from 400 or 250 to 10 mL. The effects of reducing solvent volume, changing solvent composition, and changing the order of solvent addition on soil extraction were also studied.

Recovery of unleaded gasoline from both dry and wet soil spiked at 500 mg/kg was then evaluated. The concentrations of chemicals used in this study were within the range found in actual contaminated soils.

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard 5890 gas chromatograph equipped with autosampler, flame ionization detector, and 15 m × 0.53 mm id DB-5 fused-silica capillary column (J & W Scientific) with film thickness 1.5 μm, and purified nitrogen as carrier gas. Operating conditions: injection port 240°C; detector 250°C; carrier gas flow rate 6 mL/min. Temperature program: initial temperature 40°C, held for 2.5 min; programming rate 5.3°/min to 120°C, held for 1 min.

(b) *Sonicator.*—Fisher 100 watt, tank-style.

(c) *Water bath.*—With concentric ring cover; maintains temperature within 2°C.

(d) *Glassware.*—20 mm id Pyrex chromatographic column with glass wool plug, pre-rinsed with both acetone and elution solvent; Kuderna-Danish evaporative flask, 500 mL (Kontes 570001-0500), with 10 mL graduated concentrator tube (Kontes 570050-1025), 3-ball macro-Snyder column (Kontes 503000-0121) and 2-ball micro-Snyder column (Kontes 569011-0219); Hamilton gas-tight microliter syringes; Teflon-lined screw-cap vials.

(e) *Boiling chips.*—Fisher Boileezers.

Reagents

(a) *Standard solution.*—Mixture containing all 10 hydrocarbons was prepared by adding 1 g of each compound to 25 mL volumetric flask and diluting to 25 mL with pesticide grade methanol. All reagents were obtained from Aldrich Chemical Co., 99+% pure.

(b) *Solvents.*—Methylene chloride and acetone, pesticide grade.

(c) *Sodium sulfate.*—ACS grade, granular, anhydrous. Heated 4 h at 400°C and stored in glass bottle.

Preparation of Spiked Soil

The soil used in this investigation was an Orovada fine sandy loam (coarse loamy, mixed mesic, Durixerollic Camborthid) with a pH of 8.0, cation exchange capacity of 6.8 cmol (+)/kg, organic matter content of 0.79%, column bulk density of 1.44 Mg/cu.m, porosity of 0.46, and specific surface area of 57.7 sq. m/kg. The soil was air-dried for 1 week and passed through a 1 mm sieve. Oven-dried percent moisture was 1.4% after heating to 105°C for 24 h.

For each experiment, 30 g air-dry soil was weighed into glass vials. For analysis of wet soils, 6 g water was added to the dry soil (21.6% moisture by weight on oven-dry basis, or 17.8% on wet-weight basis) and thoroughly mixed. Six soil samples were prepared for each experiment. The soil was then injected at various depths with a total of 0.15 mL grand mix per vial. The vials were sealed with Teflon-lined screw caps and shaken to mix completely. Soils were extracted by the methods indicated 20 h after spiking.

Extraction and Analysis

(a) *Water bath temperature variations.*—The first experiment was conducted to determine the effect of lowering the hot water bath temperature from 80°C (standard method) to 68°C and 58°C. For these experiments, 0.15 mL hydrocarbon mixture was added to 10 mL acetone–methylene chloride (1 + 1) in a concentrator tube. A clean boiling chip was added to the tube. The micro-Snyder column was placed on top of the concentrator tube and the apparatus was placed in a hot water bath. Three replications were run at each of the 3 temperatures. The time required for concentration to 1 mL was recorded. After concentration, samples were re-diluted to 10 mL using acetone, and were analyzed by gas chromatography. Two injections were made of each sample. A standard was prepared by placing 0.15 mL hydrocarbon mixture in a 10 mL volumetric flask and diluting to 10 mL with acetone. Percent recovery of each compound for each sample at each temperature was calculated by dividing averaged area units measured for the re-diluted, concentrated sample by the averaged area units measured for the standard.

(b) *Solvent volume concentrations.*—A second experiment was performed to determine relative recovery when concentrating differing volumes of solvent. Either 400 or 250 mL of acetone–methylene chloride (1 + 1) was placed in a 500 mL Kuderna-Danish (K-D) concentrator. Aliquots of 0.15 mL grand mix were then added to each concentrator. A clean boiling chip was added, and a 3-ball Snyder column was attached. The K-D apparatus was placed in a hot water bath at 80°C and the time required to concentrate to a final volume of 7 mL was noted. The K-D apparatus was allowed to drain and cool for a minimum of 10 min, and the joints and flask were then rinsed with 1–2 mL extraction solvent. The

Table 1. Mean percent recovery of individual hydrocarbons after concentrating from 10 to 1 mL at differing temperatures[a]

Compound	Temperature, °C		
	80	68	58
Benzene	27.5 (1.9)	37.5 (0.3)	53.9 (4.2)
n-Heptane	6.8 (1.1)	5.8 (0.7)	5.5 (0.9)
Toluene	77.8 (3.8)	85.6 (2.1)	88.1 (5.0)
m-Xylene	93.1 (3.4)	96.0 (2.9)	96.0 (2.0)
Nonane	84.1 (3.3)	90.3 (2.2)	91.3 (4.1)
n-Propylbenzene	96.3 (3.0)	97.4 (3.2)	98.2 (1.5)
1,2,4-Trimethylbenzene	96.6 (3.0)	97.8 (3.2)	98.4 (1.1)
n-Butylbenzene	97.1 (3.0)	98.7 (3.2)	99.6 (0.8)
1,2,4,5-Tetramethylbenzene	98.8 (3.0)	97.9 (3.4)	98.9 (0.4)
Dodecane	97.3 (2.9)	98.4 (3.3)	99.9 (0.4)

[a] Standard deviation in parentheses; n = 3.

Table 2. Mean percent recovery of individual hydrocarbons after concentrating to 10 mL[a]

Compound	Initial solvent volume, mL	
	400	250
Benzene	15.1 (0.7)	13.6 (2.8)
n-Heptane	0	0.4 (0.7)
Toluene	57.4 (1.2)	66.2 (4.1)
m-Xylene	90.0 (0.6)	94.6 (1.5)
Nonane	65.7 (0.6)	75.8 (4.0)
n-Propylbenzene	93.5 (1.3)	98.0 (1.1)
1,2,4-Trimethylbenzene	94.4 (1.4)	98.6 (1.3)
n-Butylbenzene	95.1 (1.5)	99.4 (1.7)
1,2,4,5-Tetramethylbenzene	95.1 (1.5)	99.1 (1.7)
Dodecane	94.9 (1.5)	99.1 (1.7)

[a] Standard deviation in parentheses; n = 4.

samples were re-diluted to 10 mL with acetone, and analyzed by gas chromatography, with 2 injections made of each sample. A standard was prepared by diluting 0.15 mL grand mix with acetone in a 10 mL volumetric flask. A total of 4 replications were made for each of the 2 solvent volumes. Recovery was measured as in (**a**).

(**c**) *Dry soil extractions.*—Sonication/extraction of the soil samples was conducted following EPA Method 3550 for low-level concentrations, with the substitution of a water bath sonicator and gravity filtration. The hydrocarbon-spiked, air-dry soil was placed in an Erlenmeyer flask. One hundred mL acetone–methylene chloride (1 + 1) was immediately added. The flask was placed in a sonicator and the sample was sonicated for 3 min. The extract was then filtered through a 10 cm column of anhydrous sodium sulfate and collected in a Kuderna-Danish (K-D) concentrator.

The sample was sonicated 2 additional times, adding 100 mL solvent mixture each time. The entire sample was poured into the filter after the final sonication, and the flask was rinsed with 50 mL of extraction solvent. The column was also rinsed with 50 mL extraction solvent, for a total solvent volume of approximately 400 mL (standard method). A boiling chip was then added to the evaporative flask and a 3-ball macro-Snyder column was attached. The K-D apparatus was placed in an 80°C water bath, and distilled until the apparent volume of liquid reached 10 mL. The K-D apparatus was then removed and allowed to cool for at least 10 min.

After cooling, the Snyder column was removed and the flask and lower joint were rinsed into the concentrator tube, using 1–2 mL extraction solvent. A 2-ball micro-Snyder column was then added to the concentrator tube, and the apparatus was placed in the 80°C water bath until the solvent was concentrated to 1 mL. The tube was then removed from the water bath and again allowed to cool for at least 10 min. The final volume was adjusted to 10 mL in a volumetric flask by adding acetone and shaking.

A portion of the final re-diluted concentrate was reserved in an autosampler vial with a Teflon-lined crimp cap for analysis. A standard was prepared by placing 0.15 mL of the hydrocarbon mixture in a 10 mL volumetric flask and diluting to volume with acetone. Aliquots (2 μL) of the extracts were analyzed by gas chromatography, with 2 injections made of each sample. Detector response was linear over the range of dilutions from 1:50 to 1:1, with a correlation coefficient of 0.999. The limit of detection was 1 ppm. Integrated values of duplicate injections were averaged for each extract. Recoveries of each constituent for each of 6 replicates were calculated by dividing the averaged area units for the extracted sample by the averaged area units for the standard.

A second soil extraction experiment was conducted to determine the effect of reducing the total extraction solvent volume from 400 to 250 mL. The method was done as previously described, except that only 50 mL of the extraction solvent mixture was used for each of the second and third extractions, and a total of 50 mL solvent was used to rinse both flask and column. Again, 6 replicates were performed. Concentration and analysis of the samples proceeded as before. Dry soil blanks were also extracted using 250 mL extraction solvent to check for soil contamination.

The effect of using methylene chloride alone instead of the methylene chloride–acetone (1 + 1) mixture as the extraction solvent was also investigated, using a total volume of 250 mL solvent as described for the second dry soil experiment. Six replicates were extracted by this method. Analysis proceeded as before.

(**d**) *Wet soil extractions.*—To test hydrocarbon recovery from wet soils, samples containing 21.6% moisture by weight on an oven-dry basis were prepared. Prior to extraction, each soil sample was mixed with 100 g anhydrous sodium sulfate to achieve a "free-flowing" texture. Extraction proceeded as before. A total of 4 experiments, each with 6 replicates, was performed. The first followed EPA Method 3550. The second used the reduced, 250 mL volume of methylene chloride–acetone (1 + 1) mixture. The third was identical to the second except that, prior to the first extraction, 50 mL acetone alone was added to the sample. The sample was then thoroughly mixed before the remaining 50 mL methylene chloride was added. Total solvent volume remained at 250 mL. The last experiment used 250 mL methylene chloride alone as the extracting solvent, following the procedure of the second wet soil experiment.

(**e**) *Gasoline extractions.*—To determine extraction efficiency (percent recovery) using an actual gasoline sample, 6 air-dry soil samples were spiked with unleaded regular gasoline at a total of 500 mg/kg (15 mg per 30 g soil). Loss by volatilization was avoided by transferring the gasoline from a gas-tight syringe into the center of the bulk soil. The sample was then thoroughly mixed. Extraction proceeded using a total of 250 mL methylene chloride alone as extracting solvent.

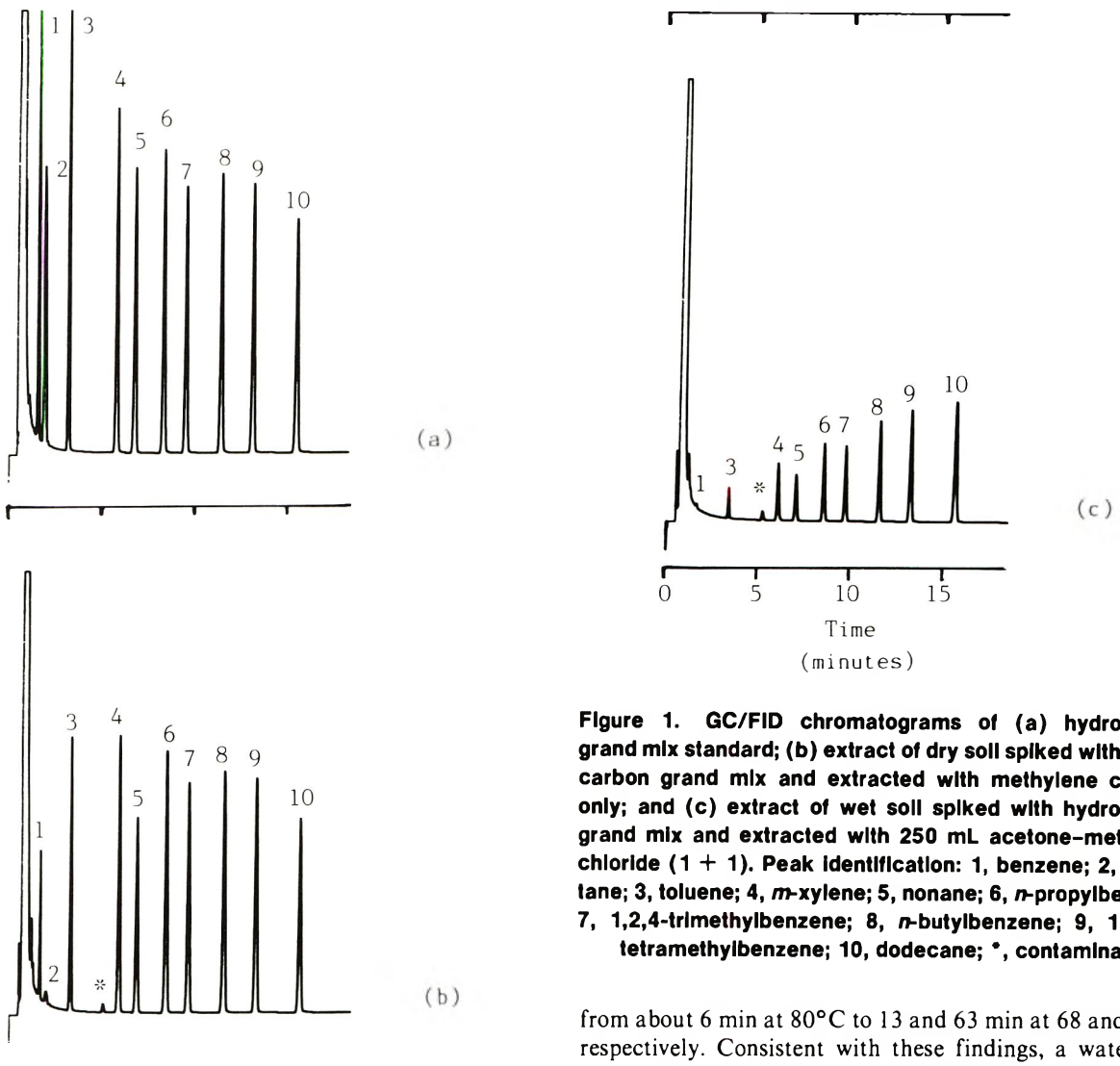


Figure 1. GC/FID chromatograms of (a) hydrocarbon grand mix standard; (b) extract of dry soil spiked with hydrocarbon grand mix and extracted with methylene chloride only; and (c) extract of wet soil spiked with hydrocarbon grand mix and extracted with 250 mL acetone–methylene chloride (1 + 1). Peak identification: 1, benzene; 2, *n*-heptane; 3, toluene; 4, *m*-xylene; 5, nonane; 6, *n*-propylbenzene; 7, 1,2,4-trimethylbenzene; 8, *n*-butylbenzene; 9, 1,2,4,5,-tetramethylbenzene; 10, dodecane; *, contaminant.

A second set of 6 soil samples was also spiked with 15 mg gasoline following the addition of 6 g water (21.6% moisture by weight on an oven-dry basis). These wet soil samples were extracted using 250 mL acetone–methylene chloride (1 + 1) following the addition of 100 g anhydrous sodium sulfate. Acetone was added separately to the samples prior to the addition of methylene chloride for the first sonication. A standard was prepared by placing 15 mg gasoline in a 10 mL volumetric flask and diluting to 10 mL with acetone.

Total petroleum hydrocarbons were quantitated by deleting any solvent or contaminant area units from the total area units for both the standard and the sample extract. Contaminants were determined from chromatograms of the soil blanks, and solvent peak retention time was used to determine solvent area units.

Results and Discussion

The effect of reducing the concentrating temperature on grand mix hydrocarbon recovery is presented in Table 1. Although reducing the temperature to 68 or 58°C generally gave an apparent increased recovery, the difference was statistically significant at $P < 0.05$ only for benzene and toluene, when tested for comparison of means by the least significant difference (LSD) method, following analysis of variance (ANOVA). The time required for concentration increased from about 6 min at 80°C to 13 and 63 min at 68 and 58°C, respectively. Consistent with these findings, a water bath temperature of 80°C was used for the remaining recovery studies.

Decreasing the solvent volume resulted in an increase in recovery, as seen in Table 2. Mean recovery was significantly

Table 3. Mean percent recovery of individual hydrocarbons from dry, spiked soil after sonication/extraction and concentration to 1 mL[a]

Compound	Method 1[b]	Method 2[c]	Method 3[d]
Benzene	2.1 (0.3)	2.7 (0.6)	20.6 (3.4)
n-Heptane	0	0	5.2 (1.8)
Toluene	31.7 (1.9)	34.3 (3.0)	67.7 (2.9)
m-Xylene	69.3 (2.3)	71.0 (4.4)	92.9 (4.1)
Nonane	42.9 (2.6)	44.4 (3.2)	78.6 (5.3)
n-Propylbenzene	81.1 (2.2)	82.4 (5.3)	99.3 (4.6)
1,2,4-Trimethylbenzene	84.3 (2.3)	85.2 (5.2)	99.2 (5.1)
n-Butylbenzene	86.4 (2.5)	87.0 (4.8)	99.2 (4.6)
1,2,4,5-Tetramethylbenzene	87.3 (2.5)	87.4 (4.1)	97.5 (4.6)
Dodecane	86.1 (2.5)	85.9 (2.9)	92.5 (4.8)

[a] Standard deviation in parentheses; n = 6.
[b] Method 1: EPA Method 3550; 400 mL acetone–methylene chloride (1 + 1) as extracting solvent.
[c] Method 2: 250 mL acetone–methylene chloride (1 + 1) as extracting solvent.
[d] Method 3: 250 mL methylene chloride alone as extracting solvent.

Table 4. Mean percent recovery of individual hydrocarbons from wet, spiked soil after sonication/extraction and concentration to 1 mL[a]

Compound	Method			
	1[b]	2[c]	3[d]	4[e]
Benzene	0.1 (0.1)	0.1 (0.1)	0 (0)	3.4 (0.8)
n-Heptane	0	0	0	0.5 (0.3)
Toluene	8.7 (1.3)	8.9 (1.9)	8.9 (1.5)	10.8 (2.1)
m-Xylene	24.8 (1.6)	21.6 (3.9)	23.3 (2.5)	20.2 (3.7)
Nonane	16.9 (2.6)	17.6 (1.5)	18.5 (3.6)	23.7 (3.3)
n-Propylbenzene	35.1 (2.2)	30.4 (4.9)	33.4 (3.2)	28.1 (4.4)
1,2,4-Trimethylbenzene	39.7 (2.5)	34.0 (5.5)	37.2 (3.4)	31.3 (4.7)
n-Butylbenzene	46.6 (3.0)	41.8 (5.9)	45.5 (3.7)	37.1 (4.9)
1,2,4,5-Tetramethylbenzene	51.8 (3.6)	48.2 (7.0)	51.9 (4.2)	40.4 (4.2)
Dodecane	60.2 (6.3)	59.2 (8.4)	63.4 (5.7)	47.4 (3.1)

[a] Standard deviation in parentheses; n = 6.
[b] Method 1: EPA Method 3550; 400 mL acetone–methylene chloride (1 + 1) as extracting solvent.
[c] Method 2: 250 mL acetone–methylene chloride (1 + 1) as extracting solvent.
[d] Method 3: 250 mL acetone–methylene chloride (1 + 1) as extracting solvent; acetone added separately prior to methylene chloride.
[e] Method 4: 250 mL methylene chloride alone as extracting solvent.

better at $P < 0.05$ for all compounds except benzene and *n*-heptane, as determined by ANOVA and LSD. The time required to concentrate was also reduced from 32 min for a 400 mL volume to 15 min for a 250 mL volume.

Soil blanks were analyzed prior to extraction of the spiked soil samples. Analysis revealed a single contaminant peak with a retention time of 5.2 min, which did not interfere with the peaks of any of the hydrocarbons used in the study. The retention time was used to identify the contaminant area units when total petroleum hydrocarbons were measured. Typical chromatograms of standards and soil extracts are presented in Figure 1.

Mean percent recovery of individual hydrocarbon constituents for air-dry and wet soil is presented in Tables 3 and 4. All means were tested for significance by using analysis of variance and comparison by least significant difference methods. Significantly reduced recovery occurred in all cases for wet soils ($P < 0.01$). This may be attributed, in part, to the development of an electrostatic hydration envelope surrounding the soil-adsorbed organic compounds. When these highly hydrated soils are subjected to the extracting solvent methylene chloride, the soil particles tend to flocculate, causing entrapment and reduced organic extraction (5, 6). The addition of sodium sulfate and subsequent mixing also allows for increased loss by volatilization.

Overall, mean hydrocarbon recovery in air-dried soils of all but the most volatile compounds was statistically equivalent when the solvent volume was decreased to 250 mL ($P < 0.05$). Use of methylene chloride alone resulted in a significant increase in recovery ($P < 0.05$). Although not as consistent, recovery from wet soil was also statistically equivalent ($P < 0.05$) when solvent volume was reduced, except for the more volatile constituents. Recovery of benzene, n-heptane, and nonane from wet soils was improved when methylene chloride was used alone as the extracting solvent, but the recovery of other constituents was significantly decreased ($P < 0.05$). Total time of concentration was also reduced from 30 to 20 min for the reduced solvent volume. Despite a decrease in precision as measured by standard deviation of the mean, the time and material saved by decreasing solvent volume coupled with comparable recovery justifies procedural modification.

Since many soil samples contaminated with gasoline are taken from very wet areas such as the capillary fringe, current estimates of total petroleum hydrocarbons based on standard analytical procedures may be understated. Unfortunately, drying of the soil samples prior to analysis is undesirable, due both to the loss of more volatile compounds and to increased adsorption which occurs as the water evaporates. However, the addition of acetone separately to wet soils prior to the first extraction when solvent volume is reduced does allow for improved soil dispersion and hence similar extraction efficiency when compared to the standard method.

Using methylene chloride alone as the extraction solvent tended to increase the recovery of the more volatile compounds (Table 3). However, since it is common to use purge-and-trap techniques to analyze for the more volatile benzene/toluene/xylene fraction, and since it is rare for field soil samples to be air-dry, use of methylene chloride alone as the extraction solvent may not be justified for total petroleum hydrocarbon analysis.

Chromatograms of the unleaded gasoline standard and the gasoline-spiked soil extract are illustrated in Figure 2. Average total petroleum hydrocarbon recovery over 6 samples of dry soil was 43.2%, with a standard deviation of 3.1. Mean recovery from wet soil was 21.8%, with a standard deviation of 4.2. Recovery was significantly greater from dry soils ($P < 0.01$) than from wet soils. As expected, recoveries of the most volatile constituents were the lowest. However, this method does allow determination of constituents with volatilities greater than or equal to xylenes. Since many fuel-contaminated soil samples may contain both gasoline and higher molecular weight hydrocarbons, such as those present in diesel fuel, this method provides a useful screening technique for total petroleum hydrocarbons, when the expected percentage of recovery is considered. An accurate determination of the most volatile constituents will require purge-and-trap or other techniques which give greater recovery of these constituents.

Acknowledgments

This research was funded in part by a grant from the Nevada Division of Environmental Protection.

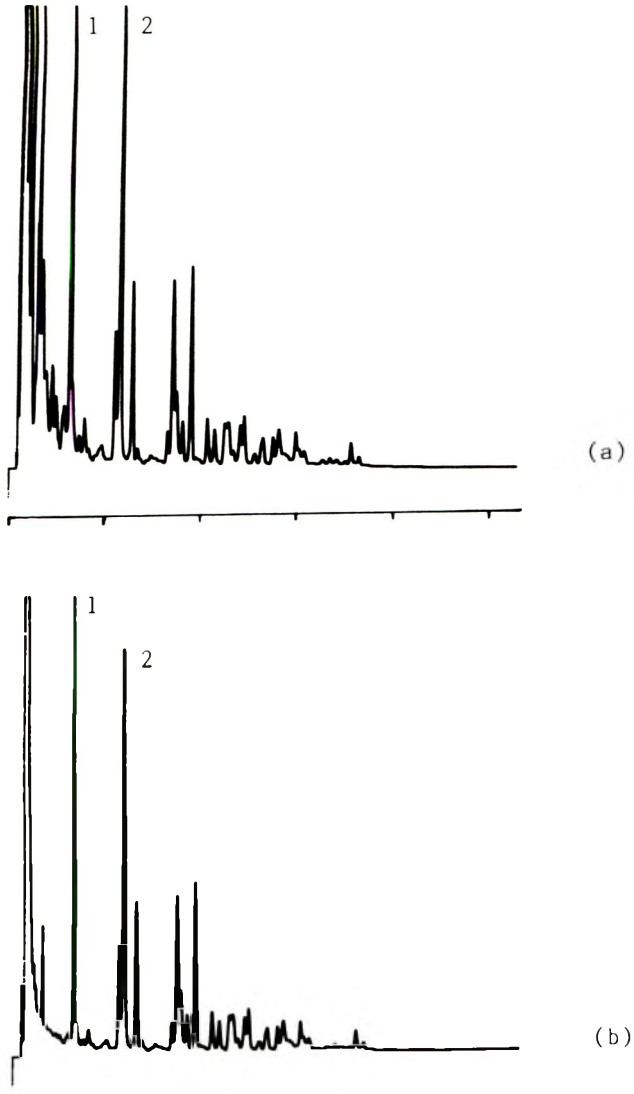


Figure 2. GC/FID chromatograms of (a) unleaded gasoline standard; (b) extract of dry soil spiked with unleaded gasoline and extracted with methylene chloride only; and (c) extract of wet soil spiked with unleaded gasoline and extracted with 250 mL acetone–methylene chloride (1 + 1). Peak identification: 1, toluene; 2, *m*-xylene.

REFERENCES

(1) U.S. Environmental Protection Agency (1986) *Test Methods for Evaluating Solid Waste*, Vol. 1B: Lab Manual, Physical/Chemical Methods, Report No. SW-846, EPA, Washington, DC

(2) Johnsen, R. E., & Starr, R. I. (1972) *J. Agric. Food Chem.* **20**, 48–51

(3) Dunnivant, F. M., & Elzerman, A. W. (1988) *J. Assoc. Off. Anal. Chem.* **71**, 551–556

(4) Verschueren, K. (1983) *Handbook of Environmental Data on Organic Chemicals*, Van Nostrand Reinhold Co., New York, NY

(5) Bohn, H. L., McNeal, B. L., & O'Connor, G. A. (1985) *Soil Chemistry*, 2nd Ed., Wiley-Interscience, New York, NY

(6) Hillel, D. (1980) *Fundamentals of Soil Physics*, Academic Press, New York, NY

PLANTS

Near Infrared Reflectance Spectroscopy. I. Calibration Techniques for Forage Quality Assessment

FRANKLIN E. BARTON II, and G. W. BURTON
U.S. Department of Agriculture, Agricultural Research Service, R. B. Russell Agricultural Research Center, PO Box 5677, Athens, GA 30613
W. G. MONSON
Coastal Plains Experiment Station, Tifton, GA 31793

It is very time consuming and resource costly to rank forages by wet laboratory methods; therefore, near infrared reflectance spectroscopy (NIRS) was used to evaluate hybrid bermudagrass (*Cynodon dactylon* (L.) Pers.) cultivars for improvements in quality. Quality was assessed by the determination of acid detergent fiber (ADF), crude protein (CP), dry matter (DM), and in vitro dry matter digestibility (IVDMD). The samples were taken from plant breeding trials from 1983, 1984, 1985, and 1986. They included 32 cultivars, 4 cuttings, and 5 replications each for a total of 640 possible observations per year. This paper describes the process by which the NIR spectrometer was calibrated for each constituent and the effect of various methods of calibration on the performance of the NIRS system to determine quality in bermudagrass hybrids. Excellent one-year equations were obtained from calibrations using the bermudagrass parents in the 1983 samples (R^2 = 0.83, 0.97, 0.70; SEC = 0.94, 0.76, 3.64, for ADF, CP, and IVDMD, respectively). Attempts to use outside populations did not produce equivalent results for the first year. The 1983 equations did not fit the 1984 and 1985 samples because of changes that were made to the spectrometer and the yearly differences in the samples. The instrument was then recalibrated with a diverse set of legumes and grass hay samples that contained some bermudagrass. These open population calibrations adequately ranked the bermudagrass entries. We documented that bermudagrass genotypes can be quickly and precisely ranked for forage quality traits via use of open population NIRS calibration equation.

In any plant breeding program, a means of assessing germplasm improvement is required. The Tilley-Terry (1963) 2-state in vitro dry matter digestibility determination has been successfully used as a determinant of quality in forage breeding programs (1–4). Near infrared reflectance spectroscopy (NIRS) was first used in a plant breeding trial by Marum et al. (5). Barton and Burdick (6) and later Burdick et al. (7) developed NIRS calibrations and validation of regression equations with NIRS for protein, fiber, and lignin in bermudagrass on a tilting filter spectrometer.

Bermudagrass presented these authors with some unique problems. First, the composition of bermudagrass could not be determined with equations that adequately represented other warm and cool season forages. Second, in vitro dry matter digestibility (IVDMD) did not correlate well with NIRS spectra for bermudagrass. The limited number of samples that could be incorporated in the calibration (40) for instruments at that time obviously contributed to the difficulty, as did some problems with acquiring reliable IVDMD data.

In his chapter on NIRS technology transfer in the *USDA Handbook 643*, Marten described how NIRS could be used in forage quality studies (8), for example, plant breeding programs involving multiple-year studies. The objective of the present study was to examine various types of calibration and establish one that could be used to rank cultivars and yield other analytical information.

Experimental

Experiment 1

This initial experiment was done to acquire a calibration that would provide analytical information on the first year's samples. A "closed population" calibration, where the samples for calibration were obtained from the same population as those to be evaluated, was obtained. The description of these sample sets follows:

1983 Calibration samples.—A set of 65 samples from the 1983 harvest representing all 32 entries (at least 2 samples of each entry) in the bermudagrass variety trial of Burton et al. (personal communication) were analyzed for in vitro dry matter digestibility (IVDMD), crude protein (CP), and acid and neutral detergent fiber (ADF and NDF) as described by Barton and Burdick (6), Barton and Windham (9), and Tilley and Terry (10). The samples were scanned on a Pacific Scientific Model 6100 near infrared spectrometer in a diffuse reflectance mode. The spectrometer was interfaced to a Digital Equipment Corp. (DEC) series PDP 11/34 mini-computer where the spectra were stored in a file through the use of the USDA/Pennsylvania State University software system operating under the DEC single-user Operating System RT-11 version 3B. Original equations were generated by programs SET and MWS (11).

1983 Samples for evaluation.—The NIR spectrum from 1100 to 2500 nm of the entire 640 bermudagrass sample set representing 32 entries, 5 replications, and 4 harvests from the 1983 season was scanned as above and stored in a file.

1984–1986 Calibration samples.—The next task was to update and/or obtain a robust calibration suitable for "all" bermudagrass samples. As such, "open population" calibrations, where the calibration samples came from a population other than the samples to be evaluated, were developed using bermudagrass only and broad-based sample sets.

Outside bermudagrass population.—The spectra of a set of 221 bermudagrass samples representing 13 cultivars, 4 harvests, and 2 years from a variety trial in Watkinsville, GA, were scanned on a Pacific Scientific Model 6350 (same instrument as above, but upgraded) NIR spectrometer and

Received December 12, 1988. Accepted August 25, 1989.

stored in the DEC computer. The software was analogous, but updated as described by Shenk in Marten et al. (8).

Broad-based population samples for calibration.—The spectra of 454 samples were combined with those of the 221 samples described above. This set (454) was a subset of some 2500 samples of legumes and grass hays collected by Abrams (12) from all 50 states and Puerto Rico that contained all the spectral variability of the parent sample set. This formed a broad-based calibration set suitable for the determination of quality in most forage species.

The analysis of the 221 bermudagrass samples for CP, ADF, and IVDMD was conducted as above (1983 calibration samples). The analysis for CP and IVDMD on the 454 samples was conducted at Pennsylvania State University as described by Abrams (12). Analysis for ADF of those samples was conducted in our laboratory as described in Barton and Windham (9).

The calibration and file transfer were performed as described in Shenk (13), using programs CAL, BEST, STAT, and NIRTRN. Computations were performed on both the DEC PDP 11/34 and a Microvax II superminicomputer.

1984–1986 Bermudagrass samples for evaluation.—The approximately 2600 samples in this part of the study were treated as above for the acquisition of spectral data. These samples were obtained from a bermudagrass breeding program and constituted 32 entries, 4 harvests, 5 replications, and 4 years (2 different trials in 1985). The samples were ground in a Udy mill in Tifton, GA, to pass a 1 mm screen, as were all other samples. Three separate Udy mills were used in the calibration and determination phases to eliminate or compensate for grinder differences that affect the particle size of the sample and its spectrum.

Experiment 2

The CAL program was used to develop true closed calibration equations for the bermudagrass files from 1983 to 1986. The samples to be used for calibration were chosen by the program SUBSET described in Shenk (13). SUBSET groups nearest neighbors from a comparison of their spectra and selects the minimum number of samples to describe the diversity of the population. The program was used in 2 ways: First, SUBSET was run on each year's data set and the samples selected were combined into a single file of 164 samples. Second, SUBSET was run on the entire file of 3195 samples, resulting in a file of 111 samples. CAL was run on each of these files as well as the 3195 sample files, reserving every 13th sample (i = 13) for use to evaluate the equations and to measure the standard error of selection (SES). The population sampling program REG70 was used on the same files to generate equations and to measure predicted error sums of square (PRESS), which is the standard error of performance (SEP) for the selected equation.

Table 1. Calibration and validation statistics for 1983 mameter of bermudagrass

Analysis[a]	SEC[b]	R^{2c}	SEP[d]
ADF	0.94	0.83	0.7
NDF	1.12	0.84	1.4
CP	0.78	0.97	0.8
IVDMD	3.64	0.68	2.7

[a] ADF = acid detergent fiber; NDF = mutual detergent fiber; CP = crude protein; IVDMD = in vitro dry matter digestibility.
[b] Standard error of calibration.
[c] Coefficient of determination.
[d] Standard error of performance corrected for bias.

The data for calibration were obtained from the co-author's laboratory. Single determinations were run on all 3195 samples. In the companion study, field replicas and years were averaged to yield 32 numbers to rank the entries. Here, each value was used as a separate determination since each sample has a separate spectra. What was lacking from multiple determinations to improve precision was compensated for by the large sample set or by the selection of the appropriate samples from their spectra to give the smallest data set that still contained all the diversity.

Calibration Methodology

Numerous steps precede a calibration that affect the character and type of calibration selected. These are discussed in the *USDA Handbook 643* (8) in general terms; in the revised edition (in press), specific guidelines are presented in an appendix chapter. The first step is to define the population to be analyzed.

The 2 basic types of populations are open and closed. In the open population, new samples are frequently added during intervals of recalibration. This leads to a calibration that is robust and able to handle variations in harvests, years, moisture, species, drying conditions, and management practices. In a closed population, all samples are available at the time of calibration and, therefore, all of the variation as well. The plant breeder's situation is intermediate between these conditions. Initially, most of the anticipated variation in study should be present in the calibration sample set and, thus, a subset of the year's trial should yield a good calibration. There are, however, multiple years to be considered as well as the overriding factor that change and outliers to the general norm of the population are what the breeder desires. If the breeder uses closed populations for assessing the selection entries, the assumption is made that all variation to be found is contained in the calibration set. Therefore, where this may or may not be the case, open population calibrations could have an advantage for breeding purposes.

Once the population has been defined, it is necessary to choose the calibration set and to collect the NIR spectral data. Concurrently, the calibration samples should be analyzed by the reference laboratory method to minimize the possibility of changes in the sample chemistry with time. This is particularly important to plant breeders who collect samples over a period of several years, as in this study. The instrument is usually calibrated by regression analysis; in this study, the analysis was accomplished with software developed for the National NIRS Research Project (8). The best equation of several possibilities is selected by its performance on a validation set. The best choice for a validation set is a separate population (open calibration) or another subset of the current population (closed calibration).

Results and Discussion

Experiment 1.—Four approaches were tried to establish the calibration. The first approach (Experiment 1) was to obtain a calibration that could be used for year one (1983). Sixty-five samples from the 1983 samples that represented at least 2 samples of each cultivar were analyzed in the laboratory. The laboratory data were regressed against their respective spectral intensities. Table 1 shows the standard errors of calibration and performance (SEC and SEP) and coefficients of determination (R^2, square of the regression

coefficient). In this case, the validation set was a randomly selected set of 30 samples from the 1983 harvest (Table 1). The SEC and SEP data obtained from this internal validation of a closed calibration were quite acceptable. The SEP value for each analysis was within laboratory error limits of Barton and Burdick (6); standard error of performance for NDF was a little higher (1.4 vs 1.0–1.2).

The necessity for an open calibration or the recalibration of a closed set became obvious when spectra of the 1984 samples were taken and the 1983 equations were used to determine ADF, CP, and IVDMD. In the interim year, the monochromator was upgraded from Model 6100 to Model 6350. The RMS (root-mean-square) noise level of the instrument was reduced significantly from the 100–150 microoptical density units (μOD) to the < 26 μOD level. While this should not have affected the equations from previous calibrations, other changes to the samples could have occurred that caused the equations no longer to be valid. A second approach was tried because the amount of sample available from the genotypes was not sufficient to allow conventional laboratory analyses to be conducted. A set of 221 bermudagrass samples from a trial at a separate location had been analyzed and their spectra were recorded on the NIRS instrument. Calibrations were obtained and these equations were used to determine the composition (ADF, CP, IVDMD) of the 1983 and 1984 samples. Because of differences in the years of harvest (221 samples were collected from 1981 to 1982) and because the sample spectra were taken on the 6100 version of the monochromator, this approach did not work well either, even though excellent calibrations were obtained as shown by the SEC and R^2 values in Table 2.

Shenk (13) described the new software (TRNFSR) for the transfer of equations from one monochromator to another. A second program NIRTRN described in the *USDA Handbook 643* (8) will match 2 instruments/monochromators wavelength-for-wavelength with the same transfer set of samples. This permitted a third and fourth approach to be tried.

A large set of forages representing many species and regimes (12) was transferred to the Model 6350, as were the 1983 samples and the outside bermudagrass sample file of 221 samples. The spectra in these files were matched to those of 1984–1986 (4 files). The third approach was to add the original 65 samples to the broad-based data set. This did not give sufficient bermudagrass character to the calibration. The fourth approach was to add the 221 bermudagrass samples to the broad-based set (454 samples). This gave a calibration population totally separate from the samples to be analyzed. The results of this calibration are in Table 3. The SEP data were virtually identical to the SEC data for ADF and CP. The samples used for validation were the sixty-five 1983 samples that were in sufficient quantity to allow conventional analyses. The SEP data for IVDMD were somewhat larger, but this reflects errors in NIRS as well as errors in spectral transfer of the 1983 samples and in the IVDMD data. The data in the calibration came from multiple laboratories whereas the 1983 samples were analyzed exclusively in our laboratory. This could have resulted in a bias that the calibration would have averaged.

Table 2. Different population—different set of bermudagrass hybrids[a]

Analysis	SEC	R^2	SEP
ADF	1.11	0.91	—
NDF	1.39	0.85	—
CP	0.63	0.85	—
IVDMD	1.67	0.82	—

[a] See Table 1 for definitions.

Table 3. Add similar set to standard set—476 samples from across United States with 221 bermudagrass samples added (total of 797 samples for calibration)[a]

Analysis	SEC	R^2	SEP
ADF	2.48	0.79	2.54
CP	0.86	0.94	0.86
IVDMD	3.10	0.82	5.25

[a] See Table 1 for definitions.

The ranking of the 32 entries by NIRS and conventional IVDMD data picked the same entry as best (Burton, personal communication). The ranking of entries 2–10 was similar, but not identical; they spanned an IVDMD difference of only 3.50 percentage units. This precision is well within the standard error of performance of 5.25 and just slightly higher than the standard error of calibration of 3.10 percentage units. Throughout the remainder of the entries, minor differences in ranking occurred between NIRS and conventional IVDMD data. There was no intent to determine accuracy per se because that is not possible for an empirical method. This is the first time that an open population NIRS equation has been used to rank genotypes in a forage breeding program. The speed and precision of the NIRS analysis were certainly an advantage in ranking digestibility of the germplasm, even though a general purpose open calibration was used. In addition, the NIRS method provided an independent check on the laboratory-generated IVDMD data.

The mean, standard deviation, and range (minimum and maximum) of the various data sets for calibration and the 5 years of the bermudagrass variety trials are given Tables 4–6. That the calibration sets are appropriate can be judged from several points: First, the average percentage IVDMD of the means for the bermudagrass trials for which the equations were developed was 55.16 (56.74 without the 1983 data). This mean and the mean of the broad-based set and that for the bermudagrass calibration set from the separate study (57.45 and 57.79, respectively) were within experimental error of the in vitro procedure (± 2.75). Also, the standard deviation of the calibration sets indicates that sufficient vari-

Table 4. Variability of data sets for IVDMD

Data set[a]	Mean	SD	Range	
			Min.	Max.
1983	48.86	5.36	30.48	63.42
1984	56.94	4.27	44.55	69.86
1985a	55.25	6.34	40.90	73.80
1985b	56.62	5.46	43.79	69.80
1986	58.13	5.20	42.11	70.89
Calibration:				
Broad-based	57.45	8.08	22.50	76.13
Bermudagrass	57.79	3.80	49.86	69.38
1983 CALSET	45.42	6.42	39.39	58.03

[a] 1983–1985a represent one trial; 1985b–1986 represent second trial.

Table 5. Variability of data sets for CP

Data set[a]	Mean	SD	Range Min.	Range Max.
1983	12.60	3.95	4.98	22.76
1984	8.50	2.14	3.58	16.89
1985a	8.36	2.67	3.32	21.21
1985b	10.56	3.16	4.08	18.27
1986	10.92	2.82	5.25	19.34
Calibration:				
Broad-based	15.51	5.41	4.21	27.76
Bermudagrass	12.57	1.59	8.26	20.06
1983 CALSET	14.52	4.33	7.30	22.60

[a] 1983–1985a represent one trial, 1985b–1986 represent second trial.

Table 6. Variability of data sets for ADF

Data set[a]	Mean	SD	Range Min.	Range Max.
1983	31.08	3.94	19.13	41.64
1984	38.31	2.95	25.96	44.77
1985a	37.97	3.68	24.80	46.96
1985b	37.35	4.68	24.73	48.20
1986	34.82	3.15	25.79	44.72
Calibration:				
Broad-based	35.20	5.61	18.71	55.20
Bermudagrass	34.20	3.35	25.84	39.72
1983 CALSET	31.51	2.30	27.08	35.72

[a] 1983–1985a represent one trial, 1985b–1986 represent second trial.

ability was present, and the ranges indicate that the IVDMD data of the sets to be determined fell within the range of the calibration. Second, the data in Tables 5 and 6 reveal that the same is true for CP and ADF data. The data sets whose values were determined by NIRS were within those used for the calibration. The fact that the ranges and means coincided for 3 variables is an indication that the population to be analyzed was well represented in the calibration set.

The average spectra of each year (1984–1986) were very similar (Figure 1). Resolution in these average spectra was enhanced by Fourier Self-Deconvolution (FSDC). This technique would make small differences more obvious than would be apparent from the normal logarithm of reciprocal reflectance. The average spectrum for the calibration set (Figure 2) is virtually identical to that of the samples whose composition was to be determined. The figures provide evidence that the calibration was adequate for the determination of IVDMD, ADF, and CP in a bermudagrass breeding trial, and that open NIRS calibration equations can be used to rank forages.

Experiment 2

The closed population calibration results from program CAL are given in Table 7. The equation selected from the use of all 3195 samples was not as good as the equation from the open population calibration with less than 1/4 the samples. The SEC values were 3.100 (open) vs 5.654 (closed) and the R^2 values were 0.82 vs 0.33, respectively (see also Table 3). The same is true for the comparison of SES (5.878) vs SEP (5.250) data between the open and closed population equations. Essentially, the 2 equations had similar levels of accuracy (SES vs SEP), but the precision of the open population equation (SEC) was better.

The program SUBSET was run in 2 ways. First, it was run to simulate the smaller mini and microcomputers. On the DEC PDP series computer, the file for SUBSET is limited to

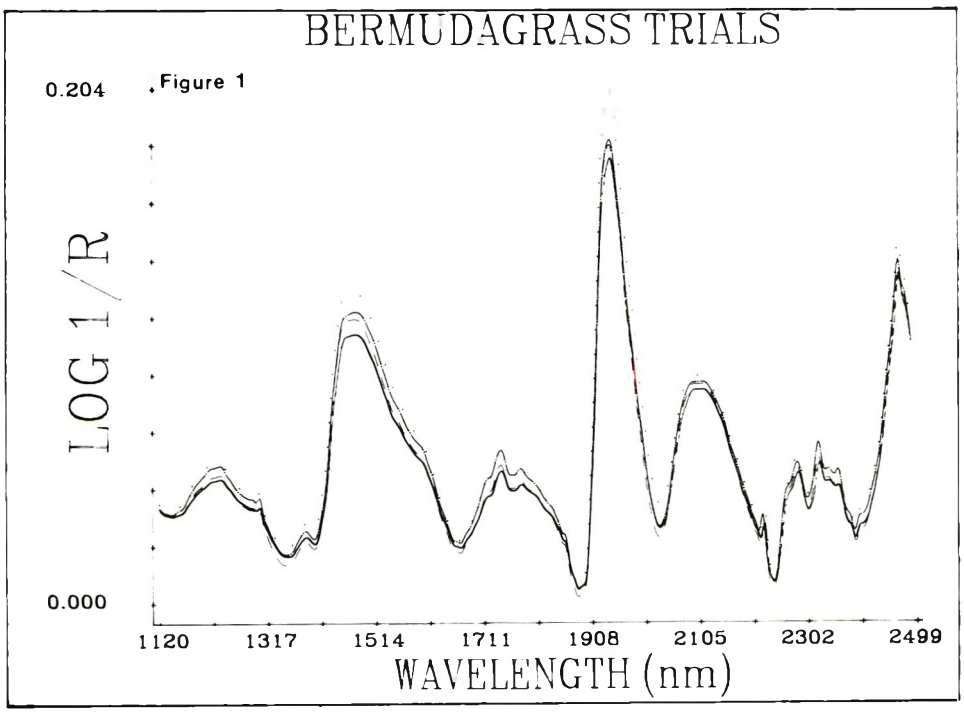


Figure 1. Plot of Fourier Self-Deconvolution of logarithm or reciprocal reflectance vs wavelength in nm of average spectrum from each of 4 years of bermudagrass breeding trials. Solid = 1984 trial 1; dashed = 1985 trial 1; dotted = 1985 trial 2; dashed-dotted = 1986 trial 2.

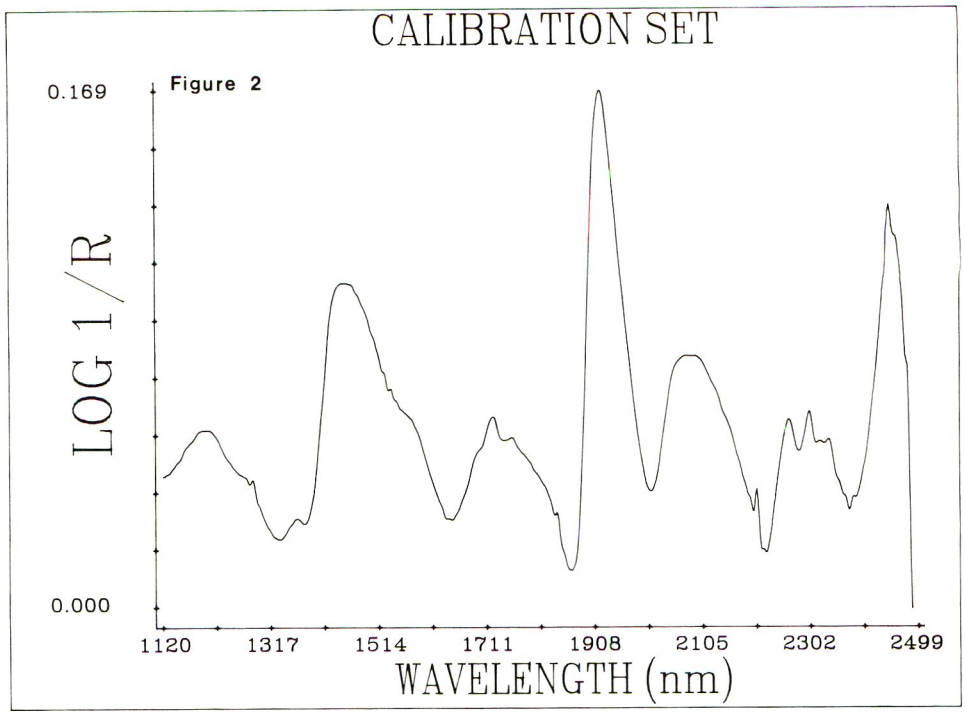


Figure 2. Plot of Fourier Self-Deconvolution of logarithm of reciprocal reflectance vs wavelength in nm for average of large calibration set.

100 samples because of available memory. This would require breaking the file for each year into 7 files and summing 35 such files for all 5 years of data. The newer computers could handle a year at a time so we would only have to sum the 5 files to generate the data set. When this was done, the file contained 164 samples, which represented all the diversity in each of the 5 years data set. It is obvious that the combined file would contain some redundancy, but not nearly as much as if each year's data set were divided into 7 separate files to accommodate a 100 sample per file program limit. There was no improvement in accuracy in the equation chosen from this file (Table 7 File/SS) over CAL on the whole file, but only 1/20th of the samples were used. The precision as measured by SEC did improve.

Second, SUBSET was run on the whole 3195 sample file on the DEC MicroVAX II, which selected only 111 samples. The equation from CAL selected here was better than any other (SEC = 2.942, SES = 1.740), which would be expected, since it should most closely represent the population to be analyzed. Note that the mean of the calibration file increased from 57.973 to 62.722 when SUBSET was used to select the calibration population. This is an indication that the original population contains redundant samples which can overly weight the calibration.

When the means, standard deviations, and ranges of IVDMD data were examined (Table 8), the means were similar for the subset compared with the entire file. The standard deviations were larger, indicating a more diverse sample set in the subset files. The ranges were somewhat narrower in the subsets, which may reflect the true variation in the laboratory data. That is, the samples for which the computer found the spectra to be identical had a variation of ±4-5 IVDMD units.

Table 7. Closed population calibration, from CAL (3195 samples)[a]

File	N	SEC	R^2	No.	EX[b]	N	SES[c]	Bias
CAL (i = 13)	2922	5.654	0.33	6	80	243	5.878	−0.002
File/SS (i = 13)	164	4.688	0.65	6	6	13	5.810	2.157
ALL/SS[d] (i = 13)[e]	111	2.942	0.85	5	5	9	1.740	0.161

[a] Subset (SS) tends to pick higher quality samples means; 57.973 < 60.571 < 62.722.
[b] EX = number of samples excluded by computer from calibration (residuals were as high as + 20 IVDMD units).
[c] SES = standard error of selection corrected for bias.
[d] One term EQA from ALL better than 6 term from File/SS, F's much smaller (subset on 5 files and then combined).
[e] Subset of large (3195) file.

Table 8. Mean, standard deviations, and ranges of IVDMD data for each year's data and the subsets[a]

File	Mean	SD	Min.	Max.
1983	63.390	5.957	44.000	76.400
1984	53.298	5.577	37.800	80.100
1985a	55.557	6.333	41.300	73.600
1985b	59.463	6.481	39.100	72.700
1986	58.227	5.723	32.500	70.800
ALL	57.975	6.940	32.500	76.400
83SS	63.824	6.956	44.200	75.800
84SS	56.818	6.790	41.500	70.100
85SS	53.317	5.919	41.600	67.200
85SS	58.146	7.228	48.600	67.800
86SS	57.075	6.880	44.200	66.800
File/SS	60.561	7.867	41.500	75.800
ALLSS	62.647	7.440	44.200	75.800

[a] 1983, 1984, 1985a are files of all samples in 1st trial for these years; 1985b, 1986 are the 2nd trial. ALL is the entire 3195 samples; SS denotes subset of above files; File/SS is the individual SS files combined; ALLSS is the subset of all 3195 samples.

Table 9. Standard errors of calibration and performance for closed population calibration equation for IVDMD by a population sampling technique[a]

File	SEC	R^2	No. of Samples	SEP
All	5.54	0.36	3195	5.59
ALLSS	2.03	0.92	111	2.60
File/SS	3.22	0.81	164	3.64
1986	4.55	0.04	638	4.69
1985b	5.26	0.06	609	5.42
1985a	6.34	0.00	639	6.61
1984	1.61	0.91	639	1.66
1983	1.83	0.90	640	1.88

[a] 1983, 1984, 1985a are files of all samples in 1st trial for these years; 1985b and 1986 are the 2nd trial. All is the entire 3195 samples; SS denotes subset of above files; File/SS is the individual SS files combined; ALLSS is the subset of all 3195 samples.

Another way to assess the data set and acquire a closed population calibration is a population sampling technique. In this technique, there are no wavelength selection criteria. Wavelengths are picked evenly across the spectrum with as many as 70 terms in the resulting regression equation, hence the name REG70. The program is best suited to closed populations and ones in which the calibration subset has been selected to represent the population diversity with the minimum of samples. Table 9 contains the SEC and SEP data for the files from each year (e.g., 1983, etc.), the total population file (ALL), the subsets of the individual years combined (File/SS), and the total population subset (ALL/SS). All of the equation were 50 terms with a second derivative math treatment with a 15 nm gap and a 15 nm smooth applied to the spectra. It is quite obvious that SUBSET in either case picks the better calibration set, better than the total population and almost as good as the files for 1983 and 1984. The problem is 1985a, 1985b, and 1986. The R^2 values for these years' data sets are virtually 0.0 with large SEC values. The IVDMD data for these 3 sets are unsuitable for use in a calibration. This is a major problem with closed population calibrations. The samples and the data are a closed set and cannot be improved upon unless the population is opened. These data, where the replicate, cutting, and year were averaged, were good for ranking but insufficient for developing a calibration since we had to use the individual IVDMD value for each spectrum. The use of an open population based on data of known error limits gave better results than the last 3 years of this data and as good as the subsets in most cases.

References

(1) Burton, G. W., Hart, R. H., & Lowrey, R. S. (1967) *Crop Sci.* **7,** 329–332

(2) Chapman, H. D., Marchant, W. H., Burton, G. W., Monson, W. G., & Utley, P. R. (1971) *J. Anim. Sci.* **32,** 374

(3) Burton, G. W. (1972) *Crop Sci.* **12,** 125

(4) Burton, G. W., & Monson, W. G. (1972) *Crop Sci.* **12,** 375–378

(5) Marum, P., Hovin, A. W., Marten, G. C., & Shenk, J. S. (1979) *Crop Sci.* **19,** 335–360

(6) Barton, F. E., II, & Burdick, D. (1979) *J. Agric. Food Chem.* **27,** 1248–1252

(7) Burdick, D., Barton, II, F. E., & Nelson, B. D. (1981) *Agron. J.* **73,** 399–403

(8) Marten, G. C., Shenk, J. S., & Barton, F. E., II (Eds) (1985) *Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality*, U.S. Dept of Agriculture Handbook No. 643, U.S. Government Printing Office, Washington, DC, 96 pp.

(9) Barton, F. E., II, & Windham, W. R. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 1162–1167

(10) Tilley, J. M. A., & Terry, R. A. (1963) *J. Brit. Glassl. Soc.* **18,** 104–111

(11) Windham, W. R., Mertens, D. R., & Barton, F. E., II (1985) in *Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality*, U.S. Dept of Agriculture Handbook No. 643, U.S. Government Printing Office, Washington, DC, 96 pp

(12) Abrams, S. M. (1985) in *Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality*, U.S. Dept of Agriculture Handbook No. 643, U.S. Government Printing Office, Washington, DC, 96 pp

(13) Shenk, J. S. (1985) in *Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality*, U.S. Dept of Agriculture Handbook No. 643, U.S. Government Printing Office, Washington, DC, 96 pp

PLANT TOXINS

Hypoglycin A Content in the Aril, Seeds, and Husks of Ackee Fruit at Various Stages of Ripeness

G. WILLIAM CHASE, JR, WILLIAM O. LANDEN, JR, and ABDEL-GAWAD M. SOLIMAN
Food and Drug Administration, Atlanta Center for Nutrient Analysis, 60 Eighth St, Atlanta, GA 30309

Recently, hypoglycin A (HG-A), a natural toxin, was detected in canned ackee fruit. To determine the source of contamination, the HG-A content in the ackee fruit components (aril, seeds, and husks) at various stages of ripeness was determined by a method using an amino acid analyzer. HG-A concentrations in the unripe ackee fruit components were 939, 711, and 41.6 mg/100 g of seed, aril, and husk components, respectively. Analysis of the ripe fruit components showed that HG-A in the seed decreased to 269 mg/100 g and remained unchanged in the husk while the concentrations in the edible ripe aril decreased below the detection limit of 1.2 mg/100 g.

The ackee tree (*Blighia sapida*) is cultivated mainly in Jamaica but has also been introduced into southern Florida. The yellowish red fruit of the ackee tree is pear size and splits open longitudinally when ripe, revealing 3 large black seeds and a yellow fleshy edible material called the aril (1). The unripe or immature fruit also appears to be yellowish red; however, it is not split open. The unripe aril appears similar to the ripe aril with the exception of being smaller and not as intense in yellow color. The unripe seeds are the same size as the ripe ones; however, they are softer and green in color. The fleshy aril is an important part of the Jamaican diet and may be prepared for consumption in many ways.

Ackee poisoning or "vomiting sickness" can also be associated with the island of Jamaica (2). Ellington (3) found evidence linking hypoglycin A (HG-A), the toxic component of ackee fruit, with vomiting sickness. Tanaka et al. (4) suggested that the effects of HG-A may be cumulative and result in cellular and liver damage.

The unripe ackee aril may contain about 1 mg HG-A/g, with the seeds containing from 2 to 3 times as much (2). As the fruit ripens, the HG-A content of the aril decreases to less than 0.01% (10 mg/100 g) (2). Ellington (3) found 0.008% (8 mg/100 g) HG-A in the ripe arillus and 0.111% (111 mg/100 g) HG-A in the unripe arillus.

Previous studies have relied on ion-exchange or thin-layer chromatographic methods that could not resolve HG-A and leucine. Application of an ion-exchange method using an amino acid analyzer, which provides for baseline resolution of HG-A and leucine, showed that canned ackee fruit contained variable quantities of HG-A (5, 6). The objective of the present investigation was to apply the improved method to study the change in levels and location of HG-A in the ackee fruit at various stages of maturity, and to determine, if possible, the source of contamination of the canned ackee fruit product.

Received October 2, 1989. Accepted November 6, 1989.
This paper was presented at the 50th annual meeting of the Institute of Food Technologists in Chicago, IL.

Experimental

Sample Description

Because ackee fruit is not available in the United States, a limited quantity of fruit at various stages of maturity was provided by Robert Knight of the Subtropical Horticulture Research Station, Miami, FL. Most of these were used for method development. Two green, 2 unripe, and 2 ripe ackee fruit were used for this study. Ripe fruit were characteristically split longitudinally, revealing 3 large black seeds and yellow aril. Unripe fruit were not split and contained large green seeds. Green fruit were small, hard, and contained either small undeveloped green seeds or no seeds at all.

Procedure

Samples for each fruit component were prepared by separating the husk, aril, and seeds from individual unripe and mature fruit. To prepare the composite, each component from each fruit was cut into pieces not exceeding 0.5 mm in diameter. Because component parts of the green fruit were undeveloped, the entire green, immature fruit was cut into small pieces for analysis.

Portions of each composite (16 g) were extracted by homogenization in 80% aqueous alcohol with a Polytron^{R} homogenizer. HG-A was quantitated by the ion-exchange chromatographic method recently developed by Chase et al. (6), using duplicate injections.

Results and Discussion

Data on the HG-A content of ackee fruit components at various stages of maturity are presented in Table 1. The HG-A content was highest in the unripe ackee fruit; the amount was largest in the seeds and least in the husk. The amount of HG-A present in the ripe aril was below the detection level of 1.21 mg/100 g. The level of HG-A in the ripe husk remained unchanged. Although the HG-A level in the seeds decreased with maturation, the ripe seeds remained a rich source of HG-A. The aril and seed of the green fruit were undeveloped and, therefore, could not be analyzed as separate components.

Previous work showed that canned ackee fruit, composed entirely of arils (Jamaican grown), contained an average of 9.4 mg/100 g (6). The difference in HG-A content between the canned aril and the ripe aril from the fresh fruit may be attributed to the quality of processing, variety of fruit, or growing conditions (southern Florida vs Jamaica). The average amount of HG-A present in the unripe arillus (711 mg/100 g) was greater than 6 times what Ellington (3) found (111 mg/100 g). This may be attributed to the same factors as mentioned above.

The study provides data relating HG-A content of ackee fruit to the level of ripeness and to the location within the fruit. These data demonstrate that the husk and the seeds of the fresh ackee fruit continue to contain significant levels of HG-A. According to Morton (1), the ackee fruit is consid-

Table 1. Amounts of hypoglycin A found at various stages of ripeness in ackee fruit components

	Hypoglycin A, mg/100 g[a]		
	Husk	Aril	Seed
Canned ackee fruit[b]	NA	9.4	NA
Ripe fruit	47.3 ± 2.9	–[c]	269 ± 145
Unripe fruit	41.6 ± 10.4	711 ± 44.0	939 ± 117
Green fruit	53.2 ± 8.2	UN	UN

[a] Each data point is the mean ± standard deviation of duplicate injections of 2 different fruit samples. NA = not applicable; UN = undeveloped fruit.
[b] See ref. 6.
[c] Below a detection limit of 1.21 mg/100 g.

ered ripe and fit for consumption when the fruit splits open or yawns into 3 sections, with each section usually containing a plump yellow fleshy aril (the edible portion) with a black seed at the tip. HG-A concentrations observed in the canned aril might be caused by contamination with the husk and seed components as a result of improper handling during the canning process. Since HG-A is a toxic compound with no established tolerance levels, further studies are recommended to establish tolerance levels and evaluate canning methods to reduce HG-A in the canned product to meet such levels.

References

(1) Morton, J. F. (1977) in *Forensic Medicine*, Vol. 3, C. G. Tedeschi, W. G. Eckert, & L. G. Tedeschi (Eds), W.B. Saunders Co., Philadelphia, PA, pp. 1530–1531
(2) Manchester, K. L. (1974) *FEBS Lett.* **40,** 5133–5139
(3) Ellington, E. V. (1975) in Pan-American Association of Biochemical Societies Symposium, *Hypoglycin*, E. A. Kean (Ed.), Academic Press, New York, NY, pp. 1–2
(4) Tanaka, K., Isselbacher, K. J., & Shih, V. (1972) *Science* **175,** 69–71
(5) Chase, G. W., Jr, Landen, W. O., Jr, & Soliman, A. M. (1988) *J. Chromatogr.* **456,** 431–434
(6) Chase, G. W., Jr, Landen, W. O., Jr, Gelbaum, L. T., & Soliman, A. M. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 374–377

TECHNICAL COMMUNICATIONS

Determination of Copper, Iron, and Nickel in Oils and Fats by Direct Graphite Furnace Atomic Absorption Spectrometry: Summary of Collaborative Study

STEPHEN G. CAPAR[1]
U.S. Food and Drug Administration, Division of Contaminants Chemistry, 200 C St, SW, Washington, DC 20204

A collaborative study of a method for the determination of copper, iron, and nickel in edible oils and fats by direct graphite furnace atomic absorption spectrometry was recently conducted by the International Union of Pure and Applied Chemistry. The quantitation limits of the method are 5 μg/kg for copper and 10 μg/kg for iron and nickel. The method has been adopted official first action as an IUPAC–AOAC method.

A method for the determination of copper, iron, and nickel in edible oils and fats by direct graphite furnace atomic absorption spectrometry was subjected to collaborative study between 1983 and 1987 by the Commission on Oils, Fats and Derivatives of the International Union of Pure and Applied Chemistry (1). The results of the collaborative study were reported for oils (soybean and groundnut) containing copper, iron, and nickel and for fats (cocoa butters) containing copper and iron at 3 concentration levels. Nickel was not studied in cocoa butters because it is not normally found in this product. Known amounts of organometallic standards in oil were added to metal-free oil and fat to obtain the 3 concentration levels shown in Table 1 (P.W. Hendrikse, Unilever Research Laboratory Vlaardingen, The Netherlands, personal communication, 1989). (The exact structures of the organometallic compounds were not provided.) Each level was represented by 2 batches. In the case of oils, the batches were soybean and groundnut oils; in the case of fats, the batches were 2 cocoa butters. Samples from each batch were provided in duplicate (blind) so that each participant received 24 samples. Participants were asked to analyze each sample in duplicate. Results were received from 32 laboratories. After data screening and statistical tests for stragglers and outliers, 25, 28, and 27 laboratories remained for final statistical calculations for copper, iron, and nickel, respectively, for each batch of oil; 25 and 28 laboratories remained for copper and iron, respectively, for each batch of fat.

Results from statistical analysis of the collaborative data for copper, iron, and nickel in edible oils are presented in Table 2. The results for each element from the same level in both batches of oil were in close enough agreement for the mean to be considered representative of both batches. Results from statistical analysis of the collaborative data for copper and iron in edible fats are presented in Table 3. Except for the results for iron at the low level, the results for each element from the same level in both batches of cocoa butter were in close enough agreement for the mean to be considered representative of both batches.

AOAC guidelines for statistical analysis (2) should be followed for the sake of harmonization and for ease of intercomparison of results from different studies. Although the values presented in Tables 2 and 3 are not based on these guidelines, they provide a sound basis for assessing the analytical chemical methods. Minor deviations from the harmonized statistical analysis procedure are not expected to alter the overall assessment of the analytical methods.

Summary of Method

A 20 μL portion of an edible oil or fat is introduced directly into the graphite furnace atomizer of an atomic absorption spectrometer equipped with deuterium background correction and a copper, iron, or nickel hollow cathode lamp. For determination of iron, the graphite tube of the graphite furnace atomizer is coated with niobium. Absorption during atomization is recorded and peak height response is measured. The mean response for duplicate determinations is compared to a calibration curve of 3 levels of organometallic standards prepared in sunflower oil. The concentrations of the copper standard working solutions are 50, 100, and 200 μg/kg; the concentrations of the iron and nickel standard working solutions are 250, 500, and 1000 μg/kg. Copper, iron, and nickel are determined sequentially at 324.7, 302.1, and 232.0 nm, respectively. The quantitation limits of the method are 5 μg/kg for copper and 10 μg/kg for iron and nickel. (The definition for these limits is unknown; the method's lowest standards are 50 μg/kg for copper and 250 μg/kg for iron and nickel.)

Recommendation

The General Referee on Metals and Other Elements recommends that this method for the determination of copper,

Table 1. Concentrations of copper, iron, and nickel in samples for collaborative study of graphite furnace AAS method

		Element concentration, μg/kg		
Sample	Level	Copper	Iron	Nickel
Oil	high	140	750	800
	medium	80	500	500
	low	40	150	140
Fat	high	150	850	
	medium	100	600	
	low	40	150	

Received for publication August 2, 1989.
This report was presented at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO.
The recommendation was approved interim official first action by the General Referee, the Committee on Residues, and the Chairman of the Official Methods Board. The method was adopted official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* 73, January/February issue.
[1] S. G. Capar is the AOAC General Referee on Metals and Other Elements.

Table 2. Results from analysis of variance of collaborative data for determination of copper, iron, and nickel in soybean and groundnut oils by graphite furnace AAS method[a]

	Copper			Iron			Nickel		
Statistic	High	Medium	Low	High	Medium	Low	High	Medium	Low
Mean, μg/kg	143	85	36	748	469	148	766	491	153
Standard deviation, μg/kg									
Repeatability (s_r)	10.7	7.8	5.4	46.5	43.5	30.7	49.3	47.5	25.8
Reproducibility (s_R)	21.4	13.5	7.6	147.1	88.7	39.7	129.9	86.3	33.7
Relative standard deviation, %									
Repeatability (RSD_r)	7.5	9.2	15.1	6.2	9.3	20.8	6.4	9.7	16.8
Reproducibility (RSD_R)	15.0	15.8	21.4	19.7	18.9	26.9	16.9	17.6	22.0
No. of labs		25			28			27	
s_r range, μg/kg		5.4–10.7			30.7–46.5			25.8–49.3	
s_R range, μg/kg		7.6–21.4			39.7–147.1			33.7–129.9	
Av. RSD_r, %		10.6			12.1			11.0	
Av. RSD_R, %		17.4			21.8			18.8	

[a] At high, medium, and low concentrations (Table 1).

Table 3. Results from analysis of variance of collaborative data for determination of copper and iron in cocoa butter by graphite furnace AAS method[a]

	Copper			Iron			
Statistic	High	Medium	Low	High	Medium	Low-1	Low-2
Mean, μg/kg	153	102	40	901	576	414	133
Standard deviation, μg/kg							
Repeatability (s_r)	9.7	5.5	4.5	66.9	50.7	39.5	17.9
Reproducibility (s_R)	27.0	19.2	7.9	183.4	118.4	93.5	36.3
Relative standard deviation, %							
Repeatability (RSD_r)	6.4	5.4	11.2	7.4	8.8	9.5	13.4
Reproducibility (RSD_R)	17.7	18.8	19.5	20.4	20.6	22.6	27.2
No. of labs		25			28		
s_r range, μg/kg		4.5–9.7			17.9–66.9		
s_R range, μg/kg		7.9–27.0			36.3–183.4		
Av. RSD_r, %		7.7			9.8		
Av. RSD_R, %		18.7			22.7		

[a] At high, medium, and low concentrations (Table 1).

iron, and nickel in edible oils and copper and iron in edible fats be adopted official first action as a new method in the chapter on Metals and Other Elements. This recommendation has the concurrence of the General Referee on Oils and Fats.

References

(1) Hendrikse, P. W., Slikkerveer, F. J., Zaalberg, J., & Hautfenne, A. (1988) *Pure Appl. Chem.* **60,** 893–900

(2) "Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis" (1988) *J. Assoc. Off. Anal. Chem.* **71,** 161–172

Adsorption of Aqueous Nonylphenol Ethoxylate Surfactants on Metal Sample Loops: Effect on Quantitation by Liquid Chromatography

B. B. SITHOLE, B. ZVILICHOVSKY,[1] C. LAPOINTE, and L. H. ALLEN
Pulp and Paper Research Institute of Canada, Process Chemistry Division, 570 St. John's Blvd, Pointe Claire, Quebec H9R 3J9, Canada

In aqueous solutions of Igepal CO 610, a nonionic nonylphenol ethoxylate surfactant, adsorption of the surfactant takes place onto stainless steel metal surfaces. This adsorption results in increased peak height of the surfactant when a sample loop is purged with increasing volumes of the surfactant. This, in turn, affects the quantitation of the surfactant. However, the adsorption is reproducible at constant purging volumes and results in calibration curves with good precision.

In a recent report, we described a procedure for the determination of Igepal CO610, a nonylphenol ethoxylate surfactant, in pulp and paper mill process samples by spectrophotometry and liquid chromatography (B. B. Sithole & L. H. Allen (1989) *J. Assoc. Off. Anal. Chem.* **72,** 273-276). For the liquid chromatographic procedure, we stated that samples were injected by syringe into the 100 μL sample loop of the Rheodyne injector. This was done by loading the sample loop with constant excess volumes of 0.6 mL. We selected this value because the Rheodyne manual (Operating Instruction for Model 7125 Syringe Loading Sample Injector) recommends injecting at least 2, but preferably 5, loop volumes for good precision. We have since found that the amount of the excess volume used is a critical factor in the quantitation of the surfactant. The peak height of the surfactant is dependent on the volume of the sample used to purge the loop and increases with increase in the purging volume used. This is totally unexpected because we should be injecting only 100 μL sample into the column regardless of the purging volume used. This present report addresses the problem which, to our knowledge, has never been reported before, and offers a solution to it.

Experimental

Apparatus

We used Waters 600 gradient module (a multisolvent pump), Waters 990+ photodiode array detector (with NEC APC IV computer), and Waters 990 plotter/recorder. Column parameter and operating conditions were as follows: 150 mm × 4.6 mm id Waters Novapak octadecyl silica column preceded by Waters Guard-Pak precolumn module packed with μBondapak C18. A 1 mL Glenco flushing syringe (Supelco) was used to introduce surfactant into Model 7125 Rheodyne injector with 100 μL loop. Isocratic elution mixture was 85% acetonitrile and 15% water at flow rate of 1.0 mL/min. Eluant was degassed by purging with prepurified helium.

Alternatively, we used Varian Vista 5500 module (a multisolvent pump) with Model 200 Varian variable wavelength detector, Model 7125 Rheodyne injector with 20 μL loop, Model 8085 Varian autosampler, Vista 402 data system, and 150 mm × 4.6 mm id Chromatography Science Co. (Montreal) ODS-2 octadecyl column.

Reagents

(a) *Surfactant solution.*—1000 mg/L. Dissolve 1.0152 g Igepal CO 610 (Domtar Chemicals Group, Mississauga, Ontario) in 1 L water. Dilute to prepare a 20 μg/mL working solution.

(b) *Acenaphthene solution.*—Prepare 100 μg/mL stock solution of acenaphthene (Supelco) in methanol and dilute to make a 5 μg/mL working solution.

(c) *Vanillin solution.*—Prepare 100 μg/mL stock solution of vanillin (Anachemia, Montreal) in water and dilute to make a 5 μg/mL working solution.

(d) *Water and acetonitrile.*—LC grade (American Burdick & Jackson).

Injection into Sample Loop

Test I: With sample loop in load mode, 0.3, 0.6, 0.9, 1.2, and 1.8 mL of 20 μg/mL Igepal was injected in triplicate.

Test II: The 0.9 mL Igepal solution was injected in 3 modes: (a) 0.3 mL + 0.3 mL + 0.3 mL; that is, 0.3 mL sample was injected through loop, syringe was withdrawn, and injection was repeated twice before valve was turned to inject position. (b) 0.6 mL + 0.3 mL; that is, 0.6 mL was injected, syringe was withdrawn, and 0.3 mL was injected. (c) 0.9 mL; that is, 0.9 mL was all injected through loop before valve was turned to inject position.

Test III: Tests I and II were repeated with 5 μg/mL solutions of acenaphthene and vanillin.

Results and Discussion

Figure 1 demonstrates the effect of increasing the volume of injections on the peak height of Igepal. We see that the

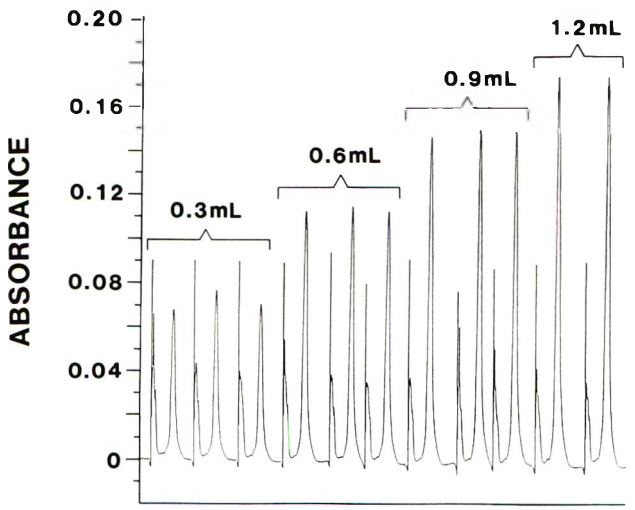


Figure 1. Chromatogram showing effect of increasing purging volumes on peak height of 20 μg/mL solution of Igepal CO-610. Monitored at 230 nm.

Received May 2, 1989. Accepted September 6, 1989.
[1] Visiting scientist, Israel Fibre Institute, Jerusalem, Israel.

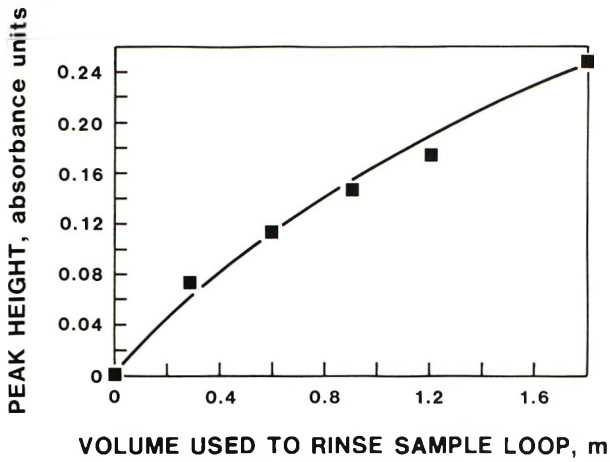


Figure 2. Plot showing effect of increase in purging volumes on peak height of Igepal CO-610.

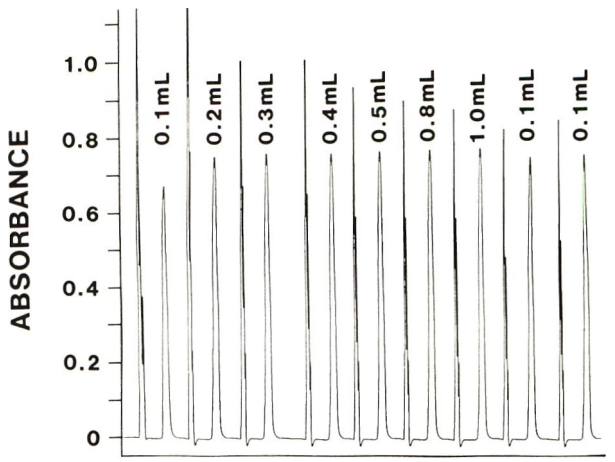


Figure 3. Chromatogram showing effect of increase in purging volumes on peak height of 5 μg/mL solution of acenaphthene. Monitored at 230 nm.

peak height increases with increase in the volume passed through the sample loop. This trend was observed when the sample was injected either by the syringe injection mode or by the syringe withdrawal mode. A sample of the surfactant from a different supplier gave identical results, thus indicating that sample composition was not the problem. Also, monitoring the peak height at different wavelengths, namely 230 and 276 nm, gave identical results. As shown in Figure 2, the increase in peak height shows no leveling off over the volume range studied. This is surprising, considering that we are using a fixed loop and the excess volumes go to waste. Only 100 μL is being transferred to the column and no more.

At first, we thought of 2 possibilities to explain the phenomenon: (a) the Rheodyne injector valve was faulty and had some kind of reservoir that accumulated excess Igepal solution and leaked it into the loop during the injection mode; and (b) the hydrodynamic effects in the sample loop were such that we needed much more than the recommended 5 loop volumes to completely fill the loop with sample (actually, the Rheodyne manual states that the optimum value should be evaluated experimentally by each user). After looking at the schematic of the sample loop and reading the manuals, we came to the conclusion that possibility (a) was not applicable because the valve is designed such that there is no needle-to-loop connecting passage and the needle directly abuts the end of the loop (Technical Notes 5, Rheodyne, Inc.).

Possibility (b) was more plausible because the peak height was not leveling off (Figure 2), but this was difficult to believe because no plateau was evident even after purging with 1.8 mL. Also, as shown in Table 1, purging the loop with a constant volume of 0.9 mL in 3 different modes affects the peak height. Purging with three 0.3 mL aliquots gives higher

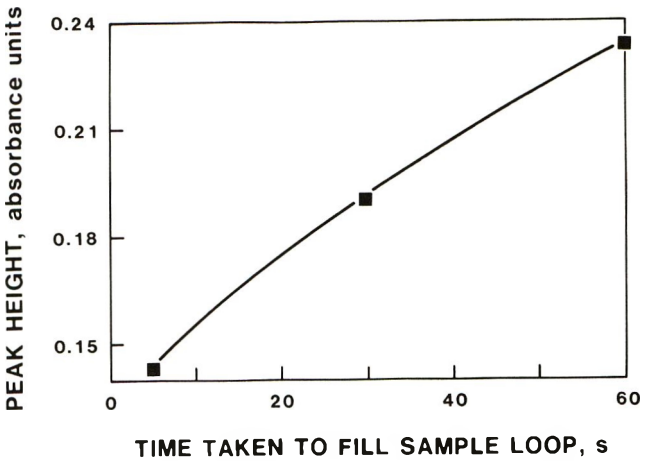


Figure 4. Effect of residence time in sample loop on peak height of Igepal CO-610.

Table 1. Effect of purging sample loop with 0.9 mL of 20 μg/mL Igepal solution and 0.9 mL of 5 μg/mL acenaphthene in 3 different modes (monitored at 230 nm)

Injection mode	Peak height, absorbance unit[a]	
	Igepal CO-610	Acenaphthene
0.3 + 0.3 + 0.3	0.1834 ± 0.0019	0.7465 ± 0.0058
0.6 + 0.3	0.1638 ± 0.0038	0.7486 ± 0.0065
0.9	0.1436 ± 0.0046	0.7542 ± 0.0056

[a] Average peak height ± standard deviation for triplicate determinations.

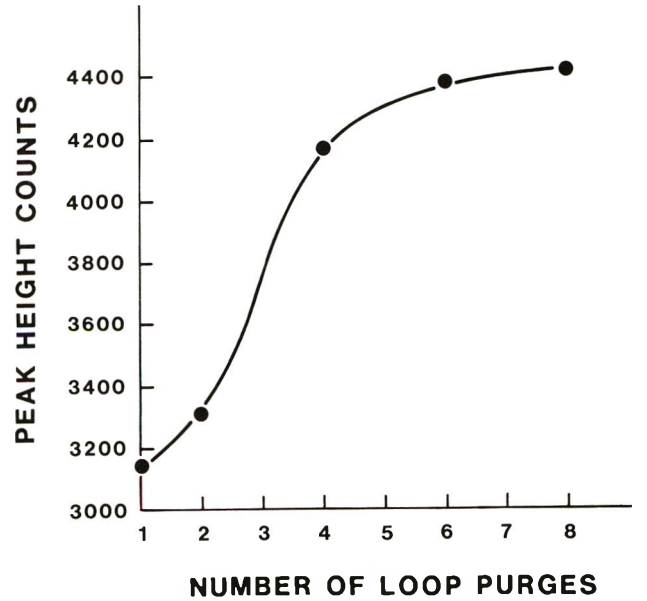


Figure 5. Plot showing effect of increasing purging volumes on peak height of 100 μg/mL solution of Igepal. Analyzed by Varian LC with Rheodyne injection valve (20 μL sample loop) and autosampler.

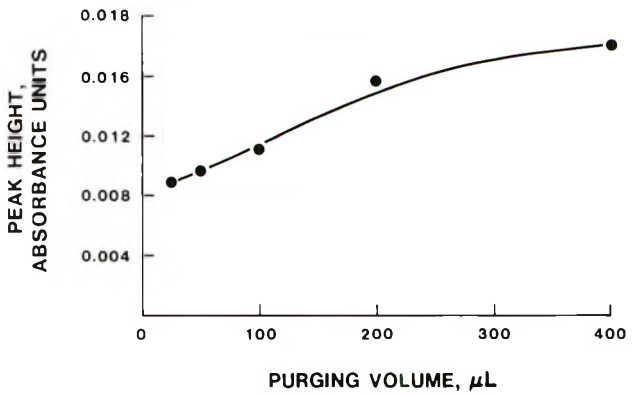


Figure 6. Plot showing increase in peak height of 100 μg/mL solution of Igepal with increase in purging volumes. A 20 μL sample loop was used.

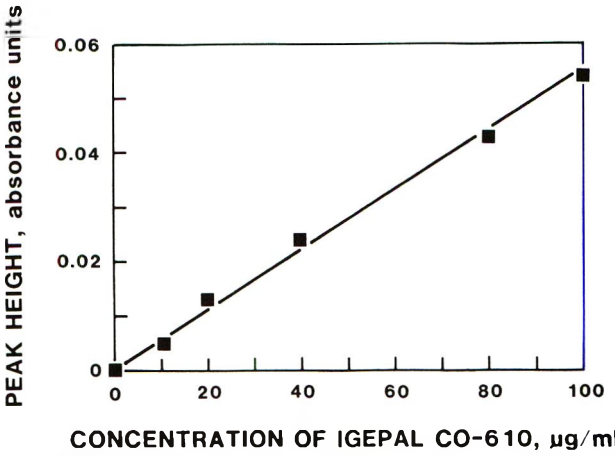


Figure 7. Calibration curve of Igepal CO610. Sample purged with 0.6 mL. Monitored at 230 nm.

peaks than purging with (0.6 mL + 0.3 mL) aliquots, which, in turn, gives higher peaks than purging with a single 0.9 mL aliquot.

The 2 possibilities we raised were soon eliminated after we observed the results for acenaphthene. As shown in Figure 3, there was no change in peak height for acenaphthene with increase in residence time in the loop and increase in the volumes of acenaphthene passed through the loop.

A much more probable explanation is based on adsorption; that is, some of the Igepal adsorbs onto the surface of the sample loop during purging and is desorbed onto the column when the injection valve is turned to the inject mode. Thus, the greater the volume that is passed through the loop, the greater the adsorption that takes place onto the loop surface with consequent increase in peak height. For injections of 0.3, 0.6, 0.9, 1.2, and 1.8 mL passed through the loop, peak height absorbance units were 0.0716 ± 0.0047, 0.1143 ± 0.0010, 0.1496 ± 0.0017, 0.1760 ± 0.0002, and 0.2483 ± 0.0029.

Results in Table 1 can be explained by the residence time of the surfactant in the loop. The total residence time is higher in the three 0.3 mL aliquots than in the single 0.9 mL aliquot. The effect of residence time is corroborated by the results shown in Figure 4 in which 0.9 mL single aliquots of a 20 μg/mL sample were loaded into the sample loop at different times (from 5 s, the normal time taken to fill the loop, to 60 s). The results show that the peak height increased with increase in the time taken to pass the sample through the loop. We did note in our previous report that Igepal had a strong propensity for adsorption onto various surfaces, but at the time, we were not aware that it also adsorbs onto metal surfaces. The adsorption mechanism is probably physical since the compound desorbs easily into the eluant. With acenaphthene, no such adsorption takes place and one loop volume suffices to completely fill the loop.

Questions arise as to whether the phenomenon observed is due to solvent effects and not to adsorption since the Igepal solution was in water while the acenaphthene was in methanol. To answer this, the experiments mentioned above were repeated with a 5 μg/mL solution of vanillin in water. The results showed that vanillin behaved similarly to acenaphthene in that the volumes used to purge the sample loop did not affect the peak height. Neither increase in residence time during purging nor purging in the 3 different modes resulted in increased peak heights for vanillin as in the Igepal case. This proves that the phenomenon we observed was due to adsorption.

Further evidence of the adsorption of Igepal onto the sample loop is shown in Figures 5 and 6. Figure 5 shows the results in which a 100 μg/mL solution of Igepal was loaded onto a 20 μL sample loop using an autosampler on a Varian 5000 LC unit. The peak height increased with increase in the number of sample purges through the sample loop. Figure 6 shows the results obtained when a 20 μL sample loop was substituted for the 100 μL sample loop in our Waters LC unit. The figure shows that the peak height increases with increase in purging volume as was the case with the 100 μL loop. The values obtained are approximately one-fifth of those obtained with the larger loop. These results demonstrate that it is the metal surface and not the plastic components in the Rheodyne sample loop that are responsible for the adsorption. In another study, the sample loop was completely cleared of solvent before purging with sample. The excess sample overflow was re-injected after the loop had been emptied again. The results show that the sample which was injected after it had passed through the metal gave lower peak heights than the original sample (0.0805 vs 0.1150 absorbance unit), indicating adsorption onto the metal surfaces. If there was no adsorption taking place, the samples would have shown the same peak heights. A similar study with vanillin showed no difference in the peak heights (0.1524 vs 0.1517 absorbance unit).

From Figure 1, we see that even though the peak heights increase with increase in volume injected, the reproducibility within a single set of experiments is very good. Thus one can correct for the adsorption problem by always injecting constant volumes of the surfactant through the sample loop at a constant rate. This technique produces linear calibration curves with good precision as shown in Figure 7.

Conclusion

Nonylphenol ethoxylate surfactants adsorb onto metal surfaces and this adsorption leads to quantitation problems in liquid chromatography. However, very good precision is obtained if constant rinsing volumes are always used. This is a precaution which should be taken when quantitatively analyzing surfactants of the alkylphenol ethoxylate type. The adsorption is reversible in that the eluant easily desorbs the adsorbed material.

Mass Spectrometric Confirmation of the Presence of *N*-Nitrosopyrrolidine in Instant Coffee

NRISINHA P. SEN, STEPHEN W. SEAMAN, and D. WEBER
Health Protection Branch, Food Directorate, Food Research Division, Bureau of Chemical Safety, Ottawa, Ontario K1A 0L2, Canada

Traces of *N*-nitrosopyrrolidine (NPYR) may occur in some samples of both instant coffee and fine-ground roasted coffee. The identity of NPYR in 2 samples of instant coffee was confirmed by mass spectrometry as well as by liquid chromatography-thermal energy analysis. A 2-step cleanup procedure, involving fractionation on basic alumina followed by gradient elution on reverse-phase C_{18} cartridge, is described that allows full-scan mass spectrometric confirmation of NPYR in tested samples.

Research during the past 10–15 years has indicated the presence of various volatile *N*-nitrosamines in a wide variety of foods and beverages (1, 2). Although the levels of these compounds in many products (e.g., cured meats, beer, and ale) have decreased significantly during the past few years, some items (e.g., fried bacon) continue to contain appreciable levels of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR), both of which are potent carcinogens in laboratory animals (3). Furthermore, traces of volatile *N*-nitrosamines, mainly, NDMA and occasionally NPYR, *N*-nitrosopiperidine (NPIP), and *N*-nitrosomorpholine (NMOR), are also present in instant skim milk powder, whey powder, spray-dried cheese, and dried soy protein concentrate (1, 2). In all of the above food items, the *N*-nitrosamines are believed to be formed during the drying process by the interaction of amines in the food with the nitrogen oxides (NO_x) present in the hot air used for drying (4). It has been suggested that such formation can take place even with the indirect drying process if the NO_x level in the ambient air is high (e.g., in or around industrial areas or large cities) (2).

In connection with a survey of air-dried foods, we previously reported the occurrence of traces of NPYR in instant coffee (5). Five of 10 samples analyzed were found to contain 0.3–1.4 ppb (mean 0.35 ppb) NPYR. Because of such low levels and the presence of many interfering volatile materials in coffee, we were unable to confirm these results by mass spectrometry (MS). The data were based only on gas chromatography-thermal energy analysis (GC-TEA). Recently, Österdahl (6) of Sweden has verified the above findings. Six of 13 samples of fine-ground coffee and 3 of 3 instant coffees analyzed in the Swedish study were positive for NPYR (0.1–0.5 ppb) by GC-TEA analysis. Again, no MS confirmation was reported.

In the present paper, we wish to report unequivocal MS confirmation of NPYR in instant coffee, and also describe an efficient cleanup procedure that was helpful in carrying out this confirmation.

Experimental

Apparatus

(a) *Gas chromatograph-thermal energy analyzer.*—Thermal energy analyzer (Thermedics, Inc., Woburn, MA, Model 502) coupled to Varian gas chromatograph (Model Vista 6000). Operated as described previously (7, 8) except that CTR gas stream filter (Thermedics, Inc.) was used instead of liquid nitrogen cold trap.

(b) *GC column.*—(i) 30 m × 0.53 mm id fused silica capillary column coated with DB-Wax (1 μm thickness) (J&W Scientific, Inc., Rancho Cordora, CA) or (ii) 2.73 m × 4 mm id coiled glass column packed with 10% Carbowax 20 M on HMDS-treated 60–80 mesh Chromosorb W. GC operating conditions: (i) Argon carrier gas flow 8 mL/min; injector 65°C; column oven 80°C for 2 min, programmed to 150°C at 6°/min with hold for 5 min at 150°C, and again programmed to 220°C at 10°/min. Under these conditions, NPYR eluted after 11.82 min. (ii) Carrier gas flow 25 mL/min; injector 220°C; column oven 120°C, programmed to 220°C at 10°/min. Under these conditions, NPYR eluted after 7.82 min.

(c) *Liquid chromatograph-TEA (LC-TEA).*—TEA coupled to LC system consisting of Waters (Model 6000) solvent delivery system. Operated as described previously (9). Alltech/Applied Science (State College, PA) stainless steel column (25 cm × 4.6 mm id) packed with Lichrosorb Si 100 (5 μm) was used for chromatographic separation. Mobile phase 5% acetone in *n*-hexane; solvent flow 2 mL/min. Under these conditions, NPYR eluted after 17.28 min.

(d) *Mass spectrometer (MS).*—VG Analytical MS system (Model 7070EQ) equipped with 11/250 VG data system and interfaced to Varian GC (Model Vista 6000) was used for MS confirmation. Front stage of MS is conventional double-focusing (forward geometry) instrument used for normal operation in electron impact (EI) mode. Both selected ion monitoring under high resolution (10 000 by 10% valley definition) and repetitive exponential scanning (0.6 s per decade) were used for MS confirmation of NPYR.

Instrument resolution for full scan analysis was 1000. Operated as described previously (10, 11). Fused silica column (30 m × 0.22 mm id) coated with DB-Wax (0.25 μm film thickness) (J&W Scientific Inc.) was used for GC separation. Temperature programming: 60°C for 1 min, heated to 140°C at 50°/min, followed by heating to 200°C at 4°/min. Other conditions: injection port (on-column injection) programmed from 60°C (held for 0.5 min) to 180°C at 40°/min; transfer line 140°C; and head pressure 12 psi (helium carrier gas). For high resolution selected ion monitoring, peak at m/z 118.9920 in perfluorokerosene was used as lock mass.

(e) *Other apparatus.*—Flash evaporator and Kuderna-Danish concentrator were reported previously (7).

Reagents

(a) *Solvents.*—All reagents used were analytical grade. Special precautions were taken to distill and test dichloromethane (DCM) (7). *n*-Pentane was glass-distilled and passed through highly activated basic alumina (<0.1% water content) to remove any nitrosamine contamination before use. Purified *n*-pentane, prepared by this method, was used throughout method.

Received June 12, 1989. Accepted September 11, 1989.

(b) *Basic alumina.*—For column chromatography (ICN Biomedicals, K&K Laboratories, Plainview, NY). Aliquots of alumina were heated overnight in 500°C oven, cooled in desiccator, and then deactivated by adding 1.5% water. Deactivated alumina was stored in glass-stopper Erlenmeyer flask and used after equilibration for ca 16 h.

(c) *Reverse-phase C_{18} solid-phase extraction tube.*—Supelclean™ SPE, 3 mL capacity (LC-18); purchased from Supelco Canada Ltd, Oakville, Ontario.

Analysis of Coffee for Volatile *N*-Nitrosamines

Caution: *N*-Nitrosamines are potent carcinogens; take adequate precautions while handling or working with these compounds.

A 20 g aliquot of instant coffee or finely ground roasted coffee was processed by low temperature vacuum distillation (LTVD) method as described previously (5). Final DCM extract was concentrated, using Kuderna-Danish (K-D) concentrator and macro- and micro-Snyder columns, to 1.0 mL (7). Suitable aliquots (2 μL for megabore column or 10 μL for packed column) were analyzed by GC-TEA to determine concentration of NPYR in the sample. Only samples containing >1 ppb NPYR were used for MS confirmation, after additional cleanup steps described below.

It should be emphasized that all concentration steps of DCM extracts, mentioned hereafter, were also carried out as above, i.e., using K-D concentrator and macro- and micro-Snyder columns. Concentration by flash evaporation or with a stream of nitrogen was avoided.

Cleanup of Sample Extract

Prior to cleanup, 4 more extracts were prepared by separately processing four 20 g aliquots of each positive sample by LTVD method as mentioned above. Final DCM extracts, 1 mL each, from the 5 replicate extractions were combined, and again concentrated to 1 mL. This extract was mixed with 9 mL *n*-pentane and then fractionated on basic alumina as described below.

(a) *Fractionation on basic alumina column.*—Column was prepared by pouring mixture of 10 g basic alumina (1.5% water content) and ca 25 mL *n*-pentane into glass chromatography column (1 × 25 cm) fitted with Teflon stopcock and containing glass wool plug at bottom. A layer of ca 1 cm anhydrous sodium sulfate was added on top of the alumina. Liquid level was never allowed to go below top of sodium sulfate layer during entire cleanup process.

The above mixture of *n*-pentane and coffee extract was gradually added, using a Pasteur pipet, to the column and allowed to pass through the column at a flow rate of 2–3 mL/min. The concentrator tube was rinsed with 3 × 2 mL portions of *n*-pentane and each rinse was added to the column as above. Column was then successively washed with 25 mL each of fresh *n*-pentane and mixture of *n*-pentane and DCM (4 + 1), and washings were discarded. Finally, column was eluted with DCM until 5 successive 10 mL eluate fractions were separately collected in 10–15 mL K-D concentrator tubes. Each eluate was concentrated to 1 mL as mentioned earlier, and 2–10 μL aliquots of each were analyzed by GC-TEA to determine which fraction(s) contained the bulk of the NPYR. Under the conditions used, NPYR eluted in the third DCM fraction. Only this fraction was used for further cleanup on C_{18} cartridge as described below.

Phase transfer.—Exactly 2 mL water and a small piece of Boileezer (Fisher Scientific) were added to the eluate containing NPYR obtained from alumina cleanup step. A micro-Snyder column was placed on top of the concentrator tube containing the mixture, and the tube was heated in a 50–60°C water bath until most of the DCM evaporated (excessive boiling avoided). In the end, the Snyder column was removed and the heating was continued for an additional 10 min until the last trace of DCM was gone. If necessary, the water bath temperature was raised to 65°C. The aqueous residue was cooled to room temperature before cleanup on the C_{18} cartridge.

(b) *Fractionation on reverse-phase C_{18} cartridge.*—*Conditioning the cartridge.*—About 5 mL of 50% methanol in water was passed through the cartridge under gravity. After 10 min, the cartridge was washed with 5 mL water, and the washing was discarded.

Cleanup.—The aqueous extract was quantitatively transferred, using a Pasteur pipet, to the C_{18} cartridge and allowed to pass through it under gravity. If the solvent flow was too slow (<0.5 mL/min), a mild pressure, using compressed nitrogen gas (ca 5 psi), was applied on top until a flow rate of ca 1 mL/min was obtained. The concentrator tube, which contained the extract, was rinsed with 2 mL water and the rinse was passed through the cartridge as above. Finally, the cartridge was successively eluted with 3 mL each of 5% methanol in water, 20% methanol in water, and 50% methanol in water and the eluates were collected in separate graduated centrifuge tubes (15 mL).

About 3 mL water was added to the tube containing the last eluate. DCM (ca 3 mL) was added to each tube, and the tubes were stoppered and mixed well for 1 min each on a vortex mixer. Aliquots (2–10 μL) of the DCM (lower) layer from each fraction were analyzed by GC-TEA to determine the fraction containing bulk of the NPYR which, under these conditions, eluted in the 20% methanol fraction.

The DCM layer from this fraction was carefully withdrawn using a Pasteur pipet and passed through ca 2 g anhydrous sulfate in a small sintered glass funnel (coarse); the dried extract was collected in a 10–15 mL K-D concentrator tube. The above extraction process was repeated twice with 3 mL DCM and each extract was collected and processed as above. The combined DCM extracts were carefully concentrated to 0.5 mL. About 0.8–1.2 μL aliquots of this solution were injected for GC-MS confirmation, and 50 μL aliquots were used for additional confirmation by LC-TEA.

Results and Discussion

Seven brands of instant coffee and 6 brands of roasted ground (both regular and finely ground varieties) coffee were analyzed for volatile nitrosamines. Of these, only 2 instant coffees and one finely ground roasted coffee were positive, containing, respectively, 1.5, 2.8, and 0.4 ppb NPYR. Only the first 2 samples were used for MS confirmation. Reagent blanks carried through all the steps gave negative results.

First, the identity of NPYR in the 2 cleaned up extracts was confirmed by the selected ion monitoring technique using a resolution of 10 000. The molecular ion at m/z 100 was used for this purpose. Both extracts produced strong peaks at the exact retention time (5 min 30 s) of NPYR standard. Next, an attempt was made to obtain a full-scan spectrum using the GC-MS exponential scanning mode. Again, at the exact retention time of NPYR standard, both extracts gave spectra that were comparable to that obtained from authentic standard.

Tracings of the above analyses are, respectively, shown in Figures 1 and 2. The 2 major fragments at m/z 100 and 41 in the mass spectrum (Figure 2) are attributed to the $M^{+\cdot}$ and

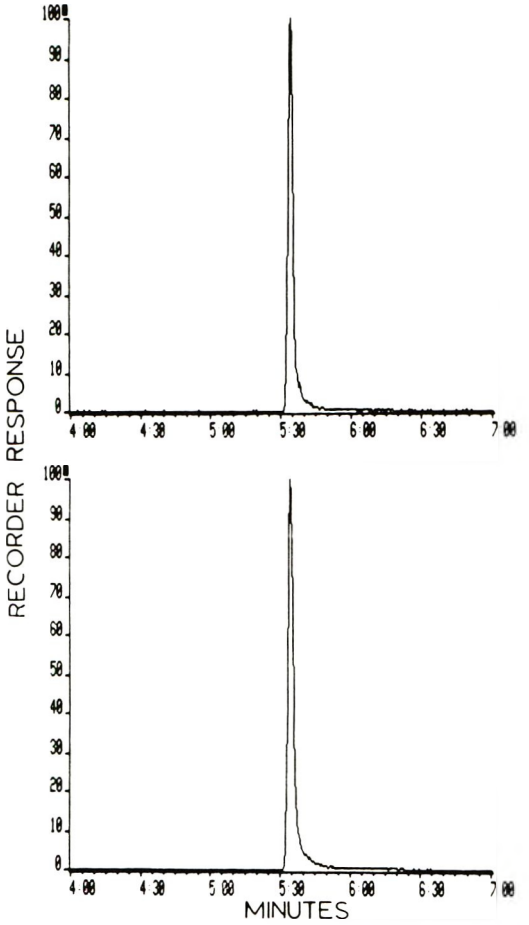


Figure 1. Selective ion monitoring for the M^+ of NPYR (at m/z 100.0637) at resolution of 10 000. Top: NPYR standard (1 ng); bottom: cleaned up extract of sample B containing 2.8 ppb NPYR.

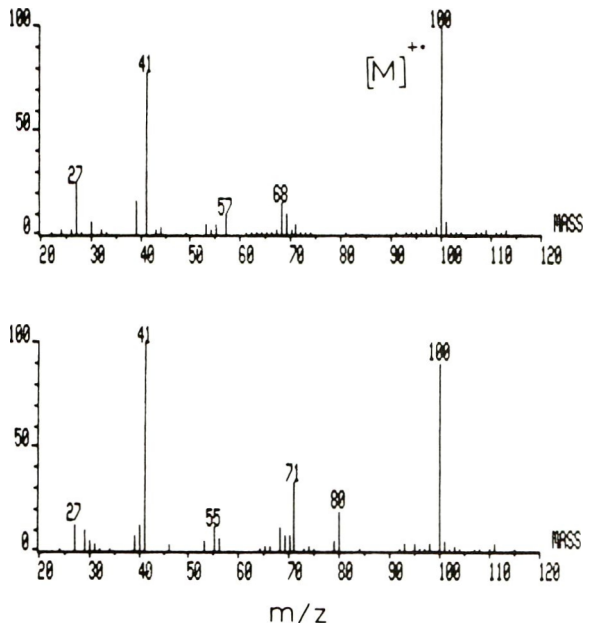


Figure 2. Background subtracted mass spectra of NPYR standard (top) and that of compound isolated from instant coffee B (bottom) as mentioned in Figure 1.

$C_3H_5^+$, respectively, although the latter assignment should be confirmed by accurate mass measurements. The fragments at m/z 70 and 30 most likely correspond to $(M\text{-}NO)^+$ and NO^+, but both peaks are extremely small. This is understandable because the relative intensities of various fragments with respect to that of the M^+ have been reported to vary widely depending on the operating conditions (14). The fragment at m/z 68 is thought to arise from $M\text{-}H_2NO$ (15).

LC-TEA analysis of these extracts also confirmed the presence of NPYR; no other volatile nitrosamines were detected in the cleaned up extracts (chromatograms not shown). In view of the positive results by both GC-TEA and LC-TEA as well as by the above 2 MS techniques, it was concluded that traces of NPYR were indeed present in the samples of 2 instant coffee analyzed.

Confirmation of trace levels of volatile nitrosamines in foods is an extremely difficult task and has posed problems in the past (12, 13), particularly if confirmation by full-scan MS is desired. Here we have described a relatively simple cleanup technique that enabled us to confirm the presence of NPYR at 1–3 ppb levels. The 2 successive cleanup techniques (alumina and C_{18} cartridge) allow one to achieve finer separations of a particular nitrosamine from various impurities and other *N*-nitroso compounds. The overall cleanup technique is also capable of handling a large sample size needed to produce an extract that is concentrated enough in NPYR for full scan MS analysis. For lipophilic *N*-nitroso compounds, however, a stronger eluant (e.g., 75% methanol in water or methanol) will be needed to elute them from the C_{18} cartridge. Further application of the technique for the MS confirmation of both volatile and nonvolatile *N*-nitroso compounds in foods is currently being investigated in our laboratories. It is hoped that other laboratories will also find the techniques useful.

References

(1) Sen, N. P. (1986) in *Diet, Nutrition and Cancer: A Critical Evaluation, Vol. II. Micronutrient, Nonnutritive Dietary Factors, and Cancer*, B. S. Reddy & R. A. Cohen (Eds), CRC Press Inc., Boca Raton, FL, pp. 135–160
(2) Hotchkiss, J. H. (1987) *Adv. Food Res.* **31,** 53–115
(3) Preussmann, R., & Stewart, B. W. (1984) in *Chemical Carcinogens*, 2nd Ed., Vol. 2, C. E. Searle (Ed.), ACS Monograph 182, American Chemical Society, Washington, DC, pp. 643–868
(4) Spiegelhalder, B., Eisenbrand, G., & Preussmann, R. (1979) *Food Cosmet. Toxicol.* **17,** 29–31
(5) Sen, N. P., & Seaman, S. W. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1238–1242
(6) Österdahl, B.-G. (1988) *Food Addit. Contam.* **5,** 587–595
(7) Sen, N. P., & Seaman, S. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 933–938
(8) Fine, D. H., Rufesh, F., Lieb, D., & Rounbehler, D. P. (1975) *Anal. Chem.* **47,** 1188–1191
(9) Sen, N. P., Seaman, S. W., Baddoo, P. A., & Weber, D. (1988) *J. Food Sci.* **53,** 731–738
(10) Sen, N. P., Kushwaha, S. C., Seaman, S. W., & Clarkson, S. G. (1985) *J. Agric. Food Chem.* **33,** 428–433
(11) Weber, D., Sen, N. P., & Lau, P.-Y. (1988) *Proc 36th ASMS Conference on Mass Spectrometry and Allied Topics*, American Society of Mass Spectrometry, San Francisco, CA, pp. 254–255
(12) Fazio, T., Havery, D. C., & Howard, J. W. (1980) *IARC Sci. Publ. No. 31*, 419–431
(13) Hotchkiss, J. H., Libbey, L. M., Barbour, J. F., & Scanlan, R. A. (1980) *IARC Sci. Publ. No. 31*, 361–373
(14) Libbey, L. M., & Scanlan, R. A. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1255–1256.
(15) ApSimon, J. W., & Cooney, J. D. (1971) *Can. J. Chem.* **49,** 1367–1371

Quantitative Multiresidue Analyses for Volatile Organics in Water and Milk, Using a Fused Silica Open-Tubular Wide-Bore Capillary Column and Automated Headspace Gas Chromatography

TIMOTHY P. McNEAL and HENRY C. HOLLIFIELD
Food and Drug Administration, Division of Food Chemistry and Technology, Washington, DC 20204

A modified multiresidue capillary gas chromatographic (GC) procedure has been developed using automated headspace sampling and a wide-bore fused silica open-tubular (FSOT) capillary column for the determination of volatiles in water and milk. Compounds are quantitated by the method of standard additions. An IBM System 9000 computer with the CAPMC3 chromatographic applications package and a BASIC linear regression program are used for data reduction. Data are presented for solutions prepared by fortifying water and milk with volatile solvents such as acetone, methyl ethyl ketone, benzene, methylene chloride, and chloroform, which are commonly used in the manufacture of packaging materials and adhesives. The wide-bore FSOT capillary columns showed dramatically improved detection for certain compounds, compared with normal-bore capillary GC columns. Data presented for various chemicals demonstrate the improved limits of detection from the use of automated headspace gas chromatography with wide-bore capillary columns and flame ionization detection.

The U.S. Food and Drug Administration (FDA) is required to regulate plastic materials used in food contact applications (1). FDA must have the necessary methodology to monitor possible migration into food of many compounds such as adhesive solvents, residual monomers, and polymer additives.

The use of plastic food packaging continues to grow rapidly, causing a greater volume of food to come in contact with a larger variety of polymeric container materials. These packaging systems consist of many chemical moieties that are subjected to a wider range of temperatures with the use of conventional and microwave ovens. Thus, there is an expanding need for multiresidue methods to analyze a greater variety of packaging materials in an efficient manner.

A semiquantitative procedure using a manual headspace injection technique and normal-bore fused silica open-tubular (FSOT) capillary columns was previously reported by Hollifield et al. (2). The present paper describes a modified method that has evolved from the earlier procedure, resulting in an improved, sensitive, automated, multiresidue headspace gas chromatographic (GC) procedure for the determination of packaging-derived volatile adjuvants in aqueous foods.

The modified procedure is based on wide-bore FSOT capillary column gas chromatography, automated headspace sampling techniques, and automated data processing. Compounds are quantitated by the method of standard additions. Data are presented for water and milk solutions prepared with selected volatile chemicals used in the production of packaging materials. These solutions represent typical aqueous foods.

Experimental

Caution: Some of the compounds used in this study are carcinogens, and others are toxic. Use appropriate safety precautions when handling these compounds.

Received December 15, 1988. Accepted September 13, 1989.

Apparatus

(a) *Headspace vials.*—22 mL, with 12 × 20 mm finished tops and butyl rubber septa with aluminum crimp seals (Shamrock Glass Co., Inc., PO Box 686, Seaford, DE 19973). Demonstrate absence of coeluting materials under GC conditions. Tuf-Bond Teflon-faced silicon discs (septa) are preferred (No. 12720, Pierce Chemical Co., Rockford, IL 61105).

(b) *Serum vials and Teflon-faced aluminum seals.*—1 mL (Nos. 223682 and 224211, Wheaton Scientific, Millville, NJ 08332).

(c) *Syringes.*—10, 50, and 100 μL Hamilton 700 series; 0.5 and 1.0 mL Hamilton 1000 series; glass, 10 mL Luer-Lok, with 3 in., 13 gauge hypodermic needle.

(d) *GC instrumentation.*—Perkin-Elmer F-42 automated headspace gas chromatograph modified for single capillary column operation with split injection and flame ionization detection (FID). Capillary column: 30 m × 0.53 mm id, 1.5 μm film, Megabore Durabond DB-1 (J & W Scientific, Inc., Rancho Cordova, CA 95670). Operating conditions: Carrier gas—helium, 0.4 bar; FID gases (bar)—hydrogen 2.4, air 3.0, vial pressure—0.4 bar; split injection—7:1 split ratio; column flow—3.5 mL/min; temperatures (°C)—headspace vial 75, transfer line 100, oven 40, detector 150; times (min)—equilibration 20, pressurization 0.5, withdrawal 0.25, cycle 12; injection time—1 s. Column efficiency was 8000 theoretical plates for benzene.

(e) *Data system.*—IBM CS 9001 laboratory computer equipped with 896 Kbyte random access memory with BASIC language interpreter, dual 8 in. double-density floppy discs (1 Mbyte each), 4-color high-resolution dot matrix printer, analog sensor board (4-channel capacity), CAPMC3 chromatographic applications package.

Reagents

(a) *Analytes.*—Acetone, methylene chloride, vinylidene chloride, acrylonitrile, methyl ethyl ketone, tetrahydrofuran, chloroform, benzene, and toluene. All analytes should be >99% pure and analytical reagent grade or distilled in glass.

(b) *Methanol and deionized water.*—Methanol suitable for purge and trap analysis (Product 232, Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442), or equivalent. Water obtained from Milli-Q water purification system (Millipore Corp., Bedford, MA 01730). Demonstrate absence of analytes and coeluting interferences under chromatographic conditions in *Apparatus*, (d).

(c) *Standard solutions.*—(*1*) *Stock solution.*—About 800–4000 μg analyte/mL methanol or water. Use methanol as holding solvent if high levels of halocarbons are suspected. Transfer 20 mL methanol or water to empty headspace vial, cap with silicon disc, crimp-seal, and weigh to nearest 0.1 mg. Remove vial from balance, and using microliter syringe, quickly add 20 μL of any analyte except acetone to vial.

Reweigh vial. Use 100 μL addition for acetone in mixed stock solution (ca 3900 μg acetone/mL solution). Repeat until desired mixed stock solution is made. Calculate weight/volume concentration for each analyte added. Refrigerate stock solution when not in use. After 2 days of use, replace pierced silicon disc and crimp seal. With new septa replacements, stock solutions are stable for 2 weeks. (*2*) *Working solution.*—Prepare daily. Transfer 1 mL water to 1 mL serum vial. Shake stock solution. Using microliter syringe, add volume of stock solution required to produce analyte concentrations of 5–50 μg/mL in water or to cover the range of expected analytical results. Quickly crimp Teflon-faced aluminum seal, and calculate final concentrations.

Preparation of Headspace Solutions for Standard Additions

Use milk in 8 and 16 oz waxed paperboard or plastic containers. Analyze test solutions before pull dates, and refrigerate except during analysis.

Analyses of milk, water, and other aqueous matrixes by the method of standard additions are identical. Using 10 mL glass syringe, transfer 2 mL aliquots of test liquid to headspace vials. (We prepared 7 aliquots of each test liquid for one analysis series.) Cap, and crimp-seal 2 vials. Fortify contents of remaining 5 vials with mixed working solution as follows: 2 with 3 μL, one each with 6, 9, and 15 μL. Cap, and seal each vial after fortification. If the working solution is a dilution of a stock solution in methanol, add 6 μL aqueous solution with the same methanol concentration as the mixed working solution to each of the first 2 vials.

Headspace Analysis and Quantitation Using Standard Additions

Automated headspace analysis.—Use chromatographic conditions described in *Apparatus*, (**d**). Place prepared vials in rack, record order of analysis, and check order of analysis. Start analysis sequence. A remote start function initiates and continues data acquisition until all analyses are completed.

Postanalysis calculations and data summarization.—Chromatograms are stored on discs and a summary report is printed out after each analysis. Postrun analysis of each chromatogram is performed by using the Reconstruct feature. This routine expands the chromatogram, allowing the user to amplify the chromatographic responses in any time frame. The way the baseline was drawn and the proper integration of the peaks of interest are also checked in this mode. If necessary, peaks in any time frame can be reintegrated and a second report printed, displaying the results. Microvolt responses of peaks at the retention times of the compounds of interest are compiled for the analysis series and entered into the BASIC linear regression program as y values. Fortification levels are represented by x values. A linear regression analysis and a dataplot for each compound are reported featuring slope, x and y intercepts, and the linear correlation coefficient, with the x intercept representing the analyte concentration in the test solution.

Results and Discussion

Before the availability of wide-bore FSOT capillary columns, our laboratory generally relied on packed column gas chromatography for determining compounds of potential regulatory concern (3–6). We typically developed specific procedures for individual analytes. When more than one compound was of interest, multiple procedures were needed to complete the determinations. Hollifield et al. (2) demonstrated that normal-bore capillary columns and manual headspace sampling could be used in multiresidue trace analyses. The high resolving power of normal-bore capillary columns often permits compounds of varying polarity to be readily determined on a single capillary column.

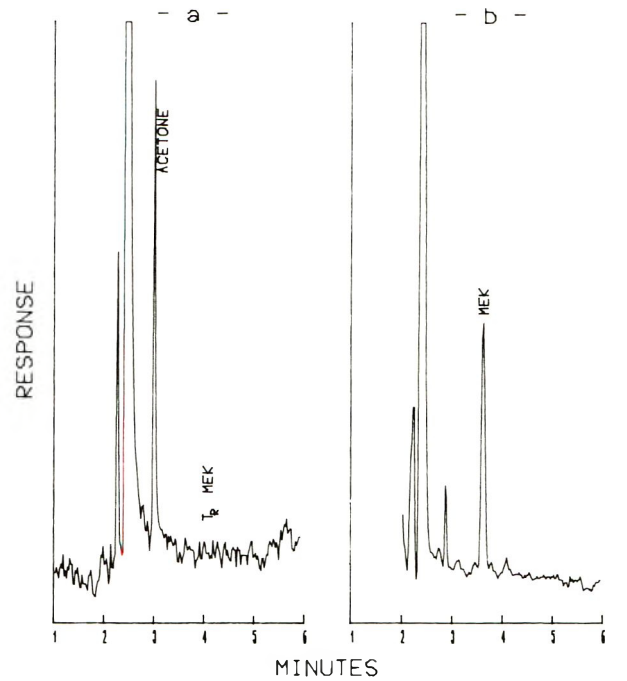


Figure 1. FID chromatograms of headspace over duplicate 2 mL aliquots of unfortified whole milk analyzed using F-42 automated headspace gas chromatograph with (a) normal-bore DB-1 FSOT capillary column (100 μV full scale) and (b) Megabore DB-1 FSOT capillary column (400 μV full scale). Acetone coelutes with interferences at 2.45 min on Megabore column. MEK = methyl ethyl ketone.

Several researchers (7–9) later demonstrated the capabilities of automated headspace gas chromatography with electron capture detection for determining trace amounts of several volatile halocarbons in multiresidue analyses. Their studies showed that repeatability problems encountered with manual headspace sampling techniques could be eliminated by automated sampling. However, in trace analysis, the limited capacity of normal-bore capillary columns is a major drawback compared with the capacity of packed GC columns. Onuska and Davies (10) have discussed limited column capacity and other considerations that confront the analyst when making the change from packed to capillary GC columns.

Hoping to overcome the column capacity limitation, improve detection limits, and demonstrate general applicability, we investigated the use of automated headspace gas chromatography with wide-bore FSOT columns. Wide-bore columns are easily installed into a Perkin-Elmer F-42 automated headspace gas chromatograph. Their use does not require a capillary injection port and carrier make-up gas.

An example of the improved sensitivity derived from the use of a wide-bore column is shown in chromatograms representing analyses for methyl ethyl ketone, a naturally occurring compound that we have found in milk at levels of about 100 ppb (Figure 1). Chromatogram a is the F-42 headspace chromatogram of milk obtained using a 30 m × 0.32 mm DB-1 FSOT column, operated isothermally at 40°C, with

Table 1. Comparison of normalized relative response factor ratios obtained by using normal-bore and wide-bore FSOT capillary columns

Compound	Relative response factor ratio[a]
Acrylonitrile	19:1
Benzene	32:1
Chloroform	29:1
Methylene chloride	28:1
Methyl ethyl ketone	26:1
Tetrahydrofuran	32:1
Toluene	27:1
Vinylidene chloride	18:1

[a] Response factor (RF) = μV response/ppb analyte added to 2 mL deionized water. Relative response factor ratio = (RF wide-bore)/(RF normal-bore).

Table 2. Recovery of selected volatile organics from fortified solutions, using a wide-bore FSOT capillary column and automated headspace gas chromatography

	Level of fortification, ppb		Response, μV		Rec., %[a]	
Compound	Water	Milk	Water	Milk	Water	Milk
Acrylonitrile	17	17	27	31	101	106
Benzene	19	19	164	144	100	101
Chloroform	32	—	31	—	106	—
Methylene chloride	29	—	40	—	97	—
Methyl ethyl ketone	18	—	30	—	109	—
Tetrahydrofuran	19	19	39	42	110	95
Toluene	19	—	94	—	105	—
Vinylidene chloride	26	26	59	59	102	100

[a] Each value is the mean of 2–7 determinations by the method of standard additions.

film coating and carrier gas conditions similar to those described by Entz and Hollifield (8) and FID parameters described in *Apparatus*, (**d**). Chromatogram b represents the same milk analyzed using a Megabore DB-1 column. No response was obtained for methyl ethyl ketone in the unfortified milk when the normal-bore column was used, but when the unfortified milk was reanalyzed using a wide-bore column, 82 ng methyl ethyl ketone/mL milk was found. The improvement in detection limits achieved with wide-bore FSOT capillary columns and automated headspace sampling is shown in Table 1, which gives ratios of relative detector responses for a number of chemicals as a measure of their relative sensitivities on wide-bore and normal-bore DB-1 capillary columns. These improved limits of detection are indicative of the increased column capacity of wide-bore columns; a wide-bore column allows injection of many more sample equivalents, compared to a narrow-bore column.

Although the capacity of the wide-bore FSOT column greatly increases sensitivity in automated headspace GC analyses, there is an accompanying loss of peak resolution. This is apparent in Figure 1, chromatogram a, which shows that acetone, a compound commonly found in milk, is well resolved on the normal-bore DB-1 column; however, chromatogram b shows that acetone is unresolved from early-eluting components on the wide-bore DB-1 column. Of course, selection of another column using a different liquid phase should produce the separation. Of the other compounds studied, vinylidene chloride and methylene chloride resolve as a doublet on the normal-bore DB-1 column but coelute on the wide-bore DB-1 column. Although peak resolution with the wide-bore column is not comparable to that of a normal-bore column of the same film thickness, it is more than adequate for resolving many multicomponent systems.

Chromatograms from automated headspace GC analyses

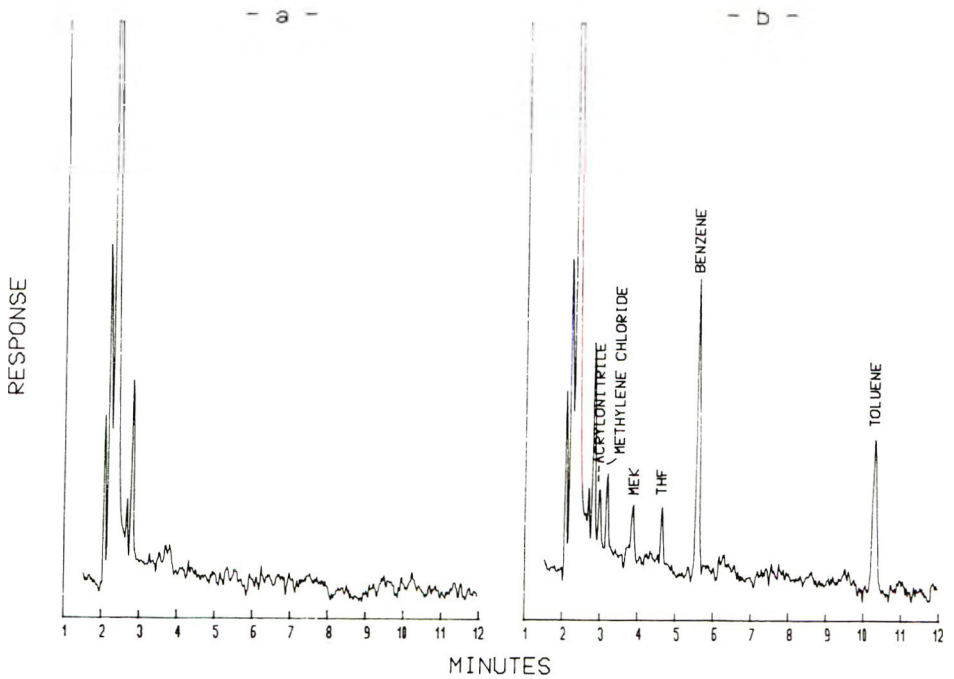


Figure 2. Consecutive FID chromatograms of the headspace over 2 mL water aliquots analyzed using F-42 automated headspace gas chromatograph and Megabore DB-1 FSOT capillary column (300 μV full scale): (a) unfortified water and (b) water fortified with 3 μL of 6-component standard solution. MEK = methyl ethyl ketone; THF = tetrahydrofuran.

using a wide-bore DB-1 FSOT capillary column and FID are shown in Figure 2. Chromatogram a represents injection of the headspace over unfortified water. Chromatogram b represents water fortified with 18, 29, 18, 19, 19, and 19 ng acrylonitrile, methylene chloride, methyl ethyl ketone, tetrahydrofuran, benzene, and toluene, respectively, per mL water. The data for these and other selected volatile organic chemicals summarized in Table 2 illustrate the good recoveries from fortified solutions, using this multiresidue automated headspace GC procedure, a Megabore DB-1 capillary column, and quantitation by the method of standard additions. It must be noted that recoveries calculated by the method of standard additions reflect only the linearity of the system and not the ability to extract the residues actually present. The typical precision of replicate analyses is indicated by the coefficients of variation, which ranged from 4 to 11% over the fortification levels listed in Table 2. Quantitation using the method of standard additions typically demonstrated good intralaboratory repeatability with milk and water matrixes. These data show that quantitative multiresidue analyses using a wide-bore capillary column can be readily performed with a high degree of sensitivity and reproducibility. In fact, low parts-per-billion determinations for many volatile chemicals are readily attained.

This procedure using wide-bore capillary gas chromatography and automated data processing gives the analyst a powerful and sensitive tool capable of analyzing complex matrixes for many trace volatile residues. Automated sampling eliminates much of the potential error associated with manual sampling and saves considerable analysis time. Even with potential losses in resolution, wide-bore FSOT capillary columns are easy to use and offer improved sensitivity due to increased column capacity, compared to normal-bore capillary columns, for the automated headspace multiresidue analysis of aqueous systems.

REFERENCES

(1) *Code of Federal Regulations* (1988) Title 21, Parts 174–177
(2) Hollifield, H. C., Snyder, R. C., & Breder, C. V. (1981) *J. Chromatogr. Sci.* **19,** 514–517
(3) Hollifield, H. C., & McNeal, T. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 537–544
(4) Varner, S. L., & Breder, C. V. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1122–1130
(5) Varner, S. L., & Breder, C. V. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 516–520
(6) McNeal, T. P., & Breder, C. V. (1987) *J. Assoc. Off. Anal. Chem.* **70,** 18–21
(7) Miller, L., & Uhler, A. D. (1988) *Bull. Environ. Contam. Toxicol.* **41,** 469–474
(8) Entz, R. C., & Hollifield, H. C. (1982) *J. Agric. Food Chem.* **30,** 84–88
(9) Entz, R. C., Thomas, K. W., & Diachenko, G. W. (1982) *J. Agric. Food Chem.* **30,** 846–849
(10) Onuska, F. I., & Davies, S. (1986) *Chromatogr. Forum* **1**(1), 45–50

Recommendations on Test Kit Methods: Task Force Report

DONALD MASTROROCCO, *Co-Chairman*
Hershey Chocolate USA, PO Box 1028, Stuarts Draft, VA 24477
MICHAEL BRODSKY, *Co-Chairman*
Ontario Ministry of Health, PO Box 9000, Terminal A, Toronto, Ontario M5W 1R5 Canada

Other Members: W. Andrews; D. Berkowitz; H. Marks; J. McNeal; H. Newsome; J. O'Rangers

Issue 1.—How can AOAC be assured that proprietary reagents and the proprietary components of test kits adopted by AOAC do not change or vary excessively from batch to batch? Should performance tests be built into the methods?

Recommendation.—Performance specifications and their rationale for reagents/components must be developed as part of the method approval process. The test kit manufacturer must assure that all future batches meet these specifications. The operational details of how the materials are tested against specifications must be included in the in-house validation process.

The manufacturer must include appropriate controls or standards where possible to provide the user adequate assurance that the kit is performing according to validated performance specifications. Actual samples with known levels of analyte should be tested by kit users to further ensure efficacy.

Issue 2.—How can AOAC assure that changes/improvements in adopted test kits, usually proprietary components, are known to AOAC? How do changes impact the official status of these methods?

Recommendation.—It is the responsibility of the test kit manufacturer to notify AOAC through the General Referee when changes to preapproved specifications for reagents, components, or procedures are anticipated or when changes are to be made in non-preapproved specifications. The General Referee will, in consultation with the test kit manufacturer, determine whether the change is substantial or minor and will recommend to the Methods Committee an appropriate validation protocol. This may be as complete as a full-scale collaborative study or may simply require in-house validation.

Changes made without AOAC approval may cause the method to lose its AOAC official status.

Issue 3.—What are acceptable performance validation parameters for pass/fail methods? What are acceptable false negative and false positive rates, especially at the level of regulation?

Recommendation.—False negative and false positive rates will be determined as part of the collaborative study of a pass/fail method. In the approval of a method, acceptable false positive/negative rates should be considered but not specified. Each method must be considered on a case-by-case basis, as judged by (*1*) intended use, (*2*) health significance of the analyte, and (*3*) sensitivity/specificity possible with current technology. The ultimate decision rests with the As-

sociate Referee, General Referee, and Methods Committee members.

Issue 4.—How many methods for the same analyte should AOAC validate?

Recommendation.—There should be no limit to the number of methods approved by AOAC. Assuring that the proper collaborative studies are conducted is AOAC's primary responsibility.

Issue 5.—Can AOAC establish guidelines for the description of test kit methods—generic vs specific, including titles?

Recommendation.—Methods should be written specifically, including titles. However, the method must include descriptive, generic principles. This includes performance specifications for proprietary components. This approach is favored over a total generic approach because (*1*) the user should know that strict detail for proprietary components will not be specified; (*2*) specific titles are easily recognized by the user; (*3*) specificity acknowledges the manufacturer for successful validation; (*4*) such acknowledgment should encourage competition and research into better methods.

All new methods, regardless of similarity to an existing method, must undergo collaborative study.

These recommendations represent general guidelines for test kits. Specific guidelines for the wide range of test kits available now and in the future were beyond the scope of the charge to the task force, and are best undertaken by committees of experts dealing with the specific packaged analytical system.

Validation of Methods Used in Crisis Situations: Task Force Report

HENRY B. S. CONACHER, *Chairman*
Health and Welfare Canada, Health Protection Branch, Food Research Division, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada

Other Members: T. Baugh; K. W. Boyer; M. Clower; E. R. Elkins; T. Gross; T. L. Jensen; D. J. McWeeny; G. Myreal; R. W. Stephany; B. Woodward

In response to an item in the AOAC Strategic Plan, a Crisis Methods Task Force was formed by the Official Methods Board in 1987 with the following as major objectives: to develop and recommend a protocol for evaluation/validation of a method for use in a crisis situation; and to assess the role that AOAC might play in such a situation.

Considerable discussion occurred regarding the definition of a crisis situation. It was essentially agreed that this would not only encompass a situation national in scope (although the terms of the original assignment would tend to imply this), but also any situation in which a laboratory (or group of laboratories) was required to generate analytical data on an unplanned, but urgent, basis *and* that decisions based on this data were likely to have considerable economic and/or public health consequences. A number of additional ground rules were established that played a major role as to the content of the final report.

These included the following: It was decided that no microbiological methods would be addressed, only chemical methods. Possibly, however, a group of microbiologists could be convened at a later date to discuss requirements in the microbiological area. It was decided that the extremely important areas of quality assurance and of sampling would not be included since they were being addressed by other groups within AOAC. It was recognized, however, that these, along with the use of validated methodology, are *essential* to the generation of valid data.

It was generally recognized that there were approximately 3 stages in the development and validation of an analytical method: estimation of acceptable performance parameters within a laboratory; demonstration of successful performance in limited interlaboratory studies; and demonstration of successful performance in recognized collaborative study.

As one progresses from the first to the third stage, the degree of confidence that one can ascribe to the validity of a particular method increases. The third stage represents what is generally accepted to be the highest degree of method validation, i.e., successful performance in a collaborative study conducted (and evaluated) according to the guidelines of AOAC (or similar organization) [H. B. S. Conacher in "Pesticide Residues in Food: Technologies for Detection," U.S. Congress, Office of Technology Assessment, OTA-F-398 (U.S. Government Printing Office, Washington, DC, October 1988), p. 136]. It was further recognized that in crisis situations an official AOAC method (or indeed that from any other international group similarly involved in the development of validated analytical methods such as ISO, IUPAC, etc.) might not exist and that insufficient time would be available for a full AOAC collaborative study, which generally cannot be completed and assessed in less than 1 year from inception.

It was considered essential, however, that any method used in a crisis situation should be based on sound scientific principles *and* be capable of meeting certain acceptable performance parameters. The main performance parameters that should be taken into account in assessing any analytical method have been outlined in several papers (ACS Committee on Environmental Improvement (1980) *Anal. Chem.* **52,** 2242, and (1983) *Anal. Chem.* **55,** 2210; W. Horwitz (1982) *J. Assoc. Off. Anal. Chem.* **65,** 525), and include accuracy, precision, specificity, limit of detection, limit of determination, linear range, and scope. While these parameters have also been thoroughly discussed in these publications, the Task Force considered it most important to reiterate them here:

(a) *Accuracy.*—Closeness of determined value to true value. This is best supported by the analysis of standard reference materials; however, the availability of such materials is usually limited. Generally, recovery of added analyte over an appropriate range of concentrations is taken as an indication

of accuracy. Whenever possible, the concentration range chosen should bracket the level of interest. It should be recognized, however, that analyte added to a substrate may behave differently (typically showing higher recovery) from endogenous analyte. Recoveries of 70–120% are generally considered acceptable at the trace (ppb, low ppm) level.

(**b**) *Precision.*—Assesses how well a method performs under different conditions of repeated use. The interlaboratory precision is the most important aspect since it is a measure of how much allowance should be made for between-laboratory variability in interpreting results produced by different laboratories. It is possible, however, to have a measure of one component of this (the repeatability, or within-laboratory precision) by multiple analyses of samples at different analyte levels on the same day or, even better, multiple analyses over different days. The latter has been termed "total within-laboratory reproducibility."

The coefficients of variation (CVs) obtained should fall within the range estimated by Horwitz [Horwitz et al. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 1344]. The repeatability (within-laboratory precision) component is generally one-half to one-third of the reproducibility (between-laboratory precision).

(**c**) *Specificity.*—Ability of method to measure only what it is intended to measure. In any method it is absolutely essential to run reagent and "field" blanks (i.e., substrate blanks) to ensure that no interfering compound, or indeed none of the analyte itself, is present. "Field" blanks should be run for each commodity examined.

To verify the identity and amount of an analyte, the ideal approach is to use 2 entirely different analytical principles. Often, however, in crisis situations, this is not possible in the time available; advantage, for organic analytes, is usually taken of the following: Mass spectrometric confirmation of identity and amount. Use of different detectors, i.e., operating under different principles, e.g., Coulson vs electron capture. Chromatography using different systems. Chemical reaction, e.g., derivatization of a functional group followed by analysis.

With inorganic analytes, recourse is generally made to use of a different wavelength (e.g., in the case of procedures based on atomic absorption (AA)) or to the use of an entirely different measurement system (e.g., inductively coupled plasma vs AA, x-ray fluorescence vs AA, etc.).

(**d**) *Limit of detection.*—The lowest concentration of an analyte that the analytical process can reliably differentiate from background levels. This has been defined as the level (background level) measured in the "field" blank plus 3 standard deviations of this level.

(**e**) *Limit of determination.*—The lowest concentration of an analyte that can be measured with a stated degree of confidence. This has been defined as the substrate blank plus 10 standard deviations; however, it is recommended that this be established in the laboratory by repeated analysis of appropriate "field" samples (spiked or endogenous).

In actual fact, in collaborative studies the limit of determination of the method should generally be considered to be the lowest level successfully analyzed in the study. On occasion results from collaborative studies are used to establish the limit of determination [Page, B.D. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 776–782].

(**f**) *Linear range.*—Generally taken as the range over which the procedure has been demonstrated to give a linear detector response. A reproducible nonlinear response curve however can also be acceptable. Nonlinearity is certainly the case with immunological procedures.

(**g**) *Scope.*—Refers to the number of different substrates to which the procedure can be successfully applied. A procedure, particularly an AOAC official method, should be considered valid for only the commodities that were successfully included in the collaborative study. To extend the scope of an official method, a mini-collaborative study may be required, demonstrating that the performance parameters generated in the main study can be met with the additional commodities.

A number of situations can exist when a crisis situation occurs. For example, these can range from a single organization/laboratory faced with 'in-house' validation, through a single organization with several laboratories at its command, to a number of organizations with a common validation need. To adequately address these various situations, it was considered that a case-by-case approach was appropriate *and* that a guideline approach was preferable to a more rigid protocol. The following situations existed within the membership of the Task Force itself and were considered to adequately cover most circumstances.

One Organization/Laboratory Operation

(i) *Existing AOAC (or AOAC equivalent) collaboratively studied method.*—(a) Same substrate(s), same analyte(s), same concentration range: Laboratory should demonstrate that it can meet the performance parameters achieved in the original collaborative study. The minimum necessary should be the demonstration of an acceptable "field" blank and recoveries from this "field" blank spiked in triplicate at or close to the level of interest. Recoveries (accuracy) should be greater than or equal to, and CVs (n = 3) should be less than or equal to, the values obtained in the collaborative study. Confirmation is desirable but not essential in analysis of violative samples.

(b) Same substrate(s), same analyte(s), lower concentration range: Laboratory should conduct minimum described in (a) *plus* determination of recoveries and precision from "field" blanks spiked in triplicate at each of 2 levels bracketing the lowest level of concern. Confirmation is necessary in case of violative samples.

(c) Same substrate(s), same analyte(s), higher concentration range: This is generally not a problem, assuming a linear range or reproducible nonlinear response curve.

(d) Different substrate(s), same analyte(s): Laboratory should analyze a number of "field" blanks of substrates (at least 3 different varieties of each commodity) *plus* that described in (b) and (c). Confirmation is necessary in case of violative samples.

(ii) *No existing AOAC (or AOAC equivalent) collaboratively studied method.*—A method selected from the literature should be employed with modifications as necessary to achieve the desired result. Step (i) (d) should be followed *as well as* data being acquired on the relevant performance parameters as described previously. In this regard it should be noted that data are not essential on *all* these performance parameters for *all* methods in *all* cases. Depending on the circumstance, some parameters may be more relevant than others.

A method that has received limited interlaboratory study is obviously preferable to one that has not. In this case, the steps (i) (a), (b), (c), or (d) should be followed as appropriate. A summary of the above requirements is presented in Table 1.

Table 1. Summary of suggested minimum data to be generated on methods used in crisis situations

AOAC collab. studied method	Accuracy (recovery)	Precision	Specificity: Reag. blank	Specificity: Field blank	Specificity: Confirm	Limit of detection	Limit of determination	Linearity	Scope
A Unchanged	Req'd (n = 3)	Req'd (n = 3)	Req'd	Req'd	Desirable	Not req'd	Not req'd	Std curve	Not req'd
B Lower concn	Req'd > (n = 3) and < (n = 3) level of conc.	Req'd > (n = 3) and < (n = 3) level of conc.	Req'd	Req'd	Yes if violative	?	Req'd	Std curve	Not req'd
C Higher concn	Req'd (n = 3)	Req'd (n = 3)	Req'd	Req'd	Desirable	Not req'd	Not req'd	Yes	Not req'd
D Diff. substrate	Req'd (n = 3)	Req'd (n = 3)	Req'd	Req'd	Yes if violative	?	Req'd	Std curve	Req'd ≥ 3 varieties
Lit. method	Req'd (n = 6)	Req'd (n = 6)	Req'd	Req'd	Yes	?	Req'd	Std curve	For all substrates (all varieties)

One Organization/Several Laboratories

In addition to the steps described above, some form of check sample program or interlaboratory study is required if the method is being applied in more than one laboratory. A suitable approach would be a minimum of 3 laboratories (or obviously 2 in a crisis situation, if only 2 are available) on a minimum of 5 samples, including one set of blind duplicates. Ideally, interlaboratory CVs should fall within values predicted by the Horwitz curve.

Violative samples can also be exchanged among laboratories for additional confirmation results.

Several Organizations/Several Laboratories

Similar considerations to those described above.

The No-Tolerance Situation

The situations referred to in the preceding sections in general pertain to instances in which the analyte(s) is (are) being determined at a level usually well above the detection limit. The one important situation not referred to relates to the case in which the presence of *any* of a particular analyte(s) may be cause for concern, i.e., the "no-tolerance" situation, which is generally encountered with trace levels of organic compounds such as pesticides, highly toxic environmental contaminants, or veterinary drug residues. In these cases, the criteria described may not be especially applicable, since the technique becomes more qualitative, and the more important parameters become specificity and limit of detection.

The laboratory should select an appropriate method, anticipated on the basis of published data or previous experience with the method, to provide a suitably low level of detection of the analyte in question. Following a demonstration of the successful application of the method at higher levels (e.g., 5×, 10× anticipated level of detection), the laboratory should make a more accurate assessment of level of detection for each separate commodity using appropriate "field" blanks. Where possible and where appropriate, positive results observed in this survey must be confirmed by an alternative technique, if possible, mass spectrometry. Other techniques such as matrix isolation Fourier transform IR for organics, ICP for inorganics, or a combination of specific derivatization procedures might also be considered. Depending on the nature of the confirmatory technique, the previously established levels of detection may not be attainable. The level of detection of the procedure is thus often limited by the limit of confirmation. The important issue is the certainty of the confirmation of identity of the analyte.

The difficulties associated with the no-tolerance situation and suggested solutions thereto have been the subject of a number of recent papers [De Ruig et al. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 487; Stephany (1989) *J. Chromatogr.* **489,** 3; De Ruig et al. (1989) *J. Chromatogr.* **489,** 89; and "Report of Scientific Group on Reference Methods of Analysis for Residues: Criteria," Commission of the European Communities, VI/1541/88-EN].

Role of AOAC

It was generally considered that the role AOAC might play in crisis situations could include publishing the current report in the *Journal* and making copies available on request; acting as a focal point in providing information (through appropriate GRs, ARs, etc.) on methods for possible use.

In crisis situations, the analyst rarely develops a method "from scratch." More often, methods for similar, or even dissimilar, matrixes are modified to work for the matrix of interest. Thus the greatest source of methods for crisis situations are methods that already exist. Since AOAC is in the business of producing, organizing, and disseminating methods, it would seem logical they would be a most valuable source of up-to-date information in this area.

General Comments

The criteria presented in this report are offered as the minimum required in support of data on which some subsequent definitive action may be taken, e.g., banning of certain items, prosecution of offenders, discontinuing production, etc., in response to a crisis. It should be pointed out, however, that in crisis situations, the analytical quality required is determined by the nature, and indeed the stage, of the crisis *or* supposed crisis. In this regard, it is important to re-emphasize that data are not necessarily essential on *all* of the performance parameters for *all* methods in *all* cases. Depending on the circumstances, some parameters may be more relevant than others. It is important that there be adequate dialogue between the analyst and user of the data to establish the "level of quality" acceptable at each stage.

Joint AOAC-AOCS-AACC-IUPAC Mycotoxin Committee

PETER M. SCOTT, *Chairman*
Health and Welfare Canada, Health Protection Branch, Ottawa, Ontario K1A 0L2, Canada

Other Members: R. Bernetti (AACC); H. Casper (AOAC); J. C. Henderson (Secretary) (AOCS); D. L. Park (IUPAC); A. E. Pohland (IUPAC); O. L. Shotwell (AACC); R. D. Stubblefield (AOCS); S. N. Tanner (AACC); M. W. Trucksess (AOAC); A. E. Waltking (AOCS)

Member organizations of the Joint Mycotoxin Committee presented the following reports at the Committee's annual meeting in St. Louis, MO, September 26, 1989.

AOAC

Peter M. Scott highlighted the AOAC General Referee report on mycotoxins and the AOAC Method Committee report on Foods I. The GR report recommended the following interim official first action methods be adopted as official first action: the ELISA screening method for aflatoxin B_1 in corn and roasted peanuts; the ELISA cup screening method for aflatoxin in corn, cottonseed, peanuts, and peanut butter; and the LC method for aflatoxins B_1, B_2, G_1, and G_2 in corn and peanut butter. The GR report also recommended that the following official first action methods be adopted as official final action: the TLC method for aflatoxin M_1 in milk and cheese; the LC method for aflatoxins M_1 and M_2 in fluid milk; and the TLC and GC methods for deoxynivalenol in wheat.

The Methods Committee report on Foods I amended the GR report because a collaborative study report on the immunoaffinity column method for aflatoxins in corn, peanuts, and peanut butter had not been received in time to consider the method for adoption at the 1989 AOAC annual meeting. In addition, 3 topics had been dropped: penicillic acid, tree nuts, and xanthomegnin and related naphthoquinones. The General Referee recommended a new topic on fumonisins.

Scott reported that 2 AOAC collaborative studies will be carried out in the United Kingdom on ELISA and immunoaffinity column methods for aflatoxins. Other AOAC collaborative studies are either in progress or planned on ochratoxin A, ergot alkaloids, aflatoxin B_1 in raw peanuts (ELISA), and aflatoxin M_1. Protocol development for collaborative studies was emphasized.

Douglas L. Park reported that the AOAC Task Force on Sampling and Sample Preparation carried out a survey of AOAC General Referees. The respondents agreed that standardization of sampling was not possible, but sample preparation procedures could be standardized. A monograph on sampling and sample preparation will be prepared, and a sampling workshop will be held by AOAC. Smaller sample sizes for analyses by rapid test kits (e.g., ELISA) are not justified.

James Pestka reviewed the AOAC recommendations on test kits and an FDA bulletin distributed to state food and laboratory officials on the use of immunoassay kits for aflatoxin screening. A workshop on immunoassays will be held at the AOAC Analytical Technology Week in Valley Forge, PA, April 2–6, 1990.

Howard Casper reported that multimycotoxin analysis is now possible for 30–40 toxins in veterinary diagnostic samples, using mass spectrometric techniques. The goal is to simultaneously analyze 50–60 toxins. Mary W. Trucksess also opened a discussion on extraction of solvents used in rapid tests. Solvent ratios and solvent/sample ratios were considered. The effectiveness of a solvent mixture may be dependent on the type of commodity. Fineness of grind is another factor affecting extraction efficiency.

AOCS

James C. Henderson reported that the AOCS Mycotoxin Committee sponsored a Mycotoxin Symposium dedicated to Leo Goldblatt, a long time colleague and pioneer researcher, at the 1989 AOCS annual meeting in Cincinnati, OH. AOCS also conducted field evaluations of ELISA and immunoaffinity column methods for aflatoxins and rewrote into AOCS format the ELISA method for aflatoxin B_1 in cottonseed products and mixed feeds.

AACC

Steven N. Tanner gave an overview of the following topics of the AACC Mycotoxin Committee: aflatoxin in the 1988 corn crop and the inadequacy of predicting such contamination, methods currently under AOAC review, AACC methods (the AOAC TLC method for deoxynivalenol in wheat has been adopted as an official AACC method), and the AACC Mycotoxin Symposium.

Raffaele Bernetti discussed an ISO collaborative study on methods for aflatoxin B_1 in animal feed.

IUPAC

A progress report on the current projects of the IUPAC Commission on Food Chemistry focused on the rationale for regulations, as well as on spectroanalytical parameters for *Fusarium* toxins, the collaborative study on an LC method for ochratoxin A, the check sample program, worldwide natural occurrence of ochratoxin A, and laboratory decontamination. New projects proposed include an evaluation of an immunoaffinity column method for aflatoxin M_1, criteria for immunological methods for mycotoxins, and methodology for fumonisin B_1. The next meeting of the Commission will be held in Vienna, Austria, August 23–25, 1990.

IDF, FGIS, and Other Business

The following topics that have occupied the attention of the IDF Group E33-Mycotoxins were reviewed by Peter M. Scott: the IDF Standard 111 for determination of aflatoxin M_1 in milk and dried milk, a collaborative study (joint with AOAC, ISO, and IUPAC) on aflatoxin M_1 by an immunoaffinity column method, and zearalenone in milk. The next meeting of Group E33 will be held in Brussels, Belgium, March 12–16, 1990.

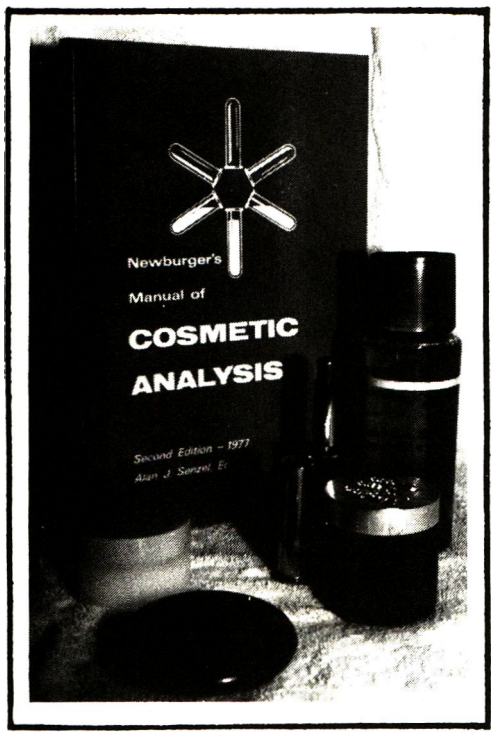
Donald E. Koeltzow of the Federal Grain Inspection Service presented results of a study in which 6 aflatoxin test kits were compared with the Holaday-Velasco mini-column method and the TLC method currently used to screen for aflatoxin in corn at FGIS field locations. Copies of the report are available on request.

Other business included: (1) a recommendation that a mini-collaborative study be conducted on toluene used in

column chromatography (the desirability of replacing benzene with toluene in AOAC official methods led to this recommendation); (2) a recommendation that an IUPAC study on 2 LC methods for determination of patulin in apple juice be considered for acceptability by AOAC; and (3) discussions that Nordic Committee on Food Analysis methods on mycotoxins are AOAC methods, and that certified reference materials, now available for aflatoxin M_1, may be a problem if used in collaborative studies because analysts could look up the mycotoxin content.

Newburger's Manual of
Cosmetic Analysis

- Chapters on analytical techniques: gas chromatography, other chromatographic techniques, and spectroscopy
- Chapters on analysis of creams and lotions, lipsticks, nail lacquers and extenders, hair fixatives and dyes, cold wave solutions and neutralizers, antiperspirants and deodorants, sunscreens
- Chapters on cosmetic microbiology, colors, perfumes and fragrances, preservatives, and toothpastes



2nd Edition — 1977
Price—Members $30.20 in U.S., $35.20 outside U.S.
Nonmembers $33.00 in U.S., $38.00 outside U.S.
To obtain book, send order with your name and address, and check or credit card information (VISA or MasterCard number, expiration date, signature and date) to:

ASSOCIATION OF

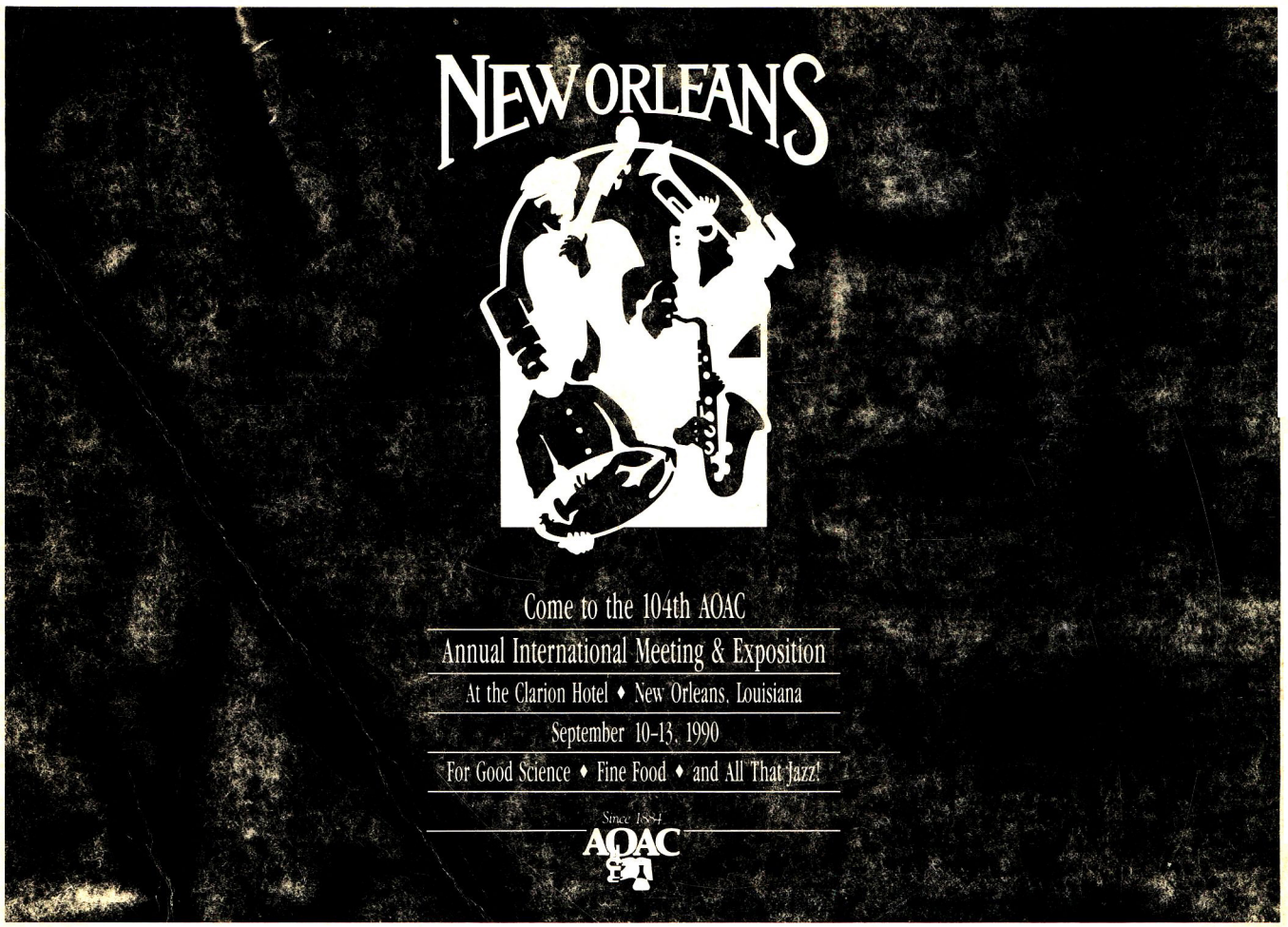
OFFICIAL ANALYTICAL

CHEMISTS

2200 Wilson Boulevard
Suite 400-J
Arlington, VA 22201-3301 USA

U.S. Funds Drawn on U.S. Banks Only

AOAC in



Technical Program

Symposia on these cutting-edge topics:

- ***Risk Management in the Laboratory – Data Quality Assessment,*** co-chaired by Nancy J. Miller and William Horwitz
- ***Laboratory Waste Disposal Technology,*** chaired by Jerry Bucklin
- ***Immunoassays for Mycotoxins and Animal Toxins,*** with computer session, co-chaired by P. Frank Ross and Robert Stubblefield
- ***Laboratory Information Management System (LIMS),*** chaired by James J. Karr
- ***Groundwater: Environmental Concerns,*** chaired by Hershel Morris

Microbiology Update: Old Friends – New Enemies, chaired by Michael Brodsky

Open Forum

Regulatory Roundtable: Pesticide Regulations, Including European Community (EC) 1992 Regulations

Laboratory Equipment Exposition

Modern Laboratory Equipment Workshop

Short Courses:

- Quality Assurance for Analytical Laboratories
- Statistics for Methodology Development
- Improving Sampling for Analysis of Food, Drugs, and Agricultural Materials
- Laboratory Safety and Health

For further information, contact: Director of Meetings and Administration,
AOAC, 2200 Wilson Blvd., Suite 400-J, Arlington, VA 22201-3301 USA. (703) 522-3032, FAX (703) 522-5468.