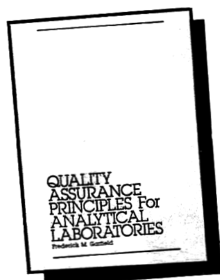
Association of Official Analytical Chemists

MARCH/APRIL 1988
VOL. 71, NO. 2

JANCA 71(2) 241–450 (1988)
ISSN 0004-5756

Keep Essential Analytical Information At Your Fingertips



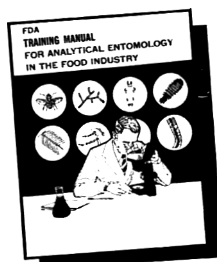
☐ **Quality Assurance Principles for Analtyical Laboratories**
1984. 224 pp. Softbound. ISBN 0-935584-26-9.
Members: $42.25 in U.S., $46.25 outside U.S.
Nonmembers: $46.50 in U.S., $50.50 outside U.S.
A handbook for initiating or improving a laboratory quality assurance program.

☐ **EPA Manual of Chemical Methods for Pesticides and Devices**
1983. 1363 pp. With spectra. Includes three supplements and binder. ISBN 0-935584-23-4.
Members: $62.95 in U.S., $66.95 outside U.S.
Nonmembers: $69.50 in U.S., $73.50 outside U.S.
EPA and State laboratory compilation of over 300 currently used methods for analyzing pesticide formulations.

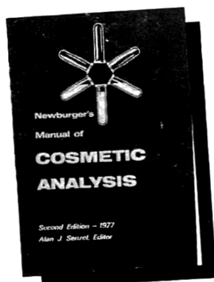
☐ **Macroanalytical Procedures Manual—FDA Technical Bulletin No. 5**
1984. 176 pp. Three hole drill with binder. ISBN 0-935534-28-5.
Members: $28.75 in U.S., $32.75 outside U.S.
Nonmembers: $31.50 in U.S., $35.50 outside U.S.
Manual for inexpensive and speedy identification of defects, filth, decomposition, and foreign matter in large quantities of food.

ORDER THESE AOAC PUBLICATIONS TODAY!

And take advantage of AOAC Membership Discounts too!



☐ **FDA Training Manual for Analytical Entomology in the Food Industry**
1978. 184 pp. Looseleaf. ISBN 0-935584-11-0.
Members: $28.72 in U.S., $32.75 outside U.S.
Nonmembers: $31.50 in U.S., $35.50 outside U.S.
With the aid of this text, organizations can set up their own in-house training.



☐ **Newburger's Manual of Cosmetic Analysis**
1977. 150 pp. Softbound. ISBN 0-935584-09-0.
Members: $29.20 in U.S., $33.20 outside U.S.
Nonmembers: $32.00 in U.S., $36.00 outside U.S.
Chromatographic and spectroscopic techniques with analyses for specific cosmetics.

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

AOAC
1111 North 19th St., Suite 210-J
Arlington, Virginia 22209 USA

Total Amount of Order: $ ____________
U.S. Funds Only

Please Print
Send to:

Name ____________

Organization ____________

Street Address ____________

City ____________

State (Country) ____________ Zip ____________

VM ____________

Member No. *(to qualify for member price, include Member Number with order.)*

Five times faster than Soxhlet.

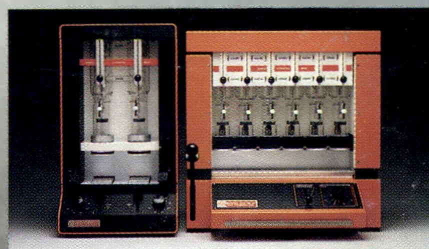
Soxtec® has revolutionized traditional solvent extractions. Extraction times are reduced from days to hours, or from hours to minutes, without loss of accuracy. This has been proven in applications such as petroleum, food and feed, textiles, plastics and rubber, chemicals and many others.

Soxtec cuts costs. The built-in solvent recovery system reduces the cost for solvent as much as 65%.

Soxtec is safe. The systems are heated indirectly by circulating oil from an electronically controlled service unit, which can be placed at a safe distance from the extraction solvent.

Soxtec is flexible. A broad range of solvents can be used which provides great flexibility. Sample holders of various materials and dimensions can hold up to 65 ml of samples containing well below 1% extractable matter.

Soxtec is the perfect tool for most extraction needs. Please call or write for product details.

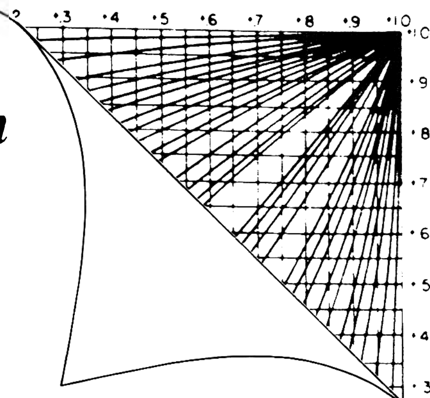


tecator

WE MAKE ROUTINE ANALYSIS SIMPLE.

Tecator Inc., P.O. Box 405, Herndon, Virginia 22070. Phone (703) 435 3300. Telex 903034.
In the USA distribution exclusively by Fisher Scientific Company.
CIRCLE 24 ON READER SERVICE CARD

AOAC Announces the Publication of an Indispensable Statistical Reference Book



USE OF STATISTICS TO DEVELOP AND EVALUATE ANALYTICAL METHODS

Grant T. Wernimont, Author
William Spendley, Editor

With the aid of this manual, the analytical investigator can, with a knowledge of only simple statistical procedures, use the experimental designs and techniques described 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 the results, includes a number of experimental plans for developing and modifying analytical procedures, and discusses evaluation of the data.

Other features include scores of specific statistical analyses of real-life data, many useful statistical tables, and very complete references.

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

Contents

1. Introduction
   - AOAC and Collaborative Studies
   - Organization and Procedures for Collaborative Studies
   - Selection of Methods of Study
   - Types of Interlaboratory Study
   - Need for This Manual
2. The Measurement Process
   - What is Measurement?
   - Measurement as a Relationship Between Properties
   - Performance Characteristics of a Measurement Process
   - Developing, Evaluating, and Using Analytical Processes
   - AOAC Methods of Analysis
3. Intralaboratory Development of an Analytical Process
   - The Need for Intralaboratory Experiments
   - Some Requisites for Sound Experimentation
   - Statistical Methodology
   - Experimental Plans
4. Interlaboratory Evaluation of an Analytical Process
   - Interlaboratory Experiments
   - Objectives for Interlaboratory Study
   - Concept of Variance Components
   - Planning an Interlaboratory Study
   - Experiments to Compare Laboratory Performance
   - Evaluating Interlaboratory Data and Formulating Precision Statements
   - Reporting the Results From an Interlaboratory Study

Appendixes: Tables, Statistical Computations, Glossary. Index.

1985. xvi + 183 pages. Softbound. ISBN 0-935584-31-5.
Price—Members: $48.55 in U.S., $52.55 outside U.S.; Nonmembers: $53.50 in U.S., $57.50 outside U.S.

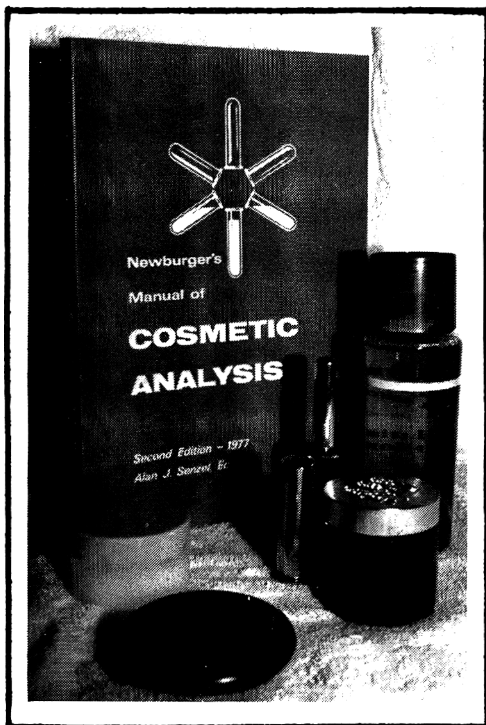
To obtain book, send order with your name and address and check to:

AOAC, 1111 N. 19th Street, Suite 210-J, Arlington, Virginia 22209 USA

(USA funds only)

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

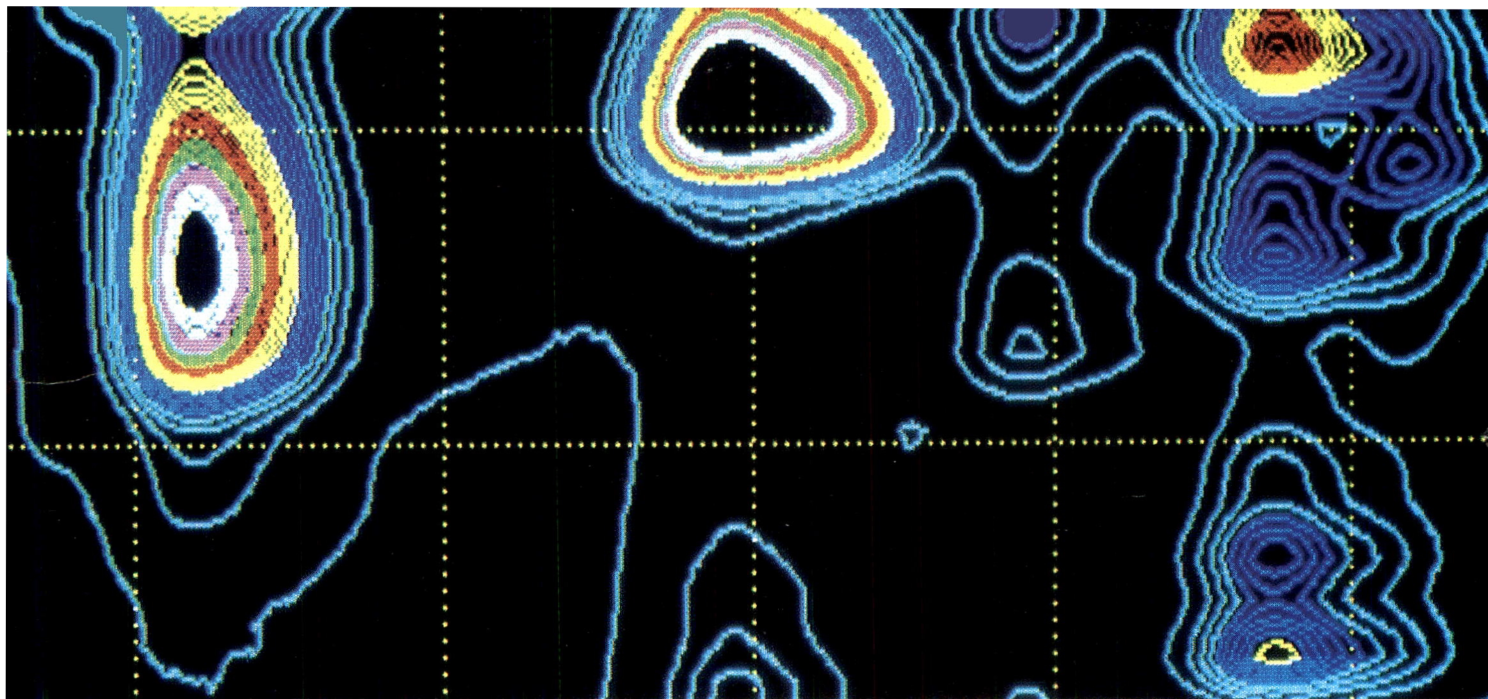


2nd Edition — 1977
Price—Members: $29.20 in U.S.,
$33.20 outside U.S.
Nonmembers: $32.00 in U.S.,
$36.00 outside U.S.
To obtain book, send order with your name and address, and payment to:

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

1111 N. 19th St., Suite 210-J
Arlington, VA 22209 USA

U.S. Funds Only



© 1988 Millipore Corporation. NEC is a registered trademark of NEC Corporation.

Power is knowledge.

Waters unveils the eye-opening new PowerLine™ Photodiode Array System.

Experience the power of total spectral information. Your view of chromatography will never be the same again.

Waters PowerLine PDA gives you instant command of technology's most advanced spectral analysis capabilities. Explore even the most subtle compositional details. Get real-time or post-run assessment of peak homogeneity. Compare chromatograms or spectra with a stored library to facilitate compound identification. Whatever you need to know about your sample, Waters PowerLine PDA gets you to the information with a couple of keystrokes.

In addition, the fully integrated Waters PowerLine system gives you single keyboard control and full documentation of all gradient, injection and detection parameters via its NEC® personal computer.

Tap into Waters PowerLine, and see your sample in a whole new light. For more information, call (617) 478-2000. Ask for Extension 2255.

CIRCLE 13 ON READER SERVICE CARD

Waters
Division of MILLIPORE

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

EDITORIAL BOARD

Raffaele Bernetti
Chairman and Editor-in-Chief
Paul R. Beljaars
Kenneth W. Boyer
Jerry A. Burke
William Y. Cobb
Claire Franklin
Ted M. Hopes
Sami W. Klein
Irwin H. Pomerantz
John K. Taylor
Albert W. Tiedemann
Charles H. Van Middelem

JOURNAL EDITORS

Agricultural Materials
Rodney J. Noel

Drugs, Cosmetics, Forensic Materials
Joseph Westheimer

Food Contaminants and Biological Methods
Louise S. Lee

Residues and Trace Elements
Joseph Sherma

Food Composition and Additives
James F. Lawrence

Chemical Contaminants Monitoring
Helen L. Reynolds

JOURNAL STAFF

Publications Manager:
Nancy Palmer
Managing Editor:
Robert E. Rathbone
Advertising: Marilyn Taub
Subscriptions: Marjorie D. Fuller

AOAC BOARD OF DIRECTORS

President: Robert C. Rund
President-Elect: Odette L. Shotwell
Secretary/Treasurer:
Thomas G. Alexander
Directors: Thomas P. Layloff
Albert W. Tiedemann
H. Michael Wehr
Frank J. Johnson

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

MARCH/APRIL 1988
VOL. 71, NO. 2
JANCA 71(2)241–450 (1988)

34A *New Products*
36A *For Your Information*
42A *Books in Brief*
45A *Information for Subscribers, Contributors, and Advertisers*
46A *Authors in This Issue*
450 *Instructions to Authors*

Focus on Sampling

241 Sampling and Preparation of Sample for Chemical Examination
William Horwitz

246 Statistical Sampling Approaches
Janet A. Springer and Foster D. McClure

Drugs in Feeds

251 Ion-Pair Reverse-Phase Liquid Chromatographic Determination of Amprolium in Complete Feeds and Premixes
Elzbieta J. Kentzer, Layna S. Cottingham, and Robert L. Smallidge

Feeds

256 Moisture Analysis of Forage by Near Infrared Reflectance Spectroscopy: Preliminary Collaborative Study and Comparison Between Karl Fischer and Oven Drying Reference Methods
William R. Windham, Franklin E. Barton II, and James A. Robertson

Drug Residues in Animal Tissue

263 Determination of Zeranol/Zearalenone and Their Metabolites in Edible Animal Tissue by Liquid Chromatography with Electrochemical Detectio– and Confirmation by Gas Chromatography/Mass Spectrometry
Jose E. Roybal, Robert K. Munns, William J. Morris, Jeffrey A. Hurlbut, and Wilbert Shimoda

Plants

272 Gas Chromatographic Determination of the Monosaccharide Composition o– Plant Cell Wall Preparations
Philip J. Harris, Anthony B. Blakeney, Robert J. Henry, and Bruce A. Sto

Copyright, 1988, by Association of Official Analytical Chemists, Inc. All rights reserved. The *Journa– of the Association of Official Analytical Chemists* (ISSN 0004-5756) is published bimonthly by the Association of Official Analytical Chemists, 1111 N 19th St, Suite 210, Arlington, VA 22209. **Subscriptions** for Vol. 71 (1988) are: Members, $84.57 in U.S., $94.57 outside U.S.; Nonmembers, $99.50 in U.S., $109.50 outside U.S. Second class postage paid at Arlington, VA, and additional mailing offices. Abstracted or indexed in 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, 1111 N 19th St, Suite 210, Arlington, VA 22209.

Sugars

276 Determination of Dextran in Raw Cane Sugar by Roberts Copper Method: Collaborative Study
Margaret A. Clarke and Mary An Godshall

Mycotoxins

280 Comparison of Four Thin-Layer Chromatographic Methods for the Determination of Aflatoxin in Raisins
Dilek Boyacioğlu and Meral Gönül

282 Potentials of a Synchronized Culture of *Tetrahymena pyriformis* for Toxicity Studies of Mycotoxins
Jan P. Bijl, Dominique M. Rousseau, Daniel G. Dive, and Carlos H. Van Peteghem

285 Interactive Effects of Duration of Storage and Addition of Formaldehyde on Levels of Aflatoxin M_1 in Milk
Susan K. Heimbecher, Karen V. Jorgensen, and Ralph L. Price

Disinfectants

288 Comparison of Stainless Steel Penicylinders Used in Disinfectant Testing
Eugene C. Cole, William A. Rutala, and Edith M. Alfano

Cosmetics

290 Stability-Indicating Liquid Chromatographic Procedure for Determination of Allantoin in Cosmetic Lotion
Ramesh J. Trivedi

Antibiotics

295 In Vitro Analytical System for Determining the Ability of Antibiotics at Residue Levels to Select for Resistance in Bacteria
Marietta Sue Brady, Robert J. Strobel, and Stanley E. Katz

299 Method to Determine Effect of Antibiotics at Residue Levels on R-Factor Transfer
Marietta Sue Brady and Stanley E. Katz

302 Extraction of Sulfamethazine from Feed Samples
W. John Blanchflower and Desmond A. Rice

304 Microbial Receptor Assay for Rapid Detection and Identification of Seven Families of Antimicrobial Drugs in Milk: Collaborative Study
Stanley E. Charm and Ruey Chi

Pesticide Formulations

317 Determination of Trichlorfon in Technical and Powder Formulations
Stephen C. Slahck

321 Simultaneous Determination of Diazinon and Chlorpyrifos Pesticide Formulations by Liquid Chromatography
Rodney J. Bushway, L. Brian Perkins, and Joan M. King

323 Analysis of Rotenone in Cubé and Derris Root Powders and Formulations by Liquid Chromatography
Rodney J. Bushway, Angela Yang, and Ali Al-Yamany

325 Liquid Chromatographic Determination of Phenolic Impurities in Technical MCPA Using Electrochemical Detection (Coulometric Mode)
Yuk Y. Wigfield and Monique Lanouette

328 Gas Chromatographic Determination of *N*-Nitrosodialkanolamines in Herbicide Di- or Trialkanolamine Formulations
Yuk Y. Wigfield, Mario D. Lacroix, Monique Lanouette, and Narine P. Gurprasad

333 Determination of *N*-Nitrosodipropylamine in Trifluralin Emulsifiable Concentrates Using Minicolumn Cleanup and Gas Chromatography with Thermal Energy Analyzer
Dave Wotherspoon and Ralph Hindle

Microbiological Methods

337 Determination of Residual Cephalexin in Chick Tissues by *Bacillus stearothermophilus* Paper Disc Assay
Junya Okada, Ikuji Higuchi, Sadao Kondo, and Bun-Ichi Saito

341 Evaluation of Abbreviated Enzyme Immunoassay Method for Detection of *Salmonella* in Low-Moisture Foods
Russell S. Flowers, Mary Joan Klatt, Barbara J. Robison, and Jerome A. Mattingly

343 Temperature-Independent Pectin Gel Method for Aerobic Plate Count in Dairy and Nondairy Food Products: Collaborative Study
Jonathan N. Roth

Vitamins and Other Nutrients

350 Liquid Chromatographic Determination of Available Lysine in Soybean and Fish Meal
Joan Rabasseda, Gemma Rauret, and Teresa Galceran

353 Evaluation of Infant Formula Protein Quality: Comparison of In Vitro with In Vivo Methods
David A. Harris, Robert A. Burns, and Rida Ali

Pesticide and Industrial Chemical Residues

358 Improved Method for Determination of Chlorothalonil and Related Residues in Cranberries
Ibrahim M. El-Nabarawy and William F. Carey

360 Determination of Tri-*n*-Butyltin and Di-*n*-Butyltin Compounds in Fish by Gas Chromatography with Flame Photometric Detection
Kumiko Sasaki, Takashi Ishizaka, Takashi Suzuki, and Yukio Saito

363 Intercomparative Study on the Determination of Polynuclear Aromatic Hydrocarbons in Marine Shellfish Tissue
John F. Uthe and Charles J. Musial

369 Multi-Laboratory Study of Measurement of Chlorobiphenyls and Other Organochlorines in Fish Oil
John F. Uthe, Charles M. Musial, and Raj K. Misra

373 Determination of Inorganic Tin in Biological Samples by Hydride Generation-Atomic Absorption Spectrometry after Silica Gel Cleanup
Taizo Tsuda, Minoru Wada, Shigeru Aoki, and Yoshihiro Matsui

375 Single-Laboratory Evaluation of EPA Method 8080 for Determination of Chlorinated Pesticides and Polychlorinated Biphenyls in Hazardous Wastes
Viorica Lopez-Avila, Sarah Schoen, June Milanes, and Werner F. Beckert

Drugs

388 Simple Determination of Ranitidine in Dosage Forms by In-Phase Selective AC Polarography
Juan A. Squella, Luis A. Zuñiga, Igor Lemus, and Luis J. Nuñez-Vergara

390 Liquid Chromatographic Determination of Carbenicillin and Ticarcillin in Injectable Dosage Forms
Barry Mopper

Indirect Food Additives

394 Gas Chromatographic-Mass Spectrometric Determination of Adipate-Based Polymeric Plasticizers in Foods
Laurence Castle, Angela J. Mercer, and John Gilbert

Meat and Poultry

397 Liquid Chromatographic Identification of Meats
Samy H. Ashoor, Woodrow C. Monte, and Philip G. Stiles

403 Liquid Chromatographic Quantitation of Chicken and Turkey in Unheated Chicken-Turkey Mixtures
Samy H. Ashoor and Magdi A. Osman

406 Detection of Poultry and Pork in Cooked and Canned Meat Foods by Enzyme-Linked Immunosorbent Assays
Ronald G. Berger, Richard P. Mageau, Bernard Schwab, and Ralph W. Johnston

Chemical Contaminants Monitoring

410 Organohalogen Residues in Human Adipose Autopsy Samples from Six Ontario Municipalities
David T. Williams, Guy L. LeBel, and Elizabeth Junkins

415 Levels and Incidences of Pesticide Residues in Various Foods and Animal Feeds Analyzed by the Luke Multiresidue Methodology for Fiscal Years 1982–1986
Milton A. Luke, Herbert T. Masumoto, Thomas Cairns, and Harvey K. Hundley

434 Ion Abundance Criteria for Gas Chromatographic/Mass Spectrometric Environmental Analysis
J. R. Donnelly, G. W. Sovocool, and R. K. Mitchum

Technical Communications

440 Direct Gas Chromatographic Analysis of Trichlorfon for Its Decomposition Products
Stephen C. Slahck

443 Semiquantitative Enzyme-Linked Immunosorbent Assay of Soy Protein in Meat Products: Summary of Collaborative Study
Jon E. McNeal

444 Development of Porcine Rapid Identification Method (PRIME) by Modified Agar-Gel Immunodiffusion
Mark E. Cutrufelli, Richard P. Mageau, Bernard Schwab, and Ralph W. Johnston

Abstracts: AOAC Europe IV/ASFILAB Seminar

446 Evolution of Vitamin Determinations in Food and Feed
Ulrich Manz

446 Fluorimetric Detection of Vitamins: Application of Fluorescence to the Analysis of Vitamins A, E, B_1, B_2, B_6, and C
J. P. Antalick and J. G. Faugere

446 The Quantitative Determination of Cyanocobalamin in Pharmaceutical Preparations by Liquid Chromatography
Jaap P. De Kleijn and Chris C. Jansen

446 Progress in Determination of Group B Vitamins
M. Bourgeay-Causse, M. Guillaumont, and M. Leclercq

447 Modern Methods for Determination of the Fat-Soluble Vitamins
Lawrence Rosner

447 New Approaches in Vitamin Analysis: Objectives and Strategy
Paul M. Finglas, David A. T. Southgate, and Michael R. A. Morgan

447 The Biospecific Determination of Biotin and Pantothenate in Food
Paul M. Finglas, David A. T. Southgate, and Michael R. A. Morgan

447 Simplification of the Sample Treatment for Vitamins A and E Determinations
Claude F. Bourgeois

448 Vitamin Supplementation of Foodstuffs: Regulatory Aspects and Outlooks
Daniel Klein

448 AOAC Overview of Conventional and Rapid Methods for Microbiological Analysis of Foods
Wallace H. Andrews

448 Use of Pattern Analysis Techniques for Lactic Bacteria Counting
Patrice Bracquart

448 New Aspects of Traditional Enrichment of *Salmonella*
Martin Busse

448 Epifluorescence Microscopy and Reflectance Colorimetry for Counting Bacteria and Somatic Cells in Raw Milk
Remy Grappin and Gary H. Richardson

449 Use of Impedance and Optical Density Instruments in Food Microbiology
Hjorleifur Einarsson

449 Some Studies on Methodology for Recuperation of *Listeria monocytogenes* from Foods with Special Reference to Soft Cheeses
Lynton J. Cox, Annemarie Renaud, and M. Van Schothorst

449 Evolution, Interests, and Limitations of Some Immunological Techniques and Food Microbiology
Cecile A. Lahellec

VOLUME 44
NUMBER 2
APR./MAY/JUN. 1988

The George E. Failing Company

P. O. BOX 872, ENID, OKLAHOMA 73702
(405) 234-4141 • CABLE GEFCO • TELEX 796524

For more information call 1-800-654-5117, wait for tone and dial 7447.
In Oklahoma: 1-800-522-4140, wait for tone and dial 7447.

Failing Organizes Hazardous Materials Safety Certification Course For Monitoring Drilling Industry

HAMMONTON, NJ — Working with Kaselaan & D'Angelo Associates, an environmental science and engineering firm, this George E. Failing Company branch offers an accredited certification course which drilling contractors must have before going on most hazardous waste sites.

Certification in the 40-hour, five day course is required by order of the Occupational Safety & Health Administration (OSHA) for anyone on a Level C or higher hazardous waste site. Failing branch manager Montia A. Rice, Jr. saw the need and the opportunity to help drillers. Rice and the training division of Kaselaan & D'Angelo got together and the first training course began in early April.

The course, "Hazardous Waste Site Health & Safety," is led by Kaselaan & D'Angelo's Corey W. Briggs, hazardous materials training director. This training program combines classroom lectures, equipment demonstrations, laboratory sessions and field exercises emphasizing hands-on activities. Classroom training is held at Failing's Hammonton, NJ branch. Failing is also promoting the course and registering people for new classes.

"You're not allowed on landfill sites without certification," Rice explained, "and I know contractors that were looking for a training school or waiting for months to get into a class. That's why Failing got involved."

Kaselaan & D'Angelo Associates, Inc. is a national environmental engineering and industrial hygiene consulting firm. They have offices in Haddon Heights as well as Boston, Los Angeles and New York.

Classes are scheduled once a minimum of 10 register. The cost is $900. For more information or to register, contact Montia A. Rice, Jr. at George E. Failing Company, 235-B N. White Horse Pike, Hammonton, NJ 08037, 609/567-1620. You can also call Failing's main toll-free number: 1-800-654-5117, wait for tone and dial 7447.

THE INSIDE STORIES

Failing F-2: The Little Rig That Could
Q&A with project engineer Gerald Nolte

Heat Pump Potential
Ground water heat pumps gaining popularity

Hydraulically Speaking
Start-up and maintenance tips

Coming Up
60th Michigan Well Drillers Association Convention
April 13-14, 1988 — Lansing, Mich.

NWWA Outdoor Action Expo
May 23-26, 1988 — Las Vegas, Nev.

1988 Northeast Ground Water Exposition
June 6-7, 1988 — Springfield, Mass.

South Atlantic JUBILEE
July 31-August 2, 1988 — Myrtle Beach, S.C.

For more information, call Lisa at National Water Well Association, 614/761-1711

Did you hear about the auto worker that got fired for taking a brake?

Two silkworms had a race and ended up in a tie.

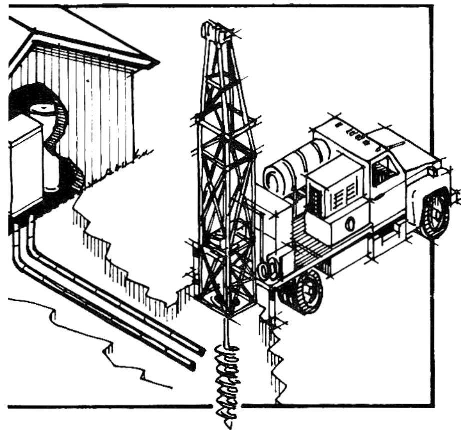
Renowned lawyer F. Lee Bailey went back to college and got a third degree.

Branch Managers Share Outlooks

ENID, OK, February, 1988 — Representatives of George E. Failing branch locations and master distributors from across the U.S. and Canada met here to give regional and industry-wide reports on the drilling market.

A small increase in oil drilling activity during 1987 was just one factor for the optimistic tone of the three-day conference. Company officials also see growing opportunities in the ground water monitoring and soil sampling business. New equipment was also discussed, including two new models of cable tool rigs, which are now being manufactured at Failing subsidiary, Wellmaster Pipe & Supply, and are available at all Failing locations. A new line of Failing vibrators is also expected to be on the market later this year.

Ground Water Heat Pumps Are Raising Opportunities



Ground water heat pumps (gwhp) re enjoying a second wave of opularity throughout the country. 'he gwhp concept has been around ɔr more than 20 years and has njoyed considerable success. But a ɜcond wave of interest is being enerated thanks to new technologies nd marketing strategies.

Around 125,000 water source heat ·umps are installed annually in the U.S. and the number continues to increase. There are several areas of country where 25% or better of all the heat pump installations were ground water source. The renewed interest is due in part to the fact that many of the gwhp technological difficulties have been worked out. Heat pump compressors are more efficient requiring less energy to run. And installation has been made easier using flexible polyethylene and polybutylene piping. Advancements have also been made in the development of smaller, more mobile drilling rigs for vertical installations.

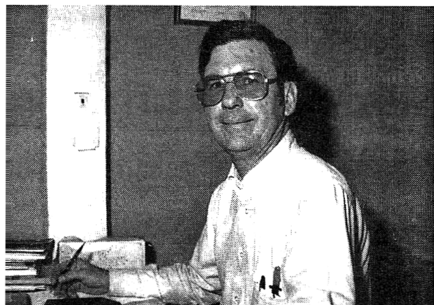
Prices of gwhp's have come down making them more attractive to customers. And as popularity grows and the number of installations increases, even lower prices are expected. The benefits to consumers are immediate. A $30 decrease in a consumer's monthly electric bill is not uncommon.

In the past, many contractors have been reluctant to get into gwhp drilling. But now that thousands of gwhp's have been installed around the country, installation is becoming easier and problems can be avoided by looking at past mistakes. Careful planning is essential. "Guesstimates" should not be used in lieu of approved calculations. Improper load sizing of the heat pump for a building can thus be avoided. Site analysis is also very important.

To get involved with gwhp installation, contractors may have to purchase additional equipment. But in the long run the investment really pays off. And drilling contractors can become involved with installation to any degree they choose. For basic involvement, the contractor can drill for the system, install the open or closed loop, and then backfill and seal the hole. In this case, a contractor is concerned with hole depth and proper installation of the piping.

To be competitive in bidding on gwhp jobs, contractors need to realize that they are not installing water wells. Gwhp drilling is less costly than water well drilling. Con-

Failing F-2: Best All-Round Rig For 24 Years Running



Gerald Nolte

Failing F-2 model was one of the ‿rst top drives built and it remains ɔne of the most popular. *Core Driller* nterviewed Failing project engineer Ɉerald Nolte and asked him about he history and future of the F-2.

ƆD: What drilling applications ꞔan the F-2 handle?
⅃olte: The F-2 is best suited to the mall augering jobs. These could include using augers up to 12" outside diameter and down to 100 feet in certain formations. It can handle monitoring wells, auger drilling, rotary drilling, diamond core drilling — conventional or wireline, foundation testing, site investigation, pre-bid test boring, seismic and cathodic work. The F-2 can also be adapted to drill shallow residential waterwells.

CD: What specific need or void in the drilling market does it fill?
Nolte: The market often calls for a small drilling unit to operate in very restrictive places as far as size is concerned, such as small backyards, service stations with very limited space, etc. Because of its compact size, the F-2 can do the job.

CD: How long has this model been manufactured?
Nolte: Failing began building the F-2 in 1964; however, up until a few years ago, it was designated as our Model FA-100. It was renamed in recent years to consolidate our expanding line of top drives into the Failing "F" series.

CD: What do you consider are its best features?
Nolte: Its small size is one of its best features. This, along with its 60,000 inch pounds of torque developed by the tophead drive and speeds of 18 to 570 rpm, make it a very versatile unit. The angle hole feature can be very advantageous for drilling into less accessible areas.

CD: Briefly, what are some of the F-2's significant options?
Nolte: The wide range of drilling

tractors do not have to guarantee water and do not have to put out the time for back flushing and well development.

Opportunities for gwhp drilling and installation are growing in leaps and bounds. The biggest growth is coming from businesses. All size companies from small businesses to hospitals and high-rise hotels are installing gwhp's. With the popularity of the system on the rise, the work is definitely there for drilling contractors in a growing number of areas.

Contractors interested in gwhp drilling should contact local heat pump dealers, home builders and the local heat pump association.

If you would like more information on drilling equipment for heat pump installation, contact Therrell Hannah at 1-800-654-5117 wait for tone and dial 7447. In Oklahoma, 1-800-522-4140, wait for tone and dial 7447.

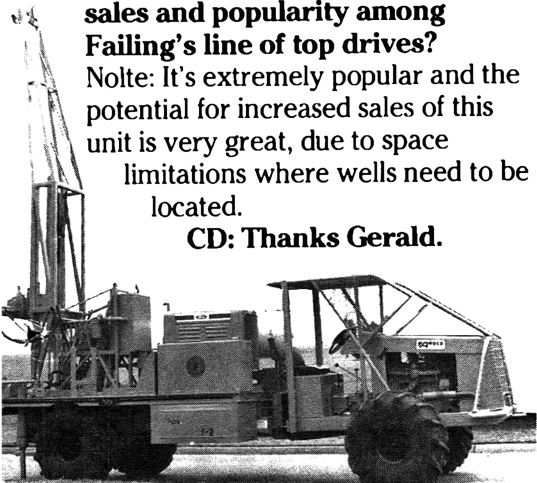
Sources consulted for this article include Dr. James Bose, Oklahoma State University, the *New England Builder*, Dec. 1984, and the *Water Well Journal*, Feb. 1988.

speeds that can be offered makes the F-2 adaptable to a variety of drilling projects. Another advantage is that the F-2 can be mounted on a variety of carriers.

CD: Where does the F-2 rank in sales and popularity among Failing's line of top drives?

Nolte: It's extremely popular and the potential for increased sales of this unit is very great, due to space limitations where wells need to be located.

CD: Thanks Gerald.



Failing F-2

The Care and Filling o Hydrostatic Systems

NOTE: *Core Driller* wishes to thank Stewart Avery of Fluid Components, Inc. in Tulsa for these valuable maintenance tips for rig owners.

First, Fluids

The hydraulic fluids used should be carefully selected according to the original manufacturer's guidelines and specifications.

Start-Up

Prior to installing both pump and motor, inspect for damage incurred during shipping and handling. Make certain all system components (reservoir, hoses, valves, fittings, heat exchanger, etc.) are clean before filling with fluid.

Fill the reservoir with recommended hydraulic fluid which should first be passed through a 10 micron (nominal no bypass) filter. Never reuse fluid.

The inlet line leading from the reservoir to the pump must be filled prior to start up. Check inlet line for properly tightened fittings and make sure it is free of restrictions and air leaks.

Be certain to fill the main pump and motor housing with clean hydraulic fluid prior to start up by pouring filtered oil in the uppermost case drain port.

Install a pressure gauge (500 psi) in the charge pressure gauge port. Leave external control linkage disconnected until after initial start up to allow pump to remain in neutral.

Start the prime mover and run at lowest possible rpm until charge pressure has been established. Air can be bled from the high pressure lines by using the high pressure gauge ports on the motor manifold.

Caution: Do not start prime mover unless pump is in neutral (0 swashplate angle). Take safety precautions to prevent machine movement in case pump is actuated during initial start up.

Once charge pressure has been established, increase speed to norr operating rpm. If charge pressure not at proper value, shut down an determine cause.

Shut down prime mover and conn external control linkage. Start prir mover, checking to be certain pum remains in neutral. With prime mover at normal operating speed, slowly check for forward and reverse machine operation.

Caution: Take necessary safety precautions before moving machine.

Shut down prime mover, remove gauges and plug ports. Check reser voir level and add fluid if necessary

The transmission is now ready fo operation.

Maintenance

For satisfactory service, regular maintenance of fluid and filters mu be performed. The recommended i tervals for changing these items are

- Sealed Type Reservoir: Every 2,000 hours
- Air Breathing Type Reservoir: Every 500 hours

Check fluid level daily. Change fluid more often if it becomes contaminated with any foreign matter (dirt, water, grease, etc.).

Check inlet filter whenever fluid i changed and whenever filter indicat shows a change is necessary. Repla all fluid lost during filter change.

Filters, Too

A machine may be equipped with the best filters available and may be positioned in the system where they do the most good. If, however, the filters are not maintained and cleane when dirty, the money spent for filters and their installation has been wasted. A filter which gets dirty afte one day and is cleaned 29 days later gives 29 days of non-filtered fluid. A filter can be no better than the maintenance afforded it.

Corporate Offices

Headquarters
2215 S. Van Buren
P.O. Box 872
Enid, Oklahoma 73702
Phone: (405) 234-4141
Call Toll Free:
1-800-654-5117
wait for tone and dial 7447
In Oklahoma
1-800-522-4140
wait for tone and dial 7447

Houston
7426 Harwin
Houston, Texas 77036
Phone: (713) 789-8400

Odessa
Unit F, 500 Country Road
1297 South, Box 1691
Odessa, Texas 79760
Phone: (915) 563-0092

Grand Junction
2514 Highway 6 & 50
P.O. Box 364
Grand Junction, Colorado 81502
Phone: (303) 243-2248

Hammonton
235-B N. White Horse Pike
Hammonton, New Jersey 08037
Phone: (609) 567-1620

New Orleans
555 S. Galvez Street
P.O. Box 53185
New Orleans, Louisiana 70153
Phone: (504) 821-2311

Conyers
521 Stanton Woods Drive
Conyers, Georgia 30208
Phone: (404) 929-1252

Largo
2101 Starkey Road
Largo, Florida 33541
Phone: (813) 531-0428

Plainfield
U.S. Highway 40 East
P.O. Box 335
Plainfield, Indiana 46168
Phone: (317) 839-2539

Bloomington
10525 Florida Avenue
Hyland Service Center
Suite 120
Bloomington, Minnesota 55438
Phone: (612) 942-0009

Edmonton
George E. Failing Supply Co.
12720 149th Street, Box 549
Edmonton, Alberta, Canada T5J2K8
Phone: (403) 447-1485

Calgary
4509 Manhattan Road S.E.
Calgary, Alberta, Canada T2G4B3
Phone: (403) 243-6071

Tillsonburg
Wellmaster Pipe and Supply Inc.
62 Goshen Street, P.O. Box 456
Tillsonburg, Ontario, Canada N4G4J1
Phone: (519) 688-0500

Master Distributor

Noland Company
1226 Center Avenue N.W.
Roanoke, Virginia 24017
Phone: (703) 345-8101

to fill out the form below and mail it to us. If you do that, we'll put you on our mailing list and send you a free copy every three months. And, if you'll mail us your comments, we'll try to include your suggestions in upcoming issues.

Please add my name to the CORE DRILLER mailing list.

Name ______
Title ______
Company ______
Address ______ City ______
State ____ Zip ______ Phone ______

Mail to:
Robert Steele
George E. Failing Co.
P.O. Box 872
Enid, OK 73702

Save time and postage.
Call Toll-Free Nationwide: 1-800-654-5117, wait for tone and dial 7447.
In Oklahoma: 1-800-522-4140, wait for tone and dial 7447.

Sunday is the day when we bow our heads. Some of us are praying and some of us are putting.

Two drillers encounter a bear in the forest. One of the men pulls his running shoes out of his knapsack and puts them on. The other man says, incredulously, "you don't think that those running shoes are going to help you outrun the bear?" The other man replies, "I don't have to outrun the bear. I only have to outrun you!"

© Copyright 1988 The George E. Failing Company 4/88, 18.5M ® Registered Trademarks U.S. and Canada

FREE
CAN DEFECTS POSTER INCLUDED

AOAC Announces the Publication of

BAM

6th Edition

A Manual for the Detection of Microorganisms in Foods and in Cosmetics

FDA Bacteriological Analytical Manual (BAM)

by the Division of Microbiology

Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration

BAM contains analytical methods for the detection of microorganisms and certain of their metabolic products, primarily in foods. The methods were developed by the U.S. Food and Drug Administration for Federal and State regulatory and industry quality control laboratories. The manual will be updated by supplements issued to users at no additional charge.

A poster for recognizing and classifying visible can defects is included *free*. It is a useful tool for those who need to analyze canned foods.

This 6th edition contains new chapters on *Campylobacter*, DNA colony hybridization as an analytical tool, and enzyme immunoassay procedures (ELISA). Most other chapters have been revised, expanded and updated.

Contents:

Chapters:

- Food Sampling Plans and Initial Sample Handling
- Food Sample Handling in the Laboratory and Preparation of the Sample Homogenate
- Microscopic Examination of Foods
- Aerobic Plate Count
- Coliform Bacteria
- Enteropathogenic *Escherichia coli*
- Isolation and Identification of *Salmonella* Species
- Fluorescent Antibody Detection of Salmonellae
- *Shigella*
- Isolation of *Campylobacter* Species
- *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*
- Recovery of *Vibrio parahaemolyticus* and Related Vibrios
- Isolation and Identification of *Vibrio cholerae*
- *Staphylococcus aureus*
- Staphylococcal Enterotoxins
- *Bacillus cereus*
- *Clostridium perfringens*: Enumeration and Identification
- *Clostridium botulinum*
- Enumeration of Yeast and Molds and Production of Toxins
- Examination of Oysters for Enteroviruses
- Parasitic Animals in Foods
- Detection of Inhibitory Substances in Milk
- Examination of Canned Foods
- Examination of Containers for Integrity
- Microbiological Methods for Cosmetics
- Detection of Pathogenic Bacteria by DNA Colony Hybridization
- Enzyme Linked Immunosorbent Assay (ELISA)
- Investigation of Food Implicated in Illness

Appendixes:

- Culture Media
- Stains, Reagents and Diluents
- MPN Determination

December 1984, 448 pages, illustrated, appendixes 3 hole drill with binder, includes Visible Can Defects poster. ISBN 0-935584-29-3. **Price — Members:** $45.85 in U.S., $49.85 outside U.S.; **Nonmembers:** $50.50 in U.S., $54.50 outside U.S.

To obtain this book, send order and remittance with your name and address to AOAC, 1111 N. 19th Street, Suite 210-J, Arlington, VA 22209 USA (US funds only).

Benchtop Shell Freezer

Labconco Corp. introduces a new benchtop shell freezer for pre-freezing samples for lyophilization. The benchtop unit rotates sample containers in a low temperature heat transfer solution. As the glassware rotates, the interior surface area of the container is coated with a uniform thin shell of the specimen material.
Circle No. 322 on reader service card.

LC System Controller

An LC system controller from Spectra-Physics Autolab Division sets up and monitors methods and analyses for multiple systems operating on Spectra-Physics' local area network. Called ChromNet, this new system manager is designed to run on an IBM XT or AT, or compatible. The menu-driven program comprises a series of screens that intelligently organize and present themselves according to the system configuration designated by the user.
Circle No. 323 on reader service card.

"Front-End" Software

Aimed at streamlining on-line searching of chemical and other scientific technical databases, STN International's new "front-end" software for personal computers enables a searcher to construct chemical structure and other queries off-line for uploading to STN International. It also supports capture of a transcript of the on-line session, including graphics, on a hard disk for off-line viewing, editing, and printing.
Circle No. 324 on reader service card.

Table Top Laminar Flow Work Stations

Table Top Laminar Flow Work Stations from Clean Room Products are designed to maintain a contamination free environment, by supplying a continuous flow of HEPA-filtered air. Horizontal flow is suited for applications where technicians bend their heads, or other parts of their bodies, over or around the product or process.
Circle No. 325 on reader service card.

Amino Acid Analysis Uses IEC Method

The new System Gold Personal Chromatograph from Beckman Instruments is now available in a configuration for amino acid analysis. With this system, the bioresearcher can utilize ion exchange chromatography and a choice of Beckman's Spherogel ion exchange columns for separating either physiological fluids or hydrolysates.
Circle No. 326 on reader service card.

Fully Integrated SEM, X-ray, and Image Analysis System

A new room-light-operable Model 1830I Analytical Scanning Microscope, developed by AMRAY, incorporates automated high-resolution SEM capabilities as well as full energy dispersive X-ray analysis, wavelength dispersive X-ray analysis, and image analysis functions. All of the instrument's subsystems are under computer control and operable from a single control panel and keyboard.
Circle No. 327 on reader service card.

Microprocessor-Controlled Spectrophotometer

Hach introduces its latest spectrophotometer, the DR/2000. The microprocessor-controlled DR/2000 has more than 95 calibrations preprogrammed in memory, eliminating manual conversion of absorbance data. Up to 50 user-generated calibrations also can be stored with the aid of prompting software.
Circle No. 328 on reader service card.

Data Sheets Describe Chromatography Hardware/Software

Dynamic Solutions announces availability of a new series of technical literature describing its Maxima Chromatography Workstations as well as its Baseline data system for the IBM and NEC families of microcomputers.
Circle No. 329 on reader service card.

Cyano-Modified HPTLC Plate

The pre-coated layer of a new cyano-modified HPTLC plate from EM Science is chemically bonded to a base of silica gel 60 with an average pore diameter of 6 nm. This widens the selectivity of the stationary phase during thin layer chromatography, according to the manufacturer, and permits separation of substances which differ only slightly in chemical structure.
Circle No. 330 on reader service card.

Scanning, Double Beam, UV/Visible Spectrophotometer

Hitachi announces the introduction of the Model U-2000 UV/Visible spectrophotometer. The U-2000 is a double beam, scanning unit that features a 9-inch CRT with graphics, non-volatile storage of methods and spectral data, wavelength programming, and quantitative analysis using either quadratic, linear, or spectral data.
Circle No. 331 on reader service card.

Silica-Based Affinity Chromatography Column

Formulated specifically for the purification of antibodies and monoclonal antibodies in biotechnology and pharmacology, ChromatoChem's HiPAC Protein A column utilizes the company's proprietary HiPAC silica support, making the column high pressure and pH tolerant while cutting purification time from several hours to 10 minutes.
Circle No. 332 on reader service card.

Laboratory and Portable Instrument Catalog

"Hach Laboratory and Portable Instruments" is a new 40-page catalog that covers many new instruments along with information about Hach systems for testing of drinking water, wastewater, foods and beverages, plating baths and effluent, and soils.
Circle No. 333 on reader service card.

New Elutriator Rotor

Applications of the new Beckman JE-5.0 Elutriator Rotor include synchronous cell growth studies, the separation of liver cells, human lymphocytes and monocytes, and other cell and particle studies. Using elutriation, cells retain their viability because they are separated by a gentle, washing action, and can be processed in an isotonic medium.
Circle No. 334 on reader service card.

Atomic Absorption Graphite Furnace Training Program

Savant announces a new audiovisual training package, AA-107, "Modern Methods of Graphite Furnace Atomic Absorption." The program, available in either slide-tape or video formats, discusses the operation of the furnace, including the requirement for

inert gas, use of a platform and autosamplers. Programming steps, including drying, charring, and optional addition of matrix modifiers, all lead to the atomization step, which is discussed in detail.
Circle No. 335 on reader service card.

Fully Integrated TG/FT-IR Analyzer
Bomem announces a new TG/FT-IR analyzer—the TG/Plus. This apparatus combines the standard features of a Dupont 951 TGA with a multipass gas cell and a Michelson FT-IR for on-line quantitative analysis of evolved products, including evolved condensates.
Circle No. 336 on reader service card.

Forensic Science Database
The Forensic Science database from Data-Star is multidisciplinary and covers literature relevant to the examination of evidential materials, analytical methods, and the presentation and interpretation of findings. Parts of the database cover drugs and toxicology, forensic biology, and forensic chemistry.
Circle No. 337 on reader service card.

Automated Glucose and L-Lactate Analyzer
The new YSI automated analyzer provides rapid real-time answers for both glucose and L-lactate levels in fermentation broths and cell cultures. The Model 2000 measures glucose and L-lactate simultaneously, prints and displays the results in 60 seconds, automatically calibrates itself, and runs 60 samples per hour.
Circle No. 338 on reader service card.

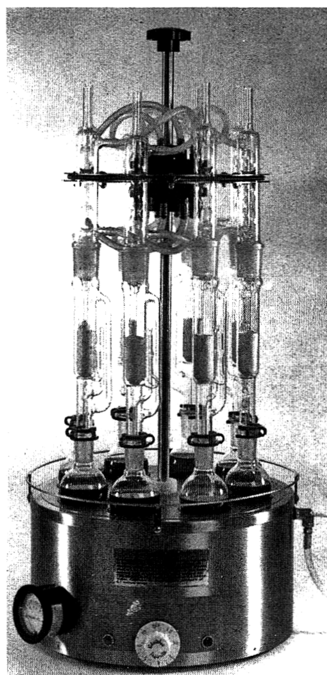
Material Safety Data Sheet Database
A new on-line database containing material safety data sheets (MSDSs) for industrial chemicals is now available from Chemical Information System. Baker, produced by the J.T. Baker Company, contains more than 1,600 OSHA-formatted MSDSs. They may be retrieved from the system by input of a chemical name or a CAS Registry Number.
Circle No. 339 on reader service card.

Sodium Measurement Electrode
While conventional sodium electrodes need time for the internal cells to reach thermal equilibrium with the sample, Ross announces that its new electrodes are virtually unaffected by temperature variation. When the ROSS sodium electrode, Model 84-11, is cycled between two portions of a 100 ppm sodium standard at 25–80°C, temperature equilibrium is reached in less than 1 minute, compared to 3–5 minutes for a conventional sodium electrode.
Circle No. 340 on reader service card.

Repetitive Micro Dispenser
The Model RD 1000 Repetitive Micro Dispenser from Monoject Scientific is a hand-held dispenser that offers the capability of rapid repetitive dispensing of samples from 10 to 1,000 μL. The units are continuously adjustable and have a click-stop mechanism to prevent accidental changes during use.
Circle No. 341 on reader service card.

Spectra-Physics' WINner Brochure
The latest in product literature on the WINner Workstation/INtegrator is now available from Spectra-Physics. This 6-page brochure describes the features of the $4,500 chromatography system controller and integrator.
Circle No. 342 on reader service card.

ROT-X-TRACT™



CIRCLE 14 ON READER SERVICE CARD

Features:
1. Up to 8 Soxhlet extractors can be handled at one time.
2. Uses standard Soxhlet glassware.
3. Compact circular arrangement reduces bench space to 14 inches.
4. Each Soxhlet set is easily accessible from the front.
5. Easily loaded and unloaded.
6. Electrical energy reduced.
7. Optional intrinsically-safe bath reduces chance of flammable solvent ignition.

Description:
Up to 8 standard Soxhlet glassware units are held in a spring holder attached to a central vertical tube. This tube fits loosely over a stand rod on which it can rotate. The assembly on its stand is placed in a boiling water bath. Each Soxhlet unit rest on a hole in a cover plate which reduces loss of steam.

Operation:
The ROT-X-TRACT will handle most commonly used extraction solvents such as Diethyl Ether, Pentane, Methylene Chloride and Petroleum Ether. For ease in loading and unloading, a spring holder allows the condensers to be held while disconnecting them from the thimble holder.

About our Firm:
Organomation Associates Inc. has been in business for over 27 years. We are proud of our reputation. Numerous repeat orders attest to the popularity of our equipment in analytical laboratories around the world. For information and prices on the ROT-X-TRACT as well as our complete line of analytical evaporators please contact us.

Organomation Associates, Inc.

Tel: (617) 393-2602, (617) 838-7378
Telex: 95-5439-ORGANOMATION
FAX: 413-733-2590

266 River Road
P.O. Box 159
South Berlin, MA 01549

FOR YOUR INFORMATION

Harvey W. Wiley Award to Frederick Kavanagh

Frederick Kavanagh, a retired analytical microbiologist with Eli Lilly, has been named to receive AOAC's 1988 Harvey W. Wiley Award, the most prestigious recognition extended by AOAC for outstanding contributions to methodology. Kavanagh will receive the award at the opening session of the 102nd AOAC Annual International Meeting and Exposition in Palm Beach, FL, August 29, 1988.

In a career spanning nearly 50 years, the last 20 years spent with Eli Lilly, Kavanagh repeatedly showed ability in conceiving theories and practices for microbiological assays of antibiotics and vitamins in pharmaceutical and agricultural commodities. His numerous publications as well as scientific instruments and electronic devices he constructed for use in industry and medicine are testimonials to his efforts.

During his career, Kavanagh first analyzed steel and paint. He then worked on the many problems of increasing yields of antibiotics. This involved shake-flask investigations and pilot plant work with the new media and strains of the producing organisms. As he neared his retirement from Eli Lilly in 1973, he was concerned, almost exclusively, with the theory and practice of assaying for antibiotics and vitamins, with instrument design, and with the development of new assays.

Within a career marked by accomplishments, 4 are particulary noteworthy for their contributions to the advancement of analytical methodology: (1) Kavanagh was a codeveloper of the AutoTurb system, an automated microbiological assay now widely used throughout the pharmaceutical industry for both antibiotic and vitamin assays and in the agricultural industry for certain feed assays; (2) he was both editor of and contributor to the books that organized the emerging use of analytical microbiology both theoretically and practically: *Analytical Microbiology*, Vol. 1, 1963, Academic Press, New York, NY, and *Analytical Microbiology*, Vol. 2, 1972, Academic Press, New York, NY; (3) he developed microbiological turbidimetric assays for monensin and tylosin in feeds; and (4) he was the author of a series of papers that deal with the theory of microbiological assays and apply mathematical and theoretical concepts to explain microbiological measurements.

Kavanagh's interest in the design of analytical instruments deserves special emphasis. Beginning in 1936 with a pH meter, consisting of electrometer, glass electrode, and reference cell, he built his own instruments at a time when commercial instruments were rare and relatively expensive. He was a consultant in instrument design to Klett Manufacturing for 30 years, designing the Klett Fluorimeter as well as a power supply, precision current measuring system, and conductivity bridge for the Klett electrophoresis equipment. For Eli Lilly, he not only codesigned the AutoTurb system but also designed photometers with self-balancing potentiometers for measuring turbidity of bacterial suspensions, a large plate assay system and reader, a recording scanner for measuring radioactivity in the spots on 2-dimensional paper chromatograms, and a Petri dish diffusion assay system that employed a special high precision incubator for the plates.

Kavanagh's career in analytical microbiology was also distinguished by a position he held for nearly 10 years at the New York Botanical Garden where he found, isolated, and characterized antibiotics produced by Basidiomycetes. He collaborated with Dr. Richard Goodwin on fluorescence of coumarin derivatives and identified scopoletin as a blue fluorescing substance in root tips by means of its characteristic fluorescence vs pH curve. Born on May 24, 1908, Kavanagh received A.B. and A.M. degrees from the University of Missouri and a doctorate from Columbia University in 1942. His hobbies include learning about history and science.

Meetings

May 19, 1988: New York/New Jersey AOAC Regional Section Meeting, Brooklyn, NY. Contact: Arthur Waltking, Best Foods Research and Engineering Center, CPC International, Inc., 1120 Commerce Ave, Union, NJ 07083, telephone 201/688-9000.

June 7, 1988: Southeast AOAC Regional Section Meeting, Atlanta, GA. Contact: Frank Allen, Environmental Protection Agency, Reg 4 ESD, Athens, GA 30613, telephone 404/546-3387.

June 20–22, 1988: Northeast AOAC Regional Section Meeting, Lowell, MA. Contact: Edmond Baratta, FDA, Winchester Engineering and Analysis Center, 109 Holton St, Winchester, MA 1890, telephone 617/729-5700.

June 20–22, 1988: Midwest AOAC Regional Section Meeting, Columbia, MO. Contact: George Rottinghaus, University of Missouri, Veterinary Medicine Diagnostic Laboratory, Columbia, MO 65211, telephone 314/882-6811.

June 23–24, 1988: Pacific Northwest AOAC Regional Section Meeting, Olympia, WA. Contact: Mike Wehr, Oregon Department of Agriculture, 635 Capitol St, NE, Salem, OR 97310, telephone 503/378-3793.

June 1988: Eastern Ontario/Quebec AOAC Regional Section Meeting. Contact: Michele Bono, HPB, Health and Welfare, 1001 St Laurent O, Longueuil, PQ J4K 1C7, Canada, telephone 514/646-1353.

June 15–17, 1988: 19th Ohio Valley Chromatography Symposium, including a minicourse on "Analytical Chemistry and Regulatory Agencies," Miami University, Oxford, OH, and sessions on bioanalytical, hybrid techniques, and recent developments, Hueston Woods State Park Lodge, OH. Contact: Richard A. Day, Department of Chemistry, University of Cincinnati, OH 45221.

August 29–September 1, 1988: 102nd AOAC Annual International Meeting and Exposition, spotlight on "Biotechnology," The Breakers, Palm Beach, FL. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

October 25–28, 1988: First Combined FGD and Dry SO_2 Control Symposium, Adam's Mark Hotel, St. Louis, MO. Sponsored by U.S. Environmental Protection Agency and Air and Energy Engineering Research Laboratory. Contact: Sharon Luongo, Electric Power Research Institute, 3412 Hillview Ave, PO Box 10412, Palo Alto, CA 94303.

October 30–November 4, 1988: FACSS XV (Federation of Analytical Chemistry and Spectroscopic Societies), Hynes Convention Center, Boston, MA. Contact: Roger Gilpin, Department of Chemistry, Kent State University, Kent, OH 44242, telephone 216/672-2032.

September 25–28, 1989: 103rd AOAC Annual International Meeting and Exposition. The Clarion Hotel, St. Louis, MO. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

September 10–13, 1990: 104th AOAC Annual International Meeting and Exposition, The Clarion Hotel, New Orleans, LA. Contact: Margaret Ridgell,

COME TO

AT THE BREAKERS, PALM BEACH, FLORIDA
AUGUST 29 - SEPTEMBER 1, 1988

The Spotlight Symposium on **BIOTECHNOLOGY** - Chairman: D.M. Hinton

Laboratory Information Management Systems (LIMS) - Chairmen: J.J. Karr and H. Morris

Fertilizer Phosphate Evaluation and Analysis - Chairmen: F. Johnson and J.R. Trimm

Drug Residues in Foods of Animal Origin - Chairmen: W.A. Moats and B. Shaikh

Pesticides in Foods - Chairman: P. Corneliussen

Over 200 **technical poster presentations** on topics such as Pesticides Formulations and Disinfectants; Foods; Residues; Microbiology; Feeds, Fertilizers and Related Topics; Drugs and Related Topics; Hazardous Substances in Waste and the Environment.

Open Forum

Regulatory Roundtable: Safety - The 1986-87 OSHA Regs

Laboratory Quality Assurance Short Course

Laboratory Equipment and Supplies Exposition

Speaker: Wiley Award winner — **Dr. Frederick Kavanagh,** eminent analytical chemist, microbiologist and instrument designer.

For further information, contact: Administrative Manager, AOAC, 1111 N. 19th St., Ste. 210, Arlington, VA 22209, or call (703) 522-3032.

AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

Short Courses

For the 25th summer, short courses in powder diffraction and x-ray spectrometry will be offered at the State University of New York at Albany. Both courses are tutorial and develop the entire field of chemical analysis. The course in powder diffraction, scheduled June 20–July 1, 1988, will emphasize quantitative methods of analysis, and the course in x-ray spectrometry, scheduled June 6–August 19, 1988, will concentrate on mathematical and computer methods to solve the matrix problem and probe into in-depth problems of the advanced spectroscopist. For course descriptions, specific dates, costs and enrollment information, contact the Department of Physics, State University of New York at Albany, 1400 Washington Ave, Albany, NY 12222, telephone 518/442-4512.

AOAC Board Approves New Collaborative Study Protocol

The AOAC Board of Directors at its January 7, 1988, meeting approved a new protocol for the design, conduct, and interpretation of collaborative studies as recommended by the Committee on Interlaboratory Studies. The new protocol replaces some of the material in the AOAC Handbook and in the Youden-Steiner Statistical Manual, although much of these documents is still valid and valuable. The new protocol recommends the minimum analysis of 5 materials by 8 laboratories. For calculating within-laboratory repeatability, blind duplicates or Youden pairs (split levels) are recommended. Certain exceptions are permitted under specified conditions. The statistical analysis should be conducted on valid data by a 1-way analysis of variance, removing outliers by the Cochran test for extreme variance and the Grubbs tests for extreme laboratories. The statistical parameters of mean and relative standard deviations within- and between-laboratories should be reported both before and after outlier removal. Associate Referees are encouraged to discuss with the collaborators apparent outliers when they are reported in order to discover reasons for aberrations.

This protocol conforms to the recommendations made by the International Union for Pure and Applied Chemistry (IUPAC) Workshop on Harmonization of Collaborative Analytical Studies, which was held in Geneva, Switzerland, on May 4–5, 1987. The recommendations of that meeting were approved by the Board.

The Committee on Interlaboratory Studies has been working on a new protocol since 1980. In the course of its work, it consulted with many of the national and international organizations that conduct collaborative studies. The Committee identified 12 points that required agreement in order to make collaborative studies interchangeable among organizations. Agreement was achieved by consensus of the representatives of about 20 organizations that participated in the IUPAC Harmonization Workshop. Collaborative studies whose design, conduct, and interpretation conform to this agreed upon protocol may be identified as conforming to the "IUPAC-1987" recommendations.

The AOAC protocol was published in the January/February 1988 issue of the *Journal of the Association of Official Analytical Chemists* as an Appendix to the Report of the Committee on Interlaboratory Studies. The more general IUPAC protocol will be published later

COMING IN THE NEXT ISSUE

SPECIAL REPORT

- Supercritical Fluid Chromatrography: A New Technique for the AOAC—*D. E. Knowles, B. E. Richter, M. B. Wygant, L. Nixon, and M. R. Anderson*

FOOD ADDITIVES

- Gas Chromatographic Determination of Monoethylene Glycol and Diethylene Glycol in Chocolate Packaged in Regenerated Cellulose Film—*L. Castel, H. R. Cloke, J. R. Startin, and J. Gilbert*
- Liquid Chromatographic Determination of L-Ascorbic Acid in Candies and Soft Drinks—*Y. Maeda, S. Ochi, T. Masui, and S. Matubara*
- Comparison of Three Methods for Determination of *N*-Nitrosopyrrolidine in Fried Dry-Cured and Pump-Cured Bacon—*A. J. Malanoski and W. J. Smith*

and

PROCEEDINGS OF THE WORKSHOP ON LISTERIA METHODOLOGY

HELD AT THE 101ST AOAC ANNUAL INTERNATIONAL MEETING SEPTEMBER 14–17, 1987, SAN FRANCISCO, CA

this year in the IUPAC journal, *Pure and Applied Chemistry*.

ISO Standards Published

The following standards have been published by the International Organization for Standardization (ISO), Technical Committee 34—Agricultural Food Products. The standards are available, at the prices indicated, from American National Standards Institute, Inc., 1430 Broadway, New York, NY 10018, telephone 212/354-3300.

ISO 1735-1987: Cheese and processed cheese products—Determination of fat content—Gravimetric method (Reference method). $22.00.

ISO 5532-1987: Durum wheat—Determination of proportion of nonwholly vitreous grains (Reference method). $14.00.

ISO 5538-1987: Milk and milk products—Sampling—Inspection by attributes. $30.00.

ISO 7555-1987: Soya beans—Specifications. $14.00.

ISO 7556-1987: Rape (colza) seeds with high erucic acid content—Specifications. $14.00.

ISO 7847-1987: Animal and vegetable fats and oil—Determination of polyunsaturated fatty acids with a cis,cis 1,4-diene structure. $20.00.

ISO 7954-1987: Microbiology—General guidance for enumeration of yeasts and molds—Colony count technique at 25°C. $18.00.

ISO 8262-1-1987: Milk products and milk-based foods—Determination of fat content by the Weibull-Berntrop gravimetric method (Reference method). Part 1: Infant foods. $20.00.

ISO 8262-2-1987: Milk products and milk-based foods—Determination of fat content by the Weibull-Berntrop gravimetric method (Reference method). Part 2: Edible ices and ice-mixes. $20.00.

ISO 8262-3-1987: Milk products and milk-based foods—Determination of fat content by the Weibull-Berntrop gravimetric method (Reference method). Part 3: Special cases. $20.00.

ISO 8381-1987: Milk-based infant foods—Determination of fat content by the Roese-Gottlieb gravimetric method (Reference method). $25.00.

ISO 8682-1987: Apples—Storage in controlled atmospheres. $20.00.

New Private Sustaining Members

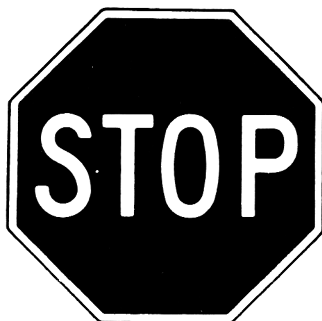
AOAC welcomes the following new private sustaining members: Cambridge/Naremco, Springfield, MO; Chesebrough-Pond's, Trumbull, CT; Swift-Eckrich, Oak Brook, IL; Penicillin Assays, Malden, MA; and Oxoid U.S.A., Columbia, MD.

Industry to Participate Fully in AOAC Voting

After years of debate on this question, the AOAC membership voted to make significant changes in its bylaws to enable members employed by industry to participate fully in association voting and governance. The vote took place at the Business Meeting held September 17, 1987, at the 101st Annual International Meeting in San Francisco, CA.

Industry has been an active participant in AOAC's analytical methods development and validation program since the establishment of the association in 1884 as the Association of Official Agricultural Chemists to approve standardized methods for regulatory analysis. Although unable, until now, to vote on adoption of methods or serve as officers of the association, industry members have served as Associate Referees, as collaborators, on special committees, and, since 1980, as General Referees and on Methods Committees.

According to AOAC's new President,



IMPORTANT DEADLINE!
NOTE IT ON YOUR
1988 CALENDAR.

For the 102nd AOAC Annual International Meeting, Palm Beach, Florida, August29-September 1, 1988.

Abstracts **April 20, 1988**

For more information, contact: Administrative Manager, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA 22209 or call 703-522-3032.

Quality Assurance Means Never Having to Say You're Sorry

Attend the
AOAC QUALITY ASSURANCE SHORT COURSE

Take this intensive comprehensive two-day course on *planning, designing, and managing* a laboratory quality assurance (QA) program.

LEARN

- **How to organize and document a QA program**
- **Why you should commit the necessary resources**
- **What it will cost**

COURSE PROGRAM

First Day

I	QA — Planning and Management
II	Basic Statistics — Applications in QA
III	Analytical Control Charting
IV	Personnel Management — Role in QA
V	Equipment and Supplies Management
VI	General Discussion and Review

Second Day

VII	QA in Sampling
VIII	QA in Sample Analysis
IX	Records and Reporting
X	Proficiency Testing — Inter- and Intralaboratory
XI	Audit Procedures for QA
XII	General Discussion and Review

COURSE SCHEDULE

Wednesday and Thursday, April 13–14, 1988
Westpark Hotel, Arlington, Virginia

Tuesday and Wednesday, July 12–13, 1988
Westpark Hotel, Arlington, Virginia

Saturday and Sunday, August 27–28, 1988
AOAC 102nd Annual International Meeting and Exposition
The Breakers, Palm Beach, Florida

The popular QA manual, *Quality Assurance for Analytical Laboratories*, is provided FREE to attendees.
Registration Fee: $475 for AOAC members, $525 for nonmembers.

REGISTER NOW! COURSE IS LIMITED TO 45 PARTICIPANTS!

REGISTRATION FORM

Please sign me up for the short course

QA for Analytical Laboratories, ☐ April 13–14, 1988, Arlington, Virginia
☐ July 12–13, 1988, Arlington, Virginia
☐ August 27–28, 1988, Palm Beach, Florida

Please Type or Print

NAME______________________ TITLE______________________

ORGANIZATION______________________

ADDRESS______________________

______________________ TELEPHONE (______)______________________

I am enclosing: ☐ $475 member registration (Member No. VM____________) ☐ $525 nonmember registration

Payment Must Accompany Registration

Return with payment to: AOAC QA Short Course, 1111 N. 19th Street, Suite 210-J, Arlington, Virginia 22209
(AOAC reserves the right to cancel courses at any time.)

For additional information, contact Margaret Ridgell at AOAC, (703) 522-3032.

Robert Rund, "By our actions ... in San Francisco, the barrier within our bylaws to full membership was removed.... No longer must we designate some as 'Associate Members' without the privilege of suffrage or the right to various offices."

This change, long favored by many members in government, academia, and industry, was also urged by FDA Associate Commissioner for Regulatory Affairs, Joseph Hile, in his address at the 1985 AOAC Annual International Meeting.

AOAC's two membership classifications, one for government and academia and another for industry, have been replaced with one classification open to all chemists, biologists, and other scientists and organizations otherwise eligible for membership in the association. This change grants full voting privileges, including voting on adoption of AOAC official methods, to all individual members. The bylaw changes also extend eligibility to all members to serve on the Board of Directors and the Official Methods Board with the requirement that government and academia together maintain a majority on these boards. In addition, the size of the Board of Directors has been increased from 7 to 9 members, and members will now vote on all matters other than adoption of methods by mail ballot.

Call for Nominations

Nominations are being sought for the 1989 Eastern Analytical Award for Outstanding Achievements in Analytical Chemistry. The award consists of a plaque and a $1000 stipend and will be presented at a special award symposium to be held at the Eastern Analytical Symposium in New York, the week of September 25–29, 1989. A nominating letter describing the nominee's specific accomplishments should be submitted along with a biographical sketch by July 1, 1988. Contact: Alvin Bober, State of Maryland, DHMH, Environmental Chemistry Division, 201 West Preston St, PO Box 2355, Baltimore, MD 21203.

APhA Establishes Research Institute

The American Pharmaceutical Association (APhA) has announced the formation of the American Pharmaceutical Institute as its new public policy research arm. The Institute will conduct and encourage policy studies on topics such as the changing environment for the delivery of health services, the role and importance of the "team" concept in providing quality care, the cost-effectiveness of modern medicines and pharmaceutical services, the role of the pharmacist in the selection and proper use of therapeutic agents, and pharmacy management in integrated health care systems. Michael R. Pollard, formerly director of policy analysis at the Pharmaceutical Manufacturers Association, has been appointed executive director.

Interim Methods

The following methods have been approved interim official first action by the respective methods committees and by the chairman of the Official Methods Board: by the Methods Committee on Pesticide Formulations and Disinfectants—(1) Gas Chromatographic Method for Determination of Fenitrothion in Fenitrothion Technical and in Water-Dispersible Powder and Emulsifiable Concentrate Formulations, submitted by D. L. Mount and F. C. Churchill (Centers for Disease Control, Atlanta, GA) and (2) Liquid Chromatographic Method for Determination of Azinphos-Methyl in Formulated Products, submitted by S. C. Slahck (Mobay Corp., Kansas City, MO); and by the Methods Committee on Feeds, Fertilizers, and Related Topics—Gas Chromatographic Method for Assay of Supplemental Vitamin E Acetate Concentrates, submitted by M. P. Labadie and C. E. Boufford (BASF Corp., Wyandotte, MI).

The methods will be submitted for adoption official first action at the 102nd AOAC Annual International Meeting, Aug. 29–Sept. 1, 1988, at Palm Beach, FL. Copies of the methods are available from the AOAC office.

Obituaries

Jacob Fitelson, 82, a Fellow of the AOAC and retired research chemist of the Food and Drug Administration, died recently at his home in West Palm Beach, FL.

Fitelson worked 25 years with FDA before his retirement as Chief Chemist in 1951. Most renowned for his work in oils, he developed tests to detect the adulteration of olive oil. During World War II, he persuaded the sole supplier of squalene, a naturally occurring hydrocarbon, to add an innocuous marker. When this was detected in oils in the marketplace, many seizures were made. His testimony in the subsequent court case was the first use of spectrophotometry to show the presence of a specific entity in a product.

He also established his own private testing and analytical laboratory and became a consultant to the Flavor and Extract Manufacturers Association. As Associate Referee for Vanilla Products, he essentially rewrote the AOAC chapter on the analysis of vanilla extract. He contributed at least six official methods, updating the official procedures by using modern analytical techniques. He was a member of the original group elected as Fellows of the AOAC in 1963 when the title was established.

Alexander P. Mathers, 76, past president of the AOAC and nationally known chemist, died November 14, 1985, at the North Carolina Baptist Hospital in Winston-Salem following a stroke he suffered during traveling. He lived in Matherville, MS.

Mathers spent most of his career with the U.S. Department of the Treasury, Bureau of Alcohol, Tobacco and Firearms, beginning as a chemist in 1941 and serving as Chief Chemist from 1962 until his retirement in 1973. He served as president of AOAC in 1967–1968.

After his retirement, Mathers returned to his native Matherville, where he established the Almarla Vineyards and Winery and was among the pioneers in the reestablishment of a viable wine industry in Mississippi. He was a graduate of the University of Florida and also earned the Master of Science degree in chemistry from Tulane University and the Doctor of Philosophy (Ph.D.) in chemistry from George Washington University.

The author of more than 30 scientific papers, Mathers received the Internal Revenue Service Gallatin Award and the Meritorious Service Award of the U.S. Treasury Department. After his retirement, he was a consultant to the Ernest and Julio Gallo Winery in matters of Food and Drug Administration and Treasury Department regulations.

Analysis of Surface Waters. By Hubert Hellmann. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1987. 275 pp. Price: $89.95 (hardcover). ISBN 0-7458-0213-3.

The complex task of analyzing surface waters is reviewed for the important provisos for a thorough presentation of results from surface water investigations, including attention to the water body itself, interactions with atmosphere, soils, and sediments, importance of hydrological factors, and meaningful expoitation of the numerical data.

Applications of Mass Spectrometry in Food Science. Edited by John Gilbert. Published by Elsevier Scientific Publishers, Inc., PO Box 211, 1000 AE Amsterdam, Netherlands, 1987. 440 pp. Price: $108.00 (hardcover). ISBN 1-85166-081-X.

A number of specialized applications are drawn together, including long-established techniques like GC/MS to the newer LC/MS and MS/MS, capillary chromatography, and computer data handling. The many illustrations describe analyses of a wide range of food contaminants and food constituents.

The Art of Scientific Writing. Hans Ebel, C. Bliefert, and W. E. Russey. Published by VCH Publishers, 220 E 23rd St, New York, NY 10010, 1987. 450 pp. Price: $24.95 (paperback), ISBN 0-89573-645-4; $59.95 (clothbound), ISBN 0-89573-495-8.

The years of experience of the authors as authors themselves and as teachers and editors range from initial notes taken in chemistry laboratories to full manuscript preparation. The authors describe the tools and materials used in scientific writing, guide the reader on correct use of units and symbols, formulation of equations, and best use of tables and figures. The many cross references and extensive index aid the daily work of authors and editors in publishing.

Principles of Environmental Sampling. Edited by Lawrence H. Keith. Published by American Chemical Society, 1155 Sixteenth St NW, Washington, DC 20036, 1987. 458 pp. (clothbound). Price: $59.95 (U.S. and Canada); $71.95 (export). ISBN 0-8412-1437-9.

Sampling may be the weakest link in the chain of analytical activities. The 30 chapters discuss overall principles, planning and design, quality assurance, and quality control for sampling water, air and stacks, biota, solids, sludges, and liquid wastes. Also discussed are instability of samples, and problems of transport and storage.

Advances in Standards and Methodology in Spectrophotometry. Edited by C. Burgess and K. D. Mielenz. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, Netherlands, 1987. 404 pp. Price: $95.00/Dfl. 195.00. ISBN 0-444-42880-1.

Proceedings from a conference in 1986 include interdisciplinary papers on high accuracy spectrometry, standards and calibration, modern spectrometry instrumentation arrays and lasers, new trends in Fourier Transform and IR spectrometry, and poster abstracts.

Selective Sample Handling and Detection in High-Performance Liquid Chromatography. Edited by R. W. Frei and K. Zech. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, Netherlands, 1987. 460 pp. Price: $117.00/Dfl. 240.00. ISBN 0-444-42881-X.

Sample handling and detection processes in an LC system are treated in an integrated fashion. Selectivity and trace enrichment in the sample preparation step place fewer demands on the quality of chromatography; on the other hand, by using a selective detection mode, the sample handling step can be simplified and automated.

Preparative Liquid Chromatography. Edited by B. A. Bidlingmeyer. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, Netherlands, 1987. 330 pp. Price: $97.50/Dfl. 200.00. ISBN 0-444-42832-1.

A focus on practical applications, and an understanding of the strategy and relationship between input and output is applied to isolation and purification problems, including efficiency of large scale packed bed columns, pharmaceutical, synthetic, and biochemical applications, enantiomers on chiral stationary phases, and size exclusion chromatography.

Handbook of Natural Pesticides: Pheromones. Edited by Bhushan Mandava. Published by CRC Press, Inc., 2000 Corporate Blvd NW, Boca Raton, FL 33431, 1988. Part A—224 pp. Price: $140.00 (U.S.), $172.00 (outside U.S.), ISBN 0-8493-3657-0. Part B—368 pp. Price $155.00 (U.S.), $172.00 (outside U.S.), ISBN 0-8493-3658-0.

Chapters are included on pheromones produced by Lepidoptera, Coleoptera, Diptera, Hymenoptera, and Isoptera, and the perception of odor and the function of molecular structure in understanding odor perception.

***Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology.** Editor-in-Chief Frederick C. Neidhardt. Published by American Society for Microbiology, 1913 Eye St NW, Washington DC 20006, 1987. Two volumes, 1654 pp. Price: softcover $66.00 (member), $90.00 (nonmember), ISBN 0-914826-85-1; hardcover $76.00 (member), $100.00 (nonmember), ISBN 0-914826-89-1.

This comprehensive treatment of the two bacteria comprises all the important data, maps, pathways, structures, and processes culled from many primary references, as well as reviews to place information into perspective and to note where new information and understanding are needed. Volume I in 2 parts discusses molecular architecture and assembly of cell parts, and metabolism and general physiology. Volume II in 4 parts discusses genome and genetics, regulation of gene expression, growth of cells and cultures, and ecology evolution and population structure.

Proceedings—International Commission for Uniform Methods of Sugar Analysis, 19th Session, Cannes, France, 1986. Published by ICUMSA, % British Sugar plc, Research Laboratories, Colney, Norwich NR4 7UB, England. Price: £28. ISBN 0-905003-11-X.

Reports on 31 subjects are included, each concerned with one particular field of sugar analysis, along with the Referee recommendations. Special discussion topics include statistics and interlaboratory tests, chromatographic and enzymatic analysis, cooperation with other organizations, and future work of ICUMSA.

AOAC MEETINGS & EXPOSITIONS

March 29, 1988
MID-CANADA REGIONAL SECTION
Winnipeg, Manitoba, Canada
Topic: Troubleshooting and
Capillary Gas Chromatography

June 20-22, 1988
MIDWEST REGIONAL SECTION
Holiday Inn West, Columbia, MO

May 19, 1988
NEW YORK-NEW JERSEY REGIONAL SECTION
Fort Hamilton Officer's Club,
Brooklyn, NY

June 23-24, 1988
PACIFIC NORTHEWST REGIONAL SECTION
(with speaker, banquet and exposition)
Evergreen College, Olympia, WA

June 14, 1988
SOUTHEAST REGIONAL SECTION
Radisson Inn & Conference Center,
Atlanta, GA

June 1988
EASTERN ONTARIO-QUEBEC REGIONAL SECTION

June 20-22, 1988
NORTHEAST REGIONAL SECTION
Lowell University, Lowell, MA

August 29-September 1, 1988
102nd ANNUAL INTERNATIONAL MEETING & EXPOSITION
The Breakers, Palm Beach, FL
Program: Analytical Methodology
including spotlight on Biotechnology

For further information, contact: The AOAC Meetings Department, AOAC, 1111 N. 19th St., Ste. 210, Arlington, VA 22209, or call in the U.S. (703) 522-3032 or outside the U.S. +1-703-522-3032.

ELIMINATING CAN DEFECTS—THE FIRST STEP

How can we keep defective cans off the market?

Botulism and other forms of food poisoning can sometimes be traced to defective cans that have leaked and thereby allowed micro-organisms to enter food. Recognizing a can defect, doing something to correct the cause, and removing the defective cans from commerce will help prevent food poisoning outbreaks. The first step is to ensure that responsible personnel know how to identify defective cans.

The Association of Official Analytical Chemists, in cooperation with the Food and Drug Administration, has published a pamphlet that unfolds to a 24″ x 36″ chart, suitable for wall display, to help food industry personnel learn to identify can defects quickly. The chart uses a combination of photographs, easy-to-follow explanations, and color coding to illustrate can defects, classify them according to their degree of potential hazard, and show what to look for in routine inspection of the finished product.

The chart is a valuable reference resource for food processors, salvage operators, retail food personnel, wholesalers and state and local government sanitarians.

For copies, send order form and check to:
Association of Official Analytical Chemists
1111 North 19th Street, Suite 210
Arlington, VA 22209 USA
Telephone: (703) 522-3032

Minimum Order:
1 package of 10 charts
$40.00 plus $4.00 postage

Second package
$30.00 plus $4.00 postage

Each additional package
$25.00 plus $4.00 postage

I enclose $________ for the following quantity of Can Defects charts:

Minimum order: _____ First package of 10 charts @ $40.00 plus $4.00 postage $________

_____ Second package of 10 charts @ $30.00 plus $4.00 postage ________

_____ Additional packages of 10 charts @ $25.00 plus $4.00 postage ________

Total ________

Name ________

Company ________

Address ________

City, State, Zip ________

INFORMATION FOR SUBSCRIBERS, CONTRIBUTORS, AND ADVERTISERS

THE ASSOCIATION

The primary objective of the Association of Official Analytical Chemists (AOAC) is to obtain, improve, develop, test, and adopt precise, accurate, and sensitive methods for analysis of foods, vitamins, food additives, pesticides, drugs, cosmetics, plants, feeds, fertilizers, hazardous substances, air, water, and any other products, substances, or phenomena affecting the public health and safety, the economic protection of the consumer, or the protection of the quality of the environment; to promote uniformity and reliability in the statement of analytical results; to promote, conduct, and encourage research in the analytical sciences related to foods, drugs, agriculture, the environment, and regulatory control of commodities in these fields; and to afford opportunity for the discussion of matters of interest to scientists engaged in relevant pursuits.

AOAC Official Methods are methods that have been validated by an AOAC-approved collaborative study, recommended by the appropriate AOAC General Referee, Methods Committee, and the Official Methods Board, and adopted and published according to the Bylaws of the Association. Published papers that include such methods are distinguished by the words Collaborative Study in the title and by footnotes that indicate Association actions.

Membership in AOAC is open to all interested persons worldwide. Sustaining memberships are available to any government agency, private company, or association interested in supporting an independent methods validation program.

European Representatives For information about AOAC and its publications, persons outside the U.S. may also contact the following: Margreet Lauwaars, PO Box 153, 6720 AD Bennekom, The Netherlands, telephone 31-8389-18725; Derek C. Abbott, Green Gables, Green Ln, Ashtead, Surrey, KT21 2JP, UK, telephone 44-3722-74856; Lars Appelqvist, Swedish University of Agricultural Sciences, Dept of Food Hygiene, S 750 07 Uppsala, Sweden, telephone 46-18-172398.

THE JOURNAL

The *Journal of the Association of Official Analytical Chemists* (ISSN 0004-5756) is published bimonthly by AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. Each volume (one calendar year) will contain about 1200 pages. The scope of the *Journal* encompasses the development and validation of analytical procedures pertaining to the physical and biological sciences related to foods, drugs, agriculture, and the environment. Emphasis is on research and development of precise, accurate, and sensitive methods for the analysis of foods, food additives and supplements, contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment.

Methods The scientific validity of published methods is, of course, evaluated as part of the peer-review process. However, unless otherwise stated, methods published in contributed papers in the *Journal* have not been adopted by AOAC and are not AOAC Official Methods.

Manuscripts Prepare manuscripts double-spaced throughout and carefully revise before submission; 4 copies should be submitted to Managing Editor, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. AOAC reserves the right to refuse manuscripts produced on dot matrix printers of less than letter quality. "Instructions to Authors" is published in the January issue of the *Journal* (may also appear in other issues) and is available on request from AOAC.

Page Charges 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. Requests for waiver may be sent to the Managing Editor by the administrative officer of the author's institution. Reports of collaborative studies performed in pursuit of approval of AOAC official methods are exempt from page charges.

Reprints Authors may order reprints of articles when they return typeset proofs. An order form and schedule of rates is included with each author proof. Readers who wish to obtain individual reprints should contact authors directly.

Responsibility AOAC and the Editors assume no responsibility for statements and opinions expressed by authors of manuscripts published in the *Journal*.

SUBSCRIPTIONS

Journal subscriptions are sold by the volume (one calendar year). Rates for 1988 are as follows: Members: $84.57 in U.S.; $94.57 outside U.S. Nonmembers: $99.50 in U.S.; $109.50 outside U.S. Two year subscriptions are also available. Airmail $56.00 additional per year. Claim for copies lost in the mail will not be allowed unless received within 30 days of the date of issue for U.S. subscribers or 90 days for all others. Claimants must state that the publication was not received at their recorded address. Address requests for replacement copies to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. For subscribers outside the U.S., copies of the *Journal* lost in transit cannot be replaced without charge because of uncertain mailing conditions.

Change of Address Notification should include both old and new addresses, with mail code, and be accompanied by a mailing label from a recent issue. Allow 4 weeks for change to become effective. Subscribers outside the U.S. should use airmail for notification.

Microfilm Volumes on microfilm are available from Princeton Microfilm Corp., PO Box 2073, Princeton, NJ 08540.

Single Issues Information on single issues is available from AOAC.

COPYING

Copyright All articles published in the *Journal* are protected by copyright. Persons requiring copies of *Journal* articles beyond the number allowed by the fair use provisions of the 1978 U.S. copyright law may request permission to copy directly from AOAC or may make the required copies and pay $1.00 per copy through the Copyright Clearance Center, Inc., 21 Congress St, Salem, MA 01970. Articles which are copied and royalties paid through the Copyright Clearance Center must be identified by the following code: 0004-5756/87$1.00, indicating the International Standard Serial Number assigned to *J. Assoc. Off. Anal. Chem.*, the year, and the copying fee per copy. Information on the use of the Copyright Clearance Center is available from the Center.

Please Note Except for photocopies prepared as described above, no part of this *Journal* may be reproduced in any form or stored in any microform or electronic form without the written permission of the Association.

ADVERTISING

Placement All space reservations and all advertising copy are due 1½ months in advance of publication at AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. Advertising inserted 3, 6, or 12 times within 1 year of first insertion earns frequency discount rates. Contact Marilyn Taub, AOAC, for size requirements and schedule of rates.

Policy Advertising is prepared by the advertisers and has been reviewed for compliance with the AOAC Policy on Advertising in Association Publications. AOAC publication of an advertisement is not an endorsement or approval, expressed or implied, by the Association or the Editors of any service, product, or claim made by the manufacturer.

New Products Notices of new products are prepared from literature received from manufacturers and other companies and are published only as a service to readers. Publication of notices of new products is not intended as an AOAC endorsement or approval of any service, product, or claim made by the manufacturers.

1/88

AUTHORS IN THIS ISSUE

Al-Yamany, Ali, 323
Alfano, Edith M., 288
Ali, Rida, 353
Andrews, Wallace H., 448
Antalick, J. P., 446
Aoki, Shigeru, 373
Ashoor, Samy H., 397, 403
Barton II, Franklin E., 256
Beckert, Werner F., 375
Berger, Ronald G., 406
Bijl, Jan P., 282
Blakeney, Anthony B., 272
Blanchflower, John W., 302
Bourgeay-Causse, M., 446
Bourgeois, Claude F., 447
Boyacioğlu, Dilek, 280
Bracquart, Patrice, 448
Brady, Marietta Sue, 295, 299
Burns, Robert A., 353
Bushway, Rodney J., 321, 323
Busse, Martin, 448
Cairns, Thomas, 415
Carey, William F., 358
Castle, Laurence, 394
Charm, Stanley E., 304
Chi, Ruey, 304
Clarke, Margaret A., 276
Cole, Eugene C., 288
Cottingham, Layna S., 251
Cox, Lynton J., 449
Cutrufelli, Mark E., 444
De Kleijn, Jaap P., 446
Dive, Daniel G., 282
Donnelly, J. R., 434
Einarsson, Hjorleifur, 449
El-Nabarawy, Ibrahim M., 358
Faugere, J. G., 446
Finglas, Paul M., 447
Flowers, Russell S., 341
Galceran, Teresa, 350
Gilbert, John, 394
Godshall, Mary An, 276
Gönül, Meral, 280
Grappin, Remy, 448
Guillaumont, M., 446
Gurprasad, Narine P., 328
Harris, David A., 353
Harris, Philip J., 272
Heimbecher, Susan K., 285
Henry, Robert J., 272
Higuchi, Ikuji, 337
Hindle, Ralph, 333
Horwitz, William, 241
Hundley, Harvey K., 415
Hurlbut, Jeffrey A., 263
Ishizaka, Takashi, 360
Jansen, Chris C., 446
Johnston, Ralph W., 406, 444
Jorgensen, Karen V., 285
Junkins, Elizabeth, 410
Katz, Stanley E., 295, 299
Kentzer, Elzbieta J., 251
King, Joan M., 321
Klatt, Mary Joan, 341
Klein, Daniel, 448
Kondo, Sadao, 337
Lacroix, Mario D., 328
Lahellec, Cecile A., 449
Lanouette, Monique, 325, 328
LeBel, Guy L., 410
Leclercq, M., 446
Lemus, Igor, 388
Lopez-Avila, Viorica, 375
Luke, Milton A., 415
Mageau, Richard P., 406, 444
Manz, Ulrich, 446
Masumoto, Herbert T., 415
Matsui, Yoshihiro, 373
Mattingly, Jerome A., 341
McClure, Foster D., 246
McNeal, Jon E., 443
Mercer, Angela J., 394
Milanes, June, 375
Misra, Raj K., 369
Mitchum, R. K., 434
Monte, Woodrow C., 397
Mopper, Barry, 390
Morgan, Michael R.A., 447
Morris, William J., 263
Munns, Robert K., 263
Musial, Charles J., 363, 369
Nuñez-Vergara, Luis J., 388
Okada, Junya, 337
Osman, Magdi A., 403
Perkins, Brian L., 321
Price, Ralph L., 285
Rabasseda, Joan, 350
Rauret, Gemma, 350
Renaud, Annemarie, 449
Rice, Desmond A., 302
Richardson, Gary H., 448
Robertson, James A., 256
Robison, Barbara J., 341
Rosner, Lawrence, 447
Roth, Jonathan N., 343
Rousseau, Dominique M., 282
Roybal, Jose E., 263
Rutala, William A., 288
Saito, Bun-Ichi, 337
Saito, Yukio, 360
Sasaki, Kumiko, 360
Schoen, Sarah, 375
Schwab, Bernard, 406, 444
Shimoda, Wilbert, 263
Slahck, Stephen C., 317, 440
Smallidge, Robert L., 251
Southgate, David A. T., 447
Sovocool, G. W., 434
Springer, Janet A., 246
Squella, Juan A., 388
Stiles, Philip G., 397
Stone, Bruce A., 272
Strobel, Robert J., 295
Suzuki, Takashi, 360
Trivedi, Ramesh J., 290
Tsuda, Taizo, 373
Uthe, John F., 363, 369
Van Peteghem, Carlos H., 282
Van Schothorst, M., 449
Wada, Minoru, 373
Wigfield, Yuk Y., 325, 328
Williams, David T., 410
Windham, William R., 256
Wotherspoon, David, 333
Yang, Angela, 323
Zuñiga, Luis A., 388

FOCUS ON SAMPLING

Sampling and Preparation of Sample for Chemical Examination

WILLIAM HORWITZ
Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, DC 20204

Sampling and methods for reducing a laboratory sample to a test sample are discussed, with particular emphasis on sampling peanuts for aflatoxin analysis as a practical example. The only way to control the total error in the analysis of this heterogeneous product is to take and to analyze many and large samples.

What happens to a laboratory sample between the time it is submitted and the time the chemist opens the test sample and proceeds to remove a test portion for analysis? Remember what a laboratory sample is—anything that is sent to the laboratory is a laboratory sample. Practically anything can be a laboratory sample—a carton of eggs, a watermelon, rotten fish, rat pellets. Sometimes it might be 50 lb of material. A chemist cannot analyze 50 lb since laboratory operations are scaled to gram quantities or at most to about 100 g test portions. The problem then is to take that laboratory sample as it is submitted and reduce it to a test sample for analysis, while maintaining the essential properties of the original laboratory sample.

Particular problems occur when the original product is segregated, or if the laboratory sample cannot be kept homogeneous. Then the laboratory must make a homogeneous mass from the heterogeneous mass so that any test portion taken from the test sample is the same as any other test portion.

Once the analysis is started, any further reduction in volume is part of the analytical process, but reduction of the laboratory sample to a test sample is a part of the sampling process that is under laboratory control.

Avoiding Unnecessary Analyses

Before an analysis is even started, it must be determined whether the results will be useful. Laboratory analysis is about the most expensive way to obtain information on which to base a decision. As an illustration, it costs about $50 an hour to maintain a Food and Drug Administration (FDA) chemist and the necessary facilities. If an average analysis takes about 1 day, the labor cost alone is $400; the capital investment in laboratory instrumentation is additional. Therefore, if the necessary information can be obtained in any other way, the alternative should be used, or if the sample is flawed, analysis should be avoided.

First, the laboratory sample must be scrutinized for damage that would impair sample integrity. If such damage is found, the laboratory sample should not be analyzed.

Second, visual examination of the laboratory sample is often sufficient to prove a point. For example, a chemical examination is not required to verify that the laboratory sample is wheat, rice, or corn as stated in the transmittal document; a visual inspection will suffice. As another example, the first thing that should be done with a white powder of unknown identity is to examine it under a microscope to see how many components are present and to determine whether any compounds, such as common salt, are recognizable from their crystalline form. Sometimes a photograph of the environment or the material is sufficient evidence for a decision. Occasionally, samples have been collected just for the purpose of documenting movement in interstate commerce or documenting that the label of a product has certain statements. In all of these cases, no chemical analysis is needed.

Analysis can be avoided by a management decision that the records are deficient. In the United States, FDA must prove that either the lot itself or the raw materials used in the product moved in interstate commerce. If this fact is not documented for an official sample, no analysis should be made because of insufficient proof of interstate commerce for a court case. Documentation is scrutinized so carefully in FDA laboratories that it is practically impossible to institute a blind quality control system. We are not able to manufacture a product that the laboratory will not recognize as unusual because of some documentation, labeling, packaging, or administrative deficiency or because of noncommercial formulation. As soon as an analyst recognizes a quality control sample submitted for analysis, special care is expended on that particular analysis.

Another way to avoid an analysis is to find that the laboratory has no responsibility for the product. For example, FDA does not regulate meat products. Meat products may be analyzed for monitoring purposes, e.g., to obtain information on pesticide residues, trace elements, and contaminants, but not for the purpose of taking legal action. If a meat product is suspected of being violative, the laboratory sample of that product should go to the Food Safety and Inspection Service of the U.S. Department of Agriculture, which has jurisdiction over meat products.

Another reason to avoid analysis is lack of experience with the analytical technique or the absence of the proper instrumentation. In such cases, arrangements can be made with another laboratory that has the experienced personnel or necessary equipment to analyze the product, in exchange for reciprocal service. If special facilities required for an analysis, such as a laminar flow hood, radioactivity counters, or incubators, are not available, makeshift facilities should not be used.

An important requirement, often overlooked, is the necessity in most cases for a valid reference standard. Today, instruments are used for most analyses. An instrument compares the response for an unknown amount (or concentration) of the analyte to the response for a standard. If a valid standard is not available, the analysis cannot be performed rigorously.

Preparation of the Test Sample from the Laboratory Sample

If the records are in order, if the laboratory sample submitted is satisfactory, and if there is no question regarding

Received May 27, 1987. Accepted July 28, 1987.
Presented at the symposium on "Field and Laboratory Sampling of Food, Drugs, and Agricultural Materials," 100th AOAC Annual International Meeting, Scottsdale, AZ, Sept. 17, 1986.

competency, then the laboratory sample usually will need preparation to make it suitable for analysis. The laboratory sample is often too large, both in bulk and in particle size, for direct analysis. If it consists of small solid particles, the simplest way to reduce the bulk is to pile the sample evenly on a clean surface, and then to flatten the pile and divide it into quarters. Two opposite quarters are combined, and the other 2 quarters are discarded. This operation is repeated several times until the laboratory sample is reduced to a manageable size. Dividers are also available that automatically and randomly divide free-flowing particles into 2, 4, or a greater number of equal streams, any one of which can be taken to represent the original laboratory sample. This is the easiest way to reduce the volume of bulk, flowable material.

Another important situation can be illustrated by a crate of spinach. Spinach must be chopped into small pieces for mixing to ensure the presence of pieces from all the different leaves in the final portion. Grains, apples, watermelons, and similar large materials must be cut into smaller pieces, mixed thoroughly, and then resampled to achieve a smaller (in volume), yet representative, test sample. The objective is to prepare a sample homogeneous enough to ensure a negligible difference between repeated test portions taken for analysis. Grinders, blenders, and food processors are useful equipment for this purpose.

Special problems may arise in handling the laboratory sample. If the product is a liquid that contains sediment, the test sample should contain the same proportion of solids to liquid as the parent product. The procedure to achieve this result will depend on the speed of sedimentation after the product has been mixed, the size of the insoluble particles, and the size of the laboratory sample. Sampling the laboratory or test sample with a wide-bore pipet while blending is a satisfactory procedure, when possible. Sometimes the sediment is deposited as a cake in the container, in which case the container must be opened completely and all of the deposit must be loosened with a policeman or spatula and incorporated into the bulk of the liquid before mixing. When, on occasion, the solid and liquid portions are analyzed separately, the weight of each must be recorded if the composition of the original product is to be reconstructed mathematically.

During the reduction of a laboratory sample to a test sample, the product must be protected from changes in composition. Some common possibilities are loss of moisture (or other volatiles) because of exposure to the atmosphere while mixing or grinding. Exposure should be kept to a minimum by keeping the product covered with plastic or aluminum foil as much as possible. The opposite phenomenon occurs if a cold product, usually at refrigerator temperature, is brought into the laboratory at room temperature. When the container is opened, moisture from the atmosphere condenses on the cold surfaces. This can be avoided by letting the container and product warm to room temperature before opening. Condensation can even occur in the closed container when it is placed in the refrigerator. Cheese shavings at room temperature will lose moisture to the walls of the jar when placed in the refrigerator. These droplets of moisture must be reincorporated into the product with a spatula or other mixer before test portions are removed for analysis.

The use of bare metal mills must be avoided if the product is to be analyzed for trace metals. Iron, copper, and manganese are common components of laboratory equipment that somehow seem to find their way into the test solutions. Erratic values for these elements can often be traced to laboratory contamination. Lead is another frequent contaminant from soldered cans, from the raw water supply, or even from the atmosphere if the laboratory is located near a busy highway. Grinders and other mechanical devices should not be overloaded; this could result in heating the product and subsequent loss of volatiles or changes in composition from chemical reactions.

Sometimes air must be excluded, by use of a blanket of nitrogen or carbon dioxide, during the mixing and grinding operations, particularly when polyunsaturated fatty acids and other easily oxidized analytes are involved. The use of these low temperature additives also prevents overheating of the product through the friction of grinding.

Ordinarily, a product is analyzed in the form in which it is commonly used. For foods, the rule of edibility generally is followed, i.e., banana peels, nut shells, orange peels, fish bones, and other parts not normally consumed are discarded. Special exceptions apply to analyses for pesticide residues: the regulations must be followed as to how to calculate the residue in or on the food. For example, for corn the regulation specifies "kernels plus cob with husks removed."

Preservation of the Laboratory or Test Sample

Often the original laboratory sample or the prepared test sample must be held for a long time, sometimes for years when a sample is the basis for a legal action. In such cases, the laboratory or test sample must be preserved without changing the essential properties of the original material.

Materials can be preserved in many ways, depending on the properties of the commodity. The easiest products to store are low-moisture solids or stable liquids, like oils, which can be kept for several years without change in sealed, airtight containers at room temperature in the dark. Wet materials can be dried to prevent growth of bacteria or molds. The mildest drying treatment is freeze-drying, but air-drying at room temperature or in an oven is also permissible if the product composition does not change with elevated temperatures. When the moisture level is reduced, however, the amount of water or other volatile materials removed should be determined so that analyte concentrations can be calculated for the original material, if necessary. Many products can be kept for a long time in refrigerated storage. Almost everything can be preserved indefinitely if the storage temperature is low enough, but this type of storage capacity in a laboratory is usually limited. Therefore, rental space in a commercial warehouse may become necessary, with the attendant problems of chain-of-custody requirements, extensive record keeping, and cost. Emulsified products with a high moisture content cannot be preserved by freezing. Freezing of such products breaks the emulsion to such an extent that the homogeneity of the original product cannot be restored. Liquid milk is one example. However, ice cream is an example of an emulsion that is stable when frozen. An awareness of the physical and chemical properties of the commodity sampled is needed for intelligent storage decisions.

Sometimes preservatives can be added to products to enable the laboratory samples to be stored unchanged at room temperature, but the added preservative must not interfere with any analysis that might be conducted in the future. Small samples of raw milk, for example, are often accumulated over a period of time so that a single analysis for fat can cover multiple deliveries. Mercuric chloride and potassium dichromate have been used for many years to preserve such samples, but the treated samples are of little use for any other analysis. Chloroform is a useful preservative for phosphatase

Lot	0	1-9	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	AVERAGE
Lot 1	7	1	2																											3.0
2	4	4		1			1																							9.3
3	4	3	1	1		1																								9.9
4	7		1			1		1																						12.5
5	1	4	3	1	1																									12.6
6	5	2		1			1		1																					15.9
7	7	1				1								1																16.7
8	4	2	3											1																18.8
9	4	1	2			2		1																						18.8
10	3	3		2			1	1																						18.9
11	2	1	3	2			1	1																						21.7
12	5	1	1		2										1															21.9
13	7	1		1																	1									22.8
14	2	1	4	1				1				1																		24.2
15	1	1	4		1	1	2																							25.8
16	7	1	1																										1	30.6
17	2	4	1					1				1						1												36.2
18	1	3	1				1	3							1															39.8
19		3	1			2	1		1	1				1																44.3
20	2				4		1	1	1						1															46.8
21	1	2	1		1	1				1	1			1				1												58.1
22			1	2	1	2				2	1							1												62.3
23		2				2	1	1	1		2														1					70.6
24			2	1	1		1				2	1			1	1														71.1
25		2	2							1	1	1	1	1		1														71.3
26	1	2	1		1					1					1	1					1		1							83.2
27		1	1				1	1	1		2				1	1		1												83.6
28			1				2		1	1		1	1	1						1	1									99.5
Lot 29			1	1	1				1		1		1					1	1	1	1									111.2

AFLATOXIN, PPB

Figure 1. Aflatoxin (ppb) distribution in 29 commercial lots of peanuts.
A 120 lb primary sample was removed from each lot, and each primary sample was subdivided into 10 aliquots of 12 lb each, from each of which a 250 g test portion was removed for analysis. Lot numbers are listed vertically. Numbers in the body of the table for each row are the number of aliquots (of the 10 for each lot) showing aflatoxin (ppb) in the concentration range listed horizontally at the top and bottom of the table. The average of the 10 aliquots for each lot is given in the column on the right. (Information taken from T. B. Whitaker et al. [1972] *J. Am. Oil Chem. Soc.* **49,** 590–593.)

determinations in milk, but it could ruin the test sample for many other analyses. The preservative must be chosen with subsequent analyses in mind.

Sampling Heterogeneous Products

One of the most difficult sampling situations is encountered in the attempt to obtain a representative sample of a raw commodity for an aflatoxin determination. In the early days of the aflatoxin problem in peanuts, when not much was known about the origin and distribution of the toxin, it was important to know how to sample a lot, which may amount to thousands of tons. The North Carolina Agricultural Experiment Station set out to determine the distribution of aflatoxin in peanuts. They took 29 commercial lots of peanuts, as listed vertically on the left axis of Figure 1. Each row represents a different commercial lot. Then they took a 120 lb sample from each of these 29 lots. The 120 lb initial or primary sample was then divided by riffling into 10 equal 12 lb portions (aliquots). A 250 g secondary sample (test sample) was removed for analysis from each of the 12 lb portions for a total of 290 analyses.

Across the top of Figure 1, and also along the bottom (for easier reading), are listed ranges of aflatoxin concentration in parts per billion (ppb, μg/kg, or ng/g). Each number in the body of the table represents how many of the 10 test portions from a specific lot contained aflatoxin in the concentration range given in the column head. The first column after the lot number is headed zero, indicating no aflatoxin found. For example, in lot 13, seven of the 250 g test portions, each taken from a 12 lb aliquot of the 120 lb sample from the lot, showed no aflatoxin. Continuing along the row of lot 13, one test portion from one 10 lb aliquot showed 1–9 ppb, one showed 20–29 ppb, and the tenth aliquot showed 190–199 ppb. The final column gives the overall average of the 10 aliquots: 22.8 ppb. But the range was 0 to about 195! The lot numbers have been arranged in increasing order of the average of the aflatoxin level of the 10 subdivisions, which is given as the final column on the right. Note that almost every lot has a wide scatter of values, which becomes even wider as the lot average increases. The smallest average is 3.0 ppb and the largest is 111 ppb. If each column is added, a distribution is obtained which is obviously non-normal, since it is constantly decreasing, with a very high peak at zero, a steep descent, and a tapering off slowly toward the high values. One type of distribution with these characteristics is the negative binomial distribution, the same type of distribution found in populations representing contagious disease epidemics. In these distributions, there are "hot spots," i.e., most people do not have the disease, but there are pockets of the disease of differing severity in various places.

We have been looking at the distribution from the point of view of the lots. Now consider it from the point of view of the analyst. The most likely value (the mode) that would be obtained on analysis of any one of the 10 aliquots from the 120 lb sample is a zero because more values are in this category than in any other. But in the example, the zero occurs only 77 times in the analyses of the 290 aliquots, or for about 27% of the aliquots. Therefore, if only one or two 12 lb aliquots from a 120 lb sample are analyzed, there is a fair possibility that aflatoxin will not be found. To obtain a picture of the aflatoxin distribution in the lot, several different portions must be analyzed. The more aliquots that are analyzed from the laboratory sample, the more likely it is that some aflatoxin will be found, if it is present. With contamination of this nature, a normal distribution cannot be assumed; it is more probable that some extreme type of distribution is present. Thus, it is apparent how difficult it is to formulate a sampling plan that will differentiate the first 14

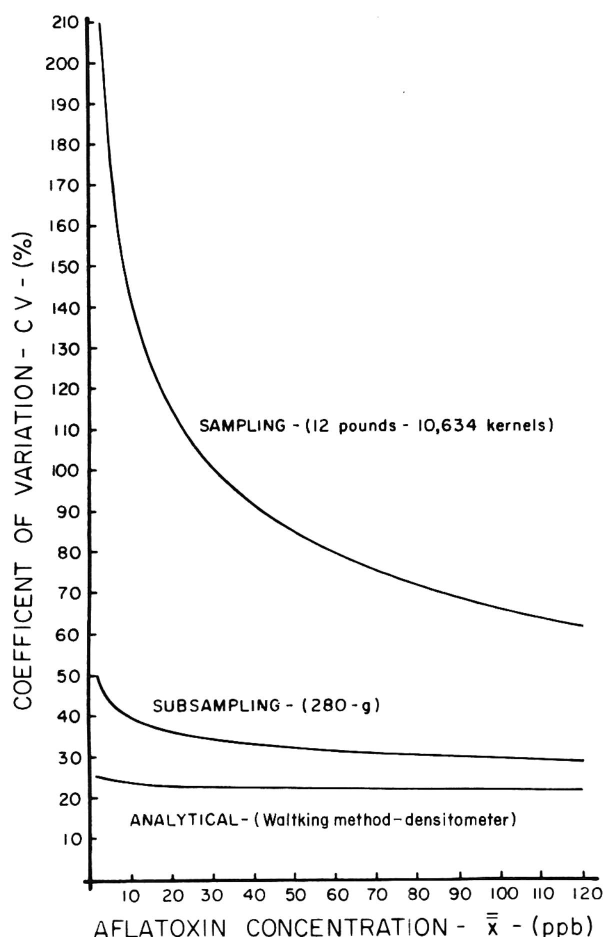


Figure 2. Coefficient of variation of components contributing to total error of aflatoxin analysis, i.e., sampling, subsampling, and analysis, as function of concentration. (Information taken from T. B. Whitaker [1974] *J. Am. Oil Chem. Soc.* 51, 214–218.)

lots whose average is below 25 ppb from the remaining 15 lots whose average is above 25 ppb. Merely looking at the broad and erratic distribution shows why it is necessary to composite many random portions and to homogenize them thoroughly to obtain a reasonable average that is stable under conditions of repeat sampling and analysis. The size of the test sample is another important consideration.

It was also discovered that aflatoxin contamination of raw peanuts was usually confined to a relatively few kernels, and that the level in an individual kernel could be as high as 0.1% of the nut meat. If, on the basis of this study, a reasonable single kernel contamination level of 0.05% is assumed, it can be calculated that 1 contaminated kernel in 10 000 would result in a level in the mixture of 50 ppb (μg/kg), exceeding the 25 ppb FDA action level in raw shelled peanuts. This ratio has been the basis for determining the minimum lot sample weight and the particle size reduction for a given test portion weight needed to obtain a representative sample of raw shelled peanuts.

Research on other commodities showed the same type of erratic distribution of toxin in corn, cottonseed, and Brazil nuts when the mold contamination had entered the crop prior to harvest as a result of plant stress or insect damage.

Another interesting result of the research on peanuts was the finding that much of the total error in aflatoxin analyses was not from the performance of the analytical method but from the sampling and subsampling procedures. Figure 2 illustrates these findings. The coefficient of variation is given on the y-axis and the concentration is plotted along the x-axis.

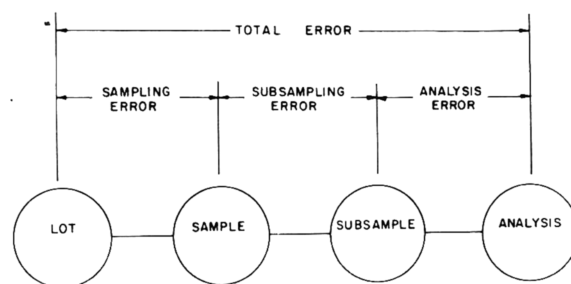


Figure 3. Experimental design for obtaining the data of Figure 2.

As the aflatoxin concentration becomes smaller toward the left, the sampling error becomes larger. Note that the analytical error, which is the bottom curve, shows the smallest error of the group that consists of sampling, subsampling, and analysis. The coefficient of variation of analysis is 20–30%. The variability from the reduction in the size of the laboratory sample is around 30–40%, but the sampling of the aflatoxin from the lot is of the order of 100%. Consequently, there is no point in having methods of analysis that are extremely precise to determine the aflatoxin concentration when we are faced with a sampling error that is several times larger than the analytical error. That is why thin-layer chromatography is still being used for this determination, although liquid chromatography (LC) is now coming into use.

The important point is that the largest error in aflatoxin determinations comes from sampling, not from analysis. Precise methods of analysis are not going to be of much help in such a situation. The best use of laboratory time would be to ensure adequate comminution and mixing before size reduction. An alternative is to increase the size of the laboratory sample, and this has been the approach of laboratories responsible for peanut certification analyses. An extract is prepared from the entire 1100 g subsample from 48 lb of peanuts comminuted in a mill equipped with a divider device. The current procedure is to form a water slurry of an 1100 g test sample from the mill. An aliquot of this slurry is the test portion.

Figure 3 shows the experimental design used in the replication of the sampling, the subsampling in the laboratory, and the analysis. In actual practice, the peanuts traveling on a conveyer belt are sampled automatically while the lot is being transferred in or out of storage by taking repetitive cuts through the stream with a sampling cup. If a large pile of peanuts is sampled, a trier or shovel is the instrument of choice. Peanuts packed in bags are also sampled with a trier, or the bag is opened and the sample is removed with a scoop.

With respect to sample size, the sample must be large enough to be representative of the lot. Random sampling is most likely to provide the most representative sample. A statistician can be helpful in selecting an appropriate sampling plan, but he or she should be informed that the distribution of aflatoxin in peanuts or other seed crops is a "negative binomial." A statistical sampling plan will reduce the risk of accepting bad lots and thus exposing consumers to high aflatoxin levels. It will also reduce the number of good lots that are unjustly destroyed because of the erratic sampling situation. If a sampling plan is extrapolated from commodity to commodity, however, the size of the kernels, or the number of kernels per unit weight, is critical. A peanut kernel weighs about a half gram; wheat grains are considerably smaller and Brazil nuts are considerably larger. Large kernel and small kernel commodities require different sample

weights, and different methods of preparation must be applied to obtain relatively homogeneous test samples. Sampling for aflatoxin analysis again provides an excellent example of an experimental approach to the problem of preparation of the test sample.

Preparation of the Test Sample

When peanuts are ground, the result is a semisolid butter, which a difficult material to mix. To determine the best way to obtain a homogeneous mass from a batch of peanuts and prepare test samples for aflatoxin analysis, investigators at FDA simulated aflatoxin contamination by irradiating several peanut kernels in the nuclear reactor at the National Bureau of Standards to make them highly radioactive. The activated peanuts were then added to batches of clean nuts to provide dilution factors of 1–16 per 10 000. The mixtures were ground in various ways with different types of grinders to determine the best way to cut the radioactive peanuts into little pieces and to distribute them uniformly through the resulting mass. They found that grinding with water or with a solvent was the best way to distribute the radioactivity through the mixture of comminuted kernels.

The final sampling plan that evolved, based on the probability of the aflatoxin in a lot exceeding an action level of 25 ppb, specifies taking three 48 lb samples from the lot. One of the characteristics of sampling statistics is that the reliability of a random sample does not depend so much on the size of the lot as it does on the size of the sample. Therefore, as a first approximation, we can ignore whether we are dealing with a million tons of peanuts or with one ton of peanuts. The current sampling plan takes a constant sample size of three 48 lb samples. The first 48 lb sample is ground in a subsampling mill, the subsample (test sample) is analyzed in duplicate, and the 2 results are averaged. If the average value is less than 16 ppb, the lot is passed. If the average value is more than 75 ppb, the lot is rejected. If the average value is between these 2 values, the second 48 pound sample is ground and analyzed in duplicate, and all 4 results are averaged. If this average is less than 22 ppb, the lot is passed; if it is more than 38 ppb, it is rejected. If the average is between the last 2 values, the third 48 pound sample is ground and analyzed in duplicate. If the average of the 6 analyses is less than 25 ppb, the lot is passed; if it is 25 ppb or more, the lot is rejected. This is called a sequential sampling plan. It is now being used by the Peanut Administrative Committee, a creation of the U.S. Department of Agriculture, for the certification of raw shelled peanuts as marketable under the 25 ppb action level agreed to by the FDA.

A sampling plan for dried milk would be considerably different from that for peanuts, because the latter product consists of discrete kernels with a heterogeneous distribution of the aflatoxin, and dried milk consists of a homogeneous, free-flowing powder prepared from a contaminated fluid milk. Considerably fewer sampling units need be taken from dried milk than from shelled peanuts to determine with the same degree of confidence whether or not aflatoxins are present. Aflatoxins occur in milk as a result of contaminated cattle feed. The feed-to-milk ratio is about 75 to 1; if there is 75 ppb aflatoxin B_1 in the feed, only about 1 ppb aflatoxin M_1 will be present in the milk. In the case of liquid milk, there will not be much of a sampling error, but in its place will be a large relative analytical error since the action level is frequently below the practical limit of determination.

With fluid milk products, samples can be taken with a dipper while the batch is being agitated. With products such as cottage cheese, where considerable mixing occurs in the manufacturing process, a single or a few containers are sufficient to determine whether or not aflatoxin is present.

Practical Aspects

In all sampling, the economic aspects must be considered. Sampling time and analytical time must be commensurate with the value of the goods and the risk of harm. The administrator must balance all these factors, as well as the practicalities, in arriving at a final sampling plan.

The aflatoxin sampling plans for raw agricultural commodities are complex because of the high degree of heterogeneity of the toxic compound in the food, the assumed high degree of risk of harm, and the relatively valuable nature of the products. For commodities with a normal distribution of a contaminant, standard plans are available. Other commodities are intermediate between these two extremes.

When a problem is encountered involving different concentration values from different samples of the same goods, it would be prudent to remember these curves and data. In many cases, the analysis is not at fault. More research and improved methods are not going to change the approach to the type of extreme distribution that occurs naturally. The only way to improve the estimate of the amount of aflatoxin in a lot is to take more and larger random samples. To reduce the sampling error, the emphasis must be placed on increasing the size of the samples, pooling multiple portions, and adequately reducing the laboratory sample to a test sample. Errors, whether they be sampling or analytical, are always large in these natural product situations.

Statistical Sampling Approaches

JANET A. SPRINGER and FOSTER D. McCLURE
Food and Drug Administration, Division of Mathematics, Washington, DC 20204

This article describes basic sampling principles and the application of statistical sampling techniques to specific problems encountered in the Food and Drug Administration (FDA). Concepts are emphasized, and theory is minimized. The basic principles of sampling from a normal and binomial population, including confidence interval calculation and sample size determination, are briefly reviewed. Stratified, random, systematic, and judgment sampling are explained. Operating characteristic curves for attribute (and perhaps variable) sampling for acceptance of lots are derived and applied to specific FDA problems. The advantages and disadvantages of single and multiple sampling plans and plans which address multiple classes of criteria such as major and minor defects are discussed. Sampling schedules such as MIL-STD-105D and Canada's Government Specifications Board CGSB-105-GP-1 are reviewed to familiarize readers with the principles involved in these plans and to give them an idea of how they could be applied to FDA problems.

The Division of Mathematics in the Center for Food Safety and Applied Nutrition of the Food and Drug Administration (FDA) often gets questions from field personnel and headquarters staff relating to very basic sampling principles and the application of these principles to FDA sampling problems. This paper will discuss basic sampling principles and can serve as a general reference for persons who need a short overview of sampling techniques and their application as used in field sampling of the type done by FDA. Sampling procedures that may be used to obtain an analytical sample from the laboratory sample are omitted here.

Sampling Definition

Essentially, sampling consists of obtaining a portion (sample) of a large group, "universe," or population on which some item or items of information are desired.

When information is collected in the field or on some study population (e.g., all the plants which make baby food, a large kettle of fish, or a train load of corn), 2 courses of action may be taken: either a complete count or sampling. With the complete count, one hopes to obtain complete and exact information on the study population; with sampling, one hopes to obtain estimates of the true values that will be sufficiently accurate for the intended purpose.

Advantages and Disadvantages of Sampling

A natural and legitimate question that many readers will ask is, "What advantages does sampling possess over a complete count?" The obvious answer is that sampling permits one to reduce costs, reduce personnel requirements, gather vital information more quickly, and obtain more comprehensive data. An apparent disadvantage is that it provides only an estimate of the required information. However, contrasted with the difficulties in making an efficient complete count and due to the technical advances that have been made in the theory and application of sampling, this disadvantage is only apparent. Sampling has come to be regarded as a reliable and efficient means of gathering information on a study population.

Received June 16, 1987. Accepted November 2, 1987.
Presented at the symposium on "Field and Laboratory Sampling of Food, Drugs, and Agricultural Materials," 100th AOAC Annual International Meeting, Sept. 16, 1986, Scottsdale, AZ.

Steps in Preparing a Sampling Plan

The primary aim of a sampling effort is to obtain a sample, subject to constraints on size, that will satisfy predefined specifications relative to the sampling plan. A sampling plan (field surveys or acceptance sampling) should evolve through a process based on input from both administrators and subject matter experts. First, the administrators and subject matter experts define the objectives, the information that is needed, and its intended use. Second, the subject matter experts develop possible approaches for collecting the necessary data to fulfill the problem objectives and determine the economic consequences or risks associated with the sampling effort. This input should define (*1*) the sampling objective, (*2*) the study population, (*3*) the statistical unit, (*4*) the selection criteria (i.e., sample size and how the sample will be collected), and (*5*) the analysis procedures (i.e., analytical and statistical). Without this input, the statistician will be operating in the dark.

Sampling Methods

The 2 primary objectives of sampling may be (*1*) to estimate, say, the average value of a characteristic or percentage possessing some characteristic or (*2*) to decide whether the average value or percentage possessing some characteristic for a lot meets a specification. The first objective is usually associated with field surveys, while the second objective deals with acceptance sampling, where a lot is accepted or rejected by inspecting a sample selected in accordance with a predetermined sampling plan. Some sampling methods that may be used to accomplish these sampling objectives will be discussed briefly below.

Estimation Methods

Four types of sampling methods considered here are simple random sampling, stratified sampling, systematic sampling, and judgment sampling. Simple random sampling is a method in which all elements of a population have an equal and independent chance of being included in the sample. A simple random sample can be drawn using a table of random numbers or other objective techniques. It is not the same as haphazard sampling.

Simple random sampling is generally used either in conjunction with other sampling methods or in cases involving small study populations.

Stratified random sampling is accomplished by separating the population elements into some overlapping groups, called strata, and selecting a simple random sample from each stratum.

The advantages of stratified sampling are several: homogeneity within each stratum is better than in the population, the cost of conducting the actual sampling tends to be less than for simple random sampling because of administrative convenience, and separate estimates of population parameters can be obtained for each stratum without additional sampling.

Reduced variability within each stratum produces stratified sampling estimates which have smaller sampling error than do the corresponding simple random sample estimates from the same sample size.

Systematic sampling is a method of drawing a 1 in K

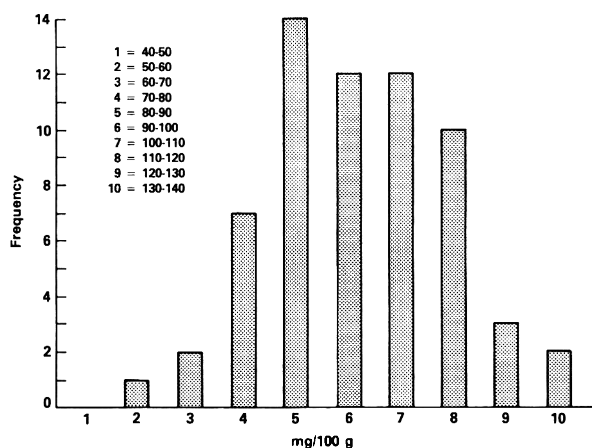


Figure 1. Histogram of potash determinations in authentic strawberry jam.

sample from a list of units, or of plants, or of houses. The procedure involves randomly selecting one of the first K units and then taking every Kth unit thereafter. For example, if there are 10 000 plants to be surveyed, every 100th plant can be drawn starting with a randomly selected number between 1 and 100. Compared to random sampling, systematic sampling is quicker, easier, and cheaper; the population size need not be known exactly; and any trends or clumping of similar units in the population will usually be more accurately represented in a systematic sample than in a simple random sample. The primary disadvantage of systematic sampling is that no valid estimate of sampling error can be calculated from a single sample.

In judgment sampling, the investigators use their own judgment and experience regarding the population, lot, or sampling frame to decide which sample units to select. Since the method is nonrandom, no statistical techniques can be validly applied to study the precision of the estimates (1). In FDA, this method of sampling is sometimes called targeted sampling, in which the inspectors target the "problem areas." This method can yield valuable results; however, care must be taken not to extrapolate the results to the population.

Sampling Error

Whenever we estimate a result from a sample, it is most unlikely that the value will be exactly the same as the value obtained by making a 100% count. The difference between the two values is sampling error. Sampling error is a disadvantage of sampling; it cannot be eliminated, but it may be reduced by proper choice of sample size and sampling method (2). Sampling error is measured in terms of the standard error of an estimate. The standard error measures the probable accuracy or precision of an estimate from sample data. The standard error of an estimate may be related to the true value using confidence intervals. A confidence interval aims at bracketing the true value of a population parameter, such as its mean or standard deviation, by taking into account the sample estimate of the parameter. The formula for the 95% confidence interval for the mean (μ) of a normal distribution, when the variance (σ^2) is unknown and is estimated by the sample variance, s^2, is:

$$\bar{x} - t_{0.025}s/\sqrt{n} \text{ to } \bar{x} + t_{0.975}s/\sqrt{n}$$

where $\bar{x}$ is the sample mean and $t_{0.975}$ is the tabular t-value for $n - 1$ and 0.975 probability. Note the importance of n, the sample size, in reducing the width of the confidence interval. The width decreases as the square root of n increases.

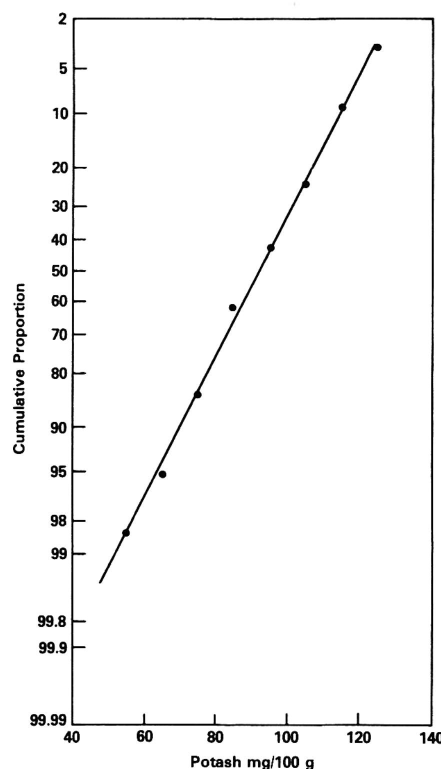


Figure 2. Normal probability plot of potash values for strawberry jam.

The following example, using frequency tables and histograms to summarize the data, shows how sampling error may be used to calculate a confidence interval.

Suppose that we have some sample data, such as potash determinations in strawberry jam, as shown in Table 1. To obtain meaningful information, this unorganized set of values must be concisely summarized, described, and presented. This can be done by arranging the data into a frequency table, as shown in Table 2, whereby the range of the data is divided into a moderate number of categories, and each observation is placed in one of the categories.

These data may now be presented in a histogram by plotting the limits (on the horizontal axis) against frequency (on the vertical axis) as shown in Figure 1. A straight line plot of data on normal probability paper (Figure 2) indicates a normal distribution. The mean of the authentic data is 96.5, with a variance of 282.24 and a standard deviation of 16.8. Since the distribution is assumed normal, the confidence interval for the authentic data is:

$$96.5 - 2(16.8/\sqrt{63}) \text{ to } 96.5 + 2(16.8/\sqrt{63}) = 92.3 \text{ to } 208.7$$

Therefore, there is 95% confidence that the true mean potash value lies within 92.3 and 208.7.

Acceptance Sampling Methods

The methods of statistical acceptance sampling used in FDA are many and varied and may be categorized as either single, double, or multiple plans involving attributes (presence or absence of a characteristic), variables (quantitative measures of a characteristic), or a combination of attributes and variables. Examples of single and multiple plans involving attributes will be considered here.

Suppose a sampling plan is desired to give a producer's risk of 5% at an acceptable quality level (AQL) of 6.5% defective items in a lot of 50–90 items. According to the Codex sampling procedures manual (3), the sampling plan would

Table 1. Analysis of strawberry jam for potash

Sample No.	Variety	Origin of fruit	Potash, mg/100 g
57329 D	Marshall	Oregon	64.2
28277 D	Marshall	Washington	66.4
28278 D	Marshall	Washington	53.1
23546 D	Marshall	Washington	77.2
57327 D	Marshall	Oregon	74.4
23544 D	Marshall	Washington	85.1
30629 E		Michigan	78.5
23551 D	Marshall	Washington	75.7
47508 D	Blakemore	Virginia	85.3
59040 D	Aroma	Ohio	91.3
59039 D	Mixed	Ohio	92.7
23549 D	Marshall	Washington	91.1
59038 D	Mixed	Tennessee	83.9
23555 D	Marshall	Washington	80.2
23548 D	Marshall	Washington	88.6
21895 D	Mixed	Michigan	78.6
57319 D	Marshall	Oregon	76.5
57330 D	Marshall	Oregon	83.2
23545 D	Marshall	Washington	80.7
57321 D	Marshall	Oregon	76.0
47507 D	Mixed	Virginia	88.7
57323 D	Marshall	Oregon	85.6
21894 D	Mixed	Michigan	88.2
57331 D	Marshall	Oregon	87.6
52524 D		New York	85.8
57325 D	Marshall	Oregon	100.5
26501 E	Marshall	Washington	100.0
35157 D	Blakemore	Maryland	93.5
23543 D	Marshall	Washington	92.0
35159 D	Blakemore	Maryland	92.3
47509 D	Mixed	Virginia	98.0
14252 B	Blakemore	Delaware	89.3
16279 E	Mixed	Michigan	80.7
57326 D	Oregon	Oregon	100.4
66622 D	Aroma	Missouri	113.6
35160 D	Blakemore	Delaware	97.1
14251 E	Blakemore	Delaware	92.5
5021 E	Blakemore	Kentucky	114.0
14253 E	Blakemore	Delaware	94.7
26043 E	Marshall	Oregon	108.2
41321 D	Marshall	Utah	102.6
26044 E	Marshall	Oregon	97.3
59041 D	Mastadong	Ohio	98.8
57324 D	Marshall	Oregon	114.3
57322 D	Marshall	Oregon	108.1
57320 D			102.0
18978 D	Klondyke	California	108.3
45158 D	Missionary	Maryland	108.3
28040 E	Missionary	North Carolina	114.3
35154 E	Eleanor Roosevelt	North Carolina	121.6
23553 D	Marshall	Washington	111.2
52525 D		New York	109.7
45126 D	Missionary	Florida	111.2
26041 E	Marshall	Oregon	120.7
57328 D		Oregon	113.3
62415 D	Klondyke	Louisiana	107.7
62416 D	Klondyke	Alabama	113.8
35152 D	Mixed	North Carolina	119.5
9725 E	Klondyke	Louisiana	130.4
62414 D	Klondyke	Louisiana	112.2
12517 E		California	133.2
62413 D	Klondyke	Louisiana	108.7
18979 D	Klondyke	California	129.4

be to sample randomly 13 items from the lot and accept the lot if 2 or fewer of the 13 items were defective. This type of sampling is called single sampling. The OC curve (the probability of accepting a given lot quality) for this plan is shown in Figure 3. The points on the curve are calculated simply from the binomial distribution. A binomial distribution (4) shows the probability of obtaining 2 or fewer defective items in a sample of 13 for assumed true proportion defectives in the lot, and it is these probabilities and assumed true proportion defectives that form the OC curve.

Table 2. Frequency distribution of data from Table 1

Range	Authentic data	Cumulative data	%
40–50	0	0	0
50–60	1	1	1.6
60–70	2	3	4.8
70–80	7	10	15.9
80–90	14	24	38.1
90–100	12	36	57.1
100–110	12	48	76.2
110–120	10	58	92.1
120–130	3	61	96.8
130–140	2	63	100.0

A fairly common sampling technique is to select a large sample of items in a rapid, inexpensive manner and use the information from this large sample as a basis for selection of a small sample for more intensive study. This procedure reduces cost when the analysis of the sample is lengthy or expensive. This type of sampling is called multiple sampling. An example of such a plan together with the equivalent single and double plans is shown in Figure 4. Multiple sampling plans have the advantage that for very good or bad lots, the average number of sampling items which needs to be examined is less than the number which would be examined under the single sampling plan. The majority of lots to be examined are usually good quality, with a smaller percentage being marginal, that is, where the probability of acceptance is near 50%. In the example in Figure 4, if the lot is, say, 1% defective, then the probability of obtaining 0 defects out of 4 items is 96%, so this lot would almost always be accepted after only 4 items were examined, and assurance equivalent to examining 10 items would be obtained under the single plan.

The calculation of OC curves and an ASN (the average number of items required to reach a decision on lot status) for multiple sampling plans is more complex than for single sampling plans but can be easily done (see your local statistician).

FDA uses a multiple sampling plan for coffee beans to examine them for insect damage (tunnels) and mold either by visual examination with a microscope or by x-ray examination. Either method is expensive and time consuming, and multiple sampling allows some savings. The plan is shown in Table 3.

The large number of samples gives an OC curve (Figure 5) that is quite steep in the region of the defect action level of 10%.

The ASN calculation for a lot with the given percentage of defects is:

Percent defects	*ASN*
6.0	542
7.0	676
8.0	990
8.5	1287
9.0	1724
10.0	2408
11.0	1854
12.0	1247

Notice how ASN is largest near the 50% probability of acceptance and is much smaller for "good" lots. In every case, it is always less than the maximum number of 3000 items.

For taking lots from long production runs of many units of products, the sampling procedures of the MIL-STD-105D

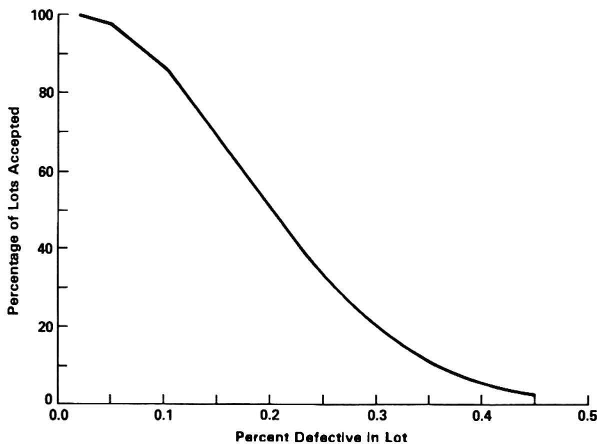


Figure 3. OC curve for n = 13, C (acceptance no.) = 2.

(Military Standard 105D) (6) are often used. The basic aim of the standard is maintenance of the outgoing quality level for a given process average. These sampling plans are developed to give assurance that if a manufacturer is producing runs consistently with quality levels at AQL, then the great majority of these lots are accepted.

Seven inspection levels are provided, covering various levels of discrimination, that is, varying "tightness" or "steepness" on OC curves at a given AQL value. The standard also provides 3 levels of inspection in terms of severity of inspection: normal, tightened, and reduced. Normal inspection is needed to start, then if quality is shown to be poor, the inspector would use a tightened level of inspection, or if quality is good, a reduced inspection. The steps for using the plans are:

1. Decide on size of lot, N. This could be smaller than a production lot size, but should not be larger.
2. Decide on inspection level: in general, II (in Table 4).
3. Using 1 and 2, find corresponding sample size code in Table 4.
4. Decide on single, double, or multiple sampling.
5. Decide whether to start with normal, tightened, or reduced sampling.
6. Decide on desired AQL for percent defectives (tables cover only up to 10%).
7. Enter the tables under the desired AQL with the sample size code to find the number of defects in the sample which will result in acceptance or rejection of the lot.

For example, suppose we had a lot of 10 000 items. Table 4, taken from MIL-STD-105D, shows sample size code L under General Inspections level II, which is normal. Table 5 (single sampling) shows a 200 sample size for sample size code letter L. If a 1% AQL had been specified, we would accept the lot on the basis of 5 or fewer defects out of the 200 items sampled, and reject the lot if 6 or more defects were found in the 200 items sampled.

The MIL-STD tables also contain the actual OC curves, the average outgoing quality limits under the various plans, and ASN calculations for double and multiple plans so that we can compare these with the single sample size.

In summary, the general approaches and the sampling plan should be selected on the basis of the program objectives and

AQL class (percent defective) **6.4-8.5**
AOQL class (percent defective)**11.0-16.0**

a. SAMPLING PLANS

Type of sampling	Sample	Sample Size	Combined samples: Size	Acceptance number	Rejection number
Single	First	10	10	2	3
Double	First	7	7	1	4
	Second	14	21	3	4
Sequential	First	4	4	0	2
	Second	4	8	0	3
	Third	4	12	1	4
	Fourth	4	16	2	5
	Fifth	4	20	4	5

b. OPERATING-CHARACTERISTIC CURVES

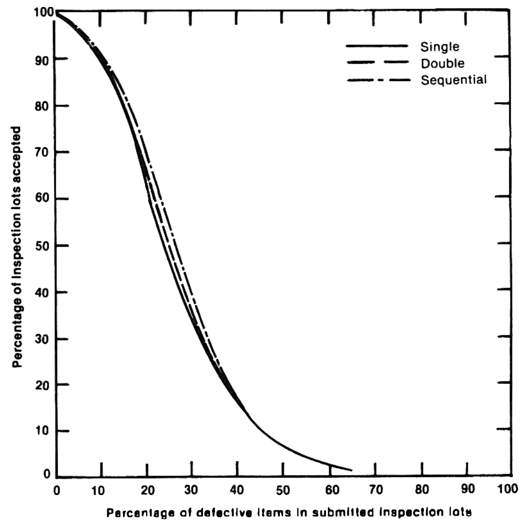


Figure 4. Equivalent sampling plans for AQL of 6.4–8.5%. (Reproduced from ref. 5 with permission.)

Table 3. Example of multiple sampling plan for coffee beans

Stage	No. of beans examined	Stop analysis and accept	No. of reject beans required to continue analysis	Stop analysis and reject
1	500	37 or less	38–115	116 or more
2	1000	85 or less	86–115	116 or more
3	1500	135 or less	136–165	166 or more
4	2000	185 or less	186–215	216 or more
5	2500	234 or less	235–264	265 or more
6	3000	283	—	284

Table 4. Sample size code letters for MIL-STD-105D sampling procedures[a]

Lot or batch size	Special inspection levels: S-1	S-2	S-3	S-4	General inspection levels: I	II	III
2 to 8	A	A	A	A	A	A	B
9 to 15	A	A	A	A	A	B	C
16 to 25	A	A	B	B	B	C	D
26 to 50	A	B	B	C	C	D	E
51 to 90	B	B	C	C	C	E	F
91 to 150	B	B	C	D	D	F	G
151 to 280	B	C	D	E	E	G	H
281 to 500	B	C	D	E	F	H	J
501 to 1200	C	C	E	F	G	J	K
1201 to 3200	C	D	E	G	H	K	L
3201 to 10 000	C	D	F	G	J	L	M
10 001 to 35 000	C	D	F	H	K	M	N
35 001 to 150 000	D	E	G	J	L	N	P
150 001 to 500 000	D	E	G	J	M	P	Q
500 001 and over	D	E	H	K	N	Q	R

[a] Data from ref. 6.

Table 5. Single sampling plans for normal inspection (master table), MIL-STD-105-D sampling procedures[a,b]

Sample size code letter	Sample size	Acceptable quality levels (normal inspection)[c]															
		0.010	0.015	0.025	0.040	0.065	0.10	0.15	0.25	0.40	0.65	1.0	1.5	2.5	4.0	6.5	10
		Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re	Ac Re
A	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0 1	—
B	3	—	—	—	—	—	—	—	—	—	—	—	—	—	0 1	—	—
C	5	—	—	—	—	—	—	—	—	—	—	—	—	0 1	—	—	1 2
D	8	—	—	—	—	—	—	—	—	—	—	—	0 1	—	—	1 2	2 3
E	13	—	—	—	—	—	—	—	—	—	—	0 1	—	—	1 2	2 3	3 4
F	20	—	—	—	—	—	—	—	—	—	0 1	—	—	1 2	2 3	3 4	5 6
G	32	—	—	—	—	—	—	—	—	0 1	—	—	1 2	2 3	3 4	5 6	7 8
H	50	—	—	—	—	—	—	—	0 1	—	—	1 2	2 3	3 4	5 6	7 8	10 11
J	80	—	—	—	—	—	—	0 1	—	—	1 2	2 3	3 4	5 6	7 8	10 11	14 15
K	125	—	—	—	—	—	0 1	—	—	1 2	2 3	3 4	5 6	7 8	10 11	14 15	21 22
L	200	—	—	—	—	0 1	—	—	1 2	2 3	3 4	5 6	7 8	10 11	14 15	21 22	—
M	315	—	—	—	0 1	—	—	1 2	2 3	3 4	5 6	7 8	10 11	14 15	21 22	—	—
N	500	—	—	0 1	—	—	1 2	2 3	3 4	5 6	7 8	10 11	14 15	21 22	—	—	—
P	800	—	0 1	—	—	1 2	2 3	3 4	5 6	7 8	10 11	14 15	21 22	—	—	—	—
Q	1250	0 1	—	—	1 2	2 3	3 4	5 6	7 8	10 11	14 15	21 22	—	—	—	—	—
R	2000	—	—	1 2	2 3	3 4	5 6	7 8	10 11	14 15	21 22	—	—	—	—	—	—

[a] When no sampling plan is available, as indicated by a dash, use the nearest acceptance/rejection values in the same column and the associated sample size and code letter in the same row.
[b] Data from ref. 6.
[c] Acceptable quality level = maximum percent defective considered satisfactory as a process average.

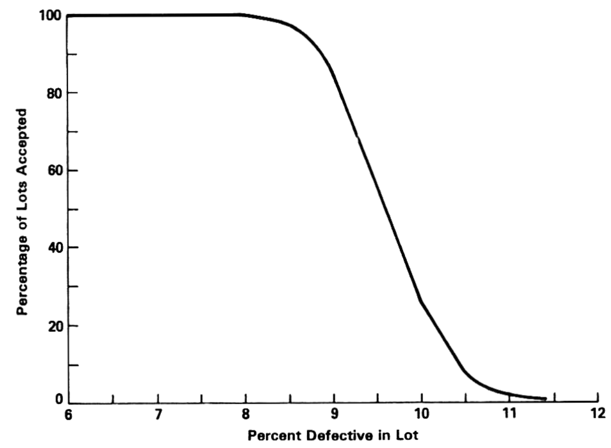


Figure 5. OC curve for multiple sampling plan for coffee beans.

the feasibility of collecting the data needed. Development of the plans should be a joint effort by administrators, subject matter experts, and the statistician.

References

(1) Puri, S. C. (1981) *Statistical Aspects of Food Quality Assurance,* Publ. 5140, Information Services, Agriculture Canada, Ottawa, Ontario, Canada
(2) Acheson, D. (1965) *Quality Control and Industrial Statistics,* Richard D. Irwin, Inc., Homewood, IL
(3) "Joint FAO/WHO Food Standards Programme," Codex Committee on Methods of Analysis and Sampling (May 1986) Instructions on Codex Sampling Procedures, Codex Alimentarius Commission, Rome, Italy
(4) *Engineering Design Handbook: Tables of the Cumulative Binomial Probabilities* (June 1972) Headquarters, U.S. Army Command, Washington, DC
(5) *Sequential Analysis of Statistical Data: Applications* (1945) Statistical Research Group, Columbia University, Columbia University Press, New York, NY
(6) *MIL-STD-105D* (April 1963) Department of Defense, U.S. Government Printing Office, Washington, DC

DRUGS IN FEEDS

Ion-Pair Reverse-Phase Liquid Chromatographic Determination of Amprolium in Complete Feeds and Premixes

ELZBIETA J. KENTZER,[1] LAYNA S. COTTINGHAM, and ROBERT L. SMALLIDGE[2]
Purdue University, Department of Biochemistry, West Lafayette, IN 47907

Amprolium is extracted with methanol–water (2 + 1) containing 5mM dioctylsulfosuccinate (DOSS) and 10mM $CaCl_2$. The analyte is separated from coextracted materials by isocratic ion-pair reverse-phase liquid chromatography, following removal of late-eluting materials on an acid alumina cleanup column, and is detected at 270 nm. The mobile phase contains 4mM DOSS with 0.3% diethylamine and 1% acetic acid in 40% acetonitrile. Linearity is satisfactory over the range of 2.5–50 μg/mL. Mean recovery, as determined by standard addition to commercial samples, is 100.1%. Accuracy was further tested in studies comparing the LC method to the official AOAC colorimetric method, using commercial samples, and was found to be satisfactory. Studies show that common poultry feed additives, grass meals, and some pelletization aids do not interfere with the analysis; however, when bentonite is present, recovery is decreased. The precision of the method, measured over several experiments on commercial samples, is satisfactory as indicated by coefficients of variation ranging from <1 to 4.5. A ruggedness test resulted in an overall CV of 3.2%, indicating the probable success of the method in a collaborative study.

Amprolium [1-(4-amino-2n-propyl-5-pyrimidinylmethyl)-2-picolinium chloride hydrochloride] is a coccidiostat used in poultry and calf feeds. The allowed levels range from 0.004 to 0.036% amprolium in complete feeds and 1.25 or 25% in premixes (1). The official AOAC colorimetric method (2) has several weaknesses, such as limited linearity and high blank values (3). Results are strongly dependent on the type of basic alumina used for extract cleanup (4; R. L. Smallidge et al. [1985] AOAC Spring Workshop, Dallas, TX). In addition, it is time consuming and hazardous because of the KCN used in color development. The official AOAC fluorometric method (5) shows a bias when certain antibiotics are present (6); it has not found widespread acceptance, as evidenced by a recent AFFCO check sample (8622) in which only 2 of 33 reported results were obtained by the fluorometric method.

Attempts have been made to modify the colorimetric method (3, 4) and to introduce new liquid chromatographic (LC) methods (7, 8), but all these attempts have certain limitations. Modification of the alumina preparation in the AOAC colorimetric method by Davis (4), although incorporated into the official method (9), does not correct the problems associated with this method (Smallidge et al., 1985). The ion-pair partition cleanup procedure proposed by Severijnen et al. (3) is time consuming and inconvenient for routine analysis. The reported LC methods make use of laboratory-prepared ion-exchange (7) or silica normal phase columns (8), which is a limitation when a method is considered for general use.

The objective in this study was to develop a reverse-phase LC method using commercially available columns and requiring minimum cleanup. The method presented meets these criteria, is accurate and precise, and gives qualitative information not available from the official methods.

Received February 24, 1987. Accepted May 31, 1987.
Presented in part at the 99th AOAC Annual International Meeting, Oct. 29, 1985, Washington, DC. E. Kentzer is Associate Referee for Amprolium.
Published as Journal Paper No. 11,066, Agricultural Experiment Station, Purdue University.
[1] Present address: Abbott Laboratories, 1400 Sheridan Rd, North Chicago, IL 60064.
[2] Address correspondence to this author.

METHOD

Principle and Scope

Finely ground feed is extracted with 100 mL methanol–water (2 + 1) containing 5mM sodium dioctylsulfosuccinate (DOSS) and 10mM $CaCl_2$. Late-eluting contaminants are removed by acid alumina cleanup. Clarified extract is assayed by reverse-phase LC using DOSS as an ion-pairing agent in the mobile phase to increase the capacity factor of amprolium beyond that of coextracted contaminants. Amprolium is detected at 270 nm. The concentration range is from 0.004% to and including premix levels of 25% amprolium.

Apparatus

(a) *Liquid chromatograph.*—Instrument capable of solvent mixing and maintaining constant pulseless flow of mobile phase. Operating conditions: flow rate, 1.5 mL/min; loop injection volume, 20 μL (<40 μL); detector wavelength, 270 nm; detector sensitivity, 0.02–0.04 or as adjusted to yield working standard peak height of ca 60% full scale.

(b) *Chromatographic column.*—Any reverse-phase C18 column with particle size ≤10 μm which will separate amprolium from coextracted materials. Guard column should be used. Acceptable columns have been (*1*) Econosphere C18, 5 μm, 150 mm (Alltech Associates, Inc., Deerfield, IL 60015, Cat. No. 70065); (*2*) American Burdick and Jackson (Muskegon, MI 49442) OD-5, 4.6 × 150 mm; (*3*) Whatman ODS-3, 10 μm, 4.6 × 250 mm (Whatman Inc., Clifton, NJ 07014). Other reverse-phase columns tested require greater adjustment of the mobile phase modifiers than these to optimize chromatography.

(c) *Sample clarification filter.*—13 mm glass fiber prefilter pads (Gelman 66073, Fisher Scientific 09-731A) inserted in 0.5 in. id, 5 mL syringe barrel (B-D syringe, No. 5603) or any other filtration device (porosity <0.5 μm) designed for clarification of aqueous chromatographic samples.

(d) *Acidic alumina cleanup column.*—(*1*) Sep-Pak alumina A cartridges (Waters Aeromatography, Milford, MA 01757, No. 51800), or (*2*) alumina, acid, Brockman Activity 1, 80–200 mesh (Fisher A-948). Wash by mixing with water (use ratio of 5 mL water to 1 g alumina) for 30 min, filter, rinse with methanol under reduced pressure, and let air dry at room temperature. Then dry for 3 h at 100°C under vacuum (ca 30 in. Hg).

Reagents

(a) *Extractant and diluent.*—Methanol–water (2 + 1) with 5mM DOSS and 10mM $CaCl_2$. Dissolve 2.22 g sodium dioctylsulfosuccinate (Aldrich D 20,117-0) and 1.47 g calcium chloride dihydrate (Sigma C-3881) in methanol–water (2 + 1) and dilute to 1 L. (Note: DOSS is hygroscopic and clumpy; however, this should not create a problem.)

(b) *Mobile phase.*—40% acetonitrile containing 4mM DOSS, 0.3% diethylamine (DEA), and 1.0% acetic acid (sta-

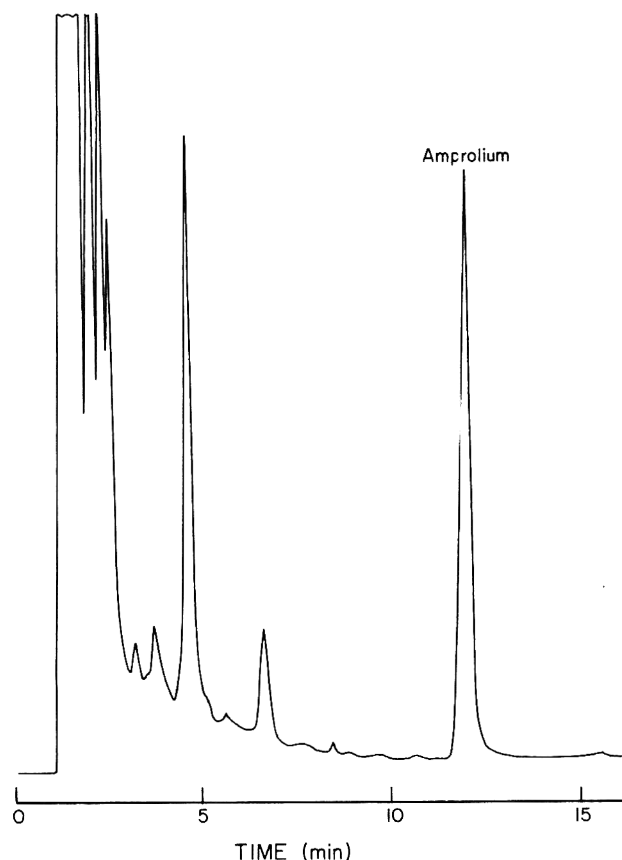


Figure 1. Separation of amprolium (k′ ≅ 10) from coextracted materials in a commercial poultry feed. Twenty mL of 0.0125% level sample was injected with 0.025 AUFS sensitivity. Column: Econosphere C18, 5 μm, 150 × 4.6 mm.

ble at least 4 weeks). When it is desirable to adjust solvent strength (as when using column for the first time), prepare the following 2 solvents: (A) 35% acetonitrile with the above-listed additives (freshly prepared because DOSS solubility in 35% acetonitrile is borderline), and (B) 45% acetonitrile with the additives. DOSS is not very soluble when mobile phase contains <30% acetonitrile. To each of two 1 L volumetric flasks, add 1.78 g DOSS (formula weight = 444.55). Then add appropriate amount of LC quality acetonitrile (350 mL for A and 450 mL for B) and ca 200 mL LC quality water. To each, add 10 mL acetic acid and mix to dissolve (sequence important). Add 3 mL DEA, dilute to volume with water, and mix.

(c) *Amprolium standard solutions.*—(*1*) *AMP stock solution.*—About 600 μg/mL. Accurately weigh 0.057–0.063 g amprolium standard (Merck and Co.) into 100 mL volumetric flask (actual weight = W_s). Dissolve in and dilute to volume with extractant. (*2*) *AMP intermediate stock solution.*—About 60 μg/mL. Transfer 10.0 mL AMP stock solution to 100 mL volumetric flask and dilute to volume with extractant. (*3*) *AMP working standard solution.*—About 15 μg/mL. Transfer 25 mL AMP intermediate standard solution to 100 mL volumetric flask. Dilute to volume with extractant and mix. Total dilution of working standard (D_s) is 4000 mL.

Recovery Test for Alumina Cleanup Column

Whenever new cartridges are received or new batch of acidic alumina is prepared, test for recovery by comparing amprolium peak response on portion of sample extract containing amprolium which has passed through cleanup column (as in *Extraction and Cleanup,* complete feed) to peak response of portion of same extract which was not passed through column. Percent recovery [(peak response through alumina/peak response of untreated extract) × 100] should be 100 ± 3%.

Mobile Phase Optimization

Retention time is quite sensitive to variation in mobile phase modifier, which may result with LC-blended mobile phase. Once optimization is accomplished, it is best to premix mobile phase. To determine best mixture, condition LC column with 1:1 instrument-mixed ratio of mobile phase A:B (equilibration takes ca 30 min). Inject water to determine void volume (t_o). Repeat injections of working standard until retention time (t_r) of amprolium peak is constant. If doubling of amprolium standard peak occurs or if peak is distorted, capacity factor ($t_r - t_o/t_o$) may be too low (k′ = 10–16 is optimum). If necessary, adjust mobile phase mixture in small increments (e.g., first try A:B (55 + 45) to increase k′, or A:B (45 + 55) to decrease k′).

It is generally not necessary to adjust mobile phase concentration of other modifiers when new column is optimized. However, if adjusting acetonitrile concentration alone is not satisfactory, increasing DOSS concentration will also increase k′. Increasing DEA concentration will reduce peak tailing and k′. Make any changes of DEA or DOSS in small increments (ca 5%). Test system with sample extract to ensure that amprolium peak is well resolved from coextracted contaminants. In certain cases where an older LC column has not been adequately cleaned following use, peak doubling of standard peak may occur. When this happens, wash column at 1 mL/min for 20 min sequentially with (*1*) water, (*2*) 0.2M oxalic acid, (*3*) water, (*4*) acetonitrile, using slow linear program between each segment. Generally, when optimized mobile phase has been determined it will remain satisfactory throughout life of column.

Extraction and Cleanup

Complete feed.—Weigh amount of well-mixed, finely ground sample to contain ca 1500 μg amprolium (sample weight = 0.15 divided by % guarantee; 12 g for 0.0125% guarantee and 6 g for 0.025% guarantee) into 250 mL Erlenmeyer flask. Do not weigh less than ca 4 g sample weight. Add by pipet 100 mL extractant, stopper, and shake mechanically 60 min. Let feed particles settle or centrifuge to clarify. Make any dilutions necessary to give ca 15 μg/mL (only needed if level is >0.04% amprolium).

Remove possible late-eluting materials from extract immediately before chromatography by one of the following techniques: (a) Attach syringe (≥10 mL) to alumina A cartridge, add 8–10 mL extract, and gently force extract through cartridge with syringe plunger. Discard first 3 mL effluent, and collect the next 4–6 mL for chromatography. (b) Alternatively, add 5 g acidic alumina to 1 cm id glass chromatographic column fitted with frit or small pledget of glass wool. Treat as in (a) except let effluent drain by gravity. Clarify by filtration (c) if necessary. Do not elute standard through cleanup column.

Premixes.—Weigh 5 g unground premix into 500 mL volumetric flask, add ca 300 mL extractant, shake 60 min on reciprocal shaker, dilute to volume with extractant, and mix. Dilute initial extract as follows: 25% premix: 10/100, then 6/100; 1.25% premix: 6/50 mL. Final amprolium concentration should be ca 15 μg/mL.

Chromatography

Refer to *Apparatus* (a) for operating conditions and to *Mobile Phase Optimization.* Condition column by pumping

premixed, optimized mobile phase until retention time and peak response for injected working standard are constant (1–2%). Bracket each 2 sample injections by working standard injections. Following the run, study chromatogram to determine if use of peak area is justified (i.e., peak integration marks are satisfactory); if not, then measure peak heights to nearest ½ mm. Use average of all standard injections in calculations if there is no drift in peak response; otherwise, use average peak response (R_s) of standards bracketing each pair of samples to calculate amprolium concentration in samples. After each use, rinse column with ca 10 column volumes of 40% acetonitrile (no other modifiers), program slowly (ca 20 min) to water, hold for ca 20 column volumes, then program slowly to acetonitrile and hold for another 20 column volumes.

Calculations

$$\text{Amprolium, \%} = (R_u \times W_s \times D_u \times 100)/(R_s \times W_u \times D_s)$$

where R_u = instrument response, unknown sample; W_s = weight (g), standard; D_u = total dilution (mL), unknown sample; R_s = instrument response, standard; W_u = weight (g), unknown sample; D_s = total dilution (mL), standard.

Dilution calculation.—Total dilution of standard (D_s), in which a certain weight of standard material (W_s) is dissolved in solvent and diluted to 100 mL and then is further diluted 10/100 and 25/100, is calculated as shown here:

$$D_s = (100 \times 100 \times 100)/(10 \times 25) = 4000 \text{ mL}$$

Common dilutions.—D_u = 100 for complete feeds (AMP < ca 0.04%). D_u = 83 333 mL for 25% premix and 4167 mL for 1.25% premix; D_s = 4000 mL.

Results and Discussion

Figure 1 shows a typical chromatogram of amprolium in a sample extract with a label claim of 0.0125%. The final version of the mobile phase was selected on the basis of elution profiles as affected by (*1*) the acetonitrile concentration, (*2*) the nature and concentration of the ion-pairing reagent, and (*3*) the diethylamine concentration.

Several alkane sulfonate counter ions, as well as dioctylsulfosuccinate (DOSS), were tried as ion-pairing reagents in the *mobile phase* in an attempt to increase the k′ of amprolium beyond that of the coextracted materials. DOSS was used by Severijnen et al. (3) as the counter ion in the ion-pair partition cleanup of amprolium in their modification of the colorimetric method. Both dodecanesulfonate and DOSS were satisfactory counter ions for reverse-phase LC ion-pair partition chromatography, but we chose the latter because it is much less expensive (even though it is quite hygroscopic and exhibits some solubility limitations in the mobile phase). Diethylamine (DEA) is added to the mobile phase to decrease the tailing of the amprolium peak. When the concentration is too high, diethylamine interferes with the counter ion and decreases the amprolium k′. Thus, a balance between acetonitrile, DEA, and DOSS must be maintained to obtain optimum chromatography.

After trying many LC columns, we found 2 which appeared to perform better than the others in this method: (*1*) OD-5 spherical C18 (4.6 mm id × 15 cm) from American Burdick and Jackson, and (*2*) Econosphere C18, 5 μm, 4.6 mm id × 15 cm, from Alltech Associates, Inc. Other columns which were tested and which showed satisfactory resolution of the amprolium peak from coextracted material peaks were Partisil ODS-3, Versapack C18 (10 μm, 25 cm), and Resolvex C8. However, minor adjustments of the mobile phase modifiers may be necessary to obtain optimum resolution and peak skew. We observed peak doubling of the amprolium standard peak with 2 of the 9 columns tested, and we could not resolve this problem by the normal means, such as reducing injection volume, altering the concentrations of the various solvent parameters, or cleaning the column. Double peaking of the amprolium was observed at times even with the more satisfactory columns if the column was not cleaned after each run as suggested in the method. When the doubling of the standard peak occurred on these columns, cleaning the column by pumping about 30 mL of 0.2% sodium oxalate followed by the same amount of water at 1–1.5 mL/min through the system was required for rejuvenation. A 20–30 min gradient between cleaning solutions is recommended. We later found a greater tendency for amprolium peak doubling at low k′.

The linearity of the system was tested by constructing a calibration curve over the range 2.5–50 μg/mL (working standard = 15 μg/mL). Least square analysis of the curve resulted in a coefficient of determination (r^2) of 0.9998. The intercept value obtained was found not to be significantly different from zero (10, 11). Multiple standard addition (MSA) using commercial test sample portions, with spiking during extraction, was also linear (r^2 = 0.9994). In MSA studies, the amount of standard amprolium in the spike was varied to be 0.0, 0.5, 1.0, 1.5, and 2.0 times the total amount of amprolium expected in the test sample, e.g., for spiking during extraction, when 1500 μg is expected in the sample, from 0 to 3000 μg spike is added to 5 sample portions in such a way that the final volume of extractant is held constant at 100 mL.

Initially, it was our desire to use the same extractant [i.e., methanol–water (2 + 1)] for the LC method that is used in the official colorimetric method (2). Preliminary studies using this extractant, with determination by LC, indicated low recovery relative to results obtained colorimetrically. Did the colorimetric method have a high bias, or was there a problem with the LC approach?

This apparent low recovery with the LC determination was studied using multiple standard addition. Several test portions of a commercial sample containing amprolium were spiked at different levels, either during extraction or by using aliquots of a pooled unspiked sample extract that were spiked after extraction; all were determined by LC. (Alumina cleanup columns were not being used when these studies were carried out.) The slopes from the linear regression treatment of the data were compared to a calibration curve constructed at the same time by calculating the slope confidence limits (11). The slopes of the 2 MSA lines were significantly different from each other, and both were less than the slope of the calibration curve (i.e., slope confidence limits did not overlap). The lower slopes indicate incomplete recovery of amprolium using this extractant prior to LC (R. L. Smallidge [1983] AOAC Spring Workshop, Indianapolis, IN). The fact that the slopes are different, and dependent on the point of spiking in the procedure, suggests that at least 2 different recovery reducing phenomena are observed. We were especially surprised that we could not completely recover all of the spike added to the clear supernate after extraction. Our working theory at the time was that amprolium in the extract was complexed so that the peak response was decreased (the peak retention time and shape were the same in sample extracts and in standard solutions).

$CaCl_2$ was added to the methanol–water extractant to "swamp out" the natural complexing agents in the extract. This helped, but we found that dioctylsulfosuccinate was also

Table 1. Standard addition recovery from commercial samples by using official method extractant or modified extractant with determination by LC

Sample	Label, %	Recovery, %	
		Official[a]	Modified[b]
1	0.00625	98.8	96.8
2	0.00625	89.9	102.4
3	0.00725	76.1	104.5
4	0.0125	84.0	101.0
5	0.0125	65.6	100.6
6	0.0125	93.5	106.5
7	0.0125	78.9	104.9
8	0.0125	104.5	96.1
9	0.0125	97.0	94.7
10	0.0125	97.1	92.7
11	0.025	95.6	101.3
Mean recovery (%)		89.2	100.1
CV (%)		13	4.5

[a] Methanol–water (2 + 1).
[b] 5 mM dioctylsulfosuccinate + 10 mM $CaCl_2$ in methanol–water (2 + 1).

needed in the extractant for complete recovery. The MSA experiment was repeated using the modified extractant (i.e., 5mM DOSS + 10mM $CaCl_2$ in methanol–water). The slopes of these MSA lines were not statistically different from that of the calibration curve, indicating complete recovery.

Single point standard addition, performed on 11 different commercial samples containing amprolium, was used to further test the effectiveness of the modified extractant for LC. The mean recovery of added spike was 89.2% for the methanol–water extractant and 100.1% for the $CaCl_2$–DOSS modified methanol–water extractant (Table 1).

The accuracy of the LC method was compared to the official colorimetric method through the analysis of a set of 11 commercial complete feed and 6 premix samples by both procedures. The experimental plan involved single determinations of each sample on 2 different days by each method for the complete feeds, and duplicates on the same day for premixes. The results were analyzed using the paired comparison plan as outlined by Wernimont (12), with the complete feed and premix data treated as separate sets. The averages of the results for each sample, by method, are presented in Table 2. In this treatment of the data, the precision of the 2 methods is first determined to be not different by comparing

Table 2. Analysis of amprolium in commercial feeds: LC vs official colorimetric method

Sample[a]	Label, %	Determination, %	
		Official	LC
1	0.00625	0.0044	0.0042
2	0.00625	0.0042	0.0042
3	0.0125	0.0100	0.0094
4	0.0125	0.0102	0.0102
5	0.0125	0.0091	0.0092
6	0.0125	0.0114	0.0115
7	0.0125	0.0094	0.0096
8	0.0125	0.0120	0.0128
9	0.025	0.0226	0.0210
10	0.025	0.0208	0.0219
11	0.03125	0.0282	0.0312
12	25	23.7	23.9
13	25	24.6	27.5
14	25	25.9	25.4
15	25	24.6	25.9
16	25	26.1	26.4
17	25	26.8	26.7

[a] 1–11 = average of single analyses on 2 different days; 12–17 = average of duplicates on the same day.

Table 3. Test for peak purity: calculation at 3 wavelengths

Label	Found, %		
	250 nm	270 nm	290 nm
0.00625	0.0049	0.0048	0.0049
0.0125	0.0127	0.0130	0.0128
0.0250	0.0232	0.0230	0.0219
0.03125	0.0303	0.0305	0.0300
Peak ht ratio[a]	0.7	1.0	0.25

[a] Standard peak response at 250 and 290 nm relative to 270 nm.

method variances in an F-test. The means of the methods are then compared by calculating the confidence limits of the mean difference between the methods. If the confidence limits include zero, the methods are not considered to be statistically different. The LC method was found to be statistically not different from the official method (alpha = 0.05).

Recovery of amprolium by the official method has been shown to be low when samples contain grass meals (3, 7). To determine if the same effect occurs when the proposed method is used, a study was conducted in which varying amounts of alfalfa were added to constant amounts of test sample. No decrease in recovery was detected until the relative amount of alfalfa was 40% of the total (much higher than is normally found in complete feeds).

Several pelletization aids were added at the 4% level to portions of a complete feed containing amprolium, the samples were assayed, and the results were compared to a portion of the feed with no pelletization aid present. Bentonite was the only aid which caused reduced recovery of amprolium. The presence of the other feed pelletization agents tested (Agri-gum, Pel-aid, Ameri-bond, and Pell-A-Firm) resulted in a small shoulder appearing on the amprolium peak tail; however, if peak heights were used for calculating results, recovery was not affected. Peak area calculations in these rare cases may result in a small positive bias.

No interference to the recovery of amprolium nor to the chromatogram was observed when the following poultry drugs and antibiotics were added to feeds: ethopabate, sulfonamides, arsanilic acid, penicillin, streptomycin, and chlorotetracycline. When thiamine was added to the extract from an amprolium-containing feed, a peak with a much smaller k′ was observed. The specificity of the proposed LC method was further tested by the analysis of 10 unmedicated poultry feeds. No peaks were observed with a k′ similar to that of amprolium.

After being well into this study, we became aware that some complete feed level samples contain late-eluting peaks. These do not interfere with the amprolium peak in the extract, but may interfere with subsequent injections. We investigated the use of several different types of commercially available cleanup columns while looking for a simple solution to this problem. At first it appeared that a pellicular C18

Table 4. Test for precision at different label claims[a]

Label claim, %	Mean, %	SD	CV, %[b]
0.00625	0.0055	0.000198	3.61
0.0075	0.00624	0.000178	2.84
0.0125	0.0146	0.000320	2.19
0.0125	0.0143	0.000493	3.46
0.250	0.0237	0.000137	0.58
0.250	0.0227	0.000314	1.39
25.0	25.2	0.5	2.0

[a] Six replicates each of 7 commercial feeds.
[b] Mean CV = 2.3%.

Table 5. Ruggedness test

Conditions[a]	Normal values	Letters	Altered values UC[b]	LC[b]	Diff.[c]
DOSS/P_m, mM	4	C, c	4.4	3.6	0.25
$CaCl_2$/Ext., mM	10	B, b	11.0	9.0	4.75
Al_2O_3 cart.	used	G, g	used	not used	11.75
DEA/P_m, %	0.3	D, d	0.33	0.27	21.75
DOSS/Ext., mM	5	A, a	6	4	26.25
CH_3CN/P_m, %	40	E, e	42	38	30.25
Wavelength, nm	270	F, f	280	260	35.25
Mean result = 0.009916% amprolium; CV = 3.17%					

[a] DOSS = sodium dioctylsulfosuccinate; P_m = mobile phase; Ext. = extractant; Al_2O_3 cart. = acid alumina cartridge; DEA = diethylamine.
[b] UC = upper case letter; LC = lower case letter.
[c] Difference = |UC mean results − LC mean results| × 10^5.

column might work, but we soon found the recovery of amprolium to be variable. After testing several of the other types of columns, including acid, neutral, and basic alumina, we found acid alumina (whether as commercial cartridges, or as laboratory-prepared acid alumina columns) to be satisfactory for removing the late-eluting peaks as well as for recovery of the amprolium peak. We recommend discarding the first 3 mL effluent from cleanup column or cartridge, and collecting the next 4–6 mL for injection onto the LC column, because, as we have found with other cleanup columns (Smallidge et al., 1985), analyte is concentrated in the first 1–2 mL effluent.

The purity of the amprolium peak from some typical feeds was tested by calculating results from chromatograms obtained at wavelengths 20 nm each side of the wavelength maximum recommended for analysis, i.e., 270 nm (Table 3). Generally good agreement was observed for all tested samples.

The within-day precision of the proposed method was studied by analyzing 6 replicates of 7 samples over the concentration range normally found in commercial feeds, i.e., 0.00625–25% (Table 4). The coefficients of variation ranged from 0.58 to 3.61%.

The proposed method was tested for ruggedness (13) to determine (*1*) if there are any procedural points where special care is needed, and (*2*) how the method might be expected to perform if subjected to a collaborative study. The following factors were selected for scrutiny: extractant (A,a): 20% each side of the proposed DOSS concentration, and (B,b): 10% each side of the proposed $CaCl_2$ concentration; mobile phase (C,c): 10% each side of the proposed DOSS concentration, (D,d): 10% each side of the proposed diethylamine concentration, and (E,e): 10% each side of the acetonitrile concentration; detector (F,f): 10 nm each side of the proposed wavelength of detection, (G,g): using or not using the alumina cleanup column. Statistical evaluation indicates negligible effects for all changes (Table 5). The overall coefficient of variation for the ruggedness test was 3.2%. This should predict the success of the method in interlaboratory use (13). The factor which is most likely to lead to problems is fine tuning the organic strength in the mobile phase.

The LC method has been used in our laboratory for more than 2 years since it has evolved to its present form. We have used the same column for much of that time with no problem as long as the suggested column wash process is followed. We have also found that once we settled on the optimum strength of the organic portion in the premixed mobile (40%), we have not needed to change it (the hygroscopic nature of the DOSS has not created any problem).

We have presented an LC method for the analysis of amprolium in feeds and feed premixes and validated it in comparison to the official colorimetric method. Besides the major advantage of specificity, the LC method also has other advantages with respect to the official method. The official method is dependent on the basic alumina selected for recovery of analyte (4; Smallidge et al., 1985), a condition which should be corrected by adding a recovery test to that procedure. The official method also uses the less than desirable KCN as part of the color developing reagent. The hands-on time for the analyst is less with the LC method than with the official method (if an autosampler is used).

Recommendations

The present plan is to subject the method to collaborative study as soon as it has been field-tested.

Acknowledgments

We thank Connie Gehrig for carrying out the official method analyses used in the manuscript.

References

(1) Leidahl, R. (Ed.) (1986) *Feed Additive Compendium*, The Miller Publishing Co., Minneapolis, MN, pp. 109–120
(2) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **42.011**-**42.015**
(3) Severijnen, M., Buyzen-Satijn, A. M. G., & Buizer, F. G. (1975) *Analyst* **100**, 328–333
(4) Davis, E. J. (1968) *J. Assoc. Off. Anal. Chem.* **51**, 129–131
(5) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **42.016**-**42.020**
(6) Kanora, J., & Szalkowski, C. R. (1964) *J. Assoc. Off. Agric. Chem.* **47**, 209–213
(7) Cox, G. B., Lascombe, C. R., Slucutt, M. J., Sugden, K., & Upfield, J. A. (1976) *J. Chromatogr.* **117**, 269–278
(8) Cox, G. B., & Sugden, K. (1976) *Analyst* **101**, 738–741
(9) Davis, E. J. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 621–623
(10) Delaney, M. F. (1985) *LC* **3**, 264–268
(11) Hunter, J. S. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 574–583
(12) Wernimont, G. T. (1985) *Use of Statistics to Develop and Evaluate Analytical Methods*, W. Spendley (Ed.), AOAC, Arlington, VA, pp. 48–50
(13) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA, pp. 5–6, 33–36, 50–55

FEEDS

Moisture Analysis of Forage by Near Infrared Reflectance Spectroscopy: Preliminary Collaborative Study and Comparison Between Karl Fischer and Oven Drying Reference Methods

WILLIAM R. WINDHAM, FRANKLIN E. BARTON II, and JAMES A. ROBERTSON
U.S. Department of Agriculture, Agricultural Research Service, Richard B. Russell Agricultural Research Center, Plant Structure and Composition Research Unit, PO Box 5677, Athens, GA 30613

A collaborative study of moisture analysis by near infrared reflectance spectroscopy (NIRS) has been completed involving 5 laboratories and 20 forage samples. Near infrared reflectance spectroscopy calibrations for moisture were developed in the Associate Referee's laboratory from Karl Fischer (KF) and AOAC air oven (AO) (135°C for 2 h) moisture methods, respectively, and transferred to each collaborating laboratory's NIRS instrument. NIRS moisture data were validated with KF data from the Associate Referee's laboratory and AO data from each collaborating laboratory. The standard error of analysis of KF data by NIRS KF determination and AO data by NIRS AO determination ranged from 0.25 to 0.48% and from 0.74 to 1.88%, respectively. The standard errors between laboratories for NIRS KF and NIRS AO determinations were 0.27 and 0.39%, respectively. The standard error between moisture analyses by NIRS KF and NIRS AO calibrations, averaged across laboratories, was 0.42%. In addition, the standard error between laboratories for the AOAC AO method was 0.63%. The increase in standard error for the AOAC AO method was due to the random and systematic errors associated with the gravimetric techniques. The results indicate that NIRS analysis can accurately and precisely determine the moisture content of forages and forage crops because of the very strong absorbance of water in the near infrared region.

Near infrared reflectance spectroscopy (NIRS) has been extensively researched and widely accepted for the analysis of crude protein, acid and neutral detergent fiber, lignin, and in vitro dry matter digestibility of forages over the last 10 years (1). However, only in the last year has the NIRS technique been proposed in the literature for routine moisture analysis of perennial forages (2, 3), even though Kaye (4) in 1954 suggested that moisture could be determined by NIRS analysis and Hoffman (5) in 1963 established the relationship between reflectance at 1450 and 1930 nm to moisture content of solid materials. In fact, NIRS analysts recommended drying samples to a 5–10% moisture range before NIRS analysis to reduce the interference from water (1). Also, NIRS analysis has been used to determine moisture content in wheat (6), corn and grain sorghum (7), oilseeds (8), and corn silage (9). Although these previous investigators demonstrated the feasibility of NIRS moisture analysis, accuracy of their data was limited by calibration with air oven moisture data.

The acceptance of NIRS for the routine analysis of moisture in forages has been hampered by the lack of a primary reference method specific for water. The Karl Fischer (KF) titration method is specific for water and has been applied to a wide variety of agricultural products, including cereal grains (10), soybeans (11), sunflower seeds (12), oils and fats (13), and corn (R. Bernetti, CPC International, private communication 1987). Application of the KF titration method to moisture analysis in perennial forages and as the reference method for NIRS calibration was first evaluated by Windham et al. (3). Those results were compared to the 2 AOAC official methods (14): air oven method, **7.007** and vacuum oven method, **7.003**, and the air oven method with drying at 105°C for 24 h. Moisture data from method **7.003** and the modified air oven method were lower ($P < 0.05$) than those obtained for method **7.007** or the KF method. No difference was found between the latter 2 methods. Examination of the 1930 nm absorption band from the vacuum oven and modified air oven residues with NIRS indicated that the lower moisture values were due to the inability of these methods to remove effectively all the water from the sample; the residue spectra from method **7.007** showed little absorption at 1930 nm, indicating that virtually all water had been removed. On the other hand, further comparison of these methods in forage populations to include silages and silage-based dairy rations resulted in higher ($P < 0.05$) moisture data from method **7.007** vs KF. This difference was probably due to the loss of volatile acids, bases, and alcohols at 135°C, resulting in an overestimation of moisture (15). We demonstrated and concluded that NIRS can be used to precisely and accurately analyze forages for moisture when calibrations are developed with a primary reference method that truly defines moisture concentration.

The analysis of moisture in forages is important in other aspects of NIRS technology. One of the 4 keys to successful use of NIRS is accurate laboratory analyses (chemical or bioassay) of the calibration samples (1). Many NIRS analysts prefer to calibrate when sample assays are expressed on a dry matter (DM) basis rather than on an ambient environment basis. As such, an equally important component of accurate laboratory analysis of the calibration samples is that of moisture determination. Variability in moisture determination will produce inaccurate estimates of all other chemical components. The analysis of moisture by NIRS has the potential to reduce or eliminate this source of variation.

This research was undertaken by the National NIRS Forage Research Project Network on behalf of the U.S. Department of Agriculture, Agricultural Research Service. The preliminary collaborative study of the proposed method of moisture analysis by NIRS provided an opportunity to establish guidelines for NIRS moisture calibration and to demonstrate the potential of NIRS to reduce analytical errors among laboratories.

Moisture in Forage and Forage Crops
Karl Fischer Method

Apparatus and Reagents

(**a**) *Karl Fischer titration assembly.*—Manual or automated, with stirrer.

(**b**) *Mill.*—Model 8000 Mixer/Mill with 65 mL capacity hardened steel vessel and supply of 0.5 in. diameter steel balls (Spex Industries, Metuchen, NJ 08840), or equivalent.

Received March 13, 1987. Accepted May 20, 1987.
Contribution from The Plant Structure and Composition Research Unit, Richard B. Russell Agricultural Research Center, Athens, GA 30613.
Mention of a trade name does not imply an endorsement or recommendation by the U.S. Department of Agriculture.

(c) *Karl Fischer reagent.*—Hydranal-Composite 5 (Crescent Chemical Co., Hauppauge, NY).

(d) *Sample solvent.*-Anhydrous distilled-in-glass methanol (Baker Chemical Co., Phillipsburg, NJ).

Sample Preparation and Extraction of Water

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

Accurately weigh ca 1 g cyclone-ground (1 mm) forage, place in milling vessel with 3 steel balls, and pipet 50 mL methanol into vessel. Seal lid to vessel, and mill grind 30 min (3). Set milling vessel aside to settle solid residue from sample. Allow 1½ h if vessel is not centrifuged. Centrifuging will greatly reduce settling time. Repeat procedure with 50 mL methanol blank. Minimize exposure of sample, methanol, and mixture to air to prevent contamination by moisture in air.

Determination

Prepare titrator for titration according to manufacturer's instructions. Standardize titrator by injecting water as outlined by Jones and Brinkenkamp (11). For best results, make titrations of methanol blank at beginning and end of a series of determinations and compute mean. Withdraw specimen of desired quantity of methanol-extracted water mixture from milling vessel and weigh on microbalance. Insert tip of needle beneath surface of solution in titration vessel, expel mixture, and start titration. Carefully wipe needle clean of solution and reweigh syringe. Record weight of specimen injected. After end point has been reached, record titration value (mL Hydranal reagent used in case of the motorized biuret type titration). Repeat titration procedure for duplicate titration values on specimen. Estimate mass of oil and/or extraneous material extracted into methanol-extracted water mixture from forage by the procedure of Jones and Brinkenkamp (11).

Calculations

Calculate moisture content of forage sample, using mass basis Karl Fischer titration equation (16).

Moisture in Animal Feed
Oven Drying at 135°C

Conduct moisture determination by AOAC official method **7.007**. For best results, control time of drying; control time needed for air oven to return to desired temperature after opening; minimize exposure of dry matter residues to air; clean and properly lubricate seals and glass surfaces of desiccator; use fresh and effective desiccant; and use low desiccator loading rate.

Moisture in Forage
Near Infrared Reflectance Spectroscopy

Apparatus

(a) *Wavelength-scanning instrument.*—Model 6100 or 6350 grating monochromator (Pacific Scientific Corp., Gardner/Neotec Instrument Div., Silver Spring, MD 20910), or equivalent. Analysts are referred elsewhere (17, 18) for detailed description of monochromator.

(b) *Computer.*—PDP 11 series computer equipped with 64 K bytes of main memory; dual RX02 double-density floppy disks; RL01 five megabyte or RL02 ten megabyte hard disks. PDP system software RT-11 V5.0 (Digital Equipment Corp., Nashua, NH 03061), or equivalent.

(c) *USDA Public Software.*—Analysts are referred to USDA NIRS Handbook 643 (1) for detailed description of software. Software consists of 14 programs written in FORTRAN IV to collect, store, and process NIRS data.

(d) *Mill.*—Tecator Cyclone Sample mill with 1 mm screen (Fisher Scientific, Springfield, NJ 07081), or equivalent. Periodically change grinding ring to ensure consistency of particle size over time.

(e) *NIRS sample holder.*—Nylon holder 2½ cm diameter and 1 cm thickness with infrared transmittance quartz window. Sample capacity ranges from 0.75 to 1.75 g. The sample is held in holder with back made of rubber or foam core (Pacific Scientific Corp.), or equivalent.

(f) *Sample storage container.*—For best result in maintaining sample moisture concentration, store in Poly-Kraft Bags-Mil-B-121 Type II Grade A, Class I. Place sample in bag and heat-seal (EDCO Supply Corp., Brooklyn, NY 11232).

Instrument Operation

(a) *Start-up.*—For best results, run instrument continuously. If instrument is turned on from cold condition, warm-up time should be no less than 15 min and may require as much as 1 h.

(b) *Monochromator diagnostic tests.*—Instrument noise: Conduct scans of ceramic reference to itself. Deviations from zero are expressed as average deviation (bias); root mean square (RMS) is expressed as $\log(1/R)/10^6$. Bias indicates any systematic change in $\log(1/R)$ level of scans taken over time. All positive or all negative bias values indicate problem with instrument. RMS value can range from a low of 10 to a high of 50 without affecting analysis. Monochromators manufactured since 1983 should have average noise level below 30 RMS over 100 scans. Wavelength accuracy: Measure wavelength repeatability and accuracy with a clear polystyrene petri dish. Place petri dish in light beam and pull out sample drawer to expose ceramic standard. Reference this scan to measurements without petri dish. Locate major polystyrene peaks and compare with known locations at 1680.3, 2164.9, and 2304.2 nm. Standard deviation of repeatability should be less than 0.05 nm, and the deviation from known location should be less than 0.5 nm. Large values usually indicate mechanical problems in monochromator.

(c) *Maintenance.*—Clean ceramic standard, all parts of drawer assembly, and windows above and below detector with vacuum, brush, or soft tissue whenever dirt is seen to accumulate. Detailed information on instrument operation is given in USDA NIRS Handbook 643 (1) and elsewhere (19).

Determination

(a) *Preparation of sample.*—Grind samples for NIRS analysis in cyclone mill through 1 mm screen. Clean mill between samples to minimize cross-contamination. Before grinding samples containing more than 25% moisture, dry 24 h in 60°C air oven. Mix milled samples well and take random test portions. Continue to take random test portions until NIRS sample holder is level full. Press back into holder. Continue to press back until it is tight and level. As a check, invert NIRS sample holder and make certain that sample is firmly pressed against window. If any abnormality is apparent, remove back and repeat this procedure. Consistency in sample handling and preparation is crucial to successful NIRS analysis.

(b) *Calibration.*—Randomly select samples for moisture calibration from either finite or infinite population. (A finite population has defined boundaries set by the analyst which limit the population, whereas an infinite population has no such boundaries.) Select as many samples as possible cov-

ering a range of moisture concentration and all other variables that may affect chemical and physical composition of the forage (stage of growth, species, preservation method, etc.). In practical terms, a minimum of 50 samples should be considered (19).

Collect reflectance (R) measurements (log 1/R) of calibration set with program SCAN (1) from 1100 to 2500 nm and record at 2 nm intervals. Conduct primary reference moisture method on NIRS test portion to ensure that the moisture concentration of calibration set is not influenced by changes in environmental conditions. Develop multiterm calibration equations by multiple linear regression of reflectance measurements to moisture concentration using program BEST (1). Before evaluating regression statistics, examine differences (residuals) between NIRS moisture data and reference moisture method for samples with large t- or H-values. Large positive or negative t-values represented by "☆" on computer printout indicate that residual is 2.5 times the standard error of a difference between NIRS determination and reference method. Reanalyze these samples by the reference method. A large H-statistic denoted as "☆" and "☆,☆" indicates that NIRS spectrum used in calibration for a given sample differs substantially from NIRS spectra of other samples.

Calculation was mathematically derived from covariance matrix according to the formula: $H = X(s'x)^{-1}x'$ (1, 20). A high value on the diagonal of the H-matrix indicates a sample that is dissimilar to the calibration set at the wavelengths used in the equation. Rescan such a sample. If the 2 scans agree and the sample belongs in the population, then retain sample. If scans disagree, then the first one was a mistake and should be discarded.

Examine standard error of calibration (SEC) to determine how well calibration samples were fit. The lower the SEC value, the better the fit. Select an equation with SEC about 2 times the laboratory repeatability error of reference moisture method. Examine coefficient of determination (R^2) to determine proportion of variability explained by regression equation. Low R^2 values often indicate that laboratory data from reference method are imprecise. If laboratory repeatability error from reference method is one-fourth the standard deviation, select equation with $R^2 \geq 0.75$.

Examine F-statistic of regression coefficients. High F-values indicate that associated coefficient is significantly different from zero. Small F-values indicate that coefficient contributes little to equation except to fit random errors. Reject equations with F-values less than 10. Examine wavelengths from equation selection process. For moisture calibration, one- to three-term equations are sufficient as long as wavelengths are in the proximity of known absorption peaks for moisture (1430 and 1930 nm).

(c) *Validation.*—Conduct NIRS analysis (program PRE) with equations selected from calibration statistics on a population of unknown samples. Examine NIRS data for samples with larger H values. A large H-value (greater than 3.0) for a few samples indicates that their NIR spectra are different from the spectra of the calibration population. A large t-value (2.5 times SED) for a few samples would indicate that laboratory values from reference method did not represent samples at the time the scan was taken. Many validation samples with large t- and H-values indicate that overfitting has occurred and that equation is specific to those samples in calibration set.

Next, use validation statistics from program STAT (1) to examine standard error of analysis (SEA) of reference method data by NIRS. SEA is a true indication of the performance of the equation on unknown samples. Select an equation with lowest bias and SEA. Unlike SEC, which must decrease with each additional term, SEA only decreases until overfitting becomes important and forces it to increase. Final equation for routine NIRS analysis is based on both calibration and validation statistics.

Collaborative Study

Twenty samples of bermudagrass (*Cynodon dactylon* (L.) Pers.) cultivars were sent to 5 collaborating laboratories including the Associate Referee's laboratory. The laboratories participating in the study were all members of the USDA/ARS National NIRS Forage Research Project Network. The samples consisted of different bermudagrass cultivars, harvest dates, and stages of regrowth. One-half of the samples were preserved by freeze drying and the remaining were sun-cured hay. Samples ranged in moisture content from 2.1 to 16.2%. Bulk quantities of each sample were ground in a cyclone mill, mixed until completely homogeneous, and subdivided into 5 pairs in the Associate Referee's laboratory. Samples were subsequently packed in 6 oz Whirl-Pak bags and heat sealed in Poly-Kraft bags to ensure the moisture concentration did not change during the collaborative study. All collaborators used compatible equipment as described in the apparatus section of the NIRS method. Collaborators received a floppy diskette which contained NIRS moisture calibration equations developed in the Associate Referee's laboratory from Karl Fischer (KF) and air oven (AO) (AOAC **7.007**) moisture methods, respectively; the test samples with KF moisture data; as well as specific instructions on sample handling. Collaborators were instructed to open one Poly-Kraft bag at a time, pack the sample in the NIRS sample holder, reseal the bag, conduct NIRS moisture determination, and then conduct moisture analysis by the AO method on the same test portion used for NIRS analysis. To obtain some measure of the homogeneity and stability of the samples, collaborative sample sets were analyzed using NIRS KF and AO calibrations developed in the Associate Referee's laboratory prior to and after the collaborative study.

The forages used for NIRS moisture calibration by the AO method were 48 bermudagrass samples described by Windham et al. (3). The samples were preserved by air oven drying at 55°C for 24 h and ranged in moisture content from 1.39 to 18.67%. The samples used for NIRS moisture calibration by the KF method were the previously described bermudagrass samples plus 10 sun-cured bermudagrass samples and 3 samples each of freeze-dried temperate grasses, legumes, silages, and silage-based dairy cattle rations, respectively. Analysts are referred to Windham et al. (3) for complete description of the additional samples. NIRS, KF, and AO analyses were conducted concurrently on 6 samples per day to ensure that the moisture data were not influenced by changes in laboratory environment.

The NIRS instrument was separately calibrated with respect to both moisture reference methods in the Associate Referee's laboratory, in each case by using the procedure described in the NIRS method. The 20 collaborative samples were then used as a "validation" set for testing the calibration equations within moisture reference methods. Comparison criteria for choosing the best NIRS moisture equations for distribution to collaborators were the moisture data from the reference method compared to the NIRS moisture data using R^2, SEA, and average deviation of the NIRS moisture data from the reference method data (bias) in the validation set.

The NIRS moisture calibration equations from the 2 reference methods were transferred from the referee's "master"

Table 1. NIRS moisture calibration statistics at Associate Referee's laboratory

Method	N[a]	Mean,[b] %	SD[b]	Math treatment[c]	Wavelength, nm	SEC,[d] %	R^2[e]
Air oven	48	6.63	4.52	1	1858, 1978, 1938	0.27	0.99
Air oven	70	7.32	4.10	2	2008, 1848	1.34	0.89
Karl Fischer	70	6.82	3.92	2	2008, 1848	0.38	0.99

[a] Number of samples in calibration set.
[b] Mean and standard deviation of calibration sets.
[c] Math treatment = 1, 30, 10, 2, where 1 = first derivative, 30 = wavelength gap in nm for computing first derivative, 10 = smoothing interval in nm for first smooth, 2 = smoothing interval in nm for second smooth. Math treatment for KF calibration = 2, 20, 20, 2.
[d] Standard error of calibration.
[e] Fraction of explained variance.

instrument to each collaborator's "slave" instrument, with the aid of the program TRANSFER, in the following manner:

(*1*) Spectra for sealed transfer samples were collected for the master and slave instrument.

(*2*) Spectral data of the 2 transfer files were transformed using the math treatment associated with the equation term. Correlations were computed between the data of the master transfer spectra and the slave transfer spectra. The wavelength in the slave file with the highest correlation was selected. Regression at the selected wavelength was used to adjust the equation coefficient and update the equation intercept.

(*3*) The slave equation, consisting of the highest correlated wavelengths, adjusted coefficients, and intercept, was written in a file for later use. Detailed description of the transfer program and procedure are given elsewhere (1, 21).

Results and Discussion

The NIRS moisture calibration statistics for the equations that were transferred to the collaborating laboratories are shown in Table 1. At most, 2 to 3 wavelengths were adequate to provide acceptable equations for the analysis of moisture. The SEC values were similar and comparable to those reported for NIRS moisture calibration for sunflower seeds (8) and forages (3). Calibration by means of AO data, using all 70 samples, resulted in high SEC and lower R^2 values. This lack of fit indicated that the moisture content of the additional 22 samples from the AO reference method were imprecise. The mean moisture content of the additional 22 samples from the KF method was 7.14% ± 2.17 vs 8.06% ± 2.99 from the AO method. The difference in the mean deviation between the 2 methods was probably due to loss of volatiles and/or loss of carbon-containing substances due to chemical reactions at 135°C. In addition, the higher standard deviation from the AO method indicates that these losses or reactions were different in nature or extent among the forage types.

The comparisons between methods of moisture analysis of the collaborative samples are shown in Table 2. Analysis of variance (22) with a model consisting of method of analysis, method of preservation, and method of analysis × method of preservation interaction gave a significant ($P <$ 0.05) interaction term. This interaction was due to the higher ($P < 0.05$) moisture values obtained with the AO method for the freeze-dried samples. However, no differences were found between the methods for the sun-cured hay. These data are in agreement with Windham et al. (3), who reported no difference between these methods for samples that were sun-cured or oven-dried.

Table 2. Comparison between methods of moisture analysis (%) in Associate Referee's laboratory

Method of analysis	Temp., °C	Time, h	Method of preservation: Sun-cured	Freeze-dried
Air oven	135	2	9.60[a]	8.70[a]
Karl Fischer	—	—	9.62[a]	8.05[b]

[a,b] Column means within method of preservation with unlike superscripts differ ($P < 0.05$).

Validation statistics for NIRS KF moisture calibration at each collaborative laboratory are listed in Table 3. No H-statistic outliers were found, indicating that the spectra of the collaborative samples did not differ from the spectra of the calibration samples. Examination of the differences between KF data and NIRS data (residuals) resulted in no significant t-outliers, indicating that the samples analyzed by the KF method were representative of those that were scanned and that the equation was not overfitted to those samples in the calibration set. The standard error of analysis (SEA) of KF data by NIRS KF calibration ranged from 0.25% on the master instrument in the Associate Referee's laboratory to 0.53% for laboratory B. These SEA values are acceptable and similar to those reported by Windham et al. (3) for NIRS KF moisture analysis of tropical and temperate grasses, legumes, silages, and silage-based dairy cattle rations. The variance component estimation procedure (22) gave variances of 0.2, 99.55, and 0.25% for differences in moisture data due to laboratory, sample, and laboratory × sample interaction. The high R^2 (Table 3) and variance components indicated that the KF and NIRS data were ranked similarly within and between laboratories.

In addition to the analysis of the collaborative samples with the NIRS KF calibration, samples were concurrently analyzed with the NIRS AO calibrations. After NIRS analysis, analysts were instructed to determine the moisture content of the same material scanned by the AOAC AO method. Validation statistics for the NIRS AO calibration using AOAC

Table 3. Validation of collaborating laboratory's NIRS KF moisture calibration with KF moisture data from Associate Referee's laboratory

Lab.	N[a]	Mean,[b] % KF	Mean,[b] % NIRS KF	SD[b] KF	SD[b] NIRS KF	Bias,[c] %	SEA,[d] %	R^2
A[e]	20	8.77	8.67	4.24	4.11	−0.10	0.25	0.99
B	20	8.77	8.60	4.24	3.80	−0.18	0.53	0.99
C	20	8.77	8.84	4.24	3.99	0.07	0.45	0.99
D	20	8.77	8.97	4.24	4.15	0.20	0.26	0.99
E	20	8.77	8.57	4.24	3.87	−0.20	0.48	0.99

[a] Number of collaborative samples.
[b] Mean and standard deviation of moisture analysis by Karl Fischer (KF) and NIRS Karl Fischer (NIRS KF) calibration.
[c] NIRS KF moisture minus KF moisture.
[d] Standard error of analysis of KF data by NIRS KF.
[e] Referee's laboratory.

Table 4. Validation of NIRS AO moisture calibration with AO moisture analysis at each collaborating laboratory

		Mean,[b] %		SD[b]				
Lab.	N[a]	AO	NIRS AO	AO	NIRS AO	Bias,[c] %	SEA,[d] %	R^2
A	20	9.16	8.56	4.69	4.12	−0.60	0.74	0.98
B	20	8.71	8.26	4.30	3.57	−0.44	0.99	0.97
C	20	9.01	8.60	4.29	3.47	−0.41	1.15	0.96
D	20	8.84	8.67	4.31	3.89	−0.18	0.79	0.97
E	20	9.77	8.41	5.18	3.53	−1.36	1.88	0.95

[a] Number of collaborative samples.
[b] Mean and standard deviation of moisture analysis by air oven (AO) and NIRS air oven (NIRS AO) calibration.
[c] NIRS AO moisture minus AO moisture.
[d] Standard error of analysis of AO data by NIRS AO equation.

AO data from each collaborating laboratory are listed in Table 4. Validation statistics for all laboratories are unacceptable. Examination of residuals showed numerous significant t-outliers from the samples that had been preserved by freeze drying. The mean moisture content across laboratories for the freeze-dried samples was 8.75% vs 9.38% for the sun-cured samples. These data are in agreement with the comparison between methods of moisture analysis conducted in the Associate Referee's laboratory (Table 2). Therefore, the negative biases and high SEA values were due to the loss of volatile substances as well as water at 135°C with the freeze-dried samples. Validation of the NIRS AO data with KF moisture values yielded statistics similar to that reported in Table 3. The success of the NIRS AO calibration when validated with a reference method that defines the moisture concentration is due to the fact that the air oven-preserved tropical forages used in calibration were not subject to volatile loss at 135°C. Therefore, users of this technology can develop accurate moisture calibrations with the AO method, but they must have some knowledge of the content of the calibration samples and whether these samples are subject to volatile loss during drying.

The standard errors of a difference (SED) for moisture analysis between collaborating laboratories are shown in Table 5. To obtain some measure of homogeneity between collaborative sample sets, samples were analyzed by NIRS KF

Table 5. Standard error of a difference associated with moisture analysis between collaborating laboratories

Methods of analysis and effects	SED,[a] %	SED(C),[b] %	Coefficient of variance, %
NIRS KF			
Between collaborative sample sets[c]	0.16	0.15	0.17
Between collaborating labs	0.27	0.23	0.24
Between collaborative sample sets[d]	0.23	0.23	0.25
NIRS AO			
Between collaborating labs	0.39	0.35	0.39
Air oven			
Between collaborating labs	0.63	0.57	0.62
NIRS KF vs NIRS AO			
Between collaborating labs	0.42	0.38	0.32

[a] Standard error of a difference due to random and systematic errors.
[b] Standard error of a difference corrected for systematic error.
[c] SED between collaborative sample sets at referee's laboratory prior to collaborative study.
[d] SED between collaborative sample sets at referee's laboratory after collaborative study.

Table 6. Types of forages and methods of preservation analyzed for moisture by KF, AO, and NIRS-KF

Type of forage	Number of samples	Method of preservation[a]
Bermudagrass	21	AD(1) FD(5) OD(5) SC(10)
Big bluestem	1	OD
Little bluestem	1	Standing dead
Old World bluestem	9	SC
Crabgrass	1	SC
Indiangrass	2	SC
Prairie grass	2	SC
Switchgrass	1	OD
Smooth bromegrass	1	OD
Kentucky-31 tall fescue	1	SC
Orchardgrass	1	OD
Red clover leaves	1	OD
Red clover stems	1	OD
Birdsfoot trefoil leaves	2	OD
Birdsfoot trefoil stems	1	OD
Interstate-76 *Sericea lespedeza*	1	SC
Reed canarygrass	1	OD
Milk vetch leaves	1	OD
Milk vetch stems	1	OD
Alfalfa forage	28	DH(2) OD(10) Pres.(1) SC(15)
Alfalfa leaves	1	OD
Alfalfa stems	1	OD
Legumes	10	SC/MV
Hays	15	SC(14) SC/MV(1)
Haylage	15	OD(5) SC(3) SC/MV(4) MV(3)
Corn silage	15	OD(6) SC/MV(4) MV(5)
Sorghum silage	2	OD(1) AO(1)
Wheat silage	1	OD
Barley forage	1	OD
Wheat forage	7	OD
Wheat straw	1	SC
Cattle feces	3	OD

[a] AO = air oven-dried; DH = dehydrated; FD = freeze-dried; OD = oven-dried; Pres. = preservative; SC = sun-cured; MV = microwave oven-dried.

in the Associate Referee's laboratory prior to and after the collaborative study. The SED calculation between sample sets prior to the study was 0.16%. Similarities between SED and standard error of a difference corrected for systematic error (SED(C)) show that no one sample set was higher or lower in moisture content from the rest. These data indicate that the samples were completely homogeneous and were acceptable to measure the precision attainable between laboratories. The SED values between collaborative laboratories from NIRS analyses based on KF and AO calibrations were 0.27 and 0.39%, respectively. In addition, SED between moisture analyses from NIRS KF and AO calibrations pooled across laboratories was 0.42%. These data are in agreement with Grattan (23, 24) who report SED values between laboratories of 0.30 and 0.43, respectively, for the AO **7.007**. There was an increase in SED and SED(C) values when NIRS KF analysis was conducted at each collaborative laboratory vs analyses prior to the collaborative study. The increase in random and systematic errors was due to a nonsignificant change in moisture content of some samples during packing of NIRS sample holders at each collaborating laboratory and the calibration transfer process. When the sample sets were subjected to NIRS KF analysis in the Associate Referee's laboratory after the collaborative study, SED was lower and SED(C) was equal to that found between collaborative laboratories. The small decrease in SED indicates a slight systematic difference between NIRS instruments. These data are typical of collaborative results which show a larger scatter

Table 7. Comparison among methods of moisture analysis on 30 different samples from each collaborating laboratory

Lab.	Method[a]	Mean, %	SD	Bias,[b] %	SED,[c] %	R^{2d}
A	KF	6.81	0.94	1.42	2.13	0.01
	AO	8.23	1.38			
B	KF	6.44	1.16	1.09	1.50	0.47
	AO	7.53	1.41			
C	KF	6.35	1.57	−0.21	0.71	0.86
	AO	6.14	1.83			
D	KF	8.41	0.98	0.76	1.18	0.50
	AO	9.17	1.30			
E	KF	6.82	0.91	0.21	1.28	0.21
	AO	7.06	1.05			

[a] Method of moisture analysis: KF = Karl Fischer; AO = air oven at 135°C for 2 h.
[b] AO mean minus KF mean.
[c] Standard error of difference between methods of moisture analysis.
[d] Fraction of explained variance.

in the data between laboratories than within laboratories. The SED values between collaborative sample sets prior to and after the study for NIRS AO analyses gave the same trends as NIRS KF analyses.

The SED between laboratories for the AOAC AO method was 0.63%. This increase in SED compared to that for NIRS is due to the random and systematic errors associated with gravimetric techniques. The SED of duplicate determinations pooled across laboratories was 0.34%. The SEDs of duplicate determinations within a laboratory were 0.11, 0.66, 0.38, 0.12, and 0.11% for laboratories A, B, C, D, and E, respectively. Therefore, the large SED of duplicate determination pooled across laboratories was due to laboratories B and C. These data clearly demonstrate that NIRS can reduce the errors of analysis between laboratories and in some cases can be lower than the errors associated with duplicate analysis within one laboratory.

To further evaluate the reference moisture methods and NIRS KF and AO calibrations, each collaborating laboratory was asked to select 30 samples that were representative of the plant material routinely analyzed by NIRS at their location. Samples were cyclone-ground and analyzed by NIRS KF and AO calibrations at each laboratory. The remaining subsample was sealed in the Poly-Kraft Bags and sent to the Associate Referee's laboratory for KF and AO analyses. Both reference methods were conducted in our laboratory to eliminate change in moisture content due to difference in relative laboratory humidity and excessive sample handlings. The types of forages analyzed for moisture by KF, AO, and NIRS KF and AO calibrations are shown in Table 6. The samples included tropical and temperate grasses, legumes, silage forage crops, plant parts, and cattle feces. There were 8 different methods of preservation in the samples, varying from harvested standing dead to microwave drying.

The comparison between KF and AO moisture analysis on the samples from each laboratory are given in Table 7. An analysis of variance (22) with a model consisting of sample, method of analysis, duplicate determination, and a sample × method of analysis interaction gave a significant ($P < 0.05$) interaction term. The mean moisture value for the KF method was 6.92% vs 7.64% for the AO method. The interaction was due to the varying magnitude of difference in moisture content of the samples between methods. Samples from laboratories A and B showed the greatest difference between methods. The majority of these samples were silages or haylages that had a mean moisture content of 6.33% by KF vs 10.11% by AO. This bias was due to a loss of volatile

Table 8. Validation by collaborating laboratories of NIRS KF moisture calibration when tested on 30 different samples from each laboratory

	Mean,[a] %		SD[a]				
Lab.	KF	NIRS KF	KF	NIRS KF	Bias,[b] %	SEA,[c] %	R^2
A	6.88	6.89	0.93	0.99	0.01	0.40	0.82
B	6.44	6.66	1.16	0.92	0.22	0.40	0.91
C	6.35	6.84	1.59	1.67	0.49	0.45	0.93
D	8.41	8.55	0.98	0.93	0.14	0.33	0.89
E	6.82	7.16	0.91	0.93	0.34	0.28	0.91

[a] Mean and standard deviation of moisture analysis by Karl Fischer (KF) in Associate Referee's laboratory and by NIRS Karl Fischer calibration (NIRS KF) at each collaborative laboratory.
[b] NIRS KF moisture minus KF moisture.
[c] Standard error of analysis of KF by NIRS KF.

acids, bases, and alcohols as well as moisture from the fermented materials, resulting in an overestimation of moisture (15). The best agreement between the methods was recorded for the samples submitted by laboratory C. The samples were primarily sun-cured hays or air oven-dried samples which were subject to little volatile loss at 135°C. Samples submitted by laboratories D and E were overestimated in moisture content by the AO method by 8.3 and 3.4%, respectively. The differences between the standard deviation from the AO method vs KF method, as well as the lack of significant correlations, indicate that the loss of volatiles and/or loss of carbon-containing substance due to chemical reactions differed in nature or extent among the samples.

Validation of NIRS KF calibration, when it was tested on the 30 different samples from each collaborating laboratory, is shown in Table 8. Moisture values from NIRS KF analyses were slightly greater than those from KF analysis, resulting in positive biases. The SEA ranged from 0.28 to 0.45%. The larger bias and SEA values for laboratory C NIRS KF analyses were due to the 1.0 percentage unit difference between methods for wheat forage samples. These SEA values are acceptable considering the diversity of the samples submitted by each laboratory. In general, NIRS calibration equations developed for most chemical components from finite populations have limited predictive value beyond those populations. The problem is not the NIRS data, but is due to chemical reactions in the primary reference method differing in nature or extent among the species in infinite and diverse populations. This problem has been clearly pointed out with the AO method in this paper. The success of the NIRS KF calibration is due to the specificity of the KF analysis for moisture.

Analysis of the 30 different samples from each laboratory with the NIRS AO calibration and validation with KF data gave biases of 0.01, 0.40, 0.32, 0.12, and 0.28 with SEA data of 0.47, 0.40, 0.52, 0.35, and 0.48% for laboratories A, B, C, D, and E, respectively. These differences compare favorably with the SED for between NIRS moisture calibrations from the 20 collaborative samples (Table 5). The success of the NIRS AO calibration is due to the fact that the air oven-preserved tropical forages used for calibration were not subject to volatile loss at 135°C. The ability of the NIRS KF and AO calibration to analyze accurately the moisture content of an infinite population is due to the very strong absorbance of water at specific wavelengths in the near infrared region, regardless of the sample type or composition.

The successful performance of moisture analysis by NIRS is limited by (*1*) selection of calibration samples that are representative of the population to be predicted; (*2*) the ac-

curacy of the primary moisture reference method; (*3*) choice of the correct data processing technique and wavelengths to extract the maximum information from the NIR spectra; and (*4*) selection of the appropriate calibration equation for validation. The next step in this work is a full collaborative study whereby all participating laboratories will be asked to perform the AOAC official method **7.007** and apply the calibration method independently. The third step is a full collaborative study to validate the Karl Fischer titration method. Meanwhile, air oven drying at 135°C for 2 h can be recommended for NIRS calibration for moisture analysis of forage samples. However, the analyst must have some knowledge of the composition of the calibration samples and whether these samples are subject to volatile loss during drying.

Acknowledgments

The first author thanks all who participated in this study as follows:

D. H. Clark, USDA-ARS, Utah State University, Logan, UT

S. W. Coleman, USDA-ARS, Livestock and Forage Research Laboratory, El Reno, OK

G. C. Marten and J. L. Halgerson, USDA-ARS, University of Minnesota, St. Paul, MN

J. S. Shenk, M. O. Westerhaus, and M. J. Briscoe, Agronomy Department, Pennsylvania State University, University Park, PA

The authors' laboratory participated on behalf of The Plant Structure and Composition Research Unit, Richard B. Russell Agricultural Research Center, USDA-ARS, Athens, GA.

References

(1) Marten, G. C., Barton, F. E., II, & Shenk, J. S. (Eds.) (1985) *Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality*, U.S. Dep. Agric. Handb. 643, 96 pp.
(2) Windham, W. R. (1987) *Crop Sci.* **27**, 773–776
(3) Windham, W. R., Robertson, J. A., & Leffler, R. G. (1987) *Crop Sci.* **27**, 777–783
(4) Kaye, W. (1954) *Spectrochim. Acta* **6**, 257–287
(5) Hoffman, K. (1963) *Chem. Eng. Technol.* **35**, 55–60
(6) Law, D. P., & Tkachuk, R. (1977) *Cereal Chem.* **54**, 874–881
(7) Stermer, R. A., Pomeranz, Y., & McGintz, R. J. (1977) *Cereal Chem.* **54**, 345–351
(8) Robertson, J. A., & Barton, F. E., II (1984) *J. Am. Oil Chem. Soc.* **61**, 543–547
(9) Shenk, J. S., & Westerhaus, M. O. (1985) *Crop Sci.* **25**, 1120–1122
(10) Fosnot, R. H., & Haman, R. W. (1945) *Cereal Chem.* **22**, 41–49
(11) Jones, F. E., & Brinkenkamp, C. S. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 1277–1283
(12) Robertson, J. A., & Windham, W. R. (1983) *J. Am. Oil Chem. Soc.* **60**, 1773–1777
(13) Bernetti, R., Kochan, S. J., & Pienksowski, J. J. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 299–301
(14) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA
(15) Mo, M., & Tjarhom, T. (1978) *Acta Agric. Scand.* **28**, 196–202
(16) James, F. E. (1985) *J. Am. Oil Chem. Soc.* **62**, 1605
(17) Landa, I. (1979) *Rev. Sci. Instrum.* **50**, 34–40
(18) Landa, I., & Morris, K. H. (1979) *Appl. Spectrosc.* **23**, 105–107
(19) Shenk, J. S., Westerhaus, M. O., & Hoover, M. R. (1978) *Proc. Am. Soc. Agric. Eng.* p. 242
(20) Shenk, J. S., Landa, I., Hoover, M. R., & Westerhaus, M. O. (1981) *Crop Sci.* **21**, 355–358
(21) Shenk, J. S., Westerhaus, M. O., & Templeton, W. C., Jr (1985) *Crop Sci.* **25**, 159–161
(22) *SAS User's Guide: Statistics* (1985) 5th Ed., SAS Institute, Inc., Cary, NC, pp. 433–507
(23) Grattan, G. E. (1930) *J. Assoc. Off. Agric. Chem.* **13**, 173–174
(24) Grattan, G. E. (1931) *J. Assoc. Off. Agric. Chem.* **14**, 152–154

DRUG RESIDUES IN ANIMAL TISSUE

Determination of Zeranol/Zearalenone and Their Metabolites in Edible Animal Tissue by Liquid Chromatography with Electrochemical Detection and Confirmation by Gas Chromatography/Mass Spectrometry

JOSE E. ROYBAL, ROBERT K. MUNNS, WILLIAM J. MORRIS, JEFFREY A. HURLBUT, and WILBERT SHIMODA
Food and Drug Administration, Animal Drug Research Center, Denver Federal Center, PO Box 25087, Denver, CO 80225-0087

A sensitive method is described for the determination and confirmation of zeranol and zearalenone, as well as their isomers and metabolites, in edible animal tissue. The analytes are extracted from tissue with methanol, hydrolyzed enzymatically, cleaned up by acid-base partitioning, determined by liquid chromatography (LC) with electrochemical (EC) detection, and confirmed by gas chromatography/mass spectrometry (GC/MS). LC analysis is performed by isocratic elution with a buffered mobile phase using a Nova-Pak reverse-phase C_{18} column with amperometric EC detection at +0.90 V. Capillary GC/MS analysis of the trimethylsilyl derivatives provides mass spectral confirmations.

Zeranol/zearalenone and their isomers/metabolites are derivatives of benzoxacyclotetradecin, having the common chemical structure shown in Figure 1. Figure 2 shows all 6 compounds with their differing chemical configurations.

Zeranol is a nonsteroidal anabolic veterinary drug used commercially in cattle and sheep for increasing the rate of weight gain and improving feed efficiency. Metabolic studies indicate that zeranol and its metabolites, zearalanone (major metabolite) and taleranol (minor metabolite), are excreted both as free compounds and as conjugates. Zearalenone, a mycotoxin produced by *Fusarium* fungi commonly found in grain products, is the industrial precursor of zeranol. The major metabolite of zearalenone when it is ingested by animals is α- and/or β-zearalenol (1).

The use of drugs in animal feeds involves possible health risks if harmful residues remain in the meat products intended for human consumption. Many countries prohibit or restrict the use of any hormone-active substance for growth promotion and fattening in food-producing animals (2). For example, in the United States no residues of zeranol are permitted in the uncooked edible tissue of cattle and sheep as determined by the official CFR 21 method (3), which is a gas chromatographic (GC) procedure with a limit of determination of 20 ppb.

Because zearalenone may be ingested through contaminated grain by livestock that are being medicated with zeranol, the meat may become tainted with both of these compounds and their metabolites. Therefore, it is important to be able to separate, identify, and quantitate these substances.

Baldwin et al. (4), in their review, state that several methods are available for the determination of zeranol in tissue. The techniques used in these methods include thin-layer chromatography, liquid chromatography (LC), the use of the estrogen receptor binding test, gas chromatography/mass spectrometry (GC/MS), and radioimmunoassay (RIA). The most promising of these methods is RIA with a limit of determination of 0.1 ppb in plasma. A variety of methods available for the determination of zearalenone include the following techniques: liquid chromatography with either UV or fluorescence detection (1, 5–9); thin-layer chromatography (10), and GC/MS (11–13). Two RIA methods have been reported for zearalenone (13, 14), with one also capable of determining zeranol (14). Frischkorn et al. (15) described an LC system with voltammetric detection of several growth-promoting hormones including zeranol and zearalenone. Although many methods are available for the determination of either zeranol or zearalenone, or in some cases both compounds, none have been reported for the separation of all 6 compounds (parents, metabolites, and diastereomers) in combination.

Liquid chromatography with electrochemical (EC) detection was chosen for the determinative step in the method reported here because there is no requirement for derivatization. Additionally, low limits of determination are obtained with minimal sample preparation. Electrochemical detection is a sensitive and selective technique that has been increasing in popularity for use in determinations of both inorganic and organic species. It has been successfully applied to growth-promoting and estrogenic hormones in biological fluids (15–17).

The method presented in this paper permits the identification and quantitation of 4 of the zeranol/zearalenone compounds by LC separation with EC detection. Subsequently, all 6 compounds can be separated and confirmed as trimethylsilyl (TMS) derivatives by a capillary GC/MS analysis.

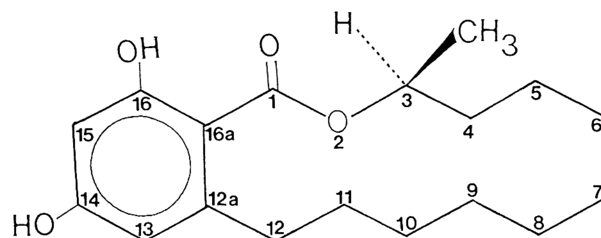
METHOD

Apparatus

(a) *Pasteur pipets.*—Disposable, 5.75 in.

(b) *Centrifuge tube.*—15 mL, graduated, ₮ g/s 13 (Kimble 45153-A).

(c) *Water bath.*—Constant temperature, 37 ± 2°C (Precision Scientific Co., Chicago, IL 60647), or equivalent.



(3S)- 3,4,5,6,7,8,9,10,11,12-decahydro-14,16-dihydroxy-3-methyl- 1H-2-Benzoxacyclotetradecin-1-one

Figure 1. Common chemical structure of zeranol/zearalenone and metabolites.

Received March 3, 1987. Accepted August 2, 1987.

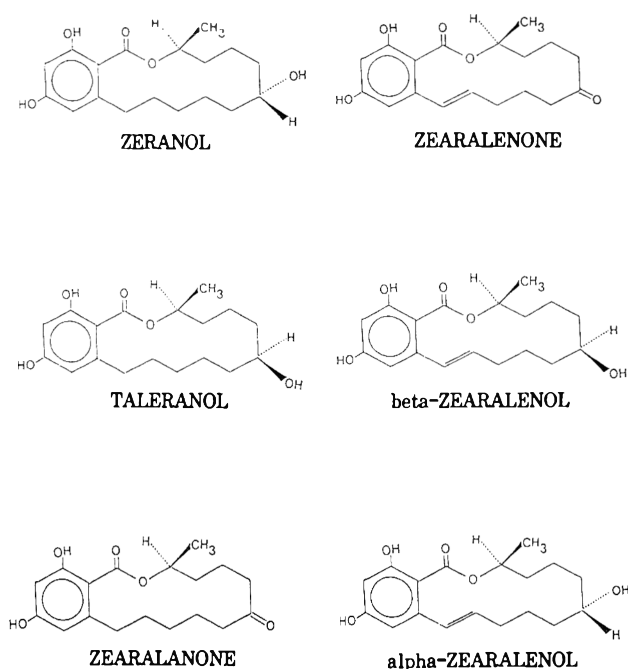


Figure 2. Chemical configurations of compounds of interest.

(d) *Homogenizer.*—High-speed, with rheostat, Tekmar Model 3412-A20 (Arthur H. Thomas, Philadelphia, PA 19105), or equivalent.

(e) *Rotary evaporator.*—Buchi/Brinkmann Rotavapor-R vacuum evaporator with water bath at 40°C, or equivalent system.

(f) *Filter paper.*—Glass microfibre, 11.0 cm, Whatman 934-AH (Whatman Ltd, Maidstone, England), or equivalent.

(g) *Boiling flasks.*—100 mL, pear-shape, acetylation, short-neck, ₮ g/s 24/40 (Kontes, Cat. No. K-608700).

(h) *Liquid chromatograph and electrochemical detector.*—*LC pumping system.*—Waters dual pump liquid chromatograph (Models 590 and 510) with Model 680 automatic gradient controller and Model U6K universal liquid chromatograph injector (Waters Associates, Milford, MA 01757). *Detector.*—Model LC4B-17D dual electrode detector (single mode used throughout method) (Bioanalytical System, Inc., Purdue Industrial Research Park, West Lafayette, IN 47906) with glassy carbon electrodes, Ag/AgCl reference; potential, +0.90 V; current, 2.0 nA FSD. Operating conditions: chart speed, 0.25 cm/min; mobile phase flow, 1.0 mL/min; column temperature, ambient; column pressure, 2500 psi; volume injected, 25 μL.

(i) *LC column.*—3.9 × 150 mm, 5 μm, RP-C_{18} Nova-Pak (Waters Associates, P/N 086344).

(j) *Recorder.*—Dual channel SE-120 strip chart recorder set at 10 mV (BBC-Metrawatt/Goerz, Broomfield, CO 80020).

(k) *Food chopper.*—Hobart, consisting of No. 12 Brite-metal chopping end (119-860-3), No. 12 stay-sharp blade (290-339), 0.25 in. stay-sharp plates (16425-2), and No. 12 stainless steel feed pan (120903) with plastic feed stomper (A-119922-1) (Hobart Corp., Denver, CO).

(l) *Mass spectrometers.*—Hewlett-Packard Model 5970B with HP-5890 gas chromatograph equipped with split/splitless capillary injector and Hewlett-Packard Model 301 workstation (Hewlett-Packard, Englewood, CO); Finnigan MAT 311A with Varian Model 2700 gas chromatograph and INCOS data system (Finnigan Corp., San Jose, CA 95134).

(m) *GC columns.*—DB-1 fused silica capillary, 20 m × 0.25 mm id (J & W Scientific, Rancho Cordova, CA 95670); DB-5 fused silica capillary, 20 m × 0.25 mm id (J & W Scientific).

(n) *Vials.*—0.3 mL mini-vials with septum screw caps (Applied Science Laboratories Inc., State College, PA, Cat. No. 13050).

Reagents

(a) *Solvents.*—Distilled-in-glass, pesticide-grade methanol and chloroform (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442). *Caution:* Chloroform is a suspected carcinogen; use only in hood or well ventilated area.

(b) *Water.*—Glass-distilled, deionized.

(c) *Sodium hydroxide.*—ACS grade; use to prepare 2N solution.

(d) *Sodium bicarbonate.*—ACS grade; use to prepare 1M solution.

(e) *Acetic acid.*—ACS grade, glacial, aldehyde-free.

(f) *Sodium acetate.*—ACS grade, anhydrous.

(g) *Sodium sulfate.*—ACS grade, anhydrous, granular.

(h) *β-Glucuronidase.*—Type B-1, 500 000 units/g (Sigma Chemical Co., St. Louis, MO 63178); use to prepare aqueous solution containing 1 mg/mL; prepare just before use.

(i) *Sodium acetate reagent.*—Prepare by adjusting 0.09M sodium acetate to pH 6.9 with acetic acid and adding ethylenediaminetetraacetic acid to obtain concentration of 10 mg/L sodium acetate reagent. Use to prepare mobile phase **(j)**.

(j) *Mobile phase.*—Methanol–sodium acetate reagent (1 + 1).

(k) *Reference standards.*—Zearalenone (available from Sigma Chemical Co.); zeranol, taleranol, zearalanone, and α- and β-zearalenol (International Minerals and Chemical Corp., Terre Haute, IN 47808).

(l) *Standard solutions.*—*Stock solutions.*—100 μg/mL. Accurately weigh 10.0 mg of each reference standard into separate 100 mL volumetric flasks, dilute to volume with methanol, and mix. *Working solutions.*—1.0 μg/mL. Pipet 1.0 mL of each stock solution into separate 100 mL volumetric flasks, dilute to volume with methanol, and mix. *Mixed standard working solution.*—1.0 μg/mL. Pipet 1.0 mL of each stock solution into common 100 mL volumetric flask, dilute to volume with methanol, and mix.

(m) *N,O-Bis(trimethylsilyl) acetamide (BSA).*—Regis Chemical Co., Morton Grove, IL 60053.

Sample Preparation

Chicken muscle (breasts).—Skin, filet, and freeze. Chop frozen muscle into cubes and grind with dry ice in Waring Blendor. Weigh 80–100 g homogenized tissue into Whirl-Pak bag. Seal bag and keep tissue frozen until analysis.

Beef muscle.—Grind muscle in food chopper by passing through 0.25 in. plate twice. Weigh 80–100 g ground muscle into Whirl-Pak bag. Seal bag and keep tissue frozen until analysis.

Beef liver.—Homogenize liver in Waring Blendor. Weigh 80–100 g homogenate into Whirl-Pak bag. Seal bag and keep tissue frozen until analysis.

Extraction Procedure

Weigh 80 g ground tissue into 500 mL centrifuge bottle and add 160 mL methanol. Thoroughly homogenize at medium-high speed for two 1 min intervals. Centrifuge 10 min at 1500 rpm. Decant supernate into 250 mL graduated cylinder and record extraction volume. Transfer extract to 250 mL flat-bottom flask with ₮ 24/40 joint. Rinse cylinder with two 10 mL portions of methanol and add rinsings to flask.

Evaporate to ca 25 mL on N-Evap, using stream of nitrogen to remove methanol.

Hydrolysis and Isolation

Adjust pH to 5.0 ± 0.2 with 20% acetic acid solution. Add 4 mL freshly prepared enzyme solution to mixture and then add 5 mL chloroform; mix by swirling. Cover flask with aluminum foil and incubate contents overnight (12–16 h) at 37 ± 2°C. Let mixture cool. Add 10 mL chloroform to flask and mix by swirling. Transfer mixture to 250 mL separatory funnel. Wash flask with 35 mL chloroform and add rinsings to 250 mL separatory funnel. Shake funnel moderately for 10 s; let phases separate and pass chloroform layer through 30–35 g anhydrous sodium sulfate (prewashed with ca 30 mL chloroform) into 125 mL separatory funnel.

Wash flask with additional 50 mL chloroform. Add washings to original 250 mL separatory funnel. Shake funnel vigorously for 30 s. Let phases separate (ca 15 min). Drain chloroform layer through sodium sulfate into 125 mL separatory funnel. Rinse sodium sulfate with two 5 mL portions of chloroform; combine all extracts and washes into 125 mL separatory funnel.

Cleanup

Add 10 mL 2N NaOH to 125 mL separatory funnel and stopper. Shake funnel vigorously for 30 s. Let phases separate (ca 15 min). Drain and discard lower (chloroform) layer, retaining emulsion. Wash aqueous layer with two 50 mL portions of chloroform. Drain and discard lower (chloroform) layer, retaining emulsion.

Buffer NaOH solution to pH 10.6–10.8 with 1M $NaHCO_3$ (ca 19–20 mL). Extract analytes from buffered solution with three 25 mL portions of chloroform. Drain each chloroform layer through sodium sulfate bed (30–35 g, prewashed with chloroform) into 100 mL pear-shape flask. Wash sodium sulfate bed with 10 mL chloroform and collect washings in 100 mL pear-shape flask. Evaporate contents of flask to dryness on rotary evaporator at 40°C.

Liquid Chromatography

Dissolve residue in 2.0 mL methanol. For EC detection, inject 25 μL sample extract followed by appropriate working standard solution (individual or mixed) into liquid chromatograph. Bracket injections of sample extract with those of standard solutions.

Calculations

Calculate concentration of each analyte in sample as follows:

$$\text{Analyte, ppm} = (A/B) \times (C/W) \times F \times (D/E)$$

where A = peak height or peak area of sample extract, B = average peak height or peak area of standard solution, C = concentration (μg/mL) of standard solution, F = final volume (mL) of sample extract, D = average volume (μL) of standard solution injected, E = volume (μL) of sample extract injected, and W = weight (g) of sample represented in final volume as determined by the following equation:

$$W = (W_1 \times V_2)/[V_1 + (W_1 \times R)]$$

where W_1 = initial sample weight (g), V_1 = initial extraction volume (mL), V_2 = recorded extraction volume (mL), and R = ratio of water/tissue = (% moisture ÷ 100)/tissue = mL/g, where % moisture is from tables found in *Composition of Foods* (18) as follows:

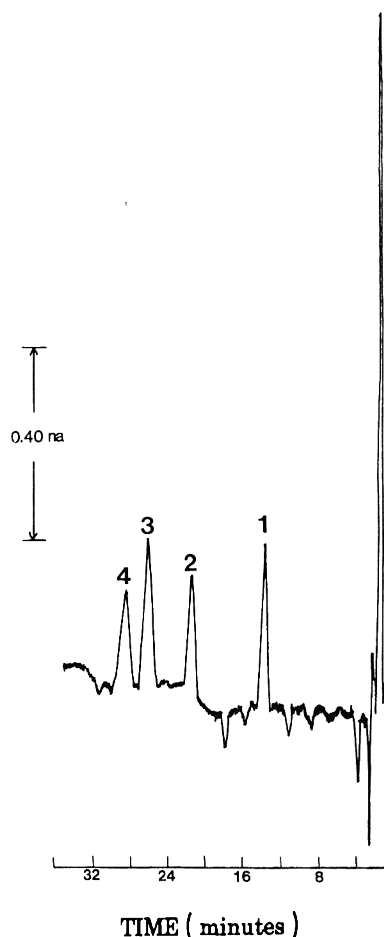


Figure 3. LC separation of mixed standards, 25 μL injected. Peaks: 1, β-zearalenol, 0.101 μg/mL, 2.5 ng; 2, zeranol, 0.109 μg/mL, 2.7 ng; 3, zearalanone, 0.136 μg/mL, 3.4 ng; 4, zearalenone, 0.101 μg/mL, 2.5 ng.

(a) *Chicken muscle.*—All classes, light meat without skin, raw: 73.7% water (ref. 18, p. 23).

(b) *Beef muscle.*—Hamburger (ground beef). (*1*) Lean, raw: 68.3% water (ref. 18, p. 15). (*2*) Regular, raw: 60.2% water (ref. 18, p. 15).

(c) *Beef liver*—Raw: 69.7% water (ref. 18, p. 37).

Derivatization (for GC/MS Determination)

Transfer 1 mL final extract solution in 0.25 mL increments to 0.3 mL mini-vial and evaporate to dryness under gentle stream of nitrogen. After evaporating final portion of sample extract, rinse sample extract container with 0.5 mL methanol. Transfer rinsings to mini-vial and evaporate to dryness, then add 50 μL hexane and 50 μL BSA to mini-vial. Tighten septum screw cap and gently shake container for 2–3 min. Inject 3–4 μL derivatized extract into gas chromatograph/mass spectrometer, using splitless capillary injector system. A 1 ppb residue results in approximately 2 ng being injected on column.

Results and Discussion

Standard plots for zeranol and zearalenone were linear in the ranges 5–500 ng and 0.5–15 ng, respectively. Detection limits for the 2 parent compounds were determined to be 100 pg for zeranol and 200 pg for zearalenone.

Initially, spiked recoveries were performed on commercially purchased chicken breasts (muscle), ground beef (muscle), and beef liver. The overall recoveries of zeranol and zearalenone from chicken muscle spiked in the 1–10 ppb

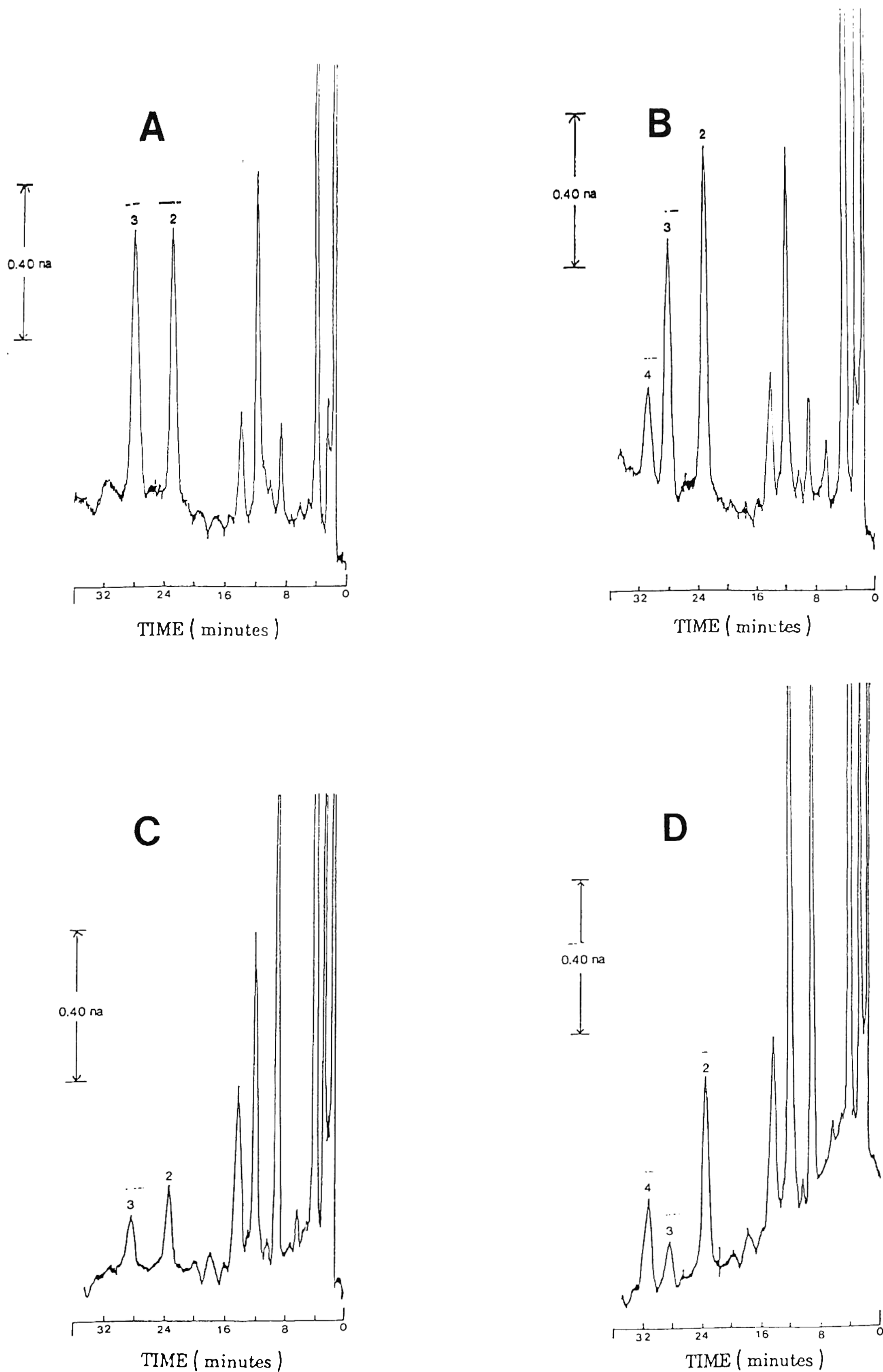


Figure 4. LC chromatograms from analyses of beef liver tissue containing incurred residues (Table 2). A, beef liver tissue sample 1, determination 4, equivalent to 21.5 g tissue/mL final solvent, 25 μL injected, peak 2 = 16.1 ppb incurred zeranol, peak 3 = 16.7 ppb incurred zearalanone; B, beef liver tissue sample 1S, equivalent to 21.5 g tissue/mL final solvent, 25 μL injected, peak 2 = zeranol, peak 3 = zearalanone, peak 4 = zearalenone; C, beef liver tissue sample 2, determination 4, equivalent to 23.4 g tissue/mL final solvent, 25 μL injected, peak 2 = 4.2 ppb incurred zeranol, peak 3 = 3.4 ppb incurred zearalanone; D, beef liver tissue sample 2S, equivalent to 23.4 g tissue/mL final solvent, 25 μL injected, peak 2 = zeranol, peak 3 = zearalanone, peak 4 = zearalenone.

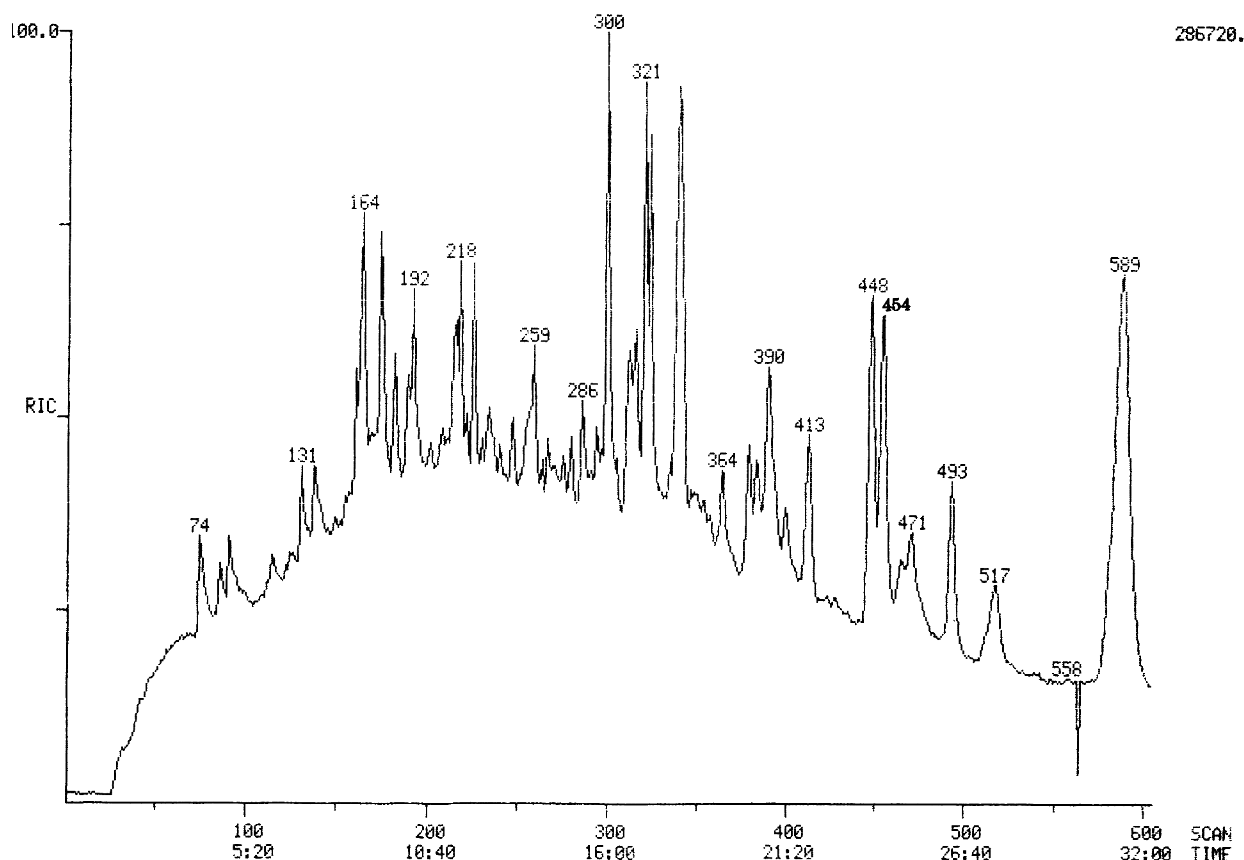


Figure 5. Total ion GC/MS chromatogram of liver sample 1 (Table 2, 1 of the 4 determinations) with incurred zeranol residues: scan 448, tri-TMS zeranol; scan 454, tri-TMS taleranol; scan 493, di-TMS zearalanone.

range are shown in Table 1. The zearalenone recovery was consistently higher than that of zeranol. Some of this variation can be attributed to interfering compounds, traced to the food chopper, with the same approximate retention time as zeranol. To correct this problem, a new food chopper (*Apparatus,* item **[k]**) was purchased and reserved for use with these samples. Recoveries from beef muscle spiked at 5–10 ppb with zeranol and zearalenone showed excellent consistency and averaged 62.1% (CV = 6.52%) and 69.6% (CV = 10.8%), respectively, for 12 determinations. Recoveries of zeranol and zearalenone from beef liver spiked at 10 ppb averaged 52.9% (CV = 11.4%) and 71.5% (CV = 3.42%), respectively, for 5 determinations. When the recoveries from the samples left overnight in base (2N NaOH) are included, not only does the average recovery value drop but the CV increases (almost doubling), indicating the effect of long exposure to base.

Beef liver tissue samples with incurred residues of zeranol were used to evaluate the proposed method. Table 2 shows the results of these analyses. Each tissue was analyzed by the proposed method in a set of 5, with 1 assigned as the spike. To each assigned spike, zeranol and zearalenone were added at 10.9 and 10.1 ppb, respectively, before analysis.

Since zearalanone was not added but was incurred, the average value reported includes the amount determined in the assigned spike sample as well. In addition, the value reported for zeranol in the tissue containing incurred residues includes the taleranol isomer, which is not resolved by LC analysis. LC/EC analytical results for all liver tissue samples found to contain incurred residues of zeranol were confirmed by GC/MS for the presence of zeranol, taleranol, and/or zearalanone.

The LC system described will resolve zearalenol, zeranol, zearalanone, and zearalenone quite well (Figure 3), but will not separate α- from β-zearalenol or zeranol from taleranol. Figure 4 shows the LC/EC chromatograms from analyses of beef liver samples with incurred zeranol residues as well as those from analyses of samples spiked with zeranol and zearalenone at 10.9 and 10.1 ppb, respectively. Since the LC/EC system cannot separate zeranol from taleranol, these 2 residues were calculated as the α-isomer, zeranol. These diastereomers are distinguished in the GC/MS determination.

Table 1. Recovery of zeranol and zearalenone from chicken muscle (breast) tissue

	Added, pbb		Av. rec., %	
No. of detns	Zeranol	Zearalenone	Zeranol	Zearalenone
15	10.1	11.8	51.4	77.5
8	5.05	5.90	71.7	75.2
8	1.01	1.18	85.0	62.7
Overall			58.4	75.1
SD			15.1	8.51
CV, %			25.9	11.3

Gas Chromatography/Mass Spectrometry

The methanol solutions (1.0 mL) containing the final tissue extracts were evaporated to dryness and derivatized with BSA. Originally, the extracts were analyzed by using a Finnigan MAT 311A mass spectrometer and a DB-5, 20 m × 0.25 mm id fused silica capillary column. Figure 5 shows the total ion chromatogram obtained from analysis of beef liver sample 1 (Table 2, determination 1), which contained average levels of 16.8 ppb incurred zeranol (zeranol + taleranol) and 16.1 ppb incurred zearalanone, as calculated from results

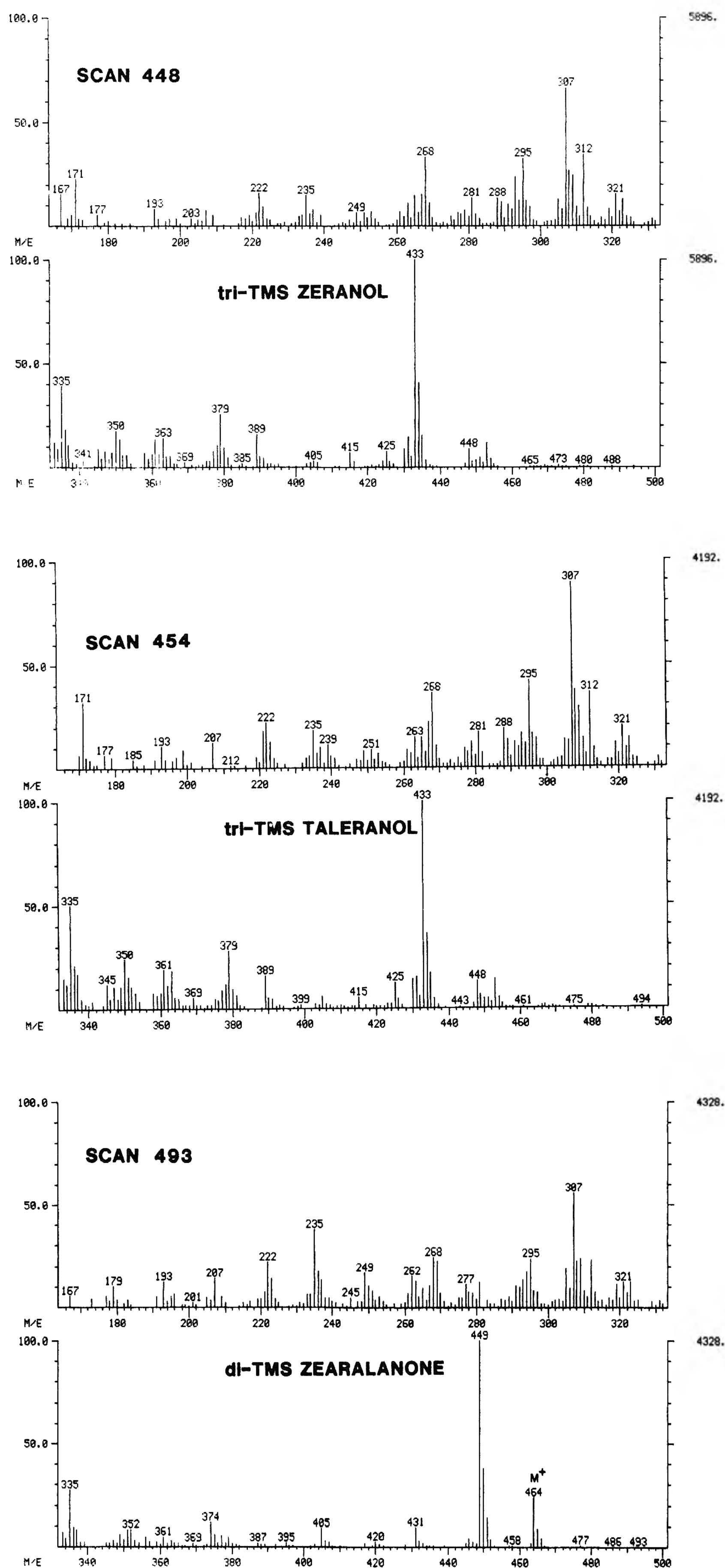


Figure 6. Mass spectra of scans 448, 454, and 493 from total ion chromatogram of liver sample 1 (Table 2, 1 of the 4 determinations).

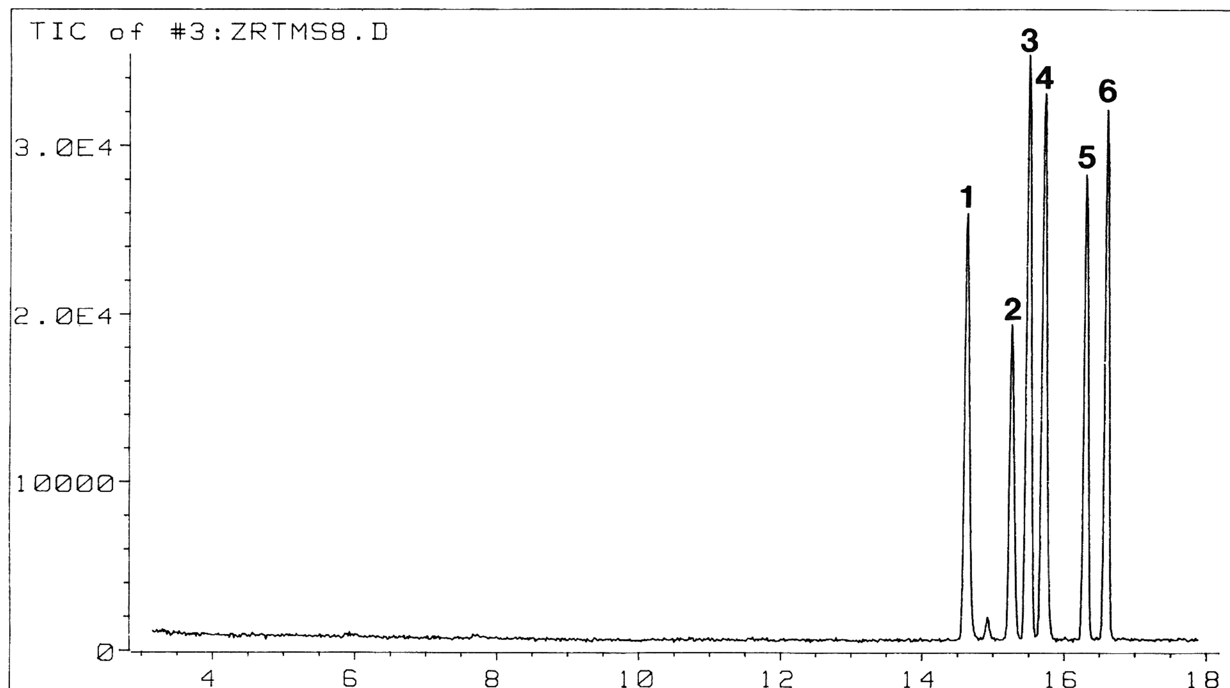


Figure 7. Total ion GC/MS chromatogram of TMS derivatives of mixed standards, obtained with Hewlett-Packard Model 5970B tabletop mass spectrometer: 1, zearalanone; 2, zearalenone; 3, zeranol; 4, taleranol; 5, alpha-zearalenol; 6, beta-zearalenol.

of LC/EC determinations. The TMS derivatives of zeranol and taleranol were not completely resolved under these chromatographic conditions (scans 448 and 454, respectively). However, the resolution was sufficient to clearly distinguish these diastereomers. Figure 6 shows the corresponding mass spectra (448, tri-TMS zeranol; 454, tri-TMS taleranol) that were recorded 6 spectra apart, with an approximate difference of 11 s in retention times. The mass range scanned was not sufficient to include the molecular ions of the TMS derivatives of these 2 diastereomers (M^+ = m/z 538). The spectra are identical and show the mass at m/z 448 [$M^+ - (OSi(CH_3)_3, H)$], which then loses CH_3 to give the most abundant ion at m/z 433. Figure 6 also shows the mass spectrum (scan 493) of di-TMS zearalanone (M^+ = m/z 464) from analysis of this tissue sample.

Table 2. Levels (> 1 ppb) of zeranol, zearalanone, and zearalenone determined in beef liver tissue

Sample	Zeranol[a] Found, ppb[b,c]	Zeranol[a] Rec., %	Zearalanone, ppb[d,e]	Zearalenone Found, ppb[b,c]	Zearalenone Rec., %
1	16.8		16.1	ND[f]	
1S	6.90	63.3	15.7	8.53	84.5
2	4.50		3.1	ND	
2S	6.80	62.4	3.0	7.24	71.7
3	2.33		1.4	ND	
3S	7.85	72.0	2.1	7.90	78.2
4	ND		ND	ND	
4S	5.54	50.8		7.09	70.2
5	ND		ND	ND	
5S	8.37	76.8		7.30	72.3
6	17.0		13.1	ND	
6S	7.8	71.6	14.0	7.59	75.1
7	14.9		12.5	ND	
7S	5.2	47.7	12.1	4.55	45.0
8	4.42		ND	ND	
8S	5.58	51.2		6.96	68.9
9	1.81		ND	ND	
9S	5.21	47.8		5.54	54.9
Overall		60.4			69.0
SD		11.4			12.0
CV, %		18.9			17.4

[a] Incurred residues include taleranol.
[b] Each value for an unlettered sample (i.e., 1–9) is the average of 4 determinations of incurred residues.
[c] Each value for a lettered sample (i.e., 1S–9S) is the amount found in the analysis of the assigned spike (10.9 ppb zeranol and 10.1 ppb zearalenone added) after subtraction of the incurred value.
[d] Each value for an unlettered sample (i.e., 1–9) is the average of 5 determinations of incurred residues.
[e] Each value for a lettered sample (i.e., 1S–9S) is the amount found in the analysis of the assigned spike (10.9 ppb zeranol and 10.1 ppb zearalenone added) without subtraction of the incurred value.
[f] ND = none detected.

The ratio of zeranol to taleranol that we found in bovine tissue extracts was approximately 60/40. The presence of taleranol in these tissue extracts is explained by the metabolism of zeranol to zearalanone and the subsequent reversal of zearalanone to both zeranol and taleranol (4).

Marked improvements (19) in the chromatographic separation of the family of zeranol-related compounds and diastereomers were obtained after our laboratory purchased a Hewlett-Packard Model 5970B tabletop mass spectrometer. Figure 7 shows the total ion chromatogram obtained from a mixture of TMS-derivatized standards which include zeranol, taleranol, zearalanone, α- and β-zearalenol, and zearalenone. The 4 diastereomers are all separated. This chromatographic separation was obtained by using a DB-1, 20 m × 0.25 mm id fused silica capillary column and a 2-stage program ramp: 80 to 150°C at 12°/min; 150 to 235°C at 8°/min.

Figure 8 shows the corresponding mass spectra that were recorded for each compound. These spectra were scanned over a mass range that clearly shows the molecular ions of the tri-TMS compounds: zeranol and taleranol (m/z 538); α- and β-zearalenol (m/z 536); as well as those of the di-TMS compounds, zearalanone (m/z 464) and zearalenone (m/z 462).

Conclusion

The combination of a highly sensitive quantitative LC screening technique that uses the specificity of EC detection

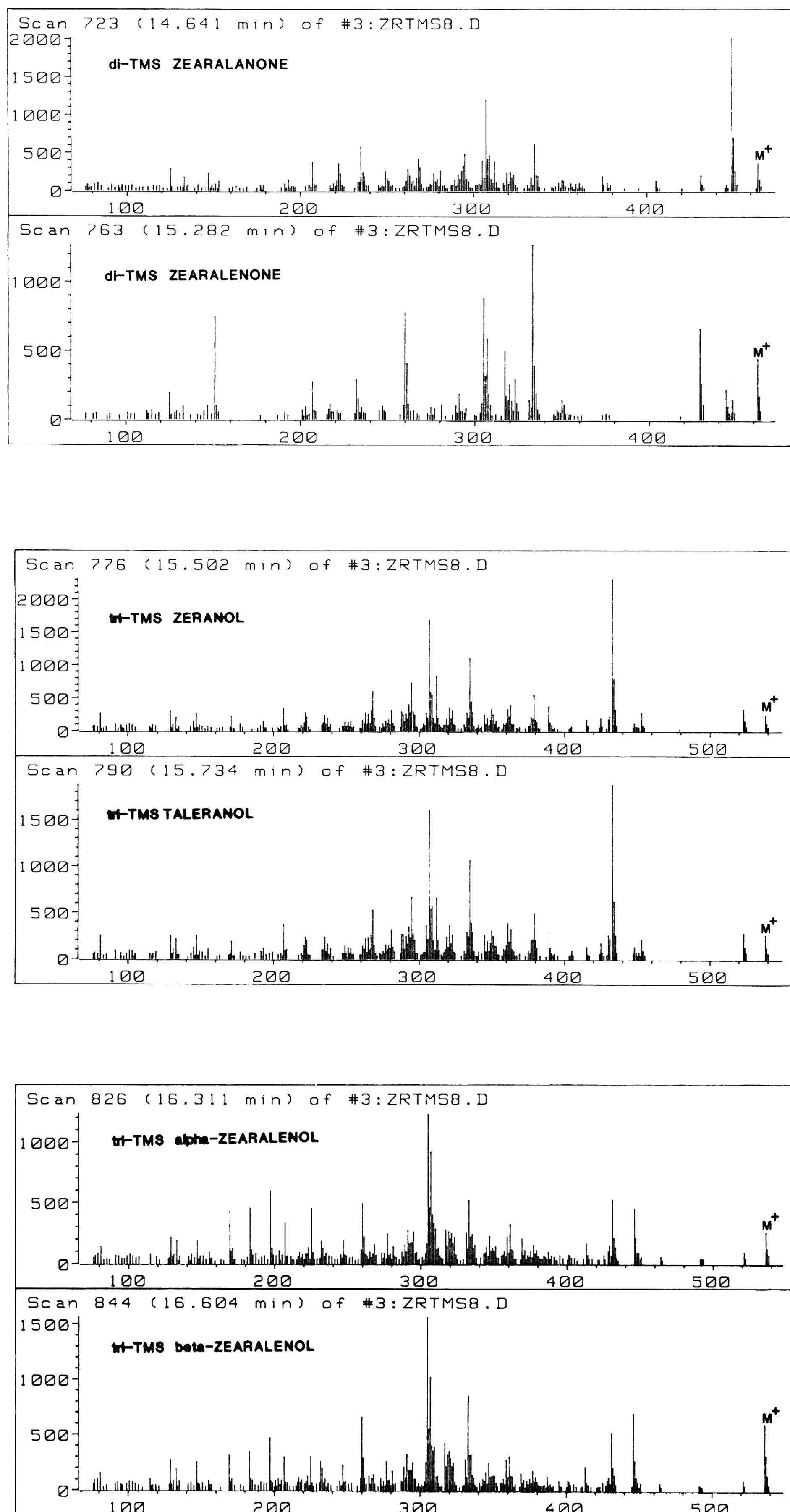


Figure 8. Mass spectra of each derivatized standard from total ion chromatogram obtained with Hewlett-Packard Model 5970B.

coupled with capillary GC/MS confirmations provides a complete and valuable method for determining all of the zeranol-related compounds at levels of ≥5 ppb in bovine and poultry tissues.

Acknowledgments

The authors express their sincere thanks to Bruce Martin and Carl J. Wassink, International Minerals and Chemical Corp., for furnishing the taleranol, α-zearalenol, and zearalanone standards; Robert M. Eppley, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Division of Food Chemistry and Technology, for supplying the zearalenone standard; and Pamela Askew, Thomas Morrison, and Henry Skinner, Food and Drug Administration, Animal Drug Research Center, for their assistance in sample analysis.

References

(1) Trenholm, H. L., Warner, R. M., & Farnworth, E. R. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 302–310
(2) Wortberg, B., & Woller, R. (1978) *J. Chromatogr.* **156**, 205–210
(3) *Code of Federal Regulations* (1986) Title 21, U.S. Government Printing Office, Washington, DC, sec. **556.760**
(4) Baldwin, R. S., Williams, R. D., & Terry, M. K. (1983) *Regul. Toxicol. Pharmacol.* **3**, 9–25
(5) Trenholm, H. L., Warner, R. M., & Fitzpatrick, D. W. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 968–972
(6) James, L. J., McGirr, L. G., & Smith, T. K. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 8–13
(7) Chang, H. L., & DeVries, J. W. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 741–744
(8) Olsen, M. E., Pettersson, H. I., Sandholm, K. A., & Kiessling, K.-H. C. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 632–635
(9) Turner, G. V., Phillips, T. D., Heidelbaugh, N. D., & Russell, L. H. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 102–104
(10) Malaiyandi, M., Barrette, J. P., & Wavrock, P. L. (1976) *J. Assoc. Off. Anal. Chem.* **59**, 959–962
(11) Stan, H. J., & Abraham, B. (1980) *J. Chromatogr.* **195**, 231–241
(12) Szathmary, Cs., Galacz, J., Vida, L., & Alexander, G. (1980) *J. Chromatogr.* **191**, 327–331
(13) Mirocha, C. J., Robison, T. S., Pawlosky, R. J., & Allen, N. K. (1982) *Toxicol. Appl. Pharmacol.* **66**, 77–87
(14) Thouvenot, D., & Morfin, R. F. (1983) *Appl. Environ. Microbiol.* **45**, 16–23
(15) Frischkorn, C. G. B., Smyth, M. R., Frischkorn, H. E., & Golimowski, J. (1980) *Fresenius Z. Anal. Chem.* **300**, 407–412
(16) Kenyhercz, T. M., & Kissinger, P. T. (1978) *J. Anal. Toxicol.* **2**, 1–2
(17) Hayashi, N., Hayata, K., & Sekiba, K. (1985) *Acta Med. Okayama* **39**, 143–153
(18) U.S. Department of Agriculture, Agriculture Research Service, Consumer and Food Economics Research Division (1963) *Composition of Foods,* Agriculture Handbook No. 8, U.S. Government Printing Office, Washington, DC, pp. 15, 23, 37
(19) Richardson, K. E., Hagler, W. M., Jr, & Mirocha, C. J. (1985) *J. Agric. Food Chem.* **33**, 862–866

AWARDS NOMINATION DEADLINES

Letters in Support of AOAC Fellows Awards — The deadline is **February 15** of each year.

Harvey W. Wiley Scholarship Nominations —
A junior and senior year scholarship of $500 per year awarded annually to sophomores majoring in scientific areas of interest to AOAC. Each year, **May 1** is the nomination deadline. The award winner is announced about six weeks later.

Harvey W. Wiley Award for the Development of Analytical Methods —
Nominations will be accepted year-round. Those received before **December 1** of any year will be eligible for the following four years' awards. Eligibility may be extended an additional four years by written request of the nominator.

For more information contact: Administrative Manager, AOAC, 1111 N. 19th Street, Arlington, Virginia 22209 or phone (703) 522-3032.

PLANTS

Gas Chromatographic Determination of the Monosaccharide Composition of Plant Cell Wall Preparations

PHILIP J. HARRIS[1]
University of Melbourne, Plant Cell Biology Research Centre, Parkville, Victoria 3052, Australia
ANTHONY B. BLAKENEY
Yanco Agricultural Institute, Yanco, New South Wales 2703, Australia
ROBERT J. HENRY
Queensland Wheat Research Institute, PO Box 5282, Toowoomba, Queensland 4350, Australia
BRUCE A. STONE[2]
La Trobe University, Department of Biochemistry, Bundoora, Victoria 3083, Australia

A simplified procedure is described for determining the monosaccharide composition of plant cell wall ("fiber") preparations. The preparations are hydrolyzed using a 2-stage sulfuric acid method. The monosaccharides released are rapidly reduced and acetylated to produce alditol acetates, which are separated by capillary gas chromatography. This procedure has been applied to the analysis of fiber preparations from a variety of animal feeds. The monosaccharide composition of the fiber preparations varied among feeds from different sources, indicating the usefulness of this method in characterizing fiber preparations in nutritional studies.

Despite recent interest in the role of plant cell walls ("fiber") in both human and animal diets, there is no generally accepted, simple method for their isolation and determination (1–3). In the past, nutritionists used crude fiber methods, which are now recognized as inadequate because of their poor recovery of plant cell wall polysaccharides (4). Extraction of plant materials with hot neutral detergent solutions has been widely used to obtain fiber preparations (5–7). These preparations (neutral detergent fiber (NDF)) appear to consist essentially of cell walls from which most of the soluble polysaccharides, such as pectin, have been removed (8). Recently, more satisfactory preparations have been obtained by enzymatic treatment of plant material (2, 9). Another approach is the direct isolation of cell walls after all cells in the plant material are broken and intracellular contents are removed (10).

The composition of the fiber preparations depends both on the method of isolation and on the source of the starting material. Cell wall composition varies widely, both among cell types within a plant and among different plant species (11). In nutritional studies, the variation in composition of fiber preparations from different sources has often been overlooked.

The major components of fiber preparations are complex polysaccharides, and because of the lack of suitable simple and rapid techniques, their determination has been attempted infrequently on a routine basis. Much of the difficulty in interpretation of nutritional studies on fiber could be overcome by obtaining more information on the specific polysaccharides present.

The starting point in any investigation of complex polysaccharides is to hydrolyze them with acid and then determine and identify the amounts of the monosaccharides released (monosaccharide composition). This can be done by direct separation of the monosaccharides by liquid chromatography (12–14), but sensitivity of detection is low and resolution of complex mixtures is a problem. More often, the monosaccharides are converted to alditol acetates, or some other volatile derivative, and separated by gas chromatography (GC) (15–23). However, previous methods for the preparation of alditol acetates were time consuming and not well suited for routine use. Recent improvements in the methods of preparation and chromatographic determination of alditol acetates (24, 25) have now made the routine analysis of fiber preparations by this approach more attractive. In this paper, we illustrate the approach by determining the monosaccharide composition of fiber preparations (NDF) from a variety of animal feeds. Results obtained with a rapid chromatographic method are compared with those obtained using a slower high resolution method (24).

METHOD

Feed Samples

(**a**) *Paspalum.*—Above-ground parts of paspalum (*Paspalum dilatatum* Poir) collected on the grounds of La Trobe University, cut into ca 1 cm lengths, and freeze-dried.

(**b**) *Canary grass.*—Above-ground parts of flowering annual canary grass (*Phalaris minor* Retz) collected and treated as in (**a**).

(**c**) *White clover.*—Above-ground parts of vegetative white clover (*Trifolium repens* L.) collected and treated as in (**a**).

(**d**) *Turnip.*—Hypocotyls of turnip (*Brassica rapa* L.) purchased from a Melbourne market.

(**e**) *Buffalo gourd.*—Roots from buffalo gourd (*Cucurbita foetidissima* HBK), field-grown at Condoboline, New South Wales.

(**f**) *Wheat straw.*—Internodes cut from wheat straw (*Triticum aestivum* L. cv. Condor) field-grown at Parkes, New South Wales.

(**g**) *Wheat bran.*—From wheat (*T. aestivum* cv. Eagle) field-grown at Yanco, New South Wales.

(**h**) *Grape seed meals (extracted for oil).*—Seeds extracted from berries of grapes (*Vitis vinifera* L. cv. Semillon and Shiraz) grown at Leeton, New South Wales.

(**i**) *Compound pelleted feeds.*—Donated by Coprice Feeds, Leeton, New South Wales. (*1*). Predominantly cereal, with 10% meat meal and 10% rice hulls; (*2*) predominantly wheat, with 5% meat meal, 5% fish meal, 5% milk solids, and 10% legume seeds; (*3*) 20% citrus pulp, 20% rice hulls, and cereal meal; (*4*) predominantly barley, 20% sunflower meal, 15% rice pollard, and 10% rice hulls.

Apparatus

(**a**) *Gas chromatograph.*—Hewlett-Packard (H-P) Model 5890 equipped with flame ionization detector used in split mode and H-P Model 3392 reporting integrator.

Received December 15, 1986. Accepted May 12, 1987.

[1] Present address: University of Auckland, Biology Department, Auckland 1, New Zealand.

[2] Address correspondence to this author.

(b) *Column.*—12 m (rapid method) or 28.5 m (high resolution method) × 0.5 mm id Silar 10C support-coated open tubular glass capillary (SGE, Melbourne, Australia).

(c) *Mill.*—Wiley, fitted with 20 mesh screen.

(d) *Ball mill.*—Ultramat Amalgamator (Southern Dental Industries, Bayswater, Victoria, Australia) fitted with stainless steel capsule (28 × 8 mm id) containing single stainless steel ball bearing (diameter 6 mm) and sealed with rubber stopper.

(e) *Hydrolysis tube.*—100 × 16 mm borosilicate glass, with Teflon-lined screw cap (Kimble, Toledo, OH 43666).

(f) *Filter.*—AP20 glass fiber prefilter disk (13 mm diameter) in Swinnex-13 filter unit (Millipore Corp., Bedford, MA 01730) mounted on all-glass 10 mL syringe.

(g) *Storage vial.*—6 mL glass sample (Wheaton Scientific, Millville, NJ 08332).

(h) *Storage bottles.*—125 mL borosilicate glass, fitted with Teflon-lined screw cap (Wheaton Scientific).

Reagents

(a) *Solvents.*—Dimethyl sulfoxide (No. 802912, stored over 4A molecular sieve) and dichloromethane (extra pure) (No. 6047, Merck, Darmstadt, FRG).

(b) *Arabinogalactan (from larch wood).*—(Sigma Chemical Co., St. Louis, MO 63178).

(c) *Bovine serum albumin.*—(Sigma Chemical Co.).

(d) *α-Amylase.*—Type VI-A from hog pancreas (Sigma Chemical Co.).

(e) *1-Methylimidazole.*—(Fluka, Buchs, Switzerland).

(f) *Carrier gas.*—High purity hydrogen (<10 ppm oxygen) (CIG, Melbourne, Australia) further purified by passage through oxygen trap (Oxy-trap; Alltech Associates, Melbourne, Australia) and drying tube (SGE).

(g) *Sulfuric acid (72% w/w).*—Weigh 20 g ice-cold water, dilute to 93.5 g with ice-cold 98% analytical grade acid (with stirring), then add ice-cold water to 100 g. Store in bottle fitted with Teflon-lined screw cap.

(h) *Sodium borohydride solution.*—Add 2 g sodium borohydride to 100 mL anhydrous dimethyl sulfoxide and heat in 100°C oven until sodium borohydride is dissolved. Store solution at room temperature in bottle fitted with Teflon-lined screw cap.

(i) *Reference alditol acetate standards.*—Dry monosaccharides (rhamnose, fucose, ribose, arabinose, xylose, allose, mannose, galactose, and glucose) (Sigma Chemical Co.) by heating at 60°C over silica gel 16 h in vacuum oven. Weigh 20 mg of each and dissolve each in 1 mL water. Mix 0.1 mL of each solution and reduce and acetylate as in *Procedure.*

Procedure

Isolation of plant cell wall (fiber) preparations.—Grind feed samples in Wiley mill to pass 20 mesh screen. Obtain fiber preparation from plant materials by method appropriate to study. (In this study we used extraction with hot neutral detergent solution to illustrate monosaccharide determination procedure.) Isolate α-amylase-treated NDF preparations according to AACC method 32-20 (26, 27). Test for starch with solution of iodine in potassium iodide; examine by bright-field microscopy (10).

Hydrolysis of plant cell wall (fiber) preparations.—Ball mill dried fiber preparations ca 3 min. Dry finely ground preparations in desiccator over P_2O_5 and then weigh 10 mg into hydrolysis tube. Add 250 μL 72% (w/w) sulfuric acid with glass pipet, flush tube with argon, screw on cap, and place in 30°C water bath. At frequent intervals, vigorously agitate tube on vortex mixer to aid dissolution of preparation in acid. After 3 h, open tube and add 2.75 mL water. Flush tube with argon, screw on cap, and place in boiling water bath for 3 h. When cool, add 600 μL 15M ammonia to neutralize solution; add 50 μL D-allose (20 mg/mL water) as internal standard and filter through glass fiber disk.

Reduction of monosaccharides.—Add 200 μL filtered hydrolysate to (29 × 100 mm) test tube; then add 1 mL sodium borohydride solution and incubate 90 min in 40°C water bath. Add 100 μL 18M acetic acid to decompose excess sodium borohydride.

Acetylation.—*Caution*: Perform this operation in fume hood. Add 200 μL 1-methylimidazole and then 2 mL acetic anhydride to reduced mixture of monosaccharides and mix. After 10 min at room temperature, add 10 mL water to decompose excess acetic anhydride and to aid phase separation. When cool, add 1 mL dichloromethane and mix on vortex mixer. After phases have separated, remove lower phase with Pasteur pipet and store in glass vial at −20°C before gas chromatography. If this phase is cloudy, add a little anhydrous sodium sulfate to dry it.

Gas chromatography.—Inject samples using H-P Model 7673A autoinjector with 2 solvent prewashes, 2 washes of sample to waste, 5 pumps of sample, and, to ensure air bubbles are removed, 2 postinjection solvent washes. Alternatively, inject samples manually (24). Operating conditions: (*1*) *Rapid method.*—Inject 1 μL sample; start with oven temperature at 170°C, then raise temperature at 10°/min to 230°C and hold for 1 min; heat injection port and detector to 250 and 300°C, respectively; carrier gas flow rate, 150 cm/s. (*2*) *High resolution method.*—As described previously (24).

Identification and quantitation of alditol acetates.—Determine retention times of reference alditol acetate standards relative to allitol hexaacetate. Reference alditol acetates elute in the order: rhamnitol acetate, fucitol acetate, ribitol acetate, arabinitol acetate, xylitol acetate, allitol acetate, mannitol acetate, galactitol acetate, and glucitol acetate. Identify alditol acetates derived from hydrolysates from their retention times relative to internal standard (allitol hexaacetate). Calculate percentage monosaccharide composition of hydrolysates from peak areas of alditol acetates by using response factors obtained from reference alditol acetate standards. Above calculations were performed by integrator that identified peaks within 0.1 min retention time windows with glucose as reference peak for drift in retention time.

Results and Discussion

The starch-free NDF found in a variety of animal feed samples is shown in Table 1. The NDF content ranged from 16.3% for buffalo gourd root to 81.3% for wheat straw. The monosaccharide composition of the NDF preparations is also shown in Table 1. Multiple samples of all NDF preparations were analyzed with replication at the hydrolysis, alditol acetate preparation, and GC steps. All hydrolysates contained xylose and glucose, and standard errors were calculated for the analyses of these monosaccharides by using a GENSTAT V general statistical computer program (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK). Standard errors due to hydrolysis were 0.22 and 0.34%, respectively, to acetylation 0.23 and 0.22%, and to injection 0.33 and 0.59%. No error was significant at the 5% level. Varying amounts of arabinose, mannose, and galactose were found in most of the hydrolysates. Traces of rhamnose, fucose, and ribose were found in some of the hydrolysates. The ribose was probably derived from cytoplasmic RNA (10).

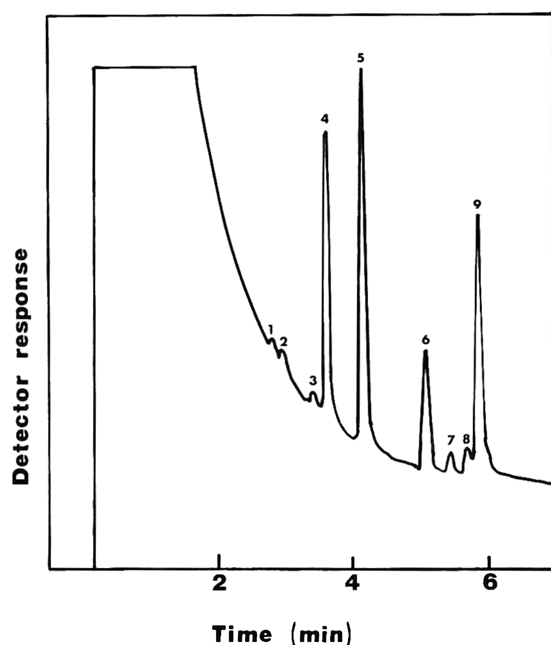


Figure 1. Separation by gas chromatography (rapid method) of alditol acetates derived from monosaccharides in acid hydrolysate of wheat bran NDF preparation. Peaks: 1, rhamnitol; 2, fucitol; 3, ribitol; 4, arabinitol; 5, xylitol; 6, allitol (internal standard); 7, mannitol; 8, galactitol; and 9, glucitol.

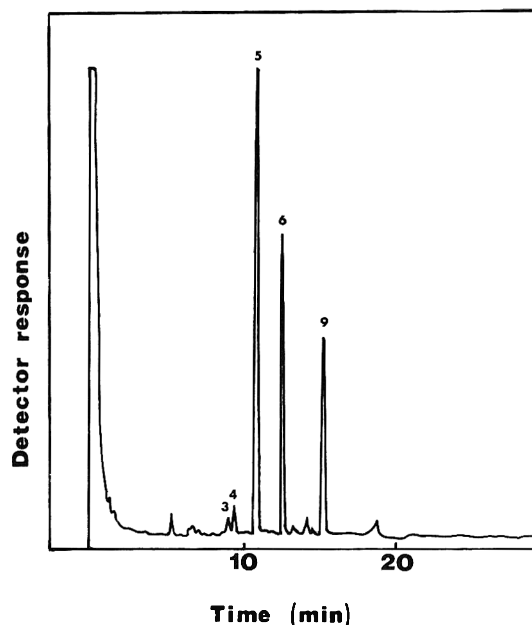


Figure 2. Separation by gas chromatography (high resolution method) of alditol acetates derived from monosaccharides in acid hydrolysate of grape seed (cv. Shiraz) NDF preparation. Identification of peaks as in Figure 1.

Because of the different rates of hydrolysis of cell wall polysaccharides, it is impossible to optimize the conditions for hydrolysis. Furthermore, the monosaccharides that are released are exposed to acid for varying periods, which makes it difficult to apply any correction factors for losses of monosaccharides due to acid degradation.

All the fiber preparations selected were readily analyzed by the improved method. The feeds from which the fiber preparations were obtained include examples of all feed types commonly encountered: pasture plants, root crops, crop residues, and commercial mixed feeds. Two of the commercial mixed feeds contained animal proteins. Wheat straw and grape seed meals have a high lignin content; grape seed meals also have a high tannin and protein content. The effects of lignin and tannin on the analysis are not known. However, the determination of an arabinogalactan was unaffected by the presence of an equal weight of the protein bovine serum albumin. Glycoproteins have also been determined by this method (28).

With the exception of the NDF preparations from grape seeds, the total monosaccharide residues recovered from the preparations accounted for a high percentage (84.2–94.5%) of the dry weight of the preparations (Table 1). The total monosaccharide residues recovered from the grape seed fiber preparations accounted for only 65.3% (cv. Semillon) and 62.8% (cv. Shiraz) of the dry weight of the preparations (Table 1). The cell walls of grape seeds have a high lignin content and the NDF preparations may also contain unextracted tannin and protein.

Only the neutral monosaccharides in the fiber preparations are determined by the procedure described; galacturonic and glucuronic acids are not reduced. These occur in plant cell walls as components of galacturonans and heteroxylans, respectively (11), and can be determined independently, for

Table 1. Monosaccharide composition of NDF preparations[a]

Fiber preparation	NDF,[b] % (w/w dry matter)	Monosaccharides, % (w/w)					Total monosaccharide residues recd[c]
		Arabinose	Xylose	Mannose	Galactose	Glucose	
Paspalum	58.9	3.8	28.0	0.3	0.9	67.0	91.2
Canary grass	63.2	5.9	27.3	0.2	3.2	63.4	92.3
White clover	24.3	4.6	11.1	2.5	5.1	76.8	87.4
Turnip	18.4	2.6	7.6	4.1	4.1	81.6	94.5
Buffalo gourd	16.3	4.4	13.0	2.8	9.0	70.9	93.8
Wheat straw	81.3	3.1	31.4	0.4	tr[d]	65.1	89.6
Wheat bran	47.7	19.9	38.5	1.3	1.8	38.6	94.2
Grape seeds (cv. Semillon)	74.8	tr	55.2	—	—	44.8	65.3
Grape seeds (cv. Shiraz)	73.2	tr	49.7	—	0.7	49.6	62.8
Compound feed 1	22.4	4.1	30.6	0.3	0.9	64.1	84.2
Compound feed 2	24.7	9.7	34.1	1.2	0.8	54.3	91.4
Compound feed 3	36.8	3.0	26.6	0.2	1.2	69.0	86.7
Compound feed 4	30.1	4.6	32.6	0.4	0.4	62.0	87.9

[a] Determined by using rapid method. Each result is the mean of 8 determinations.
[b] Starch-free.
[c] As percent of dry weight of fiber preparations.
[d] tr = trace amounts.

example, by colorimetry (29) or by decarboxylation and measurement of the carbon dioxide released (17). Lignin can also be determined independently (21). However, the dry weight of the residue after acid hydrolysis of a larger initial sample can be regarded as an estimate of Klason lignin.

Although we have not critically examined the acid hydrolysis of the fiber preparations, the sulfuric acid solution must be 72% w/w and not w/v or v/v, and the 72% w/w sulfuric acid solution must be stored in a sealed container, as it is hygroscopic. Methods of acid hydrolysis which do not hydrolyze the cellulose in fiber preparations will obviously not release all the monosaccharide residues in the preparations. For example, 2M trifluoroacetic acid hydrolyzes $<1\%$ of microcrystalline cellulose (30).

Although alditol acetates can be separated by gas chromatography using packed columns with polar stationary phases, resolution can be improved by using capillary columns. We found that a support-coated open tubular capillary column (28.5 m long) containing the polar phase Silar 10C gave baseline resolution of 13 alditol acetates (24) (high resolution method). However, in the present study we were able to use a shorter column (12 m) and a steeper temperature program (rapid method). This gave good resolution of the alditol acetates commonly found in fiber preparations. Six samples could be analyzed per hour. Figures 1 and 2 show the separation of alditol acetates using the rapid and high resolution methods, respectively.

Allose is a good internal standard because neither it nor allitol nor psicose, which yields allitol on reduction, has been encountered in plant cell wall polysaccharides. In addition, allitol acetate is well resolved from the alditol acetates derived from the monosaccharides that occur in plant cell wall polysaccharides. Allose is a better internal standard than inositol because it is a monosaccharide and hence will monitor the efficiency of the reduction step. Also, allitol acetate is eluted from Silar 10C between the pentitol and hexitol acetates rather than after the hexitol acetates. Colby et al. (31) found that the most reliable relative retention times were based on the most closely related internal standards. Allose has been used as an internal standard by Theander and Åman (15, 17).

Compounds other than alditol acetates also elute from polar phases with similar retention times, e.g., chlorinated hydrocarbon pesticides, fatty acid methyl esters, and plasticizers such as phthalate, adipate, and sebacate esters. Phthalate esters are the most frequently used plasticizers and are widespread pollutants of the environment. Although we have found that by utilizing the high resolution of capillary chromatography a large number of plasticizers can be resolved from alditol acetates on Silar 10C (32), cochromatography with contaminants is still probably the most important factor in determining the limits of reliable detection of alditol acetates. Possible sources of contamination include the reagents, the glassware, and the fiber preparations. High purity reagents should therefore be used and, if necessary, contaminating plasticizers can be removed from glassware by baking at 200°C for 24 h (32, 33) and from fiber preparations by extracting with high purity dichloromethane or chloroform. Failure to maintain optimal chromatographic conditions can lead to failure to resolve some contaminants from alditol acetates. Polar phases are sensitive to oxygen and high purity, oxygen-free carrier gas should be used to extend the column life.

The analytical procedures described here allow the monosaccharide composition of fiber preparations to be determined simply, rapidly, and on a routine basis.

Acknowledgment

We thank M. J. Neilson for critically reading the manuscript.

References

(1) James, W. P. T., & Theander, O. (1981) *The Analysis of Dietary Fiber in Food,* Marcel Dekker, New York, NY
(2) Prosky, L., Asp, N.-G., Furda, I., DeVries, J. W., Schweizer, T. F., & Harland, B. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 1044–1052
(3) Wisker, E., Feldheim, W., Pomeranz, Y., & Meuser, F. (1985) in *Advances in Cereal Science and Technology,* Vol. 7, Y. Pomeranz (Ed.), American Association of Cereal Chemists, St. Paul, MN
(4) Baker, P., Norris, K. H., & Li, B. W. (1979) in *Dietary Fibres: Chemistry and Nutrition,* G. E. Inglett & S. I. Falkehag (Eds), Academic Press, New York, NY, pp. 67–78
(5) Van Soest, P. J., & Wine, R. H. (1967) *J. Assoc. Off. Anal. Chem.* **50,** 50–55
(6) Robertson, J. B. (1980) in *Topics in Dietary Fiber Research,* G. A. Spiller (Ed.), Plenum, New York, NY, pp. 1–42
(7) Robertson, J. B., & Van Soest, P. J. (1981) in *The Analysis of Dietary Fiber in Food,* W. P. T. James & O. Theander (Eds), Marcel Dekker, New York, NY, pp. 123–158
(8) Bailey, R. W., & Ulyatt, M. J. (1970) *N. Z. J. Agric. Res.* **13,** 591–604
(9) Asp, N.-G., Johansson, C.-G., Hallmer, H., & Siljeström, M. (1983) *J. Agric. Food Chem.* **31,** 476–482
(10) Harris, P. J. (1983) in *Isolation of Membranes and Organelles from Plant Cells,* J. L. Hall & A. L. Moore (Eds), Academic Press, London, UK, pp. 25–53
(11) Bacic, A., Harris, P. J., & Stone, B. A. (1988) in *The Biochemistry of Plants: A Comprehensive Treatise Vol. 14: Carbohydrates,* J. Preiss (Ed.), Academic Press, New York, NY, pp. 297–371
(12) Windham, W. R., Barton, F. F., II, & Himmelsbach, D. S. (1983) *J. Agric. Food Chem.* **31,** 471–475
(13) Slavin, J. L., & Marlett, J. A. (1983) *J. Agric. Food Chem.* **31,** 467–471
(14) Neilson, M. J., & Marlett, J. A. (1983) *J. Agric. Food Chem.* **31,** 1342–1347
(15) Theander, O., & Åman, P. (1980) *J. Sci. Food Agric.* **31,** 31–37
(16) Chen, W. J. L., & Anderson, J. W. (1981) *Am. J. Clin. Nutr.* **34,** 1077–1082
(17) Theander, O., & Åman, P. (1981) in *The Analysis of Dietary Fiber in Food,* W. P. T. James & O. Theander (Eds), Marcel Dekker, New York, NY, pp. 51–70
(18) Englyst, H., Wiggins, H. S., & Cummings, J. H. (1982) *Analyst* **107,** 307–318
(19) Bittner, A. S., & Street, J. C. (1983) *J. Agric. Food Chem.* **31,** 7–10
(20) Englyst, H. N., & Cummings, J. H. (1984) *Analyst* **109,** 937–942
(21) Selvendran, R. R., & Du Pont, M. S. (1984) in *Developments in Food Analysis Techniques—3,* R. D. King (Ed.), Elsevier Applied Science Publishers, London, UK, and New York, NY, pp. 1–68
(22) Pitman, W. D., & Moore, J. E. (1985) *Crop Sci.* **25,** 790–792
(23) Theander, O., & Westerlund, E. A. (1986) *J. Agric. Food Chem.* **34,** 330–336
(24) Blakeney, A. B., Harris, P. J., Henry, R. J., & Stone, B. A. (1983) *Carbohydr. Res.* **113,** 291–299
(25) Blakeney, A. B., Harris, P. J., Henry, R. J., Stone, B. A., & Norris, T. (1982) *J. Chromatogr.* **249,** 180–182
(26) *Approved Methods of the AACC* (1978) Revisions: Method 32-20, American Association of Cereal Chemists, St. Paul, MN
(27) Schaller, D. R. (1981) *Cereal Foods World* **26,** 295–297
(28) Henry, R. J., Blakeney, A. B., Harris, P. J., & Stone, B. A. (1983) *J. Chromatogr.* **256,** 419–427
(29) Blumenkrantz, N., & Asboe-Hansen, G. (1973) *Anal. Biochem.* **54,** 484–489
(30) Mankarios, A. T., Jones, C. F. G., Jarvis, M. C., Threlfall, D. R., & Friend, J. (1979) *Phytochemistry* **18,** 419–422
(31) Colby, B. N., Ryan, P. W., & Wilkinson, J. E. (1983) *J. High Resolut. Chromatogr. Chromatogr. Commun.* **6,** 72–76
(32) Henry, R. J., Harris, P. J., Blakeney, A. B., & Stone, B. A. (1983) *J. Chromatogr.* **262,** 249–256
(33) Ishida, M., Suyama, K., & Adachi, S. (1980) *J. Chromatogr.* **189,** 421–424

SUGARS

Determination of Dextran in Raw Cane Sugar by Roberts Copper Method: Collaborative Study

MARGARET A. CLARKE and MARY AN GODSHALL
Sugar Processing Research, Inc., 1100 Robert E. Lee Blvd, New Orleans, LA 70124

Collaborators: W. Altenburg; H. J. Andreis; E. R. Arias; T. Balogh; W. Braunsteiner; A. Brown; R. DeStefano; J. W. Dunn; Z. J. Kimmerling; R. A. Kitchen; G. J. Leonard; D. Martin; K. McGaughey; P. Mellet; R. Plews; S. Runciman

A collaborative study was conducted using the Roberts copper method for the determination of dextran in raw cane sugars. Four samples were analyzed in duplicate, representing the range of dextran concentrations found in raw sugar. The overall repeatability and reproducibility coefficients of variation were 4.3 and 13.2%, respectively. The method has been adopted official first action.

Dextran is a soluble bacterial polysaccharide produced from sucrose by *Leuconostoc mesenteroides*. It consists of linear α-1,6-glucose units with occasional branches of α-1,4-glucose. Under certain conditions, such as poor weather, certain harvesting practices, or poor mill sanitation, dextran can form in cane stalks, cane juice, or processing syrups, and the resulting concentration of dextran in the raw sugar produced can be high enough to cause processing problems during refining. Problems caused by dextran in raw sugar include sucrose loss, increased viscosity of process syrups, poor recovery of sucrose due to inhibition of crystallization, and distorted sucrose crystals, as well as turbidity in sweetened alcoholic liqueurs. Dextran is not effectively removed in refining, so much of the dextran originally in the raw sugar ends up in the refined sugar. Levels in raw sugar range up to 5000 ppm (total dextran) and in refined sugar up to 2500 ppm (total dextran).

In recent years, dextran concentration in raw sugars has risen because of the increased use of mechanical harvesting systems which tend to damage the cane, allowing entry of the causative organism into the cane stalk.

The Roberts copper method for dextran was developed in response to the need for a test that was specific for dextran in raw cane sugar (1). The haze method was in common use and was recognized by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA) as a tentative official method (2) until 1986, when tentative status was withdrawn. However, that method is not specific for dextran because it relies on the measurement of turbidity formed in an alcoholic sugar solution. Other polysaccharides present in the sugar can interfere in the haze method as can high ash or color content of the raw sugar being tested. The haze method measures only dextran of molecular weight above 10 000 daltons.

The level of dextran in raw sugar is of commercial importance because it affects the purchase price for raw sugar. The Roberts method can be applied to raw sugars, and also to refined sugars, cane juice, and process materials. It is the only method available to determine total dextran content. This paper reports the results of a collaborative study of the method.

Collaborative Study

The sugars for this collaborative study were from Zimbabwe, Jamaica, Brazil, and South Africa. Each collaborator was sent replicate samples of each sugar sealed in plastic bottles containing approximately 100 g of sugar. The samples were labeled A1, A2, B1, B2, C1, C2, D1, D2. Sugars with the same letter label were replicates. The collaborators were asked to run each sample in duplicate. A practice (check) sample, containing 430 ppm dextran was included.

Detailed instructions were sent with the samples, as was a reporting form. The method, as originally published, required a high speed centrifuge to separate the copper-dextran complex (1). These types of centrifuges are not routinely available in all laboratories, so a modification of the method was devised for the study, in which the dextran-copper complex was separated by filtration rather than centrifugation.

Dextran in Raw Cane Sugar
Roberts Copper Method
First Action

31.D01 ***Principle***

All high MW material (>10 000 daltons) is sepd from sugar by pptn in 80% ethyl alcohol. Pptd material is filtered off, washed, and then redissolved. Alk. Cu(II) reagent is added which selectively ppts dextran from other high MW material in raw cane sugar. This ppt is filtered off, and dextran content is detd colorimetrically as carbohydrate by phenol-H_2SO_4 reaction, which breaks down dextran complex to glucose units and gives color with intensity proportional to total amt dextran in sample. Samples and reaction mixts must be kept free of contamination by sugar dust and other dust that may contain carbohydrate and react positively in phenol-H_2SO_4 color test.

31.D02 ***Apparatus***

(a) *Colorimeter or spectrophotometer.*—To read A or T at 485 nm.

(b) *Sintered glass filters.*—Coarse, pore size C, 15 mL.

(c) *Nessler tubes.*—35 mL, or flat-bottom test tubes short enough to fit inside filter flask.

31.D03 ***Reagents***

Use reagent grade chems unless otherwise specified. Use distd or deionized H_2O.

(a) *Absolute alcohol.*—Use absolute alcohol; do not use 95% alcohol.

(b) *Ethyl alcohol, 80%.*—Dil. 80 mL absolute alcohol (a) with 20 mL H_2O.

(c) *Filter aid.*—Anal. grade, acid-washed.

(d) *NaOH reagent soln.*—2.5N NaOH, satd with Na_2SO_4. Dissolve 100 g NaOH in H_2O and dil. to 1 L. Sat. with anhyd. or

Submitted for publication August 27, 1987.
This report of the Associate Referee, M. A. Godshall, was presented at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA.
The recommendation of the Associate Referee 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 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) 71, January/February issue.

hydrated Na_2SO_4, adding crystals until some are left undissolved. Store in reagent bottle that has stopper of material other than glass. Soln may be stored 1 month.

(e) *Cu stock soln.*—Dissolve 3.0 g $CuSO_4 \cdot 5H_2O$ in 50 mL H_2O. Dissolve 30.0 g Na citrate in 50 mL H_2O. Mix these 2 solns and dil. to 1 L with H_2O. Soln may be stored 2 weeks.

(f) *Cu reagent soln.*—Dil. 50 mL Cu stock soln with 50 mL H_2O. Dissolve 12.5 g anhyd. Na_2SO_4 in this soln. Reagent must be freshly prepd on each day of use and must not be stored.

(g) *Wash soln.*—To 50 mL H_2O, add 10 mL Cu reagent soln and 10 mL 2.5N NaOH reagent soln.

(h) *Phenol soln, 5%.*—Dissolve 5.0 g pure phenol in H_2O and dil. to 100 mL. Soln may be stored 2 weeks.

(i) *Dextran.*—(Pharmacia Co., Inc., 800 Centennial Ave, Piscataway, NJ 08854.) Any MW range may be used.

(j) *Sulfuric acid, 2N.*—Dissolve 98 g H_2SO_4 in H_2O and dil. to 1 L. Soln may be stored 1 month. (**Caution**: See **51.030**.)

31.D04 *Preparation of Standard Curve*

Det. moisture content of dextran to allow moisture correction when dextran is weighed for std curve. Weigh 500 mg std dextran in weighing dish and dry 4 h at 105°. Cool in desiccator over anhyd. $CaCl_2$. Weigh and calc. moisture content as follows:

$$\text{Moisture, \%} = [(\text{original wt} - \text{dry wt})/\text{original wt}] \times 100$$

Weigh 500 mg dextran (corrected for calcd moisture content), dissolve in H_2O, and dil. to 500 mL. Do not use predried dextran which may not dissolve properly. Soln contains 1.0 mg dextran/mL. Use freshly prepd soln for each std curve detn.

Dil. 100 mL 1.0 mg dextran/mL std soln to 1 L (0.1 mg dextran/mL). Use this soln in aliquots of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mL, each dild to 100 mL, to prep. std curve. Each soln contains, resp., 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1 mg dextran/mL.

Use A (or T) data to construct calibration curve with mg dextran/mL as x-axis.

31.D05 *Phenol-H_2SO_4 Test*

In series of 10 test tubes, 20 × 150 mm, place 2 mL of each dextran std soln. Place 2 mL H_2O in an 11th tube as blank. Add 1 mL 5% phenol soln to each tube. Swirl tubes gently to mix phenol and carbohydrate solns.

To each tube add 10 mL concd H_2SO_4, preferably using plastic automatic pipet, releasing acid rapidly to ensure that soln is thoroly mixed. Do not let pipet touch soln, and do not let acid remain on top of soln. Mix on Vortex mixer.

Place tubes (in rack) in boiling H_2O bath for 2 min. Let cool 30 min.

Read color as A or T, preferably A, on colorimeter or spectrophtr at 485 nm against blank soln. Set A to zero on blank and then read A of solns, using same cell (or matched cells) for blank and test solns. Make duplicate colorimetric readings and calc. av. value.

For best accuracy, A should be between 0.1 and 0.6 (T should be >25%). If A is >0.6, diln step is required. This step should not be necessary for prepn of std curve, but may be required for sugar samples.

To prep. dild soln from soln from which 2 mL aliquot was taken (final 25 mL soln in sugar sample detn), pipet 5 mL into 25 mL vol. flask and dil. to vol. Use 2 mL of this dild soln in phenol–H_2SO_4 detn. In this case, diln factor of 5 must be included in calcn of ppm dextran.

31.D06 *Determination*

Accurately weigh 40.0 g sugar in beaker and dissolve in small amt H_2O. Quant. transfer soln to 100 mL vol. flask and dil. to vol. with H_2O. Filter ≥50 mL of this soln thru qual. paper in funnel to remove coarse suspended material.

Pipet 10 mL filtrate into 100 mL beaker, add 0.3–0.4 g anal. filter aid, and stir; add 40 mL absolute alcohol and stir. Let stand 5 min to form ppt. Filter ppt off on sintered glass filter on rubber ring or rubber sleeve-topped vac. filter flask.

Wash ppt 5 times with 80% ethyl alcohol, each time filling filter with alcohol, and letting alcohol be drawn thru ppt. Do not let ppt go dry and do not let alcohol overflow filter. This step is important to remove sugar that might remain adsorbed to ppt and interfere in phenol–H_2SO_4 reaction.

When last portion of alcohol wash has been completely drawn thru ppt, quant. transfer ppt plus filter aid to 25 mL vol. flask. Use min. amt H_2O for transfer. First, place 50 or 60 mm long-stem funnel in empty 25 mL flask. Invert sintered glass funnel contg ppt into long-stem funnel; fill stem of sintered glass funnel with H_2O and blow ppt plus filter aid into long-stem funnel, using air hose or mouth. Use H_2O from wash bottle to wash remaining traces of solid from sintered glass funnel (2 times) into long-stem funnel, and wash all ppt (broken up with spatula) into 25 mL vol. flask. Amt H_2O used must not exceed vol. of 25 mL flask. Adjust to vol. with H_2O.

Filter soln thru fluted Whatman No. 42, 110 mm, paper in 60 or 80 mm funnel. Collect ≥10 mL filtrate for analysis.

Pipet 10 mL filtrate into 20 × 100 mm glass or plastic test tube. Pipet 2 mL 2.5N NaOH reagent soln into test tube. Pipet 2 mL Cu reagent soln into test tube and add 0.2 g anal. filter aid. Place test tubes (in rack) in boiling H_2O bath 5 min to ppt Cu–dextran complex on filter aid, and then let cool 20 min.

Filter ppt, which contains Cu–dextran complex, on sintered glass filter (coarse pore size, 15 mL). Rinse test tube 2 times with 10 mL portions of wash soln and pour these into sintered glass filter.

Discard filtrate.

Place filter, contg ppt, on vac. flask contg short (35 mL) Nessler tube (or flat-bottom vial) positioned so that stem of sintered glass funnel extends into Nessler tube.

Pour 2 mL 2N H_2SO_4 soln onto ppt in sintered glass funnel; turn on vac. and draw acid soln thru ppt. Repeat this procedure, then rinse ppt with 2 mL H_2O.

Quant. transfer filtrate, which contains solubilized dextran to 25 mL vol. flask and dil. to vol. with H_2O.

Pipet 2 mL of this soln into 20 × 150 mm test tube and follow procedure for *Phenol–H_2SO_4 Test*.

31.D07 *Calculation*

Read dextran concn, mg/mL, from std calibration curve.

Dextran in sample, ppm = [(mg/mL dextran from std curve) × (mL final soln of Cu–dextran complex) × (mL soln of alcohol ppt) × 10^5]/[(mL aliquot taken for Cu pptn) × (mL aliquot taken for alcohol pptn) × (wt sample solids dild to 100 mL)]

If A = wt sample solids, g, dild to 100 mL; B = aliquot taken for alcohol pptn, mL; C = soln of alcohol ppt, mL; D = aliquot taken for Cu pptn, mL; E = final soln of Cu–dextran complex, mL; F = dextran (from std curve), mg/mL, then:

$$\text{Dextran, ppm} = (F) \times (E) \times (C/D) \times (1/B) \times (1/A) \times 10^5$$

Therefore, for aliquots specified in procedure: $A = 40$, $B = 10$, $C = 25$, $D = 10$, and $E = 25$.

Ref.: JAOAC **71**, March issue (1988).

CAS-9004-54-0 (dextran)

Results and Discussion

Most collaborators reported no problems with the method although one thought that the procedure was too complex for routine analysis. All collaborators reported that they used absorbance readings.

The original method specified a centrifugation step to isolate the copper-dextran complex, but to simplify the test and remove the need for specialized equipment, a filtration step was substituted. A comparison of filtration and centrifugation in the authors' laboratories showed that the results were the same, with a precision of 5.1% for filtration and 5.2% for centrifugation. However, if time was allowed to elapse after completion of centrifugation, there was some loss of sample, which became a considerable loss after 1 h (up to 25% loss) because of resolubilization of the complex. This problem was

Table 1. Collaborative results for determination of dextran (ppm) in duplicate raw cane sugar samples.

	Sample A1		Sample A2		Sample B1		Sample B2		Sample C1		Sample C2		Sample D1		Sample D2	
Lab.	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	279	301	292	283	468	460	456	454	991	991	964	980	291	300	287	301
2	312	284	306	328	351	368	402	362	1127	1057	1039	1072	390	391	422	381
3[a]	239	296	246	271	434	414	434	452	594	588	584	621	299	304	255	278
4	255	260	236	264	686	706	700	710	1145	1052	1086	1164	453	488	479	477
5	366	352	364	308	468	474	615	542	972	978	997	995	375	383	402	409
6	349	339	351	355	479	424	436	434	934	935	895	892	323	327	332	347
7	426	349	429	442	674	555	609	589	999	1136	1074	1081	467	483	432	501
8	339	452	376	409	617	613	612	625	1016	1030	955	958	438	412	425	433
9	467	456	517	544	414	428	397	453	1016	1018	1083	1050	345	373	340	351
10[a]	626	593	558	677	665	648	687	658	1047	1161	983	1130	553	553	543	580
11	415	419	353	376	647	650	628	618	957	967	981	1042	425	352	413	416
12	387	395	345	352	505	490	560	552	998	1018	1032	1008	388	392	367	362
13	278	368	254	257	633	599	598	563	974	1070	1061	1024	340	306	345	331

[a] Laboratory classified as outlier by rankings test (3).

avoided by the use of filtration. The average recovery of added dextran to sugar solutions by both methods was also comparable and ranged from 97 to 102% for both methods.

A check sample, which had already been analyzed for dextran content, was included with the test samples for practice. The value reported for the check sample was 430 ppm dextran ±13.2 (relative standard deviation 3.07%); the value was obtained by 2 experienced analysts. A value of 436 ± 31.7 ppm was obtained for the same sample when the results of a third analyst, inexperienced with the method, were included (relative standard deviation 7.27%). Only one collaborator reported difficulty in obtaining a comparable value.

The results of the collaborative study are tabulated in Table 1. The methods of Youden and Steiner (3) were used to analyze the data. A ranking test for outlying laboratories identified 2 outliers. (The criterion for 13 laboratories and 8 samples is 26/86; the rank sum for the high laboratory was 98 and for the low laboratory 17.) These laboratories were eliminated from the subsequent analysis of variance.

A Dixon test for outlier results from the remaining laboratories was negative. A homogeneity test for experimental variation between laboratories yielded a range ratio (w_{max}/w_{min}) of 2.05, which was less than the test statistic of 3.0 for 8 samples and 11 laboratories. This indicated sufficient homogeneity of the laboratories at the 5% significance level.

Cochran's test for variation between replicates was done to determine if within variability was homogenous across laboratories and samples. The value of the test statistic was 0.22. The critical value of 0.23 for a 5% significance level allows acceptance of the homogeneity of variance assumption.

Table 2 shows the individual and overall sample means with relative reproducibility and repeatability coefficients of variation.

Table 3 shows the results of the overall analysis of variance. The laboratory-sample interaction term was significant; a P-value of 0.0001 was obtained, indicating that the results of the method will vary between laboratories more than within a single laboratory, probably due to differences in filtration techniques. The between-laboratory variance is not significant, so there is no evidence of consistent laboratory bias.

The overall repeatability of 24.6 is an estimate of the replicate variability; the reproducibility of 75.8, which includes replicate variability, between-laboratory variability, and laboratory-sample interaction, is an estimate of the total variability. This reproducibility figure indicates that a fairly wide range of results could be expected between laboratories. Part of the variability may be due to inexperience with the method.

Recommendation

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

Acknowledgments

The authors acknowledge the assistance of Margaret Chmielewski in the statistical analysis and interpretation of data. The help of the AOAC Statistics Committee is also acknowledged. The authors acknowledge the contribution of chemicals and equipment by the Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, to this study. The authors thank Earl J. Roberts for his interest and advice.

The authors thank the following collaborators who participated in the study:

Walter Altenburg, New York Sugar Trade Laboratory, Clark, NJ

Henry J. Andreis and Ronald DeStefano, U.S. Sugar Corp., Clewiston, FL

Enrique R. Arias, Sugar Cane Growers Cooperative of Florida, Belle Glade, FL

Tereza Balogh, Redpath Sugars, Toronto, Ontario, Canada

W. Braunsteiner, Zuckerforschungs-Institut, Wien, Austria

Table 2. Means and CVs for results of collaborative study on determination of dextran in raw cane sugar

Sample	Mean, ppm	Reproducibility CV, %	Repeatability CV, %
A	354.3	21.0	9.7
B	530.8	20.2	6.5
C	1018.5	6.2	3.5
D	386.3	15.5	5.1
Overall	573.7	13.2	4.3

Table 3. Analysis of variance of results of collaborative study on determination of dextran in raw cane sugar

Source of variation	Degrees of freedom	Sum of squares	F-value	PR > F
Between labs	10	163 946	1.81	0.1015
Lab-sample interaction	30	271 461	14.96	0.0001
Repeatability standard deviation		24.6		
Reproducibility standard deviation		75.8		

Alan Brown, Tate and Lyle Refineries, Plaistow Wharf, London, England

James W. Dunn, Tate and Lyle Group Research and Development, Reading, England

Z. J. Kimmerling, South African Sugar Terminals Ltd, Maydon Wharf, Natal, South Africa

Richard A. Kitchen, B.C. Sugars, Vancouver, British Columbia, Canada

G. J. Leonard, Bureau of Sugar Experiment Station, Indooroopilly, Queensland, Australia

Dennis Martin, Tate and Lyle Refineries, United Molasses, Sugar Quay, London, England

Kim McGaughey, Savannah Foods, Savannah, GA

Piet Mellet, Sugar Milling Research Institute, Durban, Natal, South Africa

Ronald Plews, Tate and Lyle Sugars, Thames Refinery, London, England

Scott Runciman, Lantic Sugar Ltd, Oshawa, Ontario, Canada

References

(1) Roberts, E. J. (1983) *Int. Sugar J.* **85**, 10–13

(2) Report of Proceedings of 16th Session, Int. Comm. Uniform Methods Sugar Anal. (1974) ICUMSA, PO Box 35, Wharf Rd, Peterborough PE2 9PU, U.K., pp. 363–367

(3) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC,* AOAC, Arlington, VA, 88 pp.

THE 102nd AOAC ANNUAL INTERNATIONAL MEETING AND EXPOSITION

AT THE BREAKERS, PALM BEACH, FLORIDA
AUGUST 29 - SEPTEMBER 1, 1988

Program Includes:

The Spotlight Symposium on **BIOTECHNOLOGY** - Chairman: D.M. Hinton

Symposia on:

Laboratory Information Management Systems (LIMS) - Chairmen: J.J. Karr and H. Morris

Fertilizer Phosphate Evaluation and Analysis - Chairmen: F. Johnson and J.R. Trimm

Drug Residues in Foods of Animal Origin - Chairmen: W.A. Moats and B. Shaikh

Pesticides in Foods - Chairman: P. Corneliussen

Speaker: Wiley Award winner — **Dr. Frederick Kavanagh,** eminent analytical chemist, microbiologist and instrument designer.

Over 200 **technical poster presentations** on topics such as Pesticides Formulations and Disinfectants; Foods; Residues; Microbiology; Feeds, Fertilizers and Related Topics; Drugs and Related Topics; Hazardous Substances in Waste and the Environment.

Open Forum

Regulatory Roundtable: Safety - The 1986-87 OSHA Regs

Laboratory Quality Assurance Short Course

Laboratory Equipment and Supplies Exposition

For further information, contact: Administrative Manager, AOAC, 1111 N. 19th St., Ste. 210, Arlington, VA 22209, or call (703) 522-3032.

MYCOTOXINS

Comparison of Four Thin-Layer Chromatographic Methods for the Determination of Aflatoxins in Raisins

DİLEK BOYACIOĞLU and MERAL GÖNÜL
University of Ege, Engineering Faculty, Food Engineering Department, 35101 Bornova, İzmir, Turkey

A study was conducted to determine the accuracy and precision of 3 AOAC methods, secs 26.026-26.031 (CB), secs 26.032-26.036 (BF), and secs 26.052-26.060 (cottonseed), and the Romer quantitative method for the thin-layer chromatographic (TLC) determination of aflatoxins B_1, B_2, G_1, and G_2 in raisins. The samples were spiked at a level of 10 μg total aflatoxins/kg. The TLC development systems were: ether–methanol–water (94 + 4.5 + 1.5) and chloroform–acetone (9 + 1). The interaction between the 4 methods and the 2 development systems was also studied. The average recoveries were 88, 80, 75, and 93% with coefficients of variation of 14.0, 10.4, 14.0, and 9.6% for aflatoxin B_1 using the CB, BF, cottonseed, and Romer methods, respectively. Statistical analysis showed no difference in the results obtained using the 2 TLC development systems.

Several methods have been developed to determine aflatoxins in various foodstuffs (1–4). However, it is necessary to evaluate the efficiency and suitability of methods for other agricultural commodities or products that may be susceptible to aflatoxin contamination. Although Romer (4) reported the determination of low levels of aflatoxins in different commodities (including raisins), the suitability of available methods has not been extensively studied in raisins, which are one of the most important export crops of the Ege area of Turkey (5).

Although aflatoxin-producing strains of *Aspergillus flavus* have been isolated from raisins (6), no aflatoxin problem in raisins has ever occurred. However, past experience has shown that we must be prepared in case an outbreak does occur. Furthermore, we wished to check the suitability and efficiency of available methods for a survey of raisins. Hence, the present study was conducted to compare the following AOAC (7) methods: secs **26.026-26.031** (CB), secs **26.032-26.036** (BF), and secs **26.052-26.060** (cottonseed), and the Romer quantitative method for the thin-layer chromatographic (TLC) determination of aflatoxins in raisins (4).

Experimental

Samples

Raisins were homogenized by adding water at the ratio 1:1 in Waring Blendor. Then, they were spiked according to AOAC methods by adding aflatoxin standards at levels of 4 μg/kg for aflatoxins B_1 and G_1 and 1 μg/kg for aflatoxins B_2 and G_2.

Aflatoxin Standards

Standards were prepared from pure crystalline aflatoxins B_1, B_2, G_1, and G_2 (Aldrich Chemical Co., Milwaukee, WI 53233) dissolved in benzene–acetonitrile (98 + 2). TLC working solutions were diluted to contain 0.5 μg B_1 and G_1/mL and 0.125 μg B_2 and G_2/mL for quantitative visual determination. The concentration of these standards was checked by measuring their ultraviolet absorption with a UV-Vis Pye Unicam SP 9 spectrophotometer, as described in secs **26.004-26.012** (7).

Received December 9, 1986. Accepted June 5, 1987.

TLC Methods

The published methods (4, 7) were used with the following modifications.

In the BF method, secs **26.032-26.036** (7), a mixture of the sample and the extraction solvent was transferred to a separatory funnel because of the lack of a large capacity centrifuge to separate the phases.

In the Romer method, the sample extracts for TLC were prepared by quantitatively transferring the eluate to a vial and then evaporating just to dryness under a stream of nitrogen in a water bath (4).

The chloroform extracts obtained in the final step of the 4 methods were filtered through a Na_2SO_4 bed to remove moisture, which would cause cloudiness.

The fluorescence intensities of the sample extracts and standards were visually compared under longwave ultraviolet light. The sample extracts were diluted to 100 μL with chloroform after they were evaporated just to dryness under a stream of nitrogen in a water bath. The TLC plates were coated with Adsorbosil (with 10% $CaSO_4$ binder, Applied Science Laboratories, State College, PA 16801). The coated plates were 500 μm thick to provide better resolution of standard spots.

Results and Discussion

Results obtained by the 4 methods are shown in Table 1 along with the means, standard deviations, coefficients of variation (CV), and average percent recoveries for aflatoxins B_1, B_2, G_1, and G_2 and total aflatoxins. No statistical difference was found between the results obtained using the chloroform–acetone (9 + 1) and ether–methanol–water (94 + 4.5 + 1.5) development systems, as shown in Table 2, but use of the latter development system is suggested because it facilitates the visual estimation by completely removing the interferences from sample spots on the TLC plates.

An evaluation of the average recoveries (Table 1) indicated that there were differences among the methods' results (Table 2). Therefore, those differences were tested by a least significant difference (LSD) test, as shown in Table 3.

The CVs were 9.6% for the Romer method and 10.4% for the BF method for aflatoxin B_1, indicating good precision or high repeatability for these 2 methods. Although the repeatability of the BF method was relatively high, its accuracy was unsatisfactory since it fell in the second group in the LSD test as did the CB method (Table 3). However, the accuracy of the Romer method was quite good for aflatoxin B_1; its average recovery was 93% (Table 1), and it alone comprises the first group in the LSD test (Table 3).

For total aflatoxins, the highest repeatability was obtained using the Romer and BF methods, with CVs of 5.7 and 7.0%, respectively (Table 1). However, the accuracy of the BF method was relatively lower than the other methods, as is shown by it alone being the second group in the LSD test (Table 3). The first group in the same test comprises the Romer, CB, and cottonseed methods, indicating good accuracy at a total aflatoxin level of 10 μg/kg (Table 3); however,

Table 1. Comparison of results (µg/kg) of analysis of spiked raisin samples using 4 TLC methods

Replicate No.	Development system: Chloroform–acetone (9 + 1)					Ether–methanol–water (94 + 4.5 + 1.5)				
	B_1	B_2	G_1	G_2	Total	B_1	B_2	G_1	G_2	Total
Romer method										
1	3.2	1.00	3.3	1.00	8.5	4.7	1.40	3.6	0.91	10.6
2	3.6	1.00	3.5	0.87	9.0	4.0	0.83	3.7	0.91	9.4
3	3.6	0.83	3.2	1.10	8.7	3.6	0.83	3.2	1.00	8.4
4	4.0	0.83	3.4	1.00	9.2	3.6	0.85	3.4	0.91	8.8
5	4.0	0.97	3.7	1.00	9.7	4.3	0.97	3.5	0.91	9.7
6	4.0	0.97	3.4	0.91	9.3	4.0	0.97	3.5	1.40	9.9
7	3.2	0.83	3.5	0.90	8.4	3.3	0.83	3.4	0.91	8.4
8	4.0	0.85	3.7	1.20	9.8	3.8	0.84	3.2	0.92	8.8
Mean	3.7	0.91	3.5	1.00	9.1	3.9	0.94	3.4	0.98	9.3
Std dev.	0.36	0.08	0.18	0.11	0.52	0.44	0.20	0.18	0.17	0.79
CV, %	9.6	8.8	5.1	11.0	5.7	11.3	20.9	5.2	17.6	8.5
Av. rec., %	93	91	88	100	91	98	94	85	98	93
CB method										
1	3.7	0.94	5.5	0.90	11.0	2.9	1.00	5.5	1.10	10.5
2	3.7	1.10	4.9	0.67	10.4	4.9	1.70	4.6	0.89	12.0
3	3.5	1.20	4.1	1.55	10.4	3.1	2.00	4.5	1.30	10.9
4	3.3	1.7	3.7	0.45	9.2	2.1	1.90	3.7	0.89	8.6
5	4.5	1.2	4.6	0.55	10.9	3.7	1.30	6.4	1.10	12.5
6	3.3	2.0	2.8	0.67	8.8	2.3	1.90	3.7	0.91	8.8
7	3.1	1.1	2.7	0.45	7.4	1.8	0.85	2.8	0.67	6.1
8	2.9	0.95	1.8	0.45	6.1	1.6	1.20	2.7	0.77	6.3
Mean	3.5	1.27	3.8	0.71	9.3	2.8	1.48	4.2	0.95	9.5
Std dev.	0.49	0.38	1.26	0.37	1.77	1.10	0.45	1.28	0.20	2.43
CV, %	14.0	29.0	33.0	52.5	19.1	39.4	30.3	30.5	21.3	25.6
Av. rec., %	88	130	95	71	93	70	148	105	85	95
BF method										
1	3.4	0.84	3.2	0.84	8.3	3.2	0.80	3.0	0.91	7.9
2	3.3	0.78	2.7	0.58	7.4	3.1	0.75	2.8	0.67	7.3
3	3.7	0.91	2.6	0.87	8.1	3.6	0.91	2.7	0.81	8.0
4	3.1	0.55	3.6	0.95	8.2	3.0	0.65	3.4	0.81	7.9
5	2.7	0.70	2.8	0.70	6.9	2.9	0.71	3.0	0.73	7.3
6	3.4	0.55	3.0	0.50	7.5	3.5	0.60	2.9	0.58	7.6
7	2.9	0.67	3.2	0.48	7.3	3.1	0.64	3.4	0.55	7.7
8	2.9	0.50	2.5	0.51	6.7	2.5	0.71	2.8	0.40	6.4
Mean	3.2	0.69	3.0	0.68	7.6	3.1	0.72	3.0	0.68	7.5
Std dev.	0.33	0.15	0.37	0.19	0.60	0.34	0.10	0.27	0.17	0.52
CV, %	10.4	21.6	12.3	27.6	7.8	11.1	13.8	8.9	24.5	7.0
Av. rec., %	80	69	75	68	76	78	72	75	68	75
Cottonseed method										
1	2.5	0.46	3.5	0.67	7.1	2.7	0.78	4.2	0.71	8.4
2	2.9	0.83	2.5	0.71	6.9	2.9	1.04	3.3	0.81	8.1
3	3.4	0.53	3.2	1.01	8.1	3.6	0.53	3.8	1.01	8.9
4	3.0	1.30	4.2	1.01	9.5	3.2	1.30	5.0	1.01	10.5
5	2.7	0.53	2.5	0.61	6.3	2.9	0.78	4.2	1.01	8.9
6	2.7	0.78	2.9	0.81	7.2	2.6	1.30	3.3	1.01	8.2
7	3.7	1.30	4.2	1.01	10.2	3.8	1.30	5.0	1.21	11.3
8	3.4	1.31	3.4	1.01	9.1	3.8	1.56	5.0	1.21	11.6
Mean	3.0	0.88	3.3	0.86	8.1	3.2	1.0	4.2	1.00	9.5
Std dev.	0.42	0.37	0.67	0.18	1.41	0.49	0.35	0.73	0.17	1.43
CV, %	14.0	42.3	20.2	20.3	17.4	15.3	32.7	17.3	17.3	15.0
Av. rec., %	75	88	83	86	81	80	107	105	100	95

Table 2. Analysis of variance of average recovery values obtained for aflatoxins B_1, B_2, G_1, and G_2 and total aflatoxins

Variation	df[a]	Average of error squares: B_1	B_2	G_1	G_2	Total
Methods	3	1.80[b]	1.21[b]	3.14[b]	0.29[b]	10.84[b]
Development systems	1	0.16	0.24	2.03	0.15	3.11
Interaction between method and solvents	3	0.70	0.05	0.77	0.06	1.81
Residue	56	0.30	0.09	0.56	0.04	1.82

[a] df = degrees of freedom.

[b] Significant at 1% level.

Table 3. Statistical comparison of the average recoveries of aflatoxins B_1, B_2, G_1, and G_2 and total aflatoxins using 4 TLC methods

Method	B_1	B_2	G_1	G_2	Total
Romer	3.81^{a}	0.93^{bc}	3.07^{bc}	0.99^{a}	9.16^{a}
CB	3.15^{b}	1.37^{a}	3.63^{a}	0.83^{bc}	9.37^{a}
BF	3.14^{bc}	0.70^{c}	2.98^{c}	0.68^{c}	7.53^{b}
Cottonseed	3.11^{c}	0.98^{b}	$3.39^{a,b}$	0.93^{ab}	8.77^{ab}
LSD	0.31	0.31	0.37	0.20	1.35

Values not sharing a common superscript in each column are significantly different, $P < 0.05$.

the repeatabilities of the CB and cottonseed methods were not satisfactory in comparison with the Romer method.

A statistical evaluation of the results indicates that the Romer quantitative method (4) is more accurate and precise than the other TLC methods for the determination of aflatoxins in raisins.

References

(1) Eppley, R. M., Stoloff, L., & Campbell, A. D. (1968) *J. Assoc. Off. Anal. Chem.* **51**, 67–73

(2) Pons, W. A., Jr, Cucullu, A. F., Franz, A. O., Jr, & Goldblatt, L. A. (1968) *J. Am. Oil Chem. Soc.* **45**, 694–699

(3) Waltking, A. E., Bleffert, G., & Kreman, M. (1969) *J. Am. Oil Chem. Soc.* **46**, 880–884

(4) Romer, T. R. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 500–506

(5) Gönül, M., & Boyacioğlu, D. (1985) Ege University, *J. Engineering Faculty* 3(1), 127–135

(6) Follstad, M. N. (1966) *Phytopathology* **56**, 1413

(7) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **26.004-26.012, 26.026-26.036, 26.052-26.060**

Potentials of a Synchronized Culture of *Tetrahymena pyriformis* for Toxicity Studies of Mycotoxins

JAN P. BIJL and DOMINIQUE M. ROUSSEAU

State University of Ghent, Pharmaceutical Institute, Laboratory of Food Analysis, Harelbekestraat 72, B-9000 Gent, Belgium

DANIEL G. DIVE

Institut National de la Santé et de la Recherche Médicale (INSERM), Domaine du CERTIA, 59650 Villeneuve d'Ascq, France

CARLOS H. VAN PETEGHEM

State University of Ghent, Pharmaceutical Institute, Laboratory of Food Analysis, Harelbekestraat 72, B-9000 Gent, Belgium

A synchronized culture of *Tetrahymena pyriformis* is proposed for toxicity testing of mycotoxins. Toxicity is evidenced through a delay in the division of the synchronized culture. Newly developed equipment for the synchronization consists of a double-walled culture flask built in an alternating water circuit at 28°C (optimum) and 34°C (sublethal), respectively, controlled by a time switch. The cultures are used for the detection of 16 mycotoxins for which the detection threshold is given. Synchronous cultures of *Tetrahymena pyriformis* appear to be very suitable for the detection of trichothecenes. Preliminary experiments on artificially contaminated corn samples demonstrate the possibility of prescreening of cereals.

In ecotoxicology, protozoa are often used for testing toxicity of chemicals that could be released in the aquatic ecosystems (1–4). The protozoan tests are based on either morphological, ultrastructural, ethological, or metabolic criteria. It has been shown that the growth rate of ciliates can be used for testing toxicity of fungal metabolites (5). Ueno and Yamakawa (6) tentatively used synchronous cultures of *Tetrahymena pyriformis* to study the toxicity of the trichothecenes diacetoxyscirpenol (DAS) and T-2 toxin. In a comparative study of bioassays (7), we have shown that the cultures of *Tetrahymena*, synchronized by the "one shock per generation" defined by Zeuthen (8), gave very good results in the detection of DAS and trichothecin. Also, because the cell cycle is less disturbed than that in the "multi shock" method used by Ueno, our method allowed a more precise analysis of the toxicity.

Plesner, Rasmussen, and Zeuthen (9) described in detail the different pieces of apparatus suitable for the synchronization of *Tetrahymena*. In the present paper, we describe a simple unit we have used to determine the detection threshold of 16 mycotoxins. The results obtained are compared with the detection levels for both chemical and biological methods. The possibilities of using this bioassay as a prescreening technique are partially explored with artificially contaminated corn samples.

Received January 15, 1987. Accepted May 4, 1987.

This work was supported by FGWO grant No. 3.0033.83.

Experimental

Reagents and Organism

All reagents used were analytical grade.

(**a**) *Ethyl acetate*.—Distilled from an all-glass apparatus.

(**b**) *Mycotoxins*.—Diacetoxyscirpenol, T-2 toxin, roridin A (Makor Chemicals, Jerusalem, Israel); neosolaniol, nivalenol, deoxynivalenol, fusarenon-X (Ueno, Tokyo, Japan); trichothecin (Freeman, Guildford, England); patulin, ochratoxin A, zearalenone, aflatoxins B_1, B_2, G_1, G_2 (Aldrich, Brussels, Belgium); paracelsin (Brückner, Stuttgart, FRG).

(**c**) *Strain*. —*Tetrahymena pyriformis* Phenoset A (Zeuthen strain) is cultivated axenically at 28°C on proteose peptone (0.75%) yeast extract (0.75%), which is enriched with salts (PPYS medium) as described by Plesner et al. (9). The culture is maintained in exponential growth phase by daily transfer of one drop of culture into 10 mL fresh medium.

Synchronization Apparatus

A schematic outline of the apparatus is given in Figure 1. For synchronization, ciliates are inoculated to 100 mL prewarmed medium in double-walled flask plugged with cotton wool. Initial cell concentration is 500–1000 cells/mL. Six 20 min heat shocks at 34°C separated by 160 min periods at 28°C allow synchronization of the culture.

Exposure of Cells to Toxins

At the end of the 6th heat shock, which is time 0 of the experiment, 10 mL aliquots of the culture are distributed in 100 mL screw-cap Erlenmeyer flasks held at 28°C. Toxins are added as either methanol or dimethyl sulfoxide (DMSO)

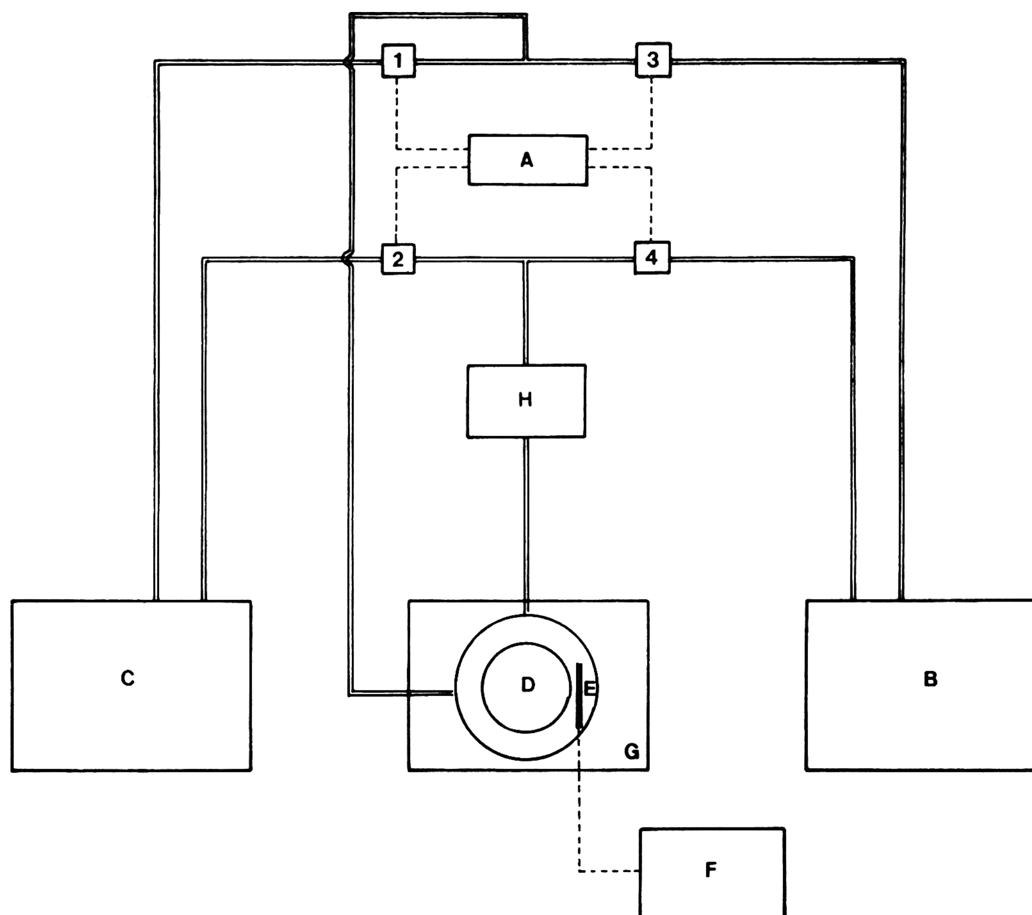


Figure 1. Schematic outline of synchronization apparatus.

A, Time switch module (Electromatic, Vel, Leuven, Belgium); B, immersion heater with thermostat (GFL, Vel) at 34.0 ± 0.1°C combined with 25 L bath; C, water bath at 28.0 ± 0.1°C with cooling capacity (Edmund Bühler, Vel); D, double-walled culture flask (Vel); E, temperature sensor (JUMO, Vel); F, temperature recorder (JUMO, Vel); G, rotary shaker (GFL, Vel); H, rotary pump, 6 L/min capacity (Eheim, Vel); 1–4, electromagnetic valves (Vel).

solutions. One mL each of cultures and a control without toxin are sampled at time 10, 40, 80, 90, 100, 110, 130, and 150 min and fixed with an equal volume of a 2.5% glutaraldehyde solution. Cells are counted electronically with Coulter Counter Model ZM (aperture 140 μm) after dilution with 10 mL 1% NaCl solution.

Preparation of Corn Extracts

The ground corn sample is extracted with ethyl acetate according to Ikediobi (10). For spiked corn, a known amount of standard solution in methanol is added to the ground sample and equilibrated ≥30 min before extraction. The extract is washed with water, filtered through anhydrous sodium sulfate, and evaporated under reduced pressure at 40°C; an aliquot equivalent to 10 g sample is dissolved in 100 μL dimethyl sulfoxide. After it is thoroughly shaken with 5 mL PPYS medium, this mixture is filtered on a membrane filter.

Exposure of Cells to Extracts

The filtered medium with extract is mixed with 5 mL synchronized culture and the cell division is followed as described under *Exposure of Cells to Toxins.*

Results and Discussion

Efficiency of Synchronization System

Zeuthen (8) has shown that a series of heat shocks spaced a normal generation time apart (which is 159 ± 2 min (n = 8) in the conditions described above) results in a good division synchrony. The temperature alternations between 28 and 34°C need to be performed in as short a time as possible. This can be realized most efficiently by abruptly changing the water content of the jacket. By using a double water circuit at the respective temperatures, this change is almost immediate because the content of the water jacket is only 300 mL. To compensate for the temperature rise in the 28°C bath by the entry of the warm water, a cooling device is built in. For the 34°C circuit, a 25 L bath is used to minimize the temperature drop by the entry of the cold water. To promote the heat exchange between the circulating water and the culture medium, the inner wall of the flask is constructed with thin glass. The culture is shaken continuously on a rotary shaker to ensure good aeration, which is essential for growth and to avoid clogging of the cells.

With the synchronization unit described here, 90% of cells have divided 40 min after the start of the division (Figure 2).

Effects of Mycotoxins on Synchronous Division

The recording of the results is simple because the division of the organisms starts within 80–90 min after the end of the last heat shock. Preliminary tests showed that within the same experiment the start of division in the different subcultures distributed into Erlenmeyer flasks differs 5 min or less. Thus, a delay of more than 10 min between start of division in a control and in a toxin-treated culture can be considered as an indication of significant toxicity and is re-

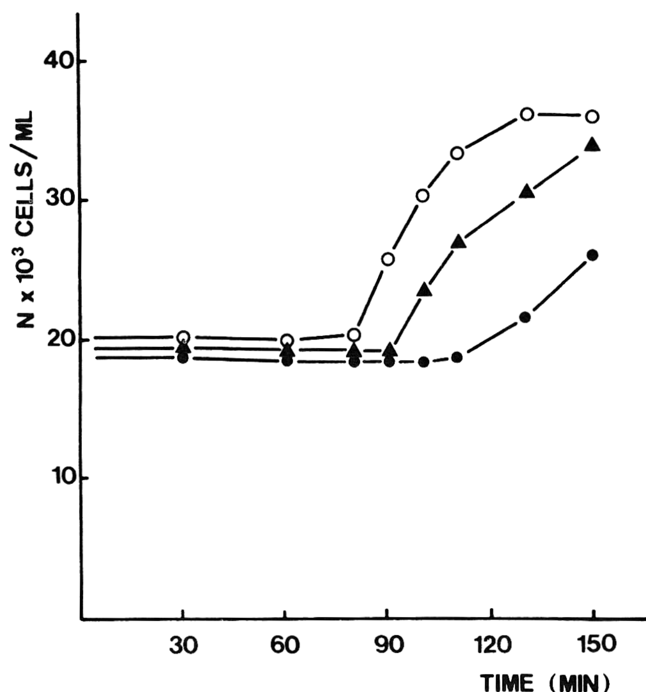


Figure 2. Growth curves of *Tetrahymena*: ○ = control; ▲ = 0.1 μg trichothecin/mL PPYS (=MAD); ● = 0.48 μg trichothecin/mL PPYS.

ferred to as the minimal active dose (MAD) (Figure 2). In comparison with exponential growth curves which need much more calculation and for which the effect is not clear for low doses, the present interpretation is quite simple. Moreover, the final test procedure takes 150 min or less.

Experiments showed that 0.1% (v/v) methanol and 1% (v/v) dimethyl sulfoxide can be added without any perceptible effect on the start of the division. However, the possible interaction between solvent and toxin was not studied during the experiment, so the substance must be added in the minimal amount of solvent.

The MAD for 16 mycotoxins studied is given in Table 1. The synchronous cultures of *Tetrahymena* are very sensitive to trichothecenes, especially diacetoxyscirpenol, T-2 toxin, and roridin A. Interesting results are also obtained with trichothecin, fusarenon-X, patulin, and vomitoxin. On the other hand, aflatoxins and ochratoxin A are not toxic up to 10 μg/mL. This result is in accordance with observations of Teunisson and Robertson (11) and Dive et al. (5). The detection threshold for other mycotoxins is too high to be suited for screening purposes.

Ueno and Yamakawa (6), using *Tetrahymena* cultures synchronized by another method, observed a similar sensitivity for DAS, nivalenol, and fusarenon-X. Concerning this latter toxin, the high sensitivity of the ciliates can be correlated with the competition of fusarenon-X with tetrahymanol for incorporation in the cell membranes, as shown by Chiba et al. (12).

Comparison with literature data shows that *Tetrahymena* is as sensitive as the mammalian cell lines HEp-2 and Chang used by Robb and Norval (13). It must be said that these authors used only 1 mL culture, which lowers the concentration that can be detected, and that the applicability of their method is not proven. Mammalian cells appear to be particularly affected by roridin A. The 2 cell lines used by Robb and Norval (13) exhibit a significant difference in their sensitivity to DAS, roridin A, and T-2 toxin. Ehrlich and Daigle (14) observed the same phenomenon when they studied the

Table 1. Minimal active doses (MAD) for 16 mycotoxins in synchronized culture toxicity studies

Mycotoxin	MAD, μg/mL PPYS
Trichothecenes:	
Diacetoxyscirpenol	0.020
T-2 toxin	0.025
Neosolaniol	1.0
Nivalenol	1.6
Deoxynivalenol	0.6
Fusarenon-X	0.4
Trichothecin	0.1
Roridin A	0.025
Patulin	0.4
Ochratoxin A	10.0
Zearalenone	2.5
Aflatoxins:	
B_1	>10.0
B_2	>10.0
G_1	>10.0
G_2	>10.0
Parace sin	>10.0

sensitivity of Vero and MELC cell lines to T-2 toxin. The other biological methods proposed in the literature, Microtox R test (15), yellow mealworm (16), and algae (17), are less sensitive. Gendloff et al. (18) developed a very sensitive enzyme-linked immunosorbent assay for detection of T-2 toxin in infected corn but, if the method is very sensitive and specific for T-2, T-2HS, and acetyl T-2 toxins, it cannot be used for the other trichothecenes. With reference to the chemical methods of detection, the results obtained by our method show that *Tetrahymena* may constitute a short-term bioassay for the detection of certain mycotoxins, particularly DAS, T-2 toxin, and roridin A.

Effects of Spiked Extracts on Synchronous Division

Extracts of corn spiked with diacetoxyscirpenol or trichothecin show the same toxicity as the equivalent quantity of pure toxins. By the method used, the concentration in the extract and the medium is the same. Blank extracts revealed no toxicity, from which it can be concluded that this test can be used as a prescreening bioassay for contaminated corn.

A problem which might be encountered is the coextraction of pesticide residues with mycotoxins and a subsequent intoxication of the ciliates. This phenomenon was apparently not observed by workers using bioassays for the direct detection of mycotoxins in cereals. The results obtained with the ciliate *Colpidium campylum* (2) indicate that the hazard is likely to be encountered with dithiocarbamates fungicides (thiram-type) because the ciliates are relatively insensitive to pesticides.

References

(1) Dive, D. (1981) *Bull. Inst. Natl. Santé Rech. Méd.* **106,** 273–294
(2) Dive, D., Persoone, G., & Leclerc, H. (1980) *Ecotoxicol. Environ. Saf.* **4,** 129–133
(3) Dive, D., & Personne, G. (1984) in *Ecotoxicological Testing for the Marine Environment,* G. Persoone, E. Jaspers, & C. Claus (Eds). State University of Ghent and Institute of Marine Scientific Research, Bredene, Belgium, pp. 281–305
(4) Persoone, G., & Dive, D. (1978) *Ecotoxicol. Environ. Saf.* **2,** 105–114
(5) Dive, D., Moreau, S., & Cacan, M. (1978) *Bull. Environ. Contam. Toxicol.* **18,** 489–495
(6) Ueno, Y., & Yamakawa, H. (1970) *Jpn. J. Exp. Med.* **40,** 385–390

(7) Bijl, J., Dive, D., & van Peteghem, C. (1981) *Environ. Pollut.* **26**, 173-182
(8) Zeuthen, E. (1971) *Exp. Cell Res.* **68**, 49-60
(9) Plesner, P., Rasmussen, L., & Zeuthen, E. (1964) in *Synchrony in Cell Division and Growth*, E. Zeuthen (Ed.), Interscience Publishers, New York, NY, pp. 543-563
(10) Ikediobi, C. (1971) *Anal. Biochem.* **43**, 327-340
(11) Teunisson, D., & Robertson, J. (1967) *Appl. Microbiol.* **15**, 1099-1103
(12) Chiba, J., Nakano, N., Morooka, N., Nakazawa, S., & Watanabe, Y. (1972) *Jpn. J. Med. Sci. Biol.* **25**, 291-296
(13) Robb, J., & Norval, M. (1983) *Appl. Environ. Microbiol.* **46**, 949-950
(14) Ehrlich, K., & Daigle, K. (1985) *Appl. Environ. Microbiol.* **50**, 914-918
(15) Yates, I., & Porter, J. (1982) *Appl. Environ. Microbiol.* **44**, 1072-1075
(16) Davis, G., Smith, J., Schieffer, B., & Loew, F. (1975) *J. Invertebr. Pathol.* **26**, 299-303
(17) Sullivan, J., Jr, & Ikawa, M. (1972) *J. Agric. Food Chem.* **20**, 921-922
(18) Gendloff, E., Pestka, J., Swanson, S., & Hart, L. (1984) *Appl. Environ. Microbiol.* **47**, 1161-1163

Interactive Effects of Duration of Storage and Addition of Formaldehyde on Levels of Aflatoxin M_1 in Milk

SUSAN K. HEIMBECHER, KAREN V. JORGENSEN, and RALPH L. PRICE[1]
University of Arizona, Department of Nutrition and Food Science, Tucson, AZ 85721

Spray-dried skim milk, naturally contaminated with aflatoxin M_1, was added to either raw or pasteurized whole milk to a final concentration of 1.1 μg aflatoxin M_1/L milk. Formalin (37% w/w) was added to the milk solutions to final concentrations of 0, 0.025, 0.05, and 0.1% formaldehyde. Samples were stored in the dark at 21°C in plastic and glass containers and were analyzed for aflatoxin M_1 at 0, 1, 2, 3, and 4 weeks. This experiment was repeated using only raw milk and glass containers. Aflatoxin M_1 analyses were done at 0, 1, and 2 weeks. Aflatoxin M_1 losses increased over time and with increased formaldehyde concentration. With both experiments, aflatoxin M_1 levels after 2 weeks were less than 0.05 μg/L in samples containing 0.1% formaldehyde.

Although it is desirable to analyze milk for aflatoxin M_1 as soon as possible after it is drawn, at times the milk must be stored before analysis. There are conflicting reports regarding the effect of storage on aflatoxin M_1 levels in dairy products. Kiermeier and Mashaley (1) noted aflatoxin M_1 losses of 11-25% in both artificially and naturally contaminated milk after 1-3 days storage at 5°C. Stoloff et al. (2) found no losses in either pasteurized or raw, artificially contaminated milk after 17 days at 4°C.

During the analysis of any commodity for very low levels of contaminant, the coefficient of variation between and even within laboratories increases as the level of the contaminant decreases (3, 4). Explanations for this variability include laboratory experience, use of different analytical methods, instability of aflatoxin M_1 extracts or standard solutions, presence of interfering substances, age of sample, storage conditions, and the low levels measured. This variability has led to studies concerning preservation of milk containing aflatoxin M_1.

Unrefrigerated storage conditions and the length of time necessary to ship analytical samples can lead to milk deterioration and to variation in the amount of aflatoxin M_1 detected. Sodium azide, potassium dichromate (5), and several oxidizing agents have been tested as preservatives for milk, but these have not been successful (R. D. Stubblefield, personal communication; 5). Historically, formaldehyde was used as a milk preservative prior to refrigeration and for this reason was chosen for use as a preservative in the 1986 Smalley Series for M_1 at a level of 0.03% (J. D. McKinney, personal communication). Formaldehyde has been shown to reduce levels of aflatoxins B_1, B_2, G_1, and G_2 during detoxification studies (6, 7) and may be expected to reduce M_1 levels. The purpose of the present study was to determine the effect of formaldehyde on aflatoxin M_1 levels in raw and pasteurized milk which is stored in glass or plastic containers for up to 4 weeks.

Experimental

Contaminated Spray-Dried Milk

Naturally contaminated, nonfat, spray-dried milk was obtained from United Dairymen of Arizona. Raw milk had been collected from Holstein dairy cows fed a 15% ration of naturally contaminated cottonseed containing total aflatoxins of 2500-5000 mg/kg. Cream had been removed from contaminated milk using a separator. Skim milk had been spray-dried using an inlet temperature of 210-227°C and an outlet temperature of 91-99°C. The resulting spray-dried milk was stored at −20°C for 1 year.

Sample Preparation

Experiment 1.—Spray-dried milk, containing approximately 7.5 μg aflatoxin M_1/L, was mixed with either raw milk or pasteurized whole milk to a final level of approximately 1.1 μg/L. Formalin solution (37% w/w) was added to the milk solutions to final concentrations of 0, 0.025, 0.05, and 0.1% formaldehyde (v/v). Two-hundred mL aliquots of the milk solutions were transferred to either glass or plastic containers, randomly coded to prevent bias, and stored in the dark at 21°C for up to 4 weeks. Samples were pulled and analyzed once a week for 1, 2, 3, and 4 weeks.

Experiment 2.—Samples were prepared as in experiment 1 except that raw milk was held up to 2 weeks in glass containers.

Milk Analysis

The original spray-dried milk and all samples were analyzed according to AOAC official method **26.095-26.099** (8). All chemicals used were AR grade, and methylene chloride was substituted for chloroform. Extracts from the experiment were stored at −20°C in benzene-acetonitrile (9 + 1) for 3 months (9) until all samples could be analyzed by liquid

Received April 21, 1987. Accepted June 30, 1987.
Arizona Agricultural Experiment Station journal article No. 4368.
[1] Address reprint requests to this author.

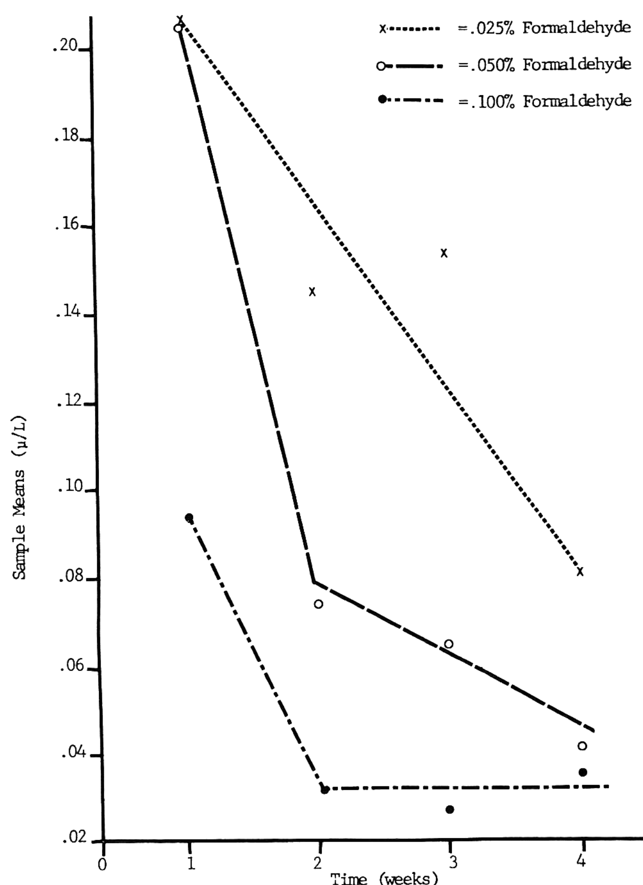


Figure 1. Aflatoxin M_1 concentration vs time and formaldehyde level in experiment 1.

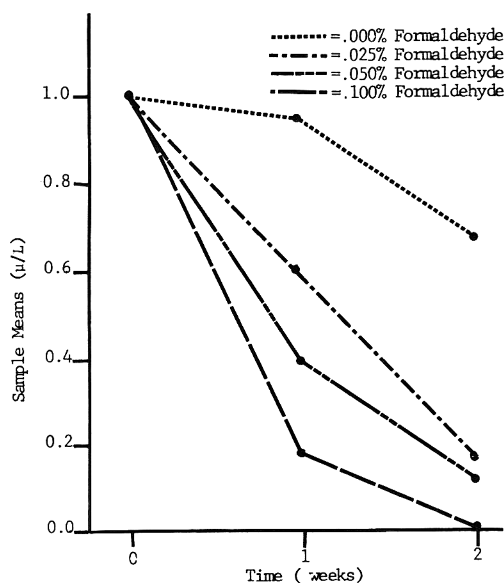


Figure 2. Aflatoxin M_1 concentration vs time and formaldehyde level in experiment 2.

chromatography. Depending on the aflatoxin M_1 level, LC analysis was performed on 10–50 μL aliquots. The LC system was a Waters modular unit that included a μC_{18} reverse-phase column, M600A pump, Wisp 710 automatic injector, 420E fluorescence detector (365–400 nm), and a data modular integrator (Waters Chromatography, Milford, MA 01757). The mobile phase was water–methanol–acetonitrile (52 + 26 + 22) with a flow rate of 0.9 mL/min and a pressure of 2000 psi for experiment 1 and 1500 psi for experiment 2. The aflatoxin M_1 standard concentration was 10 μg/L (Eureka Laboratories, Sacramento, CA 95816).

Statistical Analysis

Statistical analysis was performed on a computer using a program for a 4-way ANOVA for experiment 1 and a 2-way ANOVA for experiment 2. Means were separated by least significant difference at $P < 0.05$.

Results and Discussion

Formation of emulsions during extraction with chloroform or methylene chloride has been a continual problem when this official method for aflatoxin M_1 has been used (8, 10). Manipulations (centrifugation, addition of salt or urea, heating the chloroform or methylene chloride) used to break the emulsions, if possible, have sometimes resulted in low recoveries or loss of sample. This problem, which did not occur in this laboratory during preliminary experiments, nor in the Smalley test series, caused the loss of many samples from experiment 1, including all pasteurized samples with no added formaldehyde stored for 1 week or longer. It may have occurred because of the added solids to the fluid milk. Gentle shaking and swirling of the separatory funnel were unsuccessful in preventing emulsion formation as were the previously mentioned suggestions. The solution of this problem was to revert to the procedure previously used in this laboratory. The milk was refrigerated (4°C) and the separatory funnel was shaken in the following manner: The funnel was inverted and gas was released, then the funnel was held horizontally and shaken 60 times as hard and fast as possible. The funnel was then immediately turned upright, and the stopper was removed. No emulsions were formed using this technique. The reason that vigorous agitation at this stage promotes the breaking (or non-formation) of the emulsion is unknown.

Experiment 1

Although some data were lost in this preliminary experiment because all pasteurized samples without formaldehyde formed emulsions, some of the results were significant (Figure 1) and led to further modification in the second experiment.

Analyses for aflatoxin M_1 are usually performed on raw milk when it is delivered at the processing plant. No difference was found in the amount of aflatoxin M_1 detected in the raw and pasteurized samples containing formaldehyde.

Aflatoxin M_1 losses were greater for those samples stored in plastic than for those in glass. However, the effect was significant only for those samples containing 0.025% formaldehyde.

Aflatoxin M_1 levels decreased over time for all treatment groups, with averages of 0.17 μg/L at 1 week down to 0.05 μg/L at 4 weeks (Figure 1). No extractions were done at 0 time for samples containing 0.025–0.1% formaldehyde because it was expected that the formaldehyde level would have no effect on aflatoxin M_1 recovery at 0 time. Although this expectation was confirmed by measurements performed in experiment 2, the data for 0 time were incomplete and therefore could not be included in the 4-way ANOVA. Coagulation occurred and pH decreased after one week in samples to which formaldehyde was not added. After 4 weeks, the pH of samples had decreased from 6.5 to 5. Although samples containing formaldehyde were not coagulated and showed only minimal pH decrease after 4 weeks, a much greater decrease in aflatoxin M_1 was seen (Figure 1).

Aflatoxin M_1 levels decreased from 0.15 to 0.05 μg/L as formaldehyde levels increased from 0.025 to 0.1%. The de-

crease may be due to either degradation of aflatoxin M_1 or a decrease in its extractability. Codifer et al. (7) found that 80% of aflatoxin B_1 in contaminated peanuts was destroyed by formaldehyde treatment, and it is likely that aflatoxin M_1 would also be broken down by this treatment because the structures of B_1 and M_1 are similar. Formaldehyde, a cross-linking agent, is known to react with casein proteins, and its effects on the microenvironment of milk could lead to entrapment of aflatoxin M_1 and thereby reduce recovery.

Experiment 2

Due to the analytical problems and the lack of data at 0 time in experiment 1, the effect of formaldehyde and time on aflatoxin M_1 levels was reexamined in a second experiment. Samples containing the same levels of formaldehyde were analyzed at 0, 1, and 2 weeks. Because there was no significant difference between aflatoxin M_1 levels in milk types in experiment 1 and because raw milk is more resistant to the formation of emulsions, all experiment 2 samples were raw milk. To reduce the number of samples, only glass containers, which showed less of a negative effect on aflatoxin M_1 levels in experiment 1, were used.

The milk samples were prepared to contain approximately 1.1 μg aflatoxin M_1/L. The amount detected ranged from 91% of the original, 0 time, to nondetectable levels for samples which contained 0.1% formaldehyde and were left in storage for 2 weeks. The higher aflatoxin M_1 levels detected in samples at 0 time in this experiment were not surprising because the problems with the emulsions had been resolved. Aflatoxin M_1 levels decreased from 1.0 μg/L (0 time) to 0.025 μg/L (2 weeks), and a positive interaction occurred between formaldehyde and time (Figure 2).

Aflatoxin M_1 levels in milk samples containing no formaldehyde decreased by 34% over 2 weeks. Although formaldehyde had no immediate effect on aflatoxin M_1 levels, samples extracted at weeks 1 and 2 showed increasing losses with increasing formaldehyde concentrations. Aflatoxin M_1 levels decreased from 0.87 to 0.39 μg/L as formaldehyde levels increased from 0.0 to 0.1%. The greatest losses in formaldehyde-containing samples occurred in those stored for 1 week and may have resulted from the high initial levels of chemical reactants. These aflatoxin M_1 losses with time and formaldehyde concentration were similar to those of experiment 1.

Although formaldehyde decreased emulsions in pasteurized milk and reduced coagulation in raw and pasteurized milk, results from this research indicate that aflatoxin M_1 levels in milk samples preserved with 0.025 to 0.1% formaldehyde and stored at room temperature may be expected to decrease by 45–85% after 1 week. Because the negative effects of formaldehyde on aflatoxin M_1 in milk outweigh the benefits, it should not be used as a preservative for M_1-contaminated milk samples used for collaborative studies.

References

(1) Kiermeier, F., & Mashaley, R. (1977) *Z. Lebensm. Unters. Forsch.* **164**, 183–187
(2) Stoloff, L., Trucksess, M., Hardin, N., Francis, O. J., Hayes, J. R., Polan, C. E., & Campbell, T. C. (1975) *J. Dairy Sci.* **58**, 1789–1793
(3) Horwitz, W., Kamps, L. R., & Boyer, K. W. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 1344–1354
(4) McKinney, J. D. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 25–32
(5) Scott, P. M. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 242–248
(6) Mann, G. E., Codifer, L. P., Gardner, H. K., Jr, Koltun, S. P., & Dollear, F. G. (1970) *J. Am. Oil Chem. Soc.* **47**, 173–176
(7) Codifer, L. P., Mann, G. E., Jr, & Dollear, F. G. (1976) *J. Am. Oil Chem. Soc.* **53**, 204–206
(8) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **26.095-26.099**
(9) Stubblefield, R. D. (1980) *J. Assoc. Off. Anal. Chem.* **63**, 634–636
(10) Stubblefield, R. D. (1979) *J. Am. Oil Chem. Soc.* **56**, 800–802

DISINFECTANTS

Comparison of Stainless Steel Penicylinders Used in Disinfectant Testing

EUGENE C. COLE, WILLIAM A. RUTALA, and EDITH M. ALFANO
University of North Carolina, Department of Medicine, Division of Infectious Diseases, Chapel Hill, NC 27514

Two brands of stainless steel penicylinders, S&L Metal Products and Fisher Scientific, were simultaneously tested to determine if they provide comparable results when used in the AOAC use-dilution method of disinfectant testing. Results showed consistently more positive tubes for the Fisher brand of penicylinders than for the S&L, regardless of the surface finish of the test cylinders.

The AOAC use-dilution method (1) of disinfectant efficacy testing specifies the use of type 304 stainless steel penicylinders manufactured by S&L Metal Products, Inc., Maspeth, NY 11378. These penicylinders are meant to represent a hard, nonporous surface in disinfectant efficacy testing. In the AOAC use-dilution method, the penicylinders are inoculated with 3 specific test bacteria, which serve as disinfectant challenges. When S&L suspended the manufacture of their penicylinders for 14 months, many laboratories purchased stainless steel penicylinders from Fisher Scientific, Pittsburgh, PA 15219. Fisher penicylinders are also 304 stainless steel, but unlike the S&L model, the Fisher penicylinders are manufactured with beveled ends. Microscopic examination of both brands of penicylinders revealed defects in their surfaces where bacteria can reside and perhaps be protected from disinfectant exposure (2). In addition, the degree of polish of both brands of penicylinders has varied considerably over 2 years.

A previous study (2) showed that both types of stainless steel penicylinders retained approximately the same number of test bacteria (i.e., 10^6–10^7 bacteria/penicylinder) after inoculation. The purpose of the present study was to test simultaneously S&L and Fisher stainless steel penicylinders, using the AOAC use-dilution method, to determine if the 2 brands yielded comparable test results.

Experimental

Media and Reagents

(**a**) *Nutrient broth.*—See sec. **4.001(a)** (1), 10 mL in 20 × 150 mm culture tubes using Anatone (American Laboratories, Inc., Omaha, NE 68127), and Beef Extract (Difco Laboratories, Detroit, MI 48232) as specified. Prepare according to directions, using glass-distilled water. Adjust pH to 6.8 before sterilization.

(**b**) *Letheen broth.*—Bacto Letheen Broth (Difco), formulated to comply with sec. **4.001(d)**(*3*) (1).

(**c**) *Distilled water.*—Sterile Water for Irrigation, USP (Travenol Laboratories, Inc., Deerfield, IL 60015).

(**d**) *Asparagine.*—Bacto Asparagine (Difco) 0.1% (w/v) aqueous solution.

Organisms

Staphylococcus aureus 6538, *Salmonella choleraesuis* 10708, and *Pseudomonas aeruginosa* 15442 (American Type Culture Collection, Rockville, MD 20852).

Apparatus

(**a**) *Stainless steel penicylinders.*—8 ± 1 mm od, 6 ± 1 mm id, 10 ± 1 mm long, type 304 stainless steel (S&L Metal Products Corp., Maspeth, NY 11378; and Fisher Scientific, Pittsburgh, PA 15219).

(**b**) *Culture tubes.*—20 × 150 mm, flint glass, screw-cap (Fisher Scientific).

(**c**) *Subculture tubes.*—20 × 150 mm, flint glass (Fisher Scientific).

(**d**) *Medication tubes.*—25 × 150 mm, borosilicate glass (Fisher Scientific).

(**e**) *Sonicator.*—Ultrasonic cleaner Model 450 (RAI Research Corp., Hauppauge, NY 11787).

(**f**) *Transfer hook.*—Heavy-gauge nichrome wire with 3–5 mm right angle bend.

Procedures

Broth culture preparation.—Stock cultures of test organisms were maintained on agar slants as specified in sec. **4.002(e)** (1). Tubes of nutrient broth were inoculated from stock culture slants and incubated overnight at 37°C. At least 4 consecutive broth transfers were made before broths to be used for testing were inoculated.

Penicylinder preparation.—Penicylinders were prepared by steam sterilization (15 min at 121°C), rinsed in tap water, sonicated in distilled water for ≥5 min, rinsed in distilled water, and drained. The cylinders were then placed in 20 × 150 mm tubes (20 per tube), covered with 1.0N NaOH, and further prepared as in sec. **4.009**. Before testing, asparagine was removed from tubes of penicylinders, and penicylinders were aseptically transferred, using sterile hook, to 25 × 150 mm sterile tubes. Appropriate numbers of 48–54 h, 10 mL broth cultures used to inoculate 120 penicylinders for each day's testing were pooled and mixed. Twenty mL broth culture was added to each tube containing 20 penicylinders to be tested and allowed to remain undisturbed 15 min at room temperature. Upon removal from broth culture, penicylinders were dried at 37°C for 40 min as in sec. **4.009** (1).

Disinfectant preparation.—Disinfectants were prepared according to manufacturers' recommended use-dilutions in sterile, distilled water. Formulations for quaternary ammonium and phenolic products were as follows: *Disinfectant 1:* didecyl dimethyl ammonium chloride, 7.50%; *n*-alkyl (C14 50%, C12 40%, C16 10%) dimethyl benzyl ammonium chloride, 5.00%; tetrasodium ethylenediaminetetraacetate, 1.69%; and inert ingredients, 85.81%; use-dilution was 1:256. *Disinfectant 2: o*-benzyl-*p*-chlorophenol, 7.24%; isopropanol, 4.04%; *o*-phenylphenol, 2.23%; and inert ingredients, 86.49%; use-dilution was 1:128.

Use-dilution method.—In batches of twenty, 60 penicylinders of each brand were tested on the same day with the same broth culture against the same disinfectant. Test brand of penicylinder was alternated for each batch (S&L, Fisher, S&L, Fisher, etc.), with initial order of penicylinder brand varying on each day. Before each group of 60 penicylinders was tested, test disinfectant was freshly prepared in sterile 1 L volumetric flask. Using 10 mL volumetric pipet, disinfectant was dispensed into 25 × 150 mm medication tubes in 20°C circulating water bath. Use-dilution method was then performed. In brief, stainless steel ring carriers (i.e., penicylinders)

Received April 17, 1987. Accepted June 10, 1987.

Table 1. AOAC use-dilution method results for 2 brands of stainless steel penicylinders, recommended bacteria, and quaternary ammonium disinfectant

	Penicylinder brand			
Bacteria	S&L[a] (polished)	Fisher[a] (dull)	S&L[b] (dull)	Fisher[b] (polished)
S. choleraesuis	0/60	7/60[c]	4/60	15/60[c]
S. aureus	1/58	1/58	5/60	6/60
P. aeruginosa	0/60	13/60[c]	3/60	7/60
Total	1/178	21/178[d]	12/180	28/180[d]

[a,b] Simultaneously tested for each organism.
[c] *P*-value <0.05 as generated by Fisher's exact test (2-sided).
[d] *P*-value <0.05 as generated by Mantel-Haentzel test.

Table 2. AOAC use-dilution method results for 2 brands of stainless steel penicylinders, recommended bacteria, and phenolic disinfectant

	Penicylinder brand			
Bacteria	S&L[a] (polished)	Fisher[a] (dull)	S&L[b] (dull)	Fisher[b] (polished)
S. choleraesuis	0/60	5/60	0/60	3/60
S. aureus	4/60	14/60[c]	7/60	11/60
P. aeruginosa	0/60	2/60	0/60	1/60
Total	4/180	21/180[d]	7/180	15/180

[a,b] Simultaneously tested for each organism.
[c] *P*-value <0.05 as generated by Fisher's exact test (2-sided).
[d] *P*-value <0.05 as generated by Mantel-Haentzel test.

were inoculated by soaking 15 min in 48 h broth culture of test bacteria. Penicylinders were removed with hooked inoculating needle and dried 40 min at 37°C. After drying, inoculated penicylinders were placed individually into disinfectant solution and exposed for 10 min. Penicylinders were carefully removed and placed in 10 mL subculture tubes of letheen broth. Tubes were incubated 48 h and then examined for turbidity. Using current pass/fail specifications, >1 positive penicylinder (i.e., turbid tube) of 60 replicates indicates that dilution tested is not adequate to obtain required bactericidal activity, and disinfectant fails.

Results and Discussion

The S&L polished and the Fisher dull penicylinders were compared first. At the time of testing, these were the penicylinders that were available for purchase from the manufacturers. When tested against the quaternary ammonium disinfectant, the Fisher penicylinders yielded 7/60 and 13/60 positive for *S. choleraesuis* and *P. aeruginosa*, respectively, as opposed to the S&L penicylinders, which remained negative (Table 1). When tested against the phenolic disinfectant, Fisher penicylinders again produced more positive results than the S&L, with 5/60 vs 0/60 for *S. choleraesuis*, 14/60 vs 4/60 for *S. aureus*, and 2/60 vs 0/60 for *P. aeruginosa* (Table 2). The data comparing the S&L polished and the Fisher dull penicylinders are statistically significant for both test disinfectants.

Several months later, S&L penicylinders were produced with a dull finish, while the Fisher brand was highly polished. Another simultaneous use-dilution comparison was performed using the same quaternary ammonium and phenolic disinfectants. The Fisher polished penicylinders also yielded more positive penicylinders than the S&L dull penicylinders for the quaternary ammonium disinfectant and the 3 AOAC recommended test bacteria. A similar trend was observed (Table 2) in using the phenolic disinfectant ($P = 0.07$, Mantel-Haentzel test). Possible reasons for this difference between test penicylinders include a variation in the number of cells remaining attached to the penicylinders or protection from disinfectant exposure for bacteria that reside in the Fisher penicylinder grooves. The inner and outer surfaces of Fisher penicylinders have been shown by scanning electron microscopy to exhibit deeper grooves and more significant pitting when compared to the S&L penicylinders (2). Although the S&L polished and dull penicylinders were not directly compared, the S&L dull penicylinders failed the quaternary ammonium disinfectant for each of the 3 test bacteria while the S&L polished penicylinders yielded results that confirmed efficacy of the product against the same bacteria.

In conclusion, the Fisher penicylinders provide a more difficult challenge to the test disinfectants than do the S&L penicylinders recommended in the AOAC use-dilution method. This variation in the test procedure could partially explain the interlaboratory variability of results when laboratories test identical disinfectants. The use of polished S&L penicylinders can help standardize the use-dilution method by eliminating variability in test results due to the penicylinder.

Acknowledgment

This work was supported in part by the U.S. Environmental Protection Agency, Office of Pesticide Programs, under Cooperative Agreement CR811204-02 to the University of North Carolina at Chapel Hill.

References

(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **4.007-4.011**
(2) Cole, E. C., Rutala, W. A., & Carson, J. L. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 903–906

COSMETICS

Stability-Indicating Liquid Chromatographic Procedure for Determination of Allantoin in Cosmetic Lotion

RAMESH J. TRIVEDI
Warner-Lambert Co., Consumer Products Research and Development Center, Morris Plains, NJ 07950

A sensitive, specific liquid chromatographic (LC) procedure was developed for determination of allantoin [(2, 5-dioxo-4-imidaazolidinyl) urea or 5-ureidohydantion] in cosmetic lotion. A reverse-phase, ion-suppression mechanism separated allantoin from interfering constituents of the sample matrix, and the compound was determined with a UV detector at 240 nm with a sensitivity limit of 0.20 mg/mL. The chromatographic parameters were optimized for retention time, efficiency, and relative response to the analyte. The assay procedure was validated with spiked laboratory-prepared samples at 100 ± 15% levels. An average recovery of 99.4% with a relative standard deviation of 1.5% (n = 7) was obtained. The stability-indicating characteristics of the method were established by recovery study (99.8%) of samples spiked with known degradation products (urea, allantoic acid, and glyoxylic acid).

Because of their therapeutic properties, allantoin and its derivatives have been incorporated into various cosmetic preparations such as skin creams, lotions, lipsticks, and shaving creams (1, 2). Methods in the literature for determination of allantoin include titration (3–5), colorimetry (6), classical chromatography (7), and spectrophotometry (8, 9) at 220 nm after sample preparation by thin-layer chromatography (TLC). All these methods are time consuming or lack the specificity required for studying the chemical integrity of allantoin preparations.

Recently, 2 liquid chromatographic (LC) methods have been reported (10, 11). The former method, although able to differentiate the intact allantoin from its degradation products, is unable to separate the active ingredients from the excipients in cosmetic creams and lotions. In addition, the chromatographic column degraded after a short time. The latter, based on LC separation of an allantoin derivative, is time-consuming and subject to error in the derivative formation and is not documented to be stability indicating.

In the stability-indicating LC procedure described here, allantoin content is determined in the presence of reaction and degradation products and excipients. The assay affords complete baseline separation of peaks and offers excellent linearity, precision, and recovery.

METHOD

Reagents and Apparatus

(a) *Triethylamine.*—Reagent grade, 99% purity, 0.1% (v/v) in water. Adjust pH to 3.8 with phosphoric acid.

(b) *Acetonitrile.*—LC grade.

(c) *Reference standard.*— +98% purity, accepted as supplied (Fluka Chemical Corp., Ronponpoma, NY 11779.

(d) *Standard solutions.*—Accurately weigh ca 40, 50, and 60 mg reference standard into 3 separate 100 mL volumetric flasks. Add 30 mL water and shake mechanically 10 min. Add 50 mL acetonitrile and shake again 15 min. Dilute contents of each flask to volume with acetonitrile and mix well.

(e) *Sample solutions.*—Accurately weigh sample equivalent to 50 mg allantoin (10 g sample for 0.5% allantoin) into 100 mL volumetric flask. Disperse sample in 30 mL water for 10 min by using mechanical shaker. Add 50 mL acetonitrile and shake again 15 min. Dilute contents of flask to volume with acetonitrile and mix well. Transfer 25 mL sample into 50 mL centrifuge tube and centrifuge 20 min at 25°C. Using disposable filter unit, filter supernate into sample vial.

(f) *Disposable filter units.*—Millex HV, 0.45 μm (Millipore Co. or equivalent).

(g) *LC system.*—Perkin-Elmer, comprised of Series 3B pump, LC-75 spectrophotometric detector, LC-420B auto sampler, and Sigma 15 chromatography data station. Operating conditions: flow rate 2 mL/min; UV detector 240 nm; temperature, ambient; injection volume 100 μL loop; inlet pressure ca 2000 psi; attenuation 1; chart speed 5 mm/min.

(h) *LC column.*—250 mm × 4.5 mm id column packed with 10 μm amino (NH_2) packing (Zorbax, DuPont or equivalent).

(i) *Mobile phase.*—(*a*) 10% of 0.1% v/v triethylamine in water, pH adjusted to 4.0 with phosphoric acid; (*b*) acetonitrile. Filter (*a*) and (*b*) through appropriate 0.45 μm membrane paper. Mix 10% (v/v) (*a*) in (*b*) and degas under vacuum while stirring ca 30 min.

Procedure

Install column by connecting inlet to injection port and outlet to detector. Equilibrate column to steady baseline by pumping mobile phase at 2 mL/min. When column reaches equilibrium at chromatographic conditions, inject 100 μL aliquots of standard solution into chromatograph until con-

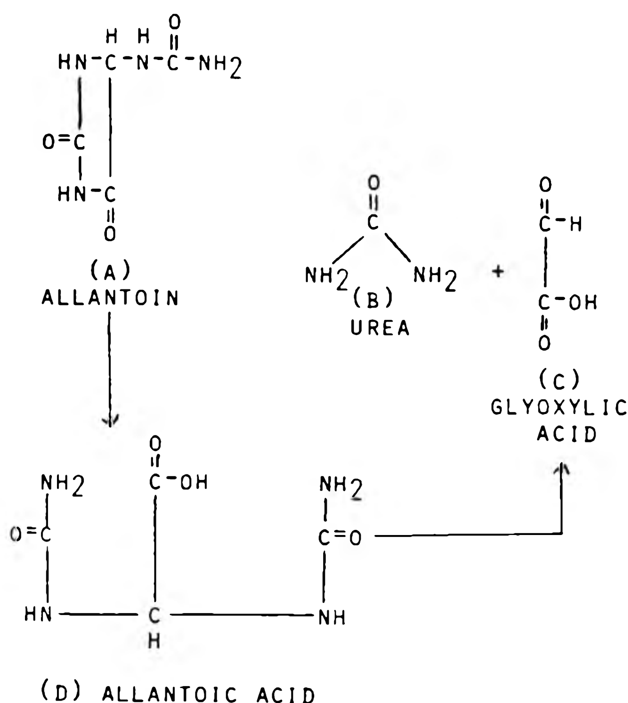


Figure 1. Stability of allantoin in alkaline conditions.

Received August 19, 1986. Accepted April 24, 1987.

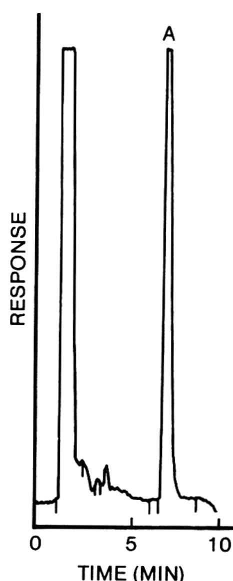


Figure 2. Chromatogram of cosmetic lotion. A, allantoin.

stant response is obtained. Response of 3 consecutive injections should agree within 2% (system suitability test).

Inject standard and sample solutions into chromatograph.

Calculations

Compute linear regression plot from standard solutions data and then calculate concentration of allantoin in sample (mg/mL) from plot. (Concentration, mg/mL, on x-axis vs area counts on y-axis.) Report allantoin content as percentage of sample, as shown below:

Allantoin, % (as is) = concn, mg/mL, × 10/sample wt, g

This equation is derived as follows:

Allantoin, % (as is) = concn, mg/mL, × 100 × 100/sample wt, g, × 1000

Results and Discussion

As shown in Figure 1, allantoin (A) is unstable in alkaline conditions and is known to hydrolyze to urea (B) and glyoxylic acid (C) via allantoic acid (D). To overcome the hydrolysis problem, the pH of the mobile phase was adjusted to 3.8 which resulted in specific determination of intact allantoin with minimum sample preparation. The detection wavelength was set at 240 nm (the maximum absorption for allantoin is 220 nm) to minimize interference from excipients. Linearity of peak area vs concentration was excellent over the concentration range 0.20 to 1.0 mg/mL (7 different concentrations, Table 1) with a correlation coefficient of 0.997. An average recovery of 99.4% (0.507% allantoin) was obtained from spiked laboratory-prepared samples with standard deviation of 0.008 and relative standard deviation of 1.5% (n = 7). The 500 g spiked sample was prepared by weighing the appropriate amount of allantoin into the placebo, and then mixing the sample well. Chromatographs of sample and standard are presented in Figures 2 and 3, respectively. The ruggedness of the method was evaluated using similar columns available from Waters and IBM. With a slight adjustment of mobile phase, K′ was obtained at a satisfactory level (5.4).

Table 1. Linearity of peak area vs concentration of allantoin[a]

No.	Concn, mg/mL	Area counts
1	0.2034	1.4699
2	0.2542	2.2048
3	0.4067	3.6081
4	0.5010	4.4320
5	0.6380	4.9988
6	0.8930	7.5532
7	1.0360	8.8547

[a] Linear regression correlation coefficient = 0.997; Slope = 8.540; Intercept = −0.0658.

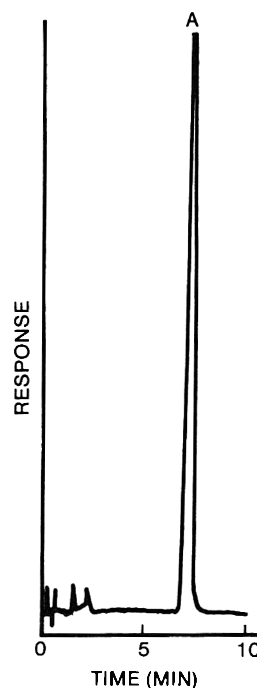


Figure 3. Chromatogram of allantoin standard. A, allantoin.

To demonstrate the stability-indicating characteristics of the method, the standard and the standard spiked with the decomposition products (urea, allantoic acid, glyoxylic acid) were subjected to LC analysis. Allantoin recovery of 99.8% was obtained (Figure 4) from the spiked sample, indicating

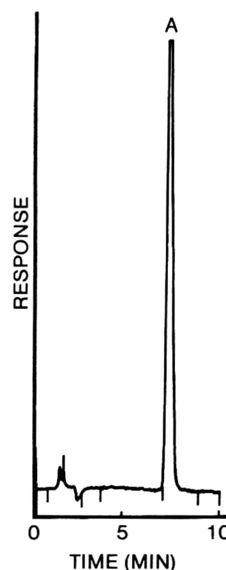


Figure 4. Chromatogram of standard solution spiked with decomposition products: urea, allantoic acid, and glyoxylic acid. A, allantoin.

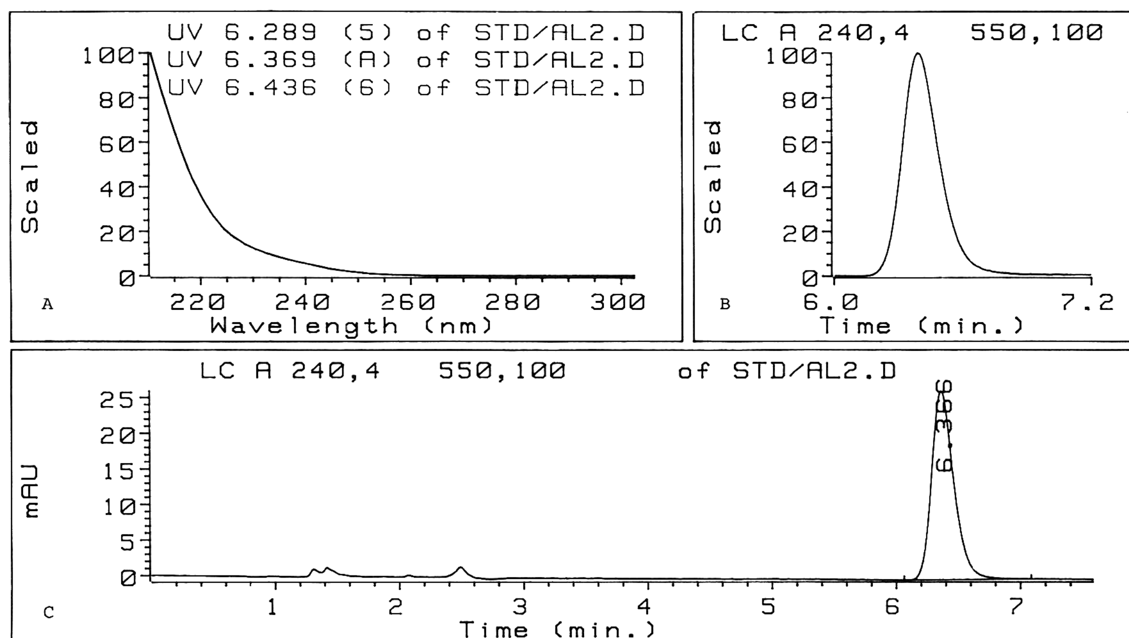


Figure 5. LC scan of allantoin standard, using Hewlett-Packard Model 1040A LC system equipped with diode array UV detector. A, UV traces of allantoin peak between 210 and 200 nm at 6.29, 6.37, and 6.44 min; B, chromatographic traces of standard; C, spiked standard.

Table 2. Recovery of allantoin (0.50% theoretical) from laboratory-prepared cosmetic lotions containing formaldehyde-contributing preservatives

Sample	Allantoin found, %
Cosmetic lotion w/glydant	0.45
Cosmetic lotion w/germall	0.43
Cosmetic lotion w/parabens	0.54
Cosmetic lotion w/glydant + 0.15% formaldehyde	0.34
Cosmetic lotion w/glydant + 0.30% formaldehyde	0.16
Cosmetic lotion w/parabens + EDTA	0.53
Cosmetic lotion w/parabens + benzoic acid	0.51
Cosmetic lotion w/parabens + sorbic acid	0.49

Table 3. Recovery of allantoin from commercial products

Sample	pH	Claimed, %	Found, %
Tegrin lotion	5.8	—	2.05
Alphosyl cream	6.4	2.0	1.48
Tegrin medicated skin cream	7.4	1.7	1.45

that the decomposition products did not coelute with the allantoin. This fact was further validated by studying the allantoin peak in the presence of degradation products and by using a Hewlett-Packard Model 1040A LC system equipped with a diode array detector (Figures 5–8).

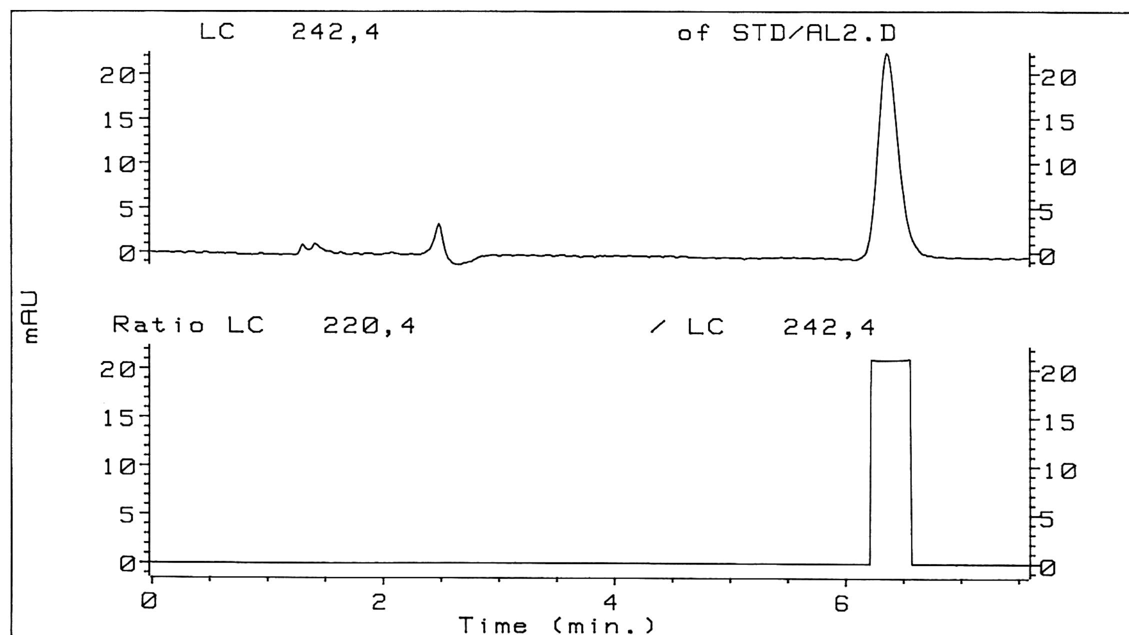


Figure 6. LC scan of allantoin standard, using Hewlett-Packard Model 1040A LC system equipped with diode array UV detector at ratio of 242/220 nm.

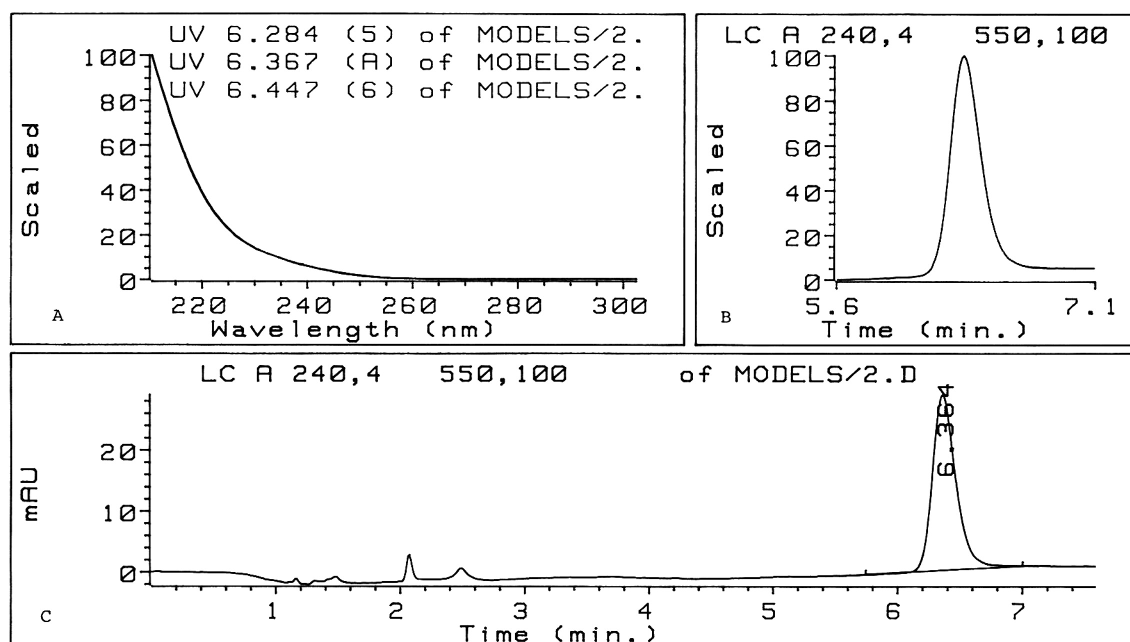


Figure 7. LC scan of allantoin standard (0.482%) spiked with degradation products (allantoic acid 0.15%, urea 0.27%, and glyoxylic acid 0.42%), using Hewlett-Packard Model 1040A LC system equipped with diode array UV detector. A, UV traces of allantoin peak between 210 and 200 nm at 6.29, 6.37, and 6.44 min; B, chromatographic traces of standard; C, spiked standard.

Figures 5A and 7A are the UV traces of the allantoin peak between 210 and 200 nm at 6.29, 6.37, and 6.44 min for the allantoin standard itself and allantoin standard (0.482%) spiked with degradation products (allantoic acid 0.15%, urea 0.27%, glyoxylic acid 0.42%). Three UV traces in each case are a single line, proving that the degradation products do not coelute with the allantoin peak. Figures 5B, 7B, 5C, and 7C are the chromatographic traces of standard and standard spiked with degradation products, respectively. Figures 6 and 8 are the chromatographic UV traces at the ratio of 242/220 nm of standard and standard spiked witih degradation products, respectively. The flat peak established the purity of the peak in each case; impurities would have resulted in multiple peaks instead of a single flat peak.

This method demonstrates the instabilty of allantoin in the presence of formaldehyde-contributing preservatives such as Glydant and Germall (Table 2). Some commercial products (Table 3) were analyzed for informational purposes.

In conclusion, the proposed method is specific for intact allantoin, and is stability indicating in the presence of interferences from degradation products and the excipient.

Acknowledgments

The author thanks R. Einig and M. Elefant of Warner-Lambert Consumer Products R&D for their useful comments and criticism.

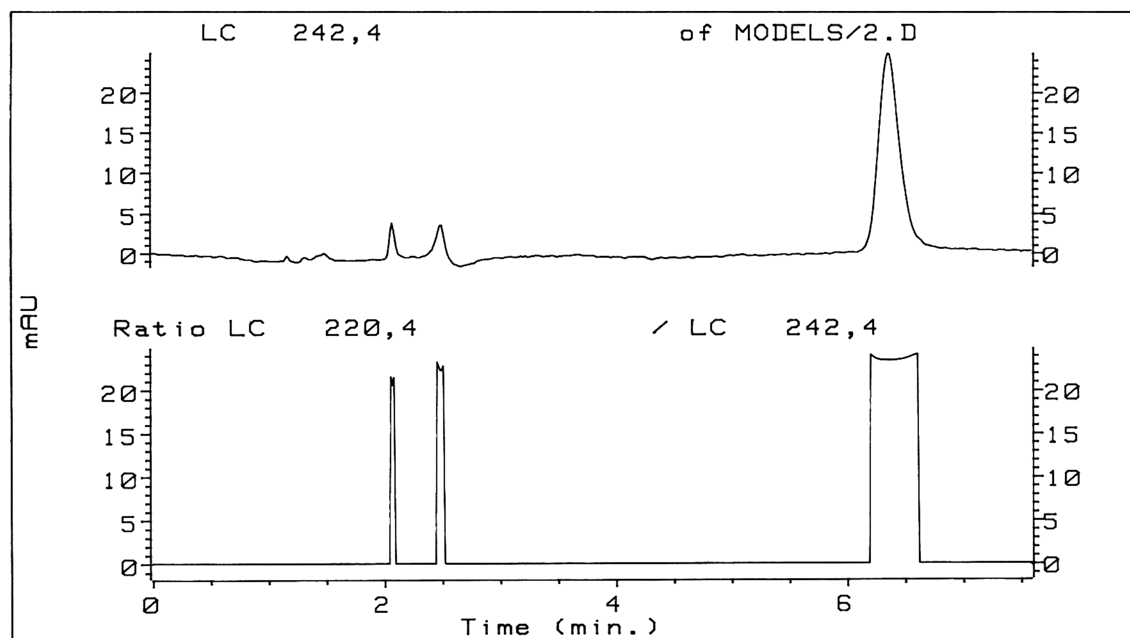


Figure 8. LC scan of allantoin standard spiked with degradation products, using Hewlett-Packard Model 1040A LC system equipped with diode array UV detector at the ratio of 242/220 nm.

References

(1) Mecca, S. B. (1955) *Proc. Sci. Sect. Toilet Goods Assoc.* **23,** 8–13
(2) Mecca, S. B. (1963) *Proc. Sci. Sect. Toilet Goods Assoc.* **39,** 7–15
(3) Cosmetic, Toiletry and Fragrance Assoc., Compendium of Cosmetic Ingredient Compositions Method D 12-1, CTFA, Washington, DC
(4) Weber, D. J., & Higgins, J. W. (1970) *J. Pharm. Sci.* **59,** 1819–1821
(5) Billabert, A., Willemot, J., & Parry, G. (1976) *Ann. Pharm. Fr.* **34,** 65–66.
(6) Katz, S. A., Turse, R., & Mecca, S. B. (1964) *J. Soc. Cosmet. Chem.* **15,** 303–310
(7) Deshusses, J., & Desbaumes, P. (1961) *Parfums, Cosmet. Savons* **4,** 192–196
(8) Bonadeo, I., & Bottazzi, G. (1968) Riv. Ital. Essenze-Profumi, Piante offic., Aromi-Saponi, Cosmet.-Aerosol. **50,** 78–80
(9) Lutomski, J., & Jernas, B. (1976) *Phamazie* **31,** 131–132
(10) Zaidi, Z. R., Sena, F. J., & Basilio, C. P. (1982) *J. Pharm. Sci.* **71,** 997–999
(11) Nakao, K., Honda, K., & Yoneya, T. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 1362–1365

ANTIBIOTICS

In Vitro Analytical System for Determining the Ability of Antibiotics at Residue Levels to Select for Resistance in Bacteria

MARIETTA SUE BRADY, ROBERT J. STROBEL, and STANLEY E. KATZ
Rutgers University, Cook College, New Jersey Agricultural Experiment Station, Department of Biochemistry and Microbiology, New Brunswick, NJ 08903

An analytical procedure, based on the concept that exposure of bacteria to antibiotics will result in the selection of a resistant population, was developed. Two strains of enteric bacteria, *Escherichia coli* CS-1 and *Enterobacter cloacae* B520, which are sensitive to a wide variety of antibiotics, were used as the test organisms. *E. coli* CS-1 were exposed to 1.00 μg antibiotic or antimicrobial/mL; *E. cloacae* B520 were exposed to 0.01, 0.10, 0.50, 1.00, and 5.00 μg/mL. Both organisms developed increased resistance to other antibiotics after exposure to chlortetracycline and oxytetracycline, as measured by the minimum inhibitory concentration (MIC). *E. cloacae* B520 showed increased resistance to ampicillin, oxytetracycline, and chloramphenicol after exposure to levels as low as 0.10 μg/mL. Exposure to streptomycin, sulfamethazine, tylosin, bacitracin, flavomycin, virginiamycin, and monensin at levels of 1.00 μg/mL did not increase the MIC. Exposure to 5.00 μg streptomycin, sulfamethazine, tylosin, and monensin/mL increased the MIC of *E. cloacae* to one of the antibiotic markers. These increased MICs exceeded the 95% confidence limits of the MIC values of the unexposed organisms.

The biological significance of antibiotic residues has been evaluated primarily for toxicological effects (1). Little if any attention has been given to some of the more subtle biological aspects, such as the potential tendency of residue levels to select for resistant bacterial populations in the intestine. Studies that involve the feeding of low levels of antibiotics to animals (0.01–5.00 ppm) to determine the effects on intestinal bacterial populations are complicated by the high percentages of drug-resistant intestinal organisms now present in animal intestinal tracts. Dinnerstein (2) found 2–3% of intestinal *Escherichia coli* were resistant to one or more antibiotics. In contrast, Mamber and Katz (3) reported that 35–38% were resistant. Because of the ubiquitousness and inordinately high numbers of antibiotic-resistant organisms in the intestinal populations of animals, nonanimal systems are required to evaluate the potential for antibiotics at residue levels to select for resistant populations.

The exposure of a bacterial population to antibiotics/antimicrobials undoubtedly results in the emergence/selection of a more drug-resistant segment of the population. This phenomenon has been well documented in both agricultural and human medical areas (4–15). Therefore, the use of a microbial system is necessary to evaluate the potential of antibiotic/antimicrobials at residue levels to cause the emergence of resistant bacterial populations that would parallel human, animal, and environmental responses.

The concept used in these studies was to isolate from the intestine Gram-negative lactose fermenters which were sensitive to most antibiotics and to use these isolates as test organisms. Exposure of these isolates to antibiotics at residue levels (0.01–5.00 μg/mL) should reflect the ability of both the drug and the concentration to select for resistant populations in the test strain.

Received December 19, 1986. Accepted May 12, 1987.
New Jersey Agricultural Experiment Station Publ. No. D01112-04-86, supported by state funds.

Experimental

Buffers

All buffer salts were dissolved and diluted to 1 L with water, then stored at room temperature (sec. **42.204** [16]).

(a) *Phosphate buffer, pH 6.0.*—8.0 g monopotassium phosphate and 2.0 g dipotassium phosphate.

(b) *Phosphate buffer, pH 8.0.*—16.73 g dipotassium phosphate and 0.523 g monopotassium phosphate.

Standard Solutions for Supplementation

(a) *Chlortetracycline and oxytetracycline.*—100 μg/mL. Dissolve hydrochloride salt of each antibiotic in 10 mL 0.1N HCl and dilute with water to 100 mL. Dilute to 25 μg/mL on day of use.

(b) *Streptomycin.*—100 μg/mL. Dissolve streptomycin sulfate in 100 mL pH 8 buffer. Dilute to 25 μg/mL on day of use.

(c) *Tylosin.*—100 μg/mL. Dissolve tylosin tartrate in 100 mL pH 8 buffer. Dilute to 25 μg/mL on day of use.

(d) *Sulfamethazine.*—100 μg/mL. Dissolve sulfamethazine in 10 mL 0.1N HCl and dilute with water to 100 mL. Dilute to 25 μg/mL on day of use.

(e) *Bacitracin.*—25 μg/mL. Dissolve bacitracin zinc salt in 10 mL 0.1N HCl and dilute with water to 100 mL. Prepare daily.

(f) *Virginiamycin.*—100 μg/mL. Dissolve virginiamycin in 10 mL ethanol and dilute with water to 100 mL. Dilute to 25 μg/mL on day of use.

(g) *Flavomycin.*—100 μg/mL. Dissolve flavomycin in 10 mL ethanol and dilute with water to 100 mL. Dilute to 25 μg/mL on day of use.

(h) *Monensin.*—100 μg/mL. Dissolve monensin in 10 mL ethanol and dilute with water to 100 mL. Dilute to 25 μg/mL on day of use.

All standards except bacitracin are stable for at least 2 weeks if stored at 4°C.

Antibiotic Standard Solutions for Minimum Inhibitory Concentration (MIC) Determination

(a) *Erythromycin.*—500 μg/mL. Dissolve erythromycin in 10 mL ethanol and dilute with water to 100 mL.

(b) *Oxytetracycline.*—500 μg/mL. Dissolve oxytetracycline hydrochloride in 10 mL 0.1N HCl and dilute with water to 100 mL.

(c) *Ampicillin.*—500 μg/mL. Dissolve ampicillin in 10 mL acetone–pH 6 buffer (5 + 5), and dilute with pH 6 buffer to 100 mL.

(d) *Neomycin.*—500 μg/mL. Dissolve neomycin in 100 mL pH 8 buffer.

(e) *Chloramphenicol.*—500 μg/mL. Dissolve chloramphenicol in 10 mL ethanol and dilute with water to 100 mL.

These standards are stable for at least 2 weeks if stored at 4°C.

Each antibiotic solution in both sets of standards was filter-

Table 1. MIC values (µg/mL) of *E. cloacae* B520: controls

Replicate	Erythromycin	Oxytetracycline	Ampicillin	Neomycin	Chloramphenicol
1	125.0	3.9	7.8	3.9	7.8
2	125.0	3.9	7.8	3.9	7.8
3	125.0	3.9	7.8	2.0	7.8
4	125.0	2.0	7.8	3.9	7.8
5	125.0	2.0	7.8	3.9	7.8
6	125.0	2.0	7.8	2.0	3.9
7	125.0	7.8	7.8	2.0	15.6
8	125.0	2.0	3.9	2.0	3.9
9	125.0	2.0	7.8	3.9	7.8
10	125.0	2.0	7.8	3.9	7.8
Av.	125.0	3.2	7.4	3.1	7.8
Std dev.	0	1.9	1.2	1.0	3.2
95% conf. upper limit	125.0	6.6	9.7	4.9	13.6

sterilized using Nalgene 0.2 µm filter, then dispensed aseptically thereafter.

Media

(a) *Maintenance medium.*—Tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI 48232) was used for maintenance of organisms.

(b) *Inoculation broth.*—Tryptic soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD 21030) was used for selection studies.

Microorganisms

A strain of *Enterobacter cloacae,* B520, was isolated from the human intestinal tract; *E. coli* CS-1 was isolated from chicken feces. The organisms were identified using the Enterotube system (Roche Diagnostic Systems, Nutley, NJ 07110). Baseline MIC values for several antibiotics were determined. For *E. cloacae* B520, the MIC values (µg/mL) were: erythromycin 125, ampicillin 7.8, oxytetracycline 3.9, neomycin 3.9, and chloramphenicol 7.8. For *E. coli* CS-1, the MIC values (µg/mL) were: ampicillin 3.1, chlortetracycline 3.1, chloramphenicol 3.1, streptomycin 12.5, kanamycin 6.3, and neomycin 6.3.

MIC Procedure

Inoculate flask of TSB with *E. cloacae* B520 or *E. coli* CS-1 and incubate with shaking for 18 h at 37°C. Dilute 18 h culture and inoculate flask of TSB so that final concentration is ca 10^3 organisms/mL. Dispense 1.0 mL seeded TSB into sufficient number of sterile test tubes for 9 dilutions per antibiotic. Add 1.0 mL antibiotic solution (500 µg/mL) to tube 1 of each 9-tube series and mix well using vortex mixer. Aseptically transfer 1.0 mL from tube 1 into tube 2, mix well, and continue dilutions for 9 tubes. Final concentrations for B520 are: 250.0, 125.0, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0, and 1.0 µg antibiotic/mL. For CS-1, concentrations are: 200.0, 100.0, 50.0, 25.0, 12.5, 6.3, 3.1, and 1.6 µg antibiotic/mL. The MIC tubes are incubated at 37°C for 18 h and examined visually for turbidity.

Selection for Resistance

At the beginning of the study, day 0, sterile flasks containing 25 mL TSB were supplemented aseptically with the sterile antibiotic solution under study. For studies using *E. cloacae* B520, concentrations were: 0.00, 0.01, 0.50, 1.00, and 5.00 µg/mL. For studies using *E. coli* CS-1, only one concentration, 1.00 µg antibiotic/mL, was used. Flasks were inoculated with test organism and incubated with shaking at 37°C for 18 h.

Each day for 14 days, flasks of TSB were aseptically supplemented with antibiotic being studied and were inoculated with previous day's growth. Growth from day 14 flasks was streaked onto TSA slants, incubated at 37°C for 18 h, and stored at 4°C until needed for MIC determination.

MICs of each substrain of test organisms to marker antibiotic were determined at day 14 and compared with profile of controls.

Results and Discussion

By definition, the MIC for an antibiotic against a specific organism is the lowest concentration at which there is no visible growth of the microorganism in a dilution series. Table 1 shows the repeatability and the variation in the MIC of *E. cloacae* B520. Each value is the MIC determined after 14 days of daily transfer of the B520. The organism showed considerable stability, having little variation in the MIC through daily transfer over many weeks.

Table 1 also shows the upper range of the 95% confidence limits, which are used to determine whether selection for resistant populations has occurred. The statistical variation is less than one tube in a series of twofold dilutions. However, a one-tube difference at the lowest level of the dilution series is a relatively small change, while at a slightly higher level the change is considerably greater. For example, the MIC of *E. cloacae* B520 for chloramphenicol is 7.8 µg/mL with the 95% upper limit of 13.6 µg/mL, an increase of 5.8 µg/mL. By comparison, the MIC for neomycin is 3.1 µg/mL with the upper limit of 4.9 µg/mL, a difference of 1.8 µg/mL. An increase in MIC is considered to be significant if it exceeds the 95% upper limit and shows a one-tube increase.

Nine compounds, representing 7 families of antibiotics and one sulfonamide, were studied for their ability to select for antibiotic resistance. The presence of residue levels of some antibiotics in the growth environment of microorganisms can select for populations which are resistant to the antibiotic present. Whether the presence of a compound at residue levels would select for a population resistant to other compounds from the same family or from different families is not known.

Table 2 shows the MIC values of *E. cloacae* B520 after 14 days of daily transfers in the presence of each of the 9 test compounds at concentrations ranging from 0.01 to 5.00 µg/mL. Chlortetracycline and oxytetracycline demonstrated similar response patterns, with consistent increases in the MIC to more than one marker antibiotic. At an exposure of 0.10 µg tetracycline/mL, the chlortetracycline-exposed B520 strain showed significant increases in the MIC to ampicillin and chloramphenicol; the oxytetracycline-exposed B520 showed significant increases in MIC to oxytetracycline, ampicillin, and chloramphenicol. After exposure to 1.00 µg tetracycline/mL, the chlortetracycline- and oxytetracycline-exposed B520 showed increased MIC to oxytetracycline, ampicillin, and chloramphenicol; at exposure to 5.00 µg/mL, the MIC to erythromycin doubled.

The B520 strain exposed to streptomycin at 5.00 µg/mL showed an increase in the MIC for neomycin (another aminoglycoside) but showed no effect on any of the other marker antibiotics. Lower concentrations of streptomycin caused no changes in the MICs.

Sulfamethazine at 5.00 µg/mL significantly increased the MIC for chloramphenicol (Tables 1 and 2). Tylosin and monensin at the 5.00 µg/mL level each caused a borderline

Table 2. MIC values (μg/mL) of *E. cloacae* B520 after exposure to various concentrations of antibiotics and antimicrobials

		Antibiotic marker				
Compound	Concn, μg/mL	Erythromycin	Oxytetracycline	Ampicillin	Neomycin	Chloramphenicol
Control[a]	baseline	125.0	6.6	9.7	4.9	13.6
Chlortetracycline	0.01	125.0	15.6	15.6	7.8	31.3
	0.10	125.0	3.9	15.6	3.9	15.6
	0.50	125.0	7.8	31.3[b]	3.9	31.3[b]
	1.00	125.0	31.3[b]	31.3[b]	3.9	62.5[b]
	5.00	250.0[b]	31.3[b]	31.3[b]	3.9	62.5[b]
Oxytetracycline	0.01	125.0	3.9	7.8	2.0	7.8
	0.10	125.0	15.6[b]	15.6	3.9	62.5[b]
	0.50	125.0	31.3[b]	31.3[b]	2.0	62.5[b]
	1.00	125.0	31.3[b]	31.3[b]	2.0	62.5[b]
	5.00	250.0[b]	31.3[b]	31.3[b]	3.9	62.5[b]
Streptomycin	0.01	125.0	3.9	7.8	3.9	7.8
	0.10	125.0	3.9	7.8	2.0	15.6
	0.50	125.0	3.9	7.8	3.9	7.8
	1.00	125.0	2.0	7.8	3.9	7.8
	5.00	125.0	2.0	3.9	31.3[b]	7.8
Tylosin	0.01	125.0	—	7.8	3.9	3.9
	0.10	125.0	2.0	7.8	2.0	7.8
	0.50	125.0	2.0	7.8	3.9	7.8
	1.00	125.0	2.0	7.8	3.9	7.8
	5.00	125.0	2.0	7.8	7.8[c]	7.8
Sulfamethazine	0.01	125.0	2.0	7.8	2.0	7.8
	0.10	125.0	3.9	7.8	2.0	7.8
	0.50	125.0	2.0	7.8	2.0	7.8
	1.00	125.0	3.9	7.8	2.0	7.8
	5.00	125.0	7.8[c]	7.8	3.9	62.5[b]
Bacitracin	0.01	125.0	2.0	7.8	3.9	7.8
	0.10	125.0	2.0	15.6	2.0	7.8
	0.50	125.0	2.0	7.8	2.0	7.8
	1.00	125.0	2.0	7.8	3.9	7.8
	5.00	125.0	2.0	7.8	3.9	7.8
Virginiamycin	0.01	125.0	2.0	7.8	3.9	7.8
	0.10	125.0	3.9	7.8	3.9	7.8
	0.50	125.0	2.0	7.8	3.9	7.8
	1.00	125.0	2.0	7.8	3.9	7.8
	5.00	125.0	3.9	7.8	3.9	7.8
Flavomycin	0.01	125.0	2.0	3.9	3.9	7.8
	0.10	125.0	2.0	3.9	3.9	7.8
	0.50	125.0	2.0	7.8	3.9	7.8
	1.00	125.0	2.0	3.9	3.9	7.8
	5.00	125.0	2.0	7.8	3.9	7.8
Monensin	0.01	125.0	2.0	3.9	3.9	7.8
	0.10	125.0	2.0	7.8	3.9	7.8
	0.50	125.0	2.0	7.8	3.9	7.8
	1.00	125.0	2.0	7.8	3.9	7.8
	5.00	125.0	2.0	3.9	3.9	15.6[c]

[a] Upper limit of 95% confidence limits.
[b] Significant, 95% confidence limits.
[c] Exceeds 95% confidence limits; MIC at one-tube difference.

increase in the MIC of one marker, neomycin and chloramphenicol, respectively, but if these results are interpreted conservatively, the response is not considered significant, being less than a one-tube increase.

Table 4. MIC values (μg/mL) of *E. coli* CS-1 after exposure to antibiotics

		Antibiotic marker				
Compound	Concn, μg/mL	Streptomycin	Chlortetracycline	Ampicillin	Chloramphenicol	Kanamycin
Control II	0.0	6.3	1.6	1.6	1.6	6.3
Oxytetracycline	1.0	6.3	12.5[a]	12.5[a]	12.5[a]	6.3
Erythromycin	1.0	12.5	1.6	3.1	3.1	6.3

[a] Significant, 95% confidence limits.

Bacitracin, virginiamycin, and flavomycin caused no significant change in the MIC of any of the antibiotic markers at any of the concentrations used.

An *E. coli* strain isolated from the excreta of chickens, CS-1, was utilized in a preliminary study of the ability of residue levels of antibiotics/antimicrobials to select for resistance. The difference between the CS-1 and B520 studies lies in the quantitation and determination of the significance of any changes in the MIC. A single control incubated with the exposed cultures was used for baseline comparisons. The data are interpreted by using a 4-fold (2-tube) increase as an indication of significance, a conservative approach considering the repeatability found using *E. cloacae* B520.

Table 3 shows the results of exposure to 1.0 μg/mL of antibiotic/antimicrobial for 10 days. The CS-1 population exposed to chlortetracycline showed increased resistance to chlortetracycline, ampicillin, chloramphenicol, and neomycin. Exposure to neomycin resulted in increases in resistance to streptomycin, kanamycin, and neomycin. Sulfamethazine and streptomycin did not increase resistance to any of the marker antibiotics.

Table 4 shows the results of a second study of 2 antibiotics, oxytetracycline and erythromycin. The *E. coli* CS-1 control demonstrated MIC values which were slightly different from those in the first study, half the concentration in some cases. Exposure of *E. coli* CS-1 to oxytetracycline caused an increase in resistance to chlortetracycline, ampicillin, and chloramphenicol. Exposure to erythromycin resulted in a doubling of the MIC for streptomycin, ampicillin, and chloramphenicol; this doubling was insufficient to meet the criterion requiring a 4-fold increase before being considered significant.

The preliminary study using *E. coli* CS-1 indicated that the 2 tetracyclines increased the MIC for ampicillin, chloramphenicol, and neomycin, as well as for tetracyclines. Exposure to neomycin increased the MICs for the other aminoglycosides but did not increase the MICs of the other marker antibiotics.

In the studies using *E. cloacae,* the results were similar. The tetracyclines increased resistance (MIC) of the assay organism to ampicillin, oxytetracycline, and chloramphenicol across a broad range of test concentrations (0.01–5.00 μg/mL). The other antibiotics/antimicrobials at an exposure of

Table 3. MIC values (μg/mL) of *E. coli* CS-1 after exposure to antibiotics and antimicrobials

		Antibiotic marker					
Compound	Concn, μg/mL	Streptomycin	Chlortetracycline	Ampicillin	Chloramphenicol	Kanamycin	Neomycin
Control I	0.0	12.5	3.1	3.1	3.1	6.3	6.3
Chlortetracycline	1.0	12.5	25.0[a]	12.5[a]	50.0[a]	6.3	25.0[a]
Streptomycin	1.0	12.5	3.1	3.1	3.1	6.3	12.5
Neomycin	1.0	25.0	6.3	6.3	6.3	25.0[a]	25.0[a]
Sulfamethazine	1.0	12.5	3.1	3.1	3.1	6.3	6.3

[a] Significant, 95% confidence limits.

1.00 μg/mL did not increase the MIC markers of B520. At the 5.00 μg/mL supplementation, there were some indications that streptomycin, tylosin, sulfamethazine, and monensin will cause some selection for resistance (increased MIC) in a single antibiotic marker.

The 2 organisms, *E. coli* CS-1 and *E. cloacae* B520, were used to measure the same basic phenomenon. Studies of both organisms indicated that, with the exception of the 2 tetracyclines, levels of 1.00 μg/mL did not select for resistant populations. Both organisms responded to the selective pressure of tetracyclines. At 5.00 μg/mL, not only tetracyclines, but also streptomycin, sulfamethazine, tylosin, and monensin selected for a more resistant population, although the latter 2 were borderline cases.

The data indicate that the potential exists for tetracyclines to select for resistant bacterial populations at low concentrations. Surprisingly, levels of 1.00 μg streptomycin and sulfamethazine/mL did not show such a potential. With the exception of the tetracyclines, concentrations of 1.00 μg/mL (1 ppm) of other antibiotics should not provide the pressure to select for resistance. The significance of the data lies in the fact that, with the exception of the tetracyclines, normal residue levels of antibiotics that might be found in foods are of no significance vis-à-vis the development of resistant populations. This finding does not obviate the toxicological considerations used to establish tolerances, but it should limit speculation on the ability of antibiotic residues to select for resistant bacterial populations in the intestinal tracts of animals and humans.

Since animal wastes containing antibiotics are often disposed of by plowing them into soil, the knowledge of the levels that could select for resistance is important. On the basis of these data, we conclude that incorporation of 1 ppm antibiotic into soil will not cause the selection toward resistance to occur. Gavalchin (17) found that the incorporation into soil of feces containing antibiotics at a concentration of 5 ppm did not result in the selection of antibiotic-resistant populations. A similar conclusion can be made for antibiotics in surface waters. There should not be any selection for resistant populations at concentrations of 1.0 ppm, excluding the tetracyclines. Metabolites of the antibiotics/antimicrobials were not studied. The ability of phase II conjugates and other metabolites to influence the emergence of resistance can be studied by the in vitro system.

The approach described in this paper can be expanded to utilize different genera of bacteria to establish as complete an overview of the effects of antibiotics as is desired.

References

(1) Livingston, R. C. (1986) in *Agricultural Uses of Antibiotics*, ACS Symp. Ser. 320, W. A. Moat (Ed.), American Chemical Society, Washington, DC, pp. 128–136

(2) Dinnerstein, P. (1977) M.S. Thesis, Rutgers University, New Brunswick, NJ

(3) Mamber, S. W., & Katz, S. E. (1985) *Appl. Environ. Microbiol.* **50**, 638–648

(4) Hays, V. W. (1986) in *Agricultural Uses of Antibiotics*, ACS Symp. Ser. 320, W. A. Moat (Ed.). American Chemical Society, Washington, DC, pp. 74–87

(5) Cooke, E. M., Shooter, R. A., Breaden, A. L., & O'Farrell, S. (1971) *Lancet* **ii**, 8–10

(6) Crawford, L. M., & Shotts, E. B. (1982) in *The Control of Antibiotic Resistant Bacteria*, C. H. Stuart-Harris & D. H. Harris (Eds), Academic Press, Inc., New York, NY, pp. 169–181

(7) Report to the Commissioner of the Food and Drug Administration on the Use of Antibiotics in Animal Feeds (1972) Food and Drug Administration, Rockville, MD, FDA Publ. 72-6008

(8) Holmberg, S. D., Osterholm, M. T., Senger, K. A., & Cohen, M. L. (1984) *N. Engl. J. Med.* **311**, 617–622

(9) *The Use of Drugs in Animal Feeds* (1969) National Academy of Sciences, Washington, DC, NAS Publ. 1679

(10) Novick, R. P. (1981) *Ann. NY Acad. Sci.* **368**, 23–59

(11) Siegel, D., Huber, W. G., & Drysdale, S. (1975) *Antimicrob. Agents Chemother.* **8**, 538–543

(12) Siegel, D., Huber, W. G., & Enloe, F. (1974) *Antimicrob. Agents Chemother.* **6**, 697–701

(13) Smith, H. W. (1968) *Nature* **218**, 728–731

(14) Smith, H. W. (1969) *Lancet* **i**, 1174–1176

(15) Smith, H. W. (1974) *Br. Vet. J.* **130**, 110–119

(16) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **42.204**

(17) Gavalchin, J. (1983) Ph.D. Thesis, Rutgers University, New Brunswick, NJ

Method to Determine Effect of Antibiotics at Residue Levels on R-Factor Transfer

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

An analytical system was developed which can assess the ability of antibiotic/antimicrobial residues (0.01-1.00 ppm) to affect the conjugal transfer of resistance among the Enterobacteriaceae. The donor strain, *Escherichia coli* RP-4 (Am^r Tc^r Nm^r Km^r Lac$^+$), and recipient strain, *E. coli* Sc-8632 (Sm^r Lac$^-$), were incubated together in a 1:9 donor : recipient ratio for 18 h with gentle shaking (50 rpm) in brain heart infusion broth in the presence of residue levels of antibiotics. The mating cultures were serially diluted and spread-plated onto MacConkey agar containing 25 μg streptomycin/mL to select the total recipient population of sensitive *E. coli* Sc-8632 and transconjugants. After an 18 h incubation at 37°C, the plates were replica-plated onto MacConkey agar containing 25 μg ampicillin/mL to select the ampicillin-resistant transconjugant population. Repeatability was good; the average transfer was 51.8%, with a coefficient of variation of 9.3%. Residue levels of tylosin (0.10 and 1.00 ppm) increased the transfer of the ampicillin marker beyond the 95% confidence limits. Oxytetracycline, bacitracin, streptomycin, penicillin, and virginiamycin did not increase the percent transfer. Oxytetracycline at 0.01 ppm decreased the percent transfer. In general, residue levels of antibiotics (0.01-1.00 ppm) did not affect the conjugal transfer of antibiotic resistance.

There has been considerable debate and conjecture concerning the role of antibiotic and antimicrobial residues as factors contributing to the relatively high levels of resistance found in human enteric bacterial populations. Enteric organisms can become resistant to relatively low levels of antibiotics, as little as 2 ppm/day (1). Whether the relatively high levels of antibiotic resistance found among enteric bacterial populations arise from medical usage, from selection due to exposure to antibiotic/antimicrobial residues, from colonization by antibiotic-resistant organisms related to food production, or from transient colonization of antibiotic-resistant species and transfer of resistance to indigenous populations is undefined (2–6).

Determining the ecological effects of antibiotic residues on the bacterial populations of the intestinal tract is an imposing task. Using either animal or human subjects requires keeping exacting records of exposure to antibiotics, frequently examining fecal samples (every 1–3 days), studying antibiotic resistance patterns, and following the molecular biology of the bacteria and plasmids used as indicators over an extended period (7). Regardless of the care taken, conclusions concerning the ecological sequence of events in the processes of selection/development of resistance and of transfer of resistance between genera and species would be limited.

Conjugal transfer of resistance occurs at relatively low frequency in nature and occurs whether or not individuals are receiving antibiotics. The frequency of transfer is the result of interactions between the donor and recipient organisms, the physiological state of both, and the locations of the transfer factor and the resistance determinant (7). In vivo plasmid transfer is apparently less efficient than in vitro transfer, although the former is reported to occur in the intestinal tract of both humans and animals (2, 8). Walton (9) fed newly hatched chicks overnight cultures of *Escherichia coli* (Tc^r Sm^r) followed in 6 h by a recipient *E. coli* culture. Converted resistant *E. coli* were found in all 24 chicks used. When recipient *Salmonella typhimurium* were used in a parallel study, converted *S. typhimurium* were found in all 12 chicks used. Smith (8) ingested donor *E. coli* after colonizing his intestinal tract with a recipient *E. coli* (Na^r) and demonstrated a successful transfer of resistance. Falkow (10) found an increased frequency of conjugal transfer in the presence of antibiotics. Only one of 10 animals showed evidence of in vivo transfer, but when streptomycin was added to the diet of the animals, 9 of 10 showed transfer within 72 h.

This paper describes an analytical procedure which can assess the ability of compounds to affect the conjugal transfer of resistance among the Enterobacteriaceae.

Received February 11, 1987. Accepted May 28, 1987.
New Jersey Agricultural Experiment Station Publ. No. D01112-01-87, supported by state funds.

Experimental

Buffers

All buffer salts were dissolved and diluted to 1 L with water, then stored at room temperature (sec. **42.204**, 11).

(**a**) *Phosphate buffer, pH 6.0.*—8.0 g monopotassium phosphate and 2.0 g dipotassium phosphate.

(**b**) *Phosphate buffer, pH 8.0.*—16.73 g dipotassium phosphate and 0.523 g monopotassium phosphate.

Antibiotic Standard Solutions

(**a**) *Oxytetracycline.*—100 μg/mL. Dissolve oxytetracycline hydrochloride in 10 mL 0.1N HCl and dilute with water to 100 mL. Dilute to 25 μg/mL on day of use.

(**b**) *Streptomycin.*—100 μg/mL. Dissolve streptomycin sulfate in 100 mL pH 8 buffer. Dilute to 25 μg/mL on day of use.

(**c**) *Bacitracin.*—25 units/mL. Dissolve bacitracin zinc salt in 10 mL 0.1N HCl and dilute with water to 100 mL.

(**d**) *Penicillin.*—25 units/mL. Dissolve procaine penicillin in 10 mL acetone–pH 6 buffer (5 + 5) and dilute with pH 6 buffer to 100 mL.

(**e**) *Tylosin.*—100 μg/mL. Dissolve tylosin tartrate in 100 mL pH 8 buffer. Dilute to 25 μg/mL on day of use.

(**f**) *Virginiamycin.*—100 μg/mL. Dissolve virginiamycin in 10 mL ethanol and dilute with water to 100 mL. Dilute to 25 μg/mL on day of use.

Each antibiotic solution was filter-sterilized using Nalgene 0.2 μm filter and dispensed aseptically thereafter. Bacitracin and penicillin standards were prepared on the day used; all others are stable for at least 2 weeks if stored at 4°C.

Media

(**a**) *Maintenance medium.*—Tryptic soy agar (Difco Laboratories, Detroit, MI 48232) was used for maintenance of the 2 strains of *E. coli*. For *E. coli* RP-4, the agar was supplemented with 25 μg neomycin/mL.

(**b**) *Inoculation broth.*—Brain heart infusion broth (BHIB) (Difco) was used for matings/incubations.

(**c**) *Assay medium.*—MacConkey agar (BBL Microbiology Systems, Cockeysville, MD 21030), supplemented with 25 μg streptomycin/mL, was used to select for all recipient *E. coli* Sc-8632. MacConkey agar containing 25 μg ampicillin/mL was used to select for transconjugants receiving the ampicillin resistance determinant.

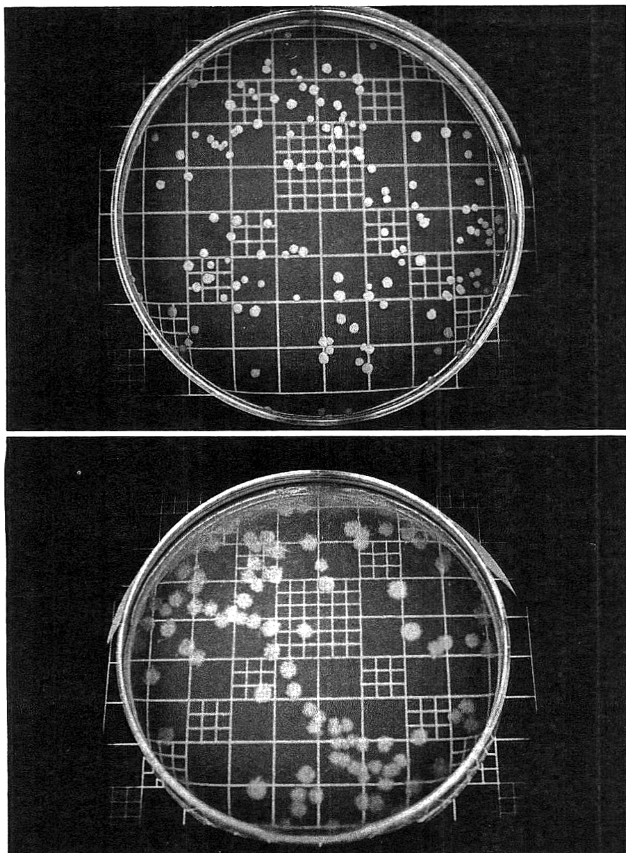


Figure 1. Top: Spread plate of total recipient population; bottom: replica plate of ampicillin-resistant transconjugants.

Microorganisms

E. coli RP-4 (Am^r Tc^r Nm^r Km^r Lac^+), the donor strain, ferments lactose and carries plasmid-determined resistance to ampicillin, neomycin, tetracycline, and kanomycin. Colonies are pink on MacConkey agar.

E. coli Sc-8632 (Sm^r Lac^-), the recipient strain, does not ferment lactose and is resistant to streptomycin. Colonies are white on MacConkey agar.

Mating and Assay Procedure

The daily protocol for the procedure was as follows:

Day 1.—Inoculate separate flasks of brain heart infusion broth with *E. coli* RP-4 (donor) and *E. coli* Sc-8632 (recipient). Incubate at 37°C with shaking for 18 h.

Day 2.—(*1*) Aseptically supplement flasks of BHIB with sterile concentrations of 0.00, 0.01, 0.10, and 1.00 μg compound/mL. (Three flasks/concentration.) (*2*) Inoculate each flask with 0.9 mL of *E. coli* Sc-8632 and 0.1 mL of *E. coli* RP-4. Incubate with gentle shaking (50 rpm) for 18 h at 37°C using a model R-2 shaker manufactured by New Brunswick Scientific Co.

Day 3.—(*1*) Make serial dilutions in sterile physiological saline of 18-h growth to 10^7 for each flask. (*2*) Spread-plate 0.5 and 1.0 mL of 10^7 dilution onto MacConkey agar containing 25 μg streptomycin/mL. (Triplicate plating at 0.5 and 1.0 mL.) Plates are allowed to absorb the spread-plated volume for 1 h before being inverted and incubated. (*3*) Incubate spread plates at 37°C for 18 h.

Day 4.—(*1*) Count total number of white colonies (Lac^-) on plates containing 100–300 colonies, using a Quebec colony counter. (*2*) Replica-plate onto MacConkey agar containing 25 μg ampicillin/mL (2 replica plates/master plate). (*3*) Incubate replicate plates for 18 h at 37°C and count colonies having received the ampicillin resistance determinant (Figure 1).

Transfer, % = [(colonies on ampicillin − supplemented plates)/(colonies on streptomycin − supplemented plates)] × 100.

Results and Discussion

Conjugal transfer of antibiotic resistance between mating strains of *E. coli* can occur within 5 min of mixing the donor and recipient strains and could be completed within 15 min. The converted recipients are capable of transferring resistance to other recipient cells, with a second round of transfer beginning within 15 min of receiving the resistance determinants. Converted cells do not become drug-resistant immediately, but require time during which the newly acquired DNA is transcribed and translated into enzymes associated with antibiotic resistance. Resistant phenotypes begin to appear 30 min after conjugal transfer. Overnight incubation of mating cells can result in transfers of 0–100% of the recipients (10).

The use of an overnight conjugal mating/incubation was thought to be more desirable than a shorter interval, such as 6 h, because the long-term population effect, rather than conjugation, was the phenomenon being evaluated. This approach should give insight into the reality of the exposure of intestinal populations to low levels of antibiotics/antimicrobials.

Preliminary studies of the transfer of specific resistance determinants indicated that the ampicillin determinant transferred at a much higher rate than kanamycin or chlortetracycline, the other antibiotics studied. Ampicillin transferred at a rate 4.4 times greater than chlortetracycline and 3.2 times greater than kanamycin. Hence, the transfer of ampicillin resistance was selected as the marker determinant for this study.

The percent transfer was related to the rate of shaking during mating. Static matings yielded transfers of 2–4% (12); at 35 rpm, an average transfer of 24.9% was obtained (13); between 45 and 50 rpm, rates of transfer were maximum. A shaking speed of 50 rpm, used in this study, yielded an average (7 replicates) transfer of 51.8% (standard deviation 4.8%). At shaking speeds >50 rpm the transfer decreased precipitously, probably due to shearing forces which disrupted the conjugation of cells. Transfer was not affected by moderate changes in the concentration of the mating/growth medium. When BHIB concentrations of 1.25 and 1.5 times the recommended strength were used, the percent transfer of ampicillin resistance remained constant.

The pooling of plate count data in determining both the total recipient population and that portion of the population receiving the ampicillin marker reduced the variation usually associated with plate counts. The repeatability, as measured by the coefficient of variation of pooled data, was 9.3%, which is good. The confidence limits (63.5–40.1%), although covering a range of 23.4%, were not excessive considering the dynamics of bacterial plate counts. The conditions for the transfer of resistance between the donor and recipient strains were repeatable. Standardization of the shaking speed, the concentration of the mating/growth medium, and the duration of incubation contributed to the consistency.

Table 1 shows the influence of residue levels of antibiotics on the transfer of ampicillin resistance between the 2 strains of *E. coli* used. With the exception of tylosin, concentrations of 0.01–1.00 μg or unit of antibiotic/mL did not increase the

Table 1. Influence of antibiotic residue levels on conjugal transfer of ampicillin resistance between *E. coli* RP-4 and *E. coli* Sc-8632

Antibiotic	Concn, unit or µg/mL	Transfer, %
Bacitracin	0.00[a]	54.2
	0.01	55.7
	0.10	51.9
	1.00	46.0
Oxytetracycline	0.00[b]	55.8
	0.01	27.4[c]
	0.10	45.5
	1.00	47.7
Penicillin	0.00[a]	52.7
	0.01	41.7
	0.10	53.9
	1.00	53.5
Streptomycin	0.00[b]	46.0
	0.01	49.3
	0.10	49.0
	1.00	55.9
Tylosin	0.00[b]	57.2
	0.01	52.3
	0.10	76.2[d]
	1.00	80.7[d]
Virginiamycin	0.00[b]	44.6
	0.01	52.5
	0.10	44.6
	1.00	55.6

[a] unit/mL.
[b] µg/mL.
[c] Transfer rate below lower limit of 95% confidence limit.
[d] Transfer rate above upper limit of 95% confidence limit.

rate of transfer of resistance beyond the 95% upper confidence limit. Tylosin residues showed a concentration-related response. A plot of the log of the concentration vs the log percent transfer yielded a straight line with a correlation coefficient of 0.92.

Oxytetracycline, at a concentration of 0.01 µg/mL, caused a decrease in the percent transfer below the 95% lower confidence limit. In general, oxytetracycline showed a dose-related rise in the percent transfer, although all transfers were below the mean of all controls. A plot of log of concentration vs log percent transfer gave a straight line with a correlation coefficient of 0.902. The reasons for the effect of the lowest concentration of oxytetracycline in decreasing transfer are unknown at this time. In contrast, increasing concentrations of bacitracin caused a decrease in the percent transfer. A plot of the log concentration vs log percent transfer gave a correlation coefficient of −0.989. This effect may be due to the surface-active properties of bacitracin, which can decrease the ability of cells to conjugate (14).

In general, residue levels of 0.01–1.00 µg/mL of representatives of 3 of the major antibiotic families implicated in the development of resistance, tetracyclines, β-lactams, and aminoglycosides, did not affect the transfer of resistance. Only tylosin, for reasons yet unknown and beyond the scope of this report, affected transfer.

The procedure described here can be used as a tool in evaluating various levels of antibiotic drugs and their effects on resistance transfer. This procedure, coupled with a system for the evaluation of the effects of residue levels of antibiotics upon selection for resistance (15), can give greater insight into the significance of antibiotic residues on bacterial populations without the problems associated with animal models. In addition, the procedures will permit an in vitro evaluation of the potential of newer antibiotics and drugs to affect bacterial populations before animal studies are undertaken.

References

(1) Huber, W. G. (1971) *Adv. Vet. Sci. Comp. Med.* **15**, 101–132
(2) Smith, H. W. (1969) *Lancet* **i**, 1174–1176
(3) Hirsh, D. C., & Wigner, N. (1978) *J. Anim. Sci.* **46**, 1437–1446
(4) Levy, S. B., Fitzgerald, G. B., & Macone, A. B. (1976) *Nature* **260**, 40–42
(5) Bettleheim, K. A., Bushrod, F. M., Chandler, M. E., Cooke, E. M., O'Farrell, S., & Shooter, R. A. (1974) *J. Hyg.* **73**, 467–471
(6) Linton, A. H., Howe, K., Bennett, P. M., Richmond, M. H., & Whiteside, E. J. (1977) *J. Appl. Bacteriol.* **43**, 465–469
(7) Richmond, M. W. (1980) *The Evolution of Antibiotic Resistance*, Squibb Institute for Medical Research, New Brunswick, NJ
(8) Smith, H. W. (1971) *Ann. NY Acad. Sci.* **182**, 80–84
(9) Walton, J. R. (1966) *Nature* **211**, 312–313
(10) Falkow, S. (1975) *Infectious Multiple Drug Resistance*, Pion Ltd, London, UK
(11) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **42.204**
(12) Bontempo, J. A. (1975) Ph.D. Thesis, Rutgers University, New Brunswick, NJ
(13) Patufka, T. M. (1981) M.S. Thesis, Rutgers University, New Brunswick, NJ
(14) Ott, J. L. (1971) *Ann. NY Acad. Sci.* **182**, 312–315
(15) Brady, M. S., Strobel, R. J., & Katz, S. E. (1988) *J. Assoc. Off. Anal. Chem.* **71**, 295–298

Extraction of Sulfamethazine from Feed Samples

W. JOHN BLANCHFLOWER and DESMOND A. RICE
Department of Agriculture, Veterinary Research Laboratories, Stormont, Belfast BT4 3SD, Northern Ireland

The efficiency of a number of published extraction solvents for sulfamethazine in feeds was evaluated. Poor recoveries were obtained with several of the solvents when sulfamethazine was extracted from commerically pelleted feed samples. Recoveries ranged from 48 to 94%. A 75% methanol acetic acid extractant gave the best results.

Sulfamethazine is frequently used as an antibacterial agent in medicated swine feeds, either alone or in combination with other antibacterial agents such as chlortetracycline or procaine penicillin. It can be determined in feed extracts by colorimetric (1, 2), liquid chromatographic (LC) (3–6), or gas chromatographic (7–9) methods.

While investigating some of these methods for possible use in our own laboratory, we found that the concentration of sulfamethazine obtained, particularly in pelleted swine feeds, was dependent on the solvent system used for extraction. We also found that when acetone–ammonia (7) was used as an extractant, the sulfamethazine levels appeared to decrease when feed samples were finely ground using a laboratory mill. This effect was also noted by others (2) when using acetone–methanol–water as an extractant but not when 50% methanol was used.

We therefore compared the efficiency of several extraction solvents used in published methods for the determination of sulfamethazine in feeds and attempted to optimize the extraction procedure. Extracts were assayed using a postcolumn derivatization method with dimethylaminobenzaldehyde (3).

Experimental

Apparatus

The LC and postcolumn reaction systems were used as described in the literature (8) with the following modifications:

(a) *Stainless steel reaction coil.*—Replaced by Teflon coil of same length and diameter.

(b) *Heating bath.*—Maintained at room temperature.

(c) *Detector.*—Wavelength set at 450 nm.

(d) *Mobile phase.*—Pumped at 1 mL/min; derivatization reagent pumped at 0.5 mL/min.

(e) *Analytical column.*—25 cm × 4.6 mm, packed with Partisil 5-ODS-3 (Whatman, Maidstone, Kent, England).

Reagents

(a) *Mobile phase and derivatization reagents.*—As described by Stringham et al. (3).

(b) *Sulfamethazine stock standard solution.*—Prepared in methanol (1 mg/mL). Dilute standards (10 μg/mL) were prepared in appropriate extractants.

(c) *Extractants.*—(A) methanol, (B) acetone–30% ammonia solution (98 + 2), (C) methanol–water (50 + 50), (D) acetone–methanol–water (85 + 10 + 5), (E) acetonitrile, (F) chloroform–methanol (3 + 1), (G) methanol–water–hydrochloric acid (25 + 73.7 + 1.3), and (H) methanol–water–glacial acetic acid (75 + 23 + 2).

(d) *Commercial swine feeds.*—Obtained from local feed compounder and used throughout. Samples were collected before and after pelleting process. Before analyses, loose as well as pelleted feed samples were ground using laboratory mill fitted with 2 mm screen.

Received May 11, 1987. Accepted November 20, 1987.

Procedure

Ten g aliquots of milled feed samples were weighed into 125 mL wide-mouth polyethylene bottles, and 100 mL appropriate extractant was added to each. Bottles were shaken 30 min in reciprocating shaker, except those containing extractant H, which were first heated 30 min at 70°C in a water bath before shaking. Bottles were capped during heating to prevent solvent evaporation.

Contents were allowed to settle, and aliquots were transferred to centrifuge tubes and centrifuged 15 min at 2500*g*. Two mL aliquots of extractant supernates, except extractant F, were placed directly in autosampler vials without further treatment. Two mL aliquots of extractant F supernates were evaporated to dryness at 50°C under nitrogen and residues were dissolved in 2 mL methanol. They were then transferred to autosample vials.

Using postcolumn derivatization method of Stringham et al. (3) for measuring sulfamethazine, 50 μL aliquots of samples and standards (10 μg/mL) were injected into LC system by an autosampler. Run time is 12 min/sample. Standards were prepared in same solvents used for extraction, except standards for extractant F, which were prepared in methanol.

Results

The sulfamethazine levels found in the 2 medicated swine feeds before and after pelleting are shown in Table 1. In general, the effect of the different extractants on loose unpelleted feed was not marked. Except for extractants D and F for feed 2, the levels found were very similar. In the pelleted feed, however, there was considerable variation. Acetonitrile gave the lowest recoveries, 60% and 48% for the high and low levels of sulfamethazine, respectively. Best recoveries were obtained when methanol–water–acetic acid was used as the extractant and bottles were heated before shaking (94% and 88%, respectively).

The effect of milling on the recovery of sulfamethazine from a feed sample is shown in Table 2. A loose feed sample was spiked with 100 mg sulfamethazine/kg, mixed, and milled using a laboratory mill fitted with 0.5 mm or 1 mm sieve. As can be seen, acetone/ammonia gave low recoveries (51% and 70%, respectively) with the milled feed whereas recoveries from the same samples were 96% and 95% when methanol–water–acetic acid was used as the extractant.

Discussion

All the examined published methods for determination of sulfamethazine in feed samples use extraction either by shaking or homogenization with a solvent, and have shown good recovery figures for sulfamethazine-spiked feed samples. One common factor, however, is that the samples have been spiked after milling, and analyses were performed on the samples without further processing.

Commercial feed samples are often supplied in pelleted form, and sulfamethazine, if used, is added before the pelleting process. It is in these samples in particular that we have found poor extraction of the added sulfamethazine. During the pelleting process the feed is subjected to a combination of heat, pressure, and moisture and this would appear to bind the sulfamethazine to the feed matrix and make it more difficult to extract.

Table 1. Comparison of sulfamethazine levels found in feeds before and after pelleting, using different extraction solvents

Extractant	Ref.	Feed 1 before pelleting, mg/kg	Feed 1 after pelleting, % rec.[a]	Feed 2 before pelleting, mg/kg	Feed 2 after pelleting, % rec.[a]
(A) Methanol	8	246	85	62	71
(B) Acetone–ammonia	7	247	69	63	60
(C) Methanol–water	4	243	91	66	74
(D) Acetone–methanol–water	2	250	82	56	82
(E) Acetonitrile	5	250	60	64	48
(F) Chloroform–methanol	6	231	81	52	67
(G) Methanol–water–HCl	3	244	72	63	68
(H) Methanol–water–acetic acid	—	245	94	66	88

[a] Expressed as percentage of level found before pelleting. Results are the means of duplicates.

From our own experiments, we found that methanol–water extractants gave higher results than methanol alone, and the highest recoveries were obtained by using 75% methanol–water containing 2% acetic acid (Table 1) and by heating 30 min at 70°C before shaking. Shaking for longer than 30 min or homogenization produced no increase in recoveries with any of the extractants.

Highest recoveries obtained (Table 1) for the 2 pelleted feed samples were 94% and 88%. This is still less than the desired 100%. Since sulfamethazine itself appears to be stable to heat and moisture, and there is no evidence that it is being destroyed, a small proportion is probably binding irreversibly to the feed matrix. However, we were unable to improve on these recovery figures by using either different extractants or extraction techniques.

Our results have also shown similar effects of sulfamethazine binding to the feed when the feed samples are finely milled through a 0.5 or 1 mm screen (Table 2). The effect in this case is extractant-dependent, with the methanol-based extractant giving better recoveries than the acetone-based extractant. This is surprising because sulfamethazine is very soluble in acetone, but it appears that acetone does not free it from the feed matrix. In any case, our studies have shown no need to mill samples using a screen size smaller than 2 mm. Another artifact reported by Munns and Roybal (7) when acetone–ammonia is used as extractant was that a 10 min extraction gave better results than a 30 min extraction. No explanation for this was offered.

Table 2. Effect of milling on recovery of sulfamethazine from feed sample spiked with 100 mg/kg

Solvent[a]	Sulfamethazine found, mg/kg[b]		
	Before milling	After milling	
		0.5 mm sieve	1 mm sieve
H	95	96	95
B	96	51	70

[a] (H) = methanol–water–acetic acid; (B) = acetone–ammonia.
[b] Results are the means of duplicates.

The postcolumn derivatization method of Stringham et al. (3) was used to assay the extractants because it is fast, requires no cleanup of extracts, and is free from interference from other feed constituents. It had an additional advantage in that it can be operated on the same analytical system of pumps, bath, and detector that we use to measure ionophore levels in feeds (8).

The low recoveries from pelleted feed samples with some of the recommended extractants highlight the need for caution in optimizing methods by spiking negative feed samples and assuming that commercially processed feeds will behave in the same way. Dependable results can only be obtained if feed samples are spiked at source.

References

(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **42.172-42.174**
(2) Schwartz, D. P. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 214–217
(3) Stringham, R. W., Mundell, E. C., & Smallidge, R. L. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 823–827
(4) Cieri, U. R. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 727–735
(5) Allred, M. C., & Dunmire, D. L. (1978) *J. Chromatogr. Sci.* **16,** 533–537
(6) McGary, E. D. (1986) *Analyst* **111,** 1341–1342
(7) Munns, R. K., & Roybal, J. E. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 1048–1053
(8) Blanchflower, W. J., Rice, D. A., & Hamilton, T. G. (1985) *Analyst* **110,** 1283–1287
(9) Holder, C. L., Thompson, H. C., & Bowman, M. C. (1981) *J. Chromatogr. Sci.* **19,** 625–633

Microbial Receptor Assay for Rapid Detection and Identification of Seven Families of Antimicrobial Drugs in Milk: Collaborative Study

STANLEY E. CHARM
Penicillin Assays, Inc., 36 Franklin St, Malden, MA 02148
RUEY CHI
Food and Drug Administration, Division of Mathematics, 200 C St, SW, Washington, DC 20204

Collaborators: G. E. Barnett; A. De Couville; J. Foos; W. Heeschen; J. Karolus; W. Kelley; L. Luckie; K. Manner; A. Marin; J.-P. Moretain; E. Reist; P. Saltzman; M. Schallibaum; L. Smith; G. Suhren; C. Tremble; R. Wallace

A microbial competitive receptor assay for detecting residues of antibiotic families in milk was studied collaboratively by 13 laboratories. The drugs and levels (ppb) tested in this study include penicillin G, 4.8; cephapirin, 5.0; cloxacillin, 100; tetracycline, 2000; chlortetracycline, 2000; oxytetracycline, 2000; erythromycin, 200; lincomycin, 400; clindamycin, 400; sulfamethazine, 75; sulfamethoxazole, 50; sulfisoxazole, 50; streptomycin, 1000; novobiocin, 50; and chloramphenicol, 800. In this method, microbial cells added to a milk sample provide specific binding sites for which ^{14}C or ^{3}H labeled drug competes with drug residues in the sample. The ^{14}C or ^{3}H binding to the specific binding sites is measured in a scintillation counter and compared with a zero standard milk. If the sample is statistically different from the zero standard, it is positive. The assay takes about 15 min. The binding reaction occurs between the receptor site and the drug functional group, so all members of a drug family are detected. In this case, beta-lactams, tetracyclines, macrolides, aminoglycosides, novobiocin, chloramphenicol, and sulfonamides, including *p*-aminobenzoic acid (PABA) and its other analogs, are detectable. The incidence of false negative determinations among samples is about 1%; the incidence of false positives is about 3%. For negative cases, the relative standard deviations for repeatability ranged from 0 to 5% and for reproducibility from 0 to 6%. For positive cases, relative standard deviations ranged from 0 to 13% for repeatability and from 0 to 14% for reproducibility. The method has been adopted official first action.

The present AOAC official methods for detecting antibiotic residues in milk are those for the beta-lactam drugs, **16.130-16.163** (1). Among these is a receptor assay method, **16.130-16.134** (1, 2), which is analogous in some respects to a radioimmunoassay (RIA). Where an RIA method uses an antibody binder, the receptor assay uses an immobilized site in a cell (in this case, a microbial cell) for the specific binder. A single cell contains the specific binding sites for many drugs. These binding reactions have low dissociation constants which are favorable for specificity and sensitivity.

Usually, although not always, microorganisms with binding sites for a particular drug are inhibited by that drug. In this receptor assay, 2 different microorganisms are required to provide the necessary sites for the 7 families. Because the functional chemical group of the drug is involved with the binding site and not a side chain as in the case of immunoassay, a single receptor detects all members of a family.

Figure 1 shows specific binding sites provided by a microbial cell. Drug tracers labeled with ^{14}C or ^{3}H compete for binding sites with the "cold" drug associated with the sample. In Figure 1, penicillin ^{14}C (P^{14}) and tetracycline ^{3}H (T^{3}) are competing for their respective sites with cold penicillin (P) and cold tetracycline (T): the greater the concentration of drug, the less binding of drug tracer to the binding sites. The amount of tracer binding is correlated with the concentration of drug in the sample.

This method detects and identifies 7 families of antibiotics in milk: beta-lactams (P), tetracyclines (T), macrolides (E), aminoglycosides (St), novobiocin (N), chloramphenicol (C), and sulfonamides (Sm) along with other *p*-aminobenzoic acid (PABA) analogs and PABA. Digital test results are expressed as positive or negative using a control point based on a predetermined standard. To identify a specific drug in a family, a confirmatory assay must be performed. The method is used to test milk tankers before they unload as well as farm samples and finished milk products.

The receptor assay may be completed in 12–15 min. This offers the opportunity for screening a wide spectrum of antibiotics before tankers are unloaded at processing plants. These antibiotics could interfere with the manufacture of cultured products or cause a large volume of milk to be in violation of state or federal regulations.

Collaborative Study

Twenty-seven coded samples representing 7 different families of antimicrobial drugs (Table 1) were sent to 13 collaborating laboratories. These included U.S. and non-U.S. federal, state, and industrial laboratories.

The 9 combinations, 3 replicates each, of 7 drug families, were distributed as 27 coded samples (1–27) (Table 2). Collaborators also received a copy of the method, instructions on tabulation of data and conversion of data to final results, and a collaborative study kit which included the following items:

(1) Zero milk powder, three 100 g packages.
(2) Reagents, freeze-dried.
(3) Positive standards (ng/mL): penicillin, 4.8; streptomycin, 500; novobiocin, 50; erythromycin, 100; sulfamethazine, 75; tetracycline, 2000; chloramphenicol, 800.
(4) Twenty-seven coded samples.
(5) Disposable pipets, tips, test tubes, 1500 mL plastic bottle for reconstituting milk powder.

Antimicrobial Drugs in Milk

Microbial Receptor Assay

First Action

(Min. detectable concns (ng/mL) are penicillin G, 3; tetracycline, 600; erythromycin, 20; streptomycin, 10; novobiocin, 10; sulfamethazine, 5; chloramphenicol, 80.)

16.D01 ***Principle***

Assay is based on binding reaction between drug functional group and receptor site on added microbial cells. ^{14}C or ^{3}H binding is

Submitted for publication August 4, 1987.
This report of the Associate Referee, S. E. Charm, was presented at the 100th AOAC Annual International Meeting, Sept. 15–18, 1986, at Scottsdale, AZ.
The recommendation of the Associate Referee was approved interim official first action by the General Referee, the Committee on Feeds, Fertilizers, and Related Materials, and the Chairman of the Official Methods Board. The method was adopted official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) **71**, January/February issue.

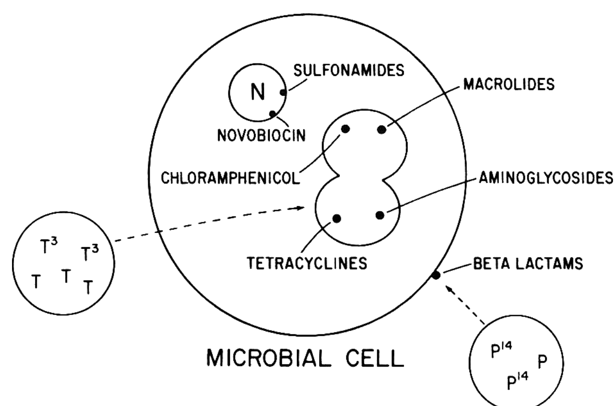


Figure 1. Sites in microbial cell used to specifically bind antibiotics. Cold antibiotic in sample competes for its specific site with radiolabeled antibiotic.

measured by scintillation counter and compared with zero std milk to detect antimicrobials. The greater the amt of antibiotic present in the sample, the lower the counts. Method does not detect metabolites, only active drugs.

16.D02 *Apparatus*

App. is available from Penicillin Assays Inc., 36 Franklin St, Malden, MA 02148.

(a) *Heaters.*—Two dry well incubators of anodized Al, each with 6 holes, 5.23 cm deep, 1.35 cm diam., controlled at 45 and 85 ± 1°, for 13 × 100 mm glass test tube.

(b) *Mixer.*—Maxi Mix II (Thermolyne Corp., 2555 Kerper Blvd, Dubuque, IA 52001), or equiv.

(c) *Bench centrifuge.*—12-place, 1300 ×g. Whisperfuge (Damon Scientific, 300 Second Ave, Needham Heights, MA 02194), or equiv.

(d) *Cold plate.*—To accommodate 7 mL plastic scintillation vials holding at −3 to −5°.

(e) *Scintillation counter.*—To accommodate 13 × 100 mm glass tube. Window settings such that <10% of ^{3}H crosses into ^{14}C channel and <30% of ^{14}C crosses into ^{3}H. Counting time is 1 min. Efficiency is >80%. Replication in counting same tube at ca 1000 cpm is ca 10%.

(f) *Scintillation fluid dispenser.*—(Oxford [Monoject Scientific, 1831 Olive St, St. Louis, MO 63103]: or equiv.).

(g) *Labware.*—Cotton swabs. Wash bottle. 13 × 100 mm test tubes, wall thickness 0.084 ± 0.005 cm. Plastic stoppers for 13 × 100 mm tubes. Plastic scintillation vials, 7 mL. Pipets capable of delivering 200 μL, 300 μL, and 5 mL.

16.D03 *Reagents*

Reagents are available from Penicillin Assays Inc.

(a) *Scintillation fluid.*—Optifluor (Packard Instrument Co., 2200 Warrenville Rd, Downers Grove, IL 60515), or equiv.

(b) *Microbial binders.*—(*1*) *Reagent D:* Freeze-dried suspension of *Bacillus stearothermophilus*. Reagent is stable 1 year at −20°. When reconstituted with Diluent D, it is stable 1 week at −20° or 24 h at −5°. About 1 × 10^8 cells are added per assay or 200 μL, used for binding beta-lactams, tetracyclines, streptomycin, macrolides, and novobiocin.

(*2*) *Reagent R:* Freeze-dried irradiated, nonviable suspension of microorganism, with receptor sites for sulfonamides and chloramphenicol (microorganisms inhibited by these drugs possess required receptor sites). Stable 1 year at −20° freeze-dried, and 6 h at −5° reconstituted. About 1 × 10^9 cells are used per 200 μL for binding sulfonamides and chloramphenicol.

(c) *Antibiotic tracer reagents.*—(*1*) Penicillin ^{14}C 125 μCi/μmol, 0.0024 μCi/test. (*2*) Tetracycline ^{3}H 0.5 μCi/μmol, 0.052 μCi/test. (*3*) Erythromycin ^{14}C 38 μCi/μmol, 0.0031 μCi/test. (*4*) Streptomycin ^{3}H 26 μCi/mmol, 0.0185 μCi/test. (*5*) Novobiocin ^{3}H 55 μCi/mmol, 0.0148 μCi/test. (*6*) Sulfamethazine ^{3}H 59 μCi/mmol, 0.0311 μCi/test. (*7*) Chloramphenicol ^{14}C 35 μCi/mmol, 0.0126 μCi/test.

Table 1. Antibiotic families, members, levels, and designation of specific antibiotics used in the study

Family	Designate	Member	Concn, ppb	Coded samples containing antibiotic
P Beta-lactams	P1	penicillin G	4.8	1, 15, 19, 24
	P2	cephapirin	5.0	2, 3, 21, 27
	P3	cloxacillin	100	6, 11, 14, 16
T Tetracyclines	T1	tetracycline	2000	12, 18, 23, 25
	T2	chlortetracycline	2000	4, 8, 10, 20
	T3	oxytetracycline	2000	5, 9, 17, 22
E Erythromycin type	E1	erythromycin	200	15, 81, 19, 23
	E2	lincomycin	400	2, 4, 20, 21
	E3	clindamycin	400	6, 9, 16, 22
Sm Sulfonamides	Sm1	sulfamethazine	75	1, 18, 19, 25
	Sm2	sulfamethoxazole	50	2, 3, 10, 20
	Sm3	sulfisoxazole	50	6, 11, 17, 22
St Streptomycin	St	streptomycin	1000	1, 3, 5, 8, 10, 11, 12, 14, 17, 24, 25, 27
N Novobiocin	N	novobiocin	50	2, 5, 6, 8, 10, 12, 15, 16, 17, 19, 21, 25
C Chloramphenicol	C	chloramphenicol	800	4, 5, 8, 9, 11, 14, 15, 16, 21, 23, 24, 25

Antibiotic tracer reagents have stabilizers added to preserve their biological activity. Freeze-dried tracer reagents are stable ≥12 months at −20°; reconstituted tracer reagents are stable 2 weeks at −20°.

In certain cases ^{14}C and ^{3}H tracers are combined to facilitate screening for more than 1 family in each tube. This is done with penicillin ^{14}C and tetracycline ^{3}H as well as erythromycin ^{14}C and streptomycin ^{3}H.

(d) *Diluent C.*—70% soln of propylene glycol for reconstituting radiolabeled tracer antibiotics.

(e) *Diluent D.*—Soln contg 5% sucrose, 10% dimethyl sulfoxide (DMSO), 1.2% NaCl for reconstitution of binding microorganisms for Reagents D and R.

(f) *Reagent NH.*—12% $(NH_4)_2SO_4$.

16.D04 *Reconstitution of Reagents*

(a) *Tracer reagents.*—Add room temp. diluent to freeze-dried tracer. Dissolution occurs immediately. Remove amt reagent needed for 1 day into plastic screw-cap vial. Place vial in cold plate at −3 to −5° for up to 24 h. Store remaining tracer as liq. at −20° for up to 2 weeks. Small plastic vials may be refilled as needed from storage vial held at −20°.

(b) *Microbial binding reagents.*—Reconstitute *Reagent D* by adding vol. of cold Diluent D (4°) indicated on label, usually 12 mL. Break up clumps of cells by using large glass test tube and vortex mixer. Mix well. Let reconstituted Reagent D warm to room temp. (25°) and hold for ca 5 min. Dispense into vials and place in cold plate at −5° for use or store at −20°. Reagent D may be stored at −20° for 1 week or 12 h at −5°. Thaw frozen Reagent D by placing in tap H_2O (ca 20°) for 5 min and cool in cold plate ≥5 min before use.

Reconstitute *Reagent R* by adding amt of Diluent D indicated on bottle. Mix well. Reagent R may be stored in cold plate 6 h at −5°.

16.D05 *General Instructions*

(a) *Exempt quantities of ^{14}C and ^{3}H.*—Amts of radioactivity used in this method are sufficiently low as to be exempt from U.S. Nuclear Regulatory Commission (NRC) and agreement state regulations. This applies to laboratories unlicensed by NRC.

Check appropriate national and local regulations for use of exempt amts of ^{14}C and ^{3}H.

Table 2. Antibiotic families and combinations of coded samples

Sample	Antibiotic family						
	P	T	E	St	C	Sm	N
A (1, 3, 11)[a]	+	−	−	+	−	+	−
B (2, 6, 19)	+	−	+	−	−	+	+
C (4, 9, 23)	−	+	+	−	+	−	−
D (5, 8, 12)	−	+	−	+	+	−	+
E (7, 13, 26)	−	−	−	−	−	−	−
F (10, 17, 25)	−	+	−	+	−	+	+
G (14, 24, 27)	+	−	−	+	+	−	−
H (15, 16, 21)	+	−	+	−	+	−	+
I (18, 20, 22)	−	+	+	−	−	+	−

[a] The numbers 1, 3, and 11 in parentheses are the coded test samples which are the 3 replicates of the A combination, etc. The A combination consists of P, St, and Sm antibiotic families. See Table 1 for family member in each coded sample.

(**b**) *Disposal of scintillation fluid*.—Liq. scintillation counting solns which are readily soluble or dispersible in H_2O (Optifluor, or equiv.) and which contain <0.05 μCi ^3H or ^{14}C per g soln can be dispersed in large vol. of H_2O and disposed thru sanitary sewerage system (in this method, 2.8 g Optifluor/assay is used). It is recommended that ratio of running H_2O to scintillation fluid exceed 10:1. Dispersion may be cloudy due to presence of high bp hydrocarbon. No special fire prevention precautions are necessary. Note that local regulations must be observed if these differ from federal regulations.

16.D06 *Performance Check*

Check reagents and equipment by testing zero std milk. Compare with previous zero milk results or with manufacturer's typical count sheet that accompanies reagents. If comparable results are obtained, proceed.

Combine tracers as follows: penicillin and tetracycline (P&T), erythromycin and streptomycin (E&St), novobiocin (N), sulfonamides and other PABA analogs (Sm), chloramphenicol (C).

(*1*) Mark 5 tubes: (P&T), (E&St), (N), (Sm), (C).

(*2*) Add 200 μL tracer reagent to corresponding tube; wipe pipet before each addn. Pipet reagent directly to bottom of tube.

(*3*) *For chloramphenicol only*, add 300 μL Reagent NH.

(*4*) Add 5 mL milk sample to each test tube.

(*5*) Gently add 200 μL *Reagent D* to (P&T), (E&St), and (N) tubes. Add *Reagent R* to (Sm) and (C) tubes.

(*6*) Mix by letting milk swirl and settle ca 10 times to ensure thoro distribution of added reagents. This should take ca 10 s.

(*7*) Incubate (P&T) and (E&St) 3 min at 85°; incubate (N), (Sm), and (C) 3 min at 45°.

(*8*) Centrf. tubes 3 min and then decant milk completely.

(*9*) Hold tubes at 45° tilt and rinse by filling tube once with H_2O from wash bottle; do not disturb ppt at bottom of tube. Tilt tubes down to drain. Wipe dry with 2 or more swabs while wiping out fat ring.

Table 3. Six zero determinations (cpm) for fixing control point (example from Lab. 5)

Sample No.	P &	T	E &	St	C	Sm	N
1	1688	4334	2371	3754	2185	3427	3172
2	1696	4081	2527	3679	2129	3166	3254
3	1581	4201	2156	3550	1972	3208	2861
4	1649	4298	2039	3594	2158	3222	3518
5	1693	4062	1935	3462	2333	3371	2652
6	1642	4089	1855	3841	2210	3305	2771
Av.	1658	4178	2147	3730	2165	3283	2965
% subtracted from av.	15	20	20	25	20	20	20
Control point[a]	1409	3342	1718	2797	1732	2626	2372

[a] Control points are about 3 standard deviations from average except P which is about 2.5.

Table 4. Control points (cpm) for known standards (example from Lab. 5)

Sample	Amt, ng/mL	P +	T	E +	St	C	Sm	N
Penicillin	4.8	1233 1252						
Tetracycline	2000		2929 3084					
Erythromycin	100			1114 1111				
Streptomycin	500				1980 2087			
Chloramphenicol	800					1386 1367		
Sulfamethazine	75						1163 1053	
Novobiocin	50							1480 1578

(*10*) Add 300 μL H_2O and resuspend pellet completely, using mixer.

(*11*) Dispense 3 mL scintillation fluid into tube, cap, and shake tube gently.

(*12*) Measure activity in analyzer ^3H channel and ^{14}C channel. Read (T), (St), (N), and (Sm) on ^3H channel and (P), (E), and (C) on ^{14}C channel.

Note whether number is above control point for neg. sample or below for pos. Control point is number about 3 std devs from av. of 6 zero samples. Reassay pos. samples.

16.D07 *Double Positive*

To det. whether double pos. in 1 tube is true double pos. or is result of single pos. and "cross talk" between ^{14}C and ^3H channels,

Table 5A. Data (cpm) for positive controls for coded samples (results from Lab. 5)

CODE NO.	P +	T	E +	St	C		Sm		N	
	C14	H3	C14	H3	C14	H3	C14	H3	C14	H3
1	1363	4176	2138	1957	2080	(894)	(214)	1015	(338)	3316
2	1260	3934	1011	3195	1890	(762)	(159)	878	(244)	1350
3	1195	3641	2023	2039	2237	(836)	(180)	814	(351)	2999
4	1330	2335	818	3003	1186	(560)	(252)	3059	(332)	3091
	*(u) 1348	2167								
	1508	2253								
	*(z) 1301	2833								
	1379	2970								
5	1484	2722	1962	1934	1274	(631)	(291)	2813	(237)	1555
6	1197	3706	971	3121	1884	(775)	(195)	828	(229)	1268
7	1665	3993	2108	3352	2128	(883)	(263)	2941	(332)	2929
8	1660	2610	2057	1928	1306	(567)	(216)	2772	(261)	1517
9	1576	2912	907	2899	1266	(588)	(222)	2629	(307)	2771
10	1464	2599	1900	1842	2021	(804)	(164)	789	(234)	1117
11	1210	3770	1985	2032	2028	(806)	(182)	757	(371)	3650
12	1573	3091	1921	1966	1366	(615)	(252)	2834	(234)	1239
13	1627	3930	1980	3557	1911	(781)	(260)	2948	(352)	2759
14	1152	3557	1633	1867	1305	(581)	(270)	2943	(329)	3073
			(u) 1828	1507						
			1913	1403						
			(z) 1972	2986						
			2001	2825						
15	1190	3701	853	2744	1303	(600)	(278)	2725	(220)	1298
			(u) 811	3103						
			710	3093						
			(z) 1851	3054						
			1942	2952						
16	1128	3600	1000	3116	1315	(586)	(271)	2716	(211)	1311
17	1679	2536	2175	1955	1893	(774)	(190)	744	(231)	1155
18	1603	2719	842	2778	1743	(769)	(179)	1020	(319)	2913
			(u) 731	2733						
			638	2495						
			(z) 1829	2734						
			1797	2737						
19	1241	3651	846	3040	1816	(846)	(162)	905	(241)	1404
20	1559	2885	905	3143	1806	(722)	(185)	773	(360)	3376
21	1177	3543	1003	3033	1241	(582)	(252)	2821	(217)	1316
22	1579	2918	866	3193	1764	(784)	(203)	805	(326)	2949
23	1522	2719	864	3101	1259	(547)	(257)	2755	(329)	3155
24	1243	3174	1734	1974	1478	(616)	(240)	2702	(313)	2996
			(u) 1079	2526						
			967	2945						
			(z) 1451	2790						
			1310	2673						
25	1705	3024	2081	2032	2072	(870)	(185)	904	(212)	1194
26	1727	3912	1706	3452	1964	(762)	(266)	2831	(285)	3062
27	1244	3574	1832	1946	1401	(649)	(250)	2536	(345)	3489

* (u) = Unknown tested with single tracer.
(z) = Zero tested with single tracer.

Table 5B. Summary of final results for each laboratory: N = negative, P = positive; * = false negative, X = false positive
Numerical readouts are compared with control points and converted to positive (below control point) or negative (above control point).

Correct Solution for Coded Samples

CODE NO.	P +	T	E +	St	C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	N	P	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Laboratory #1

CODE NO.	P +	T	E +	St	C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	N	N
14	P	N	N	P	P	N	PX
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	N	P	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Laboratory #2

CODE NO.	P +	T	E +	St	C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	PX	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	PX	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	N*	N	N	P	P	N	N
25	N	N*	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Table 5B (contd). Summary of final results for each laboratory

Laboratory #3

CODE NO.	P + T		E + St		C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	N	P	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Laboratory #4

CODE NO.	P + T		E + St		C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	PX	N
10	N	P	N	P	N	N*	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	N	P	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Laboratory #5

CODE NO.	P + T		E + St		C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	N	P	N	P	N	P	P
26	N	N	PX	N	N	N	N
27	P	N	N	P	P	PX	N

Laboratory #6

CODE NO.	P + T		E + St		C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	PX	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	PX	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	PX	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	PX	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	N	P	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Table 5B (contd). Summary of final results for each laboratory

Laboratory #7

CODE NO.	P +	T	E +	St	C	Sm	N
1	P	PX	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	PX	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	PX	N	N	N	N
8	PX	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	PX	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	PX	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	PX	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	PX	P	N	P	PX	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Laboratory #8

CODE NO.	P +	T	E +	St	C	Sm	N
1	P	PX	N	P	N	P	N
2	P	PX	P	N	N	P	P
3	P	PX	N	P	N	P	N
4	N	P	P	PX	P	N	N
5	N	P	N	P	P	N	P
6	P	PX	P	PX	N	P	P
7	N	N	PX	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	PX	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	PX	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	PX	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	PX	N*	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	N*	PX	N	P	PX
21	P	N	P	N	P	N	P
22	PX	P	P	N	PX	P	PX
23	PX	P	P	N	P	PX	PX
24	P	N	N	N*	P	N	PX
25	N	P	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	PX	N	P	P	N	N

Laboratory #9

CODE NO.	P +	T	E +	St	C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	PX	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	PX	N
8	N	P	N	P	P	PX	P
9	N	P	P	N	P	PX	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	PX	P
13	N	N	PX	N	N	PX	N
14	P	N	N	P	P	PX	N
15	P	N	P	N	P	PX	P
16	P	N	P	N	P	PX	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	PX	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	PX	N
24	P	N	N	P	P	PX	N
25	N	P	N	P	N	P	P
26	PX	N	N	N	N	N	N
27	P	N	N	P	P	PX	N

Laboratory #10

CODE NO.	P +	T	E +	St	C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	N*
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	N	P	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Table 5B (contd). Summary of final results for each laboratory

Laboratory #11

CODE NO.	P +	T	E +	St	C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	PX	P	N
19	P	N	P	N	PX	P	P
20	N	P	P	N	PX	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	N*	P	N	P	N	N
24	N*	N	N	P	N*	N	N
25	N	P	N	P	N	P	P
26	N	P*	N	N	N	N	N
27	P	N	N	P	P	N	N

Laboratory #12

CODE NO.	P +	T	E +	St	C	Sm	N
1	N*	PX	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	P	P	N	N*	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	N	N
14	P	N	N	P	P	N	N
15	P	N	P	N	P	N	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	N	P	P
20	N	P	P	N	N	P	N
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	N	P	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Laboratory #13

CODE NO.	P +	T	E +	St	C	Sm	N
1	P	N	N	P	N	P	N
2	P	N	P	N	N	P	P
3	P	N	N	P	N	P	N
4	N	N*	P	N	P	N	N
5	N	P	N	P	P	N	P
6	P	N	P	N	N	P	P
7	N	N	N	N	N	N	N
8	N	P	N	P	P	N	P
9	N	P	P	N	P	N	N
10	N	P	N	P	N	P	P
11	P	N	N	P	N	P	N
12	N	P	N	P	P	N	P
13	N	N	N	N	N	PX	N
14	P	N	N	P	P	PX	N
15	P	N	P	N	P	PX	P
16	P	N	P	N	P	N	P
17	N	P	N	P	N	P	P
18	N	P	P	N	N	P	N
19	P	N	P	N	PX	P	P
20	N	P	P	N	N	P	PX
21	P	N	P	N	P	N	P
22	N	P	P	N	N	P	N
23	N	P	P	N	P	N	N
24	P	N	N	P	P	N	N
25	N	P	N	P	N	P	P
26	N	N	N	N	N	N	N
27	P	N	N	P	P	N	N

Table 6. Results (cpm) for comparison of penicillin and tetracycline determined together and separately, demonstrating effect of "cross talk" between ^3H and ^{14}C channels

Sample	^{14}C	% Drop from zero	^3H	% Drop from zero
Zero milk	1170		5070	
	1077		4945	
	1064		4361	
	$\bar{x}$ = 1104		$\bar{x}$ = 4792	
0.008 IU P/mL	778		3413	
	716		3358	
	767		3548	
	$\bar{x}$ = 754	32	$\bar{x}$ = 3440	28
2000 ppb T	918		2036	
	968		2194	
	918		2013	
	$\bar{x}$ = 935	15	$\bar{x}$ = 2081	57
0.008 IU P/mL and 2000 ppb T	635		1758	
	726		1842	
	661		1848	
	$\bar{x}$ = 674	39	$\bar{x}$ = 1816	62

retest sample with single tracers in duplicate and compare with 2 zero detns carried out with single tracers. If single tracer result compares with zero single tracer, then that "pos." is due to cross talk.

16.D08 ***Control Point Determination***

Set control point ≥3 std devs from av. zero. For convenience, use percentage to est. 3 std devs.

For penicillin, use ca 2.5 std devs because <0.008 IU/mL must be detected to accommodate *B. stearothermophilus* disc assay std where required by U.S. Food and Drug Administration Pasteurized Milk Ordinance (1983; revision).

To det. control points, subtract following percentages from av. of 6 zero detns.

Antibiotic	*% subtracted from average zero*
Beta-lactam	15
Tetracyclines	20
Macrolides	20
Streptomycin	25
Novobiocin	20
Sulfonamides	20
Chloramphenicol	20

Results and Discussion

Statistical Evaluation

Thirteen collaborating laboratories returned results. Control points for each drug family were determined from the average of 6 zero milk determinations (Table 3). An example of the values obtained for positive controls is shown in Table 4. Coded samples are tabulated similar to the zero controls, and are compared with the control point (Table 5A). Results above the control point are negative and those below are positive, Table 5B, Laboratory 5. Table 5B contains final results for each laboratory. Numerical values are converted to positive (below control point) or negative (above control point).

In Table 5A, note the double positive in coded sample 4. Double positives are rechecked using single tracer and compared with a zero control. The "cross talk" between the ^{14}C and ^3H channels can cause a positive in one channel to draw down the result for the other channel below the control point, causing a false positive. Checking with single tracer avoids the cross-talk effect and permits determining which samples are truly positive or negative.

Table 7. False positive and false negative results for each laboratory

Lab.	Number of false pos. per 104 neg. samples	Number of false neg. per 85 pos. samples	Total false incorrect out of 189 samples	% Incorrect detns
1	1	0	1	0.53
2	2	2	4	2.12
3	0	0	0	0.00
4	1	1	2	1.06
5	2	0	2	1.06
6	4	0	4	2.12
7[a]	9	0	9	4.76
8[a]	21	3	24	12.70
9[b]	15	0	15	7.94
10	1	1	2	1.06
11	4	1	5	2.65
12	2	1	3	1.59
13	5	1	6	3.17

[a] Labs 7 and 8 excluded as procedural deviates. See text.
[b] Thirteen of 15 false positives due to Sm. Sm results excluded from study. See text.

For example, in a mixture of 0.008 IU penicillin G/mL and 2000 ppb tetracycline, the penicillin count is dropped 39% from zero and tetracycline is dropped 62%. When these antibiotics are separated, the presence of 2000 ppb tetracycline drops the zero penicillin count 15% due to cross talk and 0.008 IU penicillin/mL reduces the zero tetracycline count by 28% (Table 6).

A summary of all incorrect determinations for each laboratory is shown in Table 7. Laboratories 7 and 8 reported a number of double positives that were not checked with single tracers according to procedure. This should not be confused with simply rerunning duplicate samples, which was done by these laboratories. Laboratory 7 had 4.76% incorrect determinations, and laboratory 8 had 12.7%. These laboratories were omitted from computations as procedural deviates, i.e., they did not follow the prescribed procedure, and their results would not be representative of the method's true capability.

Laboratory 9 had 7.94% incorrect determinations. Thirteen of 15 false positives are due to sulfonamide determinations. The results for sulfonamides (Sm) from this laboratory were omitted as outliers. Faulty reagent is the suspected cause of the Sm false positives.

There is statistical validity for omitting data from laboratory 9 from the Sm results: this laboratory's success fraction for positives (8.50%) lies well outside the 95% confidence interval (89.4%, 98.1%) for the overall success fraction for positives for the remaining 10 laboratories. The Grubbs or Dixon outlier tests would not be appropriate because they assume normal distributions and, with so many identical values, the results are not normally distributed. Thus, 16% of the study data was excluded from analysis.

A summary of incorrect determinations for each laboratory and each drug combination is shown in Table 8. In all cases, there were no more than 3 incorrect determinations for any combination by any laboratory and not more than 6 total incorrect determinations for any laboratory.

For each of the 7 drug families, each laboratory analyzed 3 sets of 4 samples with the drug (positives) and 3 sets of 5 samples without the drugs (Table 2). Measures of precision

Table 8. Summary of incorrect determinations for each laboratory and each combination (see Table 2)

	Combination									
Lab.	A	B	C	D	E	F	G	H	I	Total
1							1			1
2					2	1	1			4
3										0
4			1			1				2
5					1		1			2
6			1						3	4
9[a]					2					2
10								2		2
11		1	1		1				2	5
12	2		1							3
13		1	1		1		1	1	1	6

[a] Sm determinations excluded from analysis.

Table 10. Measures of precision for positive cases[a]

Antibiotic	S_r	RSD_r, %	S_R	RSD_R, %	Success, %
P	0.075	7.7	0.075	7.7	97.7
T	0.097	10.0	0.097	10.0	97.7
E	0.044	4.4	0.044	4.4	99.2
Sm[b]	0.079	8.3	0.103	10.9	95.0
St	0.044	4.4	0.044	4.4	99.2
N	0.000	0.0	0.000	0.0	100.0
C	0.123	12.9	0.132	13.9	95.5

[a] A positive case corresponds to the situation where a member of the indicated antibiotic family is actually present. S_r and S_R are standard deviations for repeatability and reproducibility, respectively; RSD_r and RSD_R are relative standard deviations for repeatability and reproducibility, respectively.

[b] Laboratory 9 omitted as a statistical outlier (α = 0.05).

were calculated using conventional AOAC formulas (3). Table 9 summarizes results for negative cases and Table 10 for the positive cases. Note in Table 9 that there is little difference whether laboratory 9 is omitted or included in the analysis of Sm data.

The triplicate values used for each laboratory are decimal fractions corresponding to the 3 incidence or success scores of the number correct divided by the number tested. For exploratory purposes and ease of exposition, no transformations (such as Tukey arcsine transformation) were performed. Computations were done separately for the positive and negative since no a priori reasons exist for the false positive rate to equal the false negative rate. The confidence intervals for the overall success rate (positives and negatives combined) and for positives and negatives treated separately are presented in Table 11.

Conclusions of the study are: (*1*) The method is not prone to false positives. Even in the worst case (T), blank samples are identified correctly 98.2% of the time.

(*2*) At the concentrations used, the method satisfactorily detects the presence of drug residues. Even in the worst case (Sm), 95% of the time the drug residue is correctly identified.

(*3*) The efficiency of this rapid screening method is consistent among the laboratories and among the antibiotic families and members within each family.

(*4*) These conclusions are not sensitive to varying assumptions and varying statistical approaches.

Detectable Concentrations

For the concentrations used in the collaborative study (Table 1), the method precisely and accurately notes the presence and absence of members of the 7 drug families studied. Repeatability and reproducibility found at the level studied cannot be extrapolated to lower concentrations. These concentrations were selected in most cases because they give barely noticeable positives on the *Bacillus stearothermophilus* disk assay method used by state regulatory agencies.

Table 9. Measures of precision for negative cases[a]

Antibiotic	S_r	RSD_r, %	S_R	RSD_R, %	Success, %
P	0.000	0.0	0.000	0.0	100.0
T	0.049	5.0	0.059	6.0	98.2
E	0.000	0.0	0.000	0.0	100.0
Sm[b]	0.037	3.7	0.037	3.7	99.3
Sm[c]	0.035	3.5	0.035	3.5	99.4
St	0.000	0.0	0.000	0.0	100.0
N	0.035	3.5	0.035	3.5	99.4
C	0.035	3.5	0.035	3.5	99.4

[a] A negative case corresponds to the situation where a member of the indicated antibiotic family is absent. S_r and S_R are standard deviations for repeatability and reproducibility, respectively; RSD_r and RSD_R are relative standard deviations for repeatability and reproducibility, respectively.

[b] Results for laboratory 9 omitted because erratic behavior for positive cases indicates reagent problem.

[c] Results retained for all 11 laboratories (results for labs 7 and 8 excluded as procedural outliers).

Reducing False Positive Rate by Retesting Positives

In the collaborative study it was not possible to retest positives because of the large number of samples; however, in practice, positive samples are reassayed. In so doing, the false positive rate is decreased. A false positive result due to incorrect procedure is not likely to be repeated in the retest. A negative control is also assayed with the positive sample in the retest, so a positive result due to faulty reagents is readily identified.

Assay of Mixture of Antibiotics vs Antibiotics Individually

Table 12 compares results for antibiotics assayed in a mixture vs antibiotics tested individually. The tracers are not mixed together so as to avoid cross talk. There is no significant interaction between the antibiotics in the concentration range used in the study. However, it is pointed out that lincomycin binds to the same ribosomal subunit as does erythromycin, and cross-reaction between these 2 drugs is strong in this assay even though they are not in the same family. Lincomycins resemble macrolides in antibacterial activity and mode of action (4).

Assay for PABA Analogs and PABA

The assay for sulfonamides also detects other PABA analogs such as benzoic acid as well as PABA itself. None of these are allowed additives to milk in the United States. Only sulfonamides are commonly used with cows and are the only likely PABA analog causing contamination. Clorsulon, a disulfonamide, is not detected as sulfonamide, nor is analine, which has a related structure to PABA.

Aminoglycosides—Streptomycin vs Gentamycin

Although the characteristic misreading action of various aminoglycosides suggests that they bind to closely related microbial sites, these cannot be identical. Streptomycin ^3H cross-reacts with gentamycin at high concentrations in the receptor assay. Using gentamycin ^3H, sensitivity is improved about 30 fold. Gentamycin binds to multiple sites (5). Before gentamycin cross-reacts with streptomycin ^3H it may have to be in sufficient concentration to fill secondary sites. Then it may compete with streptomycin ^3H for primary sites.

Table 11. Summary of success rates[a]

	Positives		Negatives		Combined	
Antibiotic	Incidence	Interval	Incidence	Interval	Incidence	Interval
P	129/132	(93.5, 99.5)	165/165	(98.2, 100.0)	294/297	(97.9, 100.0)
T	129/132	(93.5, 99.5)	162/165	(94.8, 99.6)	291/297	(96.4, 99.6)
E	131/132	(95.9, 100.0)	165/165	(98.2, 100.0)	296/297	(99.0, 100.0)
Sm[b]	114/120	(89.4, 98.1)	149/150	(96.3, 100.0)	263/270	(95.5, 99.3)
E	131/132	(95.9, 100.0)	165/165	(98.2, 100.0)	296/297	(99.99, 100.0)
N	132/132	(97.8, 100.0)	164/165	(96.7, 100.0)	296/297	(99.0, 100.0)
C	126/132	(90.4, 98.3)	164/165	(96.7, 100.0)	290/297	(95.9, 99.4)

[a] With total number of determinations used as denominator, data are observed rates and lower and upper values for corresponding 95% confidence intervals.
[b] Results from laboratory 9 omitted throughout.

Reagent Stability

The reagent R lot used in the collaborative study was irradiated non-uniformly. This resulted in degradation of certain vials, which were associated with low counts. A performance test was suggested to collaborators to avoid the over-irradiated R vials. This problem was an aberration and has been accommodated. The sulfonamide outliers associated with laboratory 9 are thought to be the result of using a degraded reagent R.

All freeze-dried reagents are given expiration dates of 1 year at −20°C. Reagent stability actually exceeds this time.

Reconstituted receptor reagents (D and R) are quality controlled for stability at −20°C. In the collaborative study these reagents were used the same day as reconstituted and were not frozen for future use. Reconstituted reagent D may be stored at −20°C for 1 week or once thawed at 0°C for 8 h. Reagent R may experience a 10% drop in binding on freezing. Tracer reagents may be stored at −20°C for 2 weeks after reconstitution.

Other factors that can appear to be reagent problems but are not include unstable electric power and light-activating fluorescence in high-potassium test tubes. A power regulator or dedicated, shielded line accommodates the former; a performance test on empty test tubes exposed to sunlight points out the latter.

Assay Conditions

The test method is designed for rapid detection of a broad spectrum of contaminating antibiotic residues in milk. Each drug has its own optimum temperature-time relationship and its own optimum concentration of receptor cells, so only one

Table 12. Comparison of results (cpm) for mixtures of antibiotics with individual antibiotics[a]

Antibiotic	P	T	E	St	G	N	Sm	C
Mixture:								
P = 0.008 IU/mL	926 980							
T = 2 µg/mL		2804 2471						
E = 250 ng/mL			719 729					
St = 1 µg/mL				1826 1512				
G = 100 ng/mL					2543 2509			
N = 1 µg/mL						773 738		
Sm = 50 ng/mL							1153 1127	
C = 800 ng/mL								1769 1621
Individual:								
P = 0.008 IU/mL	1018 955							
T = 2 µg/mL		2730 2646						
E = 250 ng/mL			663 721					
St = 1 µg/mL				1574 1388				
G = 100 ng/mL					2705 2690			
N = 1 µg/mL						794 751		
Sm = 50 ng/mL							975 1132	
C = 800 ng/mL								1684 1691
Zero milk	1339	4110	2212	3603	4799	3407	6976	2273

[a] P = penicillin; T = tetracycline; E = erythromycin; St = streptomycin; G = gentamycin; N = novobiocin; Sm = sulfamethazine; C = chloramphenicol.

Table 13. Results (cpm) for receptor assay on individual milk samples from healthy cows

Sample	P	+ T	E	+ St	N	Sm	C	G[a]
VHT zero std								
Analyst 1	1439	4363	2405	2963	2716	6876	2373	5287
Analyst 2	1470	4040	2590	3228	—	6543	2432	4846
Individual:								
MH1	1512	4204	2420	2736	3831	4675	2414	5223
MH2	1452	4228	2641	3193	3373	5623	2582	5349
J3	1337	3907	2236	2741	2889	5302	2423	5401
J4	1507	4261	2286	2748	3339	5048	2400	4800
JS1	1391	4562	1836+[b]	2279	2977	5835	2699	6079
JS2	1388	4769	1904+	2495	3366	5053	2555	5332
173LW	1276	1706+	2843	2655	3298	3115	2033	3196
HH4	1033	3079	1804+	2363	2971	5811	2595	5576
LS2	1173	3353	2298	2468	3009	6093	2459	3899
LS3	1392	1767+	2808	2494	2675	4635	2559	5159
LS4[c]	1362	1970+	2609	2495	2173	5884	1641+	2928+
LS4	—	—	—	—	—	—	1652	2458
LS4	—	—	—	—	—	—	1975	2920
LS4	—	—	—	—	—	—	1666	2858
KM1	1262	3052	1795+	2408	2667	4522	2396	4313
KM3	1344	3150	2247	2699	3436	4914	2446	4214
109LW	1441	3324	1811	2415	3178	3655	2454	4182
KM4	1403	4318	2019	2643	3741	2419	2565	4711
Zero standard	1416	4263	2722	3252	3712	5548	2601	5341
LS4[c]								
single tracers	—	—	—	—	—	—	1295	1860
Zero							2071	3513
single tracers	—	—	—	—	—	—	1966	3394

[a] G = gentamycin type, e.g., neomycin, kanamycin.
[b] + = positive.
[c] LS4 is a positive for gentamycin and chloramphenicol. Each is tested with single tracers and compared with a zero control determined with single tracers. This sample contains about 1000 ppb chloramphenicol and the equivalent of 100 ppb gentamycin.

condition can be optimized. In this study, the beta-lactam assay was optimized for time-temperature and reagent D concentration. The sulfonamide assay was also optimized with respect to temperature and reagent R concentration. Cross talk between the ^{14}C and ^{3}H channels associated with mixing ^{14}C and ^{3}H tracers also detracts from assay sensitivity.

A quantitative assay where each drug is treated individually with optimized conditions and longer counting times could achieve higher sensitivity. For example, testing penicillin alone, i.e., not mixing tracers, it is possible to distinguish as little as 0.00065 IU penicillin/mL from zero (6).

Individual Milk Samples

The receptor assay was used to test individual milk samples from apparently healthy cows; the results of testing individual cows are shown in Table 13. Several positives were identified. Although several Sm results appear positive, they are at about the 5 ppb level of sulfamethazine. A 70% reduction from zero is about 25 ppb sulfamethazine in this case.

Table 14. Minimum detectable concentrations (MDC) using competitive receptor assay[a]

Antibiotic family	Expressed in terms of family member	MDC, ng/mL	Std dev. of test value, cpm	Test value, cpm Zero	Test value, cpm MDC
Beta-lactams	penicillin G	3	69	1109	902
Tetracyclines	tetracycline	600	317	3907	2956
Macrolides	erythromycin	20	148	1974	1530
Streptomycin	streptomycin	10	131	2274	1881
Novobiocin	novobiocin	10	91	2560	2287
Sulfonamides	sulfamethazine	5	271	5470	4657
Chloramphenicol	chloramphenicol	80	65	1975	1780

[a] Concentrations at 3 standard deviations from average zero—using single tracer.

Zero Standard Milk

Zero standard milk is prepared by passing pasteurized milk through a charcoal column. Antibiotic contaminants are removed along with certain water-soluble vitamins, e.g., B_1, B_2, B_6, C. Thus, it is conceivable that milk detected as zero by the receptor assay could have minimal detectable concentrations (MDC) (Table 14). Processing through the charcoal column can reduce the initial concentration by 99%, which results in the maximum antibiotic concentration that can be in zero milk.

In the collaborative study, a milk powder lot was obtained that closely compared with charcoal-processed zero milk. Sulfonamide was the most common contaminant in the various lots checked.

Charcoal-processed zero milk is commercially available now and is preserved by ultra high-temperature short-time sterilization (Penicillin Assays, Inc.). Zero milk may also be obtained from cows not treated with antibiotics. However, it is necessary to know that the feeds used are not contaminated with antibiotics.

Interferences

Microbiological and enzymatic activity.—A bacteria count in milk to be assayed greater than 10^6–10^7 organisms/mL can lower the measured activity of tetracycline and sulfonamide and possibly cause a false positive. It is suspected that these bacteria bind tracer, making it unavailable for the assay. Because of their size, these bacteria are not pelleted under the centrifugation conditions used. Other antibiotics are not affected by this. It takes about 1–3 weeks for normally stored pasteurized milk to show this effect and about 5–6 days for raw milk.

Mastitic milk may have a high lysozyme titer that will dissolve reagent D (*B. stearothermophilus*) during incuba-

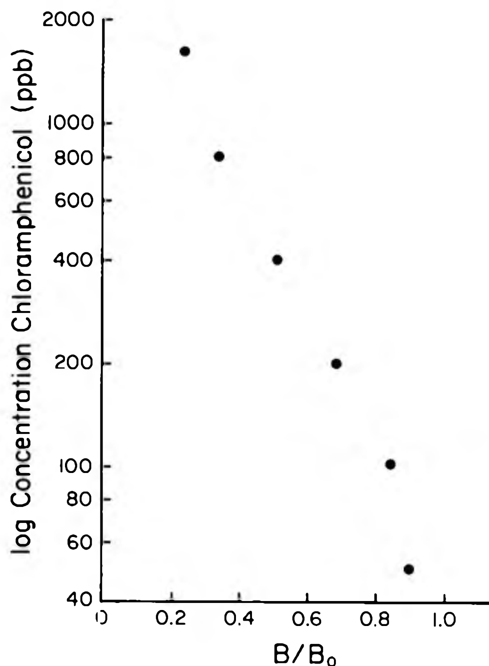


Figure 2. Chloramphenicol standard curve (5 mL wash), showing working range for quantitation.

tion. Lysozyme is associated with high somatic cell count (7). Somatic cell counts greater than 1 000 000/mL may generate sufficient lysozyme to interfere. The effect may be recognized by a "runny" or a reduced pellet size after the centrifugation step.

Normal milk has a lysozyme concentration of 0.1 μg/mL while mastitic milk may have 5 μg/mL. Tetracycline count is reduced about 15% from the zero count when 5 μg lysozyme/mL is present. The assays for other antibiotics are not significantly affected by this concentration of lysozyme.

Agricultural chemicals.—Interference of various agricultural chemicals used with cows was tested with the receptor assay (S. E. Charm, unpublished data). These include famphur (25 ppm), thiabendazole (5 ppm), chlorhexidine (5 ppm), hydrochlorthiazide (5 ppm), Clorsulon (2000 ppm), fenthion (5 ppm). None of these showed interference. Orange peel extract (100 ppm) also did not interfere; citrus peel is used sometimes in feed.

Antibiotic Metabolites

Certain antibiotics are metabolized in part, and the metabolic products are usually inactive, e.g., (8, 9). Inactive products do not bind to receptor sites in cells and are not detected by the receptor assay method, which detects only active drugs. Various inactive metabolites and approximate percentages of dose metabolized include: penicillin 20%, chloramphenicol 10%, sulfonamides 80%, erythromycin 80%, minocycline 70%. Aminoglycosides are mostly unchanged by metabolism.

Competitive Assay and Scatchard Analysis

A Scatchard analysis is often used to characterize competitive binding immuno-type assays, with respect to affinity constants and binding site concentrations (10). Immuno competitive binding assays have affinity constants in the order of 10^9 or 10^{10} L/Mole. Nonspecific binding for bovine serum albumin is in the order of 10^6 L/Mole.

When the competitive receptor assay used in the collaborative study is subjected to Scatchard analysis, the plot is not a straight line but a curve. If a straight line portion of the curve is used to calculate K, the following affinity constants (L/M) are found: P = 10^8, T = 10, E = 10^7, St = 10^6, Sm = 10^6, C = 10^4. These K values are less than what is expected for binding assays.

Table 15. Antibiotic concentration in milk and zone associated with *B. stearothermophilus*

Antibiotic standard	Concn, ppb	Zone size, mm
Beta-lactam (penicillin G)	4.8	17.7
Tetracycline	2000	15.0
Erythromycin	250	15.0
Streptomycin	1000	16.0
Novobiocin	1000	13.0
Sulfamethazine	50	No Zone
Chloramphenicol	800	No Zone
Gentamycin	100	15.0

Examining the Scatchard analysis, one major difference between immunoassay and receptor assay is that the receptor assay requires a biologically active molecule whereas immunoassay does not. The specific radioactivity associated with the receptor assay (see *Reagents*) is based on an intact chemical structure. The total radioactive tracer used in the receptor assay Scatchard analysis assumes that all the label is attached to biologically active drug corrected for nonspecific absorption. It is not possible to define this from the specific radioactivity associated with tracer used in the assay, and this is the major problem in trying to apply Scatchard analysis to these receptor assays.

For example, there are 39 000 cpm of ^{14}C chloramphenicol added in each assay, of which 2100 cpm binds to receptor cells. A wash of the cells with 5 mL water reduces the binding to 1050 cpm. Nonspecific binding is about 1000 cpm. Thus, using 39 000 cpm for tracer added is not appropriate and is the reason for the apparent low affinity constant associated with these competitive receptor assays. If the nonbinding tracer could be reduced, thereby increasing the concentration of bindable tracer, the sensitivity of the assays could be improved.

Also, the assumption of equilibrium between bound and unbound antibiotic used in the analysis is not appropriate in this case. A chloramphenicol assay using a 3 min incubation that binds 1200 cpm, in 13 min binds 2500 cpm, indicating that equilibrium is not achieved in 3 min as used in the method. Time is not taken for reaching equilibrium to permit a rapid assay.

Figure 2 is a plot of B/B_0 vs log concentration chloramphenicol, where B = bound sample tracer and B_0 = bound zero tracer. The working range, in this case between 100 and 1000 ppb, shows that specific binding does in fact occur in spite of the low apparent affinity constant. The minimum detectable concentration is 100 ppb. Saturation of receptor sites occurs at concentrations greater than 1600 ppb.

Other workers who compared Scatchard plots of ligands binding to biological receptors with bound ligand vs logarithm free ligand plots found the Scatchard-calculated constants to be incorrect (11). The lack of information concerning amount of nonbindable tracer and nonspecific binding precludes use of the Scatchard analysis in this case.

Commentary

B. stearothermophilus Disc Assay

The disc assay is a microbial inhibition assay used as a regulatory method in the dairy industry (12). In Table 15, drug concentrations and disc assay inhibition zone are noted.

A 14 mm zone is barely detectable. A 16 mm zone is the action level prescribed by the U.S. Pasteurized Milk Ordinance (PMO). It is clear from Table 15 that considerable drug residue could escape detection in this method.

The *Code of Federal Regulations* (13) denotes no tolerance or zero tolerance in milk for the drugs noted in Table 15, except for novobiocin where up to 100 ng/mL is allowed.

Identification of Antibiotic Families

Two reports on the identification of contaminating antibiotics by using the receptor assay are noted. In Switzerland, spiked samples as well as contaminated cheese were correctly identified (6).

In a second report, 23 samples found positive by the Delvo test (primary control) were sent to Institut fur Hygiene, Kiel, West Germany, for examination by the receptor assay (W. Von Heeschen & G. Suhren (1987) Institut fur Hygiene der Bundesenstalt fur Milchforschung, Kiel, FRG, internal report). Of these, 100% were positive for beta-lactam, 69.7% for tetracycline, and 31.8% for streptomycin. None of 10 samples tested for erythromycin and chloramphenicol showed positive for these.

Of another 20 samples originally marginally positive with the Delvo test, 45% were positive for beta-lactam, 75% for tetracycline, 33% for streptomycin. Eleven of these samples tested for chloramphenicol showed 9% positive, and 14 of these samples tested for erythromycin showed 43% positive. Two samples of this group were negative by receptor assay. The receptor assay was able to detect multiple contamination in this situation.

It is possible to confirm sulfonamide (or PABA analog) contamination with an inhibition assay that is reversed with PABA. This method detects sulfamethazine in raw milk at the 25–50 ppb level. The method requires heating the milk to destroy naturally present microorganisms, inoculating the milk sample with a standard culture (*B. stearothermophilus*) and nutrients (Mueller Hinton broth with trimethoprim), and noting acid production or lack thereof to denote growth or no growth. No growth without PABA and growth with PABA is the classical method for identifying sulfonamide (14; Penicillin Assays, Inc., unpublished data).

Other positive milk by the receptor assay has been reported to contain inhibitor by a cylinder plate inhibition method. These included tetracycline- and streptomycin-positive "off the shelf" milk (15). Beta-lactam positive milk by receptor assay has been confirmed extensively by the disc assay and betalactamase.

Recommendation

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

Acknowledgments

The authors thank the collaborators who participated in the study:

Gerald E. Barnett, Food and Drug Administration, Cincinnati, OH

Jane Foos and Linda Luckie, Florida Dept of Agriculture and Consumer Services, Tallahassee, FL

Walther Heeschen and Gertraude Suhren, Institut fur Hygiene, Kiel, West Germany

John Karolus, Connecticut Dept of Health Services, Laboratory Division, Hartford, CT

Wesley Kelley, South Dakota State Dairy Laboratory, Vermillion, SD

Kathleen Manner, Wisconsin Dept of Agriculture, Trade, and Consumer Protection, Bureau of Laboratory Services, Madison, WI

Arthur Marin, Dept of Agriculture and Markets, New York State Food Laboratory, Albany, NY

Jean-Pierre Moretain and Anne De Couville, Laboratoire National des Medicaments Veterinaires, Fougeres, France

Philip Saltzman, Leprino Cheese Co., Denver, CO

Melchior Schallibaum and Elsbe Reist, Swiss Federal Dairy Institute, Bern, Switzerland

Leon Smith, Mt Empire Dairy Association, Denver, CO

Cary Tremble, H. P. Hood, Newport, ME

Rebba Wallace, California Dept of Food and Agriculture, Milk and Dairy Foods, Sacramento, CA

Special acknowledgment is given to Richard Albert, Food and Drug Administration, Division of Mathematics, Washington, DC, who reviewed and supplemented the statistical analysis.

References

(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA
(2) Charm, S. E., & Chi, R. K. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 1186–1192
(3) Committee on Collaborative Interlaboratory Studies (1984) *J. Assoc. Off. Anal. Chem.* **67,** 433–440
(4) Jawetz, E., Melnick, J. L., & Adelberg, E. A. (1980) *Review of Medical Microbiology,* 14th Ed., Lange Medical Publications, Los Altos, CA, p. 119
(5) Davis, B., Dulbecco, R., Eisen, H., & Ginsberg, H. (1980) *Microbiology: Including Immunology and Molecular Genetics,* 3rd Ed., Harper & Row, Publishers, Hagerstown, MD, p. 250
(6) Schallibaum, M. (1986) *Dtsch. Molkerei-Zeitung* **24,** 787–788
(7) Fitz-Gerald, C. H., Deetle, H. C., & Kitchen, B. J. (1981) *J. Dairy Res.* **48,** 253–265
(8) Pratt, W. B., & Fetely, R. (1986) *The Antimicrobial Drugs,* Oxford University Press, Oxford, UK, p. 153
(9) Moffat, A. C. (1986) *Clarke's Isolation and Identification of Drugs,* 2nd Ed., The Pharmaceutical Press, London, UK, p. 985
(10) Berson, S. A., & Yalow, R. S. (1959) *J. Clin. Invest.* **38,** 1996
(11) Klotz, I. M. (1982) *Science* **217,** 1247–1249
(12) Food and Drug Administration (1983) Pasteurized Milk Ordinance (rev.), U.S. Dept of Health and Human Services, FDA, Washington, DC
(13) *Code of Federal Regulations* (1984) Government Printing Office, Washington, DC, Title 21, Part 556.38–556.750
(14) American Public Health Association (1985) *Standard Methods for Examination of Dairy Products,* G. Richardson (Ed.), APHA, Washington, DC, p. 286
(15) Brady, S. M., & Katz, S. E. (1988) J. Food Prot. (in press)

PESTICIDE FORMULATIONS

Determination of Trichlorfon in Technical and Powder Formulations

STEPHEN C. SLAHCK
Mobay Corp., Agricultural Chemicals Division, PO Box 4913, Hawthorne Rd, Kansas City, MO 64120

A facile procedure is described for the determination of trichlorfon in technical material and an 80% powder formulation by using liquid chromatography with ion-pairing and UV detection. Liquid chromatography allows the direct determination of trichlorfon and detection of any dichlorvos present. This method is applicable to quality control and storage stability analyses and eliminates any need for derivatization to counter the thermal lability of trichlorfon. The average standard deviations for the technical and 80% formulation determinations were 0.51 and 0.37%, respectively.

Trichlorfon (Dylox®), dimethyl-(2,2,2-trichloro-1-hydroxyethyl)phosphonate, is an effective insecticide for the control of a variety of pests that infest vegetables, fruit and field crops, and ornamentals. It is effective against many species of Diptera, Lepidoptera, Hymenoptera, Hemiptera, and Coleoptera, and it has a low order of toxicity to bees and other species of beneficial insects. The analytical technique originally applied to the analysis of trichlorfon was titrimetry (1, 2); these titrimetric procedures remain the principal methods of quality control for manufacturing (3, 4).

Although subsequent methods reported for the determination of trichlorfon have specified a variety of techniques such as thin-layer chromatography (5) and polarography (6), by far most methods report gas chromatographic determinations (GC). This, despite the fact that trichlorfon is a thermally labile compound that readily decomposes to dichlorvos (2,2-dichloroethenyl dimethyl phosphate) under GC conditions (7, 8) and can also decompose to chloral (trichloroacetaldehyde) and dimethyl phosphonate (9, 10), as shown in Figure 1.

Only limited work has been reported for the analysis of trichlorfon by liquid chromatography (LC) (11). LC analysis offers a quick and efficient separation of all known impurities and decomposition products from trichlorfon. It provides maximum specificity without requiring derivatization.

A facile LC procedure using UV detection has been developed for the analysis of trichlorfon in which sulfisoxazole is used as an internal standard (12). This method was applied to the analysis of a group of technical materials and Dylox 80 powder formulations with trichlorfon (and dichlorvos from decomposition) as the only active ingredients. An octyl sulfonic acid ion-pairing reagent with low UV absorptivity was added to sharpen the trichlorfon peak at 210 nm, resolve dichlorvos, and retain sulfisoxazole.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Able to generate >7 MPa (>1000 psi) and equipped with ultraviolet detector capable of measuring absorbances (A) at 210 nm.

(b) *Chromatographic column.*—250 × 4.6 mm id stainless steel column packed with ≤7 μm C18 bonded silica gel capable of resolving dichlorvos, trichlorfon, and internal standard peaks (Du Pont Zorbax ODS or equivalent). *Operating conditions:* Column temperature, ambient; mobile phase flow rate, 1.5 mL/min (ca 6 MPa); chart speed, 0.5 cm/min; injection volume, 20 μL; A range, 0.320 AUFS; recorder range, 10 mV; recorder chart speed, 30 cm/h; retention times: trichlorfon ca 3.5 min, sulfisoxazole ca 6.5 min, dichlorvos ca 10–15 min (mobile solvent-dependent). LC mobile phase must be pumped until system is well equilibrated (this requires ca 30 min of flat baseline).

(c) *Filters.*—0.45 μm porosity and 0.2 μm porosity, solvent compatible (Gelman Acrodisc CR, PTFE membrane, or equivalent).

(d) *Acetonitrile.*—LC grade or distilled in glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442).

(e) *Water.*—LC grade or distilled in glass (Burdick and Jackson Laboratories, Inc.).

(f) *PIC reagent.*—Waters Associates, 34 Maple St., Milford, MA 01757) PIC B8 Low UV reagent vial (Millipore Cat. No. 84283).

(g) *Mobile phase.*—Acetonitrile–water (30 + 70) with Low UV PIC B8 reagent. Combine 300 mL acetonitrile, 700 mL water, and contents of 1 reagent vial and mix thoroughly. Filter mobile phase through 0.2 μm porosity filter. Add a few glass boiling beads and degas under vacuum while sonicating (use immediately). Prepare fresh daily. Mobile phase *must* be refiltered after standing 8 h.

Warning: Allowing mobile phases containing ion-pair reagents to stand in an instrument or LC column can result in significant damage to these systems. Thoroughly flush systems with LC water after use.

(h) *Sulfisoxazole internal standard solution.*—About 30 mg/L acetonitrile.

(i) *Trichlorfon reference standard solution.*—Mobay Corp., Agricultural Chemicals Division, PO Box 4913, Hawthorne Rd, Kansas City, MO 64120. Keep standard refrigerated when not in use. Accurately weigh ca 450 mg reference standard into 100 mL volumetric flask. Pipet 10.0 mL internal standard solution into flask, dilute to half-volume with mobile phase, and sonicate 1 min. Dilute contents of flask to volume with mobile phase and mix well. Filter portion of this solution through 0.45 μm porosity filter just before performing LC analysis. Do not prepare this solution if analysis cannot be completed the same day.

Preparation of Sample

Accurately weigh amount equivalent to ca 450 mg trichlorfon into 100 mL volumetric flask. Pipet 10.0 mL internal standard solution into flask, dilute to half-volume with mobile phase, and sonicate 1 min. Dilute contents to volume with mobile phase and mix well. Filter portion of this solution through 0.45 μm porosity filter just before performing LC analysis. Do not prepare solutions if analysis cannot be completed the same day.

Determination

Inject trichlorfon standard solution and adjust operating parameters to cause trichlorfon to elute in 3–4 min. Retention times of the trichlorfon and internal standard peaks must be

Received October 7, 1986. Resubmitted April 20, 1987. Accepted June 10, 1987.

Dylox is a registered trademark of Bayer AG, Leverkusen.

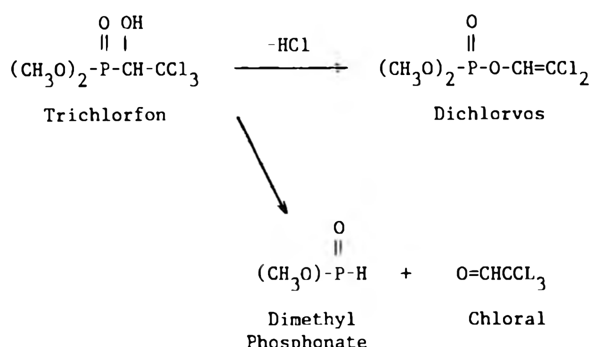


Figure 1. Decomposition routes of trichlorfon.

controlled to produce resolution shown in Figure 2 while eluting dichlorvos within a reasonable period. (Retention time of dichlorvos may vary slightly from this figure, depending on mobile solvent preparation.) If baseline resolution between trichlorfon and internal standard peaks is unobtainable, column must be changed or repacked. Adjust injection size and attenuation to give largest possible on-scale peaks.

Using same injection volume and attenuation for all sample and standard injections, make repetitive injections of the standard solution and calculate response ratios (R) by dividing the peak height of trichlorfon peak by peak height of internal standard peak. Peak area measurements are unacceptable. Response ratios of standard injections must agree within $\pm 1\%$ of their average. Average the duplicate response ratios obtained with standard injections which meet this criterion.

Perform duplicate injections of each sample solution. Response ratios must agree within $\pm 1\%$. (If they do not, the determination must be repeated.) Average the duplicate response ratios for each sample solution which meets this criterion. (Sample injections must be bracketed by standard injections whose response ratios agree within $\pm 1\%$; otherwise this standard/sample injection set must be repeated.) Average the response ratios of bracketing standards which meet this criterion.

Calculation

$$\text{Trichlorfon, wt \%} = (R/R') \times (W'/W) \times P$$

where R' and R = average response ratios for the standard and sample solutions, respectively; W' and W = weight (mg) of trichlorfon standard and sample solutions, respectively; and P = purity (%) of trichlorfon standard.

Results and Discussion

The development of an analytical method for the assay and evaluation of technical trichlorfon and its formulated products with increased specificity over titrimetry, a popular method for quality control, was undertaken. Because of the thermal lability of trichlorfon and its known impurities and decomposition products, a procedure was sought which would resolve these while preventing decomposition. These criteria made a liquid chromatographic method one of the few techniques possible which could provide the separation, detection, and specificity needed.

Although trichlorfon has very low UV absorptivity (especially relative to dichlorvos), sufficient sensitivity exists to quantitate trichlorfon by UV detection at 210 nm. Trichlorfon and dichlorvos have molar extinction coefficients of 186 and 4490 L/mole-cm, respectively.

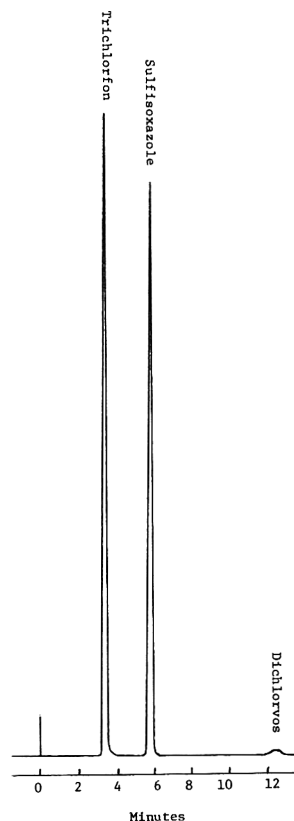


Figure 2. Typical LC chromatogram of technical trichlorfon and sulfisoxazole (trace dichlorvos indicated).

Trichlorfon was initially examined by using reverse-phase chromatography. This yielded only slight resolution between the trichlorfon and dichlorvos (Figure 3). Also, the peak shape of the trichlorfon was considerably skewed. This was evidently due to the existence of trichlorfon as a partially ionized species under these conditions.

The applicability of ion-pair chromatography was examined. The addition of ion-pairing reagent (PIC B8) successfully served several functions. It confined trichlorfon to one ionic form, which produced a sharp peak. The PIC B8 apparently paired with dichlorvos, greatly increasing retention, and it paired with the internal standard, sulfisoxazole, resulting in a suitable retention time. Figure 2 shows a typical LC chromatogram with trichlorfon, sulfisoxazole (internal standard), and dichlorvos identified. The incorporation of an acidic buffer in the mobile solvent system might provide the same improvement of trichlorfon peak shape but would not provide the desired effects of retaining the dichlorvos and internal standard.

The mechanism resulting in retention of dichlorvos appears to be that of ion-pairing. Dichlorvos is well resolved from both peaks, but its retention time is somewhat variable (ca ± 1.5 min), depending on the ion-pairing reagent concentration in the mobile phase. The lower the concentration of the ion-pairing reagent, the shorter the retention time of the dichlorvos. These effects are typical of the ion-pairing mechanism.

The internal standard's retention time is clearly a function of the ion-pairing mechanism. The sulfonic acid counter ion of the ion-pairing reagent interacts with the primary amine function of the sulfisoxazole. The size of the counter ion molecule (the octyl side-chain, "B8") provides the resolution required by retaining sulfisoxazole longer than trichlorfon. An ion-pairing reagent with a smaller side-chain, such as pentyl, shortens the retention time of the internal standard

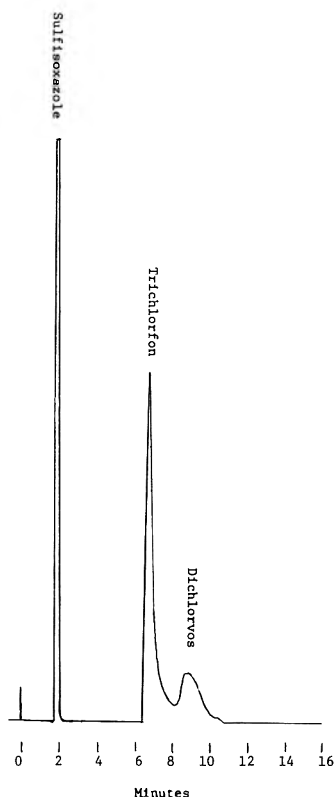


Figure 3. LC chromatogram of technical trichlorfon, sulfisoxazole, and dichlorvos, using acetonitrile–water (5 + 95) without ion-pair reagent.

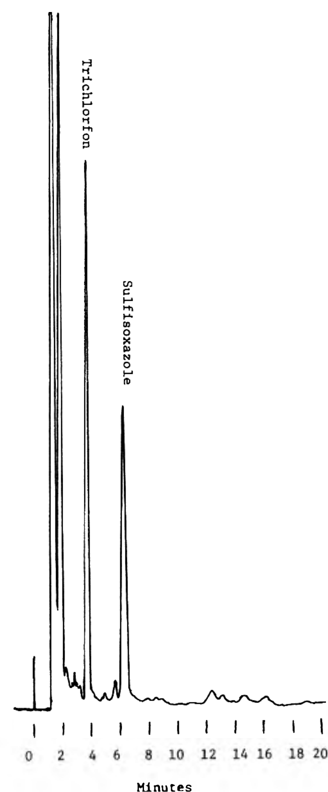


Figure 5. Typical LC chromatogram of Dylox 80 SP with sulfisoxazole.

until it elutes coincidently with the trichlorfon. In the absence of B8 ion-pairing reagent, the internal standard would elute at the dead volume. Therefore, this addition of ion-pairing reagent actually reverses the elution order of the internal standard and trichlorfon relative to conventional reverse-phase liquid chromatography. As opposed to the variance noted for dichlorvos, variance in retention time of the internal standard due to fluctuations in preparation of the mobile phase is negligible.

Figure 4 shows the linearity of the method for peak height measurements over an injection concentration of 0.45 to 8 μg/μL of trichlorfon (ca 10 and 200% w/v of the normal trichlorfon concentration). Although the method shows acceptable reproducibility and linearity by peak height measurement, peak area measurements were unacceptably imprecise due to tailing of the trichlorfon peak. Varying integration parameters does not provide acceptable reproducibility of the area integrations when multiple injections of the same solution are performed (±3–4% from the average). The LC method described here was applied to the analysis of several technical materials and Dylox 80 formulations. A typical Dylox 80 chromatogram is shown in Figure 5. Results of a variety of LC replicate analyses are shown in Tables 1 and 2. Injections of the blank materials for the formulations showed no interferences with the measured peaks of interest.

A sample of decomposed Dylox 80 material aged 11 years was analyzed. This sample showed a considerable number of peaks, but all were well resolved and no significant interferences were detected (Figure 6). With this method, desmethyl trichlorfon, a typical decomposition product, elutes near the dead volume as expected. Since this method was

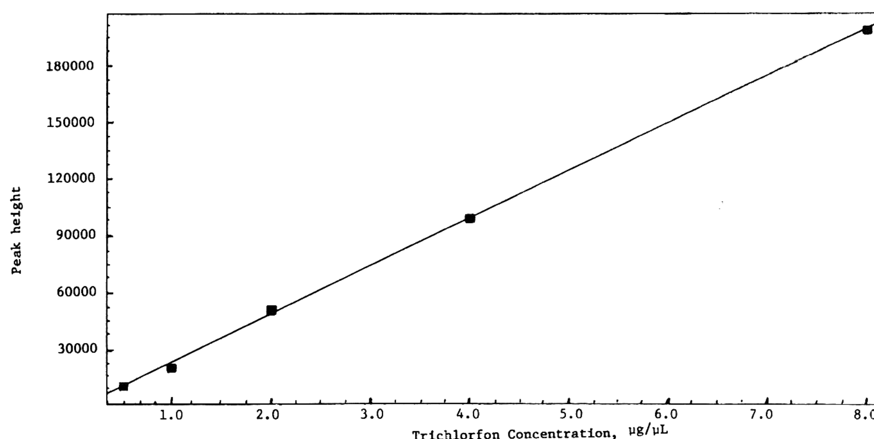


Figure 4. LC linearity plot for trichlorfon peak heights.

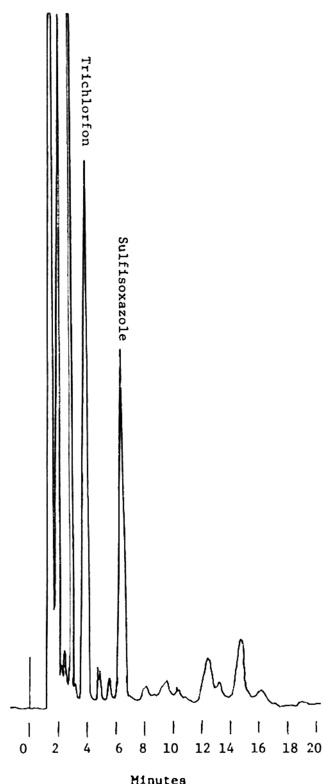


Figure 6. LC chromatogram of 11-year-old Dylox 80 SP.

Table 1. LC results for technical trichlorfon

Sample	Replicate analyses, %			Average	SD	CV, %
1	98.8	98.4	98.8	98.7	0.23	0.23
2	99.5	99.9	99.2	99.5	0.35	0.35
3	99.5	99.7	99.0	99.4	0.36	0.36
4	99.6	100	100	99.9	0.23	0.23
5	99.7	99.9	99.2	99.6	0.36	0.36
6	99.9	98.7	100	99.5	0.72	0.73
7	99.3	98.2	100	99.2	0.91	0.92

Table 2. LC results for Dylox 80 trichlorofon

Sample	Replicate analyses, %			Average	SD	CV, %
1	81.4	81.5	81.0	81.3	0.26	0.32
2	80.6	80.6	81.3	80.8	0.40	0.50
3	76.3	77.0	76.8	76.7	0.36	0.47
4	80.9	81.3	81.9	81.4	0.50	0.62
5	81.1	81.1	81.0	81.1	0.06	0.07
6	80.3	80.0	80.9	80.4	0.46	0.57

developed for a pesticide formulation assay, the sensitivity was adjusted to provide facile quantitation of the analyte down to a level of 2% (w/v). While typical detection limits are expected to be lower, this sensitivity provides for a detection limit significantly below what would be needed in this type of analysis (a sample decomposed to, or lacking, 98% of the analyte). Accurate quantitation of the pesticide formulation below such a value would be of little interest.

The LC procedure described here is a facile assay of trichlorfon in technical and formulated products where trichlorfon is the only active ingredient. The LC method provides specificity by resolving all known impurities and decomposition products in trichlorfon and provides a qualitative examination of dichlorvos.

References

(1) Bennewitz, R., & Foth, G. (1967) *Fresenius Z. Anal. Chem.* **229,** 110–117
(2) Mobay Corp., Central Analytical Test Method C-3.3, Agricultural Chemicals Division, Kansas City, MO
(3) Mobay Corp., Central Analytical Test Method C-3.4, Agricultural Chemicals Division, Kansas City, MO
(4) Zitko, V., & Sergeant, D. B. (1977) *Fish. Mar. Serv. Res. Dev. Technol.* 714
(5) Getz, M. E., & Wheeler, H. A. (1968) *J. Assoc. Off. Anal. Chem.* **51,** 1101–1107
(6) Lindner, R., & Oehler, E. (1968) *Chem. Technol. (Leipzig)* **21,** 43
(7) Flora, T. (1972) *Acta Chim. (Budapest)* **72,** 11–22
(8) Bowman, P. B., & Dame, P. W. (1974) *J. Assoc. Off. Anal. Chem.* **57,** 1128–1131
(9) Akhtar, M. H. (1982) *J. Agric. Food Chem.* **30,** 552–554
(10) Devine, J. M. (1973) *J. Agric. Food Chem.* **21,** 1095–1098
(11) Fleitman, J., Neu, D., & Benjamin, E. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 24–28
(12) Mobay Corp., Central Analytical Test Method C-3.47, Agricultural Chemicals Division, Kansas City, MO

Simultaneous Determination of Diazinon and Chlorpyrifos Pesticide Formulations by Liquid Chromatography

RODNEY J. BUSHWAY, L. BRIAN PERKINS, and JOAN M. KING
University of Maine, Department of Food Science, 102B Holmes Hall, Orono, ME 04469

A liquid chromatographic (LC) method has been developed to analyze simultaneously separate formulations of diazinon and chlorpyrifos. Samples of each formulation were mixed in a Polytron mixer with the internal standard butylated hydroxytoluene and were diluted with acetonitrile. An aliquot was injected into an Ultremex LC column. The mobile phase was acetonitrile–water–tetrahydrofuran–glacial acetic acid–monoethanolamine (480 + 230 + 55 + 2 + 0.75); all 3 compounds were monitored at 230 nm. Total analysis time was 11 min. Combinations of 11 different samples of each formulated pesticide were analyzed 9 times for coefficients of variation generally less than 3%. Purity of the diazinon, chlorpyrifos, and butylated hydroxytoluene was checked by using a photodiode array detector in the spectrum and absorbance ratio modes. No interferences were noted at 230 nm.

Diazinon and chlropyrifos are organophosphate insecticides used extensively in pesticide formulations, especially for residential applications. The pesticides are formulated separately as liquids, dusts, and granules in a wide variety of concentrations. The preferred methods of analysis are liquid chromatography (LC) (1) for chlorpyrifos and gas chromatography (GC) (2, 3) for diazinon. However, similarities in their chemical structures and properties enable diazinon and chlorpyrifos to be analyzed simultaneously, which can be advantageous.

As the importance of economic and safety management increases in laboratories, the approach to pesticide formulation analysis by LC will begin to change. One such approach that can lead to economic reductions and increased safety without sacrificing the analytical integrity of a method is to combine the analyses for different formulations that have different active ingredients. The economic savings would come in the form of fewer columns, less solvent, and more efficient use of time. Safety would be increased because less laboratory waste would be generated. Furthermore, a decrease in waste would also save money because proper waste disposal is expensive.

This paper describes an LC method for the analysis of diazinon and chlorpyrifos pesticide formulations, using the new combination approach.

METHOD

Apparatus and Reagents

(a) *Liquid chromatographs.*—Waters Associates (Milford, MA 01757) 6000A and 510 pumps and U6K septumless injectors, Valco pneumatic injector (Vici Instruments, Houston, TX 77255), Waters Associates variable wavelength UV detectors M490 and M450, Hewlett-Packard photodiode array detector (Andover, MA 01810), and Omniscribe recorders (Houston Instrument, Austin, TX 78753). Operating conditions: injection volume, 5 μL; flow rate, 1.1 mL/min; wavelength, 230 nm; absorbance range, 0.4 AUFS for the diode array detector and 1.0 AUFS for the UV detectors; recorder setting, 10 mV; chart speed, 0.5 cm/min.

(b) *Chromatographic column.*—Ultremex C18, 3 μm particle size, stainless steel, 15 cm × 4.6 mm id (Phenomenex, Rancho Palos Verdes, CA 90274). Other C18 columns tried (Zorbax, Vydac) were not effective.

Received March 31, 1987. Accepted August 18, 1987.

(c) *Mobile phase.*—Acetonitrile–water–tetrahydrofuran–glacial acetic acid–monoethanolamine (480 + 230 + 55 + 2 + 0.75). All solvents LC grade (VWR, Boston, MA 02101).

(d) *Sample extraction solvent.*—Reagent grade acetonitrile (VWR).

(e) *Internal standard solution.*—67.5 mg/25 mL. Weigh 1.35 g BHT (Sigma Chemical Co., St. Louis, MO 63178) into 500 mL volumetric flask, dilute to volume with acetonitrile, and mix.

(f) *Diazinon and chlorpyrifos standard solution.*—Accurately weigh ca 26 mg chlorpyrifos reference standard, 99.8% pure (Dow Corning Corp., Midland, MI 48686-0994), and 120 mg diazinon stabilized standard, 87.0% pure (Ciba-Geigy, Greensboro, NC 27405), into calibrated 50 mL culture tube with Teflon screw cap, pipet in 25 mL BHT internal standard solution, and mix. Mix on Polytron mixer (Brinkmann Instruments, Westbury, NY 11590) at maximum speed for 1 min. Rinse Polytron probe with ca 25 mL acetonitrile until solvent reaches calibration line on culture tube. Heat is generated from Polytron mixer so samples must be allowed to cool to room temperature. If needed, add more solvent.

Preparation of Sample

Accurately weigh amount of any liquid or solid chlorpyrifos formulation containing ca 26 mg chlorpyrifos and amount of any liquid or solid diazinon formulation containing ca 120 mg diazinon into culture tube calibrated to 50 mL. Premix granular formulations by riffle and turntable to get representative sample. Pipet in 25 mL BHT internal standard solution and mix in Polytron mixer for 1 min at maximum speed. Rinse Polytron probe with ca 25 mL acetonitrile until solvent reaches calibration line on culture tube. Let cool and add more solvent if necessary. Filter samples thru 0.45 μm filter before injection.

Note: No more than 5 g solid material can be added to each culture tube. Thus, formulations requiring 3–4 g each cannot be analyzed together.

Determination

Inject standard, followed by 2 injections of sample. Finally, inject another standard. Either peak height or area may be used to calculate content.

$$\text{Compound, \%} = (R/R') \times (W'/W) \times \text{\% purity of std}$$

where R and R' = peak height or area ratios of compound to internal standard for sample and standard, respectively; W' = mg standard; W = mg sample extracted.

Results and Discussion

Diazinon and chlorpyrifos are not formulated together; therefore, present methods (1–3) analyze them separately. However, because of the similarities in their chemical and physical properties, a procedure could be developed to extract and measure diazinon and chlorpyrifos formulations simultaneously. A typical chromatogram of a diazinon-chlorpyrifos separation is shown in Figure 1. Baseline resolution of the 2 compounds and the internal standard is completed in 9.5 min. A purity check was performed on the diazinon,

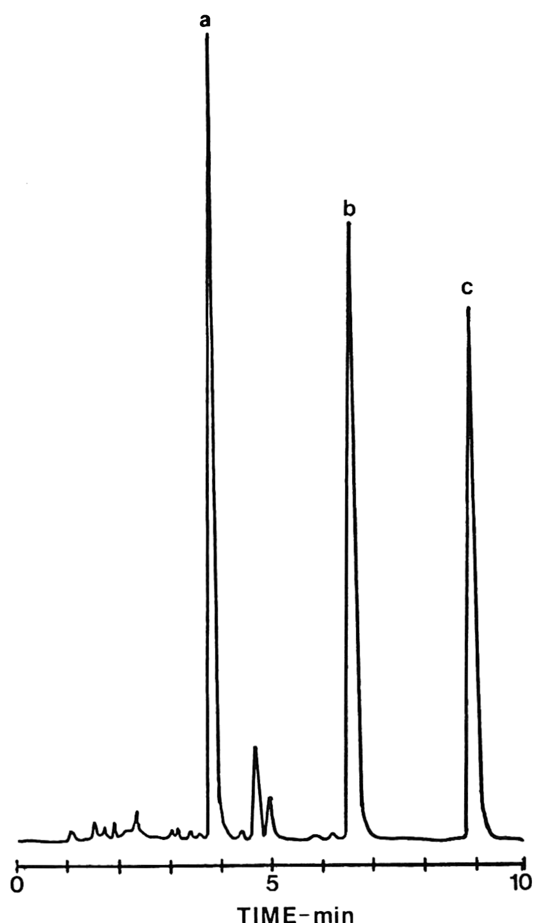


Figure 1. Liquid chromatogram of separate diazinon-chlorpyrifos formulations, analyzed simultaneously: a, diazinon; b, chlorpyrifos; c, BHT.

chlorpyrifos, and BHT peaks in all formulations by using a photodiode array detector in the spectrum and absorbance ratio modes. Spectra were scanned from 200 to 350 nm at 3 different points in each diazinon, chlorpyrifos, and BHT peak while absorbance ratios were taken at 215/230 nm, 235/230 nm, 240/230 nm, 250/230 nm, 260/230 nm, and 290/230 nm. Analyses indicated that absorbance was strong, and all 3 compounds could be analyzed at 230 nm without interferences from inert formulation ingredients.

Furthermore, it was concluded that any combination of diazinon and chlorpyrifos formulations could be analyzed, within the limitation on the amount of solid (5 g) per culture tube. The Polytron probe cannot handle a larger solid content per tube, and 50 mL solvent will not fit in the tube with more than 5 g solid material.

Table 1 presents the results of the combined diazinon-chlorpyrifos analysis. Coefficients of variation for both insecticides were mostly below 3%. Two major exceptions were the diazinon dust (6.91) and a chlorpyrifos granule formulation (5.18). These were the highest CV% and were caused by nonhomogeneous products. Results for the chlorpyrifos samples were compared to results from the official LC meth-

Table 1. Diazinon and chlorpyrifos content in separate formulations analyzed simultaneously by LC[a]

Diazinon		Chlorpyrifos		
Formulation—% concn	Found, %	Formulation—% concn	Found, %	AOAC, %
Granule—5	4.90 (2.83)	Liquid—6	5.70 (1.34)	5.79 (4.66)
Granule—5	4.55 (1.83)	Liquid—0.5	0.51 (2.87)	0.52 (2.04)
Granule—5	5.17 (1.75)	Liquid—62	61.08 (2.05)	61.18 (3.55)
Granule—2	1.87 (2.73)	Liquid—0.5	0.51 (2.28)	0.50 (0.68)
Granule—2	2.13 (1.52)	Liquid—30	30.00 (1.58)	30.85 (2.65)
Liquid—25	25.93 (1.94)	Dust—1	1.09 (2.93)	1.12 (6.25)
Dust—4	3.83 (6.91)	Liquid—5.3	5.13 (2.18)	5.11 (2.94)
Liquid—87	87.14 (1.57)	Liquid—6.7	7.26 (2.07)	7.36 (2.27)
Liquid—25	26.22 (2.42)	Granule—0.5	1.14 (3.67)	1.18 (6.78)
Liquid—25	24.80 (2.36)	Granule—0.5	0.39 (5.18)	0.36 (6.38)
Granule—5	5.23 (2.17)	Granule—1.1	0.90 (3.51)	0.90 (7.78)

[a] Each mean represents 9 determinations, except AOAC means represent 6 determinations. Coefficients of variation (%) are in parentheses.

od. As shown, the agreement between the combination analysis and official method is excellent with no significant differences. We could not compare diazinon results with official method results because of a lack of GC equipment. However, we did try an extraction procedure from one of the official methods used and an extraction procedure recommended by Ciba-Geigy, and there were no significant differences in the amount extracted by these methods vs the combined analysis for a 2 and a 5% granule formulation. Furthermore, the agreement was good between the actual formulated amount and the amount found.

To demonstrate the ruggedness of the method, combinations of 11 different formulations for each pesticide were analyzed 9 times using 3 different LC instruments and 4 different analysts. The 11 combinations were divided into sets of 5 and 6. One set of analyses was done per day per person per instrument. Also peak height and area measurements were used interchangeably among analyses to test the ruggedness of the method.

The linearity of response for all 3 compounds was checked on the basis of peak height and area at 230 nm. Both responses were linear for the ranges of 0.67–5.3 μg chlorpyrifos, 2.99–24.25 μg diazinon, and 1.69–13.50 μg BHT. These linearity ranges are more than adequate to cover formulations that are too high or low. The sensitivity of this method was 50 ng for all compounds.

In conclusion, the combined analysis for diazinon and chlorpyrifos formulations is simple, specific, and precise, and contributes to economic savings and increased safety.

Acknowledgment

This manuscript is Maine Agricultural Experiment Station No. 1213.

References

(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **6.411**
(2) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **6.425**
(3) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **6.590**

Analysis of Rotenone in Cubé and Derris Root Powders and Formulations by Liquid Chromatography

RODNEY J. BUSHWAY, ANGELA YANG, and ALI AL-YAMANY
University of Maine, Department of Food Science, 102B Holmes Hall, Orono, ME 04469

A modified liquid chromatographic (LC) procedure, including an improved extraction, has been developed to analyze rotenone in root powders of derris and cubé. The procedure is also applicable to formulations. Samples are sonicated for 5 min in chloroform or a mixed solvent of tetrahydrofuran–acetonitrile–water–glacial acetic acid, followed by liquid chromatography. Rotenone content of 4 lots of root powder samples showed good agreement between the new extraction and the AOAC official extraction; coefficients of variation ranged from 5 to 1.2%. Five formulations were also analyzed and showed good agreement with results for methanol extraction. Coefficients of variation were 4.5% or less. Purity of the rotenone peaks was tested by using a photodiode array detector in the spectrum and UV ratio modes. It is recommended that the new method be tested collaboratively.

Several liquid chromatographic (LC) methods have been developed for the analysis of rotenone formulations (1–5), but none have included the analysis of the raw material, cubé and derris root powders (ground roots of cubé and derris). Presently, these powders are analyzed by an infrared (IR) method that includes a rather lengthy extraction with either chloroform (6) or ether (7).

This paper describes a new LC method that can rapidly analyze rotenone in cubé, derris root powder, and formulations.

METHOD

Apparatus and Reagents

(a) *Liquid chromatographs.*—Waters Associates (Milford, MA 01757) 6000A and 510 pumps and U6K septumless injector; Micromeritics (Norcross, GA 30093-1877) autoinjector Model 728; Schoeffel (Westwood, NJ 07675) variable wavelength UV detector; Hewlett-Packard (Andover, MA 01810) photodiode array detector; and Omniscribe (Houston Instrument, Austin, TX 78753) recorder. Operating conditions: injection volume, 5 µL; flow rate, 1.0 mL/min; wavelength, 280 nm; absorbance range, 1.0 AUFS; recorder setting, 10 mV; chart speed, 0.5 cm/min.

(b) *Chromatographic columns.*—NovaPak C18, 4 µm particle size, stainless steel, 15 cm × 4.6 mm id (Waters Associates) or Ultremex, 3 µm particle size, stainless steel, 15 cm × 4.6 mm id (Phenomenex, Rancho Palos Verdes, CA 90274).

(c) *Mobile phase.*—Acetonitrile-water-methanol (40 + 40 + 20). All solvents LC grade (VWR, Boston, MA 02101).

(d) *Sample extraction solvent.*—Chloroform or mixed solvent of stabilized (with antioxidant, usually BHT at 0.025%) tetrahydrofuran–water–acetonitrile–glacial acetic acid (50 + 30 + 20 + 1). All solvents reagent grade (VWR).

(e) *Rotenone standard solution.*—Weigh 30 mg 99% rotenone (Penick Co., Lyndhurst, NJ 07071) into 50 mL actinic volumetric flask and dilute to volume with extraction solvent. Actinic glassware is necessary because rotenone degrades readily in most types of light.

Preparation of Sample

(a) *Cubé and derris root powders and dust formulations.*—Weigh sample equivalent to 30 mg rotenone into actinic, glass-stopper Erlenmeyer flask. Pipet in 50 mL sample extraction solvent (either chloroform or tetrahydrofuran–water–acetonitrile–glacial acetic acid) and mix in sonic bath (Bransonic 32, Fisher Scientific) 5 min while swirling gently. Let settle and filter 5–10 mL aliquot through 0.45 µm nylon filter (Gelman Sciences, Inc., Ann Arbor, MI 48106). If chloroform extraction solvent was used, evaporate aliquot and dissolve residue in mobile phase. Inject 5 µL into liquid chromatograph.

(b) *Liquid formulations.*—Weigh sample equivalent to 30 mg rotenone into actinic, glass-stopper Erlenmeyer flask. Pipet in 50 mL tetrahydrofuran–water–acetonitrile–glacial acetic acid and shake to dissolve. Filter through 0.45 µm nylon filter (Gelman Sciences). Inject 5 µL into LC system.

Determination

Inject standard solution, followed by 2 injections of sample solution. Finally inject standard solution again. Measure peak heights or areas, then average for standard and sample, and substitute into formula below:

$$\text{Rotenone, \%} = (H/H') \times (W'/W) \times \text{\% purity of std}$$

where H and H' = average peak heights or areas of sample and standard, respectively; W' = g rotenone standard/50 mL; and W = g sample extracted.

Results and Discussion

Cubé and derris root powders are presently analyzed for rotenone by lengthy chloroform or ether extractions followed by IR quantitation. Detection and measurement is nonspecific for rotenone because of the presence of similar rotenoids. These similar compounds give erroneously high values to the rotenone content of powders analyzed by IR.

To eliminate the high bias, an LC method was developed along with a less time-consuming extraction. The chromatographic conditions specified here differ from previous procedures (2–5): the reverse-phase column (NovaPak, 4 µm particles) gives a better separation and the solvent system gives better selectivity. These changes also enable both root powders and formulations to be analyzed on one LC system.

The extraction solvent was also changed from the one developed for dust formulations (5); the previously used methanol yielded lower rotenone values from root powders than did chloroform or tetrahydrofuran–acetonitrile–water–glacial acetic acid. However, the 5 min sonication used in pre-

Table 1. Rotenone content (%) of cubé and derris root powders extracted under different conditions[a]

Sample	Sonication, 5 min		Shake[b]	
	$CHCl_3$	Mixed	$CHCl_3$	Ether
Cubé 1	5.86 (2.4)	5.69 (3.2)	5.83 (3.0)	
Cubé 2	5.88 (2.6)	5.81 (1.8)	5.84 (3.8)	
Cubé 3	6.72 (4.9)	6.78 (1.2)	6.87 (2.6)	
Derris		1.27 (4.9)		1.21 (5.0)

[a] Each value is mean of 12 determinations, 2 per day. Coefficients of variation % are in parentheses.
[b] Mechanical shaking 1 h for cubé powders; Soxhlet extraction 48 h for derris powder.

Received March 25, 1987. Accepted June 26, 1987.

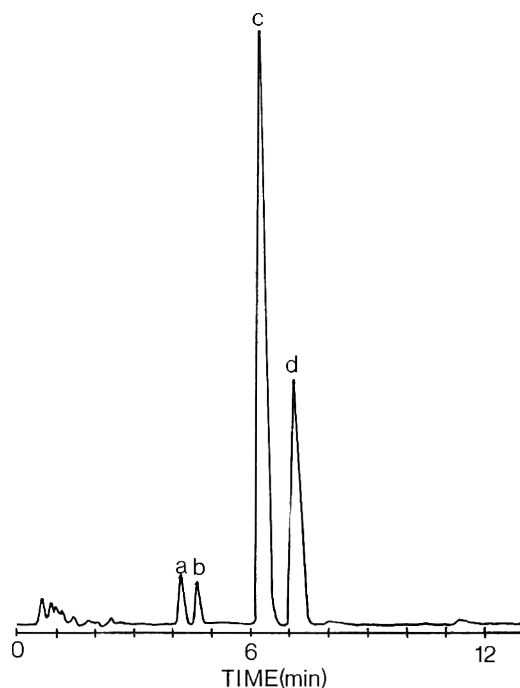


Figure 1. Liquid chromatogram of main ingredient in cubé root powder sample: a, rotenolone; b, tephrosin; c, rotenone; d, deguelin.

vious methods was adequate. Several other powder extraction solvents were tried (dimethylformamide [DMF], dimethyl sulfoxide [DMSO], and THF) but none was effective as the two chosen.

An example of a separation of rotenoids in cubé root powder is shown in Figure 1. Separation is complete in 7.5 min with baseline resolution between the 4 major rotenoids. A purity check was performed on all the rotenone peaks for the cubé and derris root powders by using a photodiode array detector in the spectrum and absorbance ratio modes. Spectra were scanned from 200 to 350 nm at 3 different points (20/100/20%) in each rotenone peak while absorbance ratios were taken at 290/280 nm, 300/280 nm, 254/280 nm, 240/280 nm, 230/280 nm, and 215/280 nm. All purity checks indicated that rotenone could be analyzed at 280 nm without interferences.

Table 1 presents the results of the rotenone content of 3 cubé powders and 1 derris powder extracted under different conditions. Each sample was analyzed 12 times (2 per day) using 2 different LC instruments and 3 different analysts. This demonstrates the ruggedness of the method. Area and peak height measurements were used interchangeably. As can be seen from Table 1, all coefficients of variation were below 5%; most were below 3.5%. The 5 min sonication technique compared favorably with the AOAC shaking method (1–4 h) for cubé powders and the ether extraction (48 h Soxhlet) for derris powders. Also, results for chloroform or mixed solvent extraction were not significantly different. However,

Table 2. Rotenone content (%) of formulations extracted by sonication and different solvents[a]

	Extraction solvent		
Sample, conc.	$CHCl_3$	Mixed	MeOH
Liquid, 5%	4.26 (2.2)	4.17 (3.4)	4.18 (1.9)
Dust, 0.75%	0.83 (4.0)	0.80 (1.7)	0.81 (1.8)
Dust A, 1%	0.98 (3.0)	0.98 (1.4)	0.96 (2.4)
Dust B, 1%	1.09 (3.6)	1.06 (1.7)	1.05 (2.5)
Dust, 33%	30.30 (4.5)	30.20 (2.7)	30.60 (3.2)

[a] Each value is mean of 12 determinations, 2 per day. Coefficients of variation are in parentheses.

the mixed solvent extraction is simpler because no evaporation step is required before injection. It should be mentioned, however, that rotenone contents of the cubé powders as determined by IR (done by each manufacturer of cubé powder) were 8–13% higher than the LC analyses. These powders contain other very similar rotenoids that interfere in the IR analysis.

Recoveries from cubé and derris powders that were spiked at 1 and 6% more than the existing amount and extracted with the mixed solvent were 98 and 95%, respectively.

Five formulations were analyzed, using the chloroform and the mixed solvent extraction, and the results were compared with the modified official LC procedure (methanol extraction) (5) (Table 2). Neither chloroform nor mixed solvent was significantly better than methanol for extraction of rotenone in formulations, although the mixed solvent was quicker and coefficients of variation were better. These results are somewhat puzzling. Some of these formulations contain cubé or derris powder, but the clay or talc used as the inert ingredients may enable the use of methanol, which is not suitable for the raw material.

In conclusion, a quick extraction of cubé and derris powders has been developed using either chloroform or tetrahydrofuran–acetonitrile–water–glacial acetic acid. Formulations can be analyzed by the same procedure. This LC procedure is needed to replace the lengthy AOAC methods (6, 7) for cubé and derris powders. We suggest that this new method be tested in a collaborative study.

Acknowledgment

Technical paper No. 1201, Agricultural Experiment Station, University of Maine.

References

(1) Bushway, R. J., Engdahl, B. S., Colvin, B. M., & Hanks, A. R. (1975) *J. Assoc. Off. Anal. Chem.* **58**, 965–969
(2) Bushway, R. J., & Hanks, A. R. (1977) *J. Chromatogr.* **134**, 210–215
(3) Bushway, R. J. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 793–795
(4) Bushway, R. J. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 796–800
(5) Bushway, R. J. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 490–491
(6) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs. **6.179-6.180**
(7) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **6.181**

Liquid Chromatographic Determination of Phenolic Impurities in Technical MCPA Using Electrochemical Detection (Coulometric Mode)

YUK Y. WIGFIELD and MONIQUE LANOUETTE
Agriculture Canada, Food Production and Inspection Branch, Laboratory Services Division, Ottawa, Ontario K1A 0C6, Canada

Fifteen samples of technical 4-chloro-2-methylphenoxy acetic acid (MCPA) from 6 manufacturers were analyzed for the presence of 13 different phenolic impurities. Reverse-phase liquid chromatography with an electrochemical (coulometric mode) detector was used for qualitative and quantitative determinations. The phenols were separated using a 40-70% methanol and 60-30% 0.02M KH_2PO_4 (pH 4.0) 35 min gradient system on a Spheri-5-RP-18 column. Confirmation of the phenols present was done by retention time comparison with the corresponding standards, using 2 other isocratic mobile phases, methanol-0.2M acetic acid (60 + 40) at a flow rate of 1.3 mL/min and acetonitrile-0.02M KH_2PO_4 (45 + 55) (pH 3.0) at a flow rate of 0.90 mL/min. The similarity of results on 3 different solvent systems demonstrates the absence of any interfering responses. 2-Methyl-4-chlorophenol (average 0.168%) and 2-methyl-4,6-dichlorophenol (average 0.004%) were detected in all 15 samples. 3-Methylphenol (0.002%) and 2,6-dimethyl-4-chlorophenol (0.002%) were detected in one sample only. The minimum detectable amount ranged from 0.1 to 0.6 ng, depending on the phenol. This corresponds to less than 0.002% when expressed relative to the weight of sample. The coefficient of variation for multiple analyses of the same sample ($n = 6$) is 1% for 2-methyl-4-chlorophenol and 3% for 2-methyl-4,6-dichlorophenol.

4-Chloro-2-methylphenoxy acetic acid (MCPA) is a systemic herbicide used to control annual and perennial weeds in cereals, grassland, and turf. It may be produced by chlorination of *o*-cresol followed by a condensation reaction with chloroacetic acid under alkaline condition (1). The identity of various phenols in MCPA is important because some of the phenols (e.g., 2,4,5- and 2,4,6-trichlorophenols) can yield chlorinated dioxins during the manufacturing process under alkaline reaction conditions. These dioxins can then be carried through to the final product in a manner analogous to the formation of dioxins in 2,4,5-T (2). Specifically, in the manufacturing process of MCPA, the condensation reaction of 2-methyl-4-chlorophenol (2-M-4CP) with chloroacetic acid under alkaline condition may favor the formation of dioxins from suitable chlorophenol precursors. 2-M-4CP itself is not a suitable dioxin precursor but may contain small amounts of other chlorophenols which generate dioxins under favorable reaction conditions.

Many approaches to the analysis of mixtures of chlorophenols have been reported, including spectrophotometric titration (3), ion exchange chromatography (4), reverse-phase liquid chromatography (LC) (5), in addition to gas chromatography (GC) of the corresponding methylated derivatives (6). The presence of 2-methyl-4-chlorophenol was detected using reverse-phase LC and an electrochemical detector (7). Direct gas chromatographic determination of 4-chloro-2-methylphenol, 6-chloro-2-methylphenol, 2,4-dichloro-6-methylphenol, and 2-methylphenol in technical and formulated products of MCPA has also been reported (8). The latter method involves dissolving the samples in a mixture of acetone and 0.1M HCl (60 + 40) and injecting the solution into a packed OV-17 column. This solvent system, due to the presence of an acid, may have some influence on the lifetime of the GC column.

This study reports the separation of 13 phenols, including 2 methylphenols, 6 methylchlorophenols, and 5 chlorophenols, using reverse-phase LC and an electrochemical detector without preliminary extraction and derivatization of the samples. The levels of various phenols detected in MCPA samples are also presented.

Received April 8, 1987. Accepted July 27, 1987.

Experimental

Apparatus

(**a**) *Liquid chromatograph.*—Spectra Physics SP8100 liquid chromatograph equipped with SP8110 autosampler and electrochemical detector Model 5100A Coulochem®, with Model 5010 analytical cell (Environmental Sciences Associates, Inc., Bedford, MA 01730). Guard cell, Model 5020, made of porous graphite is connected between pump and injector. Guard column packed with Co:Pell ODS is placed between guard cell and pump. Analytical cell is set at 0.70 V and guard cell is set at 1.1 V, sensitivity at 10 × 20 (0.5 μamp full-scale sensitivity).

(**b**) *Data acquisition.*—Spectra Physics SP4200 dual-channel computing integrator, chart speed set at 0.5 cm/min and attenuation set at 16.

(**c**) *LC column.*—25 cm × 4.6 mm Spheri-5® RP-18 reverse-phase packed with spherical 5 μm sorbent (Spheri-5) (Brownlee Labs, Santa Clara, CA 95050) connected to 7.6 cm × 2 mm Co:Pell ODS guard column (Whatman Inc., Clifton, NJ).

Solvents and Reagents

(**a**) *Buffer solution.*—0.02M solution of potassium phosphate monobasic (KH_2PO_4), LC grade (Fisher Scientific Co.).

(**b**) *Mobile phase.*—Gradient from methanol–0.02M buffer (40 + 60) to methanol–buffer (70 + 30) over 35 min, hold 15 min, 5 min to return to initial condition, and 5 min to equilibrate at flow rate of 1 mL/min. Confirmation was done with isocratic mobile phase of MeOH–0.2M acetic acid (60 + 40) at flow rate of 1 mL/min and acetonitrile–0.02M buffer (45 + 55) at flow rate of 0.90 mL/min.

(**c**) *Potassium hydroxide solution.*—0.2M KOH in water–isopropanol (60 + 40).

Table 1. Retention time and minimum detectable amounts (MDA)[a] of phenols

No.	Phenols	Abbrev.	RT, min	RRT	MDA, ng
1.	2-chlorophenol	2-CP	11.6	0.468	0.1
2.	3-methylphenol	3-MP	12.2	0.492	0.1
3.	2-methylphenol	2-MP	13.0	0.524	0.1
4.	4-chlorophenol	4-CP	16.4	0.661	0.2
5.	2,6-dichlorophenol	2,6-DCP	20.4	0.822	0.3
6.	3-methyl-6-chlorophenol	3-M-6CP	20.9	0.843	0.2
7.	3-methyl-4-chlorophenol	3-M-4CP	22.48	0.906	0.3
8.	2-methyl-6-chlorophenol	2-M-6CP	24.13	0.97	0.2
9.	2-methyl-4-chlorophenol	2-M-4CP	24.8	1.00	0.2
10.	2,4-dichlorophenol	2,4-DCP	26.91	1.08	0.1
11.	2,6-dimethyl-4-chlorophenol	2,6-DM-4-CP	31.7	1.28	0.6
12.	2-methyl-4,6-dichlorophenol	2-M-4,6-DCP	34.00	1.39	0.2
13.	2,4,6-trichlorophenol	2,4,6-TCP	35.9	1.44	2.0

[a] Expressed with signal-to-noise ratio = 2.

Table 2. Levels of active ingredient (a.i.) and phenols in MCPA samples

		Levels, %				
Manuf.	Sample	a.i.	3-MP	2-M-4CP	2-M-4, 6-DCP	2,6-DM-4-CP
A	1	87.1		0.106	0.006	
	2	91.4		0.158	0.010	
	3	90.5		0.060	0.006	
B	4	86.6	0.002	0.652	0.005	0.002
C	5	93.1		0.040	0.002	
	6	89.3		0.071	0.005	
	7	79.3		0.048	0.003	
D	8	95.1		0.277	0.002	
	9	97.1		0.303	0.002	
	10	95.8		0.260	0.002	
E	11	95.0		0.054	0.002	
	12	95.0		0.065	0.007	
	13	92.8		0.102	0.004	
F	14	88.6		0.163	0.005	
	15	93.1		0.155	0.005	
	$\bar{x}$	91.3		0.168	0.004	

(d) *Standard stock solutions.*—Accurately weigh 25 mg of each chlorophenol isomer standard and dilute each to 25 mL with KOH solution. (Compounds obtained from Canada Centre for Pesticide Analytical Standards, Laboratory Services Division, Food Protection and Inspection Branch, Ottawa, Ontario K1A 0C5, Canada.)

(e) *Analytical standard mixtures.*—Dilute appropriate aliquot of each stock solution to 10 mL with KOH solution to give final concentrations of 0.04, 0.1, 0.4, 1, 2, 4 μg/mL, respectively.

(f) *Solvents.*—Acetonitrile and methanol (LC grade, Caledon Labs Ltd, Georgetown, Ontario); water (purified with Milli-Q2 system and filtered through 0.22 μm filter); 2-propanol (LC grade, Fisher Scientific Co.).

Determination

Dissolve 300 mg MCPA sample in 25 mL KOH solution. Inject 10 μL sample, using SP8110 autosampler. Record peak areas and retention times with SP4200 computing integrator. Calculate percent compositions by using external standard mixtures. Multilevel calibration with analytical standard mixtures produced linear fit in the range 0.04 to 4 μg/mL.

Results and Discussion

Thirteen phenols, listed in Table 1, were chosen for determination in technical MCPA samples. Two of these phenols (phenols 1 and 4) have been reported as impurities in both MCPA and 2,4-dichlorophenoxy acetic acid (2,4-D), 3 phenols (phenols 5, 10, and 13) have been reported as impurities in 2,4-D only, while the remaining 8 phenols (phenols 2, 3, 6, 7, 8, 9, 11, and 12) have been reported as impurities in MCPA only (9, 10). Phenols 5, 10, and 13 were included in the determination of MCPA samples in order to investigate the possibility of cross-contamination from the production of 2,4-D technical product.

In the present study, the UV detector was not suitable for quantitative determination due to interference from responses of other impurities. Thus, an electrochemical detector was chosen for its selectivity to phenols and its advantages over an UV detector (5, 7).

The chromatographic separation of these 13 phenols, using the described LC column and mobile phase system, is shown in Figure 1A. The active ingredient content in MCPA was

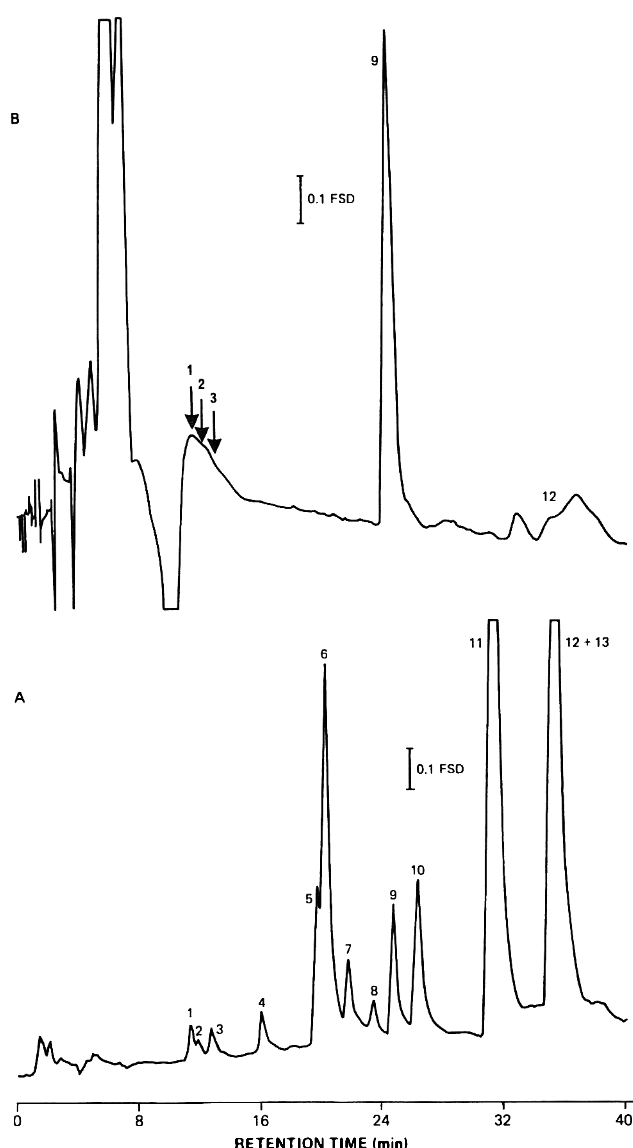


Figure 1. A, Separation of 13 phenol standards, using 40–70% methanol and 60–30% 0.2M KH_2PO_4 35 min gradient system. Numbered peaks refer to Table 1. B, Phenolic impurities present in technical MCPA sample 15, using solvent gradient system.

Table 3. Levels and retention times of 2-M-4CP, for 3 different mobile phases[a]

Sample	2-M-4CP, %		
	Solvent 1	Solvent 2	Solvent 3
1	0.106	0.108	
2	0.158	0.154	
3	0.060	0.085	
4	0.652	0.618	
5	0.040	0.040	
6	0.071	0.081	
7	0.048		0.059
8	0.277	0.231	
9	0.303		0.293
10	0.260	0.219	
11	0.054		—
12	0.065		0.074
13	0.102		0.113
14	0.163	0.183	
15	0.155	0.131	
RT (min)	23.46	6.58	8.32

[a] Solv. 1 = MeOH–buffer (40 + 60 to 70 + 30); Solv. 2 = MeOH–AcOH (60 + 40); Solv. 3 = CH_3CN–buffer (45 + 55).

Table 4. Repeatability of methyl chlorophenol determination (sample 13)

Analysis No.	2-M-4CP, %	2-M-4,6-DCP, %
1	0.103	0.0037
2	0.100	0.0037
3	0.102	0.0039
4	0.101	0.0037
5	0.103	0.0039
6	0.102	0.0037
$\bar{x}$	0.102	0.0038
SD	0.001	0.0001
CV, %	1.0	3.0

analyzed using a separate LC-UV system. The levels of phenolic impurities and active ingredient in all MCPA samples expressed relative to sample weight are reported in Table 2. Four phenolic impurities were found in the samples, 2 of which were present in all samples, i.e., 2-methyl-4-chlorophenol (2-M-4CP) and 2-methyl-4,6-dichlorophenol (2-M-4,6-DCP) at average levels of 0.168% and 0.004%, respectively. The other 2 impurities were 3-methylphenol (3-MP) and 2,6-dimethyl-4-chlorophenol (2,6-DM-4CP), each at 0.002%, found in one sample. Figure 1B represents a typical LC chromatogram of an MCPA sample. The presence of 2-M-4CP in MCPA was confirmed by eluting the samples with 2 other isocratic solvent systems: (a) MeOH–0.2M acetic acid (60 + 40) and (b) acetonitrile–0.02M KH_2PO_4 buffer (45 + 55). The similar levels of 2-M-4CP in the same MCPA sample but using different eluting solvent systems, as shown in Table 3, suggest that there are no interfering peaks from other impurities.

Using the present mobile phase, 2,6-DCP and 3-M-6CP (phenols 5 and 6) were partially resolved from each other. We have not attempted to further separate these 2 phenols by using other solvent systems because we did not find these phenols in all 15 MCPA samples analyzed.

2-M-4,6-DCP and 2,4,6-TCP (phenols 12 and 13) could not be well separated under the present analytical system. However, 2,4,6-TCP has been identified as an impurity in 2,4-D, co-existing with other phenols (phenols 1, 4, 5, and 10) but at a much lower level (10). Thus, the absence of these phenols suggests also the absence of 2,4,6-TCP. These data also suggest that there is no cross-contamination of MCPA samples from 2,4-D production.

2,6-DM-4-CP (phenol 12) was detected in sample 4 only. The response of this phenol was followed by a broad peak. The level of phenol 12 was quantitated manually by measuring peak area from baseline to valley position and calculated against the standard measured in similar fashion.

The statistical analysis shown in Table 4 was performed by analyzing the same sample (sample 13) 6 times. The LC procedure with the electrochemical detector described in this study provides a quick, sensitive, alternative procedure for the determination of phenolic impurities in MCPA.

Acknowledgments

We thank D. Chaput for assistance in sample confirmation, and Mary O'Neil for active ingredient analysis.

References

(1) Sittig, M. (1980) *Pesticide Manufacturing and Toxic Materials Control Encyclopedia,* Noyes Data Corp., Park Ridge, NJ, pp. 490–492
(2) Leng, M. L. (1979) *Proceedings of the CIPAC Symposium,* Baltimore, MD, June 5–7
(3) Hummelstedt, L. E. I., & Hume, O. E. (1960) *Anal. Chem.* **32,** 1792–1797
(4) Skelly, N. E. (1961) *Anal. Chem.* **33,** 271–273
(5) Lanouette, M., Cochrane, W. P., & Singh, J. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 494–497
(6) Harvey, D. R., & Norman, R. O. C. (1961) *J. Chem. Soc.* **III,** 3604–3610
(7) Akerblom, M., & Lindgren, B. (1983) *J. Chromatogr.* **258,** 302–306
(8) Bystricky, L. (1981) *Chem. Zvesti* **35**(5), 671–677
(9) Stevens, T., & Grorud, R. B. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 738–741
(10) Cochrane, W. P., Lanouette, M., & Singh, J. (1983) *J. Assoc. Off. Anal. Chem.* **66,** 804–809

Gas Chromatographic Determination of *N*-Nitrosodialkanolamines in Herbicide Di- or Trialkanolamine Formulations

YUK Y. WIGFIELD, MARIO D. LACROIX, MONIQUE LANOUETTE, and NARINE P. GURPRASAD
Agriculture Canada, Food Production and Inspection Branch, Laboratory Services Division, Ottawa, Ontario K1A 0C6, Canada

A modified method is presented to determine trace quantities of *N*-nitrosodiethanolamine (NDEIA) and *N*-nitrosodiisopropanolamine (NDiPIA) in the triisopropanolamine (TiPIA) formulation of a mixture of picloram and 2,4-D. Aqueous sample is extracted with dichloromethane to remove organic interferences, and then the aqueous layer is passed sequentially through chloride anion exchange column, hydrogen cation exchange column, and Clin-Elut extraction tube. The final eluate, 10% acetone in ethyl acetate, is concentrated. The isolated nitrosamines are converted to the corresponding trimethylsilyl (TMS) derivatives and determined by gas chromatography (GC) on a DB1 column coupled with a thermal energy analyzer (GC-TEA). Eight samples of commercial TiPIA formulations are analyzed. Maximum detected levels of NDEIA and NDiPIA were 0.6 and 0.9 ppm, respectively, expressed relative to total weight of active ingredients. Analysis of 13 samples of herbicide DEIA formulation using a previously established method and a DB225 column gave NDEIA results of 0.7-6.0 ppm. NDiPIA was not detected in those samples. Results are confirmed by GC-mass spectrometry (GC/MS) with oxygen negative chemical ionization (ONCI) detection. Dectection limits for both nitrosamines are 0.05 or 0.07 ng (0.1 or 0.17 ppm) for GC-TEA detection, depending on the analytical columns used, and 20 pg (0.04 ppm) for GC/MS detection. Recoveries of NDEIA are 87-109% for DEIA formulation spiked at 2.6 and 3.9 ppm and 90-115% for TiPIA formulation spiked at 0.2-0.3 ppm. Similarly, recoveries of NDiPIA are 95.7-100% for the DEIA formulation spiked at 0.24 and 0.48 ppm, and 82-118% for the TiPIA formulation spiked at 0.2-0.3 ppm.

4-Amino-3,5,6-trichloropyridine-2-carboxylic acid, commonly known as picloram, is a broad-leaf herbicide. It may be used in combination with 2,4-dichlorophenoxyacetic acid (2,4-D) against deep-rooted perennials on noncropland (1). In Canada, certain picloram products are formulated with 2,4-D as diethanolamine (DEIA) salts or triisopropanolamine (TiPIA) salts under the trade names of Tordon 202c and Tordon 101, respectively.

Diethanolamine formulations of herbicides have been reported to contain trace quantities of *N*-nitrosodiethanolamine (NDEIA) (2), in some cases, up to 2400 ppm relative to active ingredient (a.i.) weight (3, 4). NDEIA has been demonstrated to be carcinogenic to laboratory animals (5). Therefore, commercial amine formulations of a mixture of picloram and 2,4-D were investigated for nitrosamine contamination.

Several methods for the analysis of hydroxylated *N*-nitrosamines have been reported. These include liquid chromatography (LC) combined with a thermal energy analyzer (TEA) (6, 7) on the hydroxylated *N*-nitrosamine and gas chromatography (GC) with TEA or a mass spectrometer (MS) on a volatile derivative of the *N*-nitrosamine (8–10). We have previously developed cleanup techniques suitable for trace analysis of NDEIA in pesticides (2, 4). However, these techniques were unsuitable for the present investigation. Thus, in this study, we report a modified method to determine trace levels of NDEIA and NDiPIA in a mixture of 2,4-D and picloram present as TiPIA salts, as well as the levels of these nitrosamines in 21 samples of 2 formulations. Although NDiPIA has been detected in environmental samples (11), it has not previously been detected in pesticide formulations.

Received April 8, 1987. Accepted July 27, 1987.

METHOD

Apparatus

(a) *Gas chromatograph-thermal energy analyzer.*—For DEIA formulation: Varian Vista Model 6000 gas chromatograph with on-column injector (Varian Ultrabore pack mode kit) interfaced to Model 543 TEA detector (Thermedics, Inc.), equipped with Varian 8000 autosampler and Varian 402 data acquisition system. GC column: 30 m × 1.0 μm DB225 (J & W Scientific). Operating conditions: column oven temperature, 120°C for 1 min, then 120–160°C at 5°/min, followed by 160°C for 5 min; head pressure, 25 psi; injection port, 175°C; helium carrier gas flow rate, 4.0 mL/min; interface to detector, 200°C; pyrolizer temperature, 500°C with CTR® gas stream filter; vacuum, 0.5 torr; attenuation, 4; chart speed, 0.5 cm/min. Injection technique, on-column; injection volume, 2 μL. Under the given conditions, the retention time of NDEIA was 10.90 min and that of NDiPIA was 9.48 min.

For TiPIA formulation: Hewlett-Packard Model 5710A gas chromatograph with Model 18740B capillary column controller interface to Model 502 TEA detector (Thermedics, Inc.) and equipped with Spectra-Physics SP 4200 computer integrator. GC column: 30 m × 0.25 μm fused silica DB1 (J & W Scientific). Operating conditions: column temperature, 120–130°C at 1°/min; injection port, 150°C; carrier gas flow rate, 3.0 mL/min; interface to detector, 150°C; vacuum, 0.35 torr. Injection technique, spitless; purge delay time, 10 s; septum vent, 6 mL/min; split vent, 70 mL/min. All other conditions are identical to those listed for DEIA formulation. Under the given conditions, the retention time of NDEIA was 5.48 min and that of NDiPIA was 6.06 min.

(b) *Gas chromatograph/mass spectrometer.*—Finnigan Model 9610 gas chromatograph with J & W Model II capillary on-column injector and Finnigan 4500 mass spectrometer with pulsed positive ion/negative ion chemical ionization and Townsend discharge ionization. GC column: 15 m × 0.32 mm id DB5 (J & W Scientific). Data acquisition and MS control: Finnigan 2300 INCOS data system. GC operating conditions: column temperature, 85°C for 0.1 min, then 5°/min to 150°C; head pressure, 3 psi; ultra high purity helium carrier gas. Mass spectrometer source pressure, 0.48 torr; ionization mode, oxygen (UHP) negative chemical ionization (ONCI) with Townsend discharge mode; temperature 100°C; discharge current, 70 μA; voltage 1.5 kV.

(c) *Extraction tube.*—Clin Elut® columns unbuffered (part No. 1020, Analytichem International, Harbor City, CA).

(d) *Ion exchange columns.*—5 mL anion exchange material AG1-X8 (100–200 mesh) chloride ion packed with water in disposable 11 mL polypropylene Econo-Columns (Bio-Rad Laboratories) and 0.8 × 4 cm prefilled Econo-Columns packed with 100–200 mesh cation exchange resin AG50W-X8 (H^+) in water (Bio-Rad Laboratories).

Reagents

Caution: NDEIA and NDiPIA have been shown to be carcinogenic to laboratory animals; use extreme caution in handling standards and samples. Preferably, purchase dilute

standard from commercial supplier and perform all work in fume hood. Use mechanical pipetting aids for measuring all solutions and wear protective gloves while handling standards.

(a) *Solvents.*—Butanol, reagent grade; all others, glass distilled.

(b) *Nitrosamine standard solutions.*—500 μg NDEIA or NDiPIA in 5 mL acetone (Thermedics).

(c) *NDEIA analytical standard.*—Dilute appropriate aliquot of standard solution to 10 mL with acetone to give final concentrations of 50, 5.0 ng/μL, respectively.

(d) *NDiPIA analytical standard.*—Dilute appropriate aliquot of standard solution to 10 mL with acetone to give final concentration of 1.03 μg/mL, 5.14 ng/mL, respectively.

(e) *Mixed standard.*—Mix 1 mL of 50 ng/μL NDEIA and 50 μL of 1.03 μg/mL NDiPIA and dilute to 10 mL with acetone to give final NDEIA concentration of 5.00 ng/μL and NDiPIA concentration of 5.14 ng/μL.

(f) *N-Nitrosation inhibitor.*—Sodium azide solution. Weigh 10 g sodium azide (reagent grade) in 500 mL glass-distilled water (purified with Milli-Q2 system).

(g) *Silylating agent.*—*N,O-Bis*-(trimethylsilyl)acetamide (BSA), specially purified grade (Pierce Chemical Co.).

Cleanup

(a) *DEIA formulation.*—Quantitatively transfer 1.0 mL of formulation with 15 mL water to drained anion exchange column connected in series with drained cation exchange column which elutes directly to Clin-Elut extraction tube. Wait 3 min for eluted sample to be adsorbed onto dry Clin-Elut column. Add 20 mL 10% acetone in ethyl acetate to Clin Elut column and wait 2 min. Add 230 mL of the same solvent and collect eluate in 500 mL round-bottom flask. Add 0.5 mL butanol to eluate to prevent complete evaporation of solvent. Concentrate eluate to 1 mL on rotary evaporator at 36°C. Transfer resulting solution to 15 mL centrifuge tube by rinsing 3 times with 2 mL portions of dichloromethane each time. Proceed to preparation of TMS derivative.

(b) *TiPIA formulation.*—Weigh 2.0 g sample into 40 mL centrifuge tube. Add 3 mL water and 5 mL dichloromethane. Vortex 30 s and centrifuge mixture. Transfer aqueous layer to series of columns as described for DEIA formulation. Re-extract organic layer with 5 mL water and transfer resulting aqueous layer to column system. Add 7 mL water to column system. Wait 3 min, and then proceed in the same manner as described for NDEIA formulation.

Preparation of TMS Derivatives

Evaporate NDEIA extract in 15 mL centrifuge tube under nitrogen to dryness. Add 0.5 mL BSA reagent, shake, stopper, and heat 30 min at 70°C (12). Cool to room temperature and dilute to 1 mL with acetone. To prepare standard used for GC quantitation, add 200 μL standard stock solution in 15 mL centrifuge tube, evaporate to dryness, and prepare silyl derivatives as above. Proceed to GC determination within 24 h.

GC Determination

Inject 2 μL aliquot of NDEIA silyl derivative into GC apparatus. Quantitate NDEIA concentrations, using external standard:

$$\text{NDEIA (ppm a.i.)} = (A/A') \times (W'/W) \times \text{purity of std}$$

where A and A′ = area response of sample and standard, respectively; W′ and W = weight (μg) of standard, and total herbicide weight calculated from amount of picloram and 2,4-D (% w/w) contained in the herbicide amine formulation as stated on the product label.

GC/MS Confirmation

Using conditions described under apparatus, inject 3 μL aliquot of standard NDEIA silyl solution within 24 h of preparation and obtain mass spectrum, using mode of ONCI described. Repeat with silylated sample solution, and compare mass spectrum with that of standard for confirmation.

Results and Discussion

In Canada, certain picloram products are available commercially as a mixture with 2,4-D. This mixture may be present either as DEIA salts or TiPIA salts. The former product contains 12 g/L picloram and 200 g/L 2,4-D, and the latter contains 60 g/L and 240 g/L, correspondingly. The cleanup procedures used to isolate NDEIA and NDiPIA from samples of the DEIA product involve passing the aqueous samples through a series of disposable columns: a chloride anion exchange column to retain the herbicide anions, a cation exchange column to retain the dialkanolammonium cation, and a Clin-Elut column to extract the *N*-nitrosodialkanolamine into the organic phase. The cleanup procedure for TiPIA samples requires extraction with dichloromethane to remove organic ingredients in the samples prior to the use of the series of disposable columns. The resulting nitrosamines are then converted into the respective TMS derivatives and analyzed by use of a GC-TEA detection system.

We previously developed a cleanup method to isolate NDEIA from samples of 2,4-D DEIA formulation (2). Because of the similarity in herbicide compositions of all 3 products, we attempted to apply this cleanup method to the compounds in the present investigation. The method is applicable to DEIA samples, but is not suitable for TiPIA samples. Addition of TiPIA sample onto a chloride anion exchange column resulted in a very slow elution speed and a cloudy, colored eluate. The same effects were observed when a formate anion exchange column was used. Increased column size improved the color of the eluate but also increased the sample elution time and produced poor recovery results. We suspect that certain formulation ingredients may deleteriously affect the anion exchange columns. Thus a dichloromethane extraction step was introduced to remove these ingredients prior to column cleanup, resulting in clean, colorless eluates.

For TiPIA, a DB1 GC column combined with a programmed column temperature was used to separate NDEIA from NDiPIA. The TEA response time for NDEIA was 5.48 min and for NDiPIA was 6.06 min as shown in Figure 1. For DEIA samples, due to the marked difference in levels of NDEIA and NDiPIA present, a DB1 column could not give well resolved responses for these 2 nitrosamines. Thus a DB225 column was used, giving a TEA response for NDiPIA at 9.48 min and for NDEIA at 10.90 min as shown in Figure 2. This elution pattern gave well separated responses for the 2 contaminants present at widely different concentrations.

Sodium azide (NaN_3) solution has been used to prevent artifactual formation of nitrosamine in samples during cleanup (2). It also increases the ion strength of the sample and thus decreases the ion exchange column retention capacity for the sample. Therefore, the effect of NaN_3 solution on the nitrosamine levels in the samples was investigated. A sample of each formulation was analyzed for nitrosamine before and after the addition of the NaN_3 solution. Table 1 shows that the results from these 2 different conditions are the same

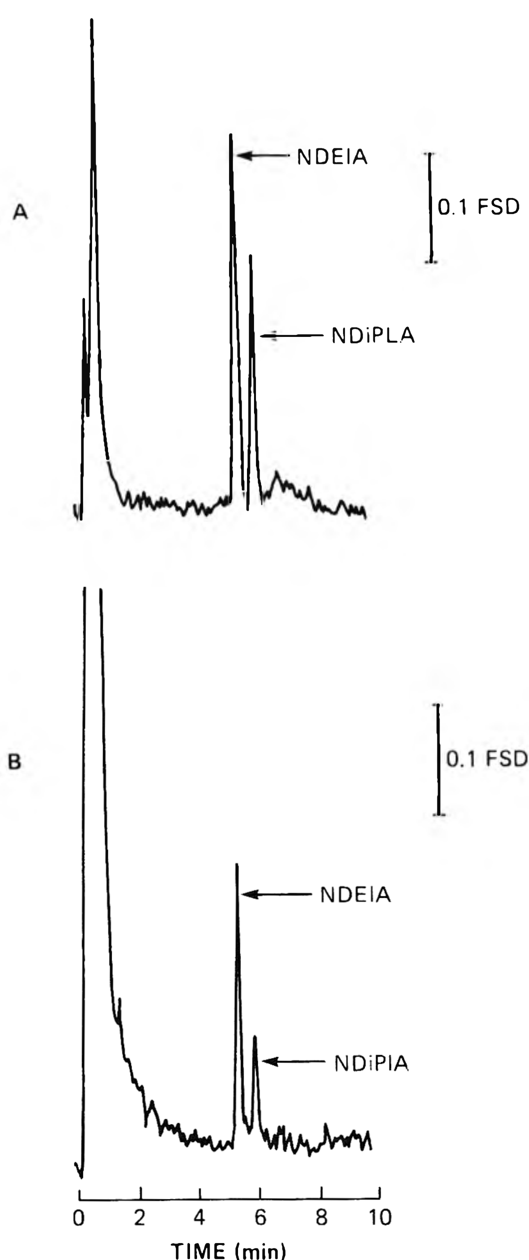


Figure 1. Typical GC-TEA chromatogram of: A, mixed standards (0.3 ng each); B, sample 20 containing NDEIA (0.2 ppm) and NDiPIA (0.1 ppm) using DB1 column.

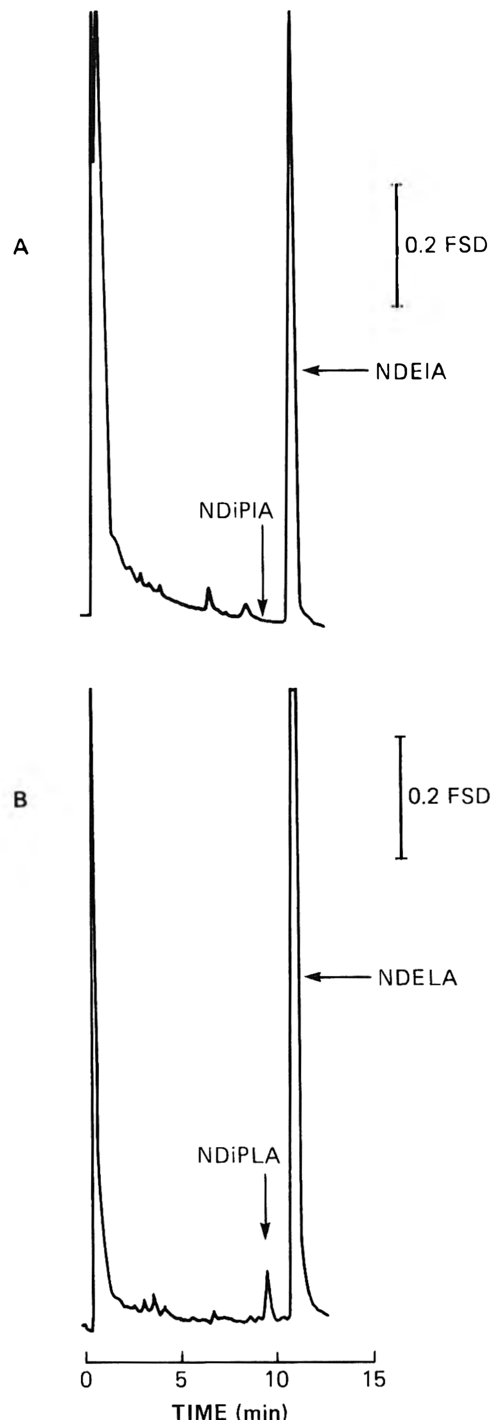


Figure 2. Typical GC-TEA chromatogram of: A, sample 5 containing NDEIA (4.16 ppm); B, sample 5 spiked with NDiPIA (0.48 ppm).

within experimental error. Thus NaN_3 was not added to the samples prior to cleanup in order to maintain the retention capacity of the ion exchange columns.

Recovery studies were performed using the described method on both formulations by spiking a mixed standard of NDEIA and NDiPIA directly into the sample, ensuring thorough mixing before extraction and cleanup. The recovery results of NDEIA and NDiPIA from the herbicide DEIA product are shown in Table 2. Recoveries of NDEIA range from 87 to 109% with an overall average value of 101% and a coefficient of variation (CV) of 4.5%, calculated at 3.9 ppm. Recoveries of NDiPIA spiked at 0.24 ppm and 0.48 ppm are 100% and 95.7%, respectively. This NDiPIA recovery study was limited because of the absence of this nitrosamine in the formulation.

Similar recovery studies were conducted on the herbicide TiPIA product spiked with NDEIA at 0.21, 0.29, 0.42, and 0.80 ppm and NDiPIA at 0.29 and 0.20 ppm, respectively. Results are shown in Tables 3 and 4. Recoveries of NDEIA range from 91 to 115% with an average of 101% and a CV of 5.0%, calculated at 0.29 ppm. Recovery results of NDiPIA are 82 to 118% with an average of 102% and a CV of 7.2%, calculated at 0.29 ppm.

Reagent blanks were analyzed after the same cleanup procedures, giving levels of both nitrosamines below the limits of detectability. Calibration curves were calculated from a mixed standard solution of NDEIA and NDiPIA in the range of 0.1 to 1.0 ng, and were linear within this range.

The minimum detectable amount (MDA), based on a signal-to-noise ratio of 2:1, was 0.07 ng for GC-TEA using the DB225 column, 0.05 ng using the DB1 column, and 20 pg for GC/MS. These limits correspond to 0.17 ppm for nitro-

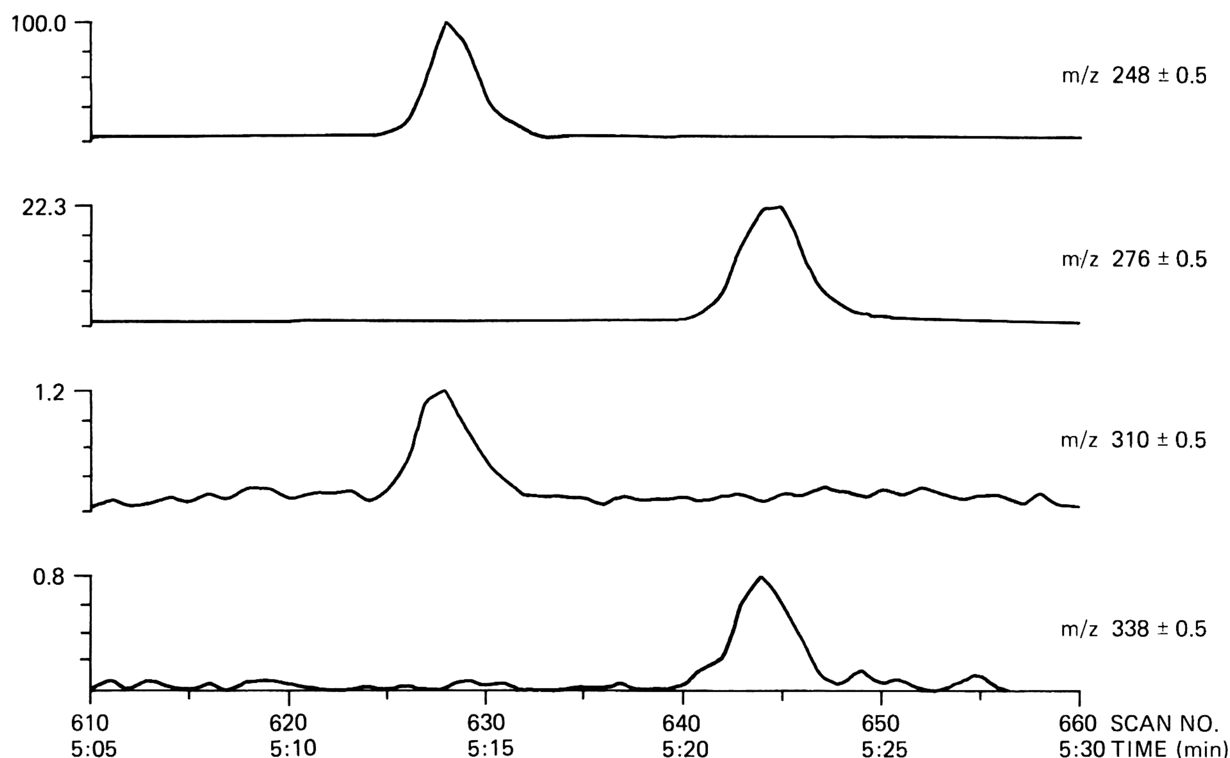


Figure 3. Typical GC/MS chromatogram of TMS derivatives of mixed standards (150 pg each).

samines in DEIA formulation analyzed by GC-TEA, 0.1 ppm for the TiPIA formulation, and 0.05 ppm for samples analyzed by GC/MS.

Analyses of 13 samples of the DEIA formulation and 8 samples of TiPIA formulation were performed in duplicate for nitrosamine contamination using GC-TEA. Averages of these duplicate results are shown in Table 5. The levels of NDEIA in the DEIA formulation range from 0.7 to 4.9 ppm and in the TiPIA product from nondetectable to 0.6 ppm. NDiPIA was not detected in all samples of the DEIA product. It was detected at low levels (max. 0.9 ppm) in some samples of the TiPIA product.

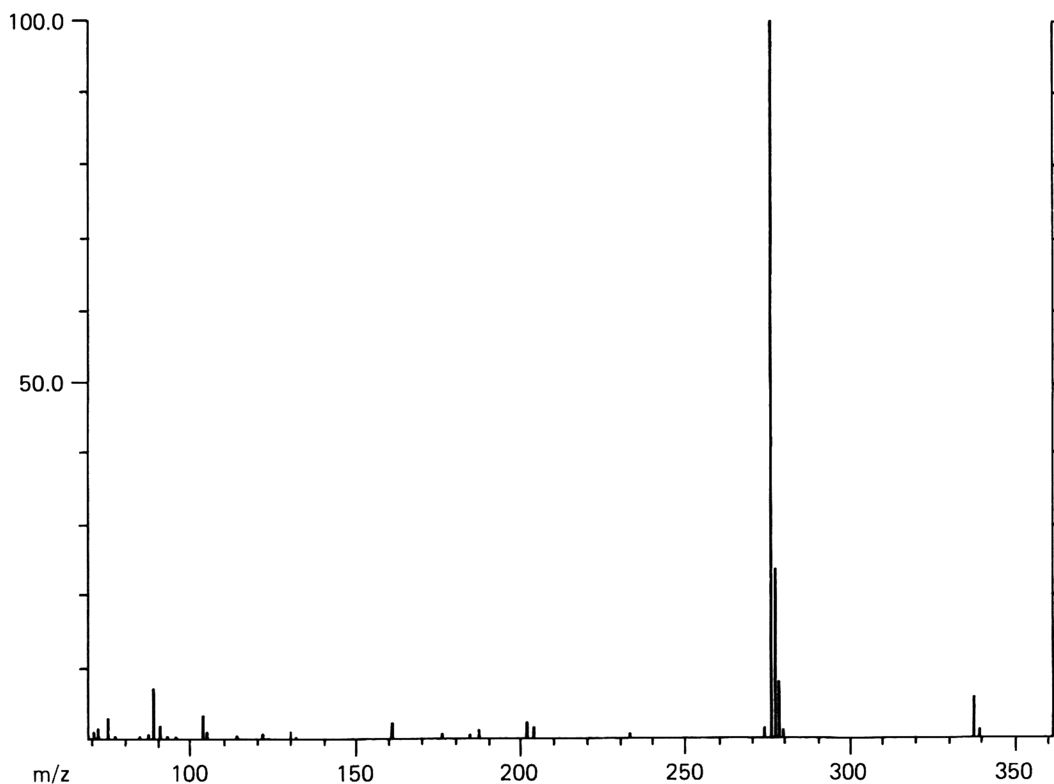


Figure 4. Typical mass fragmentation of NDiPIA-TMS standard (25 ng).

Table 1. *N*-Nitrosodialkanolamine in DEIA and TiPIA formulation before and after addition of NaN_3 solution

Analysis No.	NDEIA, ppm		NDiPIA, ppm	
	With NaN_3	Without NaN_3	With NaN_3	Without NaN_3
1	0.98	0.88	—	—
2	0.81	0.82	—	—
3	0.91	0.83	—	—
4	0.91	0.98	—	—
$\bar{x}^a$	0.90	0.88	—	—
5	0.17	0.20	0.12	0.10
6	0.21	0.20	0.10	0.10
7	0.22		0.11	0.10
$\bar{x}^b$	0.20	0.20	0.11	0.10

[a] Sample 12.
[b] Sample 19.

Table 2. Recoveries of *N*-nitrosodialkanolamine from spiked herbicide DEIA formulation, using GC-TEA

Nitrosamine	Nitrosamine level, ppm				Rec., %
	Present	Spiked	Found	Found/present	
NDEIA	4.87	3.9	8.82	3.95	100
	4.87	3.9	9.25	4.38	110
	4.87	3.9	9.08	4.21	106
	4.93	3.9	9.16	4.23	107
	4.93	3.9	8.95	4.02	102
	4.93	3.9	8.72	3.79	96
	2.73	2.6	5.14	2.41	93
	2.73	2.6	4.98	2.25	87
	2.73	2.6	5.55	2.82	109
$\bar{x}$ (overall)					101
$\bar{x}$ (at 3.9 ppm)					104
CV (at 3.9 ppm) (n = 6)					4.3
NDiPIA	ND[a]	0.24	0.24	0.24	100.0
	ND[a]	0.48	0.48	0.48	95.1

[a] ND = nondetectable at a detection limit of 0.17 ppm.

Table 3. Recoveries of *N*-nitrosodiethanolamine from spiked herbicide TiPIA formulation, using GC-TEA

NDEIA, ppm				Rec., %
Present	Spiked	Found	Found/present	
0.207	0.208	0.407	0.200	96
0.207	0.208	0.395	0.188	91
0.207	0.208	0.440	0.233	112
0.200	0.286	0.502	0.302	106
0.200	0.286	0.465	0.265	93
0.200	0.286	0.502	0.302	106
0.203	0.286	0.475	0.272	95
0.203	0.286	0.499	0.296	103
0.203	0.286	0.486	0.283	99
0.221	0.416	0.698	0.477	114
0.221	0.416	0.596	0.375	90
0.221	0.416	0.618	0.397	95
0.170	0.800	0.980	0.810	101
0.170	0.800	1.060	0.890	107
0.170	0.800	1.113	0.960	115
$\bar{x}$ (overall)				102
$\bar{x}$ (at 0.29 ppm)				100
CV (at 0.29 ppm) (n = 6)				5.0

Table 4. Recoveries of *N*-nitrosodiisopropanolamine from spiked herbicide TiPIA formulation, using GC-TEA

NDiPIA, ppm				Rec., %
Present	Spiked	Found	Found/present	
0.099	0.294	0.409	0.310	105
0.099	0.294	0.404	0.305	103
0.098	0.294	0.371	0.273	93
0.098	0.294	0.436	0.338	115
0.095	0.196	0.320	0.225	118
0.095	0.196	0.250	0.155	82
0.095	0.196	0.280	0.185	97
$\bar{x}$ (overall)				102
$\bar{x}$ (at 0.29 ppm)				104
CV (at 0.29 ppm) (n = 4)				7.2

Results in Table 5 were confirmed using ONCI GC/MS determination at m/z 248.07 ± 0.5, 276.08 ± 0.5, 310.09 ± 0.5, and 338.10 ± 0.5. The former two m/z values represent the loss of an NO group, $(m - 30)^+$, from the molecular ions NDEIA-TMS and NDiPIA-TMS. The latter 2 ions represent the oxygen adducts, $(M + 32)^+$, of these molecular ions. Figure 3 shows the GC/MS chromatogram of TMS derivatives of mixed standards demonstrating the presence of these characteristic ions. The GC/MS chromatogram and the MS fragmentation pattern of the NDEIA standard and a sample containing NDEIA have been reported (2). Figure 4 shows the MS fragmentation patterns of a sample of the NDiPIA-TMS standard.

Results in Table 5 show that samples of the DEIA product (samples 1–13) consistently contain higher levels of NDEIA than samples of the TiPIA product. The level of NDiPIA in some TiPIA samples (nos. 14, 15, 17, and 18) are higher than those in the DEIA formulation. It is suspected that the amines used to formulate these products have been contaminated with nitrosamines, probably DEIA with NDEIA and TiPIA with NDiPIA. This rationale is based on the fact that DEIA is a precursor of NDEIA. TiPIA is not a precursor of NDEIA; it is a precursor of NDiPIA only after dealkylation. However, tertiary amines may contain trace quantities of secondary amines which are nitrosamine precursors. Thus, it appears reasonable that the levels of NDEIA in samples 1–13 are significantly higher than those of NDiPIA in samples 14–21.

Table 5. *N*-Nitrosodialkanolamine levels relative to active ingredients found in mixture of picloram and 2,4-D formulations

Sample	Amine used in formulation	NDEIA, ppm		NDiPIA, ppm	
		GC-TEA	GC/MS	GC-TEA	GC/MS
1	DEIA	4.9	4.4	ND[a]	—
2	DEIA	3.4	3.4	ND	—
3	DEIA	3.9	3.7	ND	—
4	DEIA	2.4	2.5	ND	—
5	DEIA	4.2	4.1	ND	—
6	DEIA	3.7	3.3	ND	—
7	DEIA	2.9	3.1	ND	—
8	DEIA	0.8	0.7	ND	—
9	DEIA	4.6	3.5	ND	—
10	DEIA	3.9	4.3	ND	—
11	DEIA	1.0	1.0	ND	—
12	DEIA	0.7	0.7	ND	—
13	DEIA	3.1	3.5		
14	TiPIA	0.6	0.6	0.9	0.9
15	TiPIA	0.4	0.4	0.2	0.2
16	TiPIA	0.3	0.3	ND	ND
17	TiPIA	0.5	0.6	0.2	0.2
18	TiPIA	0.4	0.4	0.2	0.2
19	TiPIA	0.2	0.2	ND	0.1
20	TIPIA	0.2	—	0.1	—
21	TIPIA	ND	—	ND	—

[a] ND = nondetectable at a detection limit of 0.1 ppm.

The fact that samples 14–21 contain low levels of DEIA may be due to cross-contamination.

In conclusion, the similarity of results for the same samples determined using 2 different detection systems, as shown in Table 5, demonstrates the absence of any interfering impurities in the extract. The analytical method reported in this study is sufficiently sensitive and specific to separate and to give reproducible results of NDEIA and NDiPIA in a mixture of 2,4-D and picloram present as DEIA or TiPIA salts.

REFERENCES

(1) Worthing, C. C. (1983) *The Pesticide Manual*, 7th Ed., The British Crop Protection Council, Croydon, England, 441 pp
(2) Wigfield, Y. Y., & Lanouette, M. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 1142–1148
(3) Bontoyan, W. R., Law, M. W., & Wright, D. P., Jr (1979) *J. Agric. Food Chem.* **27**, 631–635
(4) Wigfield, Y. Y., Gurprasad, N. P., Lanouette, M., & Ripley, S. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 792–796
(5) Smith, P. A. S., & Leoppky, R. N. (1967) *J. Am. Chem. Soc.* **89**, 1147–1157
(6) Fan, T. Y., Morrison, J., Rounbehler, D. P., Ross, R., Fine, D. H., Miles, W., & Sen, N. P. (1977) *Science* **196**, 70–71
(7) Fan, T. Y., Goff, U., Song, L., Fine, D. H., Arsenault, G. P., & Biemann, K. (1977) *Food Cosmet. Toxicol.* **15**, 423–430
(8) Sen, N. P., Seaman, S., & Tessier, L. (1982) *IARC Sci. Publ.* **41**, 185–197
(9) Orshima, H., Matsui, M., & Kwabata T. (1979) *J. Chromatogr.* **169**, 279–286
(10) Sen, N. P., Miles, W. F., Seaman, S., & Lawrence, J. F. (1976) *J. Chromatogr.* **128**, 169–173
(11) Issenberg, P., Conrad, E. E., Neilson, J. W., Klein, D. A., & Miller, S. E. (1984) *IARC Sci. Publ.* **57**, 43–50
(12) Sen, N. P., Donaldson, B. A., Seaman, S., Iyengar, J. R., & Miles, W. F. (1978) *IARC Sci. Publ.* **19**, 373–393

Determination of *N*-Nitrosodipropylamine in Trifluralin Emulsifiable Concentrates Using Minicolumn Cleanup and Gas Chromatography with Thermal Energy Analyzer

DAVE WOTHERSPOON and RALPH HINDLE[1]
Agriculture Canada, Laboratory Services Division, Western Laboratory, Calgary, Alberta T2G 0X5, Canada

A quick method for determining *N*-nitrosodipropylamine (NDPA) levels in trifluralin emulsifiable concentrate formulations is described. At least 18 samples can be analyzed at one time in a minimum of fumehood space, with up to 90% savings on solvents and materials. A sample aliquot is mixed with a solution containing nitrosamine recovery standards, and nitrosamines are separated by minicolumn cleanup. Internal standard is added directly to the eluate containing the nitrosamines, and levels are determined by gas chromatography with thermal energy analyzer. Recoveries of spiked nitrosamines ranged from 98 to 102%. Coefficients of variation for samples containing <0.5 ppm NDPA are <13%. Minimum detectable limit, calculated as 3 times the noise, is 0.06 ppm. Comparison with the method formerly used by this laboratory shows no significant difference in the analytical results at 95% confidence limits, and control experiments were performed to ensure that there was no artifact formation of NDPA.

Trifluralin (α,α,α-trifluoro-2,6-dinitro-*N,N*-dipropyl-*p*-toluidine) is used extensively in Canada as a pre-emergence herbicide to control annual grasses and broad-leafed weeds among field crops, vegetables, and ornamentals. *N*-Nitrosodipropylamine (NDPA) has been detected in trifluralin formulations by Ross et al. (1), and concerns about it have been raised by Magee and Barnes (2), who have shown dialkyl nitrosamines to be carcinogenic in many species of animals. The Regulations of the Canada Pest Control Product Act (June 1982) set a maximum tolerance of 1 ppm NDPA in trifluralin formulations, and this laboratory has a monitoring program at the pre- and post-registration stages to enforce this limit.

Analysis for NDPA in trifluralin products has been described by Maybury and Grant (3); they used a silica gel column cleanup, followed by concentration of the eluate, analysis by gas chromatography, and thermal energy analyzer detection (GC/TEA). It is essentially this method that has been used in this laboratory for 2 years; 6 samples per day can be analyzed within our space limitations of one 5 ft fumehood. Significant improvements have been made that allow analyses of at least 18 samples per day in smaller space, with over 90% savings in solvents and materials. This is achieved through the use of commercially available silica gel minicolumns. The sample concentration step following cleanup has also been eliminated, thus reducing the potential for loss of the volatile nitrosamines as well as reducing analysis time by over 1 h.

During GC analysis for nitrosamines, adequate attention must be given to the possibility of artifact formation during various steps in the method, resulting in the reporting of false positives (4–6). Experimental data are provided to prove that this method does not cause nitrosamines to be formed.

METHOD

Apparatus and Reagents

Caution: Nitrosamines are carcinogenic and should only be used within a fumehood. Use of red fluorescent lighting is recommended to avoid possible photodegradation of compounds.

(a) *Gas chromatograph.*—Hewlett-Packard Model 5792 equipped with Model 7671A autosampler and fitted with 5 ft × 3 mm id glass column packed with 10% Pennwalt 223 + 4% KOH on 80–100 mesh Porapak R (Alltech Associates). Operating conditions: injection port 255°C; oven temperature 160°C for 13 min, programmed to 210°C at 30°/min, with final hold for 22 min; nitrogen carrier flow 30 mL/min.

(b) *Thermal energy analyzer.*—Model 543 with interface temperature of 200°C and pyrolyzer temperature of 550°C, equipped with CTR gas stream filter (Thermo Electron Corp., Waltham, MA).

(c) *Electronic integrator.*—Hewlett-Packard Model 3390A.

(d) *Chromatographic columns.*—3 mL silica gel Bond-Elut columns (Analytichem International). Heat overnight in 105°C oven.

Received September 24, 1986. Accepted July 31, 1987.

[1] Address correspondence to this author. Present address: Agriculture Canada, Laboratory Services Division, Pesticide Laboratory, Ottawa, Ontario K1A 0C6, Canada.

Table 1. NDPA concentration (ppm) in replicate samples of trifluralin

Replicate	Sample A	B	C	D	E	F	G	H	I
1	0.400	0.229	0.131	0.493	0.097	0.140	0.204	0.222	0.117
2	0.407	0.235	0.136	0.492	0.099	0.114	0.177	0.233	0.104
3	0.406	0.256	0.117	0.482	0.104	0.114	0.225	0.208	0.117
4	0.418	0.251	0.121	0.493	0.109	0.138	0.192	0.211	0.129
5	0.384	0.238	0.123	0.478	0.092	0.116	0.163	0.206	0.116
6	0.384	0.267	0.132	0.462	0.089	0.094	0.177	0.220	0.114
7	0.407	0.261	0.158	0.505	0.104	0.131	0.194	0.215	0.113
8	0.403	NV[a]	0.118	0.489	0.099	0.143	NV	0.241	0.136
9	0.435	NV	0.131	0.477	0.115	0.126	NV	0.190	0.118
10	NV	NV	0.142	NV	0.091	NV	NV	NV	NV
Mean	0.405	0.248	0.131	0.486	0.100	0.124	0.190	0.216	0.118
SD	0.016	0.014	0.012	0.012	0.008	0.016	0.020	0.015	0.009
RSD, %	3.95	5.65	9.16	2.47	8.00	12.9	10.5	6.94	7.63

[a] No value.

(e) *Silica gel.*—BDH Chemicals Canada Ltd, No. M07734. Preheat overnight in 105°C oven.

(f) *Sodium sulfate.*—Fisher Scientific S-421. Dry overnight in 105°C oven.

(g) *Dichloromethane.*—Distilled in glass (Caledon).

(h) *Ethanol.*—100% (Consolidated Alcohols Ltd).

(i) *Nitrosamine standards.*—Prepare NDMA, NDEA, and NDPA working standards from stock 100 μg/mL solutions of individual nitrosamines in ethanol (Thermedics, Inc.); prepare NDBA in DCM from pure standard (Sigma Chemical Co.).

Extraction and Cleanup

Prepare silica gel minicolumns by adding additional 0.3 g silica gel, and then 0.7 g sodium sulfate. Place each column in 18 × 150 mm culture test tube and prewash with 3 × 1.0 mL dichloromethane (DCM). Ridge on top of cleanup column holds it in place at top of test tube.

To separate culture test tube containing 2.0 mL DCM, pipet 1.0 mL column spiking standard containing 0.5 μg *N*-nitrosodiethylamine (NDEA) and 2.0 μg *N*-nitrosodibutylamine (NDBA) in DCM. Pipet 1.0 mL liquid trifluralin formulation and mix thoroughly using vortex mixer. Quantitatively transfer solution through cleanup column by adding two 0.5 mL DCM rinses of sample test tube to column. Elute trifluralin from column with five 0.5 mL washes of DCM, followed by 0.5 mL 10% ethanol in DCM. With cleanup completed, transfer column to clean culture tube and elute nitrosamines with three 1.0 mL portions of 10% ethanol in DCM. Following elution, discard column, and pipet 1.0 mL of 1.0 μg/mL *N*-nitrosodimethylamine (NDMA) in DCM as internal standard into final fraction, and mix using vortex mixer. Sample is now ready for GC/TEA analysis.

GC/TEA Analysis

Inject 4 μL working standard containing 330 ng/mL NDMA, 170 ng/mL NDEA, 340 ng/mL NDPA, and 670 ng/mL NDBA, using integrator in peak height mode. Retention times are 1.1, 1.8, 3.7, and 9.3 min, respectively. Calibration is complete when repeat injections are within 2% of each other, using internal standardization (ISTD). Initial temperature of 160°C for 13 min is sufficient to elute the 4 nitrosamine peaks; however, the ramp to 210°C and hold for 22.0 min is required to drive off any residual trifluralin injected, which would otherwise cause very broad peak during subsequent runs.

Calculate ppm NDPA in trifluralin, using the following formula:

NDPA, ppm = [(Ht NDPA/Ht NDPA′) × (Ht NDMA′/Ht NDMA) × (μg NDPA′/mL sample)]/% trifluralin in formulation

where Ht NDPA and Ht NDPA′ = peak height of sample and standard. respectively; μg NDPA′ = weight in standard; and mL sample = original sample size.

Results and Discussion

In order to verify the reproducibility of this method, 9 samples from various manufacturers with different formulations were analyzed up to 10 times each (Table 1). The range of sample means was 0.100–0.486 ppm NDPA, with coefficients of variation (CV) ranging from 2.57 to 12.9%. The minimum detectable limit, defined as 3 times the standard deviation (SD), is 0.06 ppm. Table 2 compares the results achieved with this method and the method previously used in this laboratory, involving cleanup on 25 g silica gel

Table 2. Comparison of NDPA recovery using different-size columns, with standard addition

Sample	NDPA, ppm Minicolumn	Large	Std addn[a]
A	0.405	0.410	0.441
B	0.248	0.254	0.238
C	0.131	0.134	0.167
D	0.486	0.449	0.458
E	0.100	0.095	0.103
F	0.124	0.124	0.124
G	0.190	0.188	0.194
H	0.216	0.203	0.204
I	0.118	0.100	0.137

[a] Each sample was spiked with 3 levels: 0.09, 0.18 and 0.36 μg/mL NDPA in xylene.

Table 3. NDPA levels observed in diluted trifluralin extracts

Trifluralin, ng/mL	NDPA, ng/mL No spike	44 ng/mL added	Rec., %
40	ND[a]	40	90
400	ND	44	100
4000	ND	42	95
40 000	ND	43	98
400 000	ND	45	102

[a] Not detected; minimum detectable limit = 6 ng/mL.

Table 4. Nitrosamine levels observed before and after addition of 6000 μg DMA

Sample	NDPA, ppm		NDMA, ppm	
	Before	After	Before	After
Blank[a]	ND[b]	ND	ND	0.028
A	0.360	0.359	ND	0.055
B	0.420	0.428	0.021	0.083
C	0.200	0.198	0.019	0.080
D	0.178	0.187	ND	0.082
E	0.435	0.433	0.008	0.070
Spike[c]	1.45	1.44	0.012	0.057
DMA	ND	ND	—	0.080

[a] Blank spiked with NDBA only.
[b] Not detected.
[c] 1.47 ppm NDPA.

Table 6. Nitrosamine levels observed in emulsifiable concentrates spiked with 6000 μg DMA before cleanup

Sample	NDPA, ppm		NDMA, ppm
	No DMA added	DMA added	
Blank	ND[a]	ND	0.015
A+[b]	—	0.344	1.021
A	0.360	0.354	0.058
B	0.420	0.418	0.048
C	0.200	0.191	0.049
D	0.178	0.183	0.046
E	0.435	0.445	0.031
Spike[c]	—	1.43	0.020
DMA	—	ND	0.026

[a] Not detected.
[b] 0.99 μg NDMA added.
[c] 1.47 ppm NDPA.

and concentrating the nitrosamine fraction in Kuderna-Danish apparatus. For comparative purposes, a standard addition experiment was also performed by spiking replicates of each sample with NDPA at 3 levels before analysis. In plotting the amount spiked vs amount found, the x-intercept value corresponds to the original amount in the sample. Using the paired t-test, there is no significant difference observed between any 2 of the methods at the 95% confidence limit.

NDEA and NDBA are added to every sample before column cleanup, so that individual column performances can be assessed. Elution order of the 3 nitrosamines from the silica gel under these conditions is NDBA, NDPA, and NDEA, with some degree of overlap for each. Complete recoveries of NDEA and NDBA give evidence that NDPA is also completely recovered. A low recovery of NDBA suggests that some has been eluted in the previous trifluralin fraction and consequently some NDPA may also have been lost. A low recovery of NDEA indicates that some of the compound is still on the column, and therefore NDPA may also be left behind to some extent. In these 2 situations, the sample should be re-analyzed. In a series of samples treated this way, recovery of NDEA was 98.8 ± 6.62% and NDBA was 102 ± 5.95% ($N = 23$). The recoveries ranged from 89 to 111% and 94 to 118% for NDEA and NDBA, respectively. Using large columns for sample cleanup, recoveries are typically 2–10% less, with corresponding differences in SDs.

To validate that NDPA results are representative of levels present in the formulation and are not artifacts formed during analysis, a series of experiments was performed. The first was simply to determine if trifluralin itself converted to NDPA when injected. The yellow color of a 50 μg/mL trifluralin solution is readily observed, and samples cleaned up by this method are not this intense, although some color is usually apparent. To show that trifluralin does not convert to NDPA in the hot injection port of the gas chromatograph following cleanup, serial dilutions of trifluralin ranging in concentration from 40 to 400 000 ng/mL were injected, and no detectable levels of NDPA were observed (Table 3). Following this, each dilution was spiked to a concentration of 44 ng/mL NDPA and re-injected. NDPA recoveries were 97 ± 4.7% of the spiked amount, indicating that the presence of even large amounts of trifluralin do not interfere with the analysis of NDPA.

The cleanup column, besides providing removal of most of the trifluralin, is used to remove any nitrosating agents and any excess dipropylamine used in the synthesis of the active ingredient from the nitrosamine fraction of the formulation. Five samples were analyzed for NDMA and NDPA, and a solvent containing only NDBA and a solvent spike containing 1.47 μg/mL NDPA were analyzed as well (Table 4). Following this determination, 6000 μg dimethylamine (DMA) was added to each sample extract, and they were re-injected. Conversion of DMA to NDMA at levels higher than those found in the DMA solution alone was not observed, indicating that if any nitrosating agents were present in the formulation, they were not carried over to the cleaned up extract. The extracts containing the added DMA were re-injected the next day, and then 0.1 mL ethanolic solution containing 11.9 μg/mL sodium nitrite was added to each sample. Samples were shaken and allowed to stand 15 min before re-injection. As expected, the concentration of NDMA increased significantly (Table 5). This simply confirmed that the presence of a nitrosating agent and available amine will readily convert to the corresponding nitrosamine. Since there was no increase in NDPA concentration, no dipropylamine was present in the extract following cleanup. Levels of NDMA

Table 5. Nitrosamine levels in extracts containing DMA before and after nitrite addition

Sample	NDPA, ppm		NDMA, ppm	
	Before	After	Before	After
Blank	ND[a]	ND	0.168	0.793
A	0.366	0.357	0.278	0.830
B	0.423	0.428	0.303	0.904
C	0.189	0.194	0.292	0.943
D	0.183	0.182	0.286	1.047
E	0.441	0.442	0.234	0.941
Spike[b]	1.44	1.47	0.190	0.967
DMA	ND	ND	0.260	0.863

[a] Not detected.
[b] 1.47 ppm NDPA.

Table 7. NDPA and NDMA concentrations after addition of nitrite to extracts of DMA-fortified emulsifiable concentrates

Sample	NDPA, ppm		NDMA, ppm	
	Before nitrite addition	After nitrite addition	Before nitrite addition	After nitrite addition
A+[a]	0.364	0.348	1.035	1.011
A	0.374	0.363	0.065	0.067
B	0.428	0.423	0.051	0.055
C	0.198	0.181	0.058	0.064
D	0.186	0.178	0.048	0.049
E	0.460	0.437	0.052	0.075
Spike[b]	1.47	1.43	0.021	0.019
DMA	ND	ND	0.221	0.909

[a] 0.99 μg NDMA added.
[b] 1.47 ppm NDPA.

Table 8. NDPA levels in samples with and without added nitrosamines

Sample	NDPA, ppm		Rec., %
	ISTD[a]	ESTD[b]	
Blank	ND[c]	ND	—
A+[d]	—	1.84	101
A	0.360	0.354	—
B	0.420	0.415	—
C	0.200	0.201	—
D	0.178	0.178	—
E	0.435	0.432	—
Spike[e]	—	1.49	101

[a] Internal standardization.
[b] External standardization.
[c] Not detected.
[d] 1.47 μg/mL NDPA added.
[e] 1.47 μg/mL NDPA.

increased in samples with added DMA when left overnight before the addition of nitrite. Presumably, this was due to nitrogen oxides from the air reacting with the amine, since the increase was also observed in the DMA solution itself.

To determine whether the column cleanup removes DPA, 6000 μg DMA was added to the same series of samples prior to column cleanup. Analysis following cleanup showed that no NDMA was formed (Table 6) and that there was no decrease in NDPA concentration. Nitrite added to these extracts caused no significant increase in NDMA levels (Table 7), indicating that all DMA was removed by the silica gel and implying that any DPA would also have been removed.

To check for the presence of nitrosating agents in the emulsifiable concentrates, sample E was analyzed with and without column cleanup, yielding NDPA levels of 0.435 and 0.431 ppm, respectively. The value of 0.435 ppm without cleanup suggests that the column does not contribute to nitrosamine formation. And since added DMA in the emulsifiable concentrates did not cause formation of NDMA, we conclude that no nitrosating agent was present in the sample before workup.

The final question to be answered is whether the added nitrosamines used for column recoveries and internal standardization are *trans*-nitrosating dipropylamine, that is, whether added nitrosamines convert to amines, and dipropylamine converts to NDPA. To test this, samples were analyzed both with and without added nitrosamines, and the latter case calculated by using external standardization (ESTD). The results in Table 8 show that *trans*-nitrosation is not taking place under these conditions.

Day and co-workers (7) used deactivated alumina in cleanup columns to separate dinitroaniline herbicides from the nitrosamine fraction, in which case the extra time required to ramp and hold the GC oven at 210°C would not be required. In our laboratory, we wanted to standardize as much of this method as possible with the method used for determining NDMA levels in 2,4-D amine formulations (8), and so we continued to use silica gel. Although the GC runs are longer, no extra analyst time is required because an autosampler is used.

The disposable silica gel columns used in this procedure are designed with a small flare at the top, which allows them to be held at the top of the culture tubes for cleanup. When a typical test tube rack with 40 positions is used, 18 samples plus one solvent blank and one standard addition spike of NDPA to a formulation are a comfortable number to work with at one time, so that as elution solvents are added to the last column, the first columns are ready for their next rinse. Fumehood space requirements are minimized, since column stands or water baths are unnecessary. Total solvent volume required per sample has been reduced from 425 mL to 12 mL, including the prewash solvent and the addition of the internal standard solution, yielding savings of over 97%. Major efficiency gains are realized by the analysis of 18 samples in the same amount of time previously required for 6. Cost of silica gel (25 g) using the method by Maybury and Grant (3) was $2.00 per sample, whereas disposable columns cost $1.60 each. Expensive glassware such as the Kuderna-Danish evaporators and 30 × 1.9 cm cleanup columns are no longer required; inexpensive culture tubes (2.5¢ each) are used instead, and are disposed of following use. The only glassware requiring washing is the pipets used for measuring samples and standards. This can also be eliminated by using pipettors with disposable tips, but at the risk of reduced accuracy and precision. For laboratories required to analyze large numbers of samples, the savings from this method can be significant.

The only specialized equipment required by the method is the thermal energy analyzer used for final detection of the nitrosamines. However, cleanup may be sufficient, even with the smaller silica gel columns, to permit detection by a nitrogen-phosphorus detector. Because of the greater fiscal savings and higher recoveries offered by this method, we recommend that it be collaboratively tested.

References

(1) Ross, R. D., Morrison, J., Rounbehler, D. P., Fan, S., & Fine, D. H. (1977) *J. Agric. Food Chem.* **25**, 1416–1418
(2) Magee, P. N., & Barnes, J. M. (1967) *Adv. Cancer Res.* **10**, 163
(3) Maybury, R. B., & Grant, R. G. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 1209–1213
(4) Krull, I. S., Fan, T. Y., & Fine, D. H. (1978) *Anal. Chem.* **50**, 698–701
(5) Fan, T. Y., & Fine, D. H. (1978) *J. Agric. Food Chem.* **26**, 1471–1472
(6) Eisenbrand, G., Archer, M., Brunnemann, K. D., Fine, D. H., Hecht, S. S., Hoffman, D., Krull, J., & Webb, K. S. (1983) *IARC Sci. Publ.* **45**, 25–34
(7) Day, E. W., Jr, West, S. D., Koenig, D. K., & Powers, F. L. (1979) *J. Agric. Food Chem.* **5**, 1081–1085
(8) Hindle, R. W., Armstrong, J. F., & Peake, A. A. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 49–51

MICROBIOLOGICAL METHODS

Determination of Residual Cephalexin in Chick Tissues by *Bacillus stearothermophilus* Paper Disc Assay

JUNYA OKADA, IKUJI HIGUCHI, and SADAO KONDO
Takeda Chemical Industries, Ltd, Research Laboratories, Animal Health Products Division, 17-85, 2-chome, Jusohonmachi, Yodogawa-ku, Osaka 532, Japan
BUN-ICHI SAITO
Japan Food Research Laboratories, 3-1, Toyotsu-cho, Suita, Osaka 564, Japan

A paper disc method is described for determination of residual cephalexin (CEX) in chick tissues. A trichloroacetic acid extract of plasma and tissues is chromatographed on a macroreticular resin (Diaion HP-20) column to remove endogenous antibacterial substances interfering with the assay. The eluate is evaporated to dryness and the residue, dissolved in methanol-water (1 + 2), is subjected to a paper disc assay using *Bacillus stearothermophilus* var. *calidolactis* C953 NIZO as a test organism. The detection limit was 0.0375 ppm in tissue; the average recovery of CEX ranged from 72.4% in skin to 90.4% in plasma. Water containing 200 or 500 mg/L of CEX was given ad libitum to 2-week-old chicks for 10 days; the highest levels of CEX were found in the kidney, and the lowest were found in muscle at 0 h of withdrawal. CEX disappeared from most tissues at 24 h after withdrawal except from skin of chicks given 500 mg/L. However, the drug was not detected in the skin at 48 h after withdrawal.

Cephalexin (CEX) is a typical cephalosporin antibiotic used orally in humans (1). It is possible that CEX may be useful for treating animal diseases because it is effective against pathogens that are resistant to antibiotics such as penicillins, tetracyclines, macrolides, and aminoglycosides. A method to determine low levels of residual CEX in tissues is required if the drug is developed as an antibacterial agent for animal use.

CEX in body fluids can be determined using microbioassay (2), liquid chromatography (3, 4), and fluorometry (5, 6). However, none of these methods possesses the sensitivity required for residue studies. This paper describes an improved assay method for CEX in chick tissues and reports profiles of the antibiotic residue in the chick, as analyzed by the method.

METHOD

Reagents and Apparatus

All ingredients were from Daigo Nutritive Chemicals, Ltd, Osaka, Japan, unless otherwise described.

(a) *Maintenance medium.*—Dissolve 10 g peptone, 5 g beef extract, 2.5 g NaCl, and 15 g agar in water and dilute to 1 L. Adjust so that pH after sterilization is 6.5 ± 0.1.

(b) *Broth medium.*—Dissolve 20 g pancreatic digest of casein (Difco Tryptone), 10 g yeast extract, and 0.5 g glucose in water and dilute to 1 L. Adjust so that pH after sterilization is 8.0 ± 0.1.

(c) *Assay medium.*—Dissolve 6 g peptone, 3 g yeast extract, 1.5 g beef extract, 1 g glucose, and 15 g agar in water and dilute to 1 L. Adjust so that pH after sterilization is 6.0 ± 0.1. (Difco antibiotic medium No. 4 was found satisfactory.)

(d) *Buffer solution.*—Mix 0.2M Na_2HPO_4 and 0.1M citric acid to give pH 4.5.

(e) *Macroreticular resin.*—Diaion HP-20 (Mitsubishi Chemical Industries, Ltd, Tokyo, Japan). Wash with methanol and store in water.

(f) *Standard solutions.*—Prepare stock solution of 500 μg CEX/mL in water. Store at ca 4°C when not in use. Dilute stock solution daily to spiking solution of 5 μg/mL with water. Dilute stock solution daily to working solutions of 0.1, 0.125, 0.1875, 0.25, 0.375, 0.5, 0.75, 1.0, 1.5, and 2.0 μg/mL with methanol–water (1 + 2). Use 0.375 μg/mL working solution as reference standard.

(g) *Homogenizer.*—Ultra-Turrax TP 18/2 (Janke & Kunkel GmbH, Staufen, FRG).

(h) *Glass column.*—25 cm × 1.1 cm id fitted with coarse fritted disc, stopcock, and solvent reservoir.

(i) *Petri dishes.*—90 mm id plastic dish (Terumo Co., Ltd, Tokyo, Japan).

(j) *Paper discs.*—8 mm, thin No. 526 (Toyo Roshi Kaisha, Tokyo, Japan).

Stock Culture of Test Organisms

Maintain test organism (*Bacillus stearothermophilus* var. *calidolactis* C953 NIZO) on maintenance medium, transferring weekly to fresh slant. Inoculate slant of maintenance medium with test organism and incubate overnight at 55 ± 2°C. Inoculate 100 mL Erlenmeyer flasks, each containing 50 mL broth medium, with loopful of test organism from slant culture. Incubate overnight at 55 ± 2°C without agitation.

Preparation of Assay Plates

Cool assay medium to 60°C, add previously prepared broth culture of test organism, and mix well. Standardize level of inoculum at ca 10^7 CFU (colony forming unit)/mL assay medium to ensure proper response of assay plates. (Usually 75–150 mL broth culture/L assay medium gives suitable response.) Pour 8 mL inoculated assay medium into Petri dish and let harden on flat, level surface.

Extraction

(a) *Kidney, muscle, and plasma.*—Place 5.0 g tissue or 5.0 mL plasma in 50 mL centrifuge tube. Add 10 mL 10% trichloroacetic acid (TCA) and homogenize 20–30 s. Centrifuge 5 min at 3000 rpm (1630 × *g*) and transfer supernatant liquid to 50 mL beaker. Repeat this extraction once. Add 25 mL buffer solution to combined TCA extracts and adjust to pH 4.5 with 1N NaOH.

(b) *Fat and skin.*—Place 5.0 g sample in 50 mL centrifuge tube. Homogenize sample with 15 mL 10% TCA 60 s. Add 10 mL *n*-hexane and stir with homogenizer an additional 20 s. Centrifuge mixture 5 min at 3000 rpm and transfer supernatant liquid to separatory funnel through funnel plugged with cotton. Let phases separate. Transfer aqueous layer to 50 mL beaker and discard *n*-hexane layer. Repeat this extraction using 10 mL 10% TCA and 5 mL *n*-hexane. Add

Received March 30, 1987. Accepted June 7, 1987.

25 mL buffer solution to combined TCA extracts and adjust to pH 4.5 with 1N NaOH.

(c) *Liver.*—Prepare extract from 10 g sample in the same manner as in **(a)**. Make volume exactly 50 mL with 10% TCA and subdivide extract into two 25 mL portions in beakers designated (U) and (T). Neutralize content of beaker (T) with 2.5N NaOH and add 3 mL 2.5N NaOH; let stand overnight at room temperature. Add 25 mL buffer solution and adjust to pH 4.5 with 1N HCl. Store beaker (U) overnight in refrigerator, then add 25 mL buffer solution and adjust to pH 4.5 with 1N NaOH.

Cleanup by Column Chromatography

Pack glass column with Diaion HP-20 to bed depth of 4 cm. Top column with cotton and prewash with 100 mL buffer solution. Add tissue extract through filter paper (Toyo filter paper No. 5B) and wash column with 150 mL water. Elute antibiotic into round-bottom flask with 10 mL 20% aqueous methanol and 30 mL 50% aqueous methanol. Remove solvent to dryness by rotary evaporation at 40°C. Redissolve residue in 1 mL methanol–water (1 + 2) for assay.

Assay

(a) *Standard curve.*—Soak 10 paper discs each with 9 working standard solutions by touching each disc to surface of solution (90 discs). Soak an additional 90 discs with reference standard solution. Use 5 plates for each standard (45 plates). Gently place 2 alternate discs of working standard solution and 2 alternate discs of reference standard solution on each agar plate. Invert plates and incubate 16–18 h at 55 ± 2°C. Measure diameters of inhibition zones to nearest 0.1 mm. Correct average diameter of each standard in relation to that of reference standard and calculate best fitted line by least-square linear regression analysis.

(b) *Tissue sample (except liver).*—Soak 10 paper discs with cleaned up sample solution. Place 2 alternate discs soaked with sample and 2 alternate discs of reference standard on each of 5 plates. Correct average value of sample in relation to that of reference standard. Calculate concentration of sample from standard curve and divide by concentration factor (5) to give final concentration. Regard discs showing inhibition zones smaller than 9 mm as negative.

(c) *Liver sample.*—Soak 10 paper discs with cleaned up untreated sample solution (U) and proceed in same manner as in **(b)**. Using an additional 5 plates and 10 discs each soaked in sample solutions (U) and (T), place 2 alternate discs of untreated and 2 alternate discs of treated solutions from same sample on each plate. Calculate concentration of sample in same manner as in **(b)** when average diameter of inhibition zone of untreated sample is larger than that of 0.1875 μg/mL standard. Judge the presence of CEX by any of the following criteria when diameter is smaller than that of 0.1875 μg/mL standard:

(*1*) No significant inhibition zones around disc of untreated sample indicate that no detectable CEX was present (no residue).

(*2*) Significant decrease of diameter of treated vs untreated sample (Student's *t*-test) indicates that CEX was present at a *trace* level.

(*3*) No significant decrease of diameter of treated vs untreated sample indicates presence of inhibitors other than CEX (no cephalexin residue).

Results and Discussion

Both liquid chromatography with fluorescence detection and thin-layer bioautography using *Bacillus subtilis* or *Bacillus stearothermophilus* to determine CEX in chick tissues were attempted. Many endogenous substances interfered in the former, and the latter lacked sensitivity. Since CEX is excreted unchanged (7), antibacterial activity in the tissues may reflect the CEX content. Therefore, a microbioassay, although it is not specific, was selected to determine CEX. The paper disc method was convenient because it is easy to handle and requires only a small quantity of sample solution.

Bacillus stearothermophilus is highly sensitive to β-lactam antibiotics and has been used as a test organism to detect low levels of residual penicillin G, ampicillin, cephapirin, and cloxacillin in milk (8–10) and tissues (11). Of 4 strains tested, C953 NIZO was selected as the test organism because it showed the greatest susceptibility to CEX and gave the clearest inhibition zones. A linear relation between the logarithms of concentration of CEX and the diameters of the inhibition zones was obtained over the range 0.25 to 2.0 μg/mL. However, it was necessary to concentrate the antibiotic to achieve the required sensitivity for assay; in general, 0.05 ppm is necessary. Thus, at least a 5-fold concentration of the antibiotic in the test solution was needed.

Cephalexin can be extracted with methanol from tissues, and the extract can be concentrated. However, an inhibition zone appeared, caused by endogenous antibacterial substances (12), even in the tissues from chicks which did not receive CEX (Table 1). Several attempts were made to eliminate the substances and to concentrate CEX. Purification of the TCA extract of the tissues through a macroreticular polystyrene adsorbent column proved to be adequate. Recovery of CEX from Diaion HP-20 was higher than that from Amberlite XAD-2. CEX is quantitatively adsorbed on HP-20 resin over the pH range 2 to 6 and is most stable at pH 4.5, its isoelectric point (13); therefore, the extract was applied to the resin at pH 4.5. To minimize the generation of air bubbles and to elute CEX quantitatively, 20% aqueous methanol followed by 50% aqueous methanol was used for the elution.

Table 1. Comparison of removal of endogenous antibacterial substances from tissues by HP-20 treatment and methanol extraction

Tissue	Inhibition zone,[a] mm	
	MeOH extraction[b]	HP-20 treatment
Liver	10.0, 10.2, 14.0	8.0, 8.4, 10.1
Kidney	11.8	8.0
Muscle	12.5	8.0
Fat	10.2	8.0
Plasma	—[c]	8.0
Skin	—	8.0

[a] Paper disc 8 mm in diameter was used; inhibition zone of 8.0 mm means no inhibition.
[b] MeOH extract of 5 g control tissue was evaporated to dryness and reconstituted with 2.5 mL MeOH–water (1 + 2).
[c] Not tested.

Table 2. Effect of tissue extract treated with HP-20 on diameter of CEX inhibition zone (mm)[a]

CEX, μg/mL	Tissue				
	None	Liver	Kidney	Muscle	Plasma
0	8.0	10.1	8.0	8.0	8.0
0.25	13.7	14.6	14.0	13.8	13.2
0.5	19.9	20.6	19.1	19.0	20.5
1.0	24.3	25.0	24.0	23.9	24.7
2.0	30.3	29.7	29.3	29.7	30.1

[a] All figures are expressed as the mean of 10 discs in a single determination.

Table 3. Comparison of inhibition zones of alkali-treated and untreated liver extracts

CEX added, μg/g	Repetition	Inhibition zone,[a] mm Untreated	Treated
0	1	9.8	10.1
	2	10.1	10.0
	3	8.0	8.0
	4	8.0	8.3
0.05	1	14.3	8.0
0.1	1	20.6	9.5

[a] Average diameters of 10 discs in a determination are shown.

Table 4. Recovery of CEX from spiked chick tissues[a]

Detn	Recovery,[b] % Liver	Kidney	Muscle	Fat	Skin	Plasma
1	80	76	82	77	71	90
2	78	74	87	77	71	94
3	74	73	74	82	75	91
4	77	75	80	76	70	83
5	78	78	88	84	75	94
Mean, %	77.4	75.2	82.2	79.2	72.4	90.4
SD	2.2	2.0	5.7	3.6	2.4	4.5

[a] Quintuplicate determinations were conducted on different days.
[b] Cephalexin was spiked to control tissues at 1 ppm.

Except for liver, the HP-20 treatment removed the endogenous antibacterial substances to such a degree that they did not show inhibition zones in controls (Table 1) and hardly affected the size of CEX zones, even at 0.25 μg/mL (Table 2). Inhibition zones due to endogenous substances were sometimes observed for liver extracts in spite of the HP-20 cleanup. Therefore, additional treatment was required to confirm low levels of CEX present. Ouderkirk (9) and Messer et al. (14) adopted penicillinase inactivation to confirm penicillins in milk. In the present study, alkali treatment at room temperature was utilized. CEX in the liver extract was completely decomposed to inactive components with 1N NaOH overnight at room temperature, and the interfering substances remained intact (Table 3). Traces of CEX in the liver could be assayed by statistically comparing inhibition zones of an alkali-treated solution and those of an untreated one. Three criteria shown in the *Method* section cover the possible results.

Recovery studies were performed in quintuplicate from each of the 6 tissues fortified with CEX at 1 ppm. Average recoveries ranged from 72.4% in skin to 90.4% in plasma; this is acceptable for residue analysis (Table 4).

Of 290 discs of each standard solution used for the tissue residue assay, all discs of 0.25 μg/mL and higher were positive; 287 (99%) and 100 (34%) discs were positive for 0.1875 and 0.125 μg/mL, respectively. A solution of 0.1 μg/mL gave

Table 5. Concentrations of CEX in tissues of chicks given CEX orally for 10 successive days

CEX given, mg/L	Withdrawal, h	Chick No.	CEX concentration,[a] ppm Liver	Kidney	Muscle	Fat	Skin	Plasma
200	0	1	tr[b]	1.15	ND[c]	ND	0.055	0.058
		2	0.039	0.814	ND	tr	tr	0.129
		3	0.053	1.17	ND	tr	0.054	0.041
		4	0.043	0.438	ND	ND	tr	0.045
		5	tr	0.908	ND	tr	tr	0.045
	24	6	ND	ND	ND	ND	ND	ND
		7	ND	ND	ND	ND	ND	ND
		8	ND	ND	ND	ND	ND	ND
		9	ND	ND	ND	ND	ND	ND
		10	ND	ND	ND	ND	ND	ND
	48	11	ND	ND	—[d]	—	ND	—
		12	ND	ND	—	—	ND	—
		13	ND	ND	—	—	ND	—
		14	ND	ND	—	—	ND	—
		15	ND	ND	—	—	ND	—
500	0	21	0.073	1.98	ND	ND	tr	0.103
		22	0.184	5.19	tr	tr	0.183	0.278
		23	0.107	4.58	ND	tr	0.174	0.212
		24	0.122	3.24	ND	tr	0.087	0.296
		25	0.145	6.22	tr	tr	0.104	0.137
	24	26	ND	ND	ND	ND	0.118	tr
		27	ND	ND	tr	ND	0.081	ND
		28	ND	ND	tr	ND	0.082	ND
		29	ND	ND	ND	ND	0.067	ND
		30	ND	ND	ND	ND	0.051	ND
	48	31	ND	ND	ND	ND	ND	ND
		32	ND	ND	ND	ND	ND	ND
		33	ND	ND	ND	ND	ND	ND
		34	ND	ND	ND	ND	ND	ND
		35	ND	ND	ND	ND	ND	ND
	72	36	—	ND	ND	—	ND	ND
		37	—	ND	ND	—	ND	ND
		38	—	ND	ND	—	ND	ND
		39	—	ND	ND	—	ND	ND
		40	—	ND	ND	—	ND	ND

[a] Figures shown are the means of duplicate assays.
[b] Trace <0.0375 ppm.
[c] Not detected.
[d] Not assayed.

only 1 positive disc. It would be reasonable to define the minimum detectable level as that which can be detected with almost certainty. This level was approximately 0.1875 μg/mL in test solution, corresponding to 0.0375 ppm in tissues.

Application of the Method to Residue Study

The study of CEX residues in chicks was conducted by giving ad libitum water containing 200 or 500 mg CEX/L to 2-week-old Hubbard broiler chicks for 10 days, with a diet containing no antibacterial agents. At 0, 24, 48, and 72 h after withdrawal of the drug from the water, plasma and tissues were collected from 5 chicks of each group. The CEX contents in each tissue are shown in Table 5. Some samples, clearly presumed to be CEX-free on the basis of the results from the previous samples, were not assayed.

The alkali treatment was applied only to liver samples. The finding that untreated samples showed inhibition zones, whereas the treated ones showed no zones, confirmed that CEX was present at a trace level in 2 of the 0-h samples (criterion 2). On the other hand, the finding that even untreated samples showed no zones confirmed that CEX was not present in the 24-h and 48-h liver samples (criterion 1).

At 0 h of withdrawal, considerable levels of CEX were found in the liver, kidney, skin, and plasma. The highest concentration of CEX was found in the kidney; the next highest concentration was in plasma; the concentrations in liver and skin were a little lower. Traces were detected in muscle and fat. The notably high concentration in kidney may indicate that in chicks, as in humans (3) and other mammals (7), the antibiotic is excreted intact mainly through the kidney.

CEX was eliminated from kidney, liver, and fat as rapidly as from plasma, and no drug residue was observed in these tissues at 24 h after withdrawal. Although CEX concentrations in skin at 0 h were a little lower than those in plasma, the disappearance rate of the drug from skin was relatively slow and, at 24 h after withdrawal, CEX still remained in the skin of chicks given 500 mg/L. The reason for the persistence of CEX in skin may be that CEX in blood is rapidly excreted from the kidney, but equilibration of CEX in skin and blood is very slow. A similar explanation was given by Waddell (15) to account for the distribution of urea to the central nervous system, eyes, and fetus of rats.

References

(1) Wick, W. E. (1967) *Appl. Microbiol.* **15,** 765–769
(2) O'Callaghan, C. H., Tootill, J. P. R., & Robinson, W. D. (1971) *J. Pharm. Pharmacol.* **23,** 50–57
(3) Nakagawa, T., Haginaka, K., Yamaoka, K., & Uno, T. (1978) *J. Antibiot.* **31,** 769–775
(4) Nahata, M. C. (1980) *J. Chromatogr.* **225,** 532–538
(5) Barbhaiya, R. H., & Turner, P. (1976) *J. Pharm. Pharmacol.* **28,** 791–792
(6) Miyazaki, K., Ogino, O., & Arita, T. (1979) *Chem. Pharm. Bull.* **27,** 2273–2280
(7) Sullivan, H. R., Billings, R. E., & McMahon, R. E. (1969) *J. Antibiot.* **22,** 195–200
(8) Ouderkirk, L. A. (1977) *J. Assoc. Off. Anal. Chem.* **60,** 1116–1118
(9) Ouderkirk, L. A. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 985–988
(10) Vilim, A. B., Moore, S., & Larocque, L. (1979) *J. Assoc. Off. Anal. Chem.* **62,** 1247–1250
(11) Vilim, A. B., & Larocque, L. (1983) *J. Assoc. Off. Anal. Chem.* **66,** 176–179
(12) Van Schothorst, M., & Pealen-Knol, G. (1970) *Neth. J. Vet. Sci.* **3,** 85–93
(13) Nightingale, C. H., Greene, D. S., & Quintiliani, R. (1975) *J. Pharm. Sci.* **64,** 1899–1927
(14) Messer, J. W., Leslie, J. E., Houghtby, G. A., Peeler, J. T., & Barnett, J. E. (1982) *J. Assoc. Off. Anal. Chem.* **65,** 1208–1214
(15) Waddell, W. J. (1968) *J. Appl. Physiol.* **24,** 828–831

Evaluation of Abbreviated Enzyme Immunoassay Method for Detection of *Salmonella* in Low-Moisture Foods

RUSSELL S. FLOWERS and MARY JOAN KLATT
Silliker Laboratories, 1304 Halsted St, Chicago Heights, IL 60411
BARBARA J. ROBISON
Organon Teknika Corp., 800 Capitola Dr, Durham, NC 27713
JEROME A. MATTINGLY
Bionetics Research, Inc., 1330 A. Piccard Dr, Rockville, MD 20850

A modified enzyme immunoassay method (EIA) utilizing an 18 h pre-enrichment, a 6-8 h selective enrichment, and a 14 h M-broth post-enrichment is compared to the standard culture method (AOAC/BAM) on selected low-moisture foods. Tested samples included 238 inoculated, 30 naturally contaminated, and 30 uninoculated foods. By EIA, 235 samples were positive (optical densities greater than 0.2 at 405 nm), 233 of which were confirmed culturally. By the culture methods, 221 samples were positive. The EIA method was more productive in detecting salmonellae in inoculated samples of dry cheese powder, chocolate, and nonfat dry milk, whereas the culture method gave better recovery from naturally contaminated meat and bone meal. The modified EIA could be completed in 40 h and required no centrifugation.

The official AOAC culture method for detection of *Salmonella* in food products requires at least 4 days to obtain negative results (1). Consequently, more rapid procedures have long been sought by the food industry (2–14). Recently, an enzyme immunoassay (EIA) method utilizing the *Salmonella* Bio-EnzaBead Screen Kit (Organon Teknika Corp., Durham, NC) was adopted official first action by AOAC for detection of *Salmonella* in foods (15, 16). This method incorporates a 24 ± 2 h pre-enrichment, an 18–24 h selective enrichment, a 6 h post-enrichment, and a centrifugation step prior to EIA. Although this method can be completed in 48 h, its practicality would be greatly improved by elimination of the centrifigation step and alteration of the incubation steps to allow the assay to be performed in the morning of a normal workday.

Experiments examining growth of *Salmonella* and competitive bacteria in selective enrichments have suggested that for low-moisture foods, the selective conditions in tetrathionate broth (TT) and selenite cystine broth (SC) are most effective in the first 6–8 h of incubation (unpublished data). During this period, little or no growth of salmonellae occurred in the selective broth, but the relative numbers of competitive bacteria were reduced. Once a more favorable ratio of salmonellae to competitive bacteria was achieved, then transfer to M-broth and overnight incubation resulted in rapid outgrowth of the salmonellae and eliminated the need for centrifugation.

Similar studies performed on high-moisture, highly contaminated foods have indicated that 6–8 h in selective enrichment is not sufficient to provide a favorable ratio of salmonellae to competitors (unpublished data). Subsequent transfer to M-broth resulted in overgrowth of the salmonellae by the competing organisms. Because the abbreviated selective enrichment was not suitable, the 48 h procedure described above must be used for these food types.

The objective of this study was to compare the abbreviated EIA method without centrifugation to standard cultural methods for detection of salmonellae in low-moisture foods. The modified EIA method incorporated an 18 h pre-enrichment, a 6–8 h selective enrichment, and a 14–18 h M-broth post-enrichment. This protocol allowed samples to be initiated in the late afternoon and to be assayed in the morning 2 days later.

Received February 2, 1987. Accepted August 3, 1987.

Experimental

Test Organisms

Six *Salmonella* species previously isolated from foods were used for inoculation (Table 1). Each culture was freeze-dried as described previously (16).

Preparation of Inoculated Foods

Dry whole egg, cheese powder, soy isolate, chocolate, nonfat dry milk, and pepper were used in the study. One serotype of *Salmonella* was used to inoculate each food type at both a high (0.4–2 cells/g) and a low (0.04–0.2 cells/g) level. The freeze-dried cell pellets were ground by mortar and pestle into a fine powder and were inoculated into the foods as described in an earlier study (16).

Analysis of Inoculated Products

Except for chocolate, 20 replicate samples (25 g) of each test product inoculated at each level (low and high) were analyzed by both the AOAC/BAM culture procedure and by the modified Bio-EnzaBead immunoassay method as described below. Only 19 replicates of each inoculation level were tested for chocolate. In addition, 5 uninoculated control samples of each product were analyzed to determine the rate of false positive reactions. A *Salmonella* most probable number (MPN) determination was performed on each of the inoculated test products on the same day that the comparative analyses were initiated (17). Sample sizes used for MPN determinations were 3 × 100 g, 3 × 10 g, and 3 × 1 g for the samples inoculated with a low level of *Salmonella* and 3 × 10 g, 3 × 1 g, and 3 × 0.1 g for samples inoculated at the higher level. MPN determinations were not performed on control samples.

Table 1. Test products, test organisms, and inoculation levels

Product	*Salmonella* organism	Dose	Most probable number/g[a]
Dry whole egg	*S. infantis*	low	0.043
		high	0.15
Cheese powder	*S. lindenberg*	low	0.07
		high	0.093
Soy isolate	*S. typhimurium*	low	>1.1
		high	>11.0
Chocolate	*S. senftenberg*	low	>1.1
		high	>11.0
Nonfat dry milk	*S. cubana*	low	0.046
		high	0.46
Pepper	*S. pretoria*	low	0.46
		high	2.4

[a] Determined on the day comparative analyses were initiated.

Table 2. Comparison of culture method and EIA method for detection of *Salmonella* in inoculated and uninoculated controls

Product	Dose	No. of samples tested	Culture	Positive samples (χ^2)[a] EIA assay[b]	EIA/confirmed[c]
Dry whole egg	Control	5	0	0 (***)[d]	0 (***)
	Low	20	8	8 (***)	8 (***)
	High	20	20	20 (***)	20 (***)
Dry cheese powder	Control	5	0	0 (***)	0 (***)
	Low	20	15	16 (0.00)	16 (0.00)
	High	20	18	20 (0.50)	20 (0.50)
Soy isolate	Control	5	0	0 (***)	0 (***)
	Low	20	20	20 (***)	20 (***)
	High	20	20	20 (***)	20 (***)
Chocolate	Control	5	0	0 (***)	0 (***)
	Low	19	18	19 (0.00)	19 (0.00)
	High	19	19	19 (***)	19 (***)
Nonfat dry milk	Control	5	0	0 (***)	0 (***)
	Low	20	15	20 (3.20)	20 (3.20)
	High	20	15	20 (3.20)	20 (3.20)
Pepper	Control	5	0	0 (***)	0 (***)
	Low	20	20	20 (***)	20 (***)
	High	20	20	20 (***)	20 (***)
Total			208	222 (7.11)	222 (7.11)

[a] χ^2 = (|Samples positive by EIA and negative by culture − samples positive by culture and negative by EIA| − 1)^2 ÷ (samples positive by EIA and negative by culture + samples positive by culture and negative by EIA).
[b] Samples reading ≥0.200 at 405 nm.
[c] Samples reading ≥0.200 at 405 nm and confirmed culturally as *Salmonella*.
[d] Statistical analysis not applicable because EIA and culture results agreed for all samples analyzed.

Analysis of Naturally Contaminated Products

Ten replicates of each product were analyzed by both test methods. Sample sizes were 375 g for pasta, 25 g for chocolate, and 25 g for meat and bone meal. Regardless of sample size, pre-enrichment in the appropriate medium represented a 1/10 dilution of product into medium. Because of the lack of availability of naturally contaminated samples, only 3 product types were tested.

AOAC/BAM Culture Method

Dry whole egg, milk chocolate, and nonfat dry milk were pre-enriched by AOAC method **46.117**. All other test products were pre-enriched according to BAM methods. Selective enrichment, isolation, preliminary screening of typical colonies, and serological and biochemical confirmations were performed as described previously (16).

Modified Bio-EnzaBead Immunoassay Method

The same sample pre-enrichments described above for the AOAC/BAM method were used for the EIA method, except that 1 mL aliquots were removed for inoculation into the selective enrichments after only 18 h inoculation. The pre-enrichment solution was re-incubated for use in the AOAC/BAM method. The selective enrichment broths for the EIA method were incubated in a 35°C water bath for 6–8 h. Portions of 1.0 mL were removed from each selective broth and added to separate 10 mL portions of sterile M-broth. The inoculated M-broths were incubated at 35°C for 14–18 h along with the associated selective enrichments. After incubation, 0.5 mL portions from each M-broth were combined in a clean screw-cap tube and heated in boiling water or flowing steam for 20 min, then cooled to 25–37°C. The EIA assay was performed according to the manufacturer's instructions. Positive EIA results required cultural confirmation. This was accomplished by streaking the corresponding M-broths and selective enrichment cultures to xylose lysine desoxycholate agar (XLD), Hektoen enteric agar (HE), and bismuth sulfite agar (BS). All subsequent steps in identification of suspicious colonies were performed as described for the cultural procedure (1).

Table 3. Contamination levels of naturally contaminated test products

Product	Organism[a]	Most probable number/g
Pasta	C_2:L; G:Z_{29}	< 0.03
Chocolate	E_4:G complex	< 0.03
Meat and bone meal	E_4:G complex; E_2:Y; C_1:Y	0.23

[a] Serotype of *Salmonella* isolated.

Results and Discussion

Artificially Inoculated and Uninoculated Control Products

Expected levels of inoculation and actual *Salmonella* MPN/g for each test product are presented in Table 1. Except for the inoculated chocolate and soy isolate, MPN values determined on the day comparative analyses were initiated were within expected ranges. The values for soy and chocolate were higher than expected.

The comparative data associated with each inoculated test product and the uninoculated controls are presented in Table 2. Samples reported as positive by EIA had optical densities of ≥0.200 at 405 nm. Samples reported as EIA/confirmed were positive by the assay and were confirmed by isolation of *Salmonella* from the M-broth or selective enrichments. Statistical analysis was performed using McNemar's test as described previously (16). A χ^2 value of >3.84 indicates a significant difference at the 5% level.

A total of 238 inoculated samples were analyzed, of which 208 were found positive for *Salmonella* by the culture method. A total of 222 samples were positive by EIA and all were culturally confirmed for the presence of *Salmonella*. Statistical analysis gave a χ^2 value of 7.11, indicating that EIA was significantly better than culture for all samples tested.

A total of 30 uninoculated control samples were analyzed. Results of these tests were negative by both methods; i.e., there were no false-positive EIA readings.

Of the inoculated samples, there was perfect agreement between the EIA method and the culture method for dry whole egg, soy isolate, and pepper. The EIA method detected an additional positive sample in the cheese powder and in the chocolate (low inoculation levels) and 10 additional positives in the nonfat dry milk (5 at the low level and 5 at the high level). There was no statistically significant difference between EIA and the culture method for any foods.

Naturally Contaminated Products

The contamination levels of the naturally contaminated foods are given in Table 3. As expected, the levels were quite low.

There was perfect agreement between the EIA method and the culture method on all the naturally contaminated samples tested (Table 4). However, 2 samples of meat and bone meal giving positive EIA results could not be confirmed culturally. However, there was no significant difference at the 5% level between the 2 methods.

Previous studies (14, 18) have shown that a short selective enrichment step is effective in recovering *Salmonella* from animal feeds and other selected foods. The present study also supports the use of a short selective enrichment to detect

Table 4. Comparison of culture method and EIA method for detection of *Salmonella* in naturally contaminated foods

Product	No. of samples tested	Culture	Positive $(\chi^2)^a$	
			EIA assay[b]	EIA confirmed[c]
Pasta	10	2	2 (***)	2 (***)
Chocolate	10	2	2 (***)	2 (***)
Meat and bone meal	10	9	9 (***)	7 (0.50)
Total		13	13 (***)	11 (0.50)

[a] χ^2 = (|Samples positive by EIA and negative by culture − samples positive by culture and negative by EIA| − 1)^2 ÷ (samples positive by EIA and negative by culture + samples positive by culture and negative by EIA).
[b] Samples reading ≥0.200 at 405 nm.
[c] Samples reading ≥0.200 at 405 nm and confirmed culturally as *Salmonella*.

Salmonella in low-moisture foods with an enzyme immunoassay. Negative samples can be screened in 40 h with this method.

A total of 298 samples were analyzed in this study, consisting of 6 inoculated foods and uninoculated controls and 3 naturally contaminated foods. By the modified EIA method, 233 samples were positive and confirmed, compared to 221 positive samples determined by the culture method. The results indicate that the modified EIA method is as productive as the culture method for detection of *Salmonella* in low-moisture foods. The timing of the culture steps used in the modified EIA method and elimination of the centrifugation step greatly improved the practicality of the procedure.

References

(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **46.117-46.127**
(2) Aleixo, J. A. G., Swaminathan, B., & Minnich, S. A. (1984) *J. Microbiol. Meth.* **2**, 135–145
(3) Anderson, J. M., & Hartman, P. A. (1985) *Appl. Environ. Microbiol.* **49**, 1124–1127
(4) Ibrahim, G. F., Lyons, M. J., Walker, R. A., & Fleet, G. H. (1985) *Appl. Environ. Microbiol.* **50**, 670–675
(5) Krysinski, E. P., & Heimsch, R. C. (1977) *Appl. Environ. Microbiol.* **33**, 947–954
(6) Fitts, R. (1985) *Food Technol.* **39**, 95–102
(7) Minnich, S. A., Hartman, P. A., & Heimsch, R. C. (1982) *Appl. Environ. Microbiol.* **43**, 877–883
(8) Rigby, C. E. (1985) *Appl. Environ. Microbiol.* **47**, 1327–1330
(9) Robison, B. J., Pretzman, C. I., & Mattingly, J. A. (1983) *Appl. Environ. Microbiol.* **45**, 1816–1821
(10) Smith, A. M., & Jones, C. (1983) *Appl. Environ. Microbiol.* **46**, 826–831
(11) Sperber, W. H., & Deibel, R. H. (1969) *Appl. Microbiol.* **17**, 533–539
(12) Stewart, B. J., Eyles, M. J., & Murrell, W. G. (1980) *Appl. Environ. Microbiol.* **40**, 223–230
(13) Thomason, B. M. (1971) *Appl. Microbiol.* **22**, 1064–1069
(14) Entis, P., Brodsky, M. H., Sharpe, A. N., & Jarvis, G. A. (1982) *Appl. Environ. Microbiol.* **43**, 261–268
(15) Andrews, W. H. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 274–277
(16) Flowers, R. S., Eckner, K., Gabis, D. A., Robison, B. J., Mattingly, J. A., & Silliker, J. H. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 786–798
(17) Andrews, W. H., Wilson, C. R., & Poelma, P. L. (1983) *J. Food Sci.* **48**, 1162–1165
(18) D'Aoust, J.-Y., Sewell, A., & Boville, A. (1983) *J. Food Prot.* **46**, 851–855

Temperature-Independent Pectin Gel Method for Aerobic Plate Count in Dairy and Nondairy Food Products: Collaborative Study

JONATHAN N. ROTH
RCR Scientific, Inc., 206 W Lincoln Ave, Goshen, IN 46526

Collaborators: J. Bonnes; M. H. Brodsky; B. S. Cieben; J. Heisick; V. Jamison; E. Johnson; J. M. Lanier; F. Montaldi; N. Moyer; E. Nath; E. Neidert; C. Okolo; G. Staat; S. Toms; F. D. McClure (*Statistical Consultant*)

Ten laboratories participated in a collaborative study to compare the pectin-based plate count (PC) Redigel method with the aerobic plate count and standard plate count agar-based standard methods for the estimation of total bacterial counts in 9 different nondairy food and dairy food products. The foods were cream, homogenized milk, raw milk, cheese, raw chicken, raw oysters, frozen broccoli, flour, and spices. Each laboratory analyzed 6 samples (3 sample pairs) of each food group. Counts obtained by the pectin-based plate count and agar-based plate count methods differed significantly ($P < 0.05$) only for homogenized milk, where the pectin gel method resulted in higher counts. The actual counts were higher in the pectin gel method in 8 of the 9 food groups. The log means for pectin gel and agar-based media, respectively, for the 9 food groups were: cream 8.106 and 7.844; homogenized milk 8.642 and 8.231; raw milk 8.711 and 8.423; chicken 7.654 and 7.645; oysters 7.201 and 7.180; broccoli 7.102 and 6.798; cheese 8.045 and 8.055; flour 4.112 and 3.988; spice 5.379 and 5.314. The repeatability standard deviations favored the pectin gel method in 6 of the 9 foods tested. The reproducibility standard deviations favored the pectin gel method in 7 of the 9 foods tested. These results strongly support the suitability of the pectin gel method as an alternative to agar-based plate count and other methods for total bacterial counts in nondairy and dairy food products. The pectin gel method has been adopted official first action.

Submitted for publication August 27, 1987.
This report of the Associate Referee was presented at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA.
The recommendation of the Associate Referee was approved interim official first action by the General Referee, the Committee on Microbiology, and the Chairman of the Official Methods Board. The method was adopted official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) 71, January/February issue.

The aerobic plate count and standard plate count methods (1, 2) are currently recognized as the official standard methods for determining total bacterial count in nondairy food and dairy food products, respectively, against which alternative methods are compared. The terms "aerobic plate count" and "standard plate count" can generally be considered synonymous, which is the reason that the title of this study simply uses the term "aerobic plate count." In this study, the appropriate protocol for each test material (either nondairy or dairy) was followed according to AOAC or APHA (1, 2) guidelines. The general term "reference plate count" will be used throughout the remainder of this paper in conjunction with aerobic plate count for nondairy foods and standard plate count for dairy food products.

The pectin gel plate count (Redigel) was developed as an alternative method and has undergone within-laboratory comparative investigations (3; and D. Fung & V. Chain,

Table 1. Replicate sample pairs tested[a]

Food	Sample 1	2	3	4	5	6
Cream	C	A	C	A	B	B
Hom. milk	B	C	C	A	A	B
Raw milk	A	C	B	A	C	B
Chicken	a	b	c	c	b	a
Oysters	a	b	c	a	c	b
Broccoli	a	a	b	c	b	c
Cheese	a	a	b	b	c	c
Flours	a	a	b	b	c	c
Spices	a	a	b	b	c	c

[a] Cream, homogenized milk, and raw milk were inoculated at 3 concentrations: A = high count; B = middle count; C = low count. For chicken and oysters, 3 different lots (a, b, c) of each were obtained. Three different brands (a, b, c) of frozen broccoli were used. Cheese samples were a, swiss; b, cheddar; c, colby. Flours were a, white; b, rye; c, wheat. Spices were a, thyme; b, onion; c, pepper.

Graduate Dept of Food Sciences, Kansas State Univeristy, Manhattan, KS, 1986, unpublished work). The pectin gel method has numerous advantages over the agar-based plate count methods and other accepted methods. Pectin gel is available as Redigel in self-contained units consisting of one tube of liquid medium and one pretreated petri plate. The liquid medium is poured into the petri plate and the sample (full strength or diluted) is added directly to give an instant pour plate. No sterilization step or temperature control is necessary. The convenience of pouring fresh plates at any time without tedious preparation is obvious. Also, the lack of temperature shock with pectin gel (compared with agar-based pour plates) results in a more accurate evaluation when psychrotrophs are present in samples (D. Fung & V. Chain, Graduate Dept of Food Sciences, Kansas State University, Manhattan, KS, 1986, unpublished work).

Collaborative Study

Ten laboratories served as collaborators in this study. Each received a full set of detailed instructions, data sheets, test materials, and samples.

The cream and milk samples were prepared as follows: Stock supplies were obtained, and samples were removed for the isolation of pure cultures of various naturally occurring bacteria. The remainder of the stock was heated in a steam chamber to 90–95°C. The samples were then cooled and refrigerated at 4°C until inoculation. Prior to distribution, the stock cream, raw milk, and homogenized milk were inoculated with mixed cultures of bacteria that had been isolated from the original samples. Three concentrations of inoculum were used for each of the stock products, so that the result was 6 inoculated samples of each product in the form of 3 sample pairs (Table 1, sample pairs A, B, C). The samples were dispensed into sterile polyethylene bottles for shipment to collaborators. Analyses were performed according to prescribed techniques in chapter 6 of *Standard Methods for the Examination of Dairy Products* (2).

To increase the probability of wider variation in counts among the sample pairs of the remaining foods, the following procedures were used: For the raw oysters, 3 different lots were obtained, and one sample pair was prepared from each lot. For the raw chicken, 3 different lots were obtained, and one sample pair was prepared from each lot. Three different brands of frozen broccoli were used, with one sample pair prepared from each brand. Single lots of 3 different cheeses (cheddar, colby, and swiss) were used to prepare the 3 sample pairs of cheese. Three different spices (thyme, onion powder, and ground black pepper) were used for the 3 sample pairs. Three different flours (white, whole wheat, and rye) were used for the 3 sample pairs. In all of these products, the naturally occurring bacterial populations were counted in the collaborative study.

The study was done over a 3 week period, with broccoli, oysters, and chicken analyzed the first week; cheese, flour, and spices analyzed the second week; and raw milk, homogenized milk, and cream analyzed the third week. The broccoli, oysters, chicken, cheese, flour, and spices were shipped by next-day air services on a Tuesday to 3 Canadian collaborators and on the next day to 7 U.S. collaborators. The broccoli, oysters, and chicken were plated on Tuesday of the following week, and the results were counted on Thursday. The cheese, flour, and spices were plated on Tuesday a week later, and the results were counted 2 days later. The raw milk, homogenized milk, and cream were sent by next-day air to the 3 Canadian collaborators on a Monday, and to the 7 U.S. collaborators the next day. They were plated by all collaborators on the following Monday, and the results were counted 2 days later. The products were shipped in cardboard-box-enclosed 1.5 in. thick foam containers with frozen "blue ice" refrigerant to ensure adequate refrigeration. The collaborators reported that the samples arrived in good condition. The shipping schedule ensured delivery that allowed time for preliminary tests by collaborators so that more accurate dilution levels could be determined for the collaborative study. On the designated setup date for each product, each laboratory performed plate counts in duplicate for each sample

Table 2 Day-of-Test control counts on representative sample series (Log base 10)

Sample	Plate Count/g or mL--reference method 1	2	3	4	5	6	Plate Count/g or mL--Redigel method 1	2	3	4	5	6
cream	5.176a	7.971	3.740a	7.903	6.618	6.695	5.978a	7.959	5.845a	7.961	6.763	6.908
raw milk	8.410	6.648	7.602	8.352	6.301a	7.663	8.470	6.785	7.643	8.447	6.591	7.854
hom. milk	7.455	7.806	7.667	7.908	7.736	7.836	8.427	8.550	8.398	8.728	8.747	8.628
cheese	8.966	8.927	6.720	6.820	7.875	7.806	8.929	8.952	7.643	7.686	7.405	7.230
chicken	6.053	5.866	6.373	6.248	6.387	6.400	6.064	6.013	6.526b	6.509b	6.712	6.538
oysters	6.681	7.225	6.574	6.423a	6.919	7.371	c	7.267	6.903	6.813a	7.146a	7.423a
spices	7.033	6.949	5.230a	5.505	4.049	3.775	7.356	7.290	5.201	5.167	4.086	4.079
flours	3.447	3.462	3.643d	3.505d	3.778d	3.686d	3.618	3.538	3.686	3.881e	3.799	3.004
broccoli	6.449	4.911	3.728	6.934b	4.886b	c	6.447	5.158	3.778a	7.146b	4.204a	c

a--estimated count--colonies/plate below reference recommendation
b--estimated count--colonies/plate above reference recommendation
c--no value due to incorrecct sample dilution
d--count of one plate due to spreaders
e--adjusted due to partial medium hydrolysis--one plate OK, one half of the other plate counted X 2

Table 3 Collaborative results for plate count in cream by reference and Redigel methods (Log base 10)

	Reference Count/mL						Redigel Count/mL					
Coll	1	2	3	4	5	6	1	2	3	4	5	6
1	6.744	8.033	5.819	7.751b	6.390	6.707	7.276	8.262	7.033	8.286	7.338	7.422b
2	7.212	8.076	7.149	8.322	7.380a	7.292	8.288	8.281	7.903	8.464b	7.916	7.872
3	c	8.477b	c	8.380	c	c	c	8.591b	c	8.462b	c	c
4	7.301a	8.258	7.602	8.362	7.389	7.498	7.491	8.346	7.892	8.534b	8.362	8.041
6	8.565b	8.919b	8.320	8.699b	8.596b	8.789b	8.748b	8.691b	8.378	8.778b	8.500b	8.576b
7	8.435b	8.778b	8.444b	8.792b	8.572b	8.626b	8.599b	8.796b	8.609b	8.732b	8.727b	8.620b
8	6.706b	c	c	c	c	c	c	c	c	c	c	c
9	8.124	8.326	8.228	8.676	8.267	8.230	8.124	8.396	8.292	8.591	8.365	8.288
10	6.524b	7.491b	6.613	7.785	7.079	7.286	6.655b	7.696b	6.924	7.820	7.179	7.358

a--estimated count--colonies/plate below 25
b--estimated count--colonies/plate above 250
c--no value provided by collaborator
d--no data from collaborator 5

Table 4 Collaborative results for plate count in raw milk by reference and Redigel methods (Log base 10)

	Reference Count/mL						Redigel Count/mL					
Coll	1	2	3	4	5	6	1	2	3	4	5	6
1	8.568	5.881	6.643	8.568	6.566b	7.338	8.944	7.458b	8.265	8.881	7.812b	7.868b
2	8.806	7.860	8.222	8.842	7.653	8.279	8.886	8.117	8.453b	8.914	8.124	8.444b
3	8.814b	c	c	8.956b	c	c	8.924b	c	c	9.013b	c	c
4	8.710b	7.597	8.097	8.797b	7.455	8.146	8.869b	8.104	8.301	8.734b	7.663	8.428b
6	9.097	8.720	9.049a	9.176a	8.607	8.978a	9.114	8.602	9.009a	9.267a	8.628	9.021a
7	8.847b	8.672b	8.767b	8.782b	8.571b	8.789b	8.840b	8.799b	8.772b	8.777b	8.660b	8.723b
8	10.083	9.398	9.949	c	9.568	8.412b	10.405b	10.322	10.413b	10.330	10.199	8.516b
9	9.117	9.029	9.104	9.146	8.944	9.134	9.158	9.033	9.140	9.212	8.944	9.179
10	8.037	7.220	7.431	8.362	6.892	7.978	8.097	7.250	7.813	8.344	7.076	8.210

a--estimated count--colonies/plate below 25
b--estimated count--colonies/plate above 250
c--no value provided by collaborator
d--no data from collaborator 5

Table 5 Collaborative results for plate count in homogenized milk by reference and Redigel methods (Log base 10)

	Reference Count/mL						Redigel Count/mL					
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
1	5.544a	6.978a	6.161a	5.978a	7.544a	6.602a	8.868b	8.627b	8.544b	8.652b	8.868b	8.473b
2	8.562	7.823	8.013	8.505	8.667	8.442b	8.653	8.338	8.387	8.748	8.760	8.618
3	c	c	c	8.944b	8.687b	c	c	c	c	8.961b	8.980b	c
4	8.534b	8.228	8.328	8.686b	8.710b	8.534b	8.630b	8.413b	8.447b	8.835b	8.950b	8.534b
6	9.037	8.792	9.217a	9.076	8.708	8.991a	9.137	8.658	9.097a	9.090	8.863	8.878a
7	8.611b	8.681b	8.619b	8.623b	8.744b	8.785b	8.709b	8.779b	8.672b	8.650b	8.788b	8.787b
8	8.477a	7.146a	6.778a	9.704b	9.556	7.255a	10.386	8.737b	c	7.176a	9.173	8.279
9	9.086	9.152	9.137	9.097	9.093	9.143	9.223	9.270	9.241	9.134	9.190	9.316
10	7.386	6.813	7.336	8.255	6.672	8.117	7.517b	7.152	7.072	8.387	6.740	8.079

a--estimated count--colonies/plate below 25
b--estimated count--colonies/plate above 250
c--no value provided by collaborator
d--no data from collaborator 5

Table 6 Collaborative results for plate count in cheese by reference and Redigel methods (Log base 10)

	Reference Count/g						Redigel Count/g					
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
1	8.740	8.643	9.111	8.531	9.155	9.176	8.505	8.613	9.725b	9.856b	7.954	8.188
2	8.752	8.597	7.432a	7.447	7.672	8.021	8.204	7.964	8.326	8.282	6.505	6.322
3	8.041	8.450b	7.638	8.041	7.314	7.279	8.274	8.447b	8.215	7.833	7.061a	6.778a
4	8.866	8.602	8.161a	8.255a	7.740a	7.602a	8.243a	8.290a	8.695	8.716	7.740a	7.020a
5	8.699	9.004	10.593b	9.470	7.748b	7.490b	8.317a	8.279a	10.916b	9.648	7.368	7.373
7	8.744b	8.663b	7.466b	6.881	8.111	8.093	8.362	8.208	7.782	7.220	7.440b	7.562b
8	9.057	8.599b	6.653b	4.643	3.114	c	9.114	9.207	7.401b	c	7.114a	c
9	8.061	7.987	7.230a	7.477	7.561b	7.185	8.061	8.000	8.290	8.274	5.301a	5.000a
10	9.320	8.497b	8.000	8.093	8.851	8.367	9.274	8.449b	7.845	7.978	8.531	8.253

a--estimated count--colonies/plate below 25
b--estimated count--colonies/plate above 250
c--no value provided by collaborator
d--data from collaborator 6 excluded due to uncertainty in differentiating among sample pairs

Table 7 Collaborative results for plate count in raw chicken by reference and Redigel methods (Log base 10)

	Reference Count/g						Redigel Count/g					
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
1	7.850b	7.797b	7.930b	7.754b	8.057b	c	8.053b	7.930b	7.936b	7.796b	8.152b	c
2	8.851	8.442	8.238	8.111	8.207	8.752	8.942	8.320	8.498	8.292	8.049	8.613
3	7.904b	7.849b	7.744b	7.756b	7.818b	7.823	8.246	7.591	8.270	8.236	8.086	8.093
4	7.918b	8.358b	7.686b	7.986b	8.484b	7.960b	7.946b	7.853b	7.777b	7.659b	7.398	7.853b
6	8.146	8.057	7.806	7.580	7.799	7.544	8.100	8.182	7.833	7.792	7.633	7.663
8	8.584b	8.556b	8.391	8.090	7.380a	8.531b	8.477	8.462	8.538b	7.987	8.236	8.508b
9	8.371	8.301	8.004	8.083	8.173	8.223	8.512b	8.111	8.004	8.009	8.083	8.250
10	4.279a	5.699	4.643	4.398a	4.342a	5.045	4.204a	5.681	4.591	4.146a	4.519	4.785

a--estimated count--colonies/plate below 30
b--estimated count--colonies/plate above 300
c--no value provided by collaborator
d--no data from collaborators 5 and 7

Table 8 Collaborative results for plate count in raw oysters by reference and Redigel methods (Log base 10)

	Reference Count/g						Redigel Count/g					
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
1	7.083	7.435	6.924	6.863	7.100	7.444	7.330	7.314	7.130	7.342	7.182	7.276
2	7.258	7.505	6.908	7.268	7.045	7.491	7.628	7.362	7.061	7.703	7.161	7.439a
3	7.497b	7.121	6.806	7.537b	6.763	7.033	7.699	7.225	6.699	7.982	6.643	7.170
4	6.695	7.534b	7.076	6.724	7.188	7.601b	7.149	7.659b	7.033	6.756	7.233	7.525b
6	6.688	7.152	6.813	6.398a	6.898	7.360	7.155	7.188	6.959	7.179	6.996	7.164
7	7.223b	7.292b	6.831b	7.220b	6.734b	7.241b	c	7.031b	6.500b	c	6.410	7.105b
8	6.820	7.679b	7.898	7.362a	7.544	8.629b	7.322	7.228	7.643	7.079a	7.301a	8.117
9	7.748	7.391	6.591	7.505	6.898	7.348	7.949	7.025	7.380a	7.580	6.398a	6.996
10	7.501b	7.526b	6.439	7.230	6.452	7.405	7.562b	7.367	6.258	7.417	6.367	7.097

a--estimated count--colonies/plate below 30
b--estimated count--colonies/plate above 300
c--no value provided by collaborator
d--no data from collaborator 5

by both the reference plate method (1, 2) and the pectin gel method. Day-of-test control counts are shown in Table 2. Collaborators calculated and reported all data in counts/g or mL, and also recorded and reported all raw data.

Duplicate negative controls were run with each 500 mL batch of plate count agar and each 50 tube batch of plate count Redigel. Additional checks on Redigel were done by visually scanning each tube for abnormal cloudiness or flocculant which might indicate contamination, and by visual checks on plates for the presence of any colonies.

Two negative environmental control plates of each medium (plate count agar and plate count pectin gel) were exposed for 15 min each morning and afternoon on days that samples were run, to verify that results were not affected by environmental contamination.

The plate count pectin gel utilized the same ingredients as the plate count agar, except that the gelling agent was pectin instead of agar. (The nutrients for both media were: 5.0 g/L of pancreatic digest of casein, 2.5 g/L of yeast extract, 1.0 g/L of glucose in accordance with **46.005(g)** (1). The plate count agar used in this study, **46.005(g)** (2), was made from the same sources (supplier, batch, lots) of ingredients as the plate count Redigel that was used in this study, which was why a premixed, commercial plate count agar was not used. This eliminated the possibility of variation due to differences in the basic ingredients.

Aliquots from the identical (same) samples were used for both the pectin gel (Redigel) and the agar-based media, and the plates of both were inoculated in the same 15 min period for each sample type.

Aerobic Plate Count
Pectin Gel Method
First Action

46.D01 ***Principle***

Method uses pretreated petri plates contg thin "hardener" layer, and liq. medium contg nutrients with pectin as only gelling agent. Liq. medium, 12–15 mL, is poured into pretreated petri plate and undild or dild sample is added. Plate is rotated and rocked to mix sample and medium. Plates are then allowed to stand on level surface 30–40 min until medium solidifies. Total process is done at ambient temp. Plates are then incubated and counted.

46.D02 ***Materials***

Note: Before pectin base medium formulated from individual ingredients is used, comparability to commercially available medium must be demonstrated.

Pectin gel tubes and plates—Pectin gel is available as sterile liq. in individual tubes contg sufficient gel to pour 1 plate. Use tubes of Redigel and pretreated petri plates (RCR Scientific, Inc., 206 W Lincoln Ave, Goshen, IN 46526), or equiv. that meet specifications.

To prep. plate count pectin gel from individual ingredients, suspend 5.0 g pancreatic digest of casein, 2.5 g yeast ext. and 1.0 g glucose, in 500 mL H_2O. Suspend 15 g low methoxyl pectin in 500 mL H_2O. Heat individual mixts until all ingredients are dissolved. Autoclave solns 15 min at 121°. Combine nutrient and pectin solns and adjust pH to 7.0 ± 0.1. To prep. pretreated petri plates, prep. hardener layer mixt. of 1% agar with 0.02 $CaCl_2$ concn. Sterilize mixt. by autoclaving 15 min at 121°. Aseptically dispense 5 mL portions of mixt. into sterile petri plates.

Table 9 Collaborative results for plate count in spices by reference and Redigel methods (Log base 10)

	Reference Count/g						Redigel Count/g					
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
1	7.322a	7.362a	5.954	5.124	3.477a	3.903a	7.663	7.680	6.519	5.188	3.301a	3.694a
2	7.215	6.911	4.618	4.607	3.703	3.744	7.481b	7.110	4.618	4.550	3.732	3.836
3	7.057	7.121	5.279a	5.415a	3,352a	3.792	7.305	7.380	5.041a	5.398a	3.477a	3.301a
4	6.924	6.993	5.342a	5.190a	3.892	3.966	7.167	7.167	5.371a	5.204a	4.041	4.097
5	6.760	6.771	5.452	4.942	3.398a	c	6.922	6.964	5.489b	4.964	3.954a	c
6	7.076	6.954	5.408	5.340	3.919	3.875	7.220	7.158	5.580	5.220	4.146	3.978
7	7.152	7.196	6.300	5.092	4.051	3.736	7.411	7.363	5.580	5.122	3.998	3.628
8	5.978	5.447a	5.090	5.100	4.415a	3.477a	6.439	5.929	6.097	5.037	3.301a	3.000a
9	6.602	6.820	4.973	5.079	3.477a	3.301a	6.681	6.851	5.179	4.944	3.602a	3.301a
10	6.412	5.587b	5.170	5.307	5.130	4.477	6.182	5.511b	5.193	5.303	4.690	5.083

a--estimated count--colonies/plate below 30
b--estimated count--colonies/plate above 300
c--no value provided by collaborator

Table 10 Collaborative results for plate count in flours by reference and Redigel methods (Log base 10)

	Reference Count/g						Redigel Count/g					
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
1	3.903a	3.301a	4.146a	4.301a	3.477a	3.778a	3.954a	3.653a	4.362a	4.279a	3.963a	4.040a
2	3.607	3.525	3.996	4.029	3.886	3.964	3.860	4.000	3.987	4.064	3.973	4.200
3	4.250	4.025	4.100	4.279	3.663	3.914	4.190a	4.114a	4.643	4.699	4.021a	4.040a
5	3.243a	3.431a	3.663	3.658	3.477	3.538	3.423a	3.703	3.778	3.848	3.648	3.803
6	3.919	4.053	4.064	4.140	3.959	3.491	4.182	>4.477b	4.236	4.083	3.996	>4.477b
7	3.618	3.643	4.182	4.061	3.959	3.942	3.712	3.771	3.854	3.898	4.000	3.929
8	3.778a	3.778a	3.380a	4.146a	4.415a	3.255a	3.778a	3.477a	3.556	4.079a	4.322a	3.389a
9	3.982	3.924	3.919	3.900	6.740	6.763	4.004	3.940	3.991	3.964	6.806	6.869
10	4.477	4.643	3.663	4.279a	4.301a	3.813	4.491	4.462a	3.833	4.431a	4.663	3.845

a--estimated count--colonies/plate below 30
b--data excluded from statistical analysis
c--no data from collaborator 4

Table 11 Collaborative results for plate count in frozen broccoli by reference and Redigel methods (Log base 10)

	Reference Count/g						Redigel Count/g					
Coll.	1	2	3	4	5	6	1	2	3	4	5	6
1	6.543b	6.569b	5.212	7.223	5.377	7.255	6.792b	6.904b	6.086	7.533	6.152	8.230b
2	6.895	7.000	7.053	7.193	6.489b	6.179	7.473	7.533b	7.373	7.279	7.130	6.398
3	6.823b	6.890b	5.121	6.914b	5.681	6.688b	7.100	7.320	6.100	7.663b	6.292	7.633b
4	6.322	6.631b	5.462	7.179b	5.336	7.450b	6.600b	6.534b	5.423a	7.045b	5.659b	7.127b
5	6.406	6.528b	4.748	6.924b	5.633	6.749b	6.627b	6.736b	6.367	7.176b	6.274	7.079b
6	6.668b	6.665b	5.672	5.987	6.193	7.062b	6.601b	d	d	6.121	6.332	d
7	c	c	7.301b	c	7.301b	c	c	c	c	c	c	c
8	6.721b	5.797b	6.498b	c	c	c	6.810b	5.320	6.508b	c	c	c
9	7.179	7.064	7.270	7.255	7.196	7.188	7.276	7.100	7.279	7.253	7.217	7.290
10	8.818b	9.619b	7.057	9.778b	8.854b	9.695b	8.789b	9.688b	7.449	9.902b	8.589b	9.740b

a--estimated count--colonies/plate below 30
b--estimated count—colonies/plate above 300
c--no value provided by collaborator
d--gross contamination, no value provided

Table 12. Analysis of variance of pectin gel and SPC agar methods

Food	Sum of sqs.	DF	Mean sq.	F	Signif. of F
Cream	1.748	1	1.748	3.739	0.0564
Raw milk	1.489	1	1.489	2.495	0.118
Homogenized milk	4.021	1	4.021	6.010	0.016
Cheese	0.202	1	0.202	0.175	0.677
Chicken	0.035	1	0.035	0.025	0.875
Oysters	0.012	1	0.012	0.073	0.788
Spice	0.120	1	0.120	0.066	0.797
Flour	0.399	1	0.399	1.011	0.317
Broccoli	2.045	1	2.045	1.927	0.168

46.D03 ***Preparation of Samples***

Prep. all decimal dilns with 90 mL sterile diluent (Butterfield's phosphate buffer) plus 10 mL of previous diln unless otherwise specified. Shake all dilns 25 times in 30 cm arc. Pipets must accurately deliver required vol. Do not use to deliver <10% of their total vol. For example, to deliver 1 mL, do not use pipet >10 mL; to deliver 0.1 mL, do not use pipet >1 mL.

(a) *Dairy products.*—Measure (or weigh) 11 mL (or g) sample and dil. in 99 mL Butterfield's diluent. For solid samples, blend 2 min at 10 000 to 12 000 rpm. Prep. addnl dilns so that total colonies/plate is in 25–250 range. Incubate plates 48 ± 3 h at 32 ± 1°.

(b) *Nondairy products.*—Weigh 50 g sample into 450 mL Butterfield's diluent and blend 2 min at 10 000 to 12 000 rpm. Prep. further dilns by dispensing 10 mL sample into 90 mL diluent so that total colonies/plate is in 30–300 range. Incubate plates 48 ± 2 h at 35 ± 1°.

Table 13. Means and precision estimates for collaborative data

Sample pair	Method	Mean log	S_r	S_R	RSD_r	RSD_R
		Cream				
1	Redigel	8.405	0.098	0.344	1.166	4.093
	Reference	8.305	0.171	0.454	2.059	5.467
2	Redigel	8.040	0.109	0.555	1.356	6.903
	Reference	7.721	0.121	0.809	1.567	10.478
3	Redigel	7.872	0.208	0.710	2.642	9.019
	Reference	7.506	0.271	0.904	3.610	12.044
Overall	Redigel	8.106	0.147	0.557	1.813	6.871
	Reference	7.844	0.198	0.748	2.524	9.536
		Raw Milk				
1	Redigel	9.048	0.087	0.615	0.961	6.797
	Reference	8.862	0.095	0.473	1.072	5.337
2	Redigel	8.660	0.495	0.632	5.716	7.298
	Reference	8.395	0.444	0.837	5.289	9.970
3	Redigel	8.424	0.157	0.964	1.864	11.443
	Reference	8.040	0.209	1.100	2.599	13.682
Overall	Redigel	8.711	0.304	0.754	3.490	8.656
	Reference	8.423	0.294	0.835	3.490	9.913
		Homogenized Milk				
1	Redigel	8.664	0.616	0.660	7.110	7.618
	Reference	8.514	0.539	0.950	6.331	11.158
2	Redigel	8.755	0.559	0.624	6.385	7.127
	Reference	8.194	0.447	1.034	5.455	12.619
3	Redigel	8.496	0.126	0.654	1.483	7.698
	Reference	7.950	0.286	1.005	3.597	12.641
Overall	Redigel	8.642	0.501	0.693	5.797	8.019
	Reference	8.231	0.441	0.999	5.358	12.137
		Cheese				
1	Redigel	8.434	0.213	0.398	2.525	4.719
	Reference	8.629	0.265	0.351	3.071	4.068
2	Redigel	8.529	0.363	1.005	4.256	11.783
	Reference	7.840	0.588	1.273	7.500	16.237
3	Redigel	7.148	0.233	0.992	3.260	13.878
	Reference	7.675	0.191	1.367	2.489	17.811
Overall	Redigel	8.045	0.276	0.840	3.431	10.441
	Reference	8.055	0.394	1.078	4.892	13.379
		Chicken				
1	Redigel	7.738	0.265	1.446	3.425	18.687
	Reference	7.719	0.266	1.347	3.446	17.450
2	Redigel	7.643	0.377	1.084	4.933	14.183
	Reference	7.707	0.464	1.154	6.020	14.973
3	Redigel	7.586	0.190	1.328	2.505	17.506
	Reference	7.512	0.147	1.229	1.957	16.360
Overall	Redigel	7.654	0.289	1.290	3.776	16.854
	Reference	7.645	0.322	1.248	4.212	16.324
		Oysters				
1	Redigel	7.427	0.169	0.338	2.275	4.551
	Reference	7.146	0.177	0.379	2.477	5.304
2	Redigel	7.294	0.223	0.274	3.057	3.756
	Reference	7.455	0.233	0.344	3.125	4.614
3	Redigel	6.909	0.254	0.411	3.676	5.949
	Reference	6.939	0.129	0.364	1.859	5.246
Overall	Redigel	7.201	0.220	0.354	3.055	4.916
	Reference	7.180	0.185	0.362	2.577	5.042
		Spice				
1	Redigel	6.979	0.211	0.589	3.023	8.440
	Reference	6.783	0.238	0.552	3.509	8.138
2	Redigel	5.280	0.432	0.449	8.182	8.504
	Reference	5.239	0.352	0.387	6.719	7.387
3	Redigel	3.798	0.197	0.519	5.187	13.665
	Reference	3.847	0.318	0.456	8.266	11.853
Overall	Redigel	5.379	0.303	0.560	5.633	10.411
	Reference	5.314	0.312	0.489	5.871	9.202

Table 13. Continued

Sample pair	Method	Mean log	S_r	S_R	RSD_r	RSD_R
		Flour				
1	Redigel	3.924	0.135	0.310	3.440	7.900
	Reference	3.389	0.167	0.385	4.350	10.029
2	Redigel	4.088	0.194	0.308	4.746	7.534
	Reference	3.995	0.241	0.256	6.032	6.408
3	Redigel	4.324	0.319	1.011	7.377	23.381
	Reference	4.130	0.330	1.024	7.990	24.794
Overall	Redigel	4.112	0.228	0.630	5.545	15.321
	Reference	3.988	0.255	0.649	6.394	16.274
		Broccoli				
1	Redigel	7.130	0.443	0.975	6.213	13.675
	Reference	6.952	0.301	0.916	4.330	13.176
2	Redigel	6.639	0.324	0.824	4.880	12.411
	Reference	6.168	0.557	1.082	9.030	17.542
3	Redigel	7.565	0.305	1.075	4.032	14.210
	Reference	7.295	0.384	1.063	5.264	14.572
Overall	Redigel	7.102	0.367	0.967	5.168	13.616
	Reference	6.798	0.423	1.019	6.222	14.990

46.D04 ***Determination***

(*1*) Lift lid of pretreated petri plate and pour liq. pectin gel from 1 tube (12–15 mL) into plate. Replace lid and swirl plate to cover bottom with pectin gel. Prep. number of plates needed for samples being run (duplicate plates for each diln). Plates *must* be used within 5 min after liq. pectin gel is poured.

(*2*) Add 1 mL inoculum (sample) to liq. pectin gel in petri plate. Touch pipet tip once to dry spot on inside wall of plate (above level of liq. pectin gel) after dispensing sample to rest point in pipet tip. *Immediately* rotate and rock plate to mix sample thoroly with pectin gel. Do not spill pectin gel over sides of plate. (*Note:* This step is primary difference in procedure between pectin gel and agar-based media. *Do not* add inoculum (sample) to pretreated petri plate and pour pectin gel over it. This would lock sample in one small area of plate without sepn of individual colonies.)

(*3*) Let inoculated plates stand on level surface until pectin gel is solid (ca 30–40 min), and then incubate 48 ± 2 h at 35 ± 1° for nondairy products and 48 ± 3 h at 32 ± 1° for dairy products.

(*4*) Count duplicate plates in suitable range (30–300 colonies for nondairy products, 25–250 colonies for dairy products). If plates do not contain proper range of colonies, record diln counted and note number of colonies found. Average counts obtained and report as aerobic plate count/g or mL.

Results and Discussion

All reported data (Tables 3–11) were converted to log (base 10) for statistical analyses. Each of the 10 collaborators ran each of the 9 food groups. Where data from a collaborator are omitted from the table in a particular food group, improper procedure dictated exclusion.

The data from each food group were subjected to analysis of variance (ANOVA), and repeatability and reproducibility precision estimates were produced for the sample pairs and the overall methods (4). The data used were determined with the approval of the AOAC Methods Committee statistician. The results of the statistical analysis (ANOVA) comparison of the 2 methods are reported in Table 12; precision estimates are recorded in Table 13.

Counts between the pectin gel and reference methods differed significantly ($P < 0.05$) only for homogenized milk, where the pectin gel method resulted in higher counts. The actual counts were higher in pectin gel than in the reference method in 8 of the 9 food groups. The log means for pectin gel- and agar-based media, respectively, for the 9 food groups

were: cream 8.106 and 7.844; homogenized milk 8.642 and 8.231; raw milk 8.711 and 8.423; chicken 7.654 and 7.645; oysters 7.201 and 7.180; broccoli 7.102 and 6.798; cheese 8.045 and 8.055; flour 4.112 and 3.988; spice 5.379 and 5.314.

The repeatability coefficient of variation for the pectin gel method was smaller than for the corresponding reference method in 6 of the 9 food groups; for 3 groups, it was similar (Table 13). The repeatability standard deviations for pectin gel- and agar-based media, respectively, for the 9 food groups were: cream 0.147 and 0.198; homogenized milk 0.501 and 0.441; raw milk 0.304 and 0.294; chicken 0.289 and 0.322; oysters 0.220 and 0.185; broccoli 0.367 and 0.423; cheese 0.276 and 0.394; flour 0.228 and 0.255; spice 0.303 and 0.312. The reproducibility coefficient of variation for the pectin gel method was smaller than the corresponding reference method in 7 of the 9 food groups; for 2 groups it was similar (Table 13). The reproducibility standard deviations for pectin gel and agar-based media, respectively, for the 9 food groups were: cream 0.557 and 0.748; homogenized milk 0.693 and 0.999; raw milk 0.754 and 0.835; chicken 1.290 and 1.248; oysters 0.354 and 0.362; broccoli 0.967 and 1.019; cheese 0.840 and 1.078; flour 0.630 and 0.649; spice 0.560 and 0.489. These results strongly support the suitability of the pectin gel method as an alternative to agar-based plate count and other methods for total bacterial counts in both dairy and nondairy food products.

It was generally noted by collaborators that colonies on pectin gel were somewhat more compact and discrete than in the reference agar-based medium, which made counting easier, with fewer problems from spreaders.

Recommendation

It is recommended that the proposed pectin gel method for aerobic plate count in nondairy food products and standard plate count in dairy food products be adopted official first action.

Acknowledgments

The author thanks the following microbiologists who took part in this study:

J. Bonnes, Ameritech Laboratories, Bayside, NY

M. H. Brodsky and B. S. Ciebin, Ontario Ministry of Health, Weston, Ontario, Canada

V. Jamison, Vermont Dept of Agriculture, Dairy Laboratory, Montpelier, VT

E. Johnson and F. Montaldi, QAL Inc., and Appalachian State University, Boone, NC

J. M. Lanier and J. Heisick, Food and Drug Administration, Minneapolis Center for Microbiological Investigation, Minneapolis, MN

N. Moyer, University Hygienic Laboratory, University of Iowa, Iowa City, IA

E. Neidert and E. Nath, Agriculture Canada, Ottawa, Ontario, Canada

C. Okolo, ConAgra Frozen Foods, Batesville, AR

G. Staat, Chem-Staat Labs, Neosho, MO

S. Toms, Agriculture Canada, Calgary, Alberta, Canada

The author thanks M. A. Roth for assistance in tabulating data and proofing, and D. R. Kauffmann, Goshen College, for preliminary statistical analyses. Also, thanks are due to F. D. McClure of the Food and Drug Administration Division of Mathematics, Washington, DC, for performing the statistical analyses and to General Referee W. H. Andrews, FDA, Division of Microbiology, Washington, DC, for his able direction throughout the study.

References

(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, Chap. 46, pp. 940–942

(2) Richardson, G. H. (Ed.) (1985) *Standard Methods for the Examination of Dairy Products,* 15th Ed., American Public Health Association, Washington, DC

(3) Slabyj, B. M., & Bolduc, G. R. (1987) in *Seafood Quality Determination,* D. E. Kramer & J. Liston (Eds), Proceedings of an International Symposium coordinated by University of Alaska Sea Grant College Program, Anchorage, AK, Nov. 10–14, 1986, Elsevier Science Publishers B.V., Amsterdam, The Netherlands, pp. 255–267

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

VITAMINS AND OTHER NUTRIENTS

Liquid Chromatographic Determination of Available Lysine in Soybean and Fish Meal

JOAN RABASSEDA, GEMMA RAURET,[1] and M. TERESA GALCERAN
University of Barcelona, Analytical Chemistry Department, Barcelona, Spain

A method for the determination of available lysine on the basis of the reactivity of the ε-amino group with fluorodinitrobenzene (FDNB) has been optimized. Hydrolysis is performed in closed vessels in an autoclave. Conditions for different meals were established by using a modified sequential simplex method. Hydrolysis for 4 h was sufficient for the meals studied—soybean and fish. The use of liquid chromatography to determine available lysine was studied, and optimum conditions were established for separation and quantitation of ε-DNB-lysine. The proposed method is faster, more accurate, and more precise than commonly used methods.

It is well known that the nutritional availability of lysine in foods depends on the amount of lysine possessing free ε-amino groups. This lysine is usually regarded as available lysine, and a variety of different methods (1) have been developed to determine it. Some methods are chemical and generally include protein derivatization, acid hydrolysis, and separation and quantitation of the lysine derivative.

The most widely used method was established by Carpenter (2) and uses 1-fluoro-2,4-dinitrobenzene (FDNB) for derivatization; the *N*-ε-dinitrophenyl-lysine (ε-DNP-lysine) is measured spectrophotometrically after acid hydrolysis and batch extraction. Problems with this method arise, first, because the ε-DNP-lysine is not completely stable during acidic hydrolysis and so recoveries are low; second, because the derivatization of the ε-amino groups of lysine is not specific; and, third, because the carbohydrate contained in the food can lead to the formation of interfering compounds that cause an overestimation of lysine content. For these reasons, Booth (3) proposed modifications to this method, and several authors (4–7) have studied how to overcome carbohydrate interferences.

The use of chromatographic techniques to separate ε-DNP-lysine from interfering amino acids and other compounds formed during the hydrolysis of carbohydrate-containing foods has been described, including ion-exchange chromatography (8, 9), adsorption on alumina (10), or liquid-liquid partitioning (17). Liquid chromatography (LC) has been proposed by several authors using dansyl or other derivatives (11–13). For available lysine, Peterson and Warthesen (12) used reverse phase LC with acetonitrile–acetate buffer.

Two interesting reviews about available lysine determination have been published (14, 15). The main limitation common to all methods described is the lack of reproducibility. The coefficient of variation (CV) of the results obtained is about 4% for the Carpenter method as modified by Booth and can be reduced only to 1.5% for cotton meal for the Rao et al. method (8).

To overcome some of these problems and to improve precision, we optimized the conditions for available lysine analysis in different high protein by-product feed ingredients. The use of closed vessels and an autoclave for acidic hydrolysis of the protein has been introduced; a modified simplex method (16), shown to have multiple applications in analytical problems (17–19), has been used to establish the best hydrolysis conditions. Reverse-phase liquid chromatography is used for separation and quantitation.

The stability of the standard was also studied, and the last peak in the chromatogram was identified as a derivatization reaction product not previously described in the literature.

Received March 14, 1986. Accepted March 23, 1987.
[1] Address correspondence to this author.

METHOD

Apparatus

(**a**) *Spectrophotometer.*—Pye Unicam SP6-500 UV.

(**b**) *Autoclave.*—Microclave S-477 Selecta.

(**c**) *LC equipment.*—Waters Associates, Inc., equipped with M-45 solvent delivery system, z-module radial compressor, Model 440 detector, and 5 μm Nova-Pak C_{18} column. Operating conditions: flow rate, 3 mL/min; detection at 254 nm.

(**d**) *Automated amino acid analyzer.*—Biotronik LC60001.

Reagents and Solutions

(**a**) *Stock standard solution.*—1 mg/mL. *N*-ε-2,4-DNP-L-lysine HCl (Sigma Chemical Co.) in methanol–water (1 + 4).

(**b**) *Internal standard solution.*—100 ng/mL. 2,6-Dimethylphenol (Merck), 97% purity in methanol.

(**c**) *Standard solutions.*—Use high purity acids and salts, double deionized water, and solvents from Merck. Add volume of stock standard solution to 1 mL ethanol and 0.5 mL internal standard solution. Dilute mixture to 50 mL with 0.01M sodium acetate pH 5 buffer.

(**d**) *Derivative reagent.*—1-Fluoro-2,4-dinitrobenzene (FDNB) solution (Sharlau-Ferosa) in 3% ethanol.

(**e**) *Mobile phase.*—Methanol–0.01M sodium acetate pH 5 buffer (1 + 1).

Derivative Formation

To an amount of sample containing 15–20 mg available lysine (ca 400 mg fish meal or 520 mg soybean meal) in 100 mL Pyrex vessel, add 8 mL of 8% $NaHCO_3$, 10 mL FDNB solution, and 2 glass beads. Close vessel and mechanically shake 2 h at room temperature (ca 20°C). Evaporate ethanol by immersing vessel in 95°C water bath.

Hydrolysis

(**a**) *Fish meal.*—To FDNB derivative solution, add 45 mL 6.9N HCl. Stir solution until CO_2 has been eliminated. Close vessel and autoclave at 133°C for 4 h.

(**b**) *Soybean meal.*—To FDNB derivative solution, add 70 mL 7.85N HCl. Proceed as for fish meal, but autoclave at 124°C, then cool solutions, filter, and adjust volume to 200 mL.

LC Determination

To 15 mL hydrolyzed solution (**a**) or (**b**) in 100 mL beaker, add 8.5 mL of 2N NaOH (for fish meal) or 17.0 mL of 2N NaOH (for soybean meal). Adjust to pH 5 with 1M $NaHCO_3$. Transfer resulting solution to 50 mL volumetric flask, rinse beaker with 10 mL methanol, transfer it to volumetric flask, add 0.5 mL internal standard solution, and dilute with water

Table 1. Coefficients of variation[a] for peak areas of various solutions of ε-DNP-lysine and different mobile phases

Solution	Mobile phase: Methanol–water (1 + 1)	Mobile phase: Methanol–buffer (1 + 1)
A. Standard solution in water	4.11	—
B. Standard solution in 0.01M buffer, pH 5	4.16	—
C. Standard solution in methanol buffer, pH 5	1.59	0.92
D. Hydrolyzed sample, pH 7	3.52	—
E. Hydrolyzed sample, pH 5	1.81	1.06

[a] Calculated on the basis of 10 replicate injections.

to 50 mL. Filter solution through 45 μm filter. Inject 20 μL sample and standard solutions into the chromatograph.

Samples

Soybean meal was typical commercial defatted meal from the United States. Fish meal was also commercial quality from the Canary Islands. For both samples, water content, crude protein, and lipid content were determined according to AOAC methods (20). For soybean meal, water content was 12.3%, crude protein 48.9%, and lipids 0.0%. For fish meal, water content was 8.0%, crude protein 67.0%, and lipids 7.7%. Total lysine content was determined using an automated amino acid analyzer: 2.95% for soybean meal and 5.23% for fish meal.

Results and Discussion

Chromatographic Conditions

Several mobile phases were tested on the Nova-Pak C_{18} stationary phase to determine optimum separation of hydrolyzed samples. Several mixtures of acetonitrile–water–acetic acid, methanol–water, and methanol–0.01 M acetic acid–sodium acetate pH 5 buffer solution were tested. Better results were obtained with the latter one when the methanol–water ratio was 1:1. At a flow rate of 3 mL/min, the analysis time is 8 min, and the retention time for ε-DNP-lysine is 2 min. ε-DNP-lysine in water (solution A), in 0.01M acetic acid–sodium acetate pH 5 buffer (solution B), and in mixtures of this buffer and methanol (1 + 1) (solution C) have been used as standards. The addition of methanol increases solubility of the lysine derivative and improves precision (Table 1). Several tests have also been performed with hydrolyzed samples, showing that the best reproducibility is obtained when the solution is adjusted to pH 5 (solution E) rather than when it is adjusted to pH 7 (solution D).

The use of an internal standard was also tested to increase precision and accuracy. The compounds tested as standards were 3,5-dinitrobenzoic acid, *o*-toluidine, 2,6-dimethylphenol, ethyl benzoate, 2,4,5-trichlorophenol, and pentachlorophenol. The best results were obtained with 2,6-dimethylphenol. Figure 1 shows a chromatogram of the hydrolyzed sample with the internal standard 2,6-dimethylphenol.

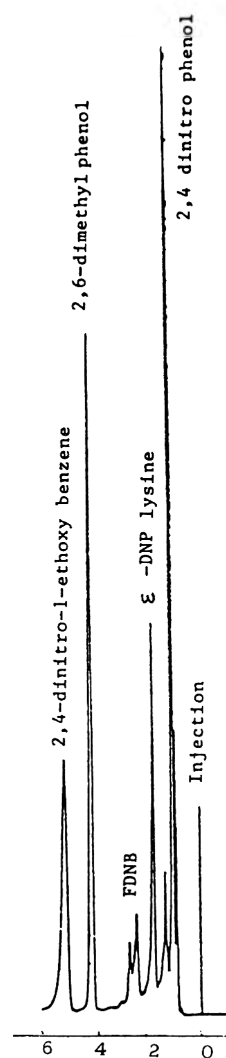


Figure 1. Chromatogram of hydrolyzed sample with the internal standard used.

In Table 1, CVs of the peak area of ε-DNP-lysine are given for 2 mobile phases and various standard and sample solutions. From the results, it can be concluded that solution C for standards and solution E for samples with an internal standard gave the best results.

Table 2. Stability of standard solutions containing 4.16 μg ε-DNP-lysine/mL over time

Time after preparation	Absorbance, nm: 268	Absorbance, nm: 274
At preparation	0.839	0.969
4 Days	0.836	0.970
1 Week	0.847	0.981
2 Weeks	0.825	0.951
1 Month	0.831	0.958
2 Months	0.830	0.962
3 Months	0.832	0.963
4 Months	0.825	0.955

Stability of Standard Solutions and Hydrolyzed Samples

To determine the stability of standard solutions, the absorbance of a solution of ε-DNP-lysine was measured periodically at 268 and 274 nm for 4 months. A solution kept in the dark at room temperature showed no significant variation in absorbance values (Table 2).

The stability of hydrolyzed samples was tested by periodic analysis of solutions immediately after hydrolysis and some days later. Available lysine in fish meal was 4.73% after hydrolysis, and 4.74% after 2 weeks. Similar stability was observed for other samples tested; therefore, samples need not be quantitated immediately after hydrolysis. However, solutions containing internal standard must be kept closed in nearly full vessels to avoid losses of 2,6-dimethylphenol through evaporation.

Identification of Unknown Peak

From their retention times, ε-DNP-lysine, FDNB, 2,4-dinitrophenol (DNP), and dimethylphenol were identified in the well-defined chromatogram of the hydrolyzed sample solution. Other peaks near DNP, not well resolved, were probably due to the 2,4-dinitrophenyl derivatives of *N*-terminal amino acids present in the sample. Another peak, with longer retention time and nonreproducible peak height, was attributed to a brown compound that precipitated slowly after the hydrolyzed sample had been filtered. It was the last peak on the chromatogram. The precipitate was characterized as follows: mp: 86–87°C; solubility: water-insoluble, ethanol-soluble, benzene-soluble, very soluble in acetone; nuclear magnetic resonance ^1H peaks: 1.5, 4.4, 7.3, 8.4, and 8.7 ppm in $CDCl_3$; IR bands: 11 621, 3125, 2941, 2857, 1613, 1515, 1315, 1282, 1149, 1053, 1010, 909, 826, and 741 cm^{-1}; UV maxima: 293, 252, and 214 nm in methanol. From this information, the compound was identified as 2,4-dinitro-1-ethoxybenzene, probably formed during evaporation of ethanol or during derivatization.

Optimal Conditions for Hydrolysis During Autoclaving

Optimal conditions would release a maximum quantity of ε-DNP-lysine with minimum losses. Lysine determinations available in the literature use a reflux time between 12 and 24 h (except cotton meal [21]) for acidic hydrolysis of protein. Matheson (7) used closed tubes for hydrolysis of small quantities of pure protein and concluded that recoveries were better.

In the present work, closed vessels and an autoclave allowed us easily and simultaneously to hydrolyze several samples and to reduce the hydrolysis time. To optimize conditions, a modified simplex method was applied and 4 parameters were studied: volume of HCl, concentration of HCl, hydrolysis time, and imposed superheated steam in the autoclave (hydrolysis temperature). The optimization was stopped when the difference between the values obtained in 2 consecutive tests were similar to the dispersion between replicated results of the same sample.

The range of variation for volume and concentration of HCl was chosen from procedures described in the literature for determining available lysine: 15–75 mL HCl, 6–9M HCl, 60–240 min hydrolysis (less than that in the literature because of the use of superheated steam), 120–133°C superheated steam. Table 3 shows the response at each successive vertex for fish and soybean meal; about 11 and 15 samples, respectively, are enough to reach the maximum.

The optimum conditions are those described in the present procedure, and are different for each meal. This could be considered a disadvantage. However, for example, the conditions for fish meal are nearly the same as those in experiment 13 for soybean meal with only 1% loss of lysine recovery. Therefore, compromise conditions could be established for different protein sources which would still yield acceptable results.

Table 3. Lysine recoveries at each successive simplex condition (conditions are given in normalized factors)

Simplex No.[a]	HCl, mL	HCl concn, M	Hydrolysis time, min	Steam press., kg/sq. cm	Lysine rec., %
		Fish meal			
1	15.0	6.00	60	1.0	1.41
2	70.5	6.65	99	1.2	3.42
3	28.1	8.78	99	1.2	3.77
4	28.1	6.65	227	1.2	4.11
5	28.1	6.65	99	1.9	3.94
6 Pref.	62.4	8.37	202	1.8	4.66
7 Pdil.	75.0	9.00	240	2.0	4.28
8 Pref.	15.0	8.89	233	1.9	4.51
9 Pdil.	15.0	9.00	240	2.0	4.88
10 Pref.	45.0	6.88	240	2.0	4.93
11 Pdil.	53.4	6.00	240	2.0	4.91
		Soybean meal			
1	15.0	6.00	60	1.0	1.10
2	70.5	6.65	99	1.2	2.05
3	28.1	8.78	99	1.2	2.04
4	28.1	6.65	227	1.2	2.32
5	28.1	6.65	99	1.9	2.28
6 Pref.	62.4	8.37	202	1.8	2.33
7 Pdil.	75.0	9.00	240	2.0	2.13
8 Pref.	66.5	6.00	214	1.9	2.23
9 Pref.	22.0	7.19	240	2.0	2.30
10 Pref.	15.0	8.45	170	1.6	2.35
11 Pcon.	25.1	7.82	181	1.7	2.28
12 Pref.	45.3	6.61	203	1.8	2.42
13 Pdil.	55.3	6.00	214	1.8	2.54
14 Pref.	55.8	7.45	240	1.5	2.55
15 Pdil.	69.7	7.85	240	1.3	2.56

[a] Pref. = reflexed vertex; Pcon. = contracted vertex; Pdil. = dilated vertex.

Detection Limit, Accuracy, and Precision

The detection limit of the proposed method at maximum chromatographic sensitivity is 4.5 ng ε-DNP-lysine, corresponding to 2 ng available lysine. The precision, calculated from 10 repeated analyses of each kind of sample, gave a mean and standard deviation of 4.96 ± 0.047% for fish meal and 2.51 ± 0.042% for soybean meal. Coefficients of variation are 0.95 and 1.66%, respectively. Accuracy was determined by comparing results with those for Carpenter's method: 5.17 ± 0.27% for fish meal and 2.45 ± 0.27% for soybean meal (average of 3 determinations each). Results for available and total lysine were 2.51 and 2.95% in soybean meal and 4.96 and 5.23% in fish meal; the availability is 85.9 and 94.8%, respectively.

Conclusions

The proposed method for available lysine determination in fish and soybean meals has 3 advantages over other commonly used methods: the precision is notably better, less time is required per analysis, and use of the autoclave improves the hydrolysis. The use of specific hydrolysis conditions for specific materials produces more accurate results.

Acknowledgments

The authors thank E. Pedroso for his help in spectroscopic analysis and Pinsos Suprem S. A. for support of this work.

References

(1) McNab, J. M. (1979) in *Recent Advances in Animal Nutrition*, W. Haresing & D. Lewis (Eds), Butherwords, London, UK, pp. 1–9
(2) Carpenter, K. H. (1960) *Biochem. J.* **77**, 604–610
(3) Booth, V. H. (1971) *J. Sci. Food Agric.* **22**, 658–666
(4) Hall, R. J., & Henderson, K. (1979) *Analyst* **104**, 1097–1100
(5) Lyman, C., & Thomas, M. C. (1965) *J. Assoc. Off. Agric. Chem.* **48**, 858–859
(6) Blom, L., Hendricks, P., & Caris, J. (1967) *Anal. Biochem.* **21**, 328–400
(7) Matheson, N. A. (1968) *J. Sci. Food Agric.* **19**, 492–502
(8) Rao, S. R., Carter, F. L., & Frankton, V. L. (1963) *Anal. Chem.* **35**, 1927–1930
(9) Roach, A. G., Sanderson, P., & Williams, D. R. (1967) *J. Sci. Food Agric.* **18**, 274–278

(10) Datta, S., & Datta, S. C. (1976) *J. Am. Oil Chem. Soc.* **54**, 117–118
(11) Warthesen, J. J., & Kramer, P. L. (1978) *Cereal Chem.* **55**, 481–488
(12) Peterson, W. R., & Warthesen, J. J. (1979) *J. Food Sci.* **44**, 994–997
(13) Kozukue, N., Kozukue, E., Terai, H., & Mizuno, S. (1982) *J. Food Sci.* **47**, 1584–1586
(14) Carpenter, K. J., & Booth, V. H. (1973) *Nutr. Abstr. Rev.* **43**, 424–451
(15) Friedman, M. (1982) *Diabetes* **31**, 5–14
(16) Nelder, J. A., & Mead, R. (1965) *Computer* **7**, 308–313
(17) Long, D. E. (1969) *Anal. Chim. Acta* **46**, 193–206
(18) Morgan, L., & Deming, S. N. (1974) *Anal. Chem.* **46**, 1170–1181
(19) Roura, M., Rauret, G., Ivern, J. R., & Sánchez, P. (1984) *Quim. Anal.* **4**, 299–310
(20) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA
(21) Hall, R. J., Trinder, N., & Guivens, D. I. (1973) *Analyst* **98**, 673–686

Evaluation of Infant Formula Protein Quality: Comparison of In Vitro with In Vivo Methods

DAVID A. HARRIS and ROBERT A. BURNS
Mead Johnson Nutritional Group, Department of Food and Nutrition Research, Evansville, IN 47721
RIDA ALI
Bristol-Myers International Group, New York, NY 10154

Two-week protein efficiency ratio (2-wk PER), net protein ratio (NPR), calculated-protein efficiency ratio (C-PER), and discriminant computed-protein efficiency ratio (DC-PER) of milk- and soy-based infant formulas were compared to a 4-week protein efficiency ratio (PER). Expressed relative to ANRC casein, 2-week PER and NPR correlated significantly ($P < 0.01$, r = 0.90) with PER. Although C-PER and DC-PER also correlated significantly ($P < 0.01$) with PER, r = 0.71 and r = 0.87, respectively, these in vitro methods did not distinguish differences in protein quality among soy-based infant formulas. C-PER and DC-PER, as currently designed, are not applicable to the measurement of protein quality for all types of infant formulas.

Federal regulations (1) specify that an appropriate modification of the AOAC protein efficiency ratio (PER) method [secs **43.253–43.257** (2)] may be used for protein quality testing of infant formulas. Modifications to the official AOAC method are required because infant formulas have relatively high-fat and low-protein contents and usually contain high concentrations of lactose. The aim of such modifications is to ensure that the ANRC casein control diet and test diets contain the same level and type of fat and carbohydrate. Without proper modifications to the reference control diet, the PER values of infant formulas may be underrated in comparison to ANRC casein (3).

Two major practical drawbacks of the PER method are the length of time (4 weeks) and expense required to conduct the assay. A more ideal protein quality method would be one that is capable of ranking the quality of proteins at least as well as PER while substantially reducing time and expense (4). Net protein ratio (NPR), a 10–14 day bioassay, and 2-week PER are possible alternatives to the PER assay although neither method drastically reduces time and expense. In 1984, the calculated-protein efficiency ratio (C-PER) and the discriminant computed-protein efficiency ratio (DC-PER) computer prediction models of Satterlee et al. (5) were adopted official first action by AOAC. These in vitro methods are very rapid and fairly inexpensive to conduct if an automatic amino acid analyzer and computer are available.

The ability of the 2-week PER, NPR, C-PER, and DC-PER methods to measure or predict the protein quality of infant formulas as compared to the PER assay was the focus of this study. Three milk-based experimental infant formulas differing in protein source and 5 soy-based experimental infant formulas differing in protein source, processing conditions, and methionine level were studied, as well as ANRC reference casein and the soy flours used to make 2 of the soy formulas. Some data from this study have been presented previously (6).

Received February 2, 1987. Accepted July 23, 1987.

Experimental

Samples

The protein quality of 11 samples was measured by the in vivo methods (2-week PER, NPR, and PER) or predicted by the in vitro methods (C-PER and DC-PER). The experimental infant formulas were made on a pilot-plant scale according to standard powder infant formula processing conditions unless specified otherwise. AOAC methods (2) were used to determine fat (sec. **7.063**), ash (sec. **7.009**), and nitrogen (sec. **7.015**) contents of the samples. Samples assayed were:

(a) *ANRC reference casein.*—88.5% protein, N × 6.25 (Humko Sheffield Chemical, Memphis, TN).

(b) *Experimental milk-based formulas I–III.*—Protein sources: I, demineralized whey and nonfat milk; II, nonfat milk; III, sodium caseinate. Coconut and corn oils, lactose.

(c) *Experimental soy-based formulas I–V.*—Protein sources: I–III, soy protein isolate and L-methionine (0.8 g/100 g protein); IV, soy flour I; V, soy flour II. Coconut and soy oils, corn syrup solids. Formulas I–III were made using levels of heat treatment considered to result in underprocessed (I), optimally processed (II), and overprocessed (III) products.

(d) *Soy flours I and II.*—I, spray-dried soy flour used to make soy-based formula IV; II, drum-dried soy flour used to make soy-based formula V.

Diet Compositions

Test diets were prepared by incorporating experimental infant formulas, soy flours, or casein at levels required to provide 10% protein (N × 6.25). Formulations of test diets used for in vivo methods are shown in Table 1. Separate pretest, ANRC casein control, and nonprotein diets were used for the milk- and soy-based samples. Pretest diets contained 19% lactalbumin (Teklad Test Diets, Madison, WI) and either 45% lactose (high lactose, HL), or 0% lactose

(nonlactose, NL). For the nonprotein diets, protein was replaced by cornstarch (Koldex, A. E. Staley, Decatur, IL).

Animals

Male weanling Sprague-Dawley rats (Charles River, Portage, MI), 21–28 days old, and weighing 45–55 g, were housed in individual stainless steel cages suspended on cage racks in a room maintained at 24 ± 2°C, 50 ± 10% relative humidity, with alternating 12-h periods of light and dark. Sixty rats were fed the HL pretest diet, and 100 rats were fed the NL pretest diet for a 3-day period. Rats were then weighed and body weight was plotted vs 3-day weight gain within the respective pretest groups. Animals at the extremes of the weight distributions were not used in the study.

In Vivo Methods

Rats that were selected after HL pretest were randomly allocated into 5 groups of 10 animals each and assigned HL diets having one of the following protein sources: ANRC casein, no protein, or experimental milk-based formula I, II, or III. Likewise, rats selected after NL pretest were randomly allocated into 9 groups of 10 animals each and assigned an NL diet having one of the following protein sources: ANRC casein; no protein; experimental soy-based formula I, II, III, IV, or V; or soy flour I or II. Diet and water, given on an ad libitum basis, were provided fresh twice a week. Food consumption was measured twice weekly, and rat weight was measured weekly. No diarrhea was evident in any groups, and thus feces were collected during the second week of the study from the first 5 rats of each group by suspending fecal collection screens under the cages. Feces from individual rats were lyophilized, weighed, ground, and analyzed for nitrogen by macrokjeldahl. In vivo protein quality and apparent N digestibility (AD) values were calculated as follows:

2-wk PER = 2 wk wt gain of test animal/
2 wk protein intake of test animal

PER = 4-wk wt gain of test animal/
4-wk protein intake of test animal

2-wk relative PER (2-wk RPER) =
(2-wk PER of test animal/
av. 2-wk PER of ANRC casein group) × 100

Relative PER (RPER) = (PER of test animal/
av. PER of ANRC casein group) × 100

NPR = (2-wk wt gain of test animal
+ av. 2-wk wt loss of nonprotein group)/
2-wk protein intake of test animal

(In this study, rats fed the nonprotein HL diet for 2 weeks lost, on average, 8.1 g with a food intake of 51.4 g. Rats fed the nonprotein diet without lactose for 2 weeks lost, on average, 7.7 g with a food intake of 60.4 g.)

Relative NPR (RNPR) = (NPR of test animal/
av. NPR of ANRC casein group) × 100

AD = [(N intake of test animal − fecal N of test animal)/
N intake of test animal] × 100

Amino Acid Analysis

All amino acids except tryptophan were measured in duplicate samples of protein sources, using the methods outlined by Satterlee et al. (5). The average coefficient of variation (CV) in the measurement of amino acids was 2.6% for the conventional acid hydrolysis procedure and 9.0% for the performic acid oxidation procedure (methionine and cystine). An average nitrogen yield of 92.8%, ranging from 88.7 to 96.0%, was obtained. Amino acid values were corrected to 95% nitrogen recovery. Tryptophan was measured (single determination) using a microbiological turbidometric assay (7).

Table 1. Formulations of milk- or soy-based diets used for in vivo methods (% w/w)

Constituent	Milk-based	Soy-based
Protein (N × 6.25)	10	10
Soy oil	13	9
Coconut oil	14	9
Lactose	45	0
Cornstarch[a]	11	45
Corn syrup solids	0	20
Cellulose[b]	1	1
Vitamin mix (AOAC)[c]	1	1
Mineral mix (USP XVII)[c]	5	5
Total	100	100

[a] Koldex brand pregelatinized cornstarch (A. E. Staley Co., Decatur, IL).
[b] Nonnutritive fiber (Teklad Test Diets, Madison, WI).
[c] Teklad Test Diets.

In Vitro Methods

In vitro apparent N digestibility was measured and C-PER and DC-PER were predicted according to the method of Satterlee et al. (5). C-PER and DC-PER were also calculated relative to a PER of 100 for reference ANRC casein.

Statistical Analysis

Statistical analyses to compute mean, standard deviation, coefficient of variation, and correlation values were conducted using Statistical Analysis System (SAS®) procedures (8).

Results and Discussion

In Vivo Methods

Results of the in vivo protein quality methods are shown in Table 2. In general, the milk-based formulas were greater than or equivalent to ANRC casein in protein quality (127–97% casein). Soy protein isolate-based formulas supplemented with methionine were intermediate in protein quality (94–86% casein), and the soy flour-based formulas and soy flours had the lowest protein quality ratings (83–68% casein). These differences in protein quality parallel the level of sulfur amino acids, methionine, and cystine present in the formulas (see Table 3).

Results for 2-week PER were similar to those from the conventional 4-week PER assay, especially when expressed relative to casein (RPER and 2-week RPER in Table 2). Correlations of 2-week PER to PER ($r = 0.86$), and 2-week RPER to RPER ($r = 0.91$) were all statistically significant ($P < 0.01$). These results suggest that for infant formulas, the standard 4-week PER assay could be shortened to 2 weeks, especially when results are expressed relative to casein (9–12).

The NPR assay is shorter (10–14 days) than the conventional PER assay and overcomes a major criticism of the PER in that it incorporates a nonprotein-fed group to provide a maintenance correction factor (13). The basic assumption is that endogenous nitrogen secretion by rats fed a nonprotein

Table 2. Comparison of 2-week PER and NPR to PER and 2-week RPER and RNPR to RPER

	PER			2-Wk PER			NPR			% of ref. casein		
Sample	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %	RPER	2-Wk RPER	RNPR
ANRC reference casein (45% lactose, 27% fat diet)	3.03	0.20	6.6	3.29	0.25	7.6	4.17	0.27	6.5	100	100	100
Milk-based formula I	3.71	0.39	10.5	4.18	0.47	11.2	4.96	0.51	10.3	122	127	119
Milk-based formula II	3.38	0.20	5.9	3.77	0.46	12.2	4.64	0.47	10.1	112	114	111
Milk-based formula III	2.94	0.26	8.8	3.33	0.51	15.3	4.33	0.60	13.9	97	101	104
ANRC reference casein (0% lactose, 18% fat diet)	3.39	0.23	6.8	3.43	0.27	7.9	4.13	0.22	5.3	100	100	100
Soy-based formula I	2.97	0.21	7.1	3.07	0.31	10.1	3.74	0.30	8.0	88	90	91
Soy-based formula II	3.19	0.26	8.2	3.22	0.37	11.5	3.84	0.31	8.1	94	94	93
Soy-based formula III	3.00	0.40	13.3	2.96	0.44	14.9	3.63	0.43	11.8	88	86	88
Soy-based formula IV	2.65	0.12	4.5	2.76	0.14	5.1	3.42	0.18	5.3	78	80	83
Soy-based formula V	2.47	0.19	7.7	2.55	0.27	10.6	3.15	0.30	9.5	73	74	76
Soy flour I	2.31	0.12	5.2	2.47	0.18	7.3	3.14	0.23	7.3	68	72	76
Soy flour II	2.43	0.19	7.8	2.51	0.32	12.7	3.18	0.30	9.4	72	73	77

diet is equivalent to that of rats fed a test protein. This assumption is not strictly valid (14). In this study, RNPR values were similar to RPER values (Table 2), and NPR correlated significantly with PER ($P < 0.01$, $r = 0.84$) as did RNPR with RPER ($P < 0.01$, $r = 0.90$). For the samples tested in this experiment, NPR offered no advantage over 2 week PER and had the disadvantage of requiring an additional group of animals fed a nonprotein diet.

Apparent Protein Digestibility—In Vivo and In Vitro

In vivo and in vitro apparent digestibility values are shown in Table 4. All protein sources were highly digestible as measured by the in vivo method. In vivo digestibilities ranged from 95.8 to 90.8% and in vitro digestibilities ranged from 90.9 to 83.8%. In vitro digestibilities, on average, were about 6% lower than in vivo, but the overall correlation with in vivo digestibility was statistically significant ($P < 0.05$; $r = 0.64$). When partitioned into milk- or soy-based groups, however, a significant correlation was found for the soy-based samples ($P < 0.05$; $r = 0.75$) but not for the milk-based samples (not significant; $r = 0.61$). Using similar methods, Rich and co-workers (15) reported that in vitro digestibility was usually within 3% of in vivo values and Bodwell and colleagues (16) reported in vitro digestibility values which were about 7% lower than in vivo values. Bodwell and co-workers (16) compared in vitro to in vivo digestibility for several animal and plant proteins and found a poor correlation overall ($r = 0.19$). Better correlations were observed after the analyses were categorized according to samples of animal ($r = 0.45$) or plant ($r = 0.95$) origin (16). The effect of the in vitro digestibility measurements on C-PER is discussed below.

In Vitro Methods

The amino acid contents of the samples corrected to 95% nitrogen recovery are shown in Table 3. Amino acid profiles (Table 3) and in vitro digestibilities (Table 4) were used in the computer program to predict the C-PER for each protein source. DC-PER was predicted from amino acid profile alone. Results for PER, C-PER, and DC-PER, absolute as well as those expressed as percent reference casein, are shown in Table 5.

The C-PER for soy-based formula V (2.77) was much higher than would be expected compared to the C-PER for all other soy formulas. In this case the computer program, although operating as designed, discriminated this sample into a "high quality" protein classification resulting in the higher C-PER value (personal communication, L. D. Satterlee, Pennsylvania State Univ.).

Comparison of In Vivo and In Vitro Methods

Overall, significant correlations with RPER were observed for C-PER ($P < 0.05$; $r = 0.71$) and DC-PER ($P < 0.01$; $r = 0.87$). For milk-based samples, the correlation coefficients for C-PER vs RPER and DC-PER vs RPER were 0.83 and 0.84, respectively, but were not significant. C-PER and DC-

Table 3. Amino acid composition of proteins used for C-PER and DC-PER analysis

	Amino acid[a]											
Sample	LYS	MET + CYS	THR	ILE	LEU	VAL	PHE + TYR	TRP	ASP	PRO	CYS	(NH_3)
ANRC reference casein	7.59	3.02	3.77	4.63	10.04	5.95	10.52	1.60	7.33	11.01	0.36	1.48
Milk-based formula I	8.60	4.59	5.43	5.59	11.18	6.20	7.12	1.37	9.78	8.61	1.93	1.57
Milk-based formula II	8.15	3.87	4.06	4.93	10.76	6.27	8.64	1.16	8.26	10.11	1.10	1.60
Milk-based formula III	7.74	3.56	3.93	4.92	10.16	6.23	9.18	1.34	7.35	11.34	0.78	1.52
Soy-based formula I	5.85	3.18	3.55	4.25	8.14	4.20	8.57	1.17	11.11	5.10	1.08	1.55
Soy-based formula II	6.10	3.62	3.47	4.32	8.21	4.28	8.53	1.16	11.09	5.12	1.03	1.56
Soy-based formula III	5.91	2.74	3.49	4.37	8.33	4.33	8.44	1.10	11.23	5.24	1.03	1.60
Soy-based formula IV	5.79	2.92	3.54	4.37	8.34	4.71	8.59	1.21	11.81	5.15	1.25	1.49
Soy-based formula V	5.30	3.01	3.47	4.36	8.38	4.69	8.38	1.10	11.79	5.23	1.35	1.48
Soy flour I	5.97	2.68	3.30	4.28	8.06	4.29	9.02	1.15	11.38	5.15	1.27	1.56
Soy flour II	6.21	2.74	3.33	4.19	8.15	4.44	9.19	1.12	11.21	5.04	1.30	1.39

[a] Grams amino acid/16 g nitrogen corrected to a nitrogen yield of 95%.

Table 4. Comparison of in vivo and in vitro apparent protein digestibility

Sample	In vivo digestibility,[a] %		In vitro digestibility,[b] %	
	Mean	SD	Mean	SD
ANRC reference casein				
(45% lactose, 27% fat diet)	92.0	1.4	90.9	1.2
Milk-based formula I	92.6	0.6	84.1	1.7
Milk-based formula II	91.6	0.7	83.8	0.2
Milk-based formula III	91.1	1.2	84.6	0.7
ANRC reference casein				
(0% lactose, 18% fat diet)	95.8	0.7	90.9	1.2
Soy-based formula I	92.6	1.5	85.4	0.2
Soy-based formula II	94.7	0.5	87.7	0.2
Soy-based formula III	94.0	1.1	88.4	0.2
Soy-based formula IV	90.8	0.7	84.6	0.5
Soy-based formula V	91.5	1.4	87.4	0.2
Soy flour I	91.0	0.9	85.2	0.6
Soy flour II	92.3	0.9	86.5	0.6

[a] Five rats per sample.
[b] Three in vitro digestions per sample.

Table 5. Comparison of in vitro methods (C-PER and DC-PER) of protein quality determination to PER[a]

Sample	PER	C-PER	DC-PER	% of reference casein: RPER	C-PER	DC-PER
ANRC reference casein						
(45% lactose, 27% fat diet)	3.03	2.50	2.50	100	100	100
Milk-based formula I	3.71	3.42	2.98	122	137	119
Milk-based formula II	3.38	2.64	2.69	112	106	108
Milk-based formula III	2.94	2.61	2.68	97	104	107
ANRC reference casein						
(0% lactose, 18% fat diet)	3.39	2.50	2.50	100	100	100
Soy-based formula I	2.97	1.97	1.97	88	79	79
Soy-based formula II	3.19	1.94	1.96	94	78	78
Soy-based formula III	3.00	1.97	1.97	88	79	79
Soy-based formula IV	2.65	1.97	1.97	78	79	79
Soy-based formula V	2.47	2.77	1.97	73	110	79
Soy flour I	2.31	1.95	1.96	68	78	78
Soy flour II	2.43	1.97	1.93	72	79	77

[a] C-PER and DC-PER data expressed as percent reference casein within respective milk- or soy-based groups.

PER methods did not predict differences among all soy proteins as measured by PER assay. In particular, C-PER and DC-PER underestimated the protein quality of soy-based formulas (I–III) that were supplemented with methionine (Table 5). For soy-based samples, results from both in vitro methods were either poorly or inversely correlated with RPER. Correlations were not improved by categorizing the soy-based samples as with or without supplemental methionine.

Thus, the in vitro methods ranked milk-based samples, but not soy-based samples in similar order as PER. The model parameter estimates used in the computer program for this study were not developed using data from protein supplemented with amino acids (17). Consequently, when protein sources of this type (soy protein isolate + supplemental methionine) were analyzed in this study, the C-PER/DC-PER methods did not predict a higher protein quality for methionine-supplemented soy samples.

In addition, the in vitro methods failed to distinguish differences in protein quality among differently processed soy proteins which were detected by the PER method (Table 5). The in vitro digestibility method used to calculate C-PER provides an overall digestibility value for the protein. Use of this value in the C-PER program may cause estimation of the availability of certain amino acids to be erroneously high or low. This could be a particular problem with total-sulfur amino acids (methionine + cystine), lysine, threonine, and tryptophan which are often limiting amino acids. These amino acids are most prone to oxidation (methionine/cystine), Maillard reactions (lysine) or other types of processing damage (18, 19). Antinutritional factors such as trypsin inhibitors could have different effects on in vitro and in vivo digestibility estimates and also contribute to differences between C-PER and PER of processed soy proteins (20, 21).

The C-PER/DC-PER model as currently designed is based on a wide range of foodstuffs and did not predict specific protein quality changes such as were measured by PER in this study. A more specific model for predicting the protein quality of a particular type of food—for example, infant formula—could be developed. This would require developing model parameter estimates based on PER and amino acid data for the specific type of food to be analyzed that would encompass variables such as changes due to protein source, processing, and amino acid supplementation. Chemical procedures to measure more accurately the bioavailability of limiting amino acids as well as overall protein digestibility could be developed and used. Techniques such as immobilized enzyme digestibility assays may be useful in this respect (22, 23).

Conclusions

Two-week PER and NPR data were in close agreement with PER for the milk- or soy-based samples used in this study. NPR offered no advantage over 2-week PER for these samples but required an additional group of rats. The C-PER and DC-PER methods did not correspond with PER as well as did 2-week PER and NPR although differences in protein quality among the milk-based samples were detected by the in vitro methods. C-PER and DC-PER, as currently designed, did not differentiate protein quality among soy-based samples, whereas in vivo procedures did. The in vitro methods, however, could potentially predict the protein quality of infant formulas if appropriate modifications are made.

Acknowledgments

We thank John D. Kissel and L. Douglas Barton for their skilled technical assistance, Mary Alice Springer of the Medical Communications Group for editorial assistance, Barbara R. Hamilton and Lorna L. Ford for typing the manuscript, and Melinda J. Tao for computer assistance.

References

(1) *Fed. Regist.* (1983) Infant Formula Quality Control Procedures **27**, 17023
(2) *Official Methods of Analysis (1984)* 14th Ed., AOAC, Arlington, VA
(3) Mitchell, G. V., & Jenkins, M. Y. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 680–683
(4) Nesheim, R. O. (1981) in *Protein Quality in Humans: Assessment and In Vitro Estimation,* C. E. Bodwell, J. S. Adkins, & D. T. Hopkins (Eds), AVI, Westport, CT, pp. 55–58
(5) Satterlee, L. D., Kendrick, J. G., Marshall, H. F., Jewell, D. K., Ali, R. A., Heckman, M. M., Steinke, H. F., Larson, P., Phillips, R. D., Sarwar, G., & Slump, P. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 798–809
(6) Harris, D. A., Burns, R. A., & Ali, R. A. (1985) in *Production, Regulation, and Analysis of Infant Formula: A Topical Conference,* AOAC, Arlington, VA, pp. 90–96
(7) Kavanagh, F. (1983) *Analytical Microbiology,* Vol. 1, Academic Press, New York, NY, pp. 618–643, 670
(8) Ray, A. A. (1982) *SAS User's Guide: Basics,* SAS Institute, Cary, NC, pp. 501–512

(9) Hackler, L. R. (1977) *Cereal Chem.* **54,** 984–995
(10) Hackler, L. R., et al. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 66–67
(11) McLaughlan, J. M., Anderson, G. H., Hackler, L. R., Hill, D. C., Jansen, G. R., Keith, M. O., Sarwar, G., & Sosulski, F. W. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 462–467
(12) Happich, M. L., et al. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 255–262
(13) Bender, A. E., & Doell, B. H. (1957) *Br. J. Nutr.* **11,** 140–148
(14) Canolty, N. L., & Koong, L. J. (1978) *J. Nutr.* **108,** 1555–1565
(15) Rich, N., et al. (1980) *Nutr. Rep. Int.* **21,** 285–300
(16) Bodwell, C. E., et al. (1980) *Am. J. Clin. Nutr.* **33,** 677–686
(17) Satterlee, L. D., Kendrick, J. G., & Miller, G. A. (1977) *Food Technol.* **31**(6), 78–81
(18) Mauron, J. (1985) *Bibl. Nutr. Dieta* **34,** 57–80
(19) Hurrell, R. F., et al. (1983) *Br. J. Nutr.* **49,** 343–354
(20) Liener, I. E. (1979) *J. Am. Oil Chem. Soc.* **56,** 121–129
(21) Rothenbuhler, E., & Kinsella, J. E. (1985) *J. Agric. Food Chem.* **33,** 433–438
(22) Porter, D. H., et al. (1984) *J. Agric. Food Chem.* **32,** 334–339
(23) Swaisgood, H. E., et al. (1982) *J. Food Prot.* **45,** 1248–1256

AOAC MEETINGS & EXPOSITIONS

March 29, 1988	**MID-CANADA REGIONAL SECTION MEETING** Winnipeg, Manitoba, Canada
May 19, 1988	**NEW YORK-NEW JERSEY REGIONAL SECTION MEETING** Fort Hamilton Officer's Club, Brooklyn, NY
June 14, 1988	**SOUTHEAST REGIONAL SECTION MEETING** Radisson Inn & Conference Center, Atlanta, GA
June 20-22, 1988	**NORTHEAST REGIONAL SECTION MEETING** Lowell University, Lowell, MA
June 20-22, 1988	**MIDWEST REGIONAL SECTION MEETING** Holiday Inn West, Columbia, MO
June 23-24, 1988	**PACIFIC NORTHWEST REGIONAL SECTION MEETING** (with banquet, speaker and exposition), Evergreen College, Olympia, WA
June 1988	**EASTERN ONTARIO-QUEBEC REGIONAL SECTION**
August 29-September 1, 1988	**102nd ANNUAL INTERNATIONAL MEETING & EXPOSITION** The Breakers, Palm Beach, FL Program: Analytical Methodology including spotlight on Biotechnology
September 25-28, 1989	**103rd ANNUAL INTERNATIONAL MEETING & EXPOSITION** The Clarion Hotel, St. Louis, MO
September 10-13, 1990	**104th ANNUAL INTERNATIONAL MEETING & EXPOSITION** The Clarion Hotel, New Orleans, LA

For further information, contact: The AOAC Meetings Department, AOAC, 1111 N. 19th St., Ste. 210, Arlington, VA 22209, or call in the U.S. (703) 522-3032 or outside the U.S. +1-703-522-3032.

PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Improved Method for Determination of Chlorothalonil and Related Residues in Cranberries

IBRAHIM M. EL-NABARAWY[1] and WILLIAM F. CAREY
New Jersey Agriculture Experiment Station, Rutgers University, Cook College, Department of Environmental Science, New Brunswick, NJ 08903

Tetrachloroisophthalonitrile (chlorothalonil) was applied under controlled conditions in 1985 to a cranberry bog for fungus control and for residue studies. Randomly selected samples of cranberries were analyzed for residues of chlorothalonil, its metabolite 4-hydroxy-2,5,6-trichloroisophthalonitrile, hexachlorobenzene, and pentachlorobenzonitrile by extraction, methylation, Florisil column cleanup, and electron capture gas chromatography. Because of interferences in the GC determinative step, previously reported methodology was modified. The total residues found in the test samples were well below the permissible limit for the parent and related compounds.

Tetrachloroisophthalonitrile (chlorothalonil) is an effective fungicide against certain pathogenic fungi that affect some economically important plants (1–3) including cranberries. Chlorothalonil was applied under controlled conditions in 1985 to a cranberry bog at the National Cranberry Research Center to study its effectiveness as a fungicide. Chlorothalonil was applied on June 25, at the rate of 5 pints (40.4% active)/acre and again on July 2 at 16 pints/acre for a worst-case fungus outbreak. After the second application, samples of cranberries were taken at random on September 19, and October 2, 10, and 15. Attempts to analyze the samples by the Ballee et al. method (1) with suggested improvements were unsuccessful. The use of alumina for cleanup of the sample interferences was ineffective. Major changes in the published method were needed to adequately measure the residues of chlorothalonil and its related compounds.

METHOD

Reagents

(a) *Solvents.*—Acetone, hexane, methanol, diethyl ether, petroleum ether, all pesticide grade.

(b) *Acidic acetone extraction solvent.*—Mix 385 mL acetone and 15 mL aqueous H_2SO_4 (1 + 2).

(c) *$NaHCO_3$ solution.*—0.8M.

(d) *Florisil.*—100–200 mesh. Activate 4 h at 650°C and keep at 140°C.

(e) *Keeper solution.*—1% hexadecane in hexane.

(f) *Methylation reagent.*—Dissolve 0.1 g 3-methyl-1-*p*-tolyltriazene in 55 mL diethyl ether. Prepare solution weekly and store at ca −20°C.

(g) *Standards.*—Chlorothalonil, 99.7% pure; pentachlorobenzonitrile (PCNB), 99.9% pure; hexachlorobenzene (HCB), 99.05% pure; 4-hydroxy-2,5,6-trichloroisophthalonitrile (metabolite SDS-3701), 99.6% pure.

(h) *Filter paper.*—S & S sharkskin, to fit Buchner funnel.

(i) *Catalyst solution.*—HCl–methanol (1 + 3 v/v).

Apparatus

(a) *Gas chromatograph.*—Tracor, with ^{63}Ni electron capture detector and 6 ft × 4 mm id column containing 5% SP-2401 (Supelco) on 100–120 mesh Supelcoport support. Conditions: 80 mL nitrogen carrier gas/min; column 170°C; inlet 220°C; detector 280°C. Columbia Scientific Industries (CSI-38) digital integrator for peak area measurement.

(b) *Homogenizer.*—Brinkmann Polytron.

(c) *Chromatographic column.*—300 × 11 mm id.

Preparation of Standard Solutions

(a) *Chlorothalonil, HCB, and PCNB.—Stock solutions.*—Prepare separate solutions by weighing 0.1 g each compound into tared weighing pan. Quantitatively transfer to 100 mL volumetric flask with hexane. *Intermediate solutions.*—Dilute aliquot of each stock solution to 10 μg/mL with hexane. *Working solutions.*—Serially dilute intermediate solutions with hexane to 1 μg/mL for chorothalonil and 0.1 μg/mL for HCB and PCNB.

(b) *Metabolite SDS-3701.*—Prepare hexane stock solution and serially dilute as above to 10 μg/mL for recovery studies, and 1 μg/mL for methylation. Methylate 10 mL of latter solution after removal of solvent as described below, beginning "Add 10 mL catalyst solution . . . ." Dissolve residue in 100 mL hexane and use to obtain working standard curve for quantitation of methylated metabolite.

Extraction of Pesticide Residues

Blend 10 g sample of cranberries 2 min in pint mason jar with 50 mL acidic acetone in homogenizer. Filter homogenate under suction through filter paper on Buchner funnel. Rinse jar and filter cake twice with 15 mL acidic acetone. Add 2–3 mL water and 1 mL keeper solution to filtrate in 125 mL glass-stopper Erlenmeyer flask. Evaporate acetone in rotary evaporator on 30°C water bath. Add 50 mL 0.8M $NaHCO_3$ to extract. Adjust pH to 4.5 with aqueous H_2SO_4 (1 + 2) and quantitatively transfer solution to 250 mL separatory funnel with 50 mL petroleum ether rinse. Shake separator 2 min and vent vapors. Let layers separate and drain aqueous phase into another separatory funnel. Pour ether phase into 125 mL Erlenmeyer flask. Repeat extraction of aqueous phase with another 50 mL petroleum ether. Drain aqueous phase back into first separator and save for extraction of metabolite. Combine ether extracts in Erlenmeyer flask, add 3 drops of keeper solution, and concentrate to ca 1 mL on rotary evaporator in 30°C water bath. Let remaining solvent evaporate in fume hood. Dissolve residue in 10 mL hexane and save for column cleanup (extract A).

Adjust pH of reserved aqueous phase to <2 by adding 5 mL aqueous H_2SO_4 (1 + 2). Extract twice with 50 mL petroleum ether–diethyl ether (1 + 1). Evaporate combined ether extracts as described above and save residue containing metabolite for methylation step (extract B).

Methylation

Add 10 mL catalyst solution to extract B. Add 5 mL methylating reagent, using reagent to rinse sides of flask. Let methylation proceed 45 min at room temperature. Evaporate ether to ca 1 mL on rotary evaporator in 30°C water bath. To assure complete methylation, add additional 3 mL methylating reagent and let sample stand another 15 min. Evaporate

Received January 15, 1987. Accepted June 27, 1987.

[1] AMIDEAST postdoctoral research scientist, Plant Protection Laboratory, National Research Centre, Dokki, Cairo, Egypt.

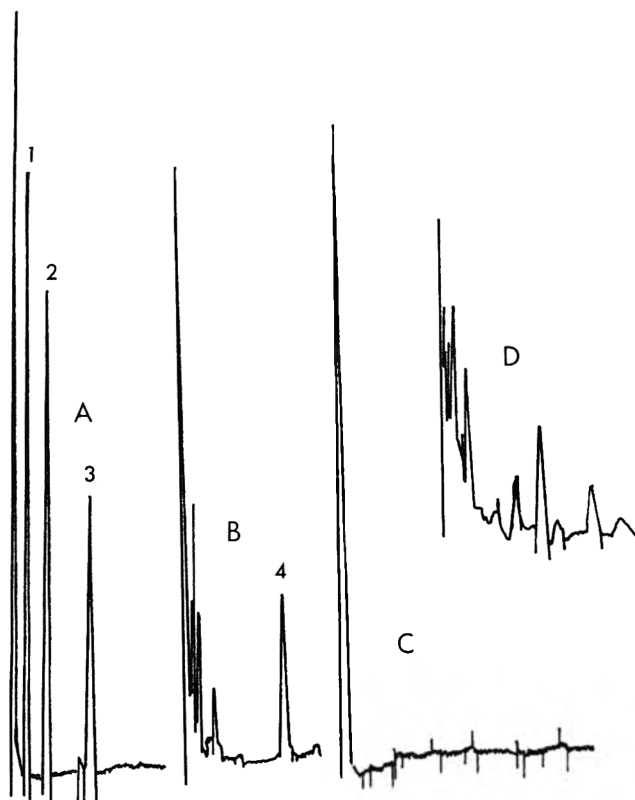


Figure 1. Typical chromatograms: A and B, standards; C, blank extract after Florisil cleanup; D, blank extract after alumina cleanup. 1, HCB; 2, PCNB; 3, chlorothalonil (2 ng each); and 4, methyl ester of SDS-3701 (1 ng).

ether again, and dissolve residue in 5 mL hexane. Save for column cleanup.

Column Cleanup

Pack chromatographic column with 0.5 cm glass wool, 4 g activated Florisil, and 1.5 cm anhydrous Na_2SO_4. Tap to settle. Wet column with 10 mL hexane. Transfer extract A onto column with the aid of two 3 mL hexane rinses. Elute HCB with 35 mL hexane into 125 mL glass-stopper Erlenmeyer flask. Elute chlorothalonil and PCNB into second flask with 65 mL diethyl ether–petroleum ether (1 + 1).

Prepare similar cleanup column for extract B. Wet with 10 mL hexane and elute with 35 mL hexane. Discard all effluents. Elute methylated metabolite with 100 mL diethyl ether–petroleum ether (1 + 1).

Quantitation

Add 3 drops keeper solution to each flask. Evaporate solvent to ca 1 mL on rotary evaporator. Let remaining solvent evaporate in fume hood. Dissolve each residue in 5 or 10 mL hexane for electron capture GC analysis.

Recovery Studies

Untreated cranberries were fortified by adding standard solution containing chlorothalonil, SDS-3701, HCB, and PCNB at levels from 0.1 to 1.0 ppm. These fortified samples were processed through all steps of the analytical method to validate the assay procedure. Results are shown in Table 1.

Results and Discussion

All glassware used in this residue study was baked overnight at 140°C and stored for use. All organic solvents were assayed for interferences before use by evaporating 50 mL to 1 mL and analyzing by electron capture GC. In the original cleanup procedure, interferences were removed by alumina column chromatography. However, this approach was not adequate for the 4 compounds being studied. Peaks appeared in the area of interest and at long retention times that obscured subsequent GC analyses. The proposed cleanup procedure eliminates both problems.

The retention times for methylated SDS-3701, PCNB, and HCB are 1.27, 0.47, and 0.2 relative to chlorothalonil (actual retention time, 7.5 min). The 4 compounds could be determined in one GC analysis except for the presence of interferences generated by the methylating procedure. Thus, it was necessary to clean up extracts A and B separately. The 2 fractions from extract A can be combined for one GC analysis if interfering peaks do not appear in the second (diethyl ether–petroleum ether) fraction.

In the proposed method, hexane was substituted for benzene for health reasons and for toluene for efficiency in the concentration steps.

Cranberry blanks analyzed by the previous procedure showed significant interferences which obscured the areas of interest. Cranberry blanks prepared by the proposed procedure showed no interfering peaks and clearly demonstrated the absence of chlorothalonil residues (Figure 1).

Residue data for chlorothalonil, HCB, PCNB, and the methylated metabolite are shown in Table 2. Residues present 2.5 months after the last application were 1.12, 0.08, 0.03, and 0.08 ppm, respectively. The total of all residues appeared to remain constant for the next month until harvest. These residues are less than the tolerance (5 ppm) adopted by the U.S. Environmental Protection Agency for chlorothalonil (4).

Table 1. Recoveries of chlorothalonil and related compounds from cranberries at indicated levels[a]

Added, ppm	Chlorothalonil	HCB	PCBN	SDS-3701
1.0	92.3 (5.5)	75.7 (3.7)	83.5 (3.0)	95.9 (3.3)
0.5	85.7 (1.3)	78.4 (1.8)	81.6 (0.5)	98.9 (—)
0.1	96.3 (4.8)	75.0 (1.0)	76.3 (5.0)	99.5 (1.0)

[a] Average of duplicate analyses, % (SD).

Acknowledgments

The authors thank the staff of the Department of Environmental Science, especially G. Winnett for facilities offered and for help during this study. The authors also express sincere gratitude to P. E. Marucci, Director of the National Cranberry Research Center, for setting up the field experiments, and thank G. L. Eilrich, Fermenta Plant Protection Co., for providing the analytical standards and advice during this research.

Table 2. Residues (ppm) of chlorothalonil and related compounds in cranberries

Sampling date	Chlorothalonil	HCB	PCBN	SDS-3701
9/19	1.17	0.08	0.03	0.08
	1.07	0.08	0.03	0.08
10/2	0.64	0.11	0.03	0.05
	0.64	0.11	0.03	0.05
10/10	0.84	0.00	0.08	0.03
	0.80	0.00	0.08	0.03
10/15	0.95	0.00	0.07	0.03
	0.97	0.00	0.07	0.03
Average	0.89	0.048	0.053	0.048
Std dev.	0.47	0.14	0.068	0.053

This project was supported by state and Hatch funds, N.J.A.E.S. publication No. D-07117-2-87.

REFERENCES

(1) Ballee, D. L., Duane, W. C., Stallard, D. E., & Wolfe, A. L. (1976) *Analytical Methods for Pesticides and Plant Growth Regulators*, Vol. 8, G. Zweig & J. Sherma (Eds), Academic Press, New York, NY, pp. 263–274
(2) Sang, F. K., Arap, X. X., & Munga, F. M. (1973) *E. Afr. Agric. Forest. J.* **39**, 41–45
(3) Vine, B. H., Vine, P. A., & Griffith, S. E. (1973) *Ann. Appl. Biol.* **75**, 359–375
(4) *Federal Register* (1985) 50, 30172, U.S. Government Printing Office, Washington, DC

Determination of Tri-*n*-Butyltin and Di-*n*-Butyltin Compounds in Fish by Gas Chromatography with Flame Photometric Detection

KUMIKO SASAKI, TAKASHI ISHIZAKA, TAKASHI SUZUKI, and YUKIO SAITO
National Institute of Hygienic Sciences, Division of Foods, 18-1, Kamiyoga 1-chome, Setagaya-ku, Tokyo 158, Japan

An analytical method is described for the simultaneous quantitative determination of tri-*n*-butyltin and di-*n*-butyltin compounds in fish. The sample was extracted with 0.5N HCl-methanol, and the methanol solution was extracted with hexane. The extract was purified by gel permeation chromatography and treated with Grignard reagent to yield the methyl derivatives, which were determined by gas chromatography with flame photometric detection operated in the tin mode (610 nm). Recoveries of tri-*n*-butyltin chloride (Bu_3SnCl) and di-*n*-butyltin dichloride (Bu_2SnCl_2) spiked to fish at the levels of 0.2 and 1.0 ppm ranged from 80 to 105%. Detection limits were 0.02 μg/g for both compounds. Tri-*n*-butyltin compounds equivalent to Bu_3SnCl levels of 0.07-2.0 ppm and di-*n*-butyltin compounds equivalent to Bu_2SnCl_2 levels of 0.02-0.11 ppm were found in reared yellowtails, and these values showed good agreement with the results from gas chromatographic-mass spectrometric analysis.

Various tri-*n*-butyltin and di-*n*-butyltin compounds have been widely used as stabilizers of poly(vinyl chloride), fungicides, and marine antifoulants, and they are known to be aquatic environmental contaminants. Biological activity and metabolic fate of tri-*n*-butyltin compounds, Bu_3SnX (X = Cl, OAc, and $OSnBu_3$), are independent of X (1). It is also well known that tri-*n*-butyltin compounds degrade in aqueous environment to di-*n*-butyltin and *n*-butyltin compounds and further to inorganic tin by photolysis and biolysis (1, 2). Dealkylation reduces the toxicity of alkyltin compounds to aquatic organisms (3) and fungus (4).

We needed a simple and sensitive analytical method for butyltin compounds in fish as a means to monitor environmental pollution. A number of procedures have been reported for determining tri- and/or di-*n*-butyltin species in water and sediments: electron capture gas chromatography for hydrogenated alkyltin (5) or chlorinated alkyltin (6), atomic absorption spectrometry for hydrogenated alkyltin (7, 8), gas chromatography with flame photometric detection for tetraalkyltin (9, 10), and liquid chromatography for di-alkyltin–Morin complex (11). Only a few reports dealt with biological samples (7, 11).

Bis(tri-*n*-butyltin)oxide (TBTO), which has been used as the most popular antifoulant in marine farms in Japan, is easily converted to Bu_3SnCl during extraction with acidified solvent (7). We therefore considered an analytical method for Bu_3SnCl and Bu_2SnCl_2, which derive from TBTO and/or other butyltin species contained in fish. Among the available methods, gas chromatography appeared to be the most sensitive and effective for simultaneous detection of tri- and di-*n*-butyltin species.

Received March 11, 1987. Accepted July 14, 1987.

Although gel permeation chromatography (GPC) has been used as a cleanup procedure to remove fats and oils from food extracts for pesticides residue analysis, no application to organotin analysis has been reported. GPC based on molecular size separation is thought to be preferable for tri- and di-alkyltin species, which are polar and easily adsorb on column packings such as silica gel. This paper reports a sensitive method for the simultaneous determination of trace levels of tri- and di-*n*-butyltin compounds in fish, using extraction with acidified solvent, GPC cleanup, derivatization with Grignard reagent, and gas chromatography with flame photometric detection.

METHOD

Reagents and Apparatus

All chemicals were analytical reagent grade; organic solvents were distilled in glass before use; water was distilled.

(a) *Standards.*—Tri-*n*-butyltin chloride (Bu_3SnCl) (Sankyo Organic Chemicals Co., Ltd, Kawasaki, Japan) and di-*n*-butyltin dichloride (Bu_2SnCl_2) (Wako Pure Chemical Industries, Ltd, Osaka, Japan), purity >97.0%.

(b) *Grignard reagent.*—1M methylmagnesium bromide solution in tetrahydrofuran (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) and pentylmagnesium bromide solution in ethyl ether; prepare latter solution by adding 25 g *n*-pentylbromide to 4 g magnesium in 125 mL ethyl ether.

(c) *Gas chromatograph.*—Shimadzu Model GC-9A (Shimadzu Co., Kyoto, Japan) equipped with flame photometric detector operated in tin (Sn, 610 nm) and sulfur (S, 394 nm) modes; Shimadzu CBP 10 fused silica capillary column, 0.53 mm id × 12 m, coated with 1.0 μm film (Shimadzu Co.). Operating conditions: injection port, 250°C; detector, 300°C; column oven—initial 90°C, heated at 20°/min to 230°C, hold 5 min; helium carrier gas, 20 mL/min; hydrogen, 150 mL/min; air, 100 mL/min; sample injection volume, 3 μL; splitless injection mode; splitter opened after 30 s.

(d) *Automated GPC system.*—AUTOPREP gel permeation chromatograph (Analytical Biochemistry Laboratories, Inc., Columbia, MO) Model 1002A, with 60 g Bio-Beads S-X3 resin (Bio-Rad Laboratories, Richmond, CA), 200–400 mesh, in 25 mm id × 60 cm column compressed to ca 44 cm bed length. Methylene chloride–cyclohexane (1 + 1) eluting solvent pumped at constant flow rate of 5 mL/min.

(e) *Gas chromatograph/mass spectrometer (GC/MS).*—JEOL Model JMS-DX 300 (JEOL Ltd, Tokyo, Japan) equipped with CBP 10 capillary column as in (c), and operated in EI mode at 70 eV and 300 μA. Operating condi-

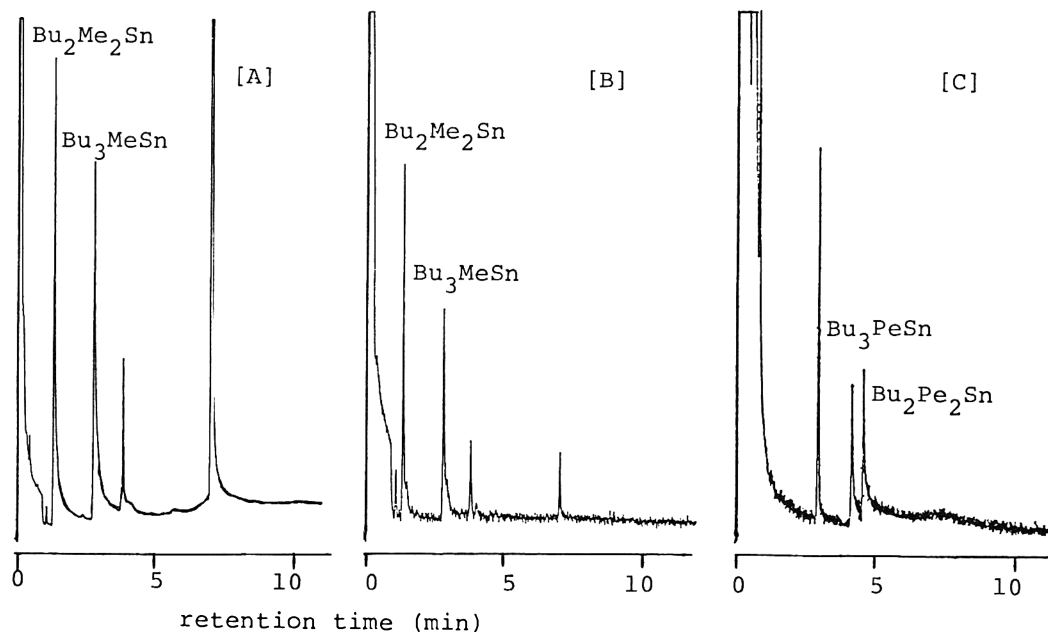


Figure 1. Gas chromatograms of methyl derivatives for S-mode (A) and Sn-mode (B), and pentyl derivatives for Sn-mode (C). Derivatives were prepared as described in the text; each is equivalent to 0.5 μg/mL of Bu_3SnCl or Bu_2SnCl_2.

tions: injection port, 240°C; column oven—initial 90°C, heated at 16°/min to 230°C, hold 5 min; separator, 200°C; ion source, 200°C; helium carrier gas, 10 mL/min. EI fragment ions m/z 193, 207, and 249 were monitored for determination of Bu_3MeSn, Bu_2Me_2Sn, and pentyl derivatives, respectively.

(f) *Samples.*—Fish were purchased from retail markets. Edible portions of tuna and yellowtails and whole body of horse-mackerel and pond smelt were used as analytical samples.

Extraction and Cleanup

Sample was extracted by slight modification of the method of Mishima et al. (7):

Homogenize fish sample with equal amount (w/v) of 0.5N HCl–methanol. Extract 10 g homogenate with 3 portions of 30 mL 0.5N HCl–methanol. Centrifuge each mixture at 3000 rpm for 5 min and concentrate combined supernate to ca 30 mL on rotary evaporator. Add 100 mL saturated aqueous NaCl and extract with three 40 mL portions of *n*-hexane. Centrifuge extracts 1 min at 2500 rpm, wash combined *n*-hexane layer with 0.1M $NaHCO_3$, and dry extract over anhydrous Na_2SO_4. Remove solvent on rotary evaporator at 35°C to just dryness. (Bu_3SnCl and methyl derivative, Bu_2Me_2Sn, are volatile; it is therefore necessary to concentrate sample solution to *just dryness* at low temperature throughout procedure.) Dissolve residue in 10 mL methylene chloride–cyclohexane (1 + 1) to give solution ready for GPC cleanup.

Load sample loop with 5 mL extract. Transfer sample from loop onto GPC column and elute with solvent mixture at 5 mL/min. Discard first 110 mL and collect second 65 mL in round-bottom flask.

Methylation

Evaporate eluate from GPC column to just dryness at 35°C on rotary evaporator and dissolve residue in 5 mL ethyl ether. Add 3 mL Grignard reagent and let stand ca 15 min. Add 10 mL water and 1 mL concentrated HCl while cooling in ice, and shake vigorously. Separate organic layer and extract aqueous layer with 3 mL ethyl ether. Add ca 0.2 g NaOH and ca 0.2 g $Na_2S_2O_3$ to combined ethyl ether extract and let stand 30 min; filter extract through paper (No. 2, Toyo Roshi Co., Ltd, Tokyo, Japan). Evaporate filtrate at 35°C to just dryness, dissolve residue in 1 mL *n*-hexane, and use 3 μL aliquots for GC and GC/MS analysis.

Prepare pentyl derivatives by same method used for methyl derivatives.

To obtain tetra-alkyltin standards for GC analysis, dissolve Bu_3SnCl and Bu_2SnCl_2 in *n*-hexane, alkylate with Grignard reagent, and process as described above.

For recovery test, add 0.5 mL acetone containing Bu_3SnCl and Bu_2SnCl_2 to 10 g homogenized sample just before extraction.

Results and Discussion

Electron capture (ECD) (5, 6), flame photometric (FPD) (1, 9, 10) and flame ionization (FID) (12) detectors have been employed in the gas chromatographic determination of trace levels of alkyltin compounds. ECD detects alkyltin hydrides and chlorides with high sensitivity but these compounds tend to adsorb on the column packing and severe sample cleanup is required for analysis by ECD/GC without detector deterioration. We used an FPD which is sensitive to tetra-alkyltins and needs only a simple cleanup compared with ECD. Figure 1A and B shows gas chromatograms of Bu_3MeSn and Bu_2Me_2Sn for FPD operated in the S and Sn modes, respectively. Figure 1C shows the gas chromatogram of Bu_3PeSn (Pe = pentyl) and Bu_2Pe_2Sn operated in the Sn mode. These tetra-alkyltins were prepared by the reaction of Bu_3SnCl or Bu_2SnCl_2 with Grignard reagents (methylmagnesium bromide and *n*-pentylmagnesium bromide). Some peaks derived from Grignard reagent appeared on each chromatogram but they did not interfere in the determination of organotins. Methyl derivatives showed high response and good shapes compared with *n*-pentyl derivatives (Figure 1B and C), so methyl derivatization was employed in our method.

Maguire and Huneault (10) reported that Bu_2Me_2Sn is fairly volatile and is lost during routine concentration procedures. Figure 2 shows that the loss of Bu_3SnCl and Bu_2Me_2Sn greatly increases as time after dryness increases. To prevent

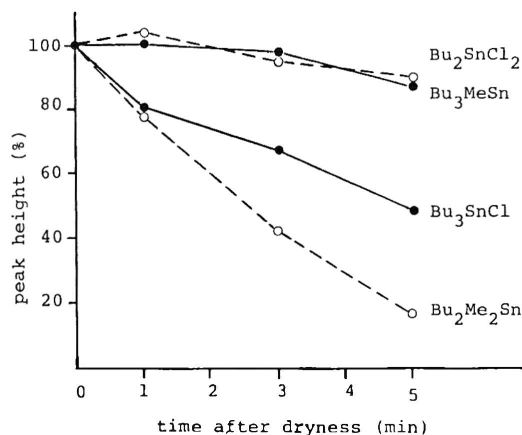


Figure 2. Stability of standards dissolved in hexane during concentration procedure. Hexane solutions of 2 μg each Bu_3MeSn, Bu_2Me_2Sn, Bu_3SnCl, and Bu_2SnCl_2 were evaporated by rotary evaporator at 35°C.

the loss of these compounds, concentration to just dryness at a low temperature was applied in the proposed method.

Aue and Flinn (13, 14) and Maguire and Huneault (10) used a modified flame photometric detector to increase sensitivity to organotins. We used a commercial detector, but replaced the S filter (394 nm) with an Sn filter (610 nm). This did not improve the sensitivity but did improve the shape of the tetra-alkyltin peaks (Figure 1A and B). Tailing of the tetra-alkyltin peaks was ascribed to the detector rather than the column (10, 13), so other columns were not considered. The FPD equipped with Sn filter gave exponential responses to both Bu_3MeSn and Bu_2Me_2Sn. The detection limits for Bu_3MeSn and Bu_2Me_2Sn were 0.1 ng, corresponding to about 0.02 μg/g each Bu_3SnCl and Bu_2SnCl_2 on a fish basis (whole body or edible portion), which is equivalent to the detection limits for the electron capture detector (5, 6).

Bu_3SnCl and Bu_2SnCl_2 were methylated by the method described by Mueller (9). Although the methyl derivatives Bu_3MeSn and Bu_2Me_2Sn in ethyl ether extract completely disappeared after standing for 7 days at room temperature without additional treatment, 70% of Bu_3MeSn and 103% of Bu_2Me_2Sn were recovered by retreatment of the ethyl ether solution with methylmagnesium bromide. Addition of about 0.2 g $Na_2S_2O_3$ or about 0.2 g NaOH to the ethyl ether solution prevented the loss of methyl derivatives during storage. Since halogen decomposes tetra-alkyltin (4), Br_2 yielded from the reaction of Grignard reagent with butyltin chlorides may have an effect on the stability of methyl derivatives. Sodium hydroxide exerts a debromination and neutralization effect on the reaction mixture.

Automated GPC system was used to clean up fish extracts. Figure 3 shows the elution profiles of fats, Bu_3SnCl, Bu_2SnCl_2, and some organochlorine pesticides subjected to GPC treatment. The elution volumes for fish oil and butter fat ranged from 70 to 120 mL. Bu_3SnCl and Bu_2SnCl_2 were eluted from the GPC system in the 120–150 mL and 140–170 mL fractions, respectively. The recovery of the former was 100% and that of the latter, 90%. The elution of these compounds preceded the organochlorine pesticides which eluted in the 150–200 mL fraction with recoveries ranging from 83 to 103%. The optimum dump fraction was determined to be 0–110 mL; the optimum collect fraction was 110–175 mL. Although the separation of butyltin chlorides from fat was not so good as that of organochlorine pesticides, the cleanup efficiency was sufficient enough for FPD/GC analysis. To exclude HCl, which is undesirable for the GPC system, fish extract was washed with 0.1M $NaHCO_3$ before GPC treatment.

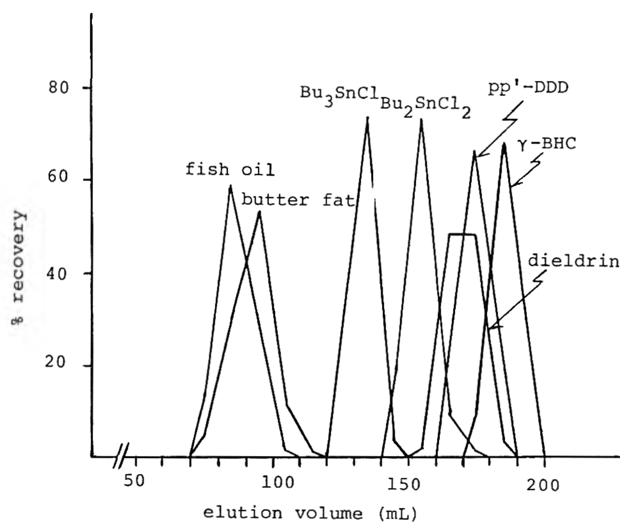


Figure 3. GPC elution profiles of fish oil, butter fat, Bu_3SnCl, Bu_2SnCl_2, and some organochlorine pesticides with methylene chloride–cyclohexane (1 + 1 v/v).

Recoveries of Bu_3SnCl and Bu_2SnCl_2 spiked to some fish at levels of 0.2 and 1.0 ppm are given in Table 1. Average recoveries of Bu_3SnCl and Bu_2SnCl_2 ranged from 80 to 104% and 92 to 105%, respectively. Levels of butyltin compounds of 0.01–0.02 ppm of Bu_3SnCl and Bu_2SnCl_2 were found in unspiked horse-mackerel and pond smelt. Tuna was not contaminated with these compounds. Extracts of fish matrix contained in the sample solution seemed to stimulate the recorder responses on GC analysis.

TBTO has been widely used as an antifoulant in fish farms; reared fish therefore are known to contain butyltin compounds at high levels compared with natural fish (15), so we analyzed reared yellowtails purchased from retail markets.

Table 1. Recovery of Bu_3SnCl and Bu_2SnCl_2 from spiked fish samples

Sample	Spiking level, μg/g	Recovery,[a] % Bu_3SnCl	Recovery,[a] % Bu_2SnCl_2
Tuna	0.2	94 ± 3	104 ± 2
	1.0	90 ± 2	105 ± 3
Horse-mackerel	0.2	104 ± 4	102 ± 6
	1.0	93 ± 6	101 ± 5
Pond smelt	0.2	80 ± 3	92 ± 8

[a] Average of triplicate analyses.

Table 2. Bu_3SnCl and Bu_2SnCl_2 levels in reared yellowtails determined as methyl and pentyl derivatives by FPD/GC and GC/MS[a]

Sample No.	Bu_3SnCl, ppm GC	Bu_3SnCl, ppm MS	Bu_2SnCl_2, ppm GC	Bu_2SnCl_2, ppm MS
1	0.67	0.58	0.11	0.16
2[b]	0.94 (0.89)	0.96 (0.94)	0.09 (−)	0.08 (0.07)
3	0.14	0.06	0.04	0.06
4	0.23	0.20	0.05	0.08
5	0.07	0.02	0.02	0.04
6[b]	2.04 (1.72)	2.40 (1.67)	0.11 (−)	0.13 (0.06)
7	0.26	0.31	0.04	0.06
8	0.53	0.65	0.06	0.09

[a] Each value is the result of a single determination. Results determined by FPD/GC (GC) and GC-mass fragmentography (MS) at m/z 193 (for Bu_3MeSn) and m/z 207 (for Bu_2Me_2Sn).

[b] Results determined as Bu_3PeSn and Bu_2Pe_2Sn; fragment ion was m/z 249.

Table 2 shows the analytical results of tri- and di-*n*-butyltin compounds in reared yellowtails with the same extraction and cleanup procedure, but with 2 detection methods, FPD/GC and GC/MS. Levels of tri- and di-*n*-butyltin compounds determined as Bu_3SnCl and Bu_2SnCl_2 in 8 samples were comparable with 2 detection methods. Correlation coefficients among results with FPD/GC and GC/MS were 0.99 and 0.91 for Bu_3SnCl and Bu_2SnCl_2, respectively. Pentyl derivatization was also applied on 2 samples, No. 2 and No. 6. Levels of tri- and di-*n*-butylin compounds determined as pentyl derivatives are in good accord with the results obtained from analysis of methyl derivatives. These 2 different alkyl derivatization procedures can be used as confirmation of tri- and di-*n*-butyltin compounds in samples containing these compounds at high levels.

TBTO and other tri-*n*-butyltin compounds which are contained in fish and are convertible to tri-*n*-butyltin chloride by the acidified extraction yield the same tetra-alkyltin derivatives. This is rather useful because the toxicity of tri-*n*-butyltin compounds (Bu_3SnX) is comparable with the structure of X (1) and the chemical forms of butyltin species in fish samples has not been clarified.

The method described here is efficient in that cleanup is simple, conventional FPD is used, and recovery is sufficient. It is suitable for residue analysis of tri- and di-*n*-butyltin compounds in fish.

References

(1) Maguire, R. J., Carey, J. H., & Hale, E. J. (1983) *J. Agric. Food Chem.* **31**, 1060–1065
(2) Maguire, R. J., & Tkacz, R. J. (1985) *J. Agric. Food Chem.* **33**, 947–953
(3) Vighi, M., & Calamari, D. (1985) *Chemosphere* **14**, 1925–1932
(4) Ingham, R. K., Rosenberg, S. D., & Gilman, H. (1960) *Chem. Rev.* **60**, 459–539
(5) Hattori, Y., Kobayashi, A., Takemoto, S., Takami, K., Kuge, Y., Sugimae, A., & Nakamoto, M. (1984) *J. Chromatogr.* **315**, 341–349
(6) Hattori, Y., Kuge, Y., & Nakamoto, M. (1984) *Bunseki Kagaku* **33**, E43–46
(7) Mishima, M., Maruyama, T., Kohiyama, M., Murakami, C., Kumagaya, M., Sumiyoshi, M., Asahi, T., Nozawa, T., Tanaka, Y., Ogawa, A., Matsuzuki, N., Nanpo, T., Ono, K., Takahashi, M., & Ito, K. (1984) *Bunseki Kagaku* **33**, T57–60
(8) Hodge, V. F., Seidel, S. L., & Goldberg, E. D. (1979) *Anal. Chem.* **51**, 1256–1259
(9) Mueller, M. D. (1984) *Fresenius Z. Anal. Chem.* **317**, 32–36
(10) Maguire, R. J., & Huneault, H. (1981) *J. Chromatogr.* **209**, 458–462
(11) Yu, T. H., & Arakawa, Y. (1983) *J. Chromatogr.* **258**, 189–197
(12) Hansen, D. R., Gilfoil, T. J., & Hill, H. H., Jr. (1981) *Anal. Chem.* **53**, 857–861
(13) Aue, W. A., & Flinn, C. G. (1977) *J. Chromatogr.* **142**, 145–154
(14) Aue, W. A., & Flinn, C. G. (1980) *Anal. Chem.* **52**, 1537–1538
(15) Chiba, H., Kato, M., Osawa, T., Kato, T., Seki, T., & Tsunoda, A. (1985) *Report of the Sendai Municipal Institute of Public Health*, **15**, 179–181

Intercomparative Study on the Determination of Polynuclear Aromatic Hydrocarbons in Marine Shellfish Tissue

JOHN F. UTHE and CHARLES J. MUSIAL

Department of Fisheries and Oceans, Marine Chemistry Division, Physical and Chemical Sciences Branch, PO Box 550, Halifax, Nova Scotia B3J 2S7, Canada

Twenty-five laboratories were sent 2 materials, one an acetone powder of lobster digestive gland, the other, the oil which had been extracted during preparation of the powder. Each laboratory was requested to measure the levels of a suite of polycyclic aromatic hydrocarbons in both materials. The response was poor with only 10 laboratories submitting results. Both intra- and interlaboratory precisions were poor; the interlaboratory error was so great as to preclude statistical analysis of the error. Relative standard deviations for oil results determined by liquid chromatography ranged from 39 to 96%.

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental contaminants, particularly in the near-shore marine environment because of various discharges and because of their use as wood preservatives in coastal marine structures (1–3). Many PAHs have been identified as carcinogens or cocarcinogens in a variety of test systems (1) and have, therefore, been a subject of analytical interest by both the Association of Official Analytical Chemists (AOAC) and the International Union of Pure and Applied Chemistry (IUPAC) (4). PAHs have been shown to accumulate in a variety of shellfish such as lobsters and mussels (3); levels 2 to 3 orders of magnitude above background have been reported in lobsters from areas receiving discharges from coal-coking facilities (5, 6).

The International Council for the Exploration of the Sea (ICES) has considered the problem of chemical contaminants in fish stocks since the early 1970s and has sponsored a number of intercomparative studies on the determination of chemical contaminants in marine compartments, including one on petroleum hydrocarbons (7). PAHs were included in the latter study but the interlaboratory agreement was extremely poor. A second study, dealing specifically with a selected number of nonalkylated PAHs, was sponsored by ICES (8), the results of which are the subject of this paper.

Received April 24, 1987. Accepted July 9, 1987.

Experimental

Preparation of Study Materials

Whole digestive glands were removed from American lobster (*Homarus americanus*) and stored frozen. The thawed glands were homogenized with ca 10 volumes of acetone (LC grade, Caledon Laboratories Ltd, Georgetown, Ontario) and stirred at room temperature overnight. Following filtration, the residue was reextracted twice with ca 5 volumes of fresh acetone. The filtered residue was air-dried under cover, broken, and ground in a parallel plate mill. After mixing, ca 5 g portions were bottled in solvent-washed, glass scintillation vials. The combined acetone extracts were extracted 3 times with 1–2 volumes of hexane (LC grade) after the addition of sufficient water (LC grade) to separate the phases. The combined hexane fractions were back-washed with water. Hexane was removed using a rotary evaporator cooled with dry ice–

Table 1. Laboratories submitting results and the methodologies used

Laboratory	Extraction; cleanup	Separation/measurement	
Continental Shelf Institute Hakon Magnussons gt. 1B N-7000 Trondheim Norway	KOH/cyclohexane; TLC on silica	GC/MS	DB-5/fused silica
Institut Scientifique et Technique des Pêches Maritimes Rue de l'Ile d'Yeu BP 1049, 44037 Nantes Cedex France	Soxhlet/pentane; Florisil	LC/UV	Micropack HCH5
Institute for Fishery Products TNO PO Box 183 1970 AD IJmuiden The Netherlands	KOH/hexane; Silica gel/alumina	LC/UV	P-E-PAH column
Woods Hole Oceanographic Institute Woods Hole, MA 02543 USA		GC/MS	SE-54 (DB-5) fused silica
Rijks-Kwaliteitsinstitut Postbus 230 6700 AE Wageningen The Netherlands	KOH/cyclohexane; Extraction with formic acid/caffeine, acid alumina	LC/UV	Lichrosorb 10 RP 18
NJIT Air Pollution Lab. 161 Warren St Newark, NJ 07012 USA	KOH/cyclohexane/powder; TLC on silica gel, oil not saponified	LC/UV	Vydac 201/TP54
U.S. Department of Commerce National Bureau of Standards Washington, DC 20234 USA	Sep-Pak/pentane; Bondapak NH	LC/UV	Vydac 201/TP54
Institute of Marine Research PO Box 1870—N 5011 Nordnes Bergen Norway	KOH/pentane; Kiselgel-60	GC/MS	SP-2100, fused silica
Institut für Meereskunde an der Universitat Kiel 23 Kiel, Dusternbrooker Weg 20 FRG	KOH/iso-octane; Florisil	GC/MS	SE52/capillary
Ministerie van Landbouw Rijksstation voor Zeevisserij 8400 Oostende, Ankerstraat 1 Belgium	KOH/iso-octane; Florisil	LC/UV (no quantitation)	RP-18
Halifax Fisheries Research Lab. PO Box 550 Halifax, Nova Scotia B3J 2S7 Canada	KOH/iso-octane; SX-3 size exclusion chromatography	LC/UV	Vydac 201/TP54

ethanol until a constant weight was obtained. After mixing, ca 3 g portions of oil were sealed in solvent-washed, glass vials (Pierce Chemical Co. Ltd, 5 mL capacity) fitted with Teflon-faced septa.

Intercomparative Study Protocol

During spring 1983, intercomparative kits were supplied to 25 participants who had requested them and agreed to measure a suite of designated PAHs, namely, phenanthrene, fluoranthene, benz[*a*]anthracene, benzo[*ghi*]perylene, indeno[1,2,3-*cd*]pyrene (*o*-phenylene pyrene), benzo[*a*]pyrene, pyrene, chrysene, and triphenylene. Participants were asked to report the concentrations on an "as received" basis. A cleanup method based on partitioning and use of Florisil chromatography (9) was included, although participants were permitted to use the method of their choice. Recipients were also requested to measure as many other PAHs as they could in the 2 materials and to analyze each material in triplicate. The final date for submitting results was December 31, 1984. The data are given exactly as reported by participants.

Results

The number of participants reporting usable results was disappointing, with only 10 of the original 25 recipients supplying quantitative information. One other participant reported that his methodology was not capable of resolving PAH compounds sufficiently for quantitation. One participant also reported that the oil contained relatively high concentrations of benzaldehyde and acetophenone along with a homologous series of methyl ketones from 2-nonanone to 2-tridecanone (M. Ehrhardt, Department of Marine Chemistry, University of Kiel, Kiel, Federal Republic of Germany). Percent recoveries were not reported by participants. Generally, analysts employed their own cleanup methodologies rather than the supplied one. The methodologies may be divided into 2 types: (*1*) liquid chromatography with ultraviolet absorption/fluorescence detection (LC/UV) and (*2*) capillary gas chromatography with mass spectometric detection (GC/MS) on cleaned up extracts (Table 1).

It is immediately apparent from inspection of the results

Table 2. Levels of individual PAH compounds (ng/g) in lobster digestive gland *OIL* reported by laboratories employing capillary gas chromatography-mass spectroscopy[a]

PAH	Lab. 1A	Lab. 1B	Lab. 3	Lab. 4A	Lab. 4B	Lab. 8	Range
Naphthalene	50.4	63.9	5.2	118.4	111.4	62	5.2–118.4
C_2-Naphthalenes	317.2	234.2	—	322.0	271.3	—	234.2–322.0
C_3-Naphthalenes	1026.7	519.7	—	467.9	425.3	—	425.3–1026.7
Phenanthrene	1650.4	994.4	852	1311.7	1459.5	672	672–1650.4
C_1-Phenanthrenes	5235.8	6055.5	—	1317.0	1342.7	—	1317.0–6055.5
C_2-Phenanthrenes	12 013.8	13 414.3	—	1796.2	1780.8	—	1780.8–13 414.3
Anthracene	1012.1	654.5	232	371.1	371.0	197	197–1012.1
Fluoranthene	1071.6	1167.1	6340	5696.2	4978.7	1967	1071.6–6340
Pyrene	903.7	968.4	3105	3555.0	3496.1	383	383–3555.0
Benz[*a*]anthracene	348.7	760.2	—	1365.4	1110.7	—	348.7–1365.4
Chrysene	17 541.8[b]	24 504.0[b]	2180	1977.9	2093.4	—	—
Benzo[e]pyrene	478.1	616.6	866	605.6	650.1	—	478.1–650.1
Benzo[*b*]fluoranthene	1791.1	2976.4	1920	2156.2[c]	1852.7[c]	—	—
Benzo[*a*]pyrene	521.6	655.8	1100	819.2	672.2	—	521.6–1100
Benzo[*ghi*]perylene	227.8	299.4	382	—	—	—	—
Indeno[1,2,3-*cd*]pyrene	421.6	502.9	—	—	—	—	—
Dibenzothiophene	28.4	39.4	38.2	149.6	142.3	42	28.4–149.6
C_1-Dibenzothiophene	227.8	187.5	—	381.0	358.0	—	187.5–381.0
C_2-Dibenzothiophene	1335.4	1203.1	—	1502.0	1285.5	—	1203.1–1502.0
Perylene	194.0	184.7	202	179.2	220.3	—	179.2–220.3
Benzo[*k*]fluoranthene	593.2	682.9	858	—	—	—	—
1-Methylnaphthalene	—	—	8.8	65.3	59.4	45	8.8–65.3
2,3,6-Trimethylnaphthalene	—	—	81.1	—	—	85	—
Acenaphthene	—	—	36.4	—	—	64	—
Fluorene	—	—	338	—	—	173	—
2-Methylphenanthrene	—	—	288	—	—	252	—
1-Methylphenanthrene	—	—	182	—	—	17	—
Dibenz[a,*h*]anthracene	—	—	—	—	—	—	—
Benzo[*j*]fluoranthene	—	—	—	—	—	—	—

[a] A and B are replicate analyses.
[b] Chrysene and triphenylene.
[c] Benzo[*b*]fluoranthene and benzo[*k*]fluoranthene.

for GC/MS analyses for the oil and powder (Tables 2 and 3, respectively) and for LC/UV analyses for oil and powder (Tables 4 and 5, respectively) that an extremely wide range of results has been reported for both methodologies and that the participants did not carry out the requested number of replicate determinations and did not report levels of all requested PAHs.

The small number and wide range in reported results precluded in-depth statistical analysis of the data.

Previous studies involving mussel homogenates (10) and sediments (11) yielded rather high estimates of intra- and interlaboratory relative standard deviations. Interlaboratory relative standard deviation for the mussel homogenate was estimated to be between 25 and 30% depending on the compound being measured, while that for the sediments was estimated to range from 4 to 28%. In both studies, the interlaboratory precision was estimated to be substantially lower (i.e., large interlaboratory relative standard deviation) than the intralaboratory precision. That this is the case with the present study is indicated by a comparison of the relative standard deviations for our own data (laboratory 11) with those calculated using a single result from each laboratory. Such a comparison could be done only for those laboratories reporting PAH concentrations in the oil as determined by LC/UV (Table 4). The limited amounts of data in the other 3 tables were judged to be insufficient for consideration. Our intralaboratory relative standard deviations ranged from 4.3 to 24.1% while the interlaboratory relative standard deviation ranged from 39 to 96%. Obviously, considerable analytical problems must be overcome before any cooperative research programs can be carried out or comparisons can be made of PAH data from different laboratories.

Some other observations may be made about this study:

(*1*) Laboratories utilizing GC/MS to measure PAH compounds reported levels of a greater number of compounds than did participants using LC/UV.

(*2*) Concentrations reported on the basis of LC/UV were generally higher than those based on GC/MS with the exception of benzo[*a*]pyrene and possibly indeno[1,2,3-*cd*]pyrene.

(*3*) A wide range in results was found irrespective of the analytical method employed.

(*4*) Many laboratories reported replicate results which differed substantially from each other, sometimes by more than 500%. In spite of this, many participants reported levels to several (an unjustified number of) significant figures.

(*5*) No laboratory submitted results based on both LC/UV and GC/MS. Therefore, no direct comparisons between the 2 methods were possible.

(*6*) The limited number and wide range in reported levels did not allow comparison to be made of extraction preceded or not preceded by saponification.

The fact that laboratories utilizing capillary GC/MS reported on more compounds than did LC/UV equipped participants probably reflects the interests of the laboratory and the availability of standards.

It is not surprising that benzo[*a*]pyrene levels determined by the 2 methodologies are in closer agreement than are results for other compounds since it is the PAH of major concern. The wide range in results from both methodologies suggests difficulty in obtaining accurate standard solutions of PAH for instrument calibration. An earlier study on the determination of chlorobiphenyls in marine oils (12) showed that many chemists were not able to prepare accurate rep-

Table 3. Levels of individual PAH compounds (ng/g) in lobster digestive gland acetone *POWDER* reported by laboratories employing capillary gas chromatography-mass spectroscopy[a]

PAH	Lab. 1A	Lab. 1B	Lab. 4A	Lab. 4B	Lab. 8	Range
Naphthalene	60.6	23.8	46.9	46.1	12	12–60.6
C_2-Naphthalenes	382.6	90.5	37.8	37.4	—	37.4–382.6
C_3-Naphthalenes	419.7	281.9	33.6	31.0	—	31.0–419.7
Phenanthrene	233.9	783.3	24.6	25.1	70	24.6–783.3
C_1-Phenanthrenes	320.5	1161.9	21.4	21.0	—	21.0–1161.9
C_2-Phenanthrenes	523.3	2038.1	13.5	13.7	—	13.5–2038.1
Anthracene	38.6	131.0	6.0	9.4	—	6.0–131.0
Fluoranthene	90.9	14.7	16.7	16.7	13	13–90.9
Pyrene	150.7	26.4	9.8	10.1	12	10.1–150.7
Benz[a]anthracene	0.8	7.3	2.9	2.9	—	0.8–7.3
Chrysene	113.7[b]	338.2[b]	7.4	7.3	—	—
Benzo[e]pyrene	55.4	11.1	—	—	—	—
Benzo[*b*]fluoranthene	40.6	10.6	—	—	—	—
Benzo[*a*]pyrene	4.6	2.7	—	—	—	—
Benzo[*ghi*]perylene	23.8	3.6	—	—	—	—
Indeno[1,2,3-*cd*]pyrene	4.4	2.0	—	—	—	—
Dibenzothiophene	58.6	6.1	2.1	2.2	—	2.1–58.6
C_1-Dibenzothiophene	175.4	20.1	4.9	6.2	—	4.9–175.4
C_2-Dibenzothiophene	418.7	78.6	9.7	11.3	—	9.7–418.7
Perylene	6.9	1.1	—	—	—	—
Benzo[*k*]fluoranthene	16.0	4.7	—	—	—	—
1-Methylnaphthalene	—	—	10.7	10.8	42	—
2,3,6-Trimethylnaphthalene	—	—	—	—	9	—
Acenaphthene	—	—	—	—	—	—
Fluorene	—	—	—	—	5	—
2-Methylphenanthrene	—	—	—	—	—	—
1-Methylphenanthrene	—	—	—	—	—	—
Dibenz[a,*h*]anthracene	—	—	—	—	—	—
Benzo[*j*]fluoranthene	—	—	—	—	—	—

[a] A and B are replicate analyses.
[b] Chrysene and triphenylene.

licate solutions from the small amounts of chlorobiphenyls usually purchased from suppliers, which is likely to be the case in this study as well.

Nevertheless, replicate values differing by as much as 500% cannot be explained by problems in preparing standard solutions. The reporting of such values, coupled with the observation on the number of significant figures, suggests carelessness as the root cause of the problem, i.e., analysts are basically honest but not particularly critical of their own results. The observation that GC/MS results were generally lower than LC/UV results suggests that the less efficient LC columns and the less selective detection system gave rise to positive interferences in the analysis. A comparison of results from saponification/extraction with those from simple extraction would be important in light of the reported necessity for saponification of insoluble samples such as meat and fish to ensure quantitative PAH removal from the tissue (13). However, it is obvious from both the reported results and our own that the major portion of the PAH content is present in the oil prepared by simple cold extraction without saponification. In the case of our own results, over 99% of the total PAH content of the 2 materials was present in the oil while less than 1% was present in the powder, probably as residuals remaining after extraction. Evidence for this is the high degree of correlation found between our PAH concentrations in the oil and those in the powder ($r = 0.975$; $r = 0.843$ after removal of the results for fluoranthene due to their high weight in the regression analysis). However, the results from other participants where sufficient data were available for study gave no significant correlations. Saponification of lobster digestive gland is not necessary for complete extractions of PAHs.

The results of this and earlier exercises have all been characterized by a large interlaboratory relative standard deviation. This must be compared to collaborative studies on foodstuffs, including smoked fish (13). Interlaboratory variation in these studies was small enough to allow the method employed in the study to be adopted as an AOAC official first action method in 1968, an AOAC official final action method in 1972, and an IUPAC commission-recommended method in 1975 (14). A general screening method for PAH (13) was adopted by the Commission on Food Additives, IUPAC, in 1976 (14). Howard and Fazio (14) believe that the use of capillary GC/MS is the only acceptable method for measuring the large number of PAHs present in most substrates due to the superior resolving capability of capillary gas chromatography and the superior detection specificity of mass spectrometry. The results of our study corroborate this conclusion.

Progress in achieving satisfactory interlaboratory error levels is likely to be made only by following a detailed standard method which has been shown to yield the required precision. It will also be necessary to ensure that standards and solutions of PAH are certified as to concentration (e.g., National Bureau of Standards SRM 1647, Priority Pollutant PAH (11)) or shown to be comparable. Obviously, certified reference materials for marine biological materials will be needed to allow laboratories to improve their methodologies to acceptable levels. It is necessary, however, to keep in mind that the use of a standard method of analysis may still present problems where levels of interferences vary from sample to sample; checks, using independent methods of analysis, will continue to be needed.

Acknowledgments

Thanks are due to J. M. Bewers and D. J. Scarratt for reviewing the manuscript and to the participants listed for their contributions to this study.

Table 4. Levels of individual PAH compounds (ng/g) in lobster digestive gland *OIL* reported by laboratories employing liquid chromatography-ultraviolet absorption/fluorescence[a]

	Lab. 2		Lab. 5		Lab. 6				Lab. 7		Lab. 9	Lab. 11			RSD,[b] %		RSD,[c]
PAH	A	B	A	B	A-1	A-2	B-1	B-2	A	B	A	A	B	C	(n = 3)	Range	%
Naphthalene	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
C_2-Naphthalenes	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
C_3-Naphthalenes	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
Phenanthrene	—	—	—	—	—	—	—	—	2300	2300	895	—	—	—			
C_1-Phenanthrenes	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
C_2-Phenanthrenes	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
Anthracene	254	245	—	—	—	—	—	—	210	240	—	—	—	—			
Fluoranthene	7466	7139	3580	3580	—	—	—	—	15 400	14 200	5650	6398	6739	6570	4.3	5650–15 400	59
Pyrene	—	—	—	—	2604.3	2838.4	2454.8	3086.1	8300	7500	3700	6146	5935	4962	10.8	2454.8–8300	49
Benz[a]anthracene	—	—	—	—	701.5	756.7	548.1	523.9	2300	2200	3680	1349	1163	1147	9.2	523.9–3680	64
Chrysene	—	—	—	—	—	—	—	—	1700	2800	—	638	675	973	24.1	638–2800	
Benzo[e]pyrene	—	—	940	940	1773.43	1756.44	720.64	928.7	—	—	—	3291	3410	2834	9.6	928.7–1773.43	—
Benzo[*b*]fluoranthene	1620	1592	1060	980	942.72	714.07	733.36	549.91	—	—	—	1818	1830	1834	4.6	549.91–1834	63
Benzo[a]pyrene	980	961	220	220	688.33	434.5	292.9	407.1	940	880	—	1078	952	886	10.0	220–1078	39
Benzo[*ghi*]perylene	325	319	152	182	140.3	152.84	188.2	159.5	—	—	—	<250	<250	<250		140.3–325	—
Indeno[1,2,3-*cd*]pyrene	68	68	440	500	—	232.17	218.96	239.24	1100	880	—	1682	1301	1100	21.3	68–1682	96
Dibenzothiophene	—	—	—	—	—	—	—	—	—	—	—	—					
C_1-Dibenzothiophene	—	—	—	—	—	—	—	—	—	—	—	—					
C_2-Dibenzothiophene	—	—	—	—	—	—	—	—	—	—	—	—					
Perylene	—	—	—	—	—	—	—	—	200	170	—	—					
Benzo[*k*]fluoranthene	810	813	720	780	565.2	661.41	387.88	393.9	1800	1400	3025	968	712	8324	15.3	565.2–1834	65
1-Methylnaphthalene	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
2,3,6-Trimethylnaphthalene	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
Acenaphthene	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
Fluorene	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
2-Methylphenanthrene	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
1-Methylphenanthrene	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
Dibenz[a,*h*]anthracene	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
Benzo[*j*]fluoranthene	—	—	<0.2	<0.2	145.95	106.1	187.22	174.78	—	—	—	—	—	—		20.2–187.22	

[a] A, B, and C are replicate analyses.
[b] Relative standard deviation, Lab. 11.
[c] Relative standard deviation for all participants, using first figure reported by that laboratory.

Table 5. Levels of individual PAH compounds (ng/g) in lobster digestive gland acetone *POWDER* reported by laboratories employing liquid chromatography-ultraviolet absorption/fluorescence

	Lab. 2		Lab. 5		Lab. 6						Lab. 9	Lab. 11		
PAH	A	B	A	B	A-1	A-2	A-3	B-1	B-2	B-3	A	A	B	Range
					—	—		—	—					
					—	—		—	—					
Naphthalene	—	—	—	—	—	—	—	—	—	—	—	—	—	
C_2-Naphthalenes	—	—	—	—	—	—	—	—	—	—	—	—	—	
C_3-Naphthalenes	—	—	—	—	—	—	—	—	—	—	—	—	—	
Phenanthrene	—	—	—	—	—	—	—	—	—	—	ND[b]	—	—	
C_1-Phenanthrenes	—	—	—	—	—	—	—	—	—	—	—	—	—	
C_2-Phenanthrenes	—	—	—	—	—	—	—	—	—	—	—	—	—	
Anthracene	<3	<3	—	—	—	163.0	—	—	85.5	—	—	—	—	
Fluoranthene	<10	<10	40	38	42.58	22.79	—	71.78	49.2	—	ND	10	16	10–40
Pyrene	—	—	—	—	84.9	156.13	109.68	—	—	—	ND	ND	ND	85.5–163.0
Benz[a]anthracene	—	—	—	—	127.88	—	39.7	113.5	131.32	—	ND	0.8	3.2	0.8–71.78
Chrysene	—	—	—	—	—	—	216.76	71.05	—	—	—	<8	<8	<8–216.76
Benzo[*e*]pyrene	—	—	6.6	6.3	—	21.04	117.0	49.94	38.89	—	—	ND	ND	6.3–131.32
Benzo[*b*]fluoranthene	<2	<2	12	10	22.25	29.46	74.79	54.0	60.34	—	—	1.1	3.2	10–74.79
Benzo[*a*]pyrene	<1	<1	2.3	1.0	15.54	11.11	33.9	42.04	42.9	47.45	—	0.6	1.1	0.6–49.94
Benzo[*ghi*]perylene	<1	<1	3.6	4.8			31.66			59.76	—	tr[c]	tr	3.6–60.34
Indelo[1,2,3-*cd*]pyrene	<6	<6	5.0	5.0			22.29			50.54	—	<3	<3	<3–50.54
Dibenzothiophene	—	—	—	—	—	—	—	—	—	—	—	—	—	
C_1-Dibenzothiophene	—	—	—	—	—	—	—	—	—	—	—	—	—	
C_2-Dibenzothiophene	—	—	—	—	—	—	—	—	—	—	—	—	—	
Perylene	—	—	—	—	—	—	—	—	—	—	—	—	—	
Benzo[*k*]fluoranthene	<2	<2	8.0	5.0	—	—	29.99	—	—	44.11	ND	1.1	3.2	1.1–29.99
1-Methylnaphthalene	—	—	—	—	—	—	—	—	—	—	—	—	—	
2,3,6-Trimethylnaphthalene	—	—	—	—	—	—	—	—	—	—	—	—	—	
Acenaphthene	—	—	—	—	—	—	—	—	—	—	—	—	—	
Fluorene	—	—	—	—	—	—	—	—	—	—	—	—	—	
2-Methylphenanthrene	—	—	—	—	—	—	—	—	—	—	—	—	—	
1-Methylphenanthrene	—	—	—	—	—	—	—	—	—	—	—	—	—	
Dibenz[*a,h*]anthracene	—	—	10	10	—	10.11	13.44	—	18.13	20.18	—	—	—	10–20.18
Benzo[*j*]fluoranthene	—	—	0.2	0.2	—	—	—	—	77.99	82.95	—	—	—	0.2–82.95

[a] A and B are replicate analyses.
[b] Not detected.
[c] Trace.

References

(1) National Academy of Sciences, U.S.A. (1972) *Particulate Polycyclic Organic Matter,* NAS, Washington, DC
(2) National Research Council of Canada (1983) *Polycyclic Aromatic Hydrocarbons in the Aquatic Environment: Formation, Sources, Fate and Effects on Aquatic Biota,* NRC Associate Committee on Scientific Criteria for Environmental Quality, Publication No. NRCC 18981, Ottawa, Ontario, Canada
(3) Uthe, J. F. (1979) "The Environmental Occurrence and Health Aspects of Polycyclic Aromatic Hydrocarbons," *Fish. Mar. Serv. Tech. Rep.* 914, 30 pp.
(4) Haenni, E. O. (1978) *Pure Appl. Chem.* **50,** 1763–1773
(5) Uthe, J. F., & Musial, C. J. (1986) *Bull. Environ. Contam. Toxicol.* **37,** 730–738
(6) Musial, C. J., & Uthe, J. F. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 462–466
(7) Law, R. J., & Portman, J. E. (1983) International Council for the Exploration of the Sea, Coop. Res. Rep. No. 117
(8) International Council for the Exploration of the Sea (1982) "Report of the 70th Statutory Meeting," C. Res. **4,** 1
(9) Sirota, G. R., Uthe, J. F., Robinson, D. G., & Musial, C. J. (1984) *Can. MS Rep. Fish. Aquat Sci.* No. 1758, 22 pp.
(10) Wise, S. A., Chelser, S. N., Guenther, F. R., Hertz, H. S., Hilpert, L. R., May, W. E., & Parris, R. M. (1980) *Anal. Chem.* **52,** 1828–1833
(11) MacLeod, W. D., Prohaskam, P. G., Gennero, D. D., & Brown, D. W. (1982) *Anal. Chem.* **54,** 386–392
(12) Uthe, J. F., & Musial, C. J. (1986) International Council for the Exploration of the Sea, Coop. Res. Rep. No. 136, pp. 81–90
(13) Grimmer, G., & Böhnke, H. (1976) *J. Assoc. Off. Anal. Chem.* **58,** 725–732
(14) Howard, J. W., & Fazio, T. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 1077–1104

Multi-Laboratory Study of Measurement of Chlorobiphenyls and Other Organochlorines in Fish Oil

JOHN F. UTHE, CHARLES J. MUSIAL, and RAJ K. MISRA[1]
Department of Fisheries and Oceans, Marine Chemistry Division, Halifax Fisheries Research Laboratory, Halifax, Nova Scotia B3J 2S7, Canada

Analysts participating in a multi-laboratory comparative study were asked to identify 4 chlorobiphenyls (CBs) supplied in neat form, to measure the amounts of these present in spiked and unspiked fish oil, to measure other CBs and organochlorines in the unspiked fish oil, and to compare results for their own standard solution and those for standard solutions prepared from the supplied CB compounds. Comparisons were done for a common supplied method and the individual methods used in each laboratory. Participants had no trouble identifying 2,2′,5,5′-tetrachlorobiphenyl, 2,2′,4,5,5′-pentachlorobiphenyl, and 2,2′,4,4′,5,5′-hexachlorobiphenyl. Most misidentified 2,2′,3,4,5-pentachlorobiphenyl. Approximately one-half of the quantitative comparisons between participants' standards and those prepared from the supplied CBs differed by more than 10%. Two standards prepared from one of the CBs differed by an average of 6.6% (range 0.0-24.5%). Recoveries of added CBs from the fish oil ranged from 24 to 294% for spikes of 63-85 ng/g with no clear distinction between results for the common method vs individual laboratory methods. The common methodology gave a lower coefficient of variation (CV) for most other CBs and organochlorines, but in most cases the CVs for the individual CBs were not smaller than that for Aroclor 1254 equivalents.

Technical mixtures of polychlorinated biphenyls (PCBs) have been shown to be widespread environmental contaminants (1). Measurement of PCBs has proven to be difficult in the past, presumably because of the unavailability of individual chlorobiphenyl (CB) standards and the use of packed-column gas chromatography (GC) which is inadequate for resolving PCB mixtures. Musial and Uthe (2) in an intercomparative study showed that the measurement of PCBs in fish oil spiked at a nominal concentration of 1–2 mg/kg was characterized by a large (25–50%) interlaboratory coefficient of variation (CV) even when participants used the same batch of PCB (Aroclor 1254) as a standard in their analysis. The measurement of CBs rather than equivalents of technical PCB formulations has been advocated (3) since the original study by Sissons and Welti (4). Recently, the availability of many individual CB compounds (5) and the application of capillary gas chromatography has led to reports of measurements of many, if not most, CBs present in environmental samples (6). However, in a small followup study using the same spiked fish oil, Uthe and co-workers (7) found that analysts who were measuring CBs at that time could not achieve a satisfactory degree of interlaboratory comparability for either CBs in fish oil or Aroclor 1254 itself. The inability of analysts to achieve satisfactory results with Aroclor 1254 indicates that fundamental analytical problems are present, because none of the variability could be ascribed to the variability characterizing trace analysis.

We investigated the problem further by requesting collaborators to identify and measure the concentrations of 4 CBs in a spiked fish oil. They were also requested to determine concentrations of CBs and other common organochlorines in the unspiked oil. To overcome problems of misidentification of the CBs by the various participants using their own CB standards, small amounts of each of the 4 pure CB compounds were distributed to each participant.

Received April 24, 1987. Accepted July 17, 1987.
[1] Biological Sciences Branch.

Experimental

Commercial herring oil from cooked, pressed herring (*Clupea harengus harengus*) was clarified by centrifugation. Four CBs, obtained from a commercial source, were added to a portion of the oil in measured amounts. Following the numerical assignations of Ballschmiter and Zell (8), they were: 2,2′,5,5′-tetrachlorobiphenyl (No. 52); 2,2′,3,4,5-pentachlorobiphenyl (No. 86); 2,2′,4,5,5′-pentachlorobiphenyl (No. 101); and 2,2′,4,4′,5,5′-hexachlorobiphenyl (No. 153). At the same time, the company supplying these CB compounds prepared vials containing 5–20 mg of neat chlorobiphenyl. Each was obtained from a single batch lot. CB No. 86 was inadvertently supplied by the company rather than the ordered No. 87 (2,2′,3,4,5′-pentachlorobiphenyl). This was not thought to pose a serious problem, although this chlorobiphenyl is present in commercial PCB mixtures only in low concentration (9).

Participants were requested to prepare standard solutions of each CB, identify each, and compare the chromatographic responses for the prepared standards with those for their own standards (3 injections of each). One CB standard (No. 101) was used to compare 2 separately prepared standard solutions (3 injections of each). In addition to determining the concentrations of the 4 CBs in the spiked and unspiked oils, participants were requested to determine other CBs and common organochlorines in the unspiked oil, using their own methodologies as well as a common cleanup methodology supplied with the intercomparison kits. This latter method was our routine one (10) which had been slightly modified from that of Reynolds and Cooper (11): CBs and many other organochlorines are separated from the fish oil by chromatography on H_2O-deactivated Florisil. Enough PR grade Florisil was supplied with the kits to allow participants to perform the determinations. Participants were also requested to calibrate their muffle furnaces by using the melting point of zinc pellets supplied in the kits. All results were reported on an "as-received" basis after quantitation on the basis of external standardization and peak height or peak area. Many participants used an internal standard to correct for injection variability and procedural losses.

Statistical Considerations

This report presents information from data expressed in ratios. However, statistical difficulties are encountered when ratio data are analyzed for tests of null hypotheses (12–16). In this presentation, the treatment of ratio data has, therefore, been restricted to the descriptive level, i.e., inferences have been drawn from the usual observation of summary statistics and are not based on formal statistical tests, except for Table 5. We recognize that quantitative chemical data are invariably presented in ratios of some sort, e.g., % by weight, parts per thousand, parts per million. The statistical difficulties arise when significant variances are associated with both the numerator and the denominator. One disadvantage with this type of ratio is that its distribution is unusual, perhaps far from normal, whereas the basic assumption underlying most tests of hypotheses is that the errors follow a normal distri-

Table 1. Comparison ratio of response factors of laboratory CB standards and standards prepared from supplied chlorobiphenyls[a]

Measurement	Total N	Number of laboratories (%)	
		Within 10%	Greater than 10%
Peak height	31	16 (52)	15 (48)
Peak area	35	19 (54)	16 (46)
		Within 20%	Greater than 20%
Peak height	31	24 (77)	7 (23)
Peak area	35	27 (77)	8 (23)
		Within 30%	Greater than 30%
Peak height	31	25 (81)	6 (19)
Peak area	35	28 (80)	7 (20)

[a] Comparison to selected tolerances (peak height and peak area comparisons used as independent comparisons because all analysts did not submit both peak height and peak area data).

Table 2. Interlaboratory performance[a] using peak height or peak area of 2 standard solutions prepared from 2 weighings of CB No. 101

Number of laboratories/Total number of laboratories				
% Difference	Peak height	Peak area	Laboratories reporting height and area	Laboratories with errors in both
> 2%	16/19	13/18	14	12
> 5%	10/19	8/18	12	4
> 10%	4/19	4/18	4	3

[a] Response factor difference/mean response factor × 100.

bution. It is our intention to present a further paper that will include an account of statistical difficulties encountered in analyzing ratio data and a detailed statistical analysis of this data set which avoids the use of ratios.

Results and Discussion

Identification of Chlorobiphenyls

Excluding ourselves, 17 of 29 participants identified the 4 CBs by chemical structure; an additional 4 identified only the number of chlorine atoms in the molecules. Most analysts used retention time criteria, but some also used mass spectroscopy. All 17 identified 3 of the 4 CBs—Nos. 52, 101, and 153. Considerable difficulty was encountered with respect to No. 86 which was correctly identified by only 2 participants. One other participant identified it as either No. 86 or No. 119. Other reported identities were Nos. 87, 97, 113, 116, and 119. Such misidentification indicates that it is not possible to recommend measurement of all or many CBs until certified CB standards are available, and unambiguous separation or detection systems can be used.

Preparation of Quantitative (Standard) Chlorobiphenyl Solutions

All participants used electron-capture detection systems for quantitation. Approximately one-fifth of the comparisons between the laboratory's own standard and the one prepared from the supplied kit differed by more than 30%, one-fourth by more than 20%, and one-half by more than 10% (Table 1). All comparisons of 2 of 14 laboratories differed by more than 30%. The underlying reason for the large discrepancies is likely to be improper preparation of the standards (weighing error) rather than factors such as solvent evaporation; adequate methods for the preservation of organochlorine solutions have been described (17, 18) and substantial amounts of solvent would have to have been lost for errors of such large magnitude. Also, all participants correctly identified the 3 CBs used in the intercomparison study, so differences cannot have been due to comparisons of different CBs. Purity of the supplied CBs was in excess of 95%. Purity of the participants' standards is unknown and may be responsible for some fraction of the observed errors.

Support for weighing error as the main source of differences between standards is found in the results for comparisons of 2 solutions prepared from separately weighed amounts of CB No. 101 (Table 2). Generally, the 2 standards differed by more than 2% with an average difference of 6.6% and a range of 0.0–24.5%. Contributors to the total error are errors associated with weighing and dilution and the error associated with the GC step. Dilution should not be a major error, and the results (3 injections of each standard) showed that chromatography was carried out consistently. Therefore, in cases where the difference exceeded 5%, the major error is most likely from preparation of the standard solution. This indicates that analysts are having difficulty preparing quantitative standard solutions from the small amounts (5–20 mg) of CBs commonly purchased. The observation that some laboratories could prepare replicate solutions of acceptable reproducibility suggests that the use of good laboratory practice, particularly in balance maintenance and use, can result in the preparation of standard solutions of acceptable quality.

Recoveries of Added Chlorobiphenyls from Herring Oil

Recoveries of added CBs were in the ranges 30–127%, 28–178%, 41–294%, and 44–283% for CB Nos. 52, 86, 153, and 101 by the common methodology, and 24–127%, 28–174%, 28–189%, and 24–132% (Table 3) by the laboratories' own methodologies. The results for CB No. 86 are included since it was felt that the problem of misidentification would not affect recovery studies when the same CB was being used for standardization and quantitation. There was no obvious advantage associated with the use of a common cleanup methodology. Results from packed-column GC are not noticeably different from capillary column results, since spiked and unspiked oils were analyzed and all 4 CBs were well separated on packed columns. It is likely that prior experience in CB trace analysis is an important factor.

Data analysis as described by Youden and Steiner (19) (Table 4) as well as large values for intralaboratory correlation (20) showed, as expected, that most of the total error associated with the results for CB Nos. 52 and 101 was due to the contribution from systematic or interlaboratory error (bias). There was no clear distinction in results between the laboratories' own cleanup methodologies and the common methodology. In the case of CB No. 101, as determined by the laboratories' own method, total error was composed of about equal amounts of systematic and random error. The lack of significant decrease in the data variances associated with the common methodology should not be construed as proof that the use of a common methodology could not result in an improved intercomparability. It more likely reflects the inexperience of the analysts with the common cleanup method.

Other Organochlorines in Unspiked Fish Oil

Analysts reported concentrations of a number of CBs other than the 4 supplied and other organochlorines in the unspiked oil (Table 5). These results allow comparison with results reported in an earlier intercomparative study (2). In general, the CVs for about one-half of the chlorobiphenyls and 5 of 6 other organochlorines determined by the common meth-

Table 3. Percent recovery of individual CBs added to herring oil[a]

	Recovery, %							
	Common methodology				Laboratory methodology			
Lab.[b]	CB No. 52	CB No. 86	CB No. 153	CB No. 101	CB No. 52	CB No. 86	CB No. 153	CB No. 101
1 P	52	28	62	49	79	56	68	42
2	82	82	98	49	112	133	108	100
3 a	91	81	79	108	127	123	92	129
3 b	83	—	78	78	—	—	126	132
5 a	88	82	80	88	109	77	72	—
5 b	—	112	98	112	—	75	88	—
5 c	—	140	148	204	—	110	119	—
6	91	92	94	86	86	85	58	69
7 P	101	45	114	146	107	56	58	83
9	—	—	—	—	48	56	71	124
10 a	110	132	294	283	82	80	84	71
10 b	—	—	—	—	87	94	92	78
12	84	91	85	97	85	101	85	68
13 a	—	96	61	79	—	92	68	79
13 b	—	121	74	106	—	112	78	41
14	—	—	—	—	89	95	106	81
15 a	79	73	84	92	—	—	—	—
15 b	63	62	72	97	—	—	—	—
16	127	29	92	110	—	—	—	—
17	85	91	95	88	92	91	90	88
18	—	121	224	103	—	—	—	—
19	39	65	49	44	70	92	42	76
20	96	104	86	83	90	108	91	96
21	110	103	129	128	59	125	35	67
22	49	76	63	103	65	72	66	96
23 a P	95	—	95	100	115	112	92	105
22 b P	77	94	88	90	93	79	84	86
24	56	178	59	62	76	174	53	52
25	30	127	58	56	35	147	60	62
26	66	71	60	78	105	84	68	76
27 a	—	—	—	—	—	—	—	—
27 b P	—	—	—	—	—	—	—	—
27 c	72	95	88	87	—	—	—	—
27 d P	95	—	95	262	—	—	—	—
28	102	82	41	68	520	29	189	114
29	—	—	—	—	24	28	28	24
30	122	116	106	117	—	—	—	—

[a] Reported concentration in spiked oil minus reported concentration in unspiked oil/concentration added to oil × 100. CB No. 52 = 82 ng/g oil; CB No. 86 = 77 ng/g oil; CB No. 153 = 85 ng/oil; CB No. 101 = 63 ng/g oil.
[b] Letters a, b, c refer to results on basis of different chromatographic analytical methodologies; P = packed-column chromatography.

odology were lower than those determined by individual laboratory methodologies. However, the variation is still large, in many cases larger than that associated with the determination of PCB concentration using Aroclor 1254 as a standard (2) (Table 5). The results for other organochlorines, measured by capillary GC, are characterized by a CV less than that reported by Uthe and Musial (21) from a similar study on fish oil by packed-column GC but are still too large to allow interlaboratory cooperative studies in marine environmental contamination programs. These results imply

Table 5. Mean concentrations of CBs in unspiked herring oil as determined with participants' own CB standards[a]

	Concn, ng/g					
CB No. or organochlorine	Common methodology			Laboratory methodology		
	Mean ± SD	RSD	*N*	Mean ± SD	RSD	*N*
28	51.3 ± 6.8	(13)	4	65.6 ± 24.4	(37)	5
44	23.9 ± 6.8	(28)	4	37.8 ± 14.9	(39)	5
49	20.2 ± 8.1	(40)	4	31.6 ± 22.4	(71)	4
52	51.3 ± 9.0	(18)	5	61.0 ± 13.6	(22)	5
70	38.0 ± 8.3	(22)	4	43.0 ± 12.9	(30)	6
87	28.0 ± 27.7	(81)	3	—		—
95	85.5 ± 41.8	(49)	5	68.8 ± 15.6	(23)	4
97	14.9 ± 6.8	(46)	4	22.3 ± 15.2	(68)	5
101	52.8 ± 12.4	(23)	8	63.0 ± 17.1	(27)	8
107	44.2 ± 25.9	(59)	3	24.7 ± 9.1	(37)	3
110	39.8 ± 13.0	(33)	4	42.1 ± 3.8	(9)	4
128	11.0 ± 7.3	(66)	8	16.9 ± 16.5	(98)	8
138	57.4 ± 20.1	(35)	11	72.3 ± 19.0	(26)	10
151	28.6 ± 20.5	(72)	3	24.2 ± 17.0	(70)	3
153	82.6 ± 32.9	(40)	7	87.1 ± 39.5	(45)	8
170	25.1 ± 36.0	(143)	4	7.6 ± 1.6	(21)	4
180	23.2 ± 13.4	(58)	3	32.3 ± 21.9	(68)	6
187	14.6 ± 5.1	(35)	3	16.1 ± 3.3	(20)	4
194	3.0 ± 2.0	(67)	3	2.8 ± 1.6	(57)	3
201	3.4 ± 1.4	(41)	3	3.3 ± 1.1	(33)	3
PCB[b]	1.05 ± 0.36	(34)	21	—		—
α-BHC	95.8 ± 27.6	(29)	4	112.8 ± 32.1	(28)	6
γ-BHC	—		—	12.4 ± 9.6	(77)	7
Hexachlorobenzene	21.7 ± 12.3	(57)	7	37.4 ± 33.5	(90)	8
Dieldrin	—		—	94.8 ± 52.0	(55)	4
p,p'-DDT	104.8 ± 36.6	(35)	5	95.5 ± 41.2	(43)	7
p,p'-DDE	161.6 ± 50.1[c]	(35)	8	188.5 ± 68.7[c]	(37)	11
α-Chlordane	91.3 ± 19.0	(21)	3	66.8 ± 28.8	(43)	4
trans-Nonachlor	60.0 ± 26.6	(44)	4	74.5 ± 36.7	(49)	4

[a] Only chlorobiphenyls and other organochlorines which at least 3 laboratories reported are considered here.
[b] Analyzed as Aroclor 1254 or equivalent formulation.
[c] Removal of results for one packed column gave common method mean of 152.6 ± 46.5 (30%) and laboratory mean of 178.9 ± 65.3 (40%).

that systematic errors are still a major problem in measurement of trace organochlorines and that the poorer resolving power of the packed-column chromatograph compared with capillary systems was not the major underlying factor for the poor interlaboratory comparability observed in earlier studies (2, 22, 23). Holden and colleagues (23) have suggested that restrictions might be needed on analysts' choice of methodologies. Tuinstra and co-workers (24) have studied the use of a detailed, comprehensive methodology for measuring 6 CBs. In that study, quantitative standard solutions of the 6 CBs were supplied to the participants, and GC conditions and columns were specified. The CV was about 14% at the 1 mg CB/kg level and about 23% at the 0.1 mg CB/kg level, comparable with the spiking level used in the present study. The wider variation found in our results for organochlorines in the unspiked oil by the common method is likely due to

Table 4. Variances associated with determination of supplied CBs[a]

	Common method			Laboratory method		
All participants	CB No. 52	CB No. 153	CB No. 101	CB No. 52	CB No. 153	CB No. 101
S_r^2 (precision variance)	203	898	758	235	170	1278
S_d^2 (variance of data)	6920	5866	10 661	6730	3116	4344
F (S_d^2/S_r^2)	34.90*	6.53*	14.06*	28.64*	18.33*	3.55
df	1,20	1,22	1,23	1,20	1,21	1,21
S_b^2 (variance of systematic error)	3358	2484	4952	3247	1473	1528

* Significant at 5% level.
[a] $S_d^2 - 2S_b^2 + S_r^2$ as defined by Youden and Steiner (19).

the inexperience of the participants with the common method and the use of individually prepared standard solutions.

Three other points were noted. Observation of chromatograms submitted by the participants showed that the baseline from the electron-capture detector was generally far less than ideal, characterized by the presence of negative responses (negative peaks) and a general profile that suggested the presence of a plethora of detector-sensitive material similar to the unresolved complex mixture underlying petroleum residue determinations (25). The presence of such materials in cleaned up sample extracts, able to interfere either positively or negatively in the analysis, is another major problem in organochlorine measurement. Second, although participants agreed to perform all of the requested investigations, a number did not comply. Furthermore, the questionnaire used in this study had certain ambiguities because of the multinational nature of the participants. Finally, some instances of simple mathematical errors occurred. Although embarrassing to the individuals, they serve as a caution to all analysts. However, the incidence of this type of error was lower than the 1 of 4 reported in an earlier interlaboratory study of trace mercury determination (26).

In conclusion, the results of this study have shown that the comparability of measurements of chlorobiphenyls and other organochlorines at an international level is rather poor, and for certain compounds, unacceptable. Causes of error range from poor preparation of standard solutions, to use of various cleanup methodologies resulting in analytical solutions that still contained interfering materials, to calculation errors. It is likely that much standardization and the use of certified reference standards and materials will be required to achieve comparability among laboratories in these measurements.

Acknowledgments

The following laboratories participated in the study:

Institute for Sea Research, Oostende, Belgium

Bermuda Biological Station for Research, Bermuda

Atlantic Research Laboratory, National Research Council of Canada, Halifax, Nova Scotia, Canada

Health Protection Branch, National Health and Welfare, Halifax, Nova Scotia, Canada

Marine Ecology Laboratory, Bedford Institute of Oceanography, Dartmouth, Nova Scotia, Canada

State Chemical Supervision Service, Lyngby, Denmark

Institute of Thermophysics and Electrophysics, Academy of Sciences of Estonian SSR, Tallinn, Estonian SSR

Centre National pour L'exploitation des Océans, Centre Oceanologique de Bretagne, Brest, France

Institute Scientifique et Technique des Pêches Maritimes, Nantes, France

Institute of Marine Research, Helsinki, Finland

Academy of Sciences of the German Democratic Republic, Institute for Sea Research, Warnemünde, German Democratic Republic

Institute for Environmental Studies, Free University, Amsterdam, The Netherlands

Netherlands Institute for Fishery Investigations, IJmuiden, The Netherlands

State Institute for Quality Control of Agricultural Products, Wageningen, The Netherlands

Institute for Marine Research, Directorate of Fisheries, Bergen, Norway

National Veterinary Institute, Oslo, Norway

Veterinary Hygiene Research Station, Gdansk, Poland

National Swedish Environment Protection Board, Special Analytical Laboratory, University of Stockholm, Wallenburg Laboratory, Stockholm, Sweden

University of Stockholm, Wallenburg Laboratory, Stockholm, Sweden

Texas A&M University, Department of Chemistry, College Station, TX

Environmental Research Laboratory, Environmental Protection Service, Narragansett, RI

National Marine Fisheries Service, Milford, CT, Gloucester, MA, and Seattle, WA

Woods Hole Oceanographic Institute, Woods Hole, MA

References

(1) Jensen, S. (1966) *New Scientist* **32**, 612
(2) Musial, C. J., & Uthe, J. F. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 22–31
(3) Duinker, J. C., Hillebrand, M. T. J., Palmork, K. H., & Wilhelmsen, K. (1980) *Bull. Environ. Contam. Toxicol.* **25**, 956–964
(4) Sissons, D., & Welti, D. J. (1971) *J. Chromatogr.* **60**, 15–32
(5) Mullin, M. D., Pochini, C. M., McCrindle, S., Romkes, M., Safe, S. H., & Safe, L. M. (1984) *Environ. Sci. Technol.* **18**, 468–476
(6) Duinker, J. C., & Hillebrand, M. T. J. (1983) *Bull. Environ. Contam. Toxicol.* **31**, 25–32
(7) Uthe, J. F., Musial, C. J., & Palmork, K. H. (1981) Statutory Meeting paper C.M. 1981.E:49, International Council for the Exploration of the Sea, Copenhagen, Denmark
(8) Ballschmiter, K., & Zell, M. (1980) *Fresenius' Z. Anal. Chem.* **302**, 20–31
(9) Duinker, J. C., & Hillebrand, M. T. J. (1983) *Environ. Sci. Technol.* **17**, 449–456
(10) Musial, C. J., Uthe, J. F., Wiseman, R. J., & Matheson, R. A. (1979) *Bull. Environ. Contam. Toxicol.* **23**, 156–261
(11) Reynolds, L. M., & Cooper, T. (1975) *Water Quality Parameters*, ASTM STP 573, American Society for Testing and Materials, Philadelphia, PA, pp. 196–205
(12) Blackith, R. E., & Reyment, R. A. (1971) *Multivariate Morphometrics* Academic Press, New York, NY
(13) Draper, N., & Smith, H. (1981) *Applied Regression Analysis*, John Wiley & Sons, New York, NY
(14) Kendall, M. G., & Stuart, A. (1958) *The Advanced Theory of Statistics*, Vol. 1, Charles Griffin and Co., London, UK
(15) Misra, R. K., & Ni, I. H. (1983) *Can. J. Fish. Aquat. Sci.* **40**, 1507–1511
(16) Pimentel, R. A. (1979) *Morphometrics*, Kendall/Hunt Publishing Co., Dubuque, IA
(17) Hodgson, D. W., & Watts, R. R. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 89–93
(18) Hodgson, D. W., Thompson, J. F., & Watts, R. R. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 94–102
(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*, Iowa State Univ. Press, Ames, IA, 507 pp.
(21) Uthe, J. F., & Musial, C. J. (1982) Cooperative Research Report No. 115, International Council for the Exploration of the Sea, Copenhagen, Denmark
(22) Sawyer, D. L. (1973) *J. Assoc. Off. Anal. Chem.* **56**, 1015–1023
(23) Holden, A. V., Topping, G., & Uthe, J. F. (1983) *Can. J. Fish. Aquat. Sci.* **40**(Supp. 2), 100–110
(24) Tuinstra, L. G. M. Th., Roos, A. H., & Werdmuller, G. A. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 756–759
(25) Farrington, J. W., Albages, J., Burns, K. A., Dunn, B. P., Eaton, P., Laseter, J. L., & Wise, S. (1980) *The International Musselwatch*, Ch. 2, National Academy of Sciences, Washington, DC, p. 777
(26) Uthe, J. F., Armstrong, F. A. J., & Tam, K. C. (1971) *J. Assoc. Off. Anal. Chem.* **54**, 866–869

Determination of Inorganic Tin in Biological Samples by Hydride Generation-Atomic Absorption Spectrometry after Silica Gel Cleanup

TAIZO TSUDA, MINORU WADA, SHIGERU AOKI, and YOSHIHIRO MATSUI
Shiga Prefectural Institute of Public Health and Environmental Science, 13-45, Gotenhama, Ohtsu, Shiga 520, Japan

A method is described for the determination of inorganic tin in biological samples by hydride generation-atomic absorption spectrometry (HG-AAS). A sample is extracted with ethyl acetate after addition of HCl and NaCl. The concentrated extract is passed through a silica gel column. The column is washed with ethanol, water, and 0.2N HCl successively, and then inorganic tin is eluted with 2N HCl and measured by HG-AAS. Recoveries from fish muscle spiked with 0.1 μg/g Sn^{4+} are 78.9 ± 4.2% (average ± standard deviation, n = 5). The detection limit is 0.01 μg/g as Sn.

In view of the increasing pollution by organotin compounds, methods of analyses are needed to measure the concentrations of inorganic tin produced by metabolism or degradation of these compounds in biological and environmental samples. At present, inorganic tin is determined in biological samples, together with butyltin or phenyltin species from the metabolism of tributyltin or triphenyltin compounds, by atomic absorption spectrometry (AAS) (1, 2) or gas chromatography (3, 4). However, if one is interested in determining only inorganic tin in biological samples, these methods are tedious and it is impossible to detect low tin concentrations.

We have developed a simple and sensitive method for the determination of inorganic tin in biological samples by hydride generation–atomic absorption spectrometry (HG-AAS). In this method, inorganic tin is extracted with ethyl acetate from biological samples after acidification with HCl. If necessary, butyltin or phenyltin species can be simultaneously determined from a portion of the ethyl acetate extract by our previous method (5).

METHOD

Apparatus and Reagents

(**a**) *Flameless atomic absorption spectrometer.*—Equipped with electrically heated quartz cell atomizer (Model AA-855 HYD-1.2, Nippon Jarrell-Ash Co. Ltd, Kyoto, Japan). Operating conditions: analytical wavelength 224.6 nm, lamp current 10 mA. *Hydride generator HYD-1.*—$NaBH_4$ solution: feed rate 10 mL/min, concentration 1% (in 12% NaOH). HCl solution: feed rate 10 mL/min, concentration HCl–H_2O (1 + 5). Sample solution: feed rate 20 mL/min. Ar flow: carrier I 0.15 L/min, carrier II 1.0 L/min. *Hydride atomizer HYD-2.*—Temperature 1100°C.

(**b**) *Reagents.*—Tin(IV) chloride pentahydrate ($SnCl_4 \cdot 5H_2O$) (Nakarai Chemicals Ltd, Kyoto, Japan); tin(II) (Sn^{2+}), 1000 μg/mL in 6N HCl (Wako Pure Chemical Industries Ltd, Osaka, Japan); butyltin and methyltin chlorides, reagent grade, used without further purification.

(**c**) *Silica gel.*—Kiesel gel 60, 70-230 mesh (Merck), used for column cleanup.

(**d**) *Standard solutions.*—*Standard stock solution.*—1000 μg Sn^{4+}/mL, prepared by dissolving 293 mg $SnCl_4 \cdot 5H_2O$ in 100 mL 10N HCl. *Working standard solutions.*—0.005–0.05 Sn^{4+} μg/mL, prepared by diluting standard stock solution with 2N HCl.

Received May 6, 1987. Accepted August 14, 1987.

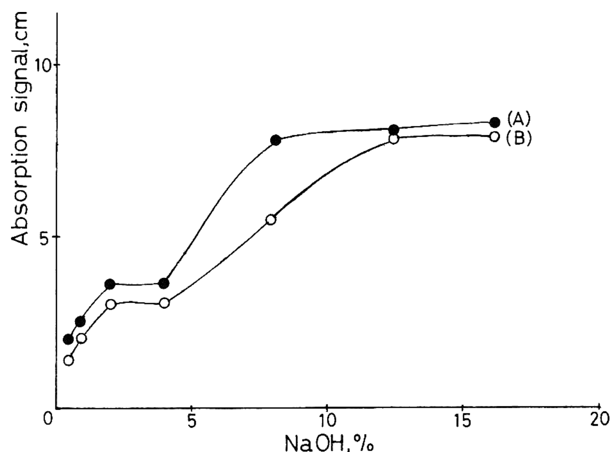


Figure 1. Effect of NaOH concentration in 1% $NaBH_4$ solutions on atomic absorption signal of tin: 0.1 μg Sn^{4+}/mL in A, HCl–H_2O (1 + 5); B, HCl–H_2O (1 + 2).

Determination of Inorganic Tin in Biological Samples

Weigh 10 g homogenized sample into 200 mL separatory funnel, and extract 30 min with 50 mL ethyl acetate after adding 20 mL water, 4 g NaCl, and 16 mL concentrated HCl (35%) according to method of Iwai et al. (1). Centrifuge mixture (2500 rpm, 5 min), and transfer 25 mL organic layer to 50 mL round-bottom flask. Evaporate organic layer to about 1 mL, using rotary-evaporator at 40°C. (Caution: Do not evaporate to dryness.) Dilute concentrated solution with 4 mL ethanol, and pass ethanol solution through 1.5 cm id × 6 cm glass cleanup column containing about 5 g ethanol-rinsed silica gel. Wash column with ethanol, 20 mL water, and 100 mL 0.2N HCl successively, and then elute inorganic tin with 30 mL 2N HCl.

Discard first 5 mL, and collect next 25 mL for analysis of inorganic tin. Simultaneously, carry out blank test in the same manner.

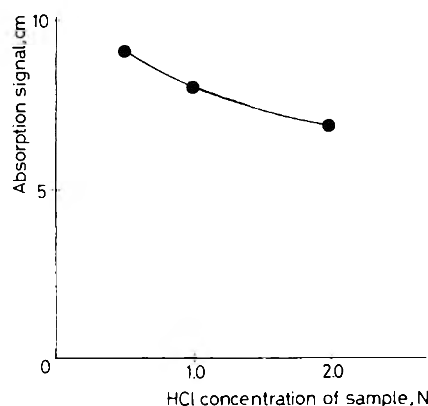


Figure 2. Effect of acidity in sample solutions on atomic absorption signal of tin: 0.1 μg Sn^{4+}/mL for HCl–H_2O (1 + 5), 1% $NaBH_4$ solution (12% NaOH).

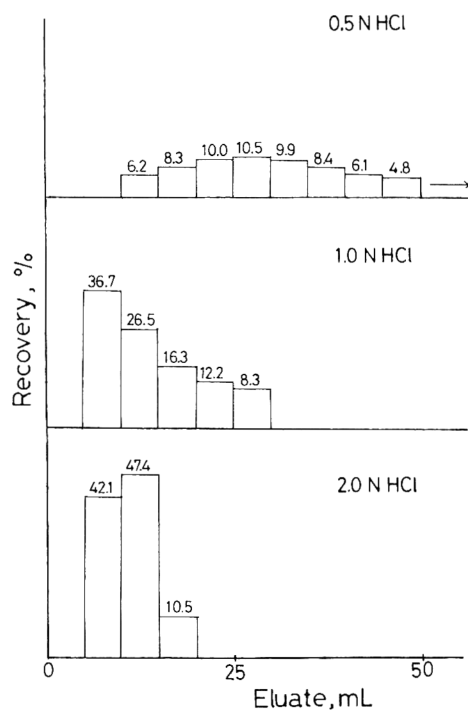


Figure 3. Elution patterns of Sn^{4+} from silica gel column: added, 1000 μg Sn^{4+}; eluate, 0.5N–2.0N HCl.

Results and Discussion

We investigated some fundamental conditions for the determination of tin. For sample solutions of tin in 1N HCl, the effect of NaOH concentration in 1% $NaBH_4$ solutions on the atomic absorption signal of tin is shown in Figure 1. From the results, 12% NaOH was sufficient to obtain the maximum atomic absorption signal of tin in both cases of HCl solutions: (A) HCl–H_2O (1 + 5) and (B) HCl–H_2O (1 + 2). Slightly better absorption signal was obtained for (A), so the concentrations of NaOH and HCl were used as described under the operating conditions.

Further, the effect of acidity of the sample solutions on the atomic absorption signal for tin is shown in Figure 2. The absorption signal was changed slightly by the difference in acidity. Therefore, standard solutions should contain the same acid concentrations as the sample solutions.

Silica Gel Cleanup

Elution patterns of inorganic tin (Sn^{4+}) are shown in Figure 3 for a silica gel column (5 g, 1.5 cm id × 6 cm). Elution of Sn^{4+} was investigated using 0.5, 1.0, and 2.0N HCl as eluants. From the results, 2.0N HCl was chosen to elute Sn^{4+} from the column. Further, Sn^{2+} eluted in the same range as Sn^{4+}. So the eluate was collected from 5 to 30 mL for inorganic tin (Sn^{4+} and Sn^{2+}).

Organotin and compounds such as butyltin or methyltin interfered with the measurement of tin by HG-AAS. To eliminate these interferences, 100 mL 0.2N HCl was passed through the column before the 2N HCl. Methyltin chlorides (mono-, di-, and tri-) eluted in the 5 to 65 mL 0.2N HCl fraction, and the butyltin chlorides (mono-, di-, and tri-) eluted in the 5 to 100 mL 0.2N HCl fraction.

Table 1. Recoveries of inorganic tin from fish sample[a]

Sn found before spiking,[b] μg/g	Added Sn^{2+}, μg/g	Added Sn^{4+}, μg/g	Total Sn found,[c] μg/g	Rec.,[d] %
0.03	0.1	—	0.103 ± 0.005	73.3 ± 3.4
0.03	—	0.1	0.109 ± 0.006	78.9 ± 4.2
0.03	—	1.0	0.811 ± 0.051	78.1 ± 4.9

[a] Crucian carp muscle, 10 g.
[b] Duplicate determinations.
[c] Average ± SD, n = 5.
[d] Average ± SD, n = 5.

Recoveries of Inorganic Tin from Biological Samples

Recoveries of inorganic tin (Sn^{4+} and Sn^{2+}) added to fish muscle were evaluated by the present procedure. The results are shown in Table 1. The recoveries were 78.9 ± 4.2% (n = 5) for Sn^{4+} and 73.3 ± 3.4% (n = 5) for Sn^{2+} at the level of 0.1 μg/g spiked to 10 g fish sample. Further, at the level of 1.0 μg/g Sn^{4+}, average recovery was 78.1 ± 4.9% (n = 5). These low recoveries are probably caused by interference from lipid in the extract of the fish sample on the adsorption of inorganic tin to silica gel. To enhance recoveries, lipids will have to be eliminated before column chromatography.

Analyses of Fish Samples

Our proposed method was applied to fish samples (crucian carp, large mouth black bass) obtained from Lake Biwa (Shiga Prefecture, Japan). Crucian carp and large mouth black bass were 12.0–16.0 and 13.0–18.5 cm in body length and weighed 54.4–132.1 and 50.2–138.2 g, respectively. Results of these analyses are shown in Table 2. Concentration ranges were 0.03–0.11 μg Sn/g for crucian carp and <0.01–0.04 μg Sn/g for large mouth black bass.

Table 2. Analyses of fish samples in Lake Biwa

Sample		Sn found, μg/g
Crucian carp	I	0.11
	II	0.04
	III	0.06
	IV	0.03
	V	0.08
Large mouth black bass	I	0.02
	II	0.01
	III	0.04
	IV	0.01
	V	ND[a]

[a] Not detectable, less than 0.01 μg/g.

Acknowledgments

We thank Y. Matsuoka and S. Okamoto in our institute for their technical assistance with the measurement of inorganic tin by HG-AAS.

References

(1) Iwai, H., et al. (1981) *J. Anal. Toxicol.* **5**, 300–306
(2) Manabe, M., et al. (1981) *Jpn. J. Ind. Health* **23**, 312–313
(3) Maguire, R. J., et al. (1984) *Can. J. Fish. Aquat. Sci.* **41**, 537–540
(4) Maguire, R. J., & Tkacz, R. J. (1985) *J. Agric. Food Chem.* **33**, 947–953
(5) Tsuda, T., et al. (1987) *J. Chromatogr.* **387**, 361–370

Single-Laboratory Evaluation of EPA Method 8080 for Determination of Chlorinated Pesticides and Polychlorinated Biphenyls in Hazardous Wastes

VIORICA LOPEZ-AVILA, SARAH SCHOEN, and JUNE MILANES
Acurex Corp., Environmental Systems Division, 485 Clyde Ave, Mountain View, CA 94039
WERNER F. BECKERT
U.S. Environmental Protection Agency, Las Vegas, NV 89109

Method 8080, as published in the Second Edition of "Test Methods for Evaluating Solid Waste," EPA Manual SW-846, has been evaluated in a single-laboratory study. The Florisil procedure recommended in Method 8080 for sample cleanup does not separate organochlorine pesticides (OCPs) from the polychlorinated biphenyls (PCBs). Consequently, gas chromatographic analysis of OCPs on the packed column recommended in the method may result in false identifications or in no identifications at all when PCBs are present. Therefore, silica gel was substituted for Florisil, a capillary column was substituted for the packed column, and a sulfur cleanup procedure was incorporated in the method. The revised method was evaluated with samples spiked at 3 concentrations. Precision and accuracy indicate that the revised method can be reliably applied to the determination of OCPs and PCBs in liquid and solid matrixes. Detection limits for liquid matrixes range from 0.02 to 0.09 μg/L for OCPs and from 0.5 to 0.9 μg/L for PCBs. Detection limits for solid matrixes range from 1 to 6 μg/kg for OCPs and from 60 to 70 μg/kg for PCBs.

Organochlorine pesticides (OCPs) can be classified in 3 major subgroups on the basis of their molecular structures: cyclopentadienes, cyclohexanes, and DDTs. Toxaphene is a mixture of chlorinated camphenes containing approximately 67–69% chlorine. Polychlorinated biphenyls (PCBs) are complex mixtures consisting of combinations of the 209 possible chlorinated biphenyls. Commercial mixtures of PCBs manufactured in the United States are known as Aroclors 1016, 1221, 1232, 1242, 1248, 1254, and 1260. The first 2 digits represent the biphenyl moiety, and the last 2 digits indicate the weight percent of chlorine in the mixture; Aroclor 1016 is an exception because it is a chlorinated biphenyl that contains 41.5% chlorine.

OCPs and PCBs have been determined in environmental samples by gas chromatography (GC) with electron-capture detection (1) and by mass spectrometry (2–5). The electron-capture detector is preferred over the mass spectrometer because it is 2–3 orders of magnitude more sensitive (6). Because PCBs are extracted along with OCPs and because they interfere with the determination of OCPs whenever electron-capture detectors are used, their presence in extracts together with the OCPs needs to be minimized. Therefore, several cleanup techniques based on Florisil (7–10), alumina (11–13), and silica gel chromatography (13–15) have been developed.

EPA Method 8080 (16) provides sample extract cleanup and GC conditions for the determination of the OCPs and the PCBs listed in Table 1 in a variety of environmental samples including groundwater, liquids, and solids. Following solvent extraction of liquid samples in a separatory funnel (Method 3510) or in a continuous liquid-liquid extractor (Method 3520) and of solid samples in a Soxhlet extractor (Method 3540) or with a sonicator (Method 3550), the extracts are cleaned up by Florisil chromatography. Compounds are eluted from the Florisil column with 6, 15, and 50% ethyl ether in hexane. All compounds listed in Table 1 except 6 OCPs elute in Fraction I (6% ether in hexane). Of those 6 pesticides, 4 (dieldrin, endosulfan I, endrin, and endrin aldehyde) elute in Fraction II (15% ether in hexane), and 2 (endosulfan II and endosulfan sulfate) elute in Fraction III (50% ether in hexane). Endrin aldehyde has also been reported in Fraction III.

There is no mention in Method 8080 of possible overlapping of compounds between fractions and of the reproducibility of the elution pattern. Moreover, the fact that OCPs are not separated from PCBs after the Florisil cleanup suggests that such a scheme may be of little use for samples containing OCPs as well as PCBs. Thus, a preliminary evaluation of Method 8080 was conducted to determine the recoveries of OCPs and PCBs in the absence of matrix interferences, to determine the extent of overlapping of compounds between fractions, and to determine the efficiency of the Florisil cleanup scheme with real samples.

In the improvement phase of this study, a fractionation procedure was developed to separate PCBs from the bulk of the OCP compounds and, at the same time, to remove interfering compounds coextracted from the samples. Several extract cleanup procedures based on silica, alumina, silica gel/Celite, and Florisil/charcoal chromatography were investigated. Furthermore, a capillary GC method was developed, and a method for sulfur removal was incorporated.

Table 1. Compounds listed in EPA Method 8080

Compound[a]	Storet No.	CAS No.
α-BHC	39337	319-84-6
β-BHC	39338	319-85-7
γ-BHC (Lindane)	39340	58-89-9
δ-BHC	34259	319-86-8
Heptachlor	39410	76-44-8
Aldrin	39330	309-00-2
Heptachlor epoxide	39420	1024-57-3
γ-Chlordane	39350	57-74-9
Endosulfan I	34361	959-98-8
4,4′-DDE	39320	72-55-9
Dieldrin	39380	60-57-1
Endrin	39390	72-20-8
Endosulfan II	34356	33212-65-9
4,4′-DDD	39310	72-54-8
Endrin aldehyde	34366	7421-93-4
Endosulfan sulfate	34351	1031-07-8
4,4′-DDT	39300	50-29-3
4,4′-Methoxychlor	NA[b]	72-43-5
Toxaphene	39400	8001-35-2
Aroclor 1016	34671	12674-11-2
Aroclor 1221	39488	1104-28-2
Aroclor 1232	39492	11141-16-5
Aroclor 1242	39496	53469-21-9
Aroclor 1248	39500	12672-29-6
Aroclor 1254	39504	11097-69-1
Aroclor 1260	39508	11096-82-5

[a] Kepone is included in Method 8080 in the second edition of SW-846 but not in the third edition.
[b] Not available.

Received May 18, 1987. Accepted August 5, 1987.
Although the research described in this article has been supported by the U.S. Environmental Protection Agency under Contract No. 68-03-3226 to the Acurex Corp., it has not been subjected to agency review and therefore does not necessarily reflect the views of the agency, and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Table 2. Distribution and percent recoveries of OCPs and PCBs in silica gel column fractions[a] (average of 3 determinations ± SD)[b]

Compound	Fraction I		Fraction II		Fraction III		Total recovery	
	Concn 1	Concn 2	Concn 1	Concn 2	Concn 1	Concn 2	Concn 1	Concn 2
α-BHC					82 (1.7)	74 (8.0)	82 (1.7)	74 (8.0)
β-BHC					107 (2.1)	98 (12.5)	107 (2.1)	98 (12.5)
γ-BHC					91 (3.6)	85 (10.7)	91 (3.6)	85 (10.7)
δ-BHC					92 (3.5)	83 (10.6)	92 (3.5)	83 (10.6)
Heptachlor	109 (4.1)	118 (8.7)					109 (4.1)	118 (8.7)
Aldrin	97 (5.6)	104 (1.6)					97 (5.6)	104 (1.6)
Heptachlor epoxide					95 (4.7)	88 (10.2)	95 (4.7)	88 (10.2)
Endosulfan I					95 (5.1)	87 (10.2)	95 (5.1)	87 (10.2)
4,4′-DDE	86 (5.4)	94 (2.8)					86 (5.4)	94 (2.8)
Dieldrin					96 (6.0)	87 (10.6)	96 (6.0)	87 (10.6)
Endrin					85 (10.5)	71 (12.3)	85 (10.5)	71 (12.3)
Endosulfan II					97 (4.4)	86 (10.4)	97 (4.4)	86 (10.4)
4,4′-DDD					102 (4.6)	92 (10.2)	102 (4.6)	92 (10.2)
Endrin aldehyde					81 (1.9)	76 (9.5)	81 (1.9)	76 (9.5)
Endosulfan sulfate					93 (4.9)	82 (9.2)	93 (4.9)	82 (9.2)
4,4′-DDT			86 (13.4)	73 (9.1)	15 (18.7)	8.7 (15.0)	101 (5.3)	82 (23.7)
4,4′-Methoxychlor					99 (9.9)	82 (10.7)	99 (9.9)	82 (10.7)
Aroclor 1016	86 (4.0)	87 (6.1)					86 (4.0)	87 (6.1)
Aroclor 1260	91 (4.1)	95 (5.0)					91 (4.1)	95 (5.0)
Tech. chlordane	14 (5.5)	22 (5.3)	19 (6.8)	39 (3.6)	29 (5.0)	37 (5.1)	62 (3.3)	98 (1.9)
Toxaphene			15 (2.4)	17 (1.4)	73 (9.4)	84 (10.7)	88 (12.0)	101 (10.1)

[a] Eluant composition: Fraction I, 80 mL hexane; Fraction II, 50 mL hexane; Fraction III, 15 mL methylene chloride; concentration 1 is 0.5 µg per column for BHCs, heptachlor, aldrin, heptachlor epoxide, endosulfan I; 1.0 µg per column for dieldrin, endosulfan II, 4,4′-DDT, endrin aldehyde, 4,4′-DDD, 4,4′-DDE, endrin, and endosulfan sulfate; 5 µg per column for 4,4′-methoxychlor and technical chlordane; 10 µg per column for toxaphene, Aroclor 1016, and Aroclor 1260. For concentration 2, amounts spiked are 10 times higher than for concentration 1.

[b] Recovery cutoff point is 5%. Data obtained with standards dissolved in 2 mL hexane.

With the use of silica gel fractionation and the added resolution achieved with a capillary column, all OCPs listed in Table 1, except toxaphene, were determined. Evaluation studies included only Aroclors 1016 and 1260.

Experimental

Apparatus and Materials

(**a**) *Glassware.*—Separatory funnel, 500 mL, with Teflon stopcock; glass drying column, 21 cm × 4.5 cm id, plugged with glass wool; Kuderna-Danish evaporative flask, 500 mL, with 10 mL graduated concentrator tube and 3-ball macro Snyder column; wide-mouth amber glass bottle, 500 mL, with Teflon-lined screw cap; glass cleanup column, 20 cm × 11 mm id, with 200 mL reservoir and Teflon stopcock.

(**b**) *Water bath.*—With concentric ring cover; heat to 98 ± 2°C.

(**c**) *Sonicator.*—Model W-375 (Heat Systems Ultrasonics, Inc.).

(**d**) *Gas chromatograph.*—Equipped with constant-current pulsed-frequency electron-capture detector and data system. Operating conditions: injection port 225°C; detector 350°C; flow rates for carrier (helium at 16 psi for DB-5 column and nitrogen at 20 psi for SPB-608 column) and detector make-up gas (nitrogen) at 30 mL/min; splitless mode with splitless valve on for 30 s.

(**e**) *GC columns.*—(*1*) DB-5 fused-silica capillary column, 30 m × 0.25 mm id (J&W Scientific Inc., Folsom, CA), film thickness 0.25 µm. Temperature program: initial 100°C, hold 2 min, programming rate 1, 15°C/min (100°C to 160°C); programming rate 2, 5°C/min (160°C to 270°C). (*2*) SPB-608 fused-silica capillary column, 30 m × 0.25 mm id (Supelco Inc., Bellefonte, PA), film thickness 0.25 µm. Temperature program: initial 160°C, hold 2 min, programming rate 5°C/min (160°C to 290°C), hold 1 min at 290°C.

(**f**) *Autosampler.*—Varian Model 8000, 2 µL injection volume.

(**g**) *Glass wool.*—Pyrex Fiberglas sliver, 8 µm. Soxhlet-extract overnight with methylene chloride.

Reagents

(**a**) *Solvents.*—Methylene chloride, hexane, acetone, toluene, 2-propanol; all pesticide grade, distilled in glass.

(**b**) *Sodium sulfate.*—ACS grade, granular, anhydrous. Purify by heating 4 h at 400°C in shallow tray.

(**c**) *Deactivated silica gel.*—3.3%. Activate silica gel PR grade (Davidson 923, 100–200 mesh, Supelco Inc., Bellefonte, PA) 16 h at 130°C. Deactivate adsorbent by adding 3.3 mL water to 96.7 g activated silica gel. Equilibrate 6 h after addition of water. Store in tightly capped glass container; do not keep for more than 1 week. Suitability test: Using procedure outlined in *Silica Gel Cleanup,* load test column with 2 mL standard in hexane and collect 3 fractions: elute Fraction I with 80 mL hexane, Fraction II with additional 50 mL hexane, and Fraction III with 15 mL methylene chloride. Concentrate fractions and determine percent recovery. Recoveries should be ≥70%, and elution patterns should match those given in Table 2. Report results of test column with all samples that were cleaned up with a given batch of silica gel.

(**d**) *Tetrabutyl ammonium sulfite reagent.*—Extract solution of 3.39 g (0.01 mol) tetrabutyl ammonium hydrogen sulfate in 100 mL water with three 20 mL portions of hexane (to remove impurities), and then add 25 g anhydrous sodium sulfite (analytical grade).

(**e**) *Pesticide and PCB standards.*—Obtain from U.S. EPA Quality Assurance Materials Bank, Research Triangle Park, NC, or from manufacturers; use without further purification (purity > 98%). Prepare stock solutions at 2000 ng/µL in toluene–hexane (1 + 1) for all compounds listed in Table 1 except methoxychlor and PCBs. Prepare methoxychlor standard in methylene chloride at 5000 ng/µL and PCB standards in hexane at 2500 ng/µL. Prepare mixture of all OCPs in

hexane at 0.05 ng/μL, 0.1 ng/μL, and 0.5 ng/μL for GC calibration. Store solutions at 4°C in the dark.

Sample Collection

Liquid waste was collected from a pesticide waste storage facility at a California agricultural field station. This waste contains high concentrations of organics (total organic carbon, 520 mg/L; total organic halogen, 30 mg/L).

The standard reference material is identified as NBS SRM-1645. According to the National Bureau of Standards, the material was dredged from the bottom of the Indiana Harbor Canal near Gary, IN. The material was screened to remove foreign objects, freeze-dried, sieved (particle size < 180 μm), and sterilized by radiation to minimize biological activity. Physicochemical characterization of the NBS SRM-1645 is given by Lopez-Avila et al. (17).

Sandy loam was obtained from Soils Inc., Puyallup, WA, and had the following physicochemical characteristics: pH 5.9–6.0; 89% sand, 7% silt, 4% clay; cation exchange capacity, 7.5 meq/100 g; total organic carbon content, 1290 ± 185 mg/kg.

Sample Extract Preparation

Extract 300 mL liquid sample at neutral pH in separatory funnel with 60 mL methylene chloride. Repeat extraction twice. Combine extracts, dry by passing through column of anhydrous sodium sulfate, and concentrate in Kuderna-Danish evaporator to less than 10 mL. Add 50 mL hexane to concentrate, and reevaporate until volume is reduced to 30 mL.

Weigh 30 g soil or sediment and place in wide-mouth bottle. Extract sample with 60 mL hexane–acetone (1 + 1) by sonicating 3 min. Repeat extraction twice, each time for 3 min and with fresh portions of hexane–acetone (1 + 1). Combine extracts, dry by passing through column of anhydrous sodium sulfate, and concentrate in Kuderna-Danish evaporator to less than 10 mL. Add 50 mL hexane to concentrated extract and reevaporate until volume is reduced to 30 mL.

Use 30-mL concentrates as follows: 2 mL for background analysis; spike three 6 mL portions with 1500 ng, 3000 ng, and 15 000 ng, respectively, with OCPs listed in Table 1 and with 15 000 ng, 30 000 ng, and 150 000 ng each of Aroclor 1016 and 1260. Keep 10 mL as backup material. Do not spike all 7 PCB mixtures listed in Table 1, because method does not allow quantitation of all PCBs in the presence of OCPs. Spike only Aroclor 1016 and Aroclor 1260 together with OCPs.

Split each spiked 6 mL extract portion into three 2 mL aliquots for triplicate GC analysis.

Silica Gel Cleanup

Fill 20 cm × 11 mm id glass cleanup column with 3 g 3.3% deactivated silica gel. Top column with 2–3 cm anhydrous sodium sulfate. Rinse column with 10 mL hexane, and discard eluate. Quantitatively transfer sample extract (2 mL) to prepared column and follow with several hexane rinses of 1–2 mL each. Elute column with 80 mL hexane at a rate of ca 5 mL/min. Remove collection flask and label as Fraction I. Elute column with additional 50 mL hexane, collect eluate, and label as Fraction II. Perform third elution with 15 mL methylene chloride (Fraction III). Concentrate 3 fractions in Kuderna-Danish evaporators. Exchange methylene chloride (Fraction III) for hexane. Adjust final volume of each fraction to 10 mL.

Elemental Sulfur Removal

Shake hexane extract (2 mL) with 1 mL 2-propanol and 1 mL tetrabutyl ammonium sulfite reagent for at least 1 min. Add ca 100 mg sodium sulfite crystals. If sodium sulfite disappears, add more sodium sulfite in 100 mg portions until solid residue remains after repeated shaking. Add 5 mL water and shake test tube for another 1 min. Centrifuge mixture and transfer hexane layer to another vial for analysis.

Gas Chromatography

Inject 1 μL aliquots into GC apparatus set up with DB-5 fused-silica capillary column (*1*). Program oven as described under apparatus **(e)**. Perform 3-level calibration using multicomponent standards containing OCPs at 0.0125 to 0.025 ng/μL (for low-level standard) and Aroclor 1016/1260 at 0.5 ng/μL (for low-level standard). Medium-level standard and high-level standards should contain OCPs or PCBs at 2 and 5 times the concentration of low-level standards, respectively. Run toxaphene standards separately at 0.1 ng/μL, 0.5 ng/μL, and 1.0 ng/μL. Alternate sample injections with OCP standards at 0.05 to 0.1 ng/μL and Aroclor 1016/1260 standards at 0.5 ng/μL. Dilute samples with hexane to bring detector response within calibration curve. Quantitate OCPs by using peak areas rather than peak heights. For multicomponent mixtures such as PCBs and toxaphene, match retention times of peaks in standards with peaks in samples. Quantitate 6–8 major peaks, add peak areas, and calculate as total response in sample vs total response in standard. Peaks that were used in quantitation of PCBs are identified in Table 3. For toxaphene, use peaks at retention times 20.564, 22.332, 23.675, 24.681, 26.246, and 27.322 min.

Method Precision, Accuracy, and Detection Limit

Process fortified extracts of environmental samples through silica gel fractionation scheme given above. Perform each experiment in triplicate at a minimum of 3 concentrations. Determine method detection limit by the procedure of Glazer et al. (18).

Results and Discussion

Florisil Fractionation

Florisil fractionation was performed as recommended in Method 8080 (16). Separate experiments were run in duplicate for PCBs, toxaphene, technical chlordane, OCP group A (γ-BHC, heptachlor, aldrin, heptachlor epoxide, endosulfan I, dieldrin, endosulfan II, 4,4′-DDT, and endrin aldehyde), and OCP group B (α-BHC, β-BHC, δ-BHC, 4,4′-DDE, endrin, 4,4′-DDD, endosulfan sulfate, and 4,4′-methoxychlor). Experimental results are presented in Table 4.

Overall recoveries are quantitative, and agreement between duplicate experiments is, in most cases, excellent. However, the following discrepancies were found between our data and the recovery data given in Method 8080: (a) dieldrin did not elute 100% in Fraction II but was recovered in Fraction I as well as II, (b) more endosulfan I was recovered in Fraction I than in Fraction II, (c) more endosulfan II was recovered in Fraction II than in Fraction III, (d) endrin overlapped in Fractions I and II, and (e) endosulfan sulfate overlapped in Fractions II and III.

A possible explanation for these discrepancies might be the fact that the Florisil calcination temperature is critical for the Florisil adsorption properties (8). Difficulties in obtaining reproducible recoveries and the overlapping of pesticides between fractions have also been reported by others (8–10).

Table 3. Summary of retention times (min) for PCBs[a]

Peak No.	Aroclor 1016	Aroclor 1221	Aroclor 1232	Aroclor 1242	Aroclor 1248	Aroclor 1254	Aroclor 1260	Pesticide eluting at same retention time
1		8.396						
2		9.149	9.146					
3		10.308	10.306					
4	10.949	10.959	10.957	10.963				
5	11.722	11.732	11.730	11.734				
6	12.060[b]	12.068	12.066	12.071				
7	12.256	12.262	12.262	12.267	12.254			
8	12.933		12.939	12.944				
9		13.573	13.574					
10	13.683[b]	13.685	13.687	13.691	13.679			
11	13.756	13.763	13.761	13.756	13.753			
12	14.048		14.053	14.057				
13	14.341[b]	14.344	14.346	14.351	14.337			
14	14.960		14.963	14.971	14.954			
15	15.055		15.061	15.066				
16	15.260[b]	14.258	15.262	15.267	15.252			
17	15.310	15.309	15.314	15.319	15.305			
18	15.652[b]	15.648	15.653	15.661	15.645			
19	15.860			15.865	15.852			
20	15.924	15.923	15.928	15.93	15.919		15.928	heptachlor (15.91 ± 0.008)
21	16.116		16.118	15.123	16.111			
22	16.398		16.400	16.406	16.390			
23	16.540[b]		16.542	16.551	16.534	16.548	16.542	
24		16.574						
25		16.604						
26	16.702	16.699	16.701	16.713	16.699	16.710	16.706	
27	16.818		16.821	16.828	16.808	16.814		
28	16.843		16.847	15.841				
29			17.142				17.141	
30	17.290		17.298	17.303	17.289	17.302	17.299	
31	17.427	17.414	17.427	17.431	17.418	17.426	17.403	
32	17.704		17.703	17.711				
33	17.755[b]		17.755	17.759	17.745	17.758	17.573	
34	18.048		18.048	18.055	18.040			
35	18.253		18.254	18.263				
36							18.356	
37				18.459	18.468	18.456		
38							18.555	
39	18.606		18.602	18.611	18.598	18.610		heptachlor epoxide (18.60 ± 0.009)
40	18.736		18.732	18.743	18.728	18.738	18.738	
41	18.881		18.875	18.886	18.868			
42			18.898				18.908	
43	19.147			19.150	19.136	19.147		
44				19.203				
45	19.503		19.500	19.514	19.498			γ-chlordane (19.48 ± 0.012)
46							19.542	
47			19.690	19.697	19.682			
48			19.749	19.760	19.742		19.757	
49	19.939		19.951	19.960	19.943	19.954	19.952	endosulfan (19.94 ± 0.010)
50				20.179		20.188	20.172	
51					20.357	20.362	20.363	
52			20.551	20.567	20.553	20.556	20.560	
53			20.745	20.755	20.738	20.744	20.757	
54	20.909		20.909	20.919	20.902	20.911	20.917	dieldrin (20.91 ± 0.008)
55							21.023	
56			21.112	21.127	21.106	21.116	21.122	
57			21.537	21.552	21.529	21.550		
58							21.587[b]	
59					21.751	21.750	21.752[b]	
60					21.894	21.897	21.887	
61		22.017			22.012			
62						22.025	22.030	
63	22.047							endosulfan II (22.05 ± 0.006)
64			22.083	22.097	22.077	22.086		
65						22.426	22.428[b]	
66					22.501	22.508		
67						22.585	22.589	
68						22.704	22.712	
69	22.752							endrin aldehyde (22.75 ± 0.007)
70		22.927		22.936	22.922	22.930	22.935	
71		23.039					23.043	
72			23.073	23.085	23.071	23.069		
73					23.395	23.401	23.407	
74							23.486	
75	23.664	23.654				23.664	23.665	

Table 3. Continued

Peak No.	Aroclor 1016	Aroclor 1221	Aroclor 1232	Aroclor 1242	Aroclor 1248	Aroclor 1254	Aroclor 1260	Pesticide eluting at same retention time
76						23.758	23.758	
77		23.955		23.969	23.950	23.959	23.962	
78						24.060	24.066[b]	
79						24.265	24.289	
80						24.509	24.504	
81				24.606	24.586	24.591	24.600	
82						24.783	24.786	
83						24.986	24.996	
84							25.185	
85						25.459	25.463[b]	
86						25.666	25.670	
87						25.857	25.841	
88						26.070	26.088	
89						26.241	26.247[b]	
90							26.368	
91				26.487	26.478	26.480	26.486	
92							26.583	
93							26.739	
94							26.954	
95							27.283	
96					27.564	27.567	27.575[b]	
97							27.777	
98							27.898[b]	
99							28.095	
100							28.639	
101							28.789	
102							29.264	
103							29.545	
104							29.643	
105					30.077		30.084[b]	
106							30.334	

[a] Single determinations on DB-5 column. GC operating conditions are given in the Experimental section.
[b] Peak used in quantitation.

Regardless of the reproducibility of the fractionation, it is apparent that the Florisil fractionation method is not suitable for samples that contain OCPs and PCBs. PCBs appear in the same fraction as the bulk of the OCP compounds, but these 2 types of compounds need to be separated from each other to a larger extent to avoid cross-interference. To exemplify this on a real sample, we extracted a liquid waste and spiked the extract with known amounts of OCPs and Aroclors 1016 and 1260. Figures 1 and 2 show GC/EC chromatograms of each of the 3 fractions (unspiked and spiked extract). Because of the complexity of this sample matrix, fractions were analyzed on a DB-5 capillary column; however, even then spiking compounds could not be determined. In view of these results, we eliminated the Florisil fractionation scheme from further evaluation.

Silica Gel Fractionation

Silica gel fractionations were performed in triplicate at 2 concentration levels according to a procedure developed by Biddleman et al. (15), with slight modifications, as detailed in the experimental section. Technical chlordane and toxaphene fractionations were performed separately. Distribution and percent recoveries of the OCPs, Aroclor 1016, Aroclor 1260, technical chlordane, and toxaphene, are given in Table 2. Distribution patterns of OCPs and PCBs in the 3 silica gel fractions were quite reproducible. Compounds found to elute in Fraction I (80 mL hexane) include heptachlor, aldrin, 4,4′-DDE, and the PCBs. Almost all other OCPs elute in Fraction III.

Total recoveries were >70%, except for technical chlordane at concentration 1, with most values ranging from 80 to 110% (Table 2). The GC/EC chromatograms of the silica gel Fractions I, II, and III for the unspiked and spiked liquid waste extract are shown in Figures 3 and 4.

Evaluation of silica gel fractionation scheme with real sample extracts (e.g., liquid waste, NBS SRM-1645, sandy loam) gave comparable results (19). Only a few compounds (4,4′-

Table 4. Recoveries (%)[a] of organochlorine pesticides and PCBs from spiked samples following Florisil fractionation

	Spike level, μg	Fraction I	Fraction II	Fraction III	Total
α-BHC	0.5	94; 92	9.7; 8.7		104; 101
β-BHC	0.5	86; 83	10; 0		96; 83
γ-BHC	0.5	76; 77	8.5; 8.1	1.5; <1.0	86; 85
δ-BHC	0.5	70; 60	31; 27		101; 87
Heptachlor	0.5	75; 78	5.3; 5.8		80; 84
Aldrin	0.5	78; 80	6.0; 6.6	2.8; 3.8	87; 90
Heptachlor epoxide	0.5	79; 80	15; 15		94; 95
Endosulfan I	0.5	59; 64	29; 30		88; 94
4,4′-DDT	1.0	95; 95	8.7; 6.7		104; 102
Dieldrin	1.0	20; 24	71; 70		91; 94
Endrin	1.0	37; 43	64; 51		101; 94
Endosulfan II	1.0		60; 79	30; 15	90; 94
4,4′-DDD	1.0	93; 90	11; 10		104; 100
Endrin aldehyde	1.0		80; 90	11; 4.4	91; 94
Endosulfan sulfate	1.0		23; 23	57; 51	80; 74
4,4′-DDE	1.0	97; 93	9.6; 8.6		107; 102
4,4′-Methoxychlor	5.0	87; 84	29; 26		116; 110
Aroclor 1016	10.0	86; 76	4.6; 4.4		91; 80
Aroclor 1260	10.0	91; 78	10; 9.0		101; 87
Tech. chlordane	5.0	93; 94	9.6; 7.6		103; 102
Toxaphene	10.0	105; 108			105; 108

[a] $N = 2$.

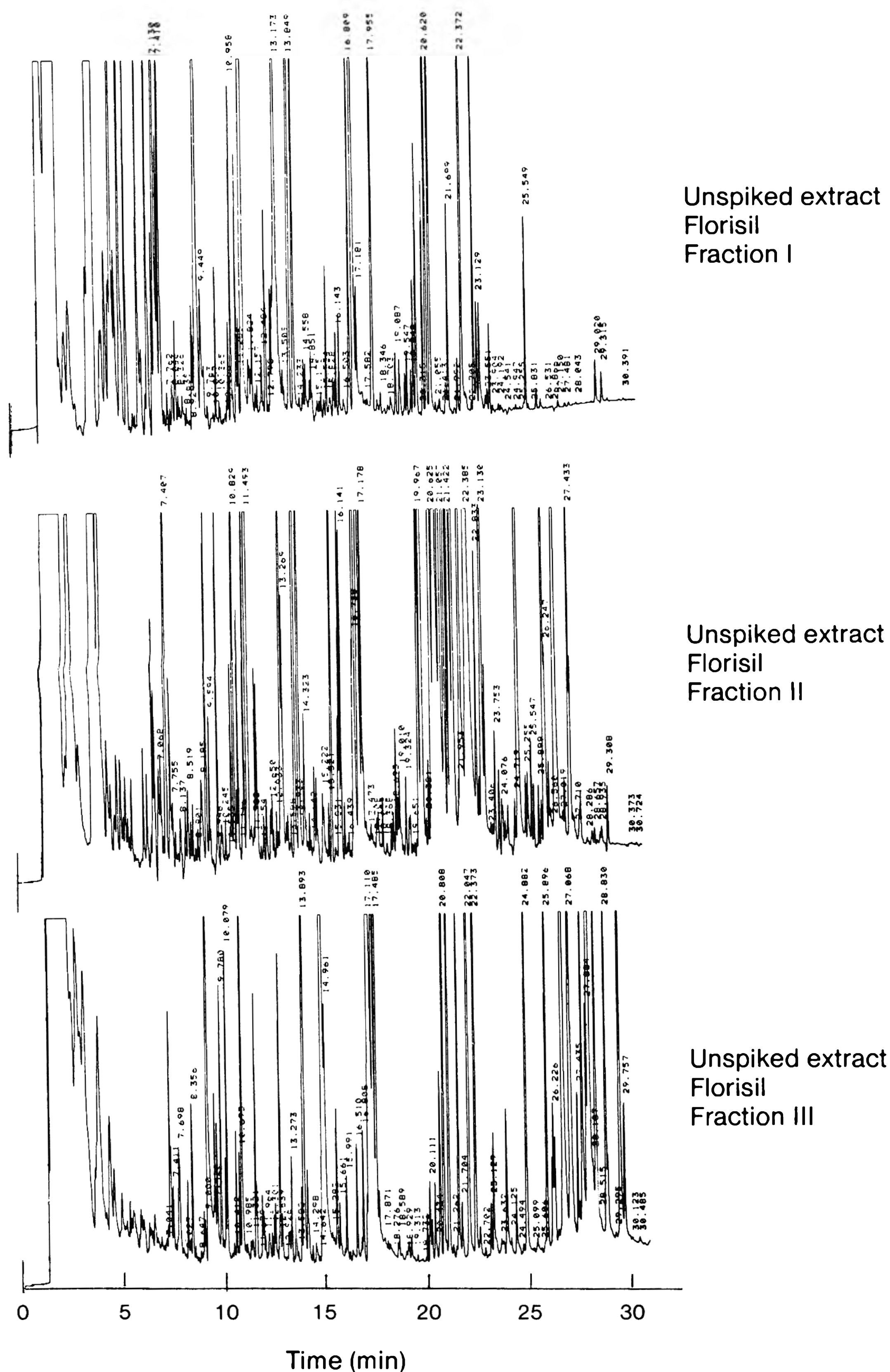


Figure 1. GC/EC chromatograms of Florisil Fractions I (top), II (middle), and III (bottom) of liquid waste extract analyzed on 30 m × 0.25 mm id DB-5 fused-silica capillary column.

DDD, α-BHC, γ-chlordane) were split between fractions when the real samples were fractionated.

These results show that Fractions II and III may be combined before concentration and analysis, when the sample matrixes are relatively simple. However, when samples with complex matrixes have to be extracted, especially matrixes containing organic solvents, more crossover between fractions may occur. In such cases, it is more advantageous to analyze the 3 fractions separately.

Sulfur Removal

Elemental sulfur, which may be present in extracts from sediments and from some industrial samples, gives GC peaks that mask the region of aldrin, BHCs, heptachlor, and hep-

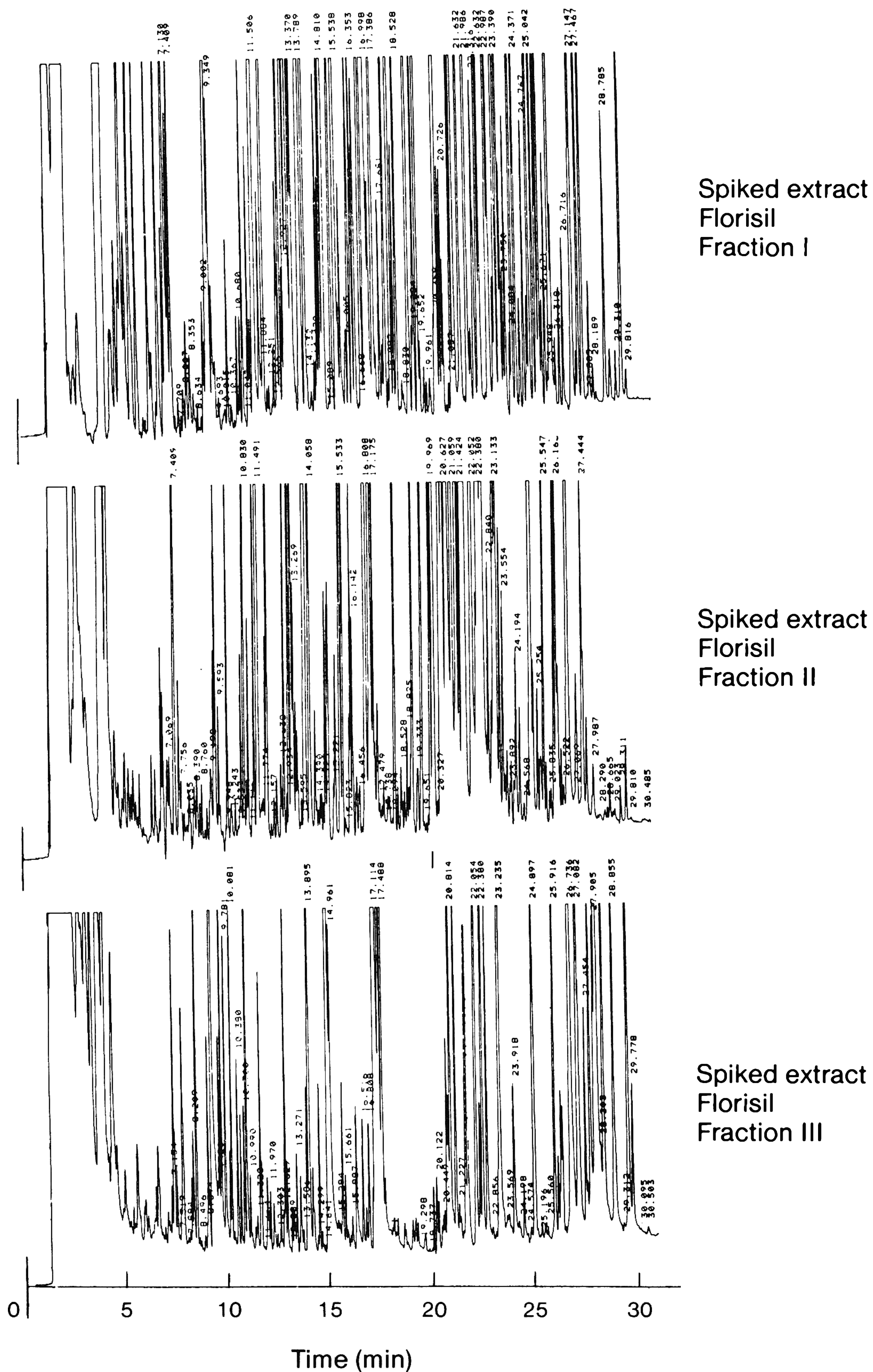


Figure 2. GC/EC chromatograms of Florisil Fractions I (top), II (middle), and III (bottom) of liquid waste extract spiked with OCPs and PCBs and analyzed on the 30 m × 0.25 mm id DB-5 fused-silica capillary column.

tachlor epoxide when the analysis is performed on the 1.5% OV-17/1.95% OV-210 on Chromosorb-WHP or on the 30 m DB-5 fused-silica capillary column.

Several methods are available for the removal of sulfur from extracts (20–24). Shaking with 0.1–0.2 mL metallic mercury (20) is simple, but it does not remove all the sulfur. Although Goerlitz and Law (20) claimed that aldrin, dieldrin, heptachlor, lindane, and 4,4′-DDT were not affected by mercury, Thompson (21) reported that recovery of heptachlor was only 40% when mercury was used for sulfur removal. Activated copper (22) and Raney nickel (23) have also been used to remove sulfur. Jensen and co-workers (24) used tetra-

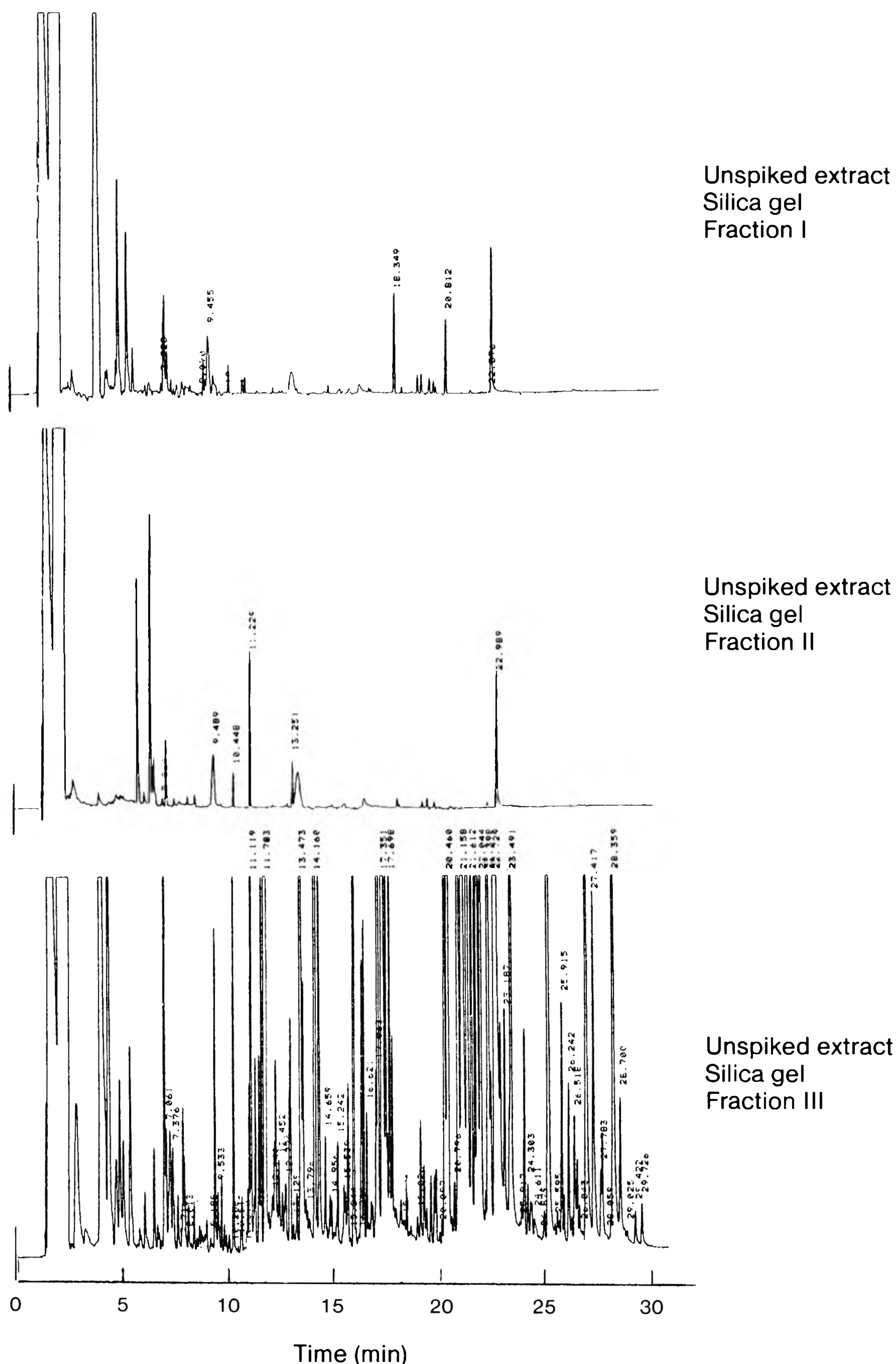


Figure 3. GC/EC chromatograms of silica gel Fractions I (top), II (middle), and III (bottom) of liquid waste extract analyzed on 30 m × 0.25 mm id DB-5 fused-silica capillary column.

butyl ammonium sulfite and reported recoveries >90% for DDT, DDD, DDE, and PCBs, and recoveries of 73–79% for aldrin and γ-BHC.

We have used the Jensen et al. procedure (24) on 5 real sample extracts fortified with OCPs and PCBs to determine if removal of sulfur is affected by matrix interferences and if OCP and PCB recoveries are acceptable (>80%). In addition to the real sample extracts, 3 pesticide standards were treated

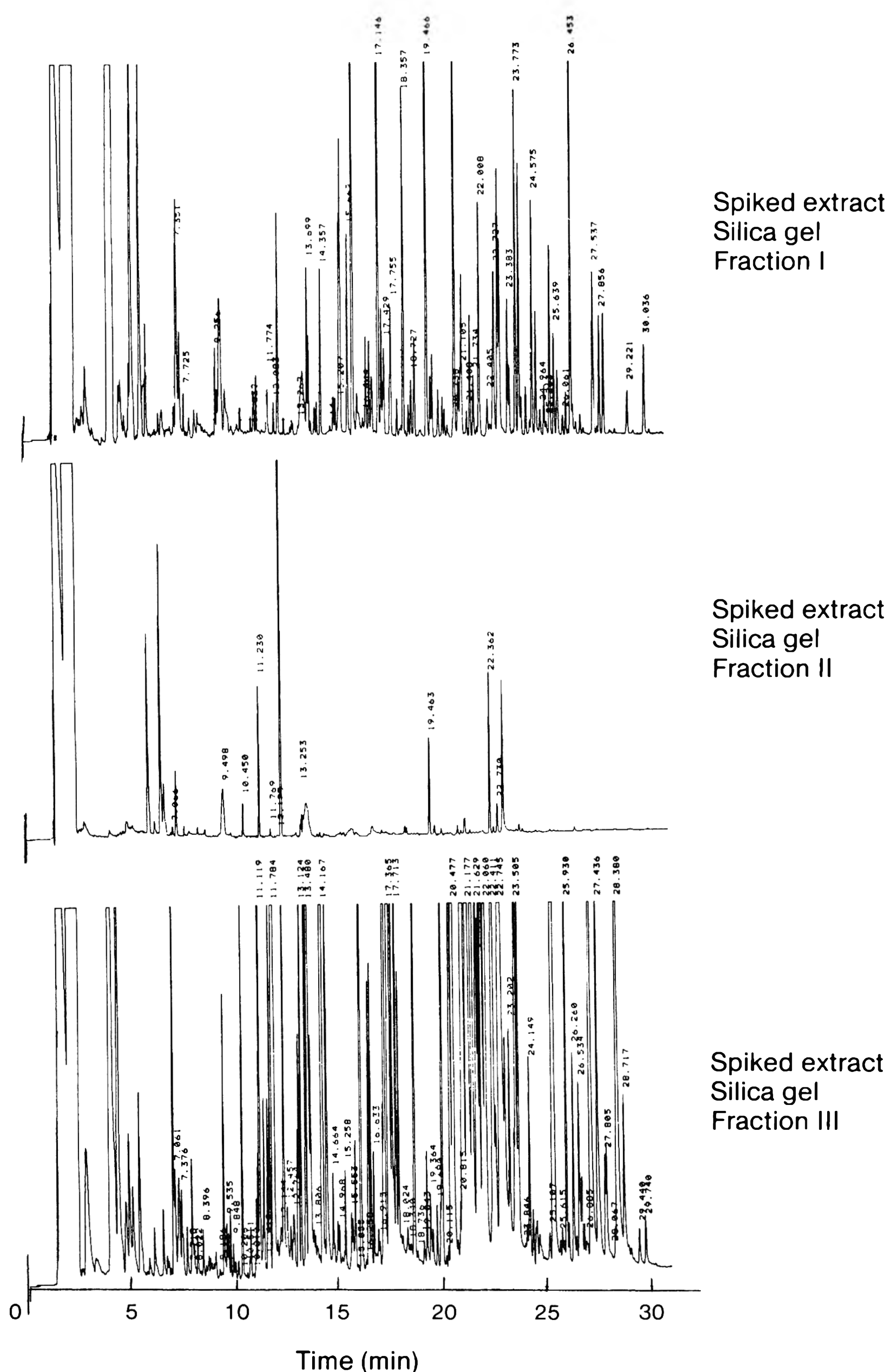


Figure 4. GC/EC chromatograms of silica gel Fractions I (top), II (middle), and III (bottom) of liquid waste spiked with OCPs and PCBs and analyzed on 30 m × 0.25 mm id DB-5 fused-silica capillary column.

with the tetrabutyl ammonium sulfite reagent to determine compound recovery in the absence of matrix interferences.

Results are presented in Table 5. Examination of the gas chromatograms showed that sulfur was removed quantitatively regardless of the matrix; therefore, this procedure is recommended for incorporation into the revised method protocol. Endrin aldehyde recovery was only about 10%, possibly because of the presence of sulfite.

Capillary GC

The GC retention times of 18 OCPs on the DB-5 and the SPB-608 fused-silica capillary columns are presented in Ta-

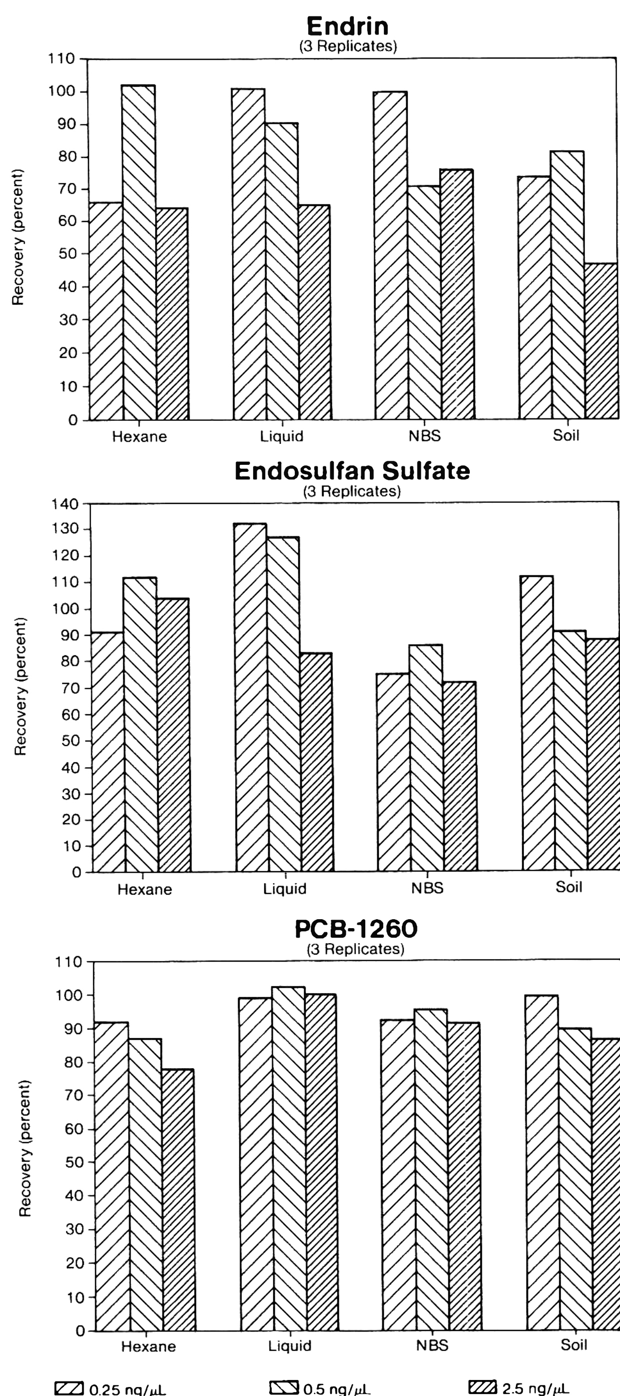


Figure 5. Distribution of average recoveries for endrin (top), endosulfan sulfate (middle), and Aroclor 1260 (bottom) for revised Method 8080.

ble 6. Toxaphene is not included because of its multipeak response. Aroclor mixtures have been analyzed individually on the DB-5 column; retention times of individual chlorinated biphenyls in these mixtures are given in Table 3.

We have indicated in Table 3 which of the OCPs elute at the same retention times as some of the components of the Aroclor mixtures. Of the 6 OCP peaks that overlap with PCB component peaks, only heptachlor and γ-chlordane are of concern, because the other 4 compounds are separated from PCBs during the silica gel chromatography step. The heptachlor peak overlaps with a PCB peak eluting at 15.91 ± 0.008 min (present in 6 of the 7 PCB mixtures), and the γ-chlordane peak overlaps with a PCB peak eluting at 19.48 ± 0.012 min (present in 4 of the 7 PCB mixtures).

Table 5. Recoveries (%) of OCPs and PCBs following reaction of standards[a] with tetrabutyl ammonium sulfite

Compound	Without sulfur added[a]	With sulfur added[b]
α-BHC	110	103
β-BHC	106	99
γ-BHC	1[illegible]2	108
δ-BHC	100	86
Heptachlor	103	97
Aldrin	104	99
Heptachlor epoxide	107	101
γ-Chlordane	98	100
Endosulfan I	108	106
4,4'-DDE and Dieldrin	91	103
Endrin	87	89
Endosulfan II	99	99
4,4'-DDD	103	106
Endrin aldehyde	10	8
Endosulfan sulfate	94	93
4,4'-DDT	86	90
4,4'-Methoxychlor	85	68
Toxaphene	93	83
Aroclor 1016	108	88
Aroclor 1260	87	68

[a] Average of 2 determinations.
[b] Single determination.

Method Performance

Method performance, as used here, includes the method precision, accuracy, and detection limit. To determine method precision and accuracy, clean hexane and extracts of environmental samples (a liquid waste, an NBS sediment SRM-1645, and a sandy loam soil sample) were spiked with OCPs listed in Table 1 (except toxaphene), with Aroclor 1016, and with Aroclor 1260 at 3 concentrations (0.25, 0.5, and 2.5 ng/μL for the OCPs and 2.5, 5.0, and 25 ng/μL for PCBs) and were processed through the method. Because of interference, δ-BHC, endosulfan II, and 4,4'-DDD could not be determined in the liquid waste when spiked at concentrations 1 and 2, endrin aldehyde could not be determined in the liquid waste at any of the 3 spike levels, and 4,4'-methoxychlor could not be determined in the NBS SRM-1645 when spiked at concentration 1. Spiked extracts rather than actual spiked samples were used in the determination of method precision and accuracy, because Method 8080 addresses only the GC analysis, including the extract cleanup step. Sample extracts were prepared according to procedures given in the 3500 series methods (16).

To summarize the significance of the results presented in Table 7, the interquartile ranges (defined by the data ranked within 25 to 75% of the total number of data points) of the average percent recoveries (method accuracy) and the relative standard deviations (RSD) (method precision) have been calculated. The interquartile range for method accuracy is 83–99% and for method precision is 5.4–14%.

No patterns between recovery and concentration of the OCPs and PCBs or the matrix were observed. Percent recovery of each compound as a function of matrix, for each of the 3 concentrations was plotted, but no trends were found. Figure 5 shows in a bar graph format how the recoveries of endrin, endosulfan sulfate, and Aroclor 1260 vary with compound concentration for each of the 4 matrixes. Likewise, RSDs of recoveries were plotted as a function of concentration for each of the 4 matrixes (Figure 6).

The method detection limits (MDL) are presented in Table 8. They were determined by subjecting 7 distilled water and 7 sandy loam soil samples containing the test compounds to the entire analytical process. Blank measurements for the distilled water and sandy loam soil were performed in trip-

Table 6. Summary of retention times (min) for organochlorine pesticides on fused-silica capillary columns[a]

Compound No.	Name	DB-5[b]	SPB-608[c]
1	α-BHC	12.29 ± 0.010	9.46
2	β-BHC	13.13 ± 0.009	11.33
3	γ-BHC	13.37 ± 0.011	10.97
4	δ-BHC	14.14 ± 0.011	12.73
5	Heptachlor	15.91 ± 0.008	12.46
6	Aldrin	17.16 ± 0.009	13.76
7	Heptachlor epoxide	18.60 ± 0.009	15.98
8	γ-Chlordane	19.48 ± 0.012	16.70
9	Endosulfan I	19.94 ± 0.010	17.40
10	4,4'-DDE	20.83 ± 0.008	18.36
11	Dieldrin	20.91 ± 0.008	18.60
12	Endrin	21.71 ± 0.007	19.96
13	Endosulfan II	22.05 ± 0.006	20.69
14	4,4'-DDD	22.38 ± 0.008	20.53
15	Endrin aldehyde	22.75 ± 0.007	21.90
16	Endosulfan sulfate	23.64 ± 0.008	22.54
17	4,4'-DDT	23.79 ± 0.008	21.72
18	4,4'-Methoxychlor	25.94 ± 0.007	24.90

[a] GC operating conditions are given in the Experimental section. Toxaphene and PCBs are not included because of their multipeak response.
[b] Values for DB-5 column are average retention time ± SD of 10 replicate determinations.
[c] Single determination.

licate. MDLs were computed from the SD of the 7 replicates and the Student's *t* value for a one-tailed test at the 99% confidence level (6 degrees of freedom). For water samples, MDLs ranged from 0.02 to 0.09 μg/L for OCPs and from 0.5 to 0.9 μg/L for PCBs. When using a 2 μL aliquot of a 10 mL extract (after silica gel fractionation) obtained from 1 L of water containing the test compounds, the amounts injected onto the GC column ranged from 4 to 18 pg for OCPs and from 100 to 180 pg for PCBs. For soil samples, MDLs ranged from 1 to 6 μg/kg for OCPs and from 60 to 70 μg/kg for PCBs. When using a 2 μL aliquot of a 10 mL extract (after silica gel fractionation) obtained from 10 g soil sample containing the test compounds, the amounts injected onto the GC column ranged from 2 to 12 pg for OCPs and from 120 to 140 pg for PCBs. These amounts are sufficient for identifying the compounds, since our low-level calibration standard contains 12.5 to 25 pg/μL for OCPs and 500 pg/μL for PCBs, and we usually inject 2 μL.

Detection limits lower than those given in Table 8 may be achievable with larger samples (typical sample volume for aqueous samples is 1 L; typical wet sample weight for solids is 10 g) and by concentrating the extracts to 0.5 mL instead of 10 mL. Except for heptachlor epoxide and endosulfan sulfate, our detection limits are higher than those reported for EPA Method 608 (1) because the final volume of the silica gel fractions was 10 mL. However, no interfering peaks coeluted with any of the test compounds to require background correction, and Aroclors 1016 and 1260 were measured in the presence of OCPs. Recoveries in the range of 58–134% were achieved for water samples, and they were similar to those measured at higher concentrations. In the case of the sandy loam soil sample, OCPs recoveries were lower (15–47%), probably because of sorption onto soil.

The revised Method 8080 presented here has been evaluated in a single laboratory with a few relevant liquid and solid wastes. However, the revised method should be evaluated in a number of different laboratories and with additional samples. This will help identify the applicability range and the limitations of the method and will define the interlaboratory method performance.

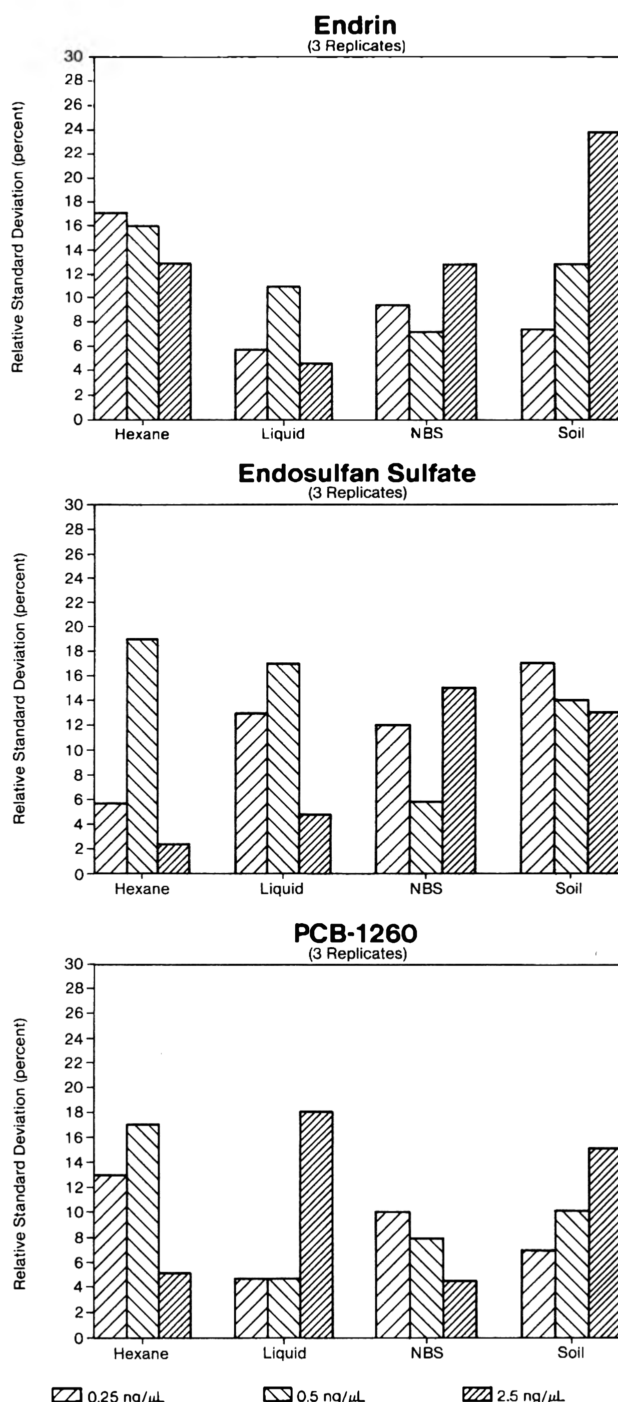


Figure 6. Distribution of relative standard deviations of average recoveries for endrin (top), endosulfan sulfate (middle), and Aroclor 1260 (bottom) for revised Method 8080.

When the revised Method 8080 is applied to samples that have not been previously characterized, confirmation of compounds identified in the sample may be required. Although the revised Method 8080 specifies a second capillary column for confirmation, additional work should be performed to determine the reliability of the identification obtained by the multicolumn technique. Determination of the OCPs and PCBs by gas chromatography/mass spectrometry (GC/MS) is an alternative that does not require fractionation of the extract by silica gel chromatography (3). With the GC/MS method, however, PCBs are identified and measured by level of chlorination. Furthermore, detection limits comparable to those obtained by using electron-capture detector may be difficult to achieve.

Table 7. Recoveries (%) of Method 8080 compounds from various spiked extracts of environmental samples

Compound	Hexane[a]			Liquid waste extract[a]		
	Concn 1	Concn 2	Concn 3	Concn 1	Concn 2	Concn 3
α-BHC	83 ± 16 (19)	106 ± 6.8 (6.4)	91 ± 4.6 (5.1)	96 ± 7.0 (7.3)	97 ± 3.5 (3.6)	79 ± 10 (13)
β-BHC	86 ± 15 (17)	110 ± 10 (9.5)	98 ± 2.0 (2.1)	92 ± 10 (11)	100 ± 4.0 (4.0)	90 ± 3.1 (3.4)
γ-BHC	85 ± 15 (18)	108 ± 11 (10)	99 ± 2.3 (2.3)	91 ± 10 (11)	100 ± 5.5 (5.5)	90 ± 4.0 (4.4)
δ-BHC	87 ± 12 (14)	109 ± 12 (11)	97 ± 1.6 (1.6)	[b]	[b]	90 ± 11 (8.8)
Heptachlor	79 ± 11 (13)	94 ± 9.5 (10)	83 ± 6.6 (7.9)	89 ± 14 (15)	94 ± 10 (11)	90 ± 11 (12)
Aldrin	88 ± 11 (13)	107 ± 9.5 (8.9)	89 ± 4.2 (4.7)	96 ± 8.9 (9.3)	98 ± 9.4 (9.6)	92 ± 9.2 (10)
Heptachlor epoxide	94 ± 6.7 (7.1)	109 ± 14 (13)	100 ± 23 (23)	111 ± 17 (15)	109 ± 14 (13)	89 ± 4.1 (4.6)
γ-Chlordane	94 ± 13 (14)	110 ± 11 (10)	91 ± 1.2 (1.3)	100 ± 8.5 (8.5)	103 ± 2.5 (2.4)	95 ± 8.0 (8.4)
Endosulfan I	89 ± 12 (14)	108 ± 13 (12)	99 ± 2.3 (23)	95 ± 6.5 (6.8)	100 ± 12 (12)	88 ± 3.8 (4.3)
4,4′-DDE	92 ± 13 (14)	107 ± 15 (14)	89 ± 4.5 (5.0)	119 ± 11 (8.9)	113 ± 2.5 (2.2)	95 ± 16 (17)
Dieldrin	89 ± 12 (14)	112 ± 13 (12)	102 ± 1.5 (1.5)	88 ± 3.2 (3.6)	86 ± 9.2 (11)	82 ± 4.3 (5.3)
Endrin	66 ± 11 (17)	65 ± 10 (16)	64 ± 8.3 (13)	101 ± 5.9 (5.8)	90 ± 10 (11)	65 ± 3.1 (4.7)
Endosulfan II	86 ± 7.9 (9.2)	111 ± 14 (13)	101 ± 0.6 (0.6)	[b]	[b]	79 ± 7.1 (9.0)
4,4′-DDD	89 ± 12 (14)	110 ± 9.8 (8.9)	97 ± 1.7 (1.8)	[b]	[b]	76 ± 16 (21)
Endrin aldehyde	83 ± 8.3 (10)	102 ± 19 (19)	95 ± 3.0 (3.2)	[b]	[b]	[b]
Endosulfan sulfate	91 ± 5.2 (5.7)	112 ± 21 (19)	104 ± 2.5 (2.4)	132 ± 17 (13)	127 ± 22 (17)	83 ± 4.0 (4.8)
4,4′-DDT	74 ± 19 (26)	88 ± 13 (15)	73 ± 5.8 (8.0)	101 ± 23 (23)	83 ± 11 (13)	88 ± 18 (21)
4,4′-Methoxychlor	98 ± 2.6 (2.7)	104 ± 18 (17)	104 ± 3.2 (3.1)	49 ± 14 (29)	58 ± 9.3 (16)	75 ± 4.6 (6.1)
Aroclor 1016	94 ± 14 (15)	93 ± 6.5 (7.0)	93 ± 2.0 (2.2)	114 ± 6.0 (5.3)	122 ± 10 (8.3)	118 ± 9.8 (8.3)
Aroclor 1260	92 ± 12 (13)	87 ± 15 (17)	78 ± 4.0 (5.1)	99 ± 4.6 (4.6)	102 ± 4.7 (4.6)	100 ± 18 (18)
	NBS SRM-1645 extract[a]			**Sandy loam soil extract[a]**		
	Concn 1	Concn 2	Concn 3	Concn 1	Concn 2	Concn 3
α-BHC	73 ± 2.1 (2.9)	75 ± 6.0 (8.0)	76 ± 5.6 (7.3)	86 ± 9.5 (11)	87 ± 4.9 (5.7)	89 ± 2.5 (2.8)
β-BHC	88 ± 4.1 (4.7)	94 ± 3.0 (3.2)	92 ± 7.1 (7.7)	94 ± 8.4 (8.9)	90 ± 4.1 (4.5)	93 ± 5.0 (5.4)
γ-BHC	83 ± 3.0 (3.6)	89 ± 4.1 (4.6)	93 ± 8.1 (8.7)	92 ± 11 (12)	91 ± 3.0 (3.3)	95 ± 4.6 (4.8)
δ-BHC	85 ± 4.6 (5.4)	92 ± 5.2 (5.6)	94 ± 8.7 (9.3)	94 ± 12 (13)	89 ± 3.6 (4.1)	93 ± 6.1 (6.5)
Heptachlor	53 ± 10 (19)	70 ± 7.7 (11)	88 ± 4.1 (4.7)	89 ± 9.6 (11)	83 ± 8.7 (11)	79 ± 17 (21)
Aldrin	69 ± 3.6 (5.2)	65 ± 4.6 (7.1)	72 ± 1.0 (1.4)	99 ± 4.4 (4.4)	88 ± 2.0 (2.3)	82 ± 12 (15)
Heptachlor epoxide	91 ± 4.9 (5.4)	91 ± 5.7 (6.3)	93 ± 8.6 (9.2)	96 ± 11 (11)	90 ± 4.6 (5.1)	94 ± 6.6 (7.0)
γ-Chlordane	77 ± 5.3 (6.9)	81 ± 4.9 (6.1)	85 ± 1.0 (1.2)	100 ± 8.3 (8.3)	93 ± 3.0 (3.2)	87 ± 11 (13)
Endosulfan I	85 ± 5.5 (6.5)	88 ± 5.1 (5.8)	91 ± 9.1 (10)	95 ± 10 (11)	89 ± 5.3 (5.9)	93 ± 7.5 (8.1)
4,4′-DDE	75 ± 5.3 (7.1)	76 ± 7.1 (9.3)	84 ± 1.0 (1.2)	105 ± 14 (13)	93 ± 5.6 (6.0)	82 ± 11 (13)
Dieldrin	92 ± 8.6 (9.4)	85 ± 9.4 (11)	94 ± 10 (11)	113 ± 12 (11)	99 ± 4.6 (4.6)	93 ± 8.2 (8.8)
Endrin	100 ± 9.5 (9.5)	87 ± 6.4 (7.3)	76 ± 9.9 (13)	74 ± 5.6 (7.5)	60 ± 7.6 (13)	47 ± 11 (24)
Endosulfan II	80 ± 7.4 (9.2)	81 ± 4.5 (5.5)	91 ± 12 (13)	97 ± 14 (14)	86 ± 6.7 (7.8)	89 ± 11 (12)
4,4′-DDD	106 ± 6.4 (6.0)	85 ± 3.1 (3.6)	90 ± 7.2 (8.0)	103 ± 9.6 (9.3)	88 ± 7.8 (8.9)	90 ± 7.8 (8.6)
Endrin aldehyde	70 ± 5.7 (8.2)	71 ± 9.2 (13)	88 ± 12 (14)	86 ± 11 (13)	82 ± 7.6 (9.3)	73 ± 20 (28)
Endosulfan sulfate	75 ± 8.7 (12)	86 ± 5.0 (5.8)	72 ± 11 (15)	112 ± 19 (17)	91 ± 13 (14)	88 ± 11 (13)
4,4′-DDT	54 ± 13 (24)	61 ± 7.9 (13)	76 ± 2.5 (3.3)	107 ± 25 (23)	83 ± 5.3 (6.5)	64 ± 14 (21)
4,4′-Methoxychlor	[b]	99 ± 17 (17)	92 ± 17 (19)	91 ± 14 (15)	89 ± 9.6 (11)	86 ± 14 (16)
Aroclor 1016	104 ± 9.0 (8.7)	104 ± 2.5 (2.4)	102 ± 4.6 (4.5)	90 ± 15 (17)	92 ± 4.5 (4.9)	85 ± 10 (12)
Aroclor 1260	92 ± 9.5 (10)	95 ± 7.5 (7.9)	91 ± 4.0 (4.4)	99 ± 6.8 (6.9)	89 ± 8.9 (10)	85 ± 13 (15)
Concn, ng/μL extract	0.25	0.5	2.5	0.25	0.5	2.5
No. of detns	3	3	3	3	3	3

[a] Triplicate determinations; value in parentheses is relative standard deviation.

[b] Unable to determine recovery because of interferences.

Table 8. Method 8080 detection limits (MDL) for distilled water and sandy loam soil[a]

	Distilled water		Sandy loam soil		EPA Method 608 (water)	
Compound	MDL, µg/L	Av. rec., %	MDL, µg/kg	Av. rec., %	MDL, µg/L	Av. rec., %
α-BHC	0.035	70	1.9	15	0.003	99
β-BHC	0.023	89	3.3	42	0.006	97
γ-BHC	0.025	91	2.0	38	0.004	103
δ-BHC	0.024	90	1.1	42	0.009	91
Heptachlor	0.040	81	2.0	38	0.003	91
Aldrin	0.034	63	2.2	26	0.004	84
Heptachlor epoxide	0.032	88	2.1	41	0.083	153
γ-Chlordane	0.037	58	1.5	32	0.014	99
Endosulfan I	0.030	90	2.1	41	0.014	72
4,4'-DDE	0.058	84	2.5	46	0.004	96
Dieldrin	0.044	96	[b]	—	0.002	100
Endrin	0.039	88	3.6	38	0.006	101
Endosulfan II	0.040	92	2.4	45	0.004	97
4,4'-DDD	0.050	96	4.2	40	0.011	100
Endrin aldehyde	0.050	76	1.6	29	[b]	[b]
Endosulfan sulfate	0.035	95	3.6	47	0.066	81
4,4'-DDT	0.081	85	3.6	46	0.012	99
4,4'-Methoxychlor	0.086	134	5.7	48	[b]	[b]
Aroclor 1016	0.54	89	57	117	[b]	[b]
Aroclor 1260	0.90	92	70	104	[b]	[b]

[a] Spiking levels are as follows: distilled water—0.1 µg/L for OCPs, 1.0 µg/L for PCBs; sandy loam soil—10 µg/kg for OCPs, 100 µg/kg for PCBs.
[b] Not available.

Use of internal standards and surrogate spiking compounds needs to be investigated in future studies. Much of the variability inherent to trace analysis in difficult samples can be reduced by the use of internal standards. Likewise, surrogate spiking compounds can provide recovery information for each sample. These surrogate spiking compounds should be selected so that they will elute into each of the 3 fractions collected from the silica gel column.

References

(1) *Fed. Regist.* (1984) Method 608, **49**(209), 89–104
(2) "Determination of Pesticides and PCBs in Water and Soil/Sediment by Gas Chromatography/Mass Spectrometry" (1985) Environmental Monitoring and Support Laboratory, EPA, Cincinnati, OH, Method 680
(3) Alford-Stevens, A. L., Bellar, T. A., Eichelberger, J. W., & Budde, W. L. (1986) *Anal. Chem.* **58**, 2022–2029
(4) Gebhart, J. E., Hayes, T. L., Alford-Stevens, A. L., & Budde, W. L. (1985) *Anal. Chem.* **57**, 2458–2463
(5) Slivon, L. E., Gebhart, J. E., Hayes, T. L., Alford-Stevens, A. L., & Budde, W. L. (1985) *Anal. Chem.* **57**, 2464–2469
(6) Pellizzari, E. D., Tomer, K. B., & Moseley, M. A. (1981) in *Advances in the Identification and Analysis of Organic Pollutants in Water,* L. H. Keith (Ed.), Ann Arbor Science, Ann Arbor, MI, Vol. 1, Chap. 12
(7) Mills, P. A., Bong, B. A., Kamps, L. R., & Burke, J. A. (1972) *J. Assoc. Off. Anal. Chem.* **55**, 39–43
(8) Burke, J. A., & Malone, B. (1966) *J. Assoc. Off. Anal. Chem.* **49**, 1003–1007
(9) Mills, P. A. (1968) *J. Assoc. Off. Anal. Chem.* **51**, 29–32
(10) McMahon, B., & Burke, J. A. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 640–652
(11) de Faubert Maunder, M. J., Egan, H., Godly, E. W., Hammond, E. W., Roburn, E. W., & Thompson, J. (1964) *Analyst* **89**, 168–174
(12) Ambrus, A., Lantos, J., Visi, E., Csatlos, I., & Sarvari, L. (1981) *J. Assoc. Off. Anal. Chem.* **64**, 733–742
(13) Armour, J. A., & Burke, J. A. (1970) *J. Assoc. Off. Anal. Chem.* **53**, 761–768
(14) Leoni, V. (1971) *J. Chromatogr.* **62**, 63–71
(15) Biddleman, T. F., Matthews, J. R., Olney, C. E., & Rice, C. R. (1978) *J. Assoc. Off. Anal. Chem.* **61**, 820–828
(16) *Test Methods for Evaluating Solid Waste* (1982) 2nd Ed., U.S. Environmental Protection Agency, Washington, DC, SW-846
(17) Lopez-Avila, V., Northcutt, R., Onstot, J., & Wickham, M. (1983) *Anal. Chem.* **55**, 881–889
(18) Glazer, J. A., Foerst, D. L., McKee, G. D., Quave, S. A., & Budde, W. L. (1981) *Environ. Sci. Technol.* **15**, 1426–1435
(19) Lopez-Avila, V., Schoen, S., & Milanes, J. (1986) "Single-Laboratory Evaluation of Method 8080 Organochlorine Pesticides and PCBs," Final Report for Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Las Vegas, NV, Contract 68-03-3226
(20) Goerlitz, D. F., & Law, L. M. (1971) *Bull. Environ. Contam. Toxicol.* **6**, 9–10
(21) Thompson, J. F. (Ed.) (1977) *Analysis of Pesticide Residues in Human and Environmental Samples,* Revision, U.S. Environmental Protection Agency, Research Triangle Park, NC
(22) Blummer, M. (1957) *Anal. Chem.* **29**, 1039–1041
(23) Ahnoff, M., & Josefsson, B. (1975) *Bull. Environ. Contam. Toxicol.* **13**, 159–166
(24) Jensen, S., Renberg, L., & Reutergardh, L. (1977) *Anal. Chem.* **49**, 316–318

DRUGS

Simple Determination of Ranitidine in Dosage Forms by In-Phase Selective AC Polarography

JUAN A. SQUELLA, LUIS A. ZUÑIGA, IGOR LEMUS, and LUIS J. NUÑEZ-VERGARA
Universidad de Chile, Facultad de Ciencias Quimicas y Farmaceuticas, PO Box 233, Santiago 1, Chile

A new AC polarographic method for the determination of pharmaceutical forms of ranitidine is proposed, based on the electroactivity of the ranitidine nitro group. Individual and composite assays as well as recovery studies are described. Results show adequate precision and accuracy. Sample preparation is easy and no excipient separation is required.

Ranitidine is a well-known antiulcerous drug which inhibits acid and pepsin secretion by reducing gastric secretion volume (1–4). Pharmacokinetic studies in humans indicate that the plasma concentration is directly proportional to dosage. Its half life ranges between 157 and 187 min. The bioavailability is higher than 50% and the plasma fraction binding to protein lies between 9.8 and 18.9%.

Several analytical procedures, such as spectrophotometry, nuclear magnetic resonance, and liquid chromatography (3, 4), have been described. Recently, electroanalytical techniques, specifically polarography, have been considered an important tool in pharmaceutical analyses (5–7). The principal advantage of this technique in formulation analysis is that excipients do not interfere to as great an extent as they do with other methods. We propose a new AC polarographic method for ranitidine in dosage forms.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Beckman DB UV-Vis, with 1 cm quartz cells.

(b) *Polarograph.*—Tacussel assembly operated in AC mode, essentially the same as described earlier (8). (In DC and DPP mode, the Tacussel assembly consisted of an EPL-3 recorder equipped with a TI-PULS module.) Operating conditions: potential scan rate 10 mV/s; drop time 1 s; frequency of superimposed AC signal 20 Hz; amplitude 10 mV; phase angle 0°; pulse amplitude (DPP) 60 mV.

(c) *Polarographic cell.*—Tacussel CPRA measuring cell with dropping mercury electrode, platinum wire counter electrode, and saturated calomel reference electrode (SCE).

(d) *McIlvaine buffer.*—Dissolve 14.7 g $Na_2HPO_4 \cdot 12H_2O$ and 16.7 g citric acid monohydrate in double-distilled water and then dilute to 1 L. Adjust to pH 3.0.

(e) *Standard solutions.*—Accurately weigh 350.9 mg ranitidine hydrochloride standard (Laboratorio Sanitas, Santiago, Chile); dissolve and dilute with double-distilled water to 100 mL (concentration = 10mM).

(f) *Solutions for calibration curve.*—*Polarography:* Accurately dilute standard solution with McIlvaine buffer to obtain solutions ranging from 0.1 to 1mM. *Spectrophotometry:* Accurately dilute standard solution with McIlvaine buffer to obtain solutions with concentrations ranging from 0.015 to 0.035mM.

(g) *Synthetic samples.*—Prepare excipient powders for recovery studies according to manufacturer's batch formulas for 150 mg ranitidine.

Received March 13, 1987. Accepted June 28, 1987.

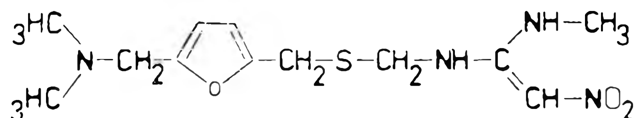


Figure 1. Structure of ranitidine.

Spectrophotometry

Dissolve amount of sample containing ca 150 mg ranitidine in 100 mL McIlvaine buffer. Take 1 mL and then dilute 200-fold with same buffer. Measure absorptivity at 313 nm, using McIlvaine buffer as blank. Calculate concentration from standard calibration curve.

Polarography

Dissolve amount of sample containing ca 150 mg ranitidine in 100 mL McIlvaine buffer. Take mL and then dilute 10-fold with same buffer.

Transfer no less than 10 mL solution to dry polarographic cell and degas by bubbling nitrogen through solution 5 min. Scan sample solution at least twice from −0.5 to −0.9 V. Calculate mg ranitidine in sample solution from prepared standard calibration curve.

Results and Discussion

Ranitidine (Figure 1) exhibits an AC polarographic peak characteristic of the 4-electron reduction of the nitro group. Two minor peaks are also observed, probably due to the reducible protonated hydroxylamine and other protonated related structures. A typical AC polarogram for ranitidine is shown in Figure 2. The reduction of the nitro group is

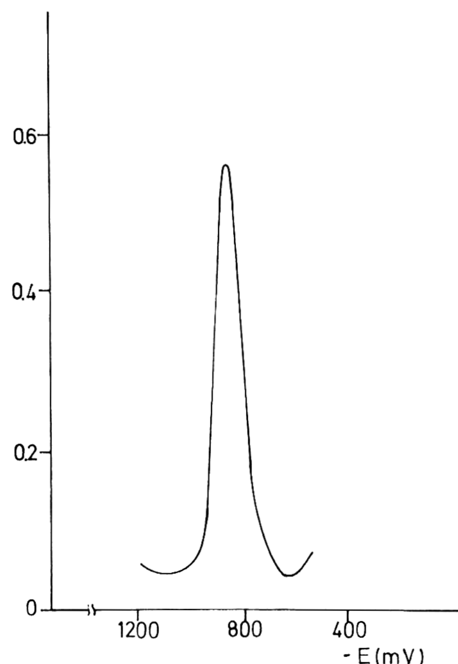


Figure 2. AC polarogram of pH 3 ranitidine solution, 0.5 mM.

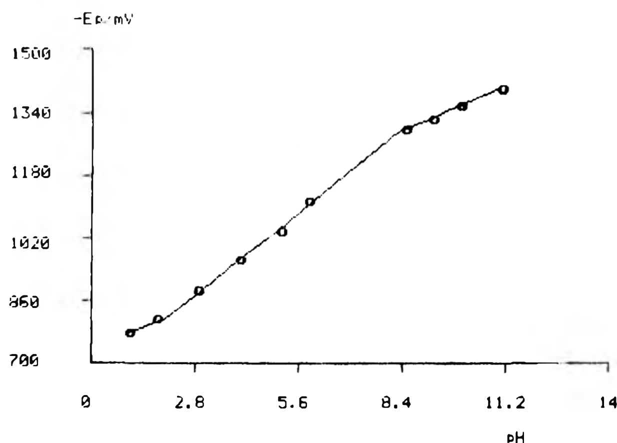


Figure 3. Dependence of peak potential of ranitidine on pH at 25°C.

irreversible and shows a strong dependence between pH and peak potential (Figure 3). The breaks in the straight line behavior at approximately pH 2.7 and pH 8.2 are in accord with the pK values described (9) for ranitidine. In spite of the above dependence, the peak height is practically unaffected by pH changes (between pH 2.5 and 12.0).

To obtain better dissolution conditions and polarographic resolution, pH 3 McIlvaine buffer is preferred. Under these conditions, a linear correlation between the square root of the mercury column height with the limiting current is found. The temperature coefficient is better than 2%/°C. The above experiments confirm that the electrode process is diffusion-controlled, limited by the diffusion of the ranitidine molecule to the electrode surface.

The peak height shows a good correlation with ranitidine concentration; it is possible to reach a detection limit of 7×10^{-4}mM, determined as 2 times noise level. For the quantitative determination, the calibration curve method was used. This curve is described by the following equation obtained by the least square linear regression method:

$$ip(\mu A) = -0.087 + 1316.21C\,(M)$$

(correlation coefficient 0.996, n = 19, concentration range 0.1–1mM, pH 3, and 25°C), where ip = peak current and C = ranitidine concentration. To obtain comparative results, we also applied a UV spectrophotometric method, based on the ranitidine absorption band at 313 nm (pH 3). The relationship between absorptivity and drug concentration is linear. For analytical determinations a calibration curve was used. This curve for ranitidine concentrations between 0.015 and 0.035mM, obtained by the least square linear regression method, is:

$$A = 0.044C\,(\mu g/mL) + 0.019$$

(correlation coefficient 0.988, n = 10), where A = absorbance of ranitidine solutions at 313 nm and C = ranitidine concentration.

Table 1 shows the recoveries from synthetic samples by both methods. Average recovery of 99.9% (SD 1.0) indicates optimal accuracy for the method developed. There were no significant differences between recovery results obtained by both methods; however, the polarographic technique provides better precision. Table 2 shows composite analyses with similar accuracy for both methods and better precision for the polarographic one. In Table 3, for individual tablet assays similar conclusions can be asserted. Comparison between the ranitidine content in 2 different pharmaceutical forms (Table 3) are in accord with accepted standards.

The proposed method proved to have adequate precision and accuracy to carry out reliable analyses of raniditine. No preparation of the sample is required before polarographic analysis; excipients present both in the tablets and in injection forms do not interfere in the analyses. Excessive dilution of the pharmaceutical forms prior to polarographic analysis is not required. With these characteristics, the polarographic method can be a useful alternative analysis of pharmaceutical forms of ranitidine.

Table 2. Composite analyses of ranitidine samples[a]

	Polarography, mg	Spectrophotometry, mg
	146.1	142.3
	149.7	156.2
	147.9	148.5
	150.6	147.4
	151.5	148.9
	147.9	149.2
Av.	149.0	148.8
SD (n − 1)	2.0	4.5

[a] Each sample contained 319.3 mg of powdered material (average weight of 10 tablets).

Acknowledgments

This research was supported by grant No. 1007 of the Fondo Nacional de Ciencias and a grant of the D.I.B., University of Chile. Furthermore, the authors express their gratitude to Claudio Telha M. for his assistance in writing this paper.

Table 1. Recovery of ranitidine from synthetic samples[a]

	Spectrophotometry		Polarography	
	Found, mg	Rec., %	Found, mg	Rec., %
	151.4	100.9	147.0	98.0
	148.4	98.9	149.0	99.3
	149.3	99.5	151.0	100.7
	143.5	95.7	150.0	100.0
	149.3	99.5	149.0	99.3
	148.3	98.9	152.0	101.3
	151.6	101.1	150.0	100.0
	154.9	103.3	152.0	101.3
	151.8	101.2	151.0	100.7
	146.3	97.5	148.0	98.7
Av.	149.5	99.6	149.9	99.9
SD (n − 1)	3.2	2.1	1.6	1.0

[a] Each synthetic mix represents a commercially available formulation, containing 150 mg ranitidine.

Table 3. Individual assays[a] for 2 dosage forms

	Polarography, mg		Spectrophotometry, mg	
	Tablets[b]	Injections[c]	Tablets	Injections
	148.8	50.6	149.3	48.9
	143.4	49.4	157.1	46.2
	145.1	49.6	153.0	47.4
	147.0	50.3	161.2	46.3
	147.0	49.4	146.7	48.0
	148.8	49.6	154.7	46.5
Av.	146.7	49.8	153.7	47.2
SD (n − 1)	2.1	0.5	5.2	1.1

[a] Assays were performed on the same unit by both methods.
[b] Ranitidine tablets (Laboratorio Chile, Santiago, Chile); declared amount = 150 mg ranitidine.
[c] Zantac® (Laboratorio Glaxo, Santiago, Chile); declared amount = 50 mg per 5 mL solution.

References

(1) *Zantac Monografia Cientifica* (1983) Laboratorio Glaxo Farmaceutica Chilena Ltda, Santiago, Chile
(2) *Ranitidina Monografia Cientifica* (1985) Laboratorio Chile S.A., Santiago, Chile
(3) Mihaly, G. W., Drumer, O. H., Marshall, A., Shall, R. A., & Louis, W. J. (1980) *J. Pharm. Sci.* **62,** 1155–1157
(4) Carey, P. F., & Martin, L. E. (1979) *J. Liq. Chromatogr.* **2,** 1291
(5) Bersier, P. M., & Bersier, J. (1985) *CRC Crit. Rev. Anal. Chem.* Vol. 16, CRC Press, Boca Raton, FL, pp. 15–128
(6) Kuta, J., & Palacek, E. (1983) in *Topics in Bioelectrochemistry and Bioenergetics,* Vol. 5, Wiley Interscience, New York, NY, pp. 1–65
(7) Browne, J. T. (1979) *Polarography of Molecules of Biological Significance,* Academic Press, London, UK, Chapter A
(8) Squella, J. A., & Nuñez-Vergara, L. J. (1986) *Elecrochim. Acta* **31,** 767–769
(9) *USP Drug Information* (1985) Vol. 1, U.S. Pharmacopeial Convention, Rockville, MD, p. 1085

Liquid Chromatographic Determination of Carbenicillin and Ticarcillin in Injectable Dosage Forms

BARRY MOPPER
Food and Drug Administration, New York Regional Laboratory, 850 Third Ave, Brooklyn, NY 11232-1593

A reverse-phase liquid chromatographic method is described for the assay of carbenicillin and ticarcillin in injectable dosage forms. These penicillins were analyzed isocratically on an octadecyl silane column using a mobile phase consisting of acetonitrile-0.01M potassium phosphate monobasic (12 + 88, v/v) with photometric detection at 225 nm. Propylthiouracil was used as an internal standard. Detector responses were rectilinearly related to concentrations of carbenicillin and ticarcillin in the ranges 40 to 130 μg/mL (r = 0.9987) and 20 to 90 μg/mL (r = 0.9998), respectively. Using the standard additions method, mean recoveries of carbenicillin and ticarcillin from commercial powders for injection were 99.8 and 100.2%, respectively, with a range of 98.1-102.2%. The coefficients of variation (CV) for repeated determinations of total carbenicillin and ticarcillin content in injectables were 0.3 and 0.2%, respectively. A procedure is included for separating the diastereoisomers of both penicillins by ion-pairing reverse-phase liquid chromatography.

Official methods for the determination of the semisynthetic penicillins, carbenicillin and ticarcillin, are based on a tedious and often inaccurate microbiological agar diffusion technique (1). These antibiotics can be more conveniently analyzed by reverse-phase liquid chromatography (RP-LC) (2–7, 9). Das Gupta and Stewart (2) used a semipolar (phenyl) column and an ammonium acetate mobile phase for the assay of the antibiotics in injectable solutions. Twomey (3) applied an ion-pair RP-LC system to separate carbenicillin from other penicillins, and to resolve the carbenicillin diastereoisomeric pair. Watson (4) determined ticarcillin and ticarcillin isomers in biological fluids by RP-LC analysis. Hoogmartens et al. (5) used a ternary aqueous mobile phase and a C_8 column to separate the diastereoisomers of carbenicillin and ticarcillin, and to monitor the epimerization of the drug. Kwan et al. (6), Aravind et al. (7), and Haginaka and Wakai (9) have described RP-LC methods for the determination of ticarcillin in serum and urine samples. The method of Haginaka and Wakai is noteworthy in that it resorts to a derivatization of the analyte to a colored product in order to improve on the sensitivity and selectivity that can be attained with methods using UV detection. In most of these LC methods, carbenicillin and ticarcillin tend to separate into their corresponding diastereoisomers. Twomey (3) has verified that the chromatographic resolution of the isomeric carbenicillins is controlled by the pH of the mobile phase, and that at precisely pH 3.35, the 2 isomers will coelute as a single peak in about 10 min. Attempts in this laboratory to duplicate these results by using a commercial C_{18} column resulted in 2 broad peaks, which exhibited considerable tailing and long retention times. The determination of carbenicillin and ticarcillin in injectable dosage forms can be performed in a simple and reliable manner by the RP-LC method described in this paper. The effects of concentration of ion-pairing reagent in the mobile phase on the chromatographic behavior of carbenicillin and ticarcillin were also studied to provide 2 sets of chromatographic conditions that will permit these drugs to be eluted, depending on the need, as single peaks or as pairs of diastereoisomeric peaks.

Received March 5, 1987. Accepted August 4, 1987.
Presented in part at the Eastern Analytical Symposium, Oct. 20–24, 1986, New York, NY.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Model 510 solvent pump, Model 481 variable wavelength detector, Model U6K injector valve, and Model 730 integrating recorder (Waters Chromatography Division, Milford, MA 01757).

(b) *Column.*—Stainless steel, 30 cm × 3.9 mm id, prepacked with 10 μm particle size μBondapak C_{18} (Waters Chromatography Division).

Reagents

(a) *Solvents.*—LC grade acetonitrile and methanol.

(b) *Potassium phosphate monobasic solution.*—Prepare 0.01M solution by weighing 1.361 g KH_2PO_4 (Fisher Scientific Co., Fair Lawn, NJ 07410) into 1 L water; prepare fresh.

(c) *1-Octanesulfonic acid sodium salt solution.*—Prepare 0.02M solution by weighing 4.325 g 1-octanesulfonic acid sodium salt (Eastman Kodak Co., Rochester, NY 14650) into 1 L 0.2% glacial acetic acid.

(d) *Internal standard solution (IS).*—Dissolve USP propylthiouracil reference standard in 50% acetonitrile to obtain a concentration of ca 0.1 mg/mL.

(e) *Carbenicillin standard preparation.*—100 μg/mL. Accurately weigh ca 10 mg USP Carbenicillin Monosodium Monohydrate Reference Standard into 100 mL volumetric flask, add 25.0 mL IS, dilute to volume with water, and mix.

(f) *Ticarcillin standard preparation.*—60 μg/mL. Accurately weigh ca 6 mg USP Ticarcillin Monosodium Mono-

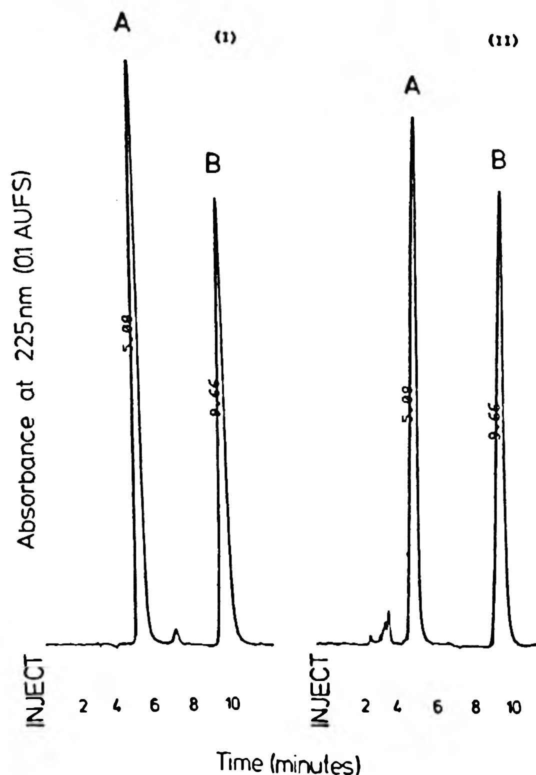


Figure 1. Typical chromatograms of (I) standard preparation and (II) sample preparation: A, carbenicillin; B, propylthiouracil IS. See assay for conditions.

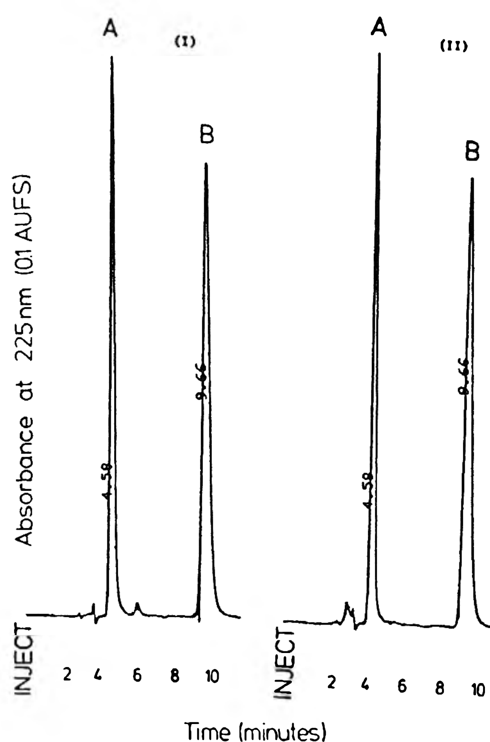


Figure 2. Typical chromatograms of (I) standard preparation and (II) sample preparation: A, ticarcillin; B, propylthiouracil IS. See assay for conditions.

hydrate Reference Standard, and prepare in same manner as carbenicillin standard preparation.

(g) *Mobile phase for assay.*—Acetonitrile–0.01M potassium phosphate monobasic (12 + 88, v/v).

(h) *Mobile phase for diastereoisomeric separation.*—Acetonitrile–methanol–0.01M potassium phosphate monobasic–0.02M octanesulfonic acid sodium salt in 0.2% glacial acetic acid (12 + 7 + 41 + 40, v/v).

Sample Preparation

Constitute powder for injection in water according to manufacturer's instructions. In a stepwise and quantitative fashion, dilute portion of constituted solution with water. Mix aliquot of dilution with 25 mL IS and dilute to 100 mL with water. Solution contains ca 60 μg/mL ticarcillin or 100 μg/mL carbenicillin.

Assay

Inject 20 μL each of standard preparation and sample preparation into chromatograph, using mobile phase flow rate of 1 mL/min, detector set at 225 nm and 0.1 AUFS, and chart speed of 0.5 cm/min.

Calculations

Obtain quantity of total penicillin (as free acid) in the injectable sample as follows:

$$\text{Total penicillin (as free acid), g} = (Ru/Rs) \times C \times D \times (V/G)$$

where Ru and Rs = the ratios of peak area of penicillin to peak area of IS for sample preparation and standard preparation, respectively; C = potency of standard preparation, in g penicillin (as free acid)/mL; D = sample dilution factor; V = total volume of sample, mL; and G = volume of sample taken for analysis, mL.

Results and Discussion

Linearity and reproducibility.—Peak area ratios were linearly related to concentrations of carbenicillin (as free acid) ranging from 40 to 130 μg/mL ($r = 0.9987$) and to concentrations of ticarcillin (as free acid) ranging from 20 to 90 μg/mL ($r = 0.9998$). The coefficients of variation (CV) for 5 replicate injections of carbenicillin and ticarcillin standards were 0.59 and 0.14%, respectively. Typical chromatograms of carbenicillin and ticarcillin standards and commercial samples are shown in Figures 1 and 2.

Recovery study and assay results.—The results of recovery studies of carbenicillin and ticarcillin standards added to composites of commercial powders for injection are shown in Table 1. The average recoveries for carbenicillin and ticarcillin were 99.8% and 100.2%, respectively. Table 2 presents LC assay results obtained on duplicates, for commercial samples of carbenicillin and ticarcillin injections. These results are within the potency limits set forth in the CFR (1) for ticarcillin (90–115% of declared) and carbenicillin (90–120% of declared).

Chromatographic behavior of carbenicillin and ticarcillin.—Carbenicillin and ticarcillin exist as diastereoisomeric pairs by virtue of an existing asymmetric carbon on the side

Table 1. Recovery study of carbenicillin and ticarcillin from commercial injectables by standard addition method[a]

Injectable[b]	Amt added, mg	Amt found, mg	Rec., %	Mean rec., %
Carbenicillin	4.973	5.053	101.6	99.8
	5.007	4.912	98.1	
Ticarcillin	3.217	3.288	102.2	100.2
	3.780	3.716	98.3	

[a] Prepared by spiking solution of constituted commercial samples with known amounts of standards.
[b] Amount declared (as free acid): carbenicillin = 2 g; ticarcillin = 3 g.

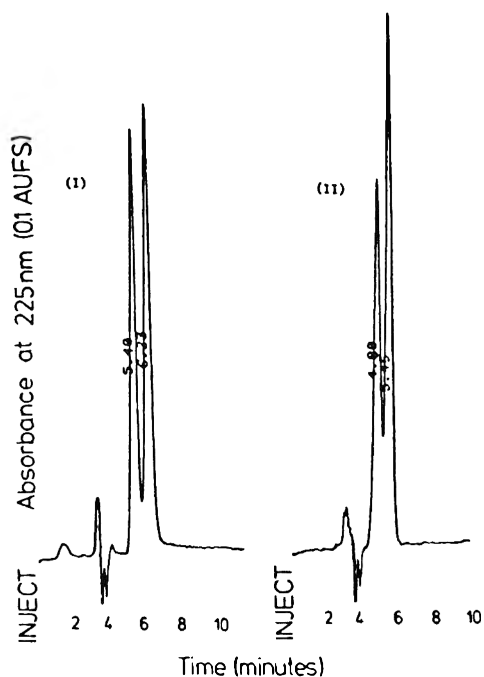


Figure 3. Typical chromatograms of resolved (I) carbenicillin and (II) ticarcillin diastereoisomers. Conditions as in text, except mobile phase is acetonitrile–methanol–0.01M potassium phosphate monobasic–0.02M octanesulfonic acid sodium salt in 0.2% glacial acetic acid (12 + 7 + 41 + 40).

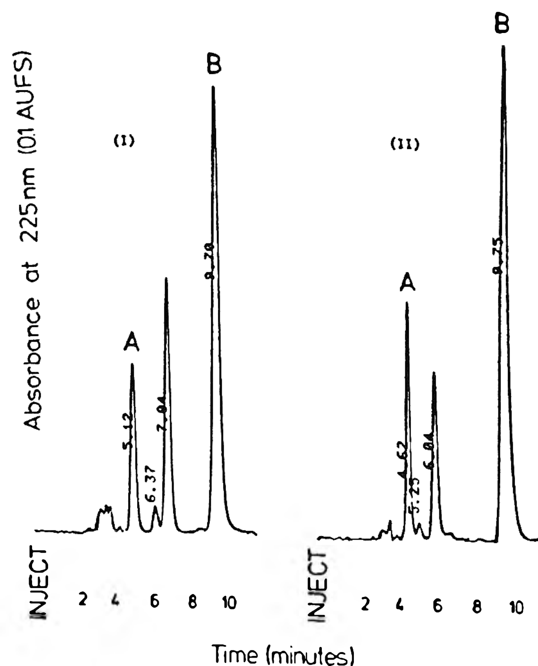


Figure 4. Typical chromatograms of partially degraded penicillin after 24 h at room temperature: (I) A, carbenicillin, B, propylthiouracil; (II) A, ticarcillin, B, propylthiouracil.

chain. Quantitative analysis of the individual isomers of ticarcillin and carbenicillin by LC on reverse-phase columns has been described in several reports (2–4, 6, 7). In these methods, peak areas, rather than peak heights, were used for the determination since the chromatographic isomeric peaks were usually both incompletely resolved and asymmetrical. In addition, individual isomeric standards are unavailable, and the relative percentage of each isomer will vary with temperature due to epimerization in solution (5, 8). It is probably unnecessary to assay isomeric content since none of the diastereoisomers of carbenicillin and ticarcillin have been shown to have therapeutically different activities. Therefore, we believe that it is more precise and convenient to analyze total carbenicillin and ticarcillin as single, reproducible chromatographic peaks along with an internal standard.

Table 2. Potency determination of carbenicillin and ticarcillin injectables

	Carbenicillin found			Ticarcillin found		
Sample[a]	µg/mg	(g)[b]	% of decl.	µg/mg	(g)[b]	% of decl.
1	826.6	(2.308)	115.4	811.7	(3.353)	111.8
	822.8	(2.297)	114.9	812.3	(3.355)	111.8
2	819.8	(2.289)	114.4	814.8	(3.365)	112.2
	819.0	(2.287)	114.3	814.4	(3.364)	112.1
3	823.9	(2.300)	115.0	809.9	(3.345)	111.5
	821.4	(2.293)	114.7	813.3	(3.359)	112.0
4	821.2	(2.293)	114.6	813.9	(3.362)	112.1
	816.6	(2.280)	114.0	810.2	(3.346)	111.5
5	823.4	(2.299)	115.0	811.4	(3.351)	111.7
	821.8	(2.295)	114.7	815.2	(3.367)	112.2
Mean	821.6			812.7		
SD	2.8			1.9		
CV, %	0.3			0.2		

[a] All samples from one manufacturer and from same lot. Amount declared (as free acid): carbenicillin = 2 g; ticarcillin = 3 g.

[b] Total grams determined by using average vial net content weight.

An initial part of the study was devoted to investigating the effects of varying the proportions of acetonitrile, methanol and 0.01M potassium phosphate monobasic in the mobile phase on the degree of resolution of the diastereoisomers of carbenicillin and ticarcillin. The incorporation of methanol in the mobile phase improved the resolution of the diastereoisomeric peaks but considerably lengthened the retention times. Because of this effect, methanol was excluded from the mobile phase recommended for potency determinations. The use of a mobile phase composed of acetonitrile–0.01M potassium phosphate monobasic (12 + 88) resulted in satisfactory and reproducible chromatographic peaks for carbenicillin (Figure 1) and ticarcillin (Figure 2).

The effect of adding an ion-pairing reagent to the mobile phase on chromatographic behavior was also investigated. As shown in Figure 3, the diastereoisomers of carbenicillin and ticarcillin can be readily resolved in 6 min by using a mobile phase consisting of acetonitrile–methanol–0.01M potassium phosphate monobasic–0.02M octanesulfonic acid sodium salt in 0.2% acetic acid (12 + 7 + 41 + 40).

The proposed method will distinguish carbenicillin and ticarcillin from their degradation products and related penicillins. For example, other types of penicillin, including methicillin, penicillins G and V, nafcillin, oxacillin, and dicloxacillin will elute much later than the analytes of interest. Likewise, typical chromatograms of partially degraded solution of carbenicillin and ticarcillin standards, as shown in Figure 4, indicate practically no interference by decomposition products.

Conclusion

The analysis of carbenicillin and ticarcillin by the proposed LC method offers significant advantages over the official microbiological method (1). These advantages include greater convenience and selectivity, less interference by related compounds and degradation products, and ability to monitor drug stability.

REFERENCES

(1) *Code of Federal Regulations* (1986) Title 21, Part 440, 342–440
(2) Das Gupta, V., & Stewart, K. R. (1980) *J. Pharm. Sci.* **69,** 1264–1267
(3) Twomey, P. A. (1981) *J. Pharm. Sci.* **70,** 824–826
(4) Watson, I. D. (1985) *J. Chromatogr.* **337,** 301–309
(5) Hoogmartens, J., Roets, E., Janssen, G., & Vanderhaeghe, H. (1982) *J. Chromatogr.* **244,** 299–309
(6) Kwan, R. H., MacLeod, S. M., Spino, M., & Teare, F. W. (1982) *J. Pharm. Sci.* **71,** 1118–1121
(7) Aravind, M. K., Miceli, J. N., & Kauffman, R. E. (1984) *J. Liq. Chromatogr.* **7,** 2887–2893
(8) Bird, A. E., Charsley, C. H., Jennings, K. R., & Marshall, A. C. (1984) *Analyst* **109,** 1209–1212
(9) Haginaka, J., & Wakai, J. (1985) *Analyst* **110,** 1185–1188

INDIRECT FOOD ADDITIVES

Gas Chromatographic–Mass Spectrometric Determination of Adipate-Based Polymeric Plasticizers in Foods

LAURENCE CASTLE, ANGELA J. MERCER, and JOHN GILBERT
Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Haldin House, Queen St, Norwich NR2 4SX, UK

A method for the quantitative determination of adipate-based polymeric plasticizers in foods is described. The procedure involves extraction from the food and transmethylation of the polymeric plasticizer to form dimethyladipate (DMA). The derivative is cleaned up by size-exclusion chromatography and determined by capillary gas chromatography-mass spectrometry with selected ion monitoring. The use of a deuterated internal standard at the extraction stage enables quantitation by stable isotope dilution. A detection limit of 0.1 mg/kg of the polymeric plasticizer in foods and a relative standard deviation of 4% have been achieved routinely. The method has been applied successfully to the analysis of cheese, sandwiches, meat, biscuits, and cake that have been in contact with polymeric plasticized poly(vinyl chloride) films.

Conventional plasticizers are organic esters of high boiling point, such as di-(2-ethylhexyl)adipate (DEHA), which are employed in food contact materials in significant amounts to impart flexibility (1). These compounds have been shown to migrate when plasticized film is in direct contact with foods, particularly those of high fat content (2–5). This problem has led the plastics and packaging industry to seek alternative plasticizers with lower migration characteristics. One approach to reducing migration is to increase the molecular size of the plasticizer; thus, the so-called polymeric plasticizers have been developed (6). These are generally polyesters of dicarboxylic acids and glycols; a typical example is a co-polymer of adipic acid and 1,3-butanediol, with a number average molecular weight some 5 times greater than that of the monomeric DEHA. The polymeric plasticizers are intended as complete or partial replacements for DEHA in plasticized PVC [poly(vinyl chloride)] "cling-film," which is used for a diversity of both retail and domestic food contact applications.

To assess migration of these polymeric plasticizers, a method of analysis has been developed that is applicable to any adipate-based plasticizer in foods. Stable isotope dilution gas chromatography–mass spectrometry (GC/MS) has previously been applied successfully to the precise and sensitive determination of DEHA in a wide variety of food types (7), and this principle has been extended to the method reported here.

METHOD

Reagents

(a) *Solvents.*—Acetone (distilled-in-glass), hexane, dichloromethane, methanol, and cyclohexane (LC grades) (Rathburn Chemicals Ltd, Walkerburn, Scotland, UK).

(b) *Boron trifluoride etherate.*—Re-distilled grade (Sigma Chemical Co., Poole, Dorset, UK).

(c) *Deuterated DEHA(d_4-DEHA) internal standard.*—Synthesized from hexanedioic-2,2,5,5-d_4 acid (MSD Isotopes, Inc.–Cambrian Gases, Croydon, UK) as described previously (7). Prepared as stock solution at 1 mg/mL in acetone–hexane (1 + 1 v/v).

Received February 24, 1987. Accepted July 8, 1987.

(d) *Sodium sulfate.*—Anhydrous, analytical grade (BDH Chemicals Ltd, Poole, UK).

(e) *Polymeric plasticizer.*—Reoplex 346 (Ciba-Geigy Industrial Chemicals, Manchester, UK).

Apparatus

(a) *Homogenizer.*—IKA-Ultra-Turrax drive T 18/10 (Janke and Kunkel GmbH and Co. KG, supplied by Sartorius Instruments Ltd, Belmont, UK).

(b) *Automated size-exclusion chromatography (SEC).*—Autoinjector WISP 710B and Waters 6000A LC pump (Waters Associates, Inc., Milford, MA); Pye LC-UV detector at 254 nm (Pye-Unicam Ltd, Cambridge, UK); and Helirac 2212 fraction collector (LKB-Produkter AB, Bromma, Sweden). Automated system connected and operated as described elsewhere (8).

(c) *Size-exclusion chromatography column.*—SR 25/100 glass column, 1 m × 25 mm id (Pharmacia, Uppsala, Sweden) packed with 800 mm bed of Bio-Beads S-X3 (Bio-Rad, Watford, UK).

(d) *Rotary evaporator.*—Buchi Rotavapor R (Orme Scientific Ltd, Manchester, UK).

(e) *Combined GC/MS.*—Carlo Erba 4160 gas chromatograph (M.S.E. Instruments, Crawley, UK) coupled directly to VG 7070H mass spectrometer with VG 11/250 data system (VG Analytical Ltd, Manchester, UK).

(f) *Capillary GC column.*—25 m × 0.23 mm id CP SIL 5CB fused silica column (Chrompack International B.V., Middleburg, Netherlands).

Extraction of Polymeric Plasticizer

Homogenize food sample in domestic food processor (Braun Type 4243), and weigh 30 g subsample into 400 mL glass beaker. Add 100 mL acetone–hexane (1 + 1 v/v) and, by syringe, add suitable volume of d_4-DEHA internal standard from appropriate stock solution (addition of d_4-DEHA should be 0.1 to 1.0 mg, depending on food type). Homogenize in beaker, using Ultra-Turrax blender, and then let stand 1 h to allow internal standard to equilibrate. Decant supernate into 500 mL conical flask and extract food residue with additional 100 mL acetone–hexane. Dry combined organic extracts overnight with 75 g sodium sulfate, filter into 500 mL round-bottom flask, and remove solvent at 50°C under vacuum. Note weight of residue to give indication of fat content of sample.

Transmethylation of Extract

Weigh portion of fat residue (ca 0.5 g) into 4 mL glass screw-cap vial (Chromacol Ltd, London, UK), add 4 mL boron trifluoride etherate in methanol (10% v/v), seal with PTFE-faced septum, and heat 2 h in 60°C oven with shaking every 30 min. Let cool, transfer by Pasteur pipet to 20 mL crimp-top glass vial (Chromacol Ltd), and add 3 mL water to quench reaction, followed by 9 mL dichloromethane–cyclohexane (3 + 7 v/v). Seal with PTFE-faced septum and

crimp on an aluminum cap, shake vigorously to extract deuterated and nondeuterated dimethyladipate, and centrifuge 5 min at 3000 rpm to break any emulsions and to separate the 2 phases. Uncap vial, and remove bottom aqueous layer by Pasteur pipet and discard. Dry organic layer with 5 g sodium sulfate for 2 h, filter into 50 mL round-bottom flask, and evaporate under vacuum to small volume (ca 2 mL). Transfer to 4 mL glass screw-cap vial (Chromacol Ltd) and dilute to 4 mL with cyclohexane.

Size-Exclusion Chromatography

Using fully automated size-exclusion chromatographic system described previously (8), load 1.5 mL lipid extract in cyclohexane onto column and elute at 3.0 mL/min with dichloromethane–cyclohexane (1 + 1 v/v). Collect fraction containing d_0-DMA and d_4-DMA (established by previous calibration) at between 243 to 264 mL. Ensure that flow rate is constant by interspersing samples with dicyclohexylphthalate as UV-active retention time marker and monitor at 254 nm. Transfer trapped fraction from collection tube to 50 mL round-bottom flask and evaporate to ca 1 mL on rotary evaporator under vacuum at 50°C.

Selected Ion Monitoring GC/MS

Carry out gas chromatographic analysis isothermally at 110°C at helium carrier gas flow rate of 1 mL/min, and make 1 μL split injections (20:1 split ratio). Operate mass spectrometer with electron ionization at 70 eV and in selected ion monitoring mode at resolution of 500 (10% valley definition). Monitor ions at m/z 143 and 101 for unlabeled dimethyladipate (d_0-DMA) and m/z 147 and 103 for deuterated dimethyladipate internal standard (d_4-DMA) with dwell times of 100 ms per mass and settling time of 20 ms to give total cycle time of 420 ms. Measure peak areas by using data system but fit baseline manually.

Calculation of Results

Prepare calibration standards from separate solutions (1 mg/mL) of polymeric plasticizer and d_4-DEHA in acetone as follows: Take 100 μL d_4-DEHA solution and add aliquots of polymeric plasticizer solution by syringe to give weight ratios ranging from 1:4 to 4:1 (7 standards in total). Take these standards through full procedure from transmethylation onward. Construct calibration curve of mass spectrometric selected ion monitoring peak area ratio (PAR) for d_0/d_4-DMA vs weight ratio of polymeric plasticizer/d_4-DEHA (R). For food samples, read value R corresponding to PAR determined by GC/MS. Calculate level of plasticizer in food from amount of d_4-DEHA added (x mg) to food subsample (y kg) according to the following:

$$\text{Polymeric plasticizer, mg/kg in food} = \mathrm{R}x/y$$

Results and Discussion

The principle of the method is equilibration of d_4-DEHA internal standard with polymeric plasticizer in a solvent slurry of the foodstuff, followed by extraction. Transmethylation converts the polymeric plasticizer to d_0-DMA and the d_4-DEHA to d_4-DMA; thereafter, the method follows a conventional stable isotope dilution approach as previously established for DEHA determinations (7). All stages of the method have been vigorously validated and conditions have been optimized as follows:

The minimum equilibration time for d_4-DEHA and polymeric plasticizer in the foodstuff was established by analysis of aliquots taken from a spiked solvent slurry of cheese at time intervals of 1, 3, 6, and 7 h. After 1 h equilibration, the ratio of d_0/d_4-DMA remained effectively constant.

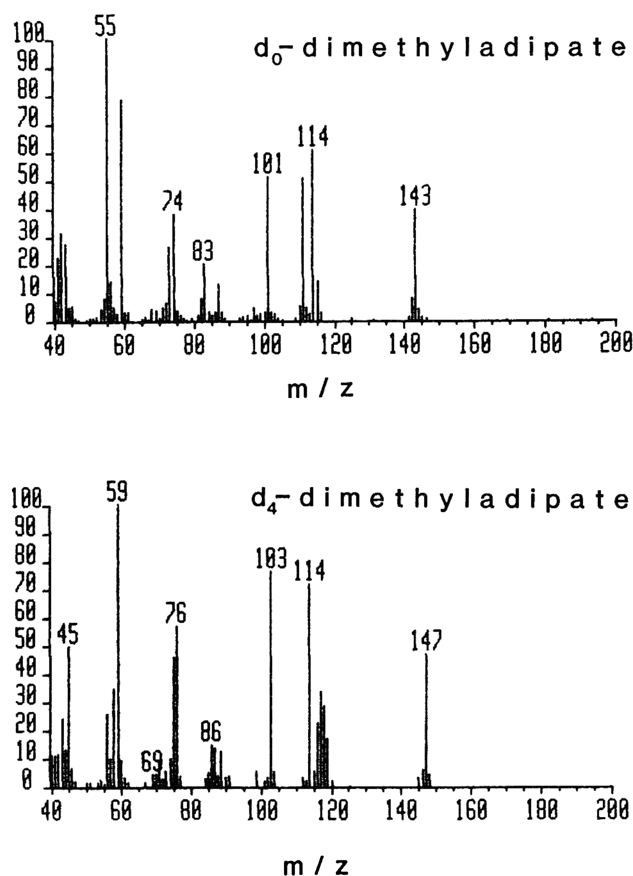


Figure 1. Mass spectra of d_0-dimethyladipate and d_4-dimethyladipate.

Quantitative extraction of polymeric plasticizer and d_4-DEHA from the food with acetone–hexane was validated by sequential extraction of a spiked cheese slurry with 100 mL portions of solvent. It was shown that 98% of both plasticizer and internal standard were extracted by the first 200 mL of solvent and that neither was extracted preferentially.

Conditions for quantitative transmethylation of both polymeric plasticizer and DEHA were established individually by using dimethylpimelate internal standard, by reaction at 60°C, and by sampling the products at time intervals of 120, 270, and 370 min. Analysis of the reaction product, in both cases, showed that the yield of DMA was complete within 120 min. When the reactions were repeated in the presence of cheese fat, it was shown that 96% of the theoretical yield of DMA was measurable after the 2 h reaction period, demonstrating that the presence of lipid did not interfere with the completeness of reaction.

The transmethylation reaction was quenched with distilled water which facilitated partition of the boron salts into the aqueous methanol phase. For subsequent extraction into dichloromethane–cyclohexane, a solvent ratio of 3:7 was established as that which enabled extraction of the maximum amount of DMA from the aqueous methanol layer while maintaining 2 immiscible liquid phases. That there were no significant differences in volatility between d_0-DMA and d_4-DMA was demonstrated by evaporating 10 mL aliquots of a solution containing both, to various volumes including dryness. Analysis showed that in no instance was there any change in the ratios of d_0-DMA to d_4-DMA.

The mass spectra of both d_0-DMA and d_4-DMA are shown in Figure 1 from which it can be seen that relatively intense

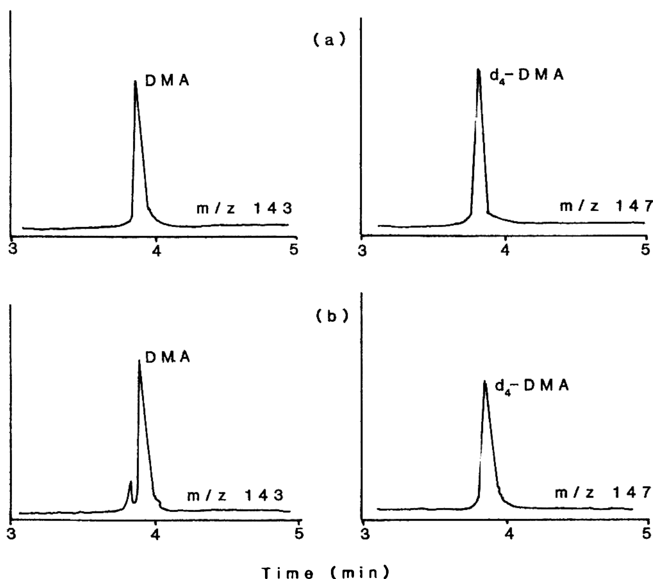


Figure 2. Selected ion chromatograms for m/z 143 (d_0-dimethyladipate) and m/z 147 (d_4-dimethyladipate) from (a) standards and (b) cheese contaminated with 12 mg/kg of polymeric plasticizer by migration from plasticized film.

ions were selected for monitoring at m/z 143 and 147 (M − 31), and the M − (CH_2COOCH_3) ion at m/z 101 and M − (CD_2COOCH_3) ion at m/z 103 from d_0-DMA and d_4-DMA, respectively. Either pair of ions can be used successfully for quantitation, and in each case measurement of the ratio of the 2 ions in both d_0-DMA and d_4-DMA provides confirmation of the adequacy of the specificity of the monitoring. Selected ion monitoring chromatograms showed smooth symmetrical peaks for both d_0-DMA and d_4-DMA and typical examples as obtained for the analysis of a cheese extract are shown in Figure 2. Calibration curves were linear with typically a correlation coefficient of 0.9998 and a standard error of 0.08095. Reagent and sample blanks from a diverse selection of foods showed no evidence of interference.

The accuracy and precision of the method was determined by spiking food samples (sandwiches, cakes, chicken, biscuits, and cheese) and solvent (as reagent blank) with polymeric plasticizer and/or d_0-DEHA. Found/added values were 100 ± 4% at spiking levels ranging between 10 and 200 mg/kg. No significant differences were experienced between different food types nor between calibration curves based on standards and those based on spiking into foods. Thus, because no matrix dependence was observed, calibration for the method can be based on standard solutions. The detection limit of the method is claimed to be 0.1 mg/kg (which was demonstrated by spiking polymeric plasticizer into cheese) but although this was the lowest level of interest for monitoring purposes, intrinsically the procedure has the capability of detection at far lower levels should this be required.

The method of analysis has been applied successfully to studies of the migration of polymeric plasticizer into cheese, cakes, and sandwiches stored wrapped in polymeric-plasticized film. Studies have also been carried out monitoring migration during the microwave cooking of chicken breasts covered in film and peanut biscuits prepared with the film as a liner. These results will be reported elsewhere. Where foodstuffs contain both DEHA and polymeric plasticizer as a result of migration from films containing both DEHA and polymeric plasticizer, then this method of analysis gives a result corresponding to the total adipate present (i.e., the level of DEHA plus polymeric plasticizer). In these instances it is necessary to carry out a separate determination of DEHA and the level of polymeric plasticizer can then be obtained by the difference of the 2 values. This has been done successfully for a wide range of food types.

The method described in this paper is applicable to any adipate-based polymeric plasticizer, and also could be of value in the determination of mixed-isomer monomeric plasticizers where conversion to single species offers considerable advantages in precision and detection limits for peak area measurement. For polymeric plasticizers based on other dicarboxylic acids, such as phthalic acid, although the method is applicable in principle, the extraction and transmethylation would need to be re-optimized, and an appropriate labeled internal standard would need to be used.

Acknowledgments

The authors gratefully acknowledge the assistance of C. Crews for the mass spectrometric analysis, J. R. Startin for helpful discussion, and Ciba-Geigy Industrial Chemicals for the supply of polymeric plasticizer.

References

(1) Tester, D. A. (1982) in *Manufacture and Processing of PVC*, R. H. Burgess (Ed.), Applied Science Publishers, London, UK, pp. 215–269
(2) Sandberg, E., Vaz, R., Albanus, L., Mattsson, P., & Nilsson, K. (1982) *Var Foda.* **34**, 470–482
(3) Startin, J. R., Sharman, M., Rose, M., Parker, I., Mercer, A. J., Castle, L., & Gilbert, J. (1987) *Food Addit. Contam.*, **4**, 385–398
(4) Castle, L., Mercer, A. J., Startin, J. R., & Gilbert, J. (1987) *Food Addit. Contam.*, **4**, 399–406
(5) Castle, L., Mercer, A. J., Startin, J. R., & Gilbert, J. (1987) *Food Addit. Contam.*, **5**, 9–20
(6) Hammer, C. F. (1978) *Polymer Blends* **2**, 219–241
(7) Startin, J. R., Parker, I., Sharman, M., & Gilbert, J. (1987) *J. Chromatogr.* **387**, 509–514
(8) Shepherd, M. J. (1984) in *Analysis of Food Contaminants*, J. Gilbert (Ed.), Elsevier Applied Science Publishers, London, UK, pp. 1–72

MEAT AND POULTRY

Liquid Chromatographic Identification of Meats

SAMY H. ASHOOR, WOODROW C. MONTE,[1] and PHILIP G. STILES
Arizona State University, Division of Agriculture, Tempe, AZ 85287

Raw beef, pork, veal, lamb, chicken, turkey, and duck have been identified with a liquid chromatographic (LC) method. Meat samples are blended in water, and soluble proteins in the aqueous blends are separated by the LC method. Meat cuts and parts from same species had similar chromatographic profiles and differed only quantitatively. However, meat cuts or parts from different species resulted in different chromatographic profiles. The qualitative and quantitative chromatographic differences among meat species were used for their identification. The LC method applies only to fresh and frozen meats. It is simple, rapid, and reliable, and can be used for quantitative detection of meat species in unheated meat blends.

It is imperative to identify meat species in the market for fair pricing, to meet government regulations, and to satisfy consumers' needs, especially for those restricted from consuming certain meat species because of religious beliefs.

Several methods are used at the present time for identification of meat species and for detection of meat adulteration with cheaper meat species, i.e., horse meat in beef and chicken in turkey. The majority of these methods involve gel electrophoresis (1–5) and gel isoelectric focusing (6–8). Immunological methods (9–11) and chromatographic methods (12–14) are also used in meat identification and adulteration. Unfortunately, these methods are not satisfactory. Electrophoretic methods require a long time to prepare, stain, and destain the gels. Even with a fast electrophoretic method such as agarose gel isoelectric focusing, results are not available before 4 h (6). Besides, electrophoretic methods in general lack the high reproducibility required for quantitative results. Immunological methods require production of high titer antisera with specific antibodies for every meat species. This was shown to be a very difficult task (11). Immunological methods are also time consuming and nonquantitative. The present chromatographic methods for meat identification involve gas chromatographic analysis of the unsaponifiable matter of meat fat after fractionation by column chromatography (12), or of methyl esters of fatty acids extracted from hydrolyzed meat fat (13). A liquid chromatographic (LC) method involving analysis of meat tryptic hydrolysates was also reported (14). All of these methods involve lengthy aration steps.

In the present study, a simple LC method for identification of beef, pork, lamb, veal, chicken, turkey, and duck is evaluated. The LC method requires a total analysis time of approximately 1 h.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Waters Associates equipped with Model 6000A pump, Model U6K injector (Waters Associates, Milford, MA 01757), and Model 111-2 one pump gradient controller (Autochrom, Milford, MA 01757).

(b) *Data module.*—Waters Associates Model 730. Set chart speed at 0.2 cm/min and run stop at 60 min.

(c) *LC column.*—250 × 4.6 mm Hi-Pore RP-304 column with Micro-Guard Hi-Pore cartridge (Bio-Rad Laboratories, Richmond, CA 94804).

(d) *Detector.*—Gilson Model 222 (Gilson Medical Electronics, Middleton, WI 53562). Set at 280 nm and sensitivity of 0.02 AUFS.

Received December 9, 1986. Accepted April 2, 1987.
[1] Department of Home Economics.

Table 1. LC analysis of beef and pork retail cuts

	Beef, % of total area[b]				Pork, % of total area[b]			
RRT[a]	Chuck	Rib	Loin	Round	Shoulder	Rib	Loin	Ham
0.61	10.8[c]	12.4	12.3	16.1	2.04	3.18	3.83	3.50
1.00	2.00	2.67	2.00	2.50	—	—	—	—
1.10	2.10	4.89	2.60	2.00	10.8	2.12	2.33	2.00
1.16	7.65	14.3	16.7	12.9	4.12	2.00	4.17	3.25
1.21	13.6	10.7	10.2	8.38	12.8	20.3	11.3	21.4
1.26	—[d]	—	—	—	32.8	19.1	25.7	17.2
1.29	3.78	4.56	2.70	2.50	—	—	—	—
1.38	—	—	—	—	2.50	8.79	5.33	4.20
1.45	12.9	10.1	11.2	14.9	—	—	—	—
1.72	—	—	—	—	6.46	9.05	9.50	11.1
1.79	4.22	6.44	6.10	4.62	—	—	—	—
1.88	2.24	2.00	2.90	2.63	—	—	—	—
2.00	—	—	—	—	2.48	3.37	2.83	3.40
2.10	—	—	—	—	6.35	4.63	5.67	4.42
2.22	4.92	4.44	4.80	3.25	4.38	7.16	8.17	8.17
2.27	6.71	5.56	5.60	6.75	—	—	—	—
2.59	—	—	—	—	3.75	6.32	6.17	—[e]
2.65	—	—	—	—	3.54	7.58	7.17	11.3
2.71	4.77	2.91	4.74	2.89	—	—	—	—
2.77	6.21	3.42	4.21	4.72	3.33	4.84	5.33	6.33
2.86	5.71	8.67	9.00	8.25	—	—	—	—

[a] Relative to retention time of BSA peak.
[b] Average of 2 determinations.
[c] Major characteristic peaks of species are underlined.
[d] Peak is absent or with area of less than 2%, unless otherwise noted.
[e] Peak overlapped with another.

Table 2. LC analysis of veal and lamb retail cuts

	Veal, % of total area[b]		Lamb, % of total area[b]			
RRT[a]	Shoulder	Round	Shoulder	Rib	Loin	Leg
0.61	6.28	5.26	11.1	11.1	11.4	13.8
1.00	3.24	3.47	—	—	—	—
1.05	—[c]	—	4.30	5.38	3.02	3.16
1.10	—	—	17.5	12.9	11.6	18.6
1.16	18.7[d]	18.8	—[e]	4.95	8.10	—[e]
1.21	6.07	6.41	24.3	14.0	9.05	14.4
1.26	10.7	10.0	—	—	—	—
1.29	3.24	3.21	14.0	18.4	19.5	19.7
1.38	3.80	3.79	5.56	4.84	6.67	3.80
1.66	6.33	5.73	—	—	—	—
1.79	3.95	4.00	—	—	—	—
2.22	6.33	5.94	—[e]	3.23	3.33	2.91
2.27	6.48	7.15	8.73	5.70	6.34	5.20
2.71	4.35	4.73	—	—	—	—
2.77	4.45	4.36	4.92	4.52	5.56	4.18
2.86	9.67	9.67	—	—	—	—

[a] Relative to retention time of BSA peak.
[b] Average of 2 determinations.
[c] Peak is absent or with area of less than 2%, unless otherwise noted.
[d] Major characteristic peaks of species are underlined.
[e] Peak overlapped with another.

Table 3. LC analysis of chicken, turkey, and duck parts

	Chicken, % of total area[b]				Turkey, % of total area[b]				Duck, % of total area[b]			
RRT[a]	Breast	Wing	Thigh	Leg	Breast	Wing	Thigh	Leg	Breast	Wing	Thigh	Leg
1.10	—[c]	—	—	—	20.0	24.1	13.0	14.2	—	—	—	—
1.16	30.0[d]	28.4	21.5	20.3	—	—	—	—	—	—	—	—
1.26	—	—	—	—	2.00	—[e]	2.60	3.30	26.7	23.7	22.5	22.4
1.40	—	—	—	—	18.8	18.2	7.90	11.2	—	—	—	—
1.45	14.5	18.6	35.3	30.8	16.7	11.5	21.4	35.5	7.33	10.6	—[e]	—[e]
1.48	—	—	—	—	—	—	—	—	7.50	11.0	28.6	24.7
1.52	16.7	7.86	7.64	22.9	8.93	13.7	14.0	9.72	18.3	11.4	10.8	12.9
1.66	3.87	2.86	2.00	2.27	3.64	2.82	2.00	2.00	—	—	—	—
1.72	—	—	—	—	—	—	—	—	7.67	8.43	5.30	4.91
1.79	—	—	—	—	—	—	—	—	5.67	5.88	4.34	3.82
2.10	8.00	7.14	6.00	4.10	5.00	5.25	4.57	4.10	6.67	6.86	4.10	4.91
2.17	—	—	—	—	—	—	—	—	3.83	2.52	2.53	2.00
2.40	6.80	8.29	6.18	4.85	5.15	7.55	4.81	5.38	—	—	—	—
2.46	6.21	5.14	3.64	4.10	5.91	—[e]	—[e]	—[e]	3.17	5.10	4.82	5.09
2.59	—[e]	6.14	5.10	2.00	—	—	—	—	—	—	—	—
2.65	11.3	7.71	8.18	6.21	6.51	10.1	7.00	3.50	9.17	10.8	10.6	9.65

[a] Relative to retention time of BSA peak.
[b] Average of 2 determinations.
[c] Peak is absent or with area of less than 2%, unless otherwise noted.
[d] Major characteristic peaks of species are underlined.
[e] Peak overlapped with another.

(e) *Blender.*—Waring, with glass jar of ca 500 mL capacity and connected to variable transformer set at 30 units (VWR Scientific, Norwalk, CA 90650).

Reagents

(a) *Water.*—Pass distilled, deionized water through 0.45 μm filter membrane (Gelman Sciences, Inc., Ann Arbor, MI 48106).

Table 4. LC analysis of beef mixtures

	Percent of total area[b]						
RRT[a]	Beef	Beef & pork	Beef & veal	Beef & lamb	Beef & chicken	Beef & turkey	Beef & duck
0.61	10.8[c]	6.75	7.17	11.8	5.54	7.04	8.68
1.00	2.00	—[d]	3.94	—	—	—	—
1.10	2.10	—	—	5.29	—	12.0	—
1.16	7.65	13.0	25.7	4.64	19.7	4.13	7.36
1.21	13.6	14.9	12.0	17.6	10.2	6.77	17.0
1.26	2.98	18.4	4.30	2.07	—	—	10.2
1.29	3.78	—	—	9.00	2.54	5.20	—
1.38	—	2.34	—	5.50	—	—	—
1.45	12.9	8.05	7.24	8.07	9.40	10.5	18.6
1.52	—	—	—	—	7.40	12.1	4.92
1.66	—	—	—	—	3.08	2.00	—
1.72	—	3.48	—	—	—	—	—
1.79	4.22	—	3.54	5.07	—	2.91	5.34
1.88	2.24	—	3.15	—	—	—	—
2.10	—	2.27	—	—	3.55	2.74	—
2.17	—	—	3.54	—	—	—	—
2.22	4.92	3.00	8.50	4.29	3.28	2.54	2.57
2.27	6.71	3.77	—	5.43	3.42	3.44	3.89
2.40	—	—	—	—	3.13	—	—
2.46	—	—	—	—	2.59	2.43	—
2.59	—	2.14	—	—	—	—	—
2.65	—	2.27	2.76	—	6.21	4.87	5.97
2.71	4.77	2.34	—	3.66	2.03	2.38	—
2.77	6.21	5.58	7.72	4.00	3.47	3.60	4.24
2.86	5.71	3.31	7.05	5.14	3.08	3.39	3.96

[a] Relative to retention time of BSA peak.
[b] Average of 2 determinations.
[c] Major characteristic peaks of species are underlined.
[d] Peak is absent or with area of less than 2%, unless otherwise noted.

(b) *Acetonitrile.*—UV grade (EM Science, Cherry Hill, NJ 08034).

(c) *Trifluoroacetic acid (TFA).*—Anhydrous, analytical grade (Sigma Chemical Co., St. Louis, MO 63178).

(d) *LC mobile phase.*—Solvent A, 0.1% TFA (c) in water (a). Solvent B, acetonitrile (b) plus water (a) plus TFA (c) (95 + 5 + 0.1). Use linear gradient programmed from 37 to 60% B in 55 min and 15 min equilibration delay at flow rate of 1.5 mL/min.

(e) *Bovine serum albumin (BSA).*—(Sigma Chemical Co.) Prepare solution of 4 mg/mL in water (a).

(f) *Sodium azide.*—(J. T. Baker Chemical Co., Phillipsburg, NJ 08865.) Prepare solution of 1% in water (a).

Samples

(a) *Meat.*—Retail cuts were purchased from local stores: beef (chuck, rib, loin, and round); pork (shoulder, rib, loin, and ham); lamb (shoulder, rib, loin, and leg); veal (shoulder and round).

(b) *Poultry.*—Whole chicken, turkey, and duck, purchased from local stores and cut into breast, wings, thighs, and legs.

Extraction of Water-Soluble Proteins

Debone, trim fat and skin (in poultry) samples, chop muscles into small pieces, and mix well. Weigh 25–50 g chopped sample and blend with twice the weight of distilled water for 5 min in Waring blender. Filter blend through Whatman No. 4 paper. Add sodium azide solution (f) to final concentration of 0.01% to preserve blend. Filter aliquot of blend through 0.45 μm membrane filter into glass vial for LC analysis.

LC Analysis

Inject 5 μL BSA solution (e) into LC 3 times and determine average retention time. Inject 10–25 μL filtered blend into LC system, and determine relative retention time (relative to BSA peak) and percent area of major peaks. For LC analysis of mixed blends, co-inject equal volumes of 2 blends simultaneously and determine relative retention time and percent area of all peaks. Identify species-specific peak(s) in mixture chromatogram by using information obtained from standard chromatograms.

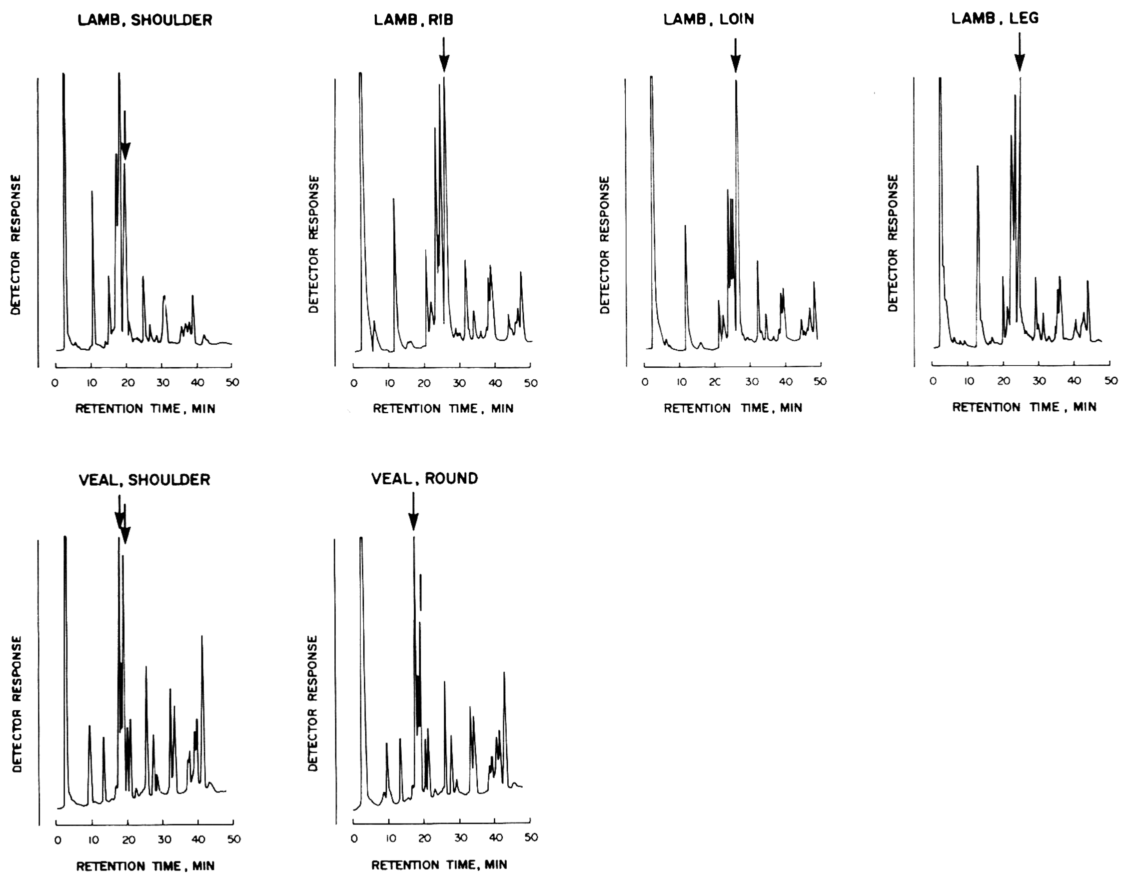
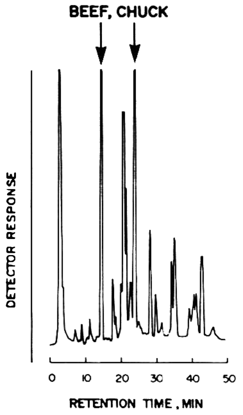
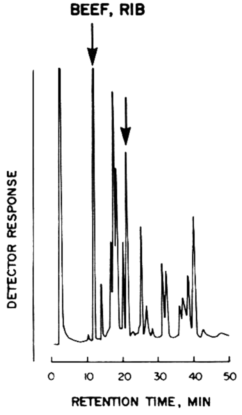
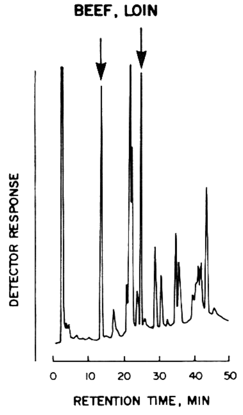
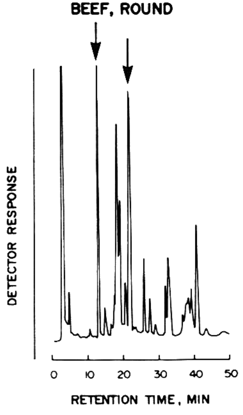


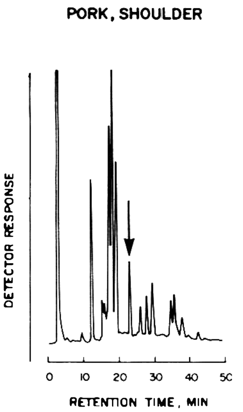
Figure 1. Chromatograms of beef, pork, lamb, and veal cuts. Arrows indicate species-specific peak(s).

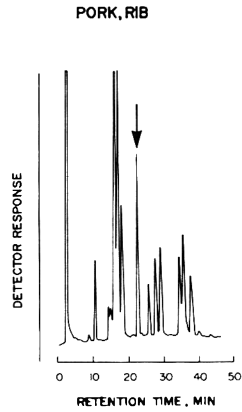

BEEF, CHUCK
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN

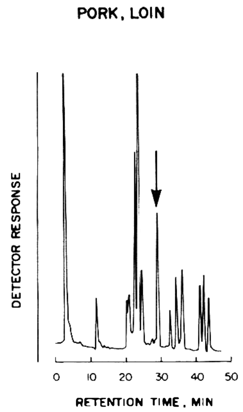

BEEF, RIB
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN

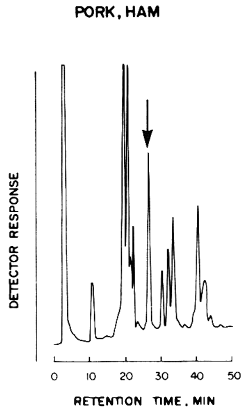

BEEF, LOIN
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


BEEF, ROUND
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


PORK, SHOULDER
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


PORK, RIB
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


PORK, LOIN
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


PORK, HAM
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN

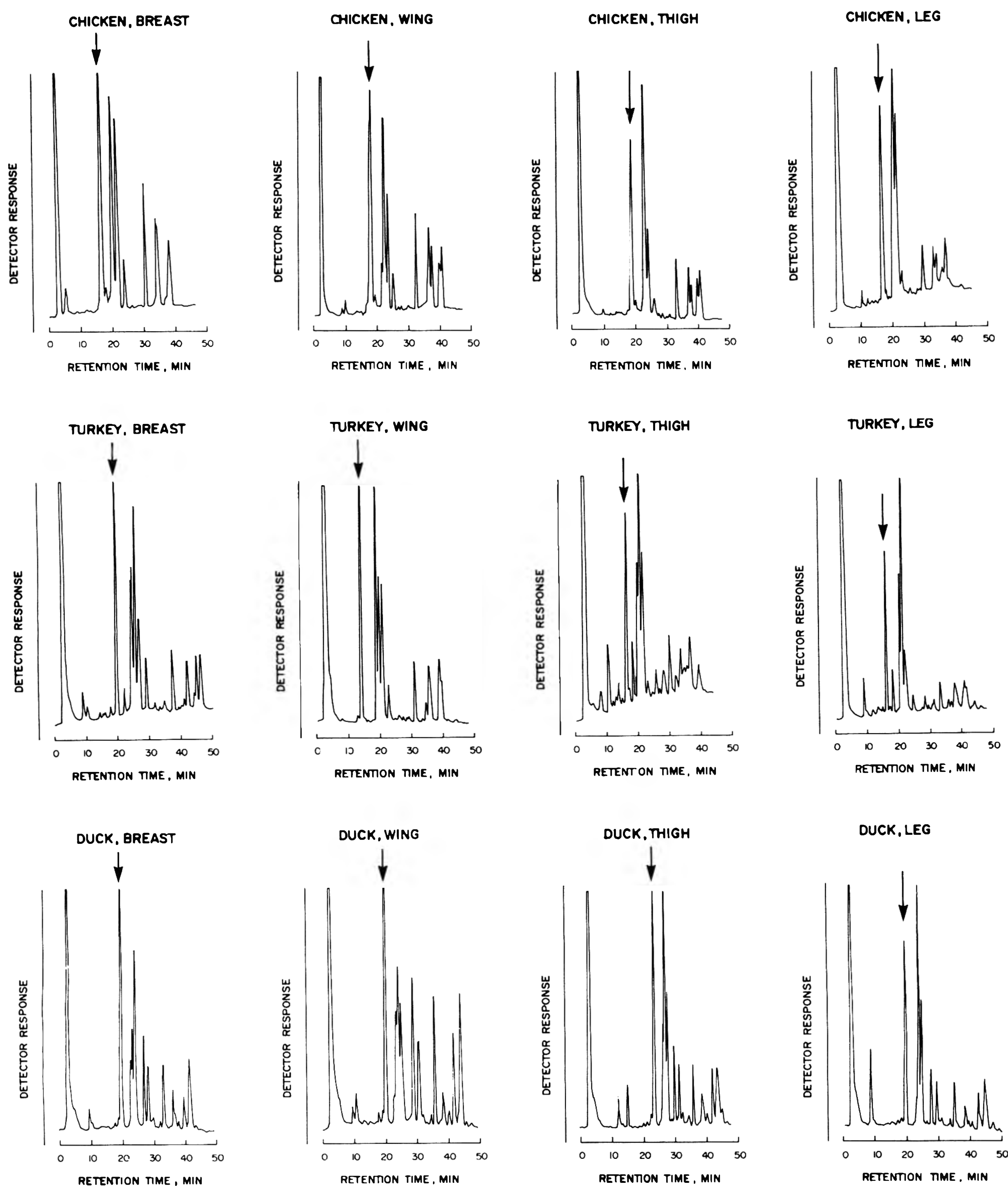


Figure 2. Chromatograms of chicken, turkey, and duck parts. Arrows indicate species-specific peak(s).

Preparation of Unknown Meat and Poultry Mixtures

To test reliability of LC method, 20 unknown mixtures were prepared in our laboratory by mixing various meat and poultry species to give the following compositions: 5, 10, and 50% pork in beef; 10 and 30% veal in beef; 5 and 50% pork in veal; 10 and 50% pork in chicken; 5, 15, and 75% chicken in turkey; 10 and 60% duck in chicken; and 5 and 50% duck in turkey. These mixtures were coded, and then prepared as indicated above, and the identity of each species was determined by the LC method.

Results and Discussion

The average retention time of BSA was 15.8 ± 0.7 min. When co-injected, BSA interfered with peaks in most meat samples; therefore, it was injected separately and used as an external standard. This made qualitative identification of various chromatograms much easier and more accurate.

Data obtained from LC analysis of various meat and poultry species, alone and in mixtures, are presented in Tables 1–6 and Figures 1–3. The data indicated that cuts or parts from the same species have similar chromatograms with peaks

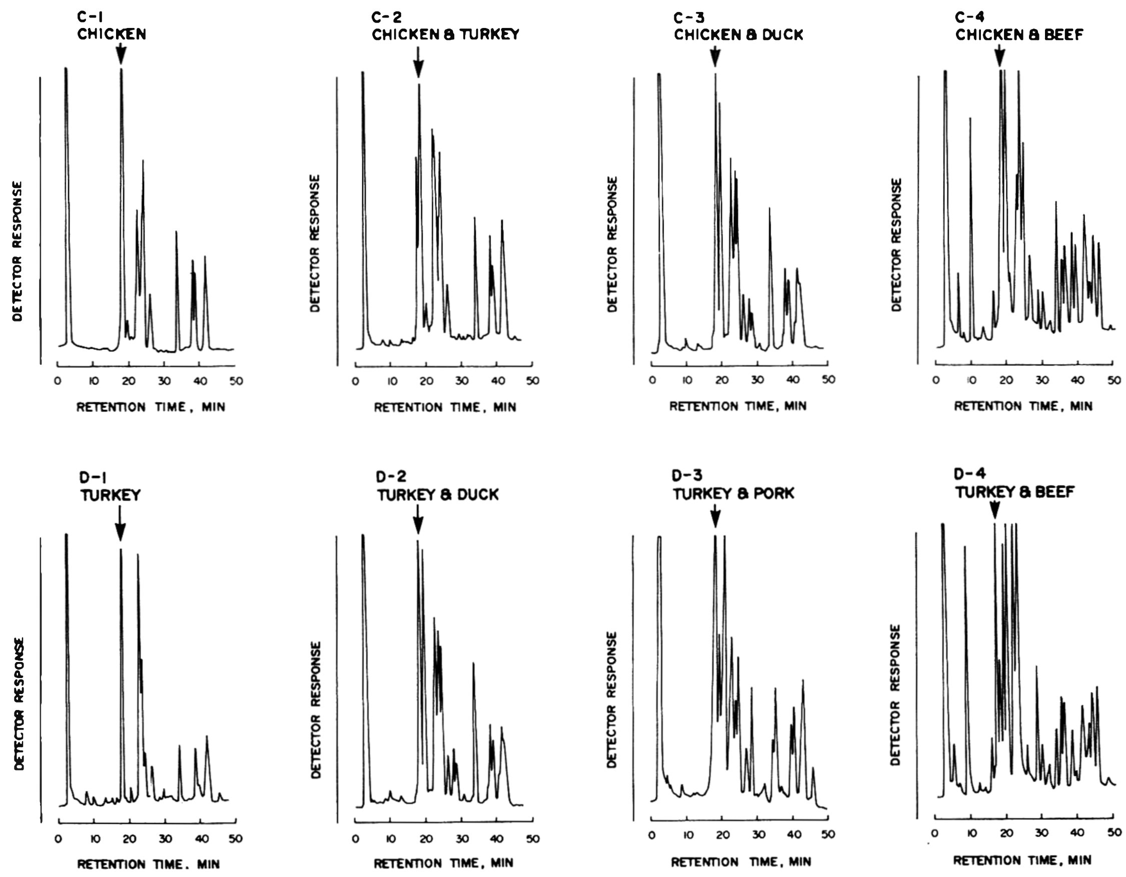
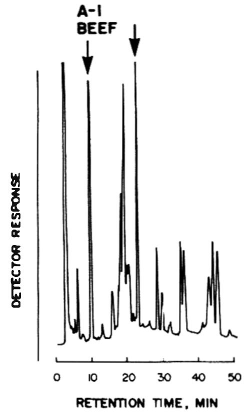
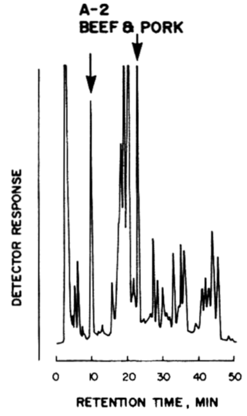
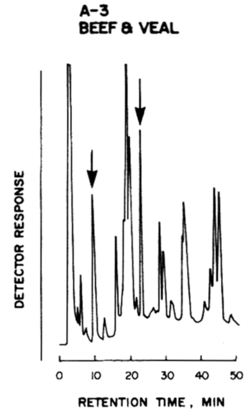
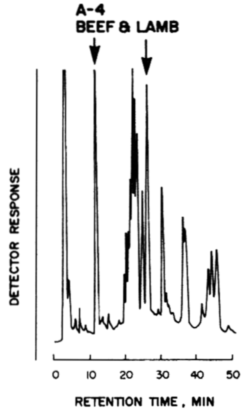


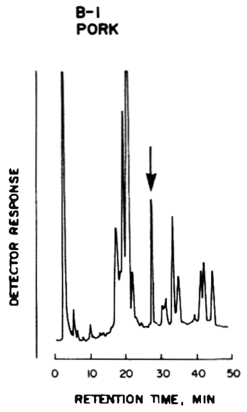
Figure 3. Chromatograms of meat and poultry mixtures. Arrows indicate peak(s) specific to beef, pork, chicken, and turkey.

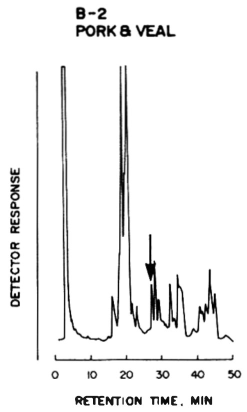

A-1
BEEF
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN

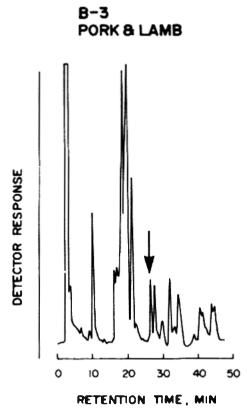

A-2
BEEF & PORK
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


A-3
BEEF & VEAL
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


A-4
BEEF & LAMB
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


B-1
PORK
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


B-2
PORK & VEAL
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN


B-3
PORK & LAMB
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN

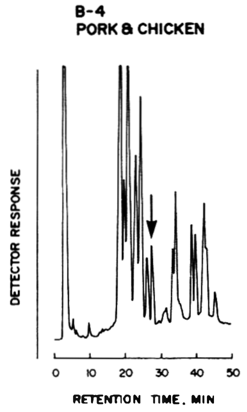

B-4
PORK & CHICKEN
DETECTOR RESPONSE
0 10 20 30 40 50
RETENTION TIME, MIN

Table 5. LC analysis of pork mixtures

	Percent of total area[b]					
RRT[a]	Pork	Pork & veal	Pork & lamb	Pork & chicken	Pork & turkey	Pork & duck
0.61	2.04	3.13	6.97	—	—	—
1.00	—[c]	2.72	—	—	—	—
1.05	—	—	2.69	—	—	—
1.10	10.8	—	6.27	—	21.8	4.42
1.16	4.12	27.0	14.8	22.0	—	—
1.21	12.8	—	—[e]	6.80	7.76	25.9
1.26	32.8[d]	30.2	31.0	16.2	17.7	22.2
1.38	2.50	2.80	6.67	—	—	2.42
1.45	—	—	—	11.2	11.3	6.97
1.52	—	—	—	10.3	5.33	6.06
1.66	—	—	3.77	2.86	2.31	—
1.72	6.46	4.63	3.98	3.86	2.93	5.96
1.79	—	3.38	—	—	—	2.22
2.00	2.48	3.83	4.76	2.24	—	—
2.10	6.35	6.00	—	6.39	2.31	3.23
2.17	—	—	—	—	—	2.14
2.22	4.38	2.00	6.47	—	—	2.14
2.40	—	—	—	3.61	—	—
2.46	—	—	2.07	3.20	3.13	—
2.59	3.75	4.15	2.00	—	3.54	—
2.65	3.54	3.30	4.23	8.84	8.36	7.47
2.77	3.33	3.54	2.04	—	—	2.32

[a] Relative to retention time of BSA peak.
[b] Average of 2 determinations.
[c] Peak is absent or with area of less than 2%, unless otherwise noted.
[d] Major characteristic peaks of species are underlined.
[e] Peak overlapped with another.

Table 6. LC analysis of poultry mixtures

	Percent of total area[b]					
RRT[a]	Chicken	Turkey	Chicken & turkey	Duck	Chicken & duck	Turkey & duck
1.10	—[c]	20.0	8.00	—	—	14.1
1.16	30.0[d]	—	18.9	—	18.5	—
1.26	—	2.00	—	26.7	12.0	12.3
1.40	—	18.8	—	—	—	15.3
1.45	14.5	16.7	22.8	7.33	16.2	8.16
1.48	—	—	—	7.50	7.93	—
1.52	16.7	8.93	13.3	18.3	8.12	9.70
1.66	3.87	3.64	4.38	—	2.45	2.52
1.72	—	—	—	7.67	2.41	2.43
1.79	—	—	—	5.67	2.98	2.33
2.10	8.00	5.25	6.86	6.67	7.16	7.77
2.17	—	—	—	3.83	—	—
2.40	6.80	7.55	5.81	—	4.40	4.85
2.46	6.21	—	4.57	3.17	4.14	4.56
2.65	11.3	6.51	9.21	9.17	10.7	11.8

[a] Relative to retention time of BSA peak.
[b] Average of 2 determinations.
[c] Peak is absent or with area of less than 2%, unless otherwise noted.
[d] Major characteristic peaks of species are underlined.

that have the same relative retention times even though different in area. Qualitative similarities of cuts or parts from the same species made the LC method applicable to all meat samples made from various cuts or parts. The data also indicated that each species has specific peak(s) which may be used for detection and quantitation of the species in meat and poultry products. However, the LC method is applicable only to fresh and frozen meats. Chromatograms of heated meats were not as distinctive as those of fresh or frozen meats and could not be used for identification. Apparently, heat denatured the water-soluble proteins that are specific to meat species. Freezing for 6 months at −40°C did not change chromatograms of the species studied.

All beef cuts used in this study had 2 specific peaks with relative retention times of 0.61 and 1.45. These 2 peaks were present in beef mixtures with all other meats (Tables 1 and 4; Figures 1 and 3). Among species tested, only veal and lamb had a major peak at a relative retention time of 0.61; however, neither species had a major peak at a relative retention time of 1.45 as in beef. The presence of these 2 major peaks was characteristic of beef and was used in the correct identification of beef in all unknown meat mixtures made in our laboratory.

Major peaks specific to pork had relative retention times of 1.26 and 1.72. However, the peak with a relative retention time of 1.72 was well resolved in all pork chromatograms (Tables 1 and 5; Figures 1 and 3) and was chosen for identification of pork in unknown meat mixtures prepared in our laboratory. Duck was the only other species with a major peak at a relative retention time of 1.72. However, duck has another major peak with a relative retention time of 1.79 which is absent in pork.

Lamb cuts had specific peaks at relative retention times of 0.61, 1.10, 1.21, and 1.29 (Tables 2 and 4; Figures 1 and 3). Using the peak with relative retention time of 1.29, lamb was identified in all prepared unknown meat mixtures.

Only 2 veal cuts were available in local stores, shoulder and round. These cuts yielded chromatograms similar to those of beef (Tables 1, 2, and 4; Figures 1 and 3). Veal, however, had a specific peak at a relative retention time of 1.26 and did not have the peak with relative retention time of 1.45, specific to beef. Veal was also identified correctly in unknowns.

Analysis of chicken, turkey, and duck major parts by the LC method yielded species-specific peaks which can be used for their reliable identification (Tables 3 and 6; Figures 2 and 3). All of the poultry unknowns made in our laboratory were identified correctly using the specific peaks with relative retention times of 1.16 for chicken, 1.10 for turkey, and 1.26 for duck (Table 6; Figure 3).

Factors such as age and sex of animal, different breeds within the same species, geographical location, and seasonal variation may affect the species-specific profile. However, this method has a great potential of being a simple and rapid identification tool for common meat and poultry species. Moreover, in a just-completed detailed study on quantitative detection of chicken and/or turkey in unheated poultry mixtures, the results indicated that the method yields reliable quantitative data. The data also indicated that the method is applicable to mixtures containing chicken, turkey, beef, and pork. Many more quantitative applications of the LC method are needed, and it is our hope that these will be done in the near future.

References

(1) Thompson, R. R. (1961) *J. Assoc. Off. Agric. Chem.* **44**, 787–788
(2) Scopes, R. K. (1968) *Biochem. J.* **107**, 139–150
(3) Hoyem, T., & Thorson, B. (1970) *J. Agric. Food Chem.* **18**, 737–739
(4) Babiker, S. A., Glover, P. A., & Lawrie, R. A. (1982) *Meat Sci.* **5**, 473–477
(5) Kim, H., & Shelef, L. A. (1986) *J. Food Sci.* **51**, 731 et seq.
(6) Kaiser, K. P., Matheis, G., Kmita-Durrman, C., & Belitz, H. D. (1981) *Sci. Tools* **28**, 5–7
(7) King, N. L., & Kurth, L. (1982) *J. Food Sci.* **47**, 1608–1612
(8) Sinclair, A. J., & Slattery, W. J. (1982) *Aust. Vet. J.* **58**, 79–80.

(9) Hayden, A. R. (1977) *J. Food Sci.* **42,** 1189–1192
(10) Hayden, A. R. (1978) *J. Food Sci.* **43,** 476 et seq.
(11) Hayden, A. R. (1979) *J. Food Sci.* **44,** 494–500
(12) Cook, H. R., & Sturgen, J. D. (1966) *J. Assoc. Off. Anal. Chem.* **49,** 877–880
(13) Saeed, T., Abu-Dagga, F., & Abdul-Rahman, H. (1986) *J. Assoc. Off. Anal. Chem.* **69,** 999–1002
(14) Medina, M. B., & Philips, J. G. (1982) *J. Agric. Food Chem.* **30,** 1250–1253

Liquid Chromatographic Quantitation of Chicken and Turkey in Unheated Chicken-Turkey Mixtures

SAMY H. ASHOOR and MAGDI A. OSMAN
Arizona State University, Division of Agriculture, Tempe, AZ 85287

A simple and rapid liquid chromatographic (LC) method has been used to quantitate chicken and turkey in unheated chicken-turkey mixtures. The LC method is sensitive and detects as little as 1% chicken or turkey. It reliably quantitates 5–100% chicken or turkey in unheated poultry mixtures. The method applies also to chicken or turkey which has been frozen, but does not apply to heat-treated poultry meats.

Chicken and turkey are commonly used, separately or together, in poultry meat products. Determination of the identity of poultry species and the amount of each poultry species used in making these poultry products is important for several reasons. Government regulations require the declaration of the identity of poultry species on the packages of poultry meat products. Consumers may have a preference to either chicken or turkey, or certain combinations of both, and therefore they need to know the identity of poultry meat(s) in poultry meat products before buying them. Moreover, turkey meat is more expensive than chicken, and there may be a tendency to adulterate turkey products with chicken.

To our knowledge, only 2 studies are reported in the literature on identifying chicken meat (1, 2) and only one study is reported on the identification of chicken and turkey meats (3). These methods are chemical (1), immunological (2), or electrophoretic (3). However, they lack specificity or reproducibility, and they all require many preparation steps.

We have reported a simple and rapid LC method for identification of meat species (4). In the present report, the LC method was used to identify and quantitate chicken and turkey in unheated chicken–turkey mixtures. The sensitivity and reliability of the method were also tested on unheated mixtures of 0–100% chicken in turkey.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Waters Associates equipped with Model 6000A pump, Model U6K injector (Waters Associates, Milford, MA 01757), and Model 111-2 one pump gradient controller (Autochrom, Milford, MA 01757).

(b) *Data module.*—Waters Associates Model 730. Set chart speed at 0.2 cm/min and run stop at 55 min.

(c) *LC column.*—250 × 4.6 mm Hi-Pore RP-304 column with Micro-Guard Hi-Pore cartridge (Bio-Rad Laboratories, Richmond, CA 94804).

(d) *Detector.*—UV, Gilson Model 222 (Gilson Medical Electronics, Middleton, WI 53562). Set at 280 nm and sensitivity of 0.02 AUFS.

(e) *Blender.*—Waring, connected to variable transformer (VWR Scientific, Norwalk, CA 90650). Set transformer reading at 30 units.

Reagents

(a) *Water.*—Pass distilled, deionized water through 0.45 μm membrane filter.

(b) *Acetonitrile.*—Analytical grade.

(c) *Trifluoroacetic acid (TFA).*—Anhydrous, analytical grade.

(d) *LC mobile phase.*—Solvent A, 0.1% TFA (c) in water (**a**). Solvent B, acetonitrile (**b**) plus water (**a**) plus TFA (**c**) (95 + 5 + 0.1). Run linear gradient of 37–60% B in 55 min with flow rate of 1.5 mL/min.

(e) *Bovine serum albumin (BSA) solution.*—4 mg/mL in water (**a**).

(f) *Sodium azide solution.*—1% in water (**a**).

Preparation of Samples

Skin, trim fat, debone, and cut into small pieces the parts (breast, wings, thighs, and legs) of 3 chickens or 3 turkeys. Prepare 11 mixtures, 30 g each, from the above chicken and turkey samples with chicken content of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%.

Extraction of Water-Soluble Proteins

Blend each of the chicken–turkey mixtures with twice the weight of water for 5 min in Waring blender (**e**). Filter blend through Whatman No. 4 paper. Add sodium azide solution (**f**) to final concentration of 0.01% to preserve blend, and pass aliquot of blend through 0.45 μm membrane filter.

LC Analysis

Inject 5 μL BSA solution (**e**) into LC system 3 times and determine average retention time. Check BSA retention time periodically during LC analysis of samples. Inject 10–25 μL filtered blend into LC system, and determine relative time (relative to BSA peak) and percent area of all peaks. Identify peak(s) specific to chicken and to turkey from chromatograms of 100% chicken and 100% turkey.

Chicken–Turkey Standard Curves

Construct standard curves by plotting average area of specific peak to chicken (relative retention time of 1.16) against percent chicken in mixture or average area of specific peak to turkey (relative retention time of 1.10) against percent turkey in mixture.

Received March 6, 1987. Accepted June 2, 1987.

Figure 1. Chromatograms of chicken and turkey (top); 10% chicken in turkey (A), 50% chicken in turkey (B), and 90% chicken in turkey (C). Arrows indicate specific peak for chicken (C) and turkey (T).

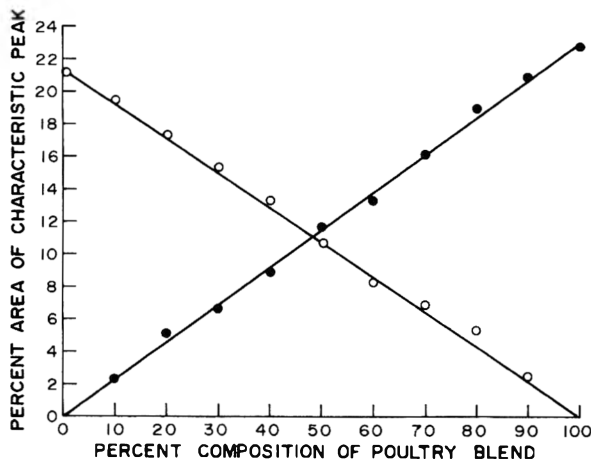


Figure 2. Standard curves for chicken in turkey mixtures, using specific peak for chicken with relative retention time of 1.16 (●——●), and specific peak for turkey with relative retention time of 1.10 (○——○).

Table 1. LC analysis of chicken and turkey blends

	Percent of total area[b]				
RRT[a]	Chicken	Turkey	10% Chicken in turkey	50% Chicken in turkey	90% Chicken in turkey
1.10	—[c]	21.4[d]	19.5	10.4	2.5
1.16	22.2	—[c]	2.4	12.1	21.1
1.40	—[e]	10.8	9.3	6.2	—[e]
1.45	30.4	37.2	32.5	30.4	31.4
1.52	13.0	—[e]	3.1	6.6	11.1
1.66	3.2	3.2	3.0	2.4	2.1
2.10	5.1	5.4	5.9	5.0	6.1
2.40	10.4	2.8	9.0	10.5	5.6
2.46	—[e]	—[e]	—[e]	—[e]	5.5
2.65	12.2	9.7	11.4	11.3	11.3

[a] Relative to retention time of BSA peak.
[b] Average of 2 determinations.
[c] Peak is absent or area is less than 1%.
[d] Species-specific peaks are underlined.
[e] Peak overlapped with another.

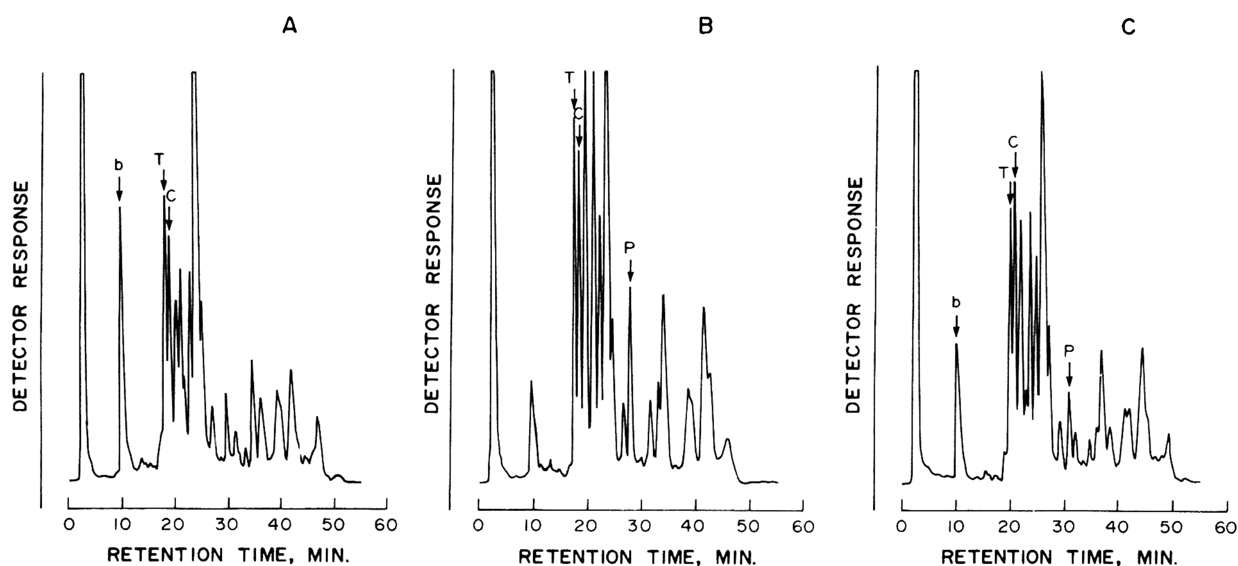


Figure 3. Chromatograms of chicken–turkey mixtures containing beef (A), pork (B), and both beef and pork (C). Arrows indicate specific peaks for chicken (C), turkey (T), beef (b), and pork (p).

Determination of Chicken and Turkey in Unknown Mixtures

Prepare 20 unknown mixtures, 30 g each, containing 0–100% chicken in turkey, and identify by code. Blend and analyze as mentioned above. Identify peaks specific to chicken or to turkey by comparing with standard chicken and turkey chromatograms. Determine percent chicken or turkey in unknowns from constructed standard curves. Decode mixtures and check with original identity and composition.

Results and Discussion

Chromatograms of chicken and turkey standard samples and 3 of the prepared chicken–turkey mixtures are shown in Figure 1. Relative retention times and percent area of major peaks in these chromatograms are presented in Table 1. These results confirm findings reported elsewhere (4) and indicate that chicken and turkey chromatograms have major specific peaks which can be used for species identification and quantitation. However, for simplicity, only one major (area over 20%), well resolved peak in each chromatogram was chosen for detection and quantitation of chicken or turkey in mixtures. The specific peak chosen for chicken has a relative retention time of 1.16, and that for turkey has a relative retention time of 1.10 as shown in Table 1.

When percent area of the specific peak to chicken or turkey was plotted against percent chicken or turkey in the prepared chicken–turkey mixtures, straight lines were obtained over a composition range of 0–100% as shown in Figure 2. This finding indicates that the LC method is applicable to all chicken–turkey mixtures and that either one of the standard curves (Figure 2) can be used for quantitative purposes. However, it should be noted that percent area of specific peak varies among major chicken parts and also among turkey parts (4). Percent area of the specific peak of chicken was 20.3, 21.5, 28.4, and 30.0 in legs, thighs, wings, and breast, respectively. The specific peak of turkey had percent area of 13.0, 14.2, 20.0, and 24.1 in thighs, legs, breast, and wings, respectively (4). If, for example, turkey breast was adulterated separately with equal weights of chicken breast and chicken legs, area of the specific peak of chicken in the turkey–chicken breast mixture would be approximately 1.5 times that in the turkey–chicken legs mixture. Therefore, individual standard curves may have to be constructed for various chicken–turkey products with different parts. Generally, several parts of poultry species are added to formulate a commercial poultry mixture. In this study, composite samples of major parts of chicken and of major parts of turkey were used to obtain curves representative of major parts of chicken or turkey. As low as 1% chicken or turkey was detected in this manner. It is important to note that the standard curves presented in Figure 2 may be used for the quantitative analysis of chicken–turkey mixtures made from any parts with a variability factor of less than 2.

LC analysis of raw chicken and turkey samples that had been frozen for 3 months at −40°C resulted in chromatograms and standard curves similar to those of unfrozen raw samples presented in Figures 1 and 2. Evidently, freezing did not alter chromatograms significantly, and the LC method can be used for detection and quantitation of chicken and turkey in frozen poultry products with raw chicken and turkey meats. However, boiling chicken–turkey mixtures for even 5 min resulted in different chromatograms that could not be used for chicken or turkey identification and quantitation.

The results obtained from LC analysis of chicken–turkey unknown mixtures made in our laboratory indicated that the LC method is reliable. The identity of all unknown mixtures was determined correctly. The results also indicated that the LC method yields accurate reproducible results (CV less than 5%) over a range of 5–100% chicken in turkey. It applies to fresh and frozen raw chicken–turkey mixtures and does not suffer serious interference from the presence of other common meats such as beef and pork as shown in Figure 3.

References

(1) Medina, M. B., & Phillps, J. G. (1982) *J. Agric. Food Chem.* **30,** 1250–1253
(2) Hayden, A. R. (1977) *J. Food Sci.* **42,** 1189–1192
(3) Kim, H., & Shelef, L. A. (1986) *J. Food Sci.* **51,** 731–741
(4) Ashoor, S. H., Monte, W. C., & Stiles, P. G. (1988) *J. Assoc. Off. Anal. Chem.* **71,** 396–401

Detection of Poultry and Pork in Cooked and Canned Meat Foods by Enzyme-Linked Immunosorbent Assays

RONALD G. BERGER, RICHARD P. MAGEAU, BERNARD SCHWAB, and RALPH W. JOHNSTON[1]
U.S. Department of Agriculture, Food Safety and Inspection Service, Microbiology Division, Medical Microbiology Branch, Beltsville, MD 20705

Enzyme-linked immunosorbent assays (ELISA) are described for the detection of poultry and pork in cooked and canned meat foods. These assays are based on species-specific, polyclonal antibodies raised against heat-resistant antigens. The heat-resistant antigens were isolated from raw skeletal muscle tissue of pork and chicken and were found to be immunoreactive even after heating to 120°C for 15 min. The poultry ELISA could detect chicken or turkey at the 126 ppm level, and the pork ELISA could detect pork at the 250 ppm level. Samples of frankfurters, bolognas, pressed meats, canned baby foods, and canned spreads were prepared by simple aqueous extractions.

The U.S. Department of Agriculture's Food Safety and Inspection Service is charged with assuring consumers that the meat and poultry they purchase is safe, wholesome, unadulterated, and accurately labeled. Preventing adulteration of meat foods with less desirable or objectionable meat species is important for economic, religious, and health reasons. Laboratory procedures currently available for determining the species composition of raw meat products include: agar gel immunodiffusion assay (1), enzyme-linked immunosorbent assay (ELISA) (2), and isoelectric focusing (3). Attempts to identify the component species of cooked meat products have met with limited success. King (4) demonstrated that the species of meat heated to 120°C could be identified by isoelectric focusing of the extracts and staining the gels specifically for the activity of adenylate kinase and creatine kinase. The extraction procedure was rather involved. The selection of carrier ampholytes varied depending on the species being sought, and the complexity of the band distribution in mixed-species samples made interpretation difficult. Milgrom and co-workers (5) found heat-stable antigens in boiled, ethanol-precipitated, saline extracts of adrenal glands which were species-specific and present at low levels in muscle tissue. Using similar antigens, Hayden (6) and Kang'ethe and colleagues (7) developed agar gel immunodiffusion assays capable of species identification in cooked meat products. Manz (8) isolated heat-resistant antigens for use in an ELISA by extracting acetone-dried meat powders with a hot urea solution. These assays suffered, however, because they required extreme efforts in preparing suitable antisera as well as samples. All the heat-resistant antigens described (5–8) were subjected to 100°C prior to their use as immunogens.

In the present study, similar antigens were partially purified from chicken and porcine skeletal muscle tissue without heating. These antigens, being relatively free of extraneous antigens and not having been subjected to the denaturing heat treatment, were used to produce potent, species-specific antisera. The antisera were used to develop 2 ELISAs for detecting pork and poultry in cooked and canned meat products.

METHOD

Immunizing Antigen

Divide 1 kg fresh lean ground skeletal muscle tissue (either pork or chicken) into 4 aliquots of 250 g each. To one 250 g aliquot, add 500 mL 0.14M NaCl and homogenize 3 min in a blender. Let slurry stand 1 h at room temperature; then centrifuge at 10 000*g* for 20 min at 4°C. Filter supernatant liquid through one layer of Kimwipes (Kimberly-Clark, Neenah, WI) and Whatman No. 42 filter paper (Wand R. Balston, Maidstone, Kent, UK). Dilute filtrate to 950 mL with distilled water; then add 50 mL 1.0M sodium acetate followed by the slow addition, with stirring, of 361 g granular ammonium sulfate. After ammonium sulfate has dissolved, let extract stand overnight at 4°C. Repeat centrifugation as above. Discard pellet and adjust pH of supernatant liquid to 4.9 by adding 1N HCl; let stand overnight at 4°C. Repeat centrifugation. Discard pellet and adjust pH of supernatant liquid to 3.7, as above, then let stand overnight at 4°C. Repeat centrifugation. Discard pellet and to the supernatant liquid add slowly, with stirring, 357 g granular ammonium sulfate. After ammonium sulfate has dissolved, let extract stand overnight at 4°C. Centrifuge at 27 000*g* for 10 min at 4°C. Discard liquid and suspend pellet in 60 mL distilled water. Repeat centrifugation to remove any insoluble material. Dialyze extract overnight against distilled water, then concentrate to ca 10 mL by dialysis against 30% aqueous solution of 20M polyethylene glycol (PEG) (Union Carbide, Danbury, CT). Dialyze concentrate exhaustively against 0.01M sodium acetate buffer pH 3.7 (prepare buffer by adding glacial acetic acid to 0.01M sodium acetate until pH reaches 3.7). Remove insoluble material by centrifugation. Repeat the above steps for the remaining 3 aliquots of ground skeletal muscle tissue. Pool the 4 harvests.

Prepare carboxymethyl cellulosic cation exchanger according to the manufacturer's specifications (Whatman, Wand R. Balston), equilibrating with starting buffer (0.01M sodium acetate, pH 3.7). Pack 1.6 × 35 cm column with CM cellulose and check equilibration; run more starting buffer through column if necessary. Apply pooled harvest to column and elute with linear gradient composed of 180 mL starting buffer and 167 mL limit buffer (0.1M sodium acetate) at 20 mL/h. Collect 3 mL fractions while monitoring absorbance at 280 nm. To ensure complete elution of major peak, apply additional limit buffer following completion of gradient. Harvest major peak, which elutes as gradient runs out. Concentrate harvest by dialysis against 30% PEG to ca 2 mg protein/mL as determined by Lowry assay (9), using bovine serum albumin as standard. Total yield should be ca 25 mg antigen.

Antisera

Inoculate New Zealand albino rabbits subcutaneously at multiple sites on their backs with 1 mg each of immunizing antigen emulsified in an equal volume of Freund's Complete Adjuvant. After 4 weeks, boost similarly, except use Freund's Incomplete Adjuvant. One week later, test sera for affinity and specificity in the assay. If necessary, continue to boost and test bleed on alternate weeks until satisfactory antisera are obtained.

Antibody

Dilute antiserum with equal volume of 0.01M phosphate-buffered saline (PBS), pH 7.2. At 4°C, add saturated ammonium sulfate solution dropwise, with stirring, to one-third

Received March 23, 1987. Accepted July 24, 1987.
[1] Microbiology Division, Washington, DC 20250.

Table 1. Poultry ELISA of control tissue extracts (mean ± SD)[a] (20 test wells)

Species	Unheated	100°C for 15 min	120°C for 15 min
Horse	0.068 ± 0.015	0.024 ± 0.006	0.027 ± 0.007
Beef	0.086 ± 0.021	0.024 ± 0.004	0.024 ± 0.007
Pork	0.035 ± 0.008	0.020 ± 0.004	0.019 ± 0.003
Sheep	0.068 ± 0.020	0.022 ± 0.004	0.022 ± 0.003
Deer	0.048 ± 0.014	0.022 ± 0.008	0.019 ± 0.004
Kangaroo	0.036 ± 0.005	0.041 ± 0.010	0.042 ± 0.009
Chicken	0.779 ± 0.030	0.742 ± 0.038[b]	0.638 ± 0.031
Turkey	0.676 ± 0.025	0.552 ± 0.023	0.505 ± 0.025

[a] OD 414–OD 492 nm.
[b] 40 test wells.

Table 2. Pork ELISA of control tissue extracts (mean ± SD)[a] (20 test wells)

Species	Unheated	100°C for 15 min	120°C for 15 min
Horse	0.167 ± 0.017	0.064 ± 0.004	0.088 ± 0.016
Beef	0.131 ± 0.059	0.053 ± 0.009	0.048 ± 0.012
Pork	0.795 ± 0.033	0.719 ± 0.060[b]	0.683 ± 0.068
Sheep	0.243 ± 0.018	0.062 ± 0.006	0.059 ± 0.009
Deer	0.130 ± 0.077	0.058 ± 0.007	0.056 ± 0.009
Kangaroo	0.054 ± 0.007	0.045 ± 0.006	0.047 ± 0.006
Chicken	0.056 ± 0.010	0.044 ± 0.008	0.042 ± 0.008
Turkey	0.059 ± 0.004	0.048 ± 0.007	0.052 ± 0.010

[a] OD 414–OD 492 nm.
[b] 38 test wells.

saturation. Let stand at least 1 h at 4°C. Centrifuge at 10 000g for 10 min at 4°C. Discard liquid and dissolve pellet in PBS to original volume of serum. Reprecipitate gammaglobulin twice more at one-third saturation with ammonium sulfate, as above. Dissolve final pellet in PBS to one-half original volume of serum and dialyze against PBS.

Pack 2.0 × 68 cm column with Sephadex G200 (Pharmacia Fine Chemicals, Piscataway, NJ) swollen in PBS. Apply 4 mL aliquot of gammaglobulin and elute at 30 mL/h with PBS. Collect 4 mL fractions while monitoring absorbance at 280 nm. Harvest second peak, which contains IgG. Concentrate IgG to ca 3 mg/mL; then dialyze against 0.1M sodium bicarbonate and store at −20°C or below. The IgG so obtained is used either as coating antibody or as biotinylated antibody.

Biotinylated Antibody

Dilute 1 mg IgG in 0.1M sodium bicarbonate to 1 mg/mL. Dissolve biotin-N-hydroxysuccinimide ester (Miles Scientific, Naperville, IL) in dimethyl sulfoxide to 1 mg/mL. Add 100 μL biotin ester solution, dropwise with mixing, to IgG. Let stand at room temperature for 4 h; dialyze overnight against PBS containing 0.04% sodium azide at 4°C. Add glycerine to the biotinylated IgG to 50% and store at −20°C.

Controls

Add 20 g lean ground skeletal muscle tissue to 60 mL normal saline, stomach for 10 s in Colworth Model 80 stomacher (Tekmar Co., Cincinnati, OH), and let stand 1 h at room temperature. Place mixture into boiling water bath for 15 min and then centrifuge at 10 000g for 15 min at 4°C. Filter liquid through 0.45 μm filter. Prepare control extracts from horse, beef, pork, sheep, deer, kangaroo, chicken, and turkey meat.

Prepare unheated tissue extracts and those heated to 120°C in similar fashion, except, omit heating step or replace it with autoclaving for 15 min.

Samples

Add 5 g finely diced cooked or canned meat food to 10 mL distilled water, stomach 1 min, and let stand 1 h at room temperature. Centrifuge extract at 15 600g. Test clear supernatant liquid in ELISA. All samples in the present study were purchased at a local supermarket, except the horse, sheep, and deer frankfurters, which were provided by the University of Connecticut Meat Sciences Laboratory, Storrs, CT.

ELISA

Determine optimal dilutions of coating antibody and biotinylated antibody by cross-titrations against control extracts, using excess streptavidin-peroxidase conjugate (Zymed Laboratories, Inc., South San Francisco, CA) such that optical density (OD) at 414 nm minus OD at 492 nm is >0.600 for homologous control and <0.060 for heterologous controls. Absorbance maximum for reacted ABTS is at 414 nm, and absorbance minimum is at 492 nm. (Subtracting minimum from maximum negates any nonspecific components of optical density due to scratches, fingerprints, or bubbles.)

To each well of flat-bottomed, 96 well microtitration plates (Immulon I. Dynatech Laboratories Inc., Alexandria, VA), add 100 μL optimally diluted IgG in 0.05M Tris-HCl buffer (pH 8.4) containing 0.01% merthiolate. Seal plates with adhesive plastic and let stand at least 24 h, but not more than 6 months at 4°C in a high-humidity atmosphere. Perform all subsequent incubations at room temperature.

Wash all wells of a plate by filling and aspirating 3 times with 300 μL/well of 0.05M PBS (pH 7.2) containing 0.05% Tween 80 (PBST). Add 100 μL normal saline to each well of the first column for blanks. Add 100 μL sample or control extract per well; use replicates of 4 wells each. Cover plate and after a 1 h incubation, wash all wells 3 times with PBST as above. Add 25 μL biotinylated antibody, optimally diluted in PBST containing 10% heat-inactivated normal rabbit serum, to each well except those in the first column, which receive diluent only. Cover plate and incubate 1 h; then wash 3 times with PBST. To each well, add 25 μL streptavidin-peroxidase conjugate, diluted 1:2000 in PBST containing 10% heat-inactivated normal rabbit serum. Cover plate and incubate 30 min; then wash 4 times with PBST, leaving last wash in wells to incubate 1 h. Aspirate PBST from wells and then add 50 μL substrate consisting of 0.4mM 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) and 1.3mM H_2O_2 in 0.1M Na_2HPO_4–citric acid buffer pH 4.0 (ABTS) to each well. Cover plate and incubate 30 min; then add 50 μL 0.1M citric acid to each well. Determine OD 414 nm minus OD 492 nm for each well, blanking the instrument (Titertek Multiscan, Flow Laboratories, McLean, VA) on column one.

All control extracts and all sample extracts were assayed on at least 5 separate occasions by both the poultry ELISA and the pork ELISA.

Results

The antibody currently used in the poultry ELISA was titered to 1:500 for the coating antibody and 1:70 for the biotinylated antibody. The antibody for the pork ELISA was titered to 1:2000 for the coating antibody and 1:90 for the biotinylated antibody. A 1:2000 dilution of the streptavidin-peroxidase conjugate was found adequate for both ELISAs.

Data in Tables 1 and 2 demonstrate the specificity of pork and poultry ELISA systems as well as the effect of heating on control tissue extracts. The poultry ELISA (Table 1) shows virtually no cross-reactions with the red meat species extracts, whereas a strong reaction is apparent under all conditions with chicken and turkey extracts. The pork ELISA shows no cross-reactions with heated, heterologous species

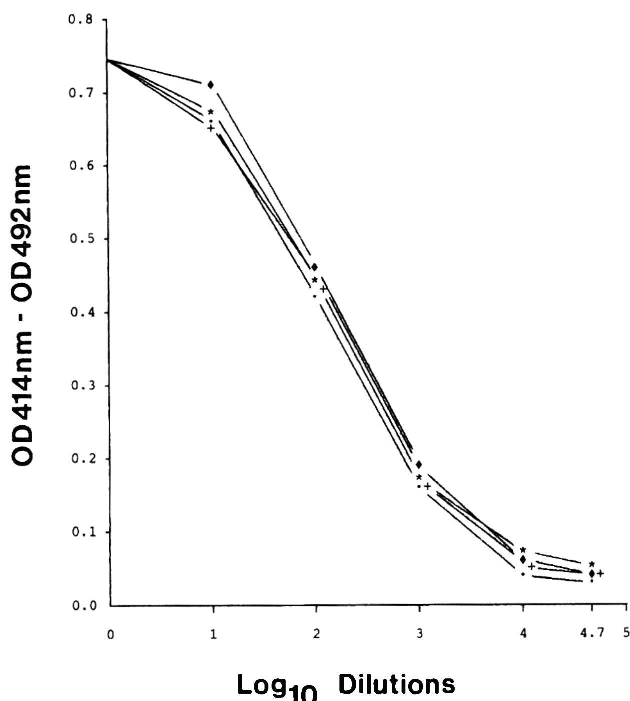


Figure 1. Effect of diluting chicken control extract (100°C for 15 min) in control extracts (100°C for 15 min) of horse (♦), beef (*) and pork (+) and beef frankfurter sample extract (●).

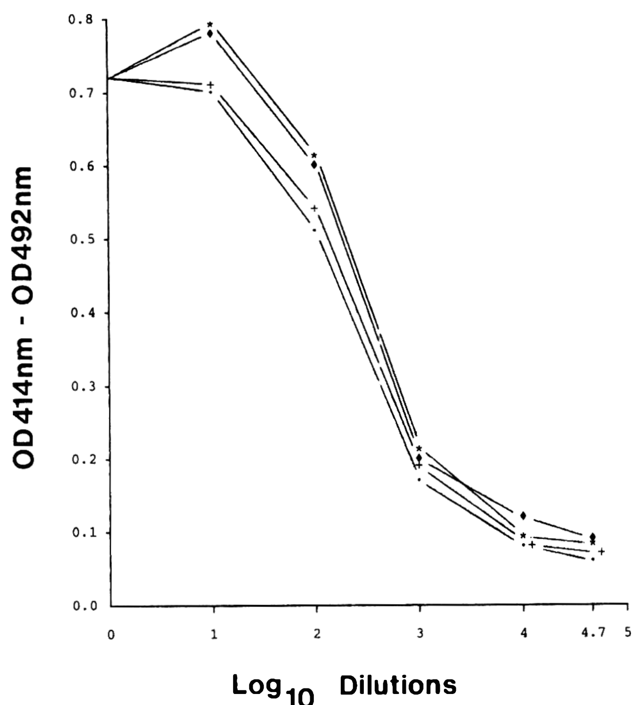


Figure 2. Effect of diluting pork control extract (100°C for 15 min) in control extracts (100°C for 15 min) of horse (♦), beef (*) and chicken (+) and beef frankfurter sample extract (●).

control extracts, whereas the pork control extracts react strongly under all conditions (Table 2). A slight cross-reaction with unheated control extracts of horse, beef, sheep, and deer occurs with the pork ELISA. The homologous extract reactions are reduced only slightly with heating increased from 100°C for 15 min to 120°C for 15 min, for the poultry ELISA as well as the pork ELISA.

Table 3. Poultry and pork ELISAs of cooked and canned meat food sample extracts (mean ± SD)[a] (20 test wells)

Product	Poultry ELISA	Pork ELISA
Frankfurters		
Horse	0.029[a] ± 0.002	0.076 ± 0.011
Beef	0.025 ± 0.006	0.060 ± 0.006
Pork	0.027 ± 0.002	0.752 ± 0.082
Sheep	0.036 ± 0.005	0.111 ± 0.008
Deer	0.040 ± 0.003	0.113 ± 0.010
Chicken	0.840 ± 0.045	0.065 ± 0.007
Turkey	0.664 ± 0.030	0.048 ± 0.004
Bolognas		
Beef	0.024 ± 0.004	0.061 ± 0.011
Pork	0.026 ± 0.003	0.754 ± 0.089
Chicken	0.813 ± 0.057	0.071 ± 0.011
Turkey	0.640 ± 0.060	0.044 ± 0.006
Chopped, pressed, sliced meats		
Beef	0.014 ± 0.003	0.054 ± 0.004
Ham	0.015 ± 0.005	0.647 ± 0.023
Chicken	0.691 ± 0.019	0.046 ± 0.004
Turkey	0.567 ± 0.033	0.063 ± 0.003
Canned baby foods		
Beef	0.017 ± 0.005	0.053 ± 0.005
Pork	0.015 ± 0.005	0.463 ± 0.064
Lamb	0.015 ± 0.005	0.051 ± 0.005
Chicken	0.446 ± 0.037	0.045 ± 0.003
Turkey	0.395 ± 0.030	0.044 ± 0.004
Canned spreads		
Beef	0.020 ± 0.002	0.053 ± 0.002
Ham	0.020 ± 0.002	0.650 ± 0.045
Chicken	0.445 ± 0.027	0.053 ± 0.006

[a] OD 414–OD 492 nm.

ELISA results obtained with sample extracts of cooked and canned meat foods are presented in Table 3. The presence of pork or poultry in a product was confirmed by a strong reaction in the appropriate assay. No significant reactions occurred with the heterologous species samples. The canned products, which had been subjected to a more severe heat treatment during retort, reacted with less intensity.

The data represented in Figures 1 and 2 were generated to give an estimate of the sensitivity of the assays in 4 matrices. The matrix appears to have little effect when the analyte is added following extraction, as is the case here. Sensitivities of 126 ppm for the poultry ELISA and 250 ppm for the pork ELISA were determined by extrapolation from Figures 1 and 2, using the beef frankfurter matrix curves. All heated heterologous species control tissue extracts (100°C for 15 min) and all heterologous species sample extracts were considered en masse, and a mean OD of 0.025 with a standard deviation (SD) of 0.009 (sample size of 400 wells) was calculated for the poultry ELISA and a mean of 0.061 ± 0.021 (500 wells) for the pork ELISA. Adding 3 SDs to the means yielded 0.052 for poultry and 0.124 for pork. Any sample with an OD value higher than these is considered to contain poultry or pork. By placing these values on the ordinate of Figure 1 or 2, the corresponding $\log_{10}$ dilution yielding that OD can be read off the abscissa. For the poultry ELISA, a significant OD of 0.052 is obtained at a $\log_{10}$ dilution of 3.9, or a 1:7943 dilution, which can be represented as 126 ppm. The pork ELISA sensitivity was calculated to be 250 ppm.

Discussion

The ELISAs for detection of poultry and pork, described herein, are the only methods for fast, simple, accurate, and sensitive determination of these species in cooked and canned meat foods. The assays can be completed in 5 h and use a simple aqueous extraction of samples. The ELISAs are very sensitive and accurate when suitable antibodies are used. The key to the successful development of these ELISAs is the isolation of native heat-resistant immunizing antigens. The

physical and chemical characteristics of these antigens have not been pursued any further than what can be derived from the fractionation procedure and the experiments described. The present fractionation procedure is similar to that used for the isolation of serum α_1-acid glycoprotein (10). Of all the serum proteins, α_1-acid glycoprotein is the most heat resistant. The immunizing antigens are not serum α_1-acid glycoprotein, however, since antibodies specific for one do not react with the other (data not shown).

The immunizing antigens are heat resistant as well as species specific. However, neither the species specificity nor the heat resistance is absolute. Rabbits immunized with these antigens often yield antisera with a high IgM/IgG ratio. These antisera will appear highly reactive when assayed by immunodiffusion against unheated antigens (data not shown), but prove unsuitable for the ELISAs which require IgG with high affinity for the antigens. Multiple boosters may be required to induce the IgM-to-IgG switch, but there is a danger with that procedure in that cross-reactive antibodies may be produced against common epitopes. A possible solution might be to produce the antisera in goats, which are more closely related to the potentially cross-reacting species than are rabbits. Another possible solution would be to produce monoclonal antibodies which have absolute specificity for the desired species.

The antisera used in the poultry ELISA showed no cross-reactivity except between chicken and turkey, both of which reacted strongly (Tables 1 and 3); this ELISA has therefore been generalized to detect poultry. The antiserum in the pork ELISA showed some cross-reactivity with the raw tissue extracts (Table 2). Because the present ELISAs were developed for use with cooked and canned meat foods only, marginal cross-reactivity with raw meats would not be significant. These assays should not be applied to raw meat foods. Any cross-reactions are greatly reduced with heating to 100°C (Table 2) and with the processing of cooked or canned meat foods (Table 3). Although the sheep and deer frankfurters showed some reactivity in the pork ELISA, comparable results might not be expected from commercially available frankfurters. The former were prepared from lean tissue with no added fat; the resulting extracts therefore contained higher concentrations of solubilized protein than did the latter.

The antigens are not completely heat stable because increased heating results in a concomitant reduction of reactions intensities (Tables 1–3). The heating no doubt caused some denaturation of the antigens, but it is also possible that heating caused molecular interactions within the matrix which rendered the antigens less extractable. The intensity of the reaction, then, would depend not only on the amount of analyte, but also on the severity of heat used in processing. The minimum level of detection would therefore depend on the type of product being tested. There are too many different types of meat food products for these ELISAs to be applied quantitatively. Accurate quantitative results could only be obtained for a particular product that has been carefully formulated with varying levels of adulteration and for which a standard curve has been generated. It would be impractical to attempt this for all possible products. For applications then, these ELISAs should be used qualitatively. The meat and poultry food products included in the present study were chosen as being representative of the extremely wide variety available. Only those products for which a poultry, a pork, and other heterologous species representatives are commercially available were included, although many other products have been assayed (data not shown).

The present ELISAs incorporate the biotin-streptavidin amplification system. Besides making the ELISAs more sensitive, this system has several important advantages. Because of the mild conditions used and the low molecular weight of biotin (244 daltons), the binding of biotin to the probe antibody lacks the deleterious effects that occur when an enzyme is directly conjugated to the antibody (11). Most important, the specificity of the antibody for the antigen is maintained. The biotinylation of antibody is a much simpler laboratory procedure, and the product is more stable than an antibody-enzyme conjugate. The use of streptavidin, an extracellular product of *Streptomyces avidinii*, rather than egg white avidin in the enzyme conjugate ensures specificity. Unlike egg white avidin, streptavidin has a neutral isoelectric point under physiological conditions and is free of carbohydrate side chains (12), thus excluding nonspecific binding to plastic or lectins.

The sensitivities of the methods were estimated to be 126 ppm for the poultry ELISA and 250 ppm for the pork ELISA. These values were extrapolated from Figures 1 and 2, which show the effect of diluting the analyte in 4 matrices. The values were taken from the beef frankfurter matrix curves. The end-point ODs were calculated as the mean plus 3 SDs for all heterologous samples and controls. Although these are only rough estimates, for regulatory purposes they are more than adequate. When considering the amount of analyte that might be expected in mislabeled or fraudulently prepared meat foods, where adulteration at less than 1% would not provide an economic incentive, OD values much higher than 0.052 for the poultry ELISA and 0.124 for pork could be set as cutoff values. Any sample that produces a value higher than the cutoff value would be considered violative. At these higher levels, the accuracy of the assays would be such that the possibility of false positives would be virtually nil.

In conclusion, the specificity and the sensitivity of the cooked poultry ELISA and the cooked pork ELISA have been deemed sufficient for them to have been incorporated as regulatory tools by the Food Safety and Inspection Service of the U.S. Department of Agriculture.

Acknowledgments

The authors thank Donald Kinsman of the University of Connecticut Animal Industries Department for providing the horse, sheep, and deer frankfurters, and Darlene Worsham for her help in preparing this manuscript.

References

(1) Fugate, H. G., & Penn, S. R. (1971) *J. Assoc. Off. Anal. Chem.* **54,** 1152–1156
(2) Whittaker, R. G., Spencer, T. L., & Copland, J. W. (1983) *J. Sci. Food Agric.* **34,** 1143–1148
(3) Sinclair, A. J., & Slattery, W. J. (1982) *Aust. Vet. J.* **58,** 79–80
(4) King, N. L. (1984) *Meat Sci.* **11,** 1–14
(5) Milgrom, F., Tuggac, Z. M., & Witebsky, E. (1964) *J. Immunol.* **93,** 902–909
(6) Hayden, A. R. (1981) *J. Food Sci.* **46,** 1810–1813
(7) Kang'ethe, E. K., Lindquist, K. J., & Gathuma, J. M. (1985) in *Biochemical Identification of Meat Species,* R. L. S. Patterson (Ed.), Elsevier Science Publishing Co., Inc., New York, NY, pp. 129–144
(8) Manz, J. (1985) *Fleischwirtsch* **65,** 497–499
(9) Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
(10) Weimer, H. E., Mehl, J. W., & Winzler, R. J. (1950) *J. Biol. Chem.* **185,** 561–568
(11) Wilchek, M., & Bayer, E. A. (1984) *Immunol. Today* **5,** 39–43
(12) Chaiet, L., & Wolf, F. J. (1964) *Arch. Biochem. Biophys.* **106,** 1–5

CHEMICAL CONTAMINANTS MONITORING

Organohalogen Residues in Human Adipose Autopsy Samples from Six Ontario Municipalities

DAVID T. WILLIAMS, GUY L. LEBEL, and ELIZABETH JUNKINS
Health and Welfare Canada, Environmental Health Centre, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada

Human adipose tissue samples obtained during autopsies in 6 Canadian Great Lakes municipalities were analyzed for chlorobenzenes, polychlorobiphenyls, and organohalogen pesticide residues. The frequency of occurrence and the range and mean for 28 organohalogen residues are reported for male and female donors in each municipality. Overall mean residue values in females were significantly higher than those in males for hexachlorobenzene, β-HCH, *p,p'*-DDE, *p,p'*-DDD + *o,p'*-DDT, and *p,p'*-DDT. The means and ranges of residue values were similar to those reported in previous Canadian surveys.

The Canada-United States Great Lakes Agreement was negotiated to address perceived and emerging problems related to pollution of the Great Lakes Basin. One of the programs under this agreement recognized the need to generate data that would provide insight into human exposure to toxic chemicals and would aid in the assessment of the total intake of contaminants from all sources. One approach to addressing this need is to determine the levels of potentially toxic chemicals present in human tissue samples. An initial study (1) had investigated the levels of organochlorine contaminants in human adipose tissues obtained from 2 municipalities, Ottawa and Kingston, located at the eastern end of the Great Lakes Basin. The present study was designed to extend the investigation and compare contaminant levels in adipose tissue samples from municipalities in other areas of the Great Lakes Basin. The tissues were analyzed for organohalogen residues by using a method recently developed for this purpose (2).

Experimental

Reagents and Apparatus

Reagents, equipment, and operating conditions for analysis of halogenated contaminants in adipose tissue have been described previously (2).

Analytical Procedure

Procedures for adipose tissue extraction and analysis have been described previously (2). Extracts were analyzed simultaneously on 2 capillary columns of differing polarity (DB-17 and DB-5) to confirm the identity of each residue. Residues were quantitated using data from the DB-17 column except for mirex, where data were used from the DB-5 column.

Quality Assurance/Quality Control

Immediately before and after the study, recovery studies were done on clean fat fortified at 10, 100, and 500 ng/g with the analytes of interest. Recoveries for all detected analytes were >90%, which was in agreement with recovery data previously published (2). A method blank was run with each set of 14 samples, and 10% of the samples were analyzed in duplicate; duplicate results agreed within ±5%. The identity of residues in 25% of the samples was qualitatively confirmed by gas chromatography/mass spectroscopy GC/MS (2).

Received March 13, 1987. Accepted June 13, 1987.

Tissue Collection

Adipose tissue samples were obtained in 1984 from unembalmed cadavers during autopsies at one hospital in each municipality. Tissues were obtained from all patients where it was legally appropriate to release tissue samples. A single tissue sample (5–20 g) was taken from the greater omentum of each donor and placed in a separate precleaned glass vial, which was then sealed with a Teflon-lined screw cap and frozen (−20°C) until analyzed. To minimize possible contamination of the samples, they were collected using instruments reserved for this purpose.

Available patient data were restricted to age, sex, and body weight. For some patients, the place of residence immediately prior to death was known; the occupational history was not known.

Statistical Methods

For each residue in the statistical analysis, results reported as not detected were assigned a value of half of the detection limit. In some instances the level of the residue could not be calculated because of interference in the GC analysis, and these were treated as missing values throughout the statistical analysis. Estimates of mean residue levels were calculated for males and females separately within each community, along with the corresponding standard deviation. The chemical residue values were transformed using natural logarithms to address the non-normality of the data. The transformed data were tested for normality using the Kolmogorov-Smirnov test (3). Differences among the municipalities and between the sexes were examined using analysis of variance and analysis of covariance (3). To exert some control over experimental error rate for comparisons between municipalities, Bonferroni-type inequalities were used to attain an overall error rate (4).

Results and Discussion

Adipose tissue samples from 92 male and 49 female donors were obtained from 6 Ontario municipalities—Cornwall, London, St. Catharines, Toronto, Welland, and Windsor—located in the Canadian section of the Great Lakes Basin. The numbers of male/female donors in each municipality and their age and body weight distributions are shown in Table 1.

Each tissue sample was analyzed for the organohalogen residues listed in Table 2. The detection limit for each residue and the frequency of occurrence of each residue for the 6 municipalities are reported in Table 2. The ranges and mean values found for those residues detected in the majority of the tissues are listed by area in Table 3. Levels of α-HCH just above the detection limit were found in 84% of the adipose tissues, but because accurate quantitation was not feasible for this compound at these levels, data on means and ranges were not calculated. Occasional tissue samples contained relatively high levels of one, but seldom two, of the following compounds: γ-HCH was found in 12 tissue samples at <20 ng/g but 3 tissues had levels of 109, 148,

Table 1. Ages and body weights of autopsy patients

Location	Sex	No. of autopsy patients	Age, years		Body weight, kg	
			Mean ± SD	Range	Mean ± SD	Range
Cornwall	M	14	67 ± 17	27–90	78 ± 12	40–94
	F	7	64 ± 16	48–91	66 ± 15	50–90
	M + F	21	65 ± 17	27–91	73 ± 14	40–94
London	M	25	64 ± 15	34–88	75 ± 20	44–132
	F	12	75 ± 7	61–84	57 ± 11	42–80
	M + F	37	68 ± 14	34–88	69 ± 19	42–132
St. Catharines	M	11	60 ± 17	21–81	N/A[a]	
	F	6	77 ± 8	66–88	N/A	
	M + F	17	66 ± 16	21–88	N/A	
Toronto	M	4	67 ± 7	59–76	N/A	
	F	3	61 ± 17	47–80	N/A	
	M + F	7	64 ± 11	47–80	N/A	
Welland	M	14	60 ± 17	28–81	81 ± 15	60–120
	F	9	64 ± 17	28–83	64 ± 12	40–80
	M + F	23	61 ± 17	28–83	74 ± 16	40–120
Windsor	M	24	53 ± 20	6–85	70 ± 17	23–113
	F	12	64 ± 19	12–81	54 ± 8	39–68
	M + F	36	57 ± 20	6–85	65 ± 17	23—113

[a] N/A: data not available.

and 174 ng/g. Octachlorostyrene was present in 5 of the tissues, but all residue values were <15 ng/g. Pentachlorobenzene was detected in 9 of the tissue samples, but only 2 of these tissues contained residue levels (43 and 15 ng/g) greater than 10 ng/g. Hexabromobiphenyl was found in 27% of the samples at <25 ng/g, but 4 tissues contained levels of 80, 97, 158, and 208 ng/g.

The overall ranges and mean residue levels for tissues from males and females in the 6 municipalities are given in Table 4. Mean residue values for females were significantly ($P < 0.01$) greater than for males for hexachlorobenzene (109 vs 71 ng/g) and β-HCH (118 vs 67 ng/g) and significantly ($P < 0.05$) greater for *p,p'*-DDE (3819 vs 2937 ng/g), *p,p'*-DDD + *o,p'*-DDT (6.7 vs 4.8 ng/g), and *p,p'*-DDT (101 vs 74 ng/g). Mes et al. (5) reported significantly higher hexachlorobenzene residues in adipose tissue from females (130 ng/g) compared to those from males (66 ng/g), but they found no significant sex differences with other residues. Focardi et al. (6) have reported significantly higher levels in females for residues of lindane, *p,p'*-DDE, and *p,p'*-DDT. However, Mussalo-Rauhamaa et al. (7) reported no significant differences in males and females for residues of total DDT, PCB, chlordanes, or HCB.

The total mean residue levels for males and females for each municipality are compared in Figure 1. Since residue levels of *p,p'*-DDE and PCB are an order of magnitude greater than levels of other residues, the total mean residue levels have been plotted both without (Figure 1a) and with (Figure

Table 2. Percentage frequency of residue occurrence

Residue	MDL[a]	Cornwall	London	St. Catharines	Toronto	Welland	Windsor
Trichlorobenzene	11	0	0	0	0	0	0
Tetrachlorobenzene	13	0	0	0	0	0	0
Pentachlorobenzene	1.9	4	3	12	0	0	14
Hexachlorobenzene	1.4	100	100	100	100	100	100
HCBD	1.2	0	0	0	0	0	0
α-HCH	1.2	83	97	94	86	83	67
Chlordene	1.2	0	0	0	0	0	0
γ-HCH	1.4	8	19	6	0	17	3
β-HCH	3.0	100	100	100	100	100	100
Heptachlor	1.4	0	0	0	0	0	0
Aldrin	1.2	0	0	0	0	0	0
Octachlorostyrene	1.1	8	0	0	0	0	8
Oxychlordane	1.2	100	100	100	100	100	100
Heptachlor epoxide	1.1	100	100	100	100	100	100
γ-Chlordane	1.3	0	0	0	0	0	0
trans-Nonachlor	1.3	100	100	100	100	100	100
α-Chlordane	1.0	0	0	0	0	0	0
α-Endosulfan	1.2	0	0	0	0	0	0
o,p'-DDE	2.6	0	0	0	0	0	0
p,p'-DDE	1.2	100	100	100	100	100	100
Dieldrin	0.9	96	100	100	100	100	100
Endrin	2.4	0	0	0	0	0	0
cis-Nonachlor	1.3	100	100	100	100	100	100
p,p'-DDD + *o,p'*-DDT	4.0	62	97	29	86	52	78
p,p'-DDT	1.7	100	100	100	100	100	100
Mirex	1.8	100	95	94	100	70	94
Hexabromobiphenyl	6.6	8	41	18	57	13	44
PCB	100	100	100	100	100	100	100

[a] Minimum detection limit (ng/g) based on area reject of 3000 counts for 1 g sample in 2 mL extract.

Table 3. Organohalogen residues (ng/g) in adipose tissue from 6 municipalities

Residue	Male		Female		All
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
			Cornwall		
Hexachlorobenzene	62 ± 29	26–109	85 ± 47	37–178	71 ± 36
β-HCH	36 ± 14	16–59	81 ± 62	34–214	51 ± 40
Oxychlordane	32 ± 11	11–59	37 ± 17	16–170	32 ± 13
Heptachlor epoxide	17 ± 10	6–39	16 ± 11	3.3–39	16 ± 10
trans-Nonachlor	60 ± 20	22–110	62 ± 37	31–142	58 ± 25
cis-Nonachlor	7.2 ± 2	4.1–10	7.2 ± 4.3	3.5–17	7.2 ± 2.9
p,p'-DDE	1553 ± 835	502–3312	3100 ± 3331	466–8069	2163 ± 2031
Dieldrin	21 ± 13	8.8–51	17 ± 10	ND–32	19 ± 12
p,p'-DDD + *o,p'*-DDT	3.7 ± 1.6	ND[a]–6.4	4.7 ± 3.4	ND–11	4.3 ± 2.3
p,p'-DDT	57 ± 40	15–159	85 ± 74	13–234	70 ± 56
Mirex	13 ± 17	2.7–55	10 ± 10	3–32	11 ± 14
PCB	2133 ± 1480	604–5866	2094 ± 1526	628–4773	2169 ± 1433
			London		
Hexachlorobenzene	79 ± 49	25–244	108 ± 53	50–225	89 ± 51
β-HCH	99 ± 82	19–297	139 ± 1[illegible]2	32–430	112 ± 93
Oxychlordane	57 ± 26	20–108	54 ± 24	19–93	56 ± 26
Heptachlor epoxide	42 ± 27	7.9–109	46 ± 3[illegible]	6.9–107	44 ± 28
trans-Nonachlor	92 ± 45	31–179	80 ± 4[illegible]	24–141	88 ± 43
cis-Nonachlor	12 ± 8.1	3.7–34	8.8 ± 4.3	2.5–15	11 ± 7.2
p,p'-DDE	3090 ± 1863	490–7217	3124 ± 2263	364–6733	3101 ± 1970
Dieldrin	63 ± 42	11–151	61 ± 43	6.5–151	63 ± 42
p,p'-DDD + *o,p'*-DDT	4.7 ± 3.7	ND–18	8.2 ± 9.2	ND–36	5.9 ± 6.2
p,p'-DDT	53 ± 49	8.4–202	77 ± 66	6.6–218	61 ± 55
Mirex	6.6 ± 4.7	ND–17	8.5 ± 12	ND–43	7.2 ± 7.6
PCB	2207 ± 1255	834–5159	1618 ± 644	618–2845	2011 ± 1115
			St. Catharines		
Hexachlorobenzene	88 ± 46	39–176	157 ± 118	51–373	114 ± 85
β-HCH	89 ± 65	28–209	140 ± 128	27–366	107 ± 91
Oxychlordane	72 ± 62	31–237	66 ± 35	36–132	70 ± 53
Heptachlor epoxide	45 ± 44	8.6–150	35 ± 23	4.9–76	42 ± 38
trans-Nonachlor	136 ± 128	68–467	110 ± 81	45–271	126 ± 111
cis-Nonachlor	14 ± 14	4.2–52	11 ± 8.1	4–27	13 ± 12
p,p'-DDE	3760 ± 2843	453–10 326	5843 ± 2283	2705–8141	4355 ± 2784
Dieldrin	56 ± 55	11–201	52 ± 46	3.8–120	54 ± 50
p,p'-DDD + o,*p'*-DDT	4.6 ± 5.6	ND–20	3.4 ± 3.3	ND–10	4.1 ± 4.8
p,p'-DDT	87 ± 107	14–369	94 ± 93	7.6–258	90 ± 99
Mirex	22 ± 19	ND–51	18 ± 16	6–43	20 ± 18
PCB	2145 ± 1184	939–4469	2872 ± 2152	940–6269	2418 ± 1587
			Toronto		
Hexachlorobenzene	57 ± 34	28–102	60 ± 31	33–95	58 ± 30
β-HCH	64 ± 56	29–148	75 ± 47	34–126	69 ± 48
Oxychlordane	48 ± 19	31–69	42 ± 22	18–62	45 ± 19
Heptachlor epoxide	24 ± 9	13–33	24 ± 15	12–41	24 ± 11
trans-Nonachlor	92 ± 55	47–165	72 ± 37	34–107	83 ± 45
cis-Nonachlor	13 ± 9	5.5–26	11 ± 4.8	6.5–16	12 ± 7
p,p'-DDE	1617 ± 1268	809–3487	2445 ± 1707	1363–4412	1972 ± 1404
Dieldrin	29 ± 11	15–40	37 ± 36	15–79	32 ± 23
p,p'-DDD + *o,p'*-DDT	4.5 ± 2.2	ND[a]–7.2	6.8 ± 2.7	4.3–9.7	5.5 ± 2.5
p,p'-DDT	61 ± 58	27–148	76 ± 34	41–110	67 ± 46
Mirex	30 ± 45	3.8–98	4.9 ± 1.3	3.5–6	19 ± 35
PCB	2626 ± 2594	789–6368	1688 ± 1090	918–2935	2224 ± 2003
			Welland		
Hexachlorobenzene	58 ± 43	18–199	123 ± 72	48–258	84 ± 64
β-HCH	45 ± 49	16–211	123 ± 155	36–530	76 ± 108
Oxychlordane	38 ± 29	14–105	51 ± 25	26–90	43 ± 28
Heptachlor epoxide	23 ± 21	2–84	25 ± 13	8.6–50	24 ± 18
trans-Nonachlor	71 ± 51	26–220	80 ± 45	41–158	74 ± 48
cis-Nonachlor	7.3 ± 4.3	ND–18	6.8 ± 2.8	3.2–13	7.1 ± 3.8
p,p'-DDE	3077 ± 2461	245–7548	3930 ± 2954	1548–11 161	3411 ± 2633
Dieldrin	30 ± 22	7.3–84	37 ± 25	11–83	33 ± 23
p,p'-DDD + *o,p'*-DDT	4 ± 2.3	2–8.9	3.5 ± 2.4	ND–9	3.8 ± 2.3
p,p'-DDT	102 ± 100	17–332	117 ± 96	34–311	108 ± 97
Mirex	8.3 ± 6.6	ND–19	9.5 ± 8.9	ND–24	8.8 ± 7.4
PCB	1456 ± 370	861–2133	2473 ± 3318	745–11 209	1854 ± 2084

Table 3. Continued

Residue	Male		Female		All
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
			Windsor		
Hexachlorobenzene	70 ± 49	25–238	102 ± 49	19–169	80 ± 50
β-HCH	54 ± 31	17–128	113 ± 85	14–270	74 ± 61
Oxychlordane	60 ± 33	25–142	64 ± 41	7.7–143	61 ± 35
Heptachlor epoxide	34 ± 27	6.5–103	36 ± 28	8.4–100	35 ± 27
trans-Nonachlor	111 ± 82	47–374	115 ± 71	14–231	112 ± 78
cis-Nonachlor	12 ± 11	3.8–55	13 ± 9	3.5–34	12 ± 10
p,p'-DDE	3379 ± 3025	138–12 167	4518 ± 3647	146–11 425	3758 ± 3239
Dieldrin	52 ± 49	7.2–235	59 ± 46	14–166	54 ± 47
p,p'-DDD + *o,p'*-DDT	6.2 ± 3.9	2–17	10 ± 9.5	ND–30	7.6 ± 6.6
p,p'-DDT	86 ± 79	8.5–282	133 ± 108	18–343	101 ± 91
Mirex	10 ± 6.6	ND–31	7.7 ± 5.4	ND–19	9.5 ± 6.3
PCB	2198 ± 1150	907–5699	2530 ± 1346	197–5396	2309 ± 1210

[a] ND: not detected.

Table 4. Overall mean residues (ng/g) in adipose tissue

Residue	Male		Female		All
	Mean ± SD[a]	Range	Mean ± SD	Range	Mean ± SD
Hexachlorobenzene	71 ± 44	18–244	109 ± 66	19–373	84 ± 56
β-HCH	67 ± 60	16–297	118 ± 106	14–530	84 ± 82
Oxychlordane	52 ± 35	11–237	54 ± 30	8–143	53 ± 33
Heptachlor epoxide	33 ± 28	2–150	33 ± 25	3–107	33 ± 27
trans-Nonachlor	94 ± 71	22–467	89 ± 57	14–271	92 ± 66
cis-Nonachlor	11 ± 8.9	5.5–26	9.6 ± 6.3	6.5–16	10 ± 8.1
p,p'-DDE	2937 ± 2369	138–12 167	3819 ± 2941	146–11 425	3237 ± 2602
Dieldrin	47 ± 42	7–235	47 ± 40	ND–166	47 ± 41
p,p'-DDD + *o,p'*-DDT	4.8 ± 3.6	ND[b]–20	6.7 ± 7.2	ND–36	5.5 ± 5.2
p,p'-DDT	74 ± 75	8–369	101 ± 86	7–343	84 ± 80
Mirex	12 ± 15	ND–98	9.6 ± 10	ND–43	11 ± 13
PCB	2088 ± 1241	604–6368	2224 ± 1835	197–11 209	2136 ± 1473
Age	60 ± 17	6–90	68 ± 15	12–91	63 ± 17
Body weight	75 ± 17	23–132	60 ± 13	39–97	70 ± 17

[a] Mean ± standard deviation for 92 males and 49 females.
[b] ND: not detected.

Table 5. Mean chlorinated residues (ng/g) in adipose tissues of Canadians

Compound	Southern Ontario[a]	Rest of Ontario[b]	Canada[c]	Kingston[d]	Ottawa[e]
PCB	3200	2500	944	2950	2000
p,p'-DDE	—	—	1721	3260	2560
p,p'-DDT	—	—	311	160	130
ΣDDT	5560	4360	2064	—	—
Dieldrin	100	110	49	36	43
Hexachlorobenzene	100	90	95	110	78
Heptachlor epoxide	—	—	37	35	37
Oxychlordane	—	—	55	42	39
β-HCH	—	—	151	140	65

[a] Based on extractable fat for 126 samples collected in 1972 (9).
[b] Based on extractable fat for 59 samples collected in 1972 (9).
[c] Based on wet weight for 99 samples collected in 1976 (5).
[d] Based on wet weight for 91 samples collected in 1979–1981 (1).
[e] Based on wet weight for 84 samples collected in 1980–1981 (1).

1b) DDE and PCB values included in the total. In general, the total residue levels for females excluding PCB and DDE (Figure 1a) are higher than those for males except in Toronto. However, the number of tissues from this municipality is so small that meaningful interpretation of the data is difficult. These results are similar to those found in the previous Ottawa-Kingston study (Figure 1a). Total residue values including DDE and PCB (Figure 1b), again, in general, are higher for females than for males. This differs from the Ottawa-Kingston study (Figure 1b), in which males had higher PCB and DDE residues compared to females, particularly in Kingston (1).

Comparison of individual residue values between the municipalities (Table 3) did not reveal statistically significant differences ($P < 0.05$). Visual comparison of total residues of individual municipalities against each other (Figure 1b) suggests that St. Catharines total residues might be higher than those for other municipalities, but these differences were not statistically significant ($P < 0.05$). Comparison of total residues for each municipality against a pooled value for the other 5 municipalities, however, did show St. Catharines total residues to be significantly ($P < 0.05$) higher, primarily because of high *p,p'*-DDE residue values, especially for females (cf. Tables 3 and 4). We assume that the route of human exposure to *p,p'*-DDE is principally through the food chain (8), but since information on the diets of the tissue donors was not available, this assumption could not be evaluated in the present study.

The mean residue levels (Table 4) in adipose tissue from these 6 municipalities are comparable to those found in other Canadian studies (Table 5) and appear to represent typical tissue residue levels found in Canada.

Acknowledgments

We thank C. Shewchuk and R. Frazer for technical assistance and the pathologists at each hospital for the tissue

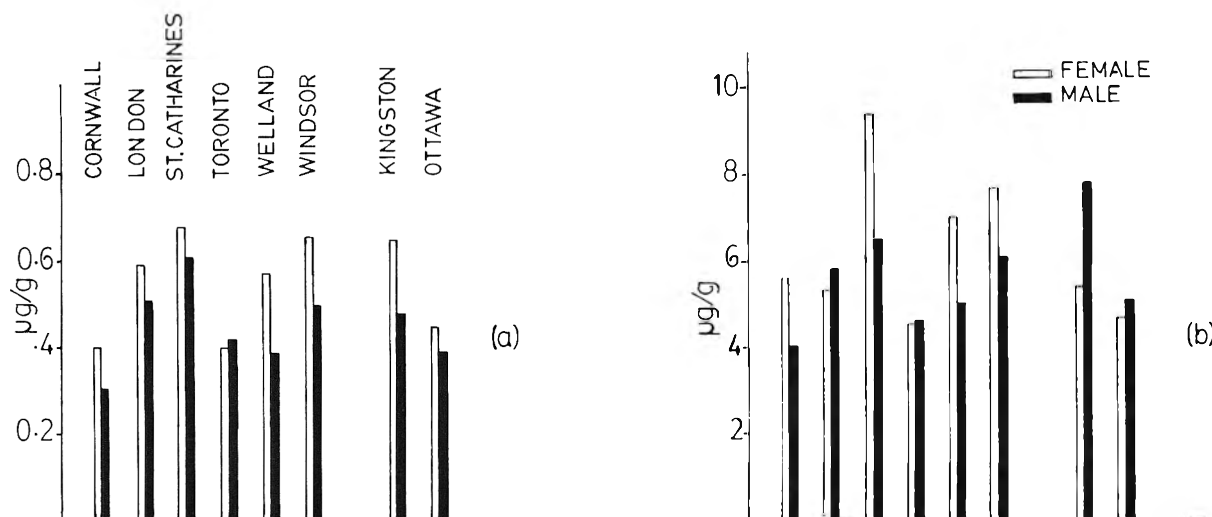


Figure 1. The mean total residue level for males and females in each municipality: (a) excluding PCB and DDE; (b) including PCB and DDE. The city identification in a is the same for b.

samples. Some statistical analyses were performed by M. Goddard.

References

(1) Williams, D. T., LeBel, G. L., & Junkins, E. (1984) *J. Toxicol. Environ. Health* **13**, 19–29
(2) LeBel, G. L., & Williams, D. T. (1986) *J. Assoc. Off. Anal. Chem.* **66**, 451–458
(3) Ostle, B., & Mensing, R. (1975) *Statistics in Research*, Iowa State University Press, Ames, IA
(4) Millar, R. G. (1966) *Simultaneous Statistical Inference*, McGraw-Hill Book Co., New York, NY
(5) Mes, J., Davies, D. J., & Turton, D. (1982) *Bull. Environ. Contam. Toxicol.* **28**, 97–104
(6) Focardi, S., Fossi, C., Leonzio, C., & Romei, R. (1986) *Bull. Environ. Contam. Toxicol.* **36**, 644–650
(7) Mussalo-Rauhamaa, H., Pyysalo, H., & Moilanen, R. (1984) *J. Toxicol. Environ. Health* **13**, 689–704
(8) World Health Organisation (1979) *DDT and Its Derivatives*, Environmental Health Criteria 9, WHO, Geneva, Switzerland
(9) Holdrinet, M. V. H., Braun, H. E., Frank, R., Stopps, G. J., Smout, M. S., & McWade, J. W. (1977) *Can. J. Public Health* **68**, 74–80

Levels and Incidences of Pesticide Residues in Various Foods and Animal Feeds Analyzed by the Luke Multiresidue Methodology for Fiscal Years 1982-1986

MILTON A. LUKE, HERBERT T. MASUMOTO, THOMAS CAIRNS, and HARVEY K. HUNDLEY
Food and Drug Administration, Office of Regulatory Affairs, Los Angeles District Laboratory, 1521 W. Pico Blvd, Los Angeles, CA 90015

During a 5 year period from 1982 to 1986, the FDA Los Angeles District Laboratory analyzed 19 851 samples of domestic and imported food and feed commodities for pesticide residues. A single, rapid, multiresidue method was used. The resultant data have been compiled showing the commodities sampled and the identity and range of levels of pesticide residues detected, including an indication of those residue findings that did not comply with U.S. federal tolerance levels. The residue data presented should not be viewed as being representative of the U.S. food supply; rather, the results are indicative of a surveillance- and compliance-oriented sampling of various food shipments collected by the Los Angeles District.

Although pesticide chemicals provide numerous benefits in the production of food, residues in foods resulting from the use of pesticides can pose a potential health risk to consumers. The estimated annual retail value of pesticide sales within the United States alone is in excess of 5 billion dollars (U.S. Department of Commerce statistics, 1985). Government agencies responsibile for the protection of the public health from possible adverse effects of pesticide residues in foods face a formidable task of monitoring and regulating affected commodities. Residues of pesticides and their metabolites can find their way into the food chain from several sources; the purposeful application of pesticides on crops to control insect, weed, and fungal infestations is the most common. Another is the indirect contamination of food with pesticides that persist in the environment.

The U.S. Food and Drug Administration (FDA) is responsible for ensuring that food and feed commodities shipped in U.S. interstate commerce (including imported comodities) comply with the pesticide tolerances established by the U.S. Environmental Protection Agency (EPA). FDA carries out this responsibility by conducting an ongoing pesticide surveillance program, the primary objective of which is to sample and analyze food and feed shipments for residues and to restrain from commerce those shipments found to contain pesticide residues of regulatory significance (i.e., residues that exceed a tolerance or for which there is no tolerance). Restraint from commerce is accomplished through various mechanisms: voluntary destruction, FDA regulatory action (seizure and injunction), or regulatory action by state and local agencies. A second objective of the FDA surveillance program is to obtain data on the levels and incidences of pesticide residues found in food and feed.

FDA has a network of district offices located throughout the nation charged with implementation of this pesticide surveillance program. The pesticide monitoring responsibilities of the FDA Los Angeles District extend to food and feed produced in and shipped from Arizona and southern California as well as food and feed imported into the United States at ports of entry located within this geographic area. In the Los Angeles District, a pesticide coordination team (Figure 1) composed of investigators (sample collection), chemists (sample analysis), and compliance officers (legal processing) provides the selected sampling plan based on current information about the types of crops, their production volume, their harvest times, the pesticides used, and the potential pest problems.

To accomplish the complex task of monitoring the billions of pounds of food, mainly fresh agricultural produce, for a wide range of pesticides, the Los Angeles District has played a key role in developing the analytical methods (1; FDA internal memorandum, 1984). Analytical methodologies were developed by Luke et al. (2, 3) to provide rapid procedures for simultaneous analysis of several hundred pesticides. Analysis typically takes about 2 h per sample and provides an extract that can contain residues ranging from the highly polar pesticides (e.g., methamidophos) to the nonpolar ones (e.g., DDT). Optional determinations of the resultant extracts by a variety of analytical techniques such as liquid chromatography, derivative formation, and gas chromatography/mass spectrometry (GC/MS) can further extend the number of residues detected for a minimum of additional time.

To enhance this procedure, an analytical team is used to expedite the preparation, extraction, and determination of the samples. With current resources, a team of 6 analysts can completely process 50–80 samples within the normal working day. The sample tracking and reporting requirements of the basic FDA program were redesigned to minimize the time spent on paperwork. Computer software to track the samples collected and analyzed and residues found was part of the newly designed system. Data summaries allow easy evaluation by the pesticide coordination team to redirect program resources and coverage as required (Figure 1) and to make available to district management pertinent information to provide program oversight.

For fresh produce, one carton of produce is usually collected as the surveillance sample. Because of this sampling approach, a single investigator can collect and deliver 20–30 samples per day for analysis.

Using the overall approach outlined above, the Los Angeles District Office has collected and analyzed about 20 000 samples in the 5 year period from October 1981 to September 1986.

This paper reports the findings of pesticide residues in a wide variety of domestic and imported food and feed samples examined during that time period; a single multiresidue method was used (a modified extraction procedure was used for analyzing fish samples [4]). The paper indicates those residue findings that were in violation of the U.S. Federal Food, Drug, and Cosmetic Act (FFDCA).

Experimental

Sample Preparation, Extraction, and Analysis

(a) *High moisture products (lettuce, fruits, etc.).*—Sample preparation as described in the Code of Federal Regulations, 40 CFR 180.1(j), was used. The extraction procedure employed has been described in detail elsewhere (2, 3, 5).

For the most part, determinations were performed by gas chromatographic (GC) systems equipped with flame photometric detectors (FPD), Hall electrolytic conductivity detectors (HECD) Model 700A, and various polar and nonpolar packed columns as described previously (2, 3, 5).

Received April 24, 1986. Resubmitted August 9, 1986. Accepted June 5, 1987.

Cyhexatin was analyzed as a bromine derivative, using an electron capture detector in the constant current mode (6).

Captan, folpet, and captafol were analyzed by a liquid chromatographic (LC) system equipped with a Tracor 965 photoconductivity detector (PCD). For these determinations, the extract (or portion) provided by the general scheme was evaporated to dryness under a stream of dry nitrogen and then diluted with acetonitrile (UV grade with absorbance cutoff at 188 nm) and injected onto a 15 cm ODS column; the mobile solvent system was acetonitrile–water (50 + 50) (S. Gonzales, FDA, private communication).

Aldicarb sulfoxide was analyzed with an HECD in the nitrogen mode, a 2% DEGS column (122 cm × 2 mm i.d.) set at 110°C, and also the postcolumn derivatization LC system previously described by Krause (7). The extract provided by the general scheme was evaporated to dryness at 37°C under dry nitrogen and diluted with 0.5 mL methanol; a 0.4 mL portion was then eluted through a C-18 Sep-Pak cartridge with acetonitrile–water (50 + 50) (W. Langham, FDA, private communication) to provide a final volume of 0.5 mL which was then filtered and injected onto the LC system.

(b) *Dry products (grains, hay, cereals, etc.).*—Bulk products were first reduced by grinding to pass a 20 mesh sieve. A portion was extracted for pesticide screening by the modification described by Luke and Doose (3); where necessary, samples were cleaned up by carbon and Florisil chromatography.

(c) *Products with high fat content.*—Fish samples were prepared by removing inedible bone, scales, skin, fins, and head before grinding in a Hobart food chopper. Then, 15 g sample was extracted by the alumina blending method of Luke and Doose (4).

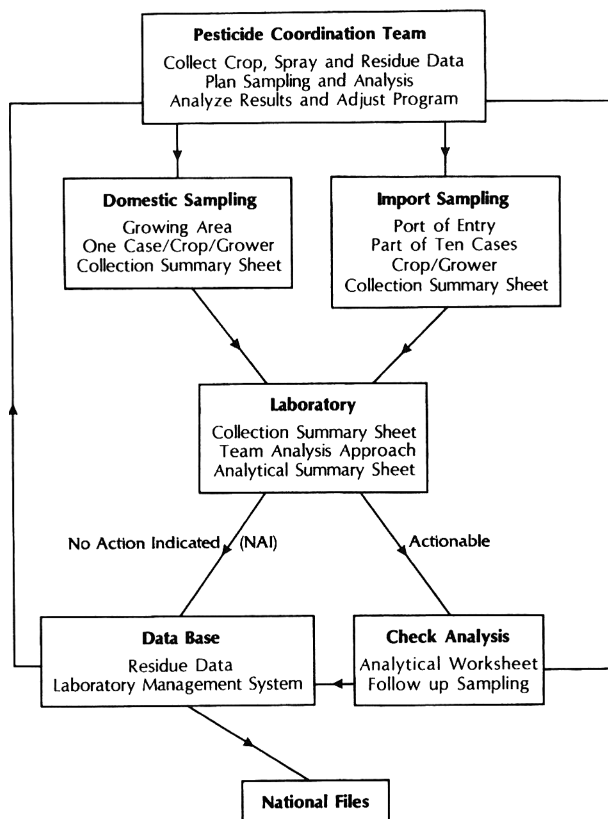


Figure 1. Flow diagram illustrating relationships among operational functions of the pesticide surveillance program.

Results and Discussion

The pesticide residues listed in Table 1 and the findings by commodity listed in Appendix 1 represent the results obtained by the FDA Los Angeles District using the methods described in carrying out its part of the agency's pesticide surveillance program. This database is a product of selective sampling as well as selective analyses. The samples were not necessarily collected at random but were selected on the basis of residue history, current pest problems, and harvest dates. This selection process provided samples most likely to contain pesticide residues. The analyses were also selective in that each extract was examined by using a limited number of GC detection systems to detect residues anticipated or most likely to be present. Few samples were examined by all the systems available and some were examined for only one pesticide. While only 85 different pesticides residues were detected during this 5 year period, the Luke procedure had the demonstrated ability (8) to recover and detect an additional 149 pesticide residues (Table 2).

The use of the Luke et al. procedure (2) to replace several traditional multiresidue methods has proven by experience in the Los Angeles laboratory and throughout this 5 year study to be an effective addition to the pesticide residue program. The data accumulated during this period illustrate the wide range of pesticides that are detected by this single, multiresidue approach as well as the wide range of commodities on which they were found. Residues levels have been underlined in Appendix 1 where the pesticide level detected did not comply (at the time of analysis/detection) with the tolerances established by EPA or was considered of regulatory significance (i.e., residue for which there was no tolerance or permitted exemptions). The absence of EPA registration(s) does not necessarily render residues of those pesticides as "illegal."

For regulatory analysis of pesticides, it was sometimes necessary to confirm the presence of a particular contaminant by mass spectrometry before initiating regulatory action. The added confirmation by GC/MS analysis was required to provide unambiguous proof of the violation. The unique role that mass spectrometry can play in supporting the scientific evidence from this single multiresidue protocol used for regulatory purposes in this laboratory has been reported elsewhere (9, 10).

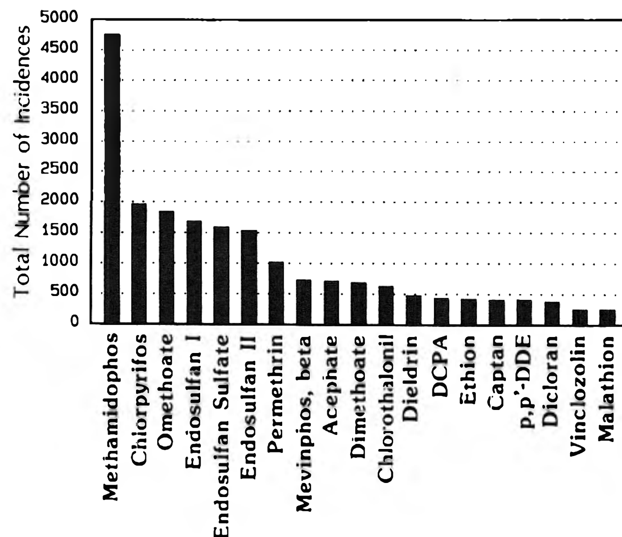


Figure 2. Total incidence levels for the most commonly encountered pesticides and/or metabolites in 19 851 samples of various agricultural commodities examined.

Table 1. Summary of pesticide residue findings on agricultural commodities, October 1, 1981–September 30, 1986

Pesticide	No. of findings in various ppm ranges[a]						
	0.05	0.10	0.50	1.0	2.0	>2.0	Total
Acephate	234	111	287	55	12	6	705
Aldicarb sulfoxide			7	3			10
Anilazine	4	3	1	2	1	[b]	11
Azinphos-methyl	12	14	33	6	3		68
BHC	3		1	1			5
BHC, alpha isomer	29	9	6				44
BHC, beta isomer	52	1					53
BHC, delta isomer	17	1	1				19
Captafol		1		2	1	2	6
Captan	47	50	112	77	45	76	407
Carbaryl	8	20	78	29	14	15	164
Carbophenothion			1				1
Chlorobenzilate				1	1		2
Chlorothalonil	165	113	237	64	30	18	627
Chlorpyrifos	1282	349	327	7	1	3	1969
Chlorthiophos	5	6	5				16
Cyhexatin		11	49	27	26	29	139
Cypermethrin		1	1				2
DCPA	219	90	123	6	1	1	440
DDE	15	8	5	1			29
DDE, *o,p'*-	1						1
DDE, *p,p'*-	288	68	47		1		408
DDT	5			1			6
DDT, *o,p'*-	3	3	1				7
DDT, *p,p'*-	9	4	7	1			21
DEF		1	4	2		4	11
Demeton-S-sulfone	16	12	22	4			54
Diazinon	71	30	29	2			132
Dichlofluanid	1		2		3	2	8
Dicloran	70	53	155	58	26	22	384
Dicofol	1	1	11	2			15
Dieldrin	457	12					469
Dimethoate	319	117	192	29	20	8	685
Diphenyl 2-ethylhexyl phosphate	9	10	15	1		1	36
Diphenylamine				1	2		3
Disulfoton sulfone	15	17	13	2			47
Endosulfan I	1266	236	145	17	11	8	1683
Endosulfan II	1178	187	143	23	5	2	1538
Endosulfan sulfate	1234	174	150	19	8	4	1589
Endrin	182	11	4				197
Ethion	167	94	137	14	11	2	425
Ethyl carbamate	39	8	9				56
Fenvalerate	9	7	8	1			25
Folpet	25	16	60	48	33	41	223

Pesticide	No. of findings in various ppm ranges						
	0.05	0.10	0.50	1.0	2.0	>2.0	Total
Gardona		1	1				2
Heptachlor	3	1					4
Heptachlor epoxide	11	0	1				12
Hydroxy diazinon		1	1		1		3
Imazalil	1		10	10	24	11	56
Iprodione	7	7	42	25	21	10	111
Lindane	22	2					24
Linuron	3	8	19				30
Malathion	78	34	92	18	12	15	249
Malathion oxygen analog			1	1			2
Metalaxyl		1	1	1	1		4
Methamidophos	2665	860	1059	129	30	21	4764
Methidathion	1	1	6	1	1		10
Methomyl	6	12	65	29	10	4	126
Methoxychlor					1	1	2
Methyl parathion	50	20	19	3	2		94
Mevinphos	39	15	16	3			73
Mevinphos, alpha	22	13	46	14	6	2	104
Mevinphos, beta	251	128	289	44	8	1	721
Monocrotophos	12	18	21	5			57
Omethoate	779	396	552	60	32	13	1832
Parathion	68	15	25	2	5	2	119
Paraoxon	1		2				3
Pentachloroaniline	7	1	3				11
Permethrin	309	155	283	104	51	17	919
Perthane				1	1		2
Phosalone	4	3	2		1		10
Phosmet	4	3	21	15	13	18	74
Pirimicarb	1	3					4
Pirimiphos-methyl	7	3	10	1		2	23
Procymidone	79	48	49		1		177
Pronamide	5	3	8	1			17
Quintozene	50	12	15	9	2		88
TDE, *p,p'*-	2	2					4
Tetradifon	3	3					6
Thiabendazole	6	1	22	20	39	57	146
Toxaphene		1	8	3		2	14
Triadimefon	6	2	1				9
Triadimenol	4	2	4				10
Trifluralin		2	1	1			4
Vinclozolin	17	20	84	67	50	19	257

[a] The count given in a column covers a range that starts at a value greater than the previous column heading (in ppm) and includes the given column heading.
[b] Blanks = none detected.

Table 2. Pesticides known to be recovered by the Luke procedure but not detected in the 19 851 samples analyzed during the period October 1, 1981–September 30, 1986

Pesticide	Pesticide
Alachlor	Isopropalin
Aldrin	Kelevan
Ametryn	Leptophos
Amitraz	Leptophos oxygen analog
Atrazine	Leptophos photo product
Azinphos-ethyl	Mecarbam
Azinphos-methyl oxygen analog	Mephosfolan
Bensulide	Methiocarb
Biphenyl	Methiocarb sulfone
Bromacil	Methiocarb sulfoxide
Bromophos	Methyl trithion
Bromophos-ethyl	Methyl-4-chloroindolyl-3-acetate
Bromopropylate	Metribuzin
Butralin	Mirex
Carbofuran	Myclobutanil naled
Carbophenothion sulfone	Napropamide
Carboxin	Nitrofen
Chlorbenside	Nonachlor, cis
Chlordane	Nonachlor, trans
Chlordane, cis	Norea
Chlordecone	Norflurazon
Chlordimeform	*o*-Phenylphenol
Chlorfenvinphos	Octachlor epoxide
Chlorfenvinphos, alpha	Oxadiazon
Chlorfenvinphos, beta	Oxamyl
Chloro-3-methyl-4-nitro-1H-pyrazole	Oxydemeton-methyl
Chlorothalonil trichloro impurity	Oxydemeton-methyl sulfone
Chlorpyrifos-methyl	Oxythioquinox
Chlorthiophos	Parathion-methyl
Cyanazine	Pendimethalin
Cycloate	Pentachlorobenzonitrile
4-Cyclohexene-1,2-dicarboximide	Phenthoate
DCPA	Phorate
Deltamethrin	Phorate sulfone
Demeton-S	Phorate sulfoxide
Des N-isopropyl isofenphos	Phosphamidon
Dialifor	Phoxim
3,4-Dichloroaniline	Phoxim oxygen analog
3,5-Dichloroaniline	Phthalimide
2,6-Dichlorobenzamide	Pirimiphos-ethyl
3-(3, 4-Dichlorophenyl)-1-methoxyurea	Pirimiphos-ethyl oxygen analog
Dichlorvos	Polychlorinated biphenyls
Diclofop-methyl	Profenophos
Dicrotophos	Profluralin
Dimethazone	Prometryn
Dinocap	Propanil
Dioxathion	Propargite
Diphenamid	Propham
Ditalimfos	Propoxur
Diuron	Pyrazon
EPN	Pyrazophos
Ethion oxygen analog	Pyrimidinol
Ethoprop	Ronnel
Ethoxyquin	Schradan
Ethyl p-toluene sulfonamide	Simazine
Ethylene thiourea	Sulfallate
Etrimfos	Sulfotep
Famphur	Sulprofos
Famphur oxygen analog	Sulprofos sulfone
Fenamiphos	Sulprofos sulfoxide
Fenarimol	TDE, *p,p'*-, olefin
Fenbutatin-oxide	Terbacil
Fenitrothion	Terbufos
Fenitrothion oxygen analog	Tetrahydrophthalimide
Fensulfothion	Thionazin
Fenthion	Triallate
Fenthion oxygen analog	Triazophos
Fenthion oxygen analog sulfoxide	2,4,5-Trichloro-alpha-methylbenzene-methanol
Fenthion sulfone	Trichlorfon
Fluchloralin	Triforine
Flucythrinate	Trimethacarb
Fonofos	Triphenyl phosphate
Iprodione isomeric metabolite	Tris(beta-chloroethyl) phosphate
Isofenphos	

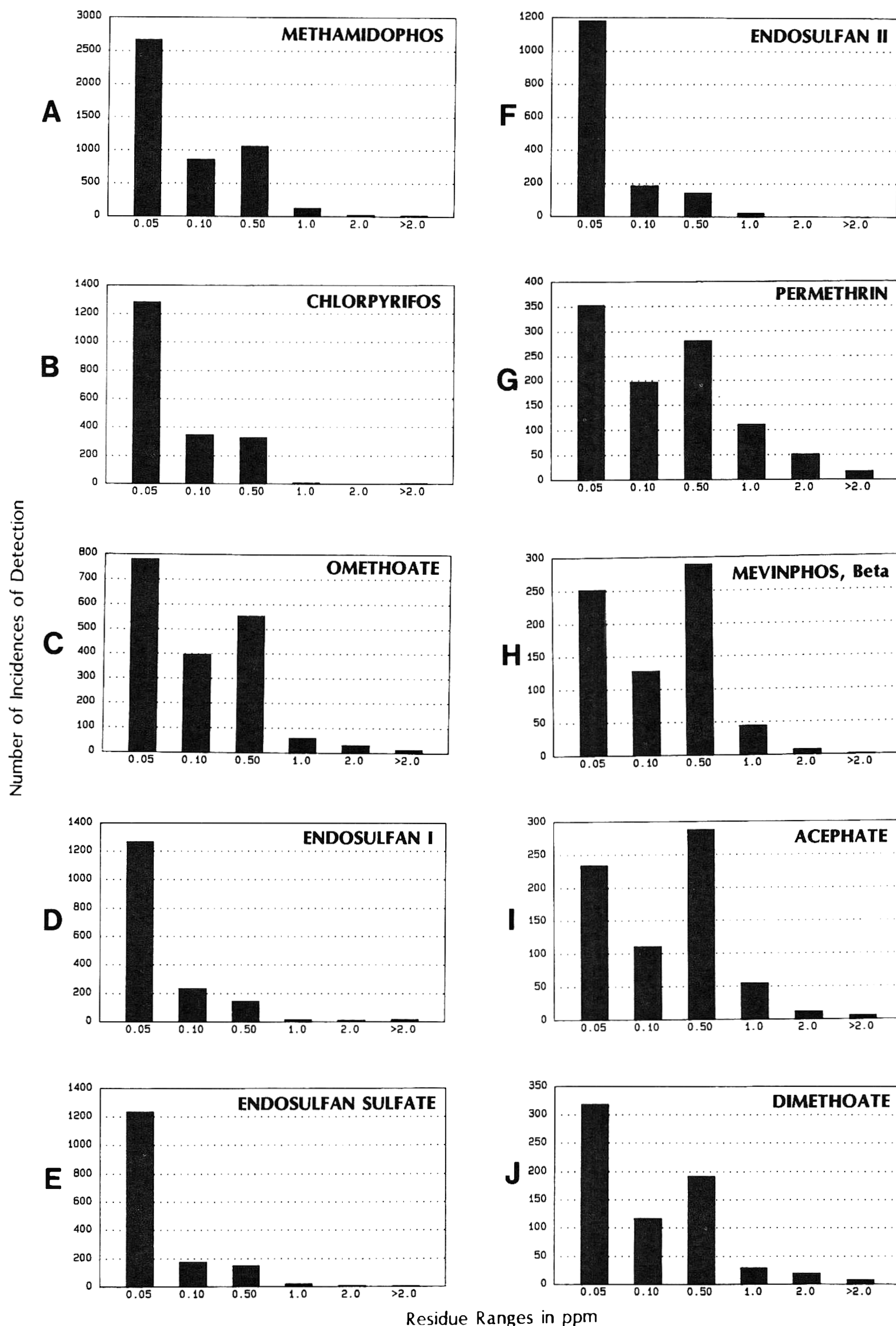


Figure 3. Distribution of findings and residue levels of the 10 pesticides and/or metabolites most commonly found in agricultural commodities.

The most frequently encountered pesticide residues found in the 19 851 samples examined during the 5 year period are listed in Figures 2 and 3. Additional information to aid in understanding these data is appropriate. Because the oxygen analog of dimethoate (omethoate) may occur as a residue in food from the use of dimethoate or from the direct use of omethoate, which is a pesticide in its own right, it would be incorrect to categorize all findings of this compound as a "commonly encountered pesticide," because the oxygen analog of dimethoate is not always a pesticide per se and should not be automatically counted as such. Instead, when it occurs as a metabolite of dimethoate, the amount of oxygen analog present should be added to the amount of the parent compound to determine the total residues of dimethoate present in the sample. If the oxygen analog is present as a residue from the use of omethoate, then reporting such findings as an "incidence" would be correct. The reporting scheme adopted for these 2 pesticides has been to separate them to provide raw data rather than cumulative. A similar problem exists with methamidophos; this compound can be present in food as a pesticide or as a metabolite from the pesticide acephate. Similarly for regulatory purposes, the levels of en-

dosulfan and its metabolites and DDT and its metabolites should be added together to obtain the total residues of parent compound or similarly related chemical.

Because of the approach outlined above, the incidence information was biased and did not represent a true picture of the frequency of individual pesticides occurring in the sampled domestic and imported agricultural commodities. This bias may be magnified by the fact that some of the 19 851 samples were commodities collected on a compliance basis, which represented intensified sampling of a commodity suspected of containing a specific illegal pesticide residue. In a future paper, the data on domestic and imported foods will be reported separately. Also, the bias introduced by combining compliance and surveillance samples will be eliminated by assessing these data separately.

Conclusions

The residue findings reported in this paper demonstrate the importance of using multiresidue analytical methods for meeting the agency's responsibility of assuring that pesticide residues in food and feed comply with federal laws and regulations. It is not unusual or unexpected that food and feed commodities may contain residues of more than one pesticide. The majority of the violative samples (75%) listed in Appendix 1 were violative because no tolerances are established for the particular pesticide-commodity combination. Therefore, using a method that can detect only a single residue would obviously not show the presence of residues of other pesticides. Also, because such methods are primarily intended to measure a specific pesticide residue in a food or feed subject to a tolerance, they would not detect the kinds of violative residues most frequently encountered by FDA (i.e., residues not permitted by tolerances). The use of multiresidue methods gives FDA the capability to deal with both of these residue problems.

The multiresidue procedure described in this paper is especially useful because, as shown in Table 1, it is capable of detecting a relatively large number of pesticides of different chemical classes in fruits, vegetables, and other types of food and feeds. It also provides a mechanism for comprehensive monitoring of the levels and incidences of various pesticides being used in agriculture as well as those pesticides that can occur in the food supply from environmental sources of contamination.

Acknowledgments

The authors gratefully acknowledge the analysts who have worked as members of the FDA Los Angeles District Laboratory pesticide analytical group (Marie A. Bennett, Ken S. Chiu, Gregory M. Doose, Jerry E. Froberg, Steve Gonzales, Theron James, David Kodama, William S. Langham, Emil S. Siegmund, and Dorothy J. Wade) for their efforts in obtaining the data reported here and also Raymond Nelson of the district investigations branch for coordinating the collection of samples.

References

(1) *Pesticide Analytical Manual* (1982) U.S. Food and Drug Administration, Washington, DC
(2) Luke, M. A., Froberg, J. E., Doose, G. M., & Masumoto, H. T. (1981) *J. Assoc. Off. Anal. Chem.* **64,** 1187–1195
(3) Luke, M. A., & Doose, G. M. (1983) *Bull. Environ. Contamin. Toxicol.* **30,** 110–116
(4) Luke, M. A., & Doose, G. M. (1984) *Bull. Environ. Contamin. Toxicol.* **32,** 651–656
(5) "*Changes in Official Methods of Analysis*" (1985) 1st Supplement, 14th Ed., AOAC, Arlington, VA, secs **29.A01-29.A04**
(6) James, T., & Langham, W. (1985) *Laboratory Information Bulletin,* No. 2292, U.S. Food and Drug Administration, Washington, DC
(7) Krause, R. T. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 1114–1124
(8) Sawyer, L. D. (1985) *J. Assoc. Off. Anal. Chem.* **68,** 64–71
(9) Cairns, T., Siegmund, E. G., Doose, G. M., & Oken, A. C. (1985) *Anal. Chem.* **57,** 572A–576A
(10) Cairns, T., Siegmund, E. G., Jacobson, R. A., Barry, T., Petzinger, G., Morris, W., & Heikes, D. (1983) *Biomed. Mass Spectrom.* **10,** 301–315

Appendix 1. Pesticide residues on 19851 samples, listed by various commodities, found with the Luke multiresidue methodology, October 1, 1981, to September 30, 1986

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a] 0.05	0.10	0.50	1.0	2.0	>2.0	Total
Alfalfa sprouts (1)							
Apple (54)							
Acephate	1						1
Azinphos-methyl	2	3	2				7
Captan	1	3	3	3			10
Carbaryl			3				3
Chlorpyrifos	4	2					6
Cyhexatin		1					1
Diazinon	2						2
DDT, p,p'-			1				1
DDT, o,p'-	1						1
Dimethoate		1					1
Diphenylamine				1	2		3
Ethion			1				1
Malathion			1				1
Methamidophos	1						1
Omethoate	2						2
Parathion	3						3
Phosmet			1				1
Tetradifon	3	3					6
Thiabendazole			2				2
Vinclozolin				2[b]	1[b]		3
Apple juice conc. (7)							
Dimethoate	1						1
Omethoate	1						1
Apricot (6)							
Azinphos-methyl		1					1
Captan			1				1
Carbaryl			1				1
Iprodione			1				1
Vinclozolin		1					1
Apricot, dried (1)							
Arrowroot (2)							
Artichoke (9)							
Endosulfan I		1					1
Endosulfan II		1					1
Endosulfan sulfate	1						1
Mevinophos	1						1
Quintozene	1						1
Asparagus (74)							
DCPA		1					1
Methamidophos	1						1
Avocado (13)							
Babaco (2)							
Banana (82)							
Thiabendazole				1			1
Basil, whole (7)							
Pirimiphos-methyl	3	1					4
Bean, Chinese (18)							
Dimethoate	1				2		3
Endosulfan I	2		1				3
Endosulfan II	1	1	1				3
Endosulfan sulfate	1	1	1				3
Malathion			1				1
Methamidophos	1		1				2
Monocrotophos					1		1
Omethoate	1	1	2	1			5
Bean, fava (11)							
DDE, p,p'-	1						1
Methamidophos	3					1	4
Quintozene		1					1
Bean, French (3)							
Bean, garbanzo (12)							
Diazinon	2						2
Bean, green (496)							
Acephate	4	2	8	1			15
BHC, alpha	2						2
Captan	2		1				3
Carbaryl		1	5	2			8
Chlorothalonil	5		4	1			10
Chlorpyrifos	8	2	3				13
DCPA	10	1	5				16
Demeton-S-sulfone		2					2
Diazinon	1						1
Dicloran	2	1	1		1		5
Dieldrin	1						1
Dimethoate	13	9	18	2	1		43
Endosulfan I	47	10	8				65
Endosulfan II	49	9	3				61
Endosulfan sulfate	40	15	8				63
Ethion			1				1
Folpet			1	1			2
Heptachlor	1						1
Heptachlor epoxide			1				1
Malathion	1						1
Methamidophos	42	10	7	1	2		62
Omethoate	40	27	31	4	7	2	111
Parathion	1						1
Pentachloroaniline	1						1
Quintozene	10	6	4	3			23
Bean, lima (9)							
Acephate			1				1
Malathion					2		2
Methamidophos		1					1
Bean, miscellaneous (2)							
Bean, mung (12)							
BHC, alpha	2						2
BHC, beta	2						2
BHC, delta	1						1
Dieldrin	1						1
Endrin	4	2	2				8
Pirimiphos-methyl			1				1
Bean, pinktos dry (5)							
Diazinon	2	1					3
Bean, pinto (1)							
Bean, red dry (1)							
Bean, white (2)							
Beer (22)							
Beets (40)							
DCPA	2						2
DDE, p,p'-	8						8
Dieldrin	1						1
Methamidophos	3			1			4
Mevinphos, alpha			1				1
Mevinphos, beta	1		1				2
Omethoate				1	1		2
Blackberry (50)							
Captan	1	1	2	3	6	9	22
Dichlorfluanid	1		1				2
Dicofol	1						1
Iprodione	2	3	5	3	4	8	25
Procymidone			1				1
Vinclozolin						1	1

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Blueberry (34)							
Captan	3	6	8	4			21
DDT, o,p'-	1						1
DDT, p,p'-			1				1
Dicloran		1					1
Iprodione		1	1		1		3
Bok choy (55)							
DCPA	1	2					3
DDE	1						1
DDE, p,p'-	1						1
Endosulfan I	4	3	5				12
Endosulfan II	4	1	4	2			11
Endosulfan sulfate	4	1	6	3			14
Folpet			1				1
Permethrin				1	1	1	3
Borage, flower (1)							
Boysenberry (4)							
Captan				1	2		3
Folpet					1		1
Broccoli (315)							
Acephate	10	1					11
BHC, alpha		1					1
Chlorothalonil		1					1
DCPA	32	5					37
DDE	1						1
Demeton-S-sulfone	1		3				4
Diazinon	1		1				2
Disulfoton sulfone		3	1				4
Endosulfan I	1		1				2
Endosulfan II	1		1				2
Endosulfan sulfate	1		1				2
Fenvalerate		1					1
Methamidophos	1	1				1	3
Omethoate		1	2				3
Quintozene					1		1
Broccoli, Chinese (2)							
DCPA		1					1
Brussels sprouts (144)							
Acephate			1				1
Dimethoate	1	1	1				3
Endosulfan I	1	1	1	1			4
Endosulfan II		2	2				4
Endosulfan sulfate	4						4
Methamidophos			3		1		4
Omethoate	1	2	1	1			5
Parathion	1		3				4
Permethrin			1				1
Quintozene	7		5	2			14
Cabbage (146)							
Demeton-S-sulfone	1						1
Dimethoate	2	1	1	1			5
Disulfotan sulfone			1				1
Endosulfan I	4	1					5
Endosulfan II	2	2	1				5
Endosulfan sulfate	4	1	1				6
Iprodione			1				1
Malathion	1						1
Methamidophos	1		2				3
Omethoate	3		3	1	1		8
Permethrin	1		1				2
Endosulfan I			2				2
Endosulfan II			2				2
Endosulfan sulfate			2				2
Methyl parathion			1				1
Parathion			1				1
Cabbage, Napa (87)							
Chlorpyrifos	1						1
Dimethoate	1						1
Endosulfan I	2	1	5				8
Cabbage, Napa (continued)							
Endosulfan II	2		3	2			7
Endosulfan sulfate	1	1	5	1			8
Methamidophos	5	2	1				8
Omethoate	1						1
Cabbage, red (74)							
Acephate			2				2
DDE, p,p'-	2						2
Endosulfan I	1						1
Endosulfan II	1						1
Endosulfan sulfate	1						1
Folpet			1				1
Methamidophos	2						2
Omethoate		1					1
Permethrin	1	1					2
Cabbage, savoy (2)							
Cactus (8)							
Omethoate	1						1
Cactus fruit (4)							
Carrot (142)							
Carbaryl			1				1
Chlorothalonil		1					1
DCPA	1	1					2
DDE			1	1			2
DDE, o,p'-	1						1
DDE, p,p'-	29	19	19		1		68
DDT, o,p'-		1					1
DDT, p,p'-	2		1				3
Dicloran			1	1	1		3
Endosulfan I	6	1					7
Endosulfan II	5	2					7
Endosulfan sulfate	7						7
Linuron	3	7	19				29
Parathion	2						2
Pentachloroaniline		1					1
TDE, p,p'-	1	1					2
Toxaphene			3				3
Trifluralin		2	1	1			4
Cassava (1)							
Cauliflower (281)							
Acephate	3	1					4
DCPA	1						1
Dimethoate	1		1				2
Endosulfan I	1	1					2
Endosulfan II	1	1					2
Endosulfan sulfate	1						1
Methamidophos	2						2
Omethoate	2						2
Celery (265)							
Acephate	20	8	33	10	1		72
Anilazine	4	3	1	2	1		11
BHC, alpha	1						1
Captan		1					1
Chlorothalonil	13	26	75	42	21	11	188
Diazinon	8	3	3	1			15
Dicloran	16	16	72	26	15	7	152
Endosulfan I	10	11	7	1	2	1	32
Endosulfan II	11	6	5	1			23
Endosulfan sulfate	16	3	3				22
Folpet	2		1	1			4
Malathion			1				1
Methamidophos	40	2	1				43
Methyl parathion	8	4	2	1	1		16
Omethoate		1					1
Parathion	1	1			1		3
Permethrin	11	18	21	1			51
Chayote (55)							
Dimethoate	1						1

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Chayote (continued)							
Methamidophos	1	1					2
Monocrotophos				1			1
Omethoate	1						1
Thiabendazole			1	4	1	1	7
Cherry (9)							
Dicloran				2			2
Iprodione			2	2	1		5
Cherry, dried (1)							
Diazinon			1				1
Choy sum (1)							
Cider, carbonated (1)							
Citrus, grapefruit (81)							
Acephate	1						1
Dimethoate	1						1
Ethion	2						2
Imazalil	1		3	4	8	4	20
Methamidophos	1						1
Methidathion			1				1
Omethoate	1						1
Parathion	1						1
Thiabendazole			2	8	18	20	48
Citrus, grapefruit juice (1)							
Citrus, lemon (30)							
Carbaryl					1		1
Dicloran		4					4
Imazalil			1	2	5	3	11
Parathion	3	1	1				5
Thiabendazole				3	8	4	15
Citrus, lime (12)							
Chlorobenzilate					1		1
Diazinon	1						1
Ethion	1						1
Malathion				1			1
Thiabendazole				1		1	2
Citrus, mandarin orange (16)							
Imazalil			1			1	2
Parathion			1				1
Thiabendazole					2	9	11
Citrus, orange (67)							
Carbaryl		1		1			2
Dimethoate		2	4				6
Imazalil			5	4	8	3	20
Malathion	1		4				5
Methidathion	1	1	5		1		8
Methyl parathion	1		5				6
Omethoate	6						6
Parathion	1		1				2
Thiabendazole	1			1	4	11	17
Citrus, orange juice (2)							
Diphenyl 2-ethylhexyl phosphate		1					1
Citrus, orange conc. (1)							
Citrus, tangello (1)							
Chlorobenzilate				1			1
Imazalil					1		1
Citrus, tangerine (14)							
Imazalil					1		1
Methyl parathion		1					1
Thiabendazole			1		1	7	9
Coconut (3)							
Collard (87)							
DCPA	8	16	21				45
DDE		1	1				2
DDE, p,p'-	19	4	1				24
Diazinon	1						1
Dimethoate		2					2
Endosulfan I	2	1		2	1		6
Endosulfan II	3		1	1	1		6
Endosulfan sulfate			2		2	1	5
Mevinphos, alpha	1		5	3	2		11
Mevinphos, beta	1	2	5	4	2		14
Omethoate	2						2
Permethrin					2	6	8
Coriander (205)							
Acephate	1						1
Chlorothalonil			1				1
Chlorpyrifos			3				3
DCPA			2	1			3
DDE	5	1					6
DDE, p,p'-	10	2	1				13
DDT	3						3
DDT, o,p'-	1						1
DDT, p,p'-	3	2					5
Endosulfan I			2		1	1	4
Endosulfan II			2	1			3
Endosulfan sulfate			2		1		3
Malathion		2	1				3
Methamidophos	11	2	4	2	1		20
Methyl parathion	1			1			2
Monocrotophos	2						2
Omethoate			1				1
Parathion	1			1			2
Pentachloroaniline	2		1				3
Quintozene	3	1			1		5
Corn, dry (12)							
Malathion			1				1
Corn, sweet (43)							
Cucumber (1200)							
Acephate	4	4	6	1			15
BHC	1						1
BHC, alpha	3						3
BHC, beta	31						31
BHC, delta	1						1
Captan	1		1				2
Chlorothalonil	3	1	2				6
Chlorpyrifos	190	52	52				294
DCPA	1						1
DDE, p,p'-	4						4
Diazinon	6	5	4				15
Dicloran	5	8	16	3			32
Dieldrin	231	6					237
Dimethoate	24	9	7				40
Endosulfan I	261	26	7				294
Endosulfan II	254	16	3				273
Endosulfan sulfate	255	7	2				264
Endrin	8						8
Ethion	18	10	18				46
Folpet	1						1
Heptachlor epoxide	4						4
Lindane	1						1
Methamidophos	280	211	208	1			701
Omethoate	58	16	20	2			96
Parathion	1						1
Thiabendazole	2		4		1	3	10
Cucumber, Chinese (4)							
Omethoate		1					1
Cucumber, European (6)							
Captan		1					1
Dieldrin	1						1

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Cucumber, European (continued)							
Quintozene	1						1
Cucumber, pepino (1)							
Cucumber, pickling (126)							
Captan	1						1
Chlorothalonil		1	1		1		3
Chlorpyrifos	20	4	2				26
DDE, p,p'-	1						1
Dieldrin	15	1					16
Endosulfan I	20	2					22
Endosulfan II	21	1					22
Endosulfan sulfate	23						23
Endrin	2						2
Ethion	1	1	1				3
Methamidophos	25	20	24				69
Monocrotophos		2					2
Omethoate	13	2	2				17
Thiabendazole	2		2				4
Currants (3)							
Folpet		1					1
Iprodione					1		1
Pirimicarb		1					1
Dandelion (2)							
Date (3)							
Malathion	1		1				2
Parathion	1						1
Eggplant (415)							
Acephate	1	1	3				5
Chlorpyrifos	4						4
DCPA	1						1
Dicloran	4	1	2				7
Dimethoate	1	1					2
Endosulfan I	9	2	1				12
Endosulfan II	9	1	2				12
Endosulfan sulfate	10	2					12
Ethion	12	7	12				31
Malathion	1	1					2
Methamidophos	159	47	32	1			239
Omethoate	8	4	9				21
Parathion		1					1
Eggplant, Japanese (47)							
Acephate	3	2	2				7
Dimethoate	3	1					4
Endosulfan I	1						1
Endosulfan II	1						1
Endosulfan sulfate	1						1
Ethion	2		5				7
Methamidophos	10	2	3				15
Omethoate	5		2	2			9
Endive (65)							
Chlorothalonil		1	1				2
DCPA			1				1
DDE	1						1
DDE, p,p'-	3	1					4
Demeton-S-sulfone	1						1
Diazinon	1						1
Dicloran			2				2
Dimethoate	3	2	4	2			11
Endosulfan I	5	3	2				10
Endosulfan II	4	2	2	1			9
Endosulfan sulfate	5	2	1	1			9
Malathion	1		1				2
Methamidophos	1						1
Methomyl			2				2
Methyl parathion		1					1
Mevinphos, beta	2	1	5	1			9
Omethoate	2	2	7				11
Pronamide	1						1
Escarole (27)							
Chlorothalonil	1	1					2
Dicloran	1			1			2
Dimethoate	2	1	1				4
Endosulfan I	1		1				2
Endosulfan II	1		1				2
Endosulfan sulfate	1		1				2
Malathion					1		1
Methyl parathion	1						1
Mevinphos, alpha			1				1
Mevinphos, beta	4	1	1				6
Omethoate	2	1	1				4
Parathion		1					1
Pronamide			1				1
Feed, almond hulls (8)							
Azinphos-methyl				1			1
Parathion	1						1
Phosmet				1			1
Feed, barley (7)							
Malathion		1					1
Feed, beet pulp (5)							
DDE, p,p'-		1					1
Feed, bran (1)							
Malathion						1	1
Feed, cattle (4)							
Malathion			2	1			3
Feed, citrus pulp (2)							
Azinphos-methyl					1		1
Methidathion				1			1
Parathion			1				1
Phosmet				1			1
Thiabendazole						1	1
Feed, corn (9)							
Malathion		1	2				3
Feed, cotton seed (1)							
Feed, cotton seed hull (3)							
DEF			2				2
Feed, cotton seed meal (2)							
Feed, dairy (7)							
Malathion		1	1		1		3
Feed, dog (3)							
Malathion			2			1	3
Feed, grain pellets (1)							
Feed, grain mixed (5)							
Malathion				2			2
Feed, hay alfalfa (77)							
Azinphos-methyl			1				1
Chlorpyrifos	4	1	3	2		3	13
DEF			2	2		4	8
Diazinon			1				1
Dimethoate		1		1	1		3
Endosulfan I		1	1				2
Endosulfan II		1	1				2
Endosulfan sulfate		1	1				2
Malathion			4			4	8
Methamidophos					1		1
Methyl parathion			2		1		3
Omethoate			1				1
Parathion	3		4	1	3	1	12
Permethrin			1				1
Phosmet						1	1
Pronamide				1			1

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Feed, hay alfalfa meal (1)							
Chlorpyrifos		1					1
Feed, hay sudan (1)							
Hay pellets (9)							
Malathion		1	3				4
Feed, kelp (1)							
Feed, meat & bone meal (1)							
Feed, mill run (1)							
Malathion					1		1
Feed, milo (13)							
Captan						1	1
Methoxychlor						1	1
Feed, oats (6)							
Chlorpyrifos		1					1
Malathion	1				1		2
Feed, orange peel (1)							
Imazalil					1		1
Feed, poultry w/fish (1)							
Permethrin			1				1
Feed, pig (7)							
Malathion		1	4				5
Feed, poultry (67)							
Chlorpyrifos	2	2	3				7
DDE, p,p'-	2						2
Malathion	1	5	18	6	1	2	33
Parathion			1				1
Feed, rape seed (1)							
Feed, safflower (1)							
Feed, sheep (1)							
Malathion		1					1
Feed, silage misc. (4)							
Feed, silage sorghum (2)							
DEF		1					1
Feed, soy bean (1)							
Feed, soy bean flakes (1)							
Feed, soy bean meal (3)							
Feed, tomato pomace (8)							
DDE, p,p'-		1	7				8
Dicofol		1	7				8
Feijoa (12)							
Chlorpyrifos	1	2					3
Diazinon	2	2					4
Fig (3)							
Captan	1	1					2
Fig, dry (1)							
Flour, mung bean (1)							
Garlic (4)							
Ginger root (4)							
Gooseberry (8)							
Captan	2						2

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Grape (1276)							
Azinphos-methyl	2	1	2				5
Captan	16	20	31	16	5	1	89
Carbaryl	1	4	15	3		3	26
DDE, p,p'-	2						2
Dimethoate	30	2	9				41
Endosulfan I		1					1
Endosulfan II		1					1
Ethion			1				1
Folpet	2	2	2			1	7
Iprodione	1	2	9	5	8	1	26
Methamidophos	4						4
Monocrotophos	2	1	3				6
Omethoate	107	56	74	3	1	1	242
Parathion	2						2
Phosalone	4	3	2		1		10
Phosmet			1				1
Procymidone	75	48	46				169
Triadimefon	6	2	1				9
Triadimenol	4	2	4				10
Vinclozolin	1						1
Grain, brewer's (1)							
Guava paste (1)							
Hops (27)							
Folpet						12	12
Hops, extract (2)							
Jicama (47)							
Kale (68)							
Acephate	1		1	2	1		5
DCPA	5	4	14				23
DDE		1					1
DDE, p,p'-	8	2					10
Dimethoate		1					1
Endosulfan I	3		4	1			8
Endosulfan II	3		1	2	1		7
Endosulfan sulfate	1		2	1	1		7
Folpet				1		1	2
Methamidophos	4	1					5
Mevinphos, alpha			3				3
Mevinphos, beta		2	3	2			7
Omethoate	1						1
Paraxon	1		2				3
Parathion			2		1	1	4
Permethrin			1				1
Kiwi fruit (95)							
Azinphos-methyl			1				1
Captan	2						2
Chlorpyrifos	7	2	13	2			24
Diazinon	11	9	5				25
Dicloran	1						1
Iprodione	1		9	10	5		25
Phosmet	3		14	14	12	17	60
Pirimiphos-methyl	4	1	7				12
Vinclozolin	5	3	15	17	6		46
Kohlrabi (2)							
DDE, p,p'-	1						1
Kumquat (2)							
Malathion			1				1
Lavender, flower (1)							
Chlorpyrifos			1				1
Leek (24)							
DCPA	1						1
DDE, p,p'-	2	2					4
Lettuce, Boston (68)							
Acephate			1	1			2

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Lettuce, Boston (continued)							
Chlorothalonil		3					3
DCPA	3	4					7
DDE, p,p'-	3						3
Demeton-S-sulfone				1			1
Dicloran	2						2
Dimethoate	2	2	2		1		7
Disulfoton sulfone			1				1
Endosulfan I	13	3	3				19
Endosulfan II	14	2	3				19
Endosulfan sulfate	13	4	2				19
Folpet		3	1	1		1	6
Methamidophos	1						1
Methomyl			2	1			3
Methyl parathion		1					1
Mevinphos				1			1
Mevinphos, alpha	3		1				4
Mevinphos, beta	4	6	10	3			23
Omethoate	3	1	1		1		6
Permethrin	1		1		1	1	4
Pronamide		1					1
Lettuce, butter (114)							
Acephate	1		1				2
Captan			1				1
Carbaryl						1	1
Chlorothalonil	2	3	2				7
DCPA	1						1
DDE	1	1					2
DDE, p,p'-	1						1
Demeton-S-sulfone	1		1				2
Diazinon		1					1
Dicloran			3				3
Dimethoate	4		2		1	2	9
Disulfoton sulfone							1
Endosulfan I	24	3	6	1			34
Endosulfan II	23	2	6				31
Endosulfan sulfate	18	6	6	1			31
Folpet			2				2
Hydroxy diazinon		1					1
Methamidophos	5	1					6
Methomyl			4	1	1		6
Mevinphos	2						2
Mevinphos, alpha		1	1				2
Mevinphos, beta	4	3	9	3			19
Omethoate	6		1	2			9
Permethrin	1	1	5		2		9
Pronamide		1	2				3
Lettuce, green leaf (266)							
Captan	1	1		1			3
Carbaryl				1			1
Chlorothalonil	4	3	6	1			14
Chlorpyrifos	5	1	2				8
DCPA	14		2				16
DDE, p,p'-	7	1					8
DDT, p,p'-		1					1
Demeton-S-sulfone	2	1	1				4
Diazinon	4	1					5
Dicloran	3	1	7				11
Dimethoate	5	4	5	3	1		18
Disulfoton sulfone	2	2					4
Endosulfan I	55	14	10	2		1	82
Endosulfan II	54	12	7	2			75
Endosulfan sulfate	56	9	8	1			74
Folpet	1		2		1	3	7
Malathion	1		1	1			3
Methamidophos	5						5
Methomyl		1	3	4	1	1	10
Mevinphos	2		2	1			5
Mevinphos, alpha	1		6				7
Mevinphos, beta	26	13	26	5		1	71
Omethoate	10	3	2				15
Permethrin	2	2	1	1	5	1	12
Pronamide	1	1	2				4
Lettuce, iceberg (1273)							
Acephate	91	28	40	8	2	1	170
Captan	6	2	14	8	6	5	41
Carbaryl			1			1	2
Chlorothalonil	3	6	3				12
Chlorpyrifos	16	4	1				21
DCPA	6	1					7
DDE, p,p'-	2						2
Demeton-S-sulfone	6	5	14				25
Diazinon	1	1					2
Dicloran	4	1	3	1			9
Dimethoate	67	25	49	4			145
Diphenyl 2-ethylhexyl phosphate	1						1
Disulfoton sulfone	6	9	9				24
Endosulfan I	168	31	29	5	3	1	236
Endosulfan II	147	35	27	5	2	1	217
Endosulfan sulfate	131	41	39	2	1		214
Fenvalerate		1	1				2
Folpet	11	6	32	25	19	11	104
Malathion	2		2				4
Metalaxyl			1				1
Methamidophos	98	4	1				103
Methomyl	6	9	38	13	7	2	75
Methoxychlor					1		1
Methyl parathion	2						2
Mevinphos	30	12	11	1			54
Mevinphos, alpha	8	3	3				14
Mevinphos, beta	155	68	156	10	1		390
Omethoate	84	33	23	1			141
Parathion	1		4				5
Permethrin	68	45	158	78	36	8	393
Perthane				1	1		2
Pronamide	3						3
Quintozene				1			1
Metalaxyl		1		1	1		3
Vinclozolin		1					1
Lettuce, red leaf (217)							
Acephate	2		1				3
Captan	1	2					3
Carbaryl			1				1
Chlorothalonil	6	2	7				15
DCPA	9	4	2				15
DDE		1					1
DDE, p,p'-	5	2					7
DDT, p,p'-	1						1
Demeton-S-sulfone	1	2					3
Diazinon	2						2
Dicloran	3						3
Dimethoate	8	3	6	1			18
Disulfoton sulfone	2	1					3
Endosulfan I	54	7	7		1		69
Endosulfan II	52	5	5		1		63
Endosulfan sulfate	45	11	5				61
Folpet	4	1	1	3		2	11
Methamidophos	4	1					5
Methomyl			6	4			10
Mevinphos	3	1					4
Mevinphos, alpha	2	1	1				4
Mevinphos, beta	21	7	18	2	1		49
Omethoate	10	2	5		1		18
Permethrin	1	1	2	5	1	1	11
Pronamide			3				3
Quintozene	1						1
Lettuce, romaine (201)							
Acephate	3	3	1				7
Captan		1		1			2
Chlorothalonil	3	3	7	1			14
Chlorpyrifos	2						2
DCPA	3	2					5
DDE, p,p'-	2						2
Demeton-S-sulfone	2	1	2	2			7
Diazinon	1		1				2
Dicloran	1	3	3			1	8
Dimethoate	4	2	4	2			12

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Lettuce, romaine (continued)							
Disulfoton sulfone	3	1		2			6
Diphenyl 2-ethyl-hexyl phosphate	1						1
Endosulfan I	97	7	3			1	50
Endosulfan II	38	4	4			1	47
Endosulfan sulfate	29	8	8	1			46
Ethion	1						1
Folpet	1	1			1	2	5
Methamidophos	3	1	1				5
Methomyl		2	5	2			9
Mevinphos	1		1				2
Mevinphos, alpha		1	3				4
Mevinphos, beta	22	10	15	7	1		55
Omethoate	4	3	2				9
Permethrin	2	1	10	5	1		19
Lichee nut (3)							
Lo bok (1)							
DCPA	1						1
Loquat (2)							
Lotus seed (1)							
Mache (greens) (4)							
Iprodione				1	1	1	3
Permethrin			1				1
Vinclozolin						1	1
Malanga (1)							
Mango (63)							
Dimethoate	1						1
Omethoate		1					1
Thiabendazole				1			1
Mango juice (2)							
Melon, cantaloupe (593)							
Acephate	2	1					3
BHC, alpha	1						1
Chlorothalonil	24	9	17				50
Chlorpyrifos	14	2					16
DCPA	4	1	2				7
DDE, p,p'-	1						1
Diazinon	1						1
Dieldrin	19						19
Dicloran	1						1
Dimethoate	7	1	1				9
Endosulfan I	116	17	2				135
Endosulfan II	104	22	4				131
Endosulfan sulfate	147	4	1				152
Endrin	2						2
Ethion	6	4					10
Malathion	27	9	6	1			43
Methamidophos	121	46	26	1	1		195
Methyl parathion	2						2
Omethoate	12	10	4				26
Permethrin	2						2
Thiabendazole		2	4		3		9
Melon, casaba (8)							
Chlorothalonil	1		1				2
Dieldrin	1						1
Methamidophos	1						1
Melon, crenshaw (31)							
Acephate	1						1
Chlorothalonil	3						3
Dieldrin	2						2
Endosulfan I	3						3
Endosulfan II	2						2
Endosulfan sulfate	12	2					14
Methamidophos	4						4
Melon, honeydew (191)							
Acephate	3	1	2				6
Captan		1					1
Chlorothalonil	1	2	4		1		8
Chlorpyrifos	3						3
Dieldrin	1						1
Endosulfan I	7						7
Endosulfan II	5						5
Endosulfan sulfate	31	31	14	1			77
Ethion			1				1
Methamidophos	38	10	11	1			61
Omethoate	5	2					7
Melon, misc. (16)							
Acephate		1					1
Chlorothalonil		1					1
Dieldrin	2						2
Methamidophos	1						1
Omethoate	1						1
Melon, papaya (1)							
Carbaryl				1			1
Melon, sharline (3)							
Chlorothalonil	1						1
Dieldrin	1						1
Endosulfan I	1						1
Endosulfan II	1						1
Endosulfan sulfate	1						1
Melon, water (462)							
Acephate		2	2	1			5
Aldicarb sulfoxide			7	3			10
Carbaryl						1	1
Chlorothalonil	2	2	2				6
Diazinon	1						1
Dicloran			1				1
Endosulfan sulfate	1						1
Ethion		1					1
Methamidophos	60	10	4				74
Monocrotophos	1		2				3
Omethoate	5	3	2				10
Permethrin		1					1
Mushroom, whole (3)							
Mustard greens (101)							
DCPA	12	12	27	1			52
DDE	1		1				2
DDE, p,p'-	23	5	1				29
Dimethoate	2		4				6
Endosulfan I	3	1					4
Endosulfan II	1		2				3
Endosulfan sulfate	1		1				2
Malathion	1						1
Methamidophos		1					1
Methyl parathion		1					1
Mevinphos		2	1				3
Mevinphos, alpha	1	3	6	4	3	2	19
Mevinphos, beta		5	15	2	2		24
Omethoate	2	1	3				6
Parathion	1	2					3
Permethrin		1	1				2
Nectarine (30)							
Acephate	1						1
Azinphos-methyl	2						2
Captan	1						1
Carbaryl						1	1
Chlorpyrifos	1						1
Dicloran		2	8	5	1	4	20
Endosulfan I	1						1
Endosulfan II	1						1
Ethion			1				1
Methamidophos	1						1
Parathion	1						1
Phosmet		1					1

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Nectarine (continued)							
Pirimicarb	1						1
Okra (33)							
DDT, o,p'-		1					1
DDT, p,p'-			1				1
Diazinon	1						1
Dimethoate		1					1
Methyl parathion		1					1
Monocrotophos		1					1
Omethoate	1	1	3	1			6
TDE, p,p'-	1						1
Olives, fresh (1)							
Onion, bulb (32)							
DCPA	2						2
DDE, p,p'-	2						2
Onion, green (150)							
Chlorothalonil			1				1
DCPA	44	15	9				68
DDE	1	1	2				4
DDE, p,p'-	37	6	5				48
Diazinon	1		2				3
Oregano (1)							
DDE, p,p'-			1				1
DDT, o,p'-			1				1
DDT, p,p'-			1				1
Papaya (9)							
Thiabendazole				1			1
Parsley (62)							
DCPA	10	6	7				23
DDE	1						1
DDE, p,p'-	3	1	1				5
DDT, p,p'-	1			1			2
DDT, o,p'-			1				1
Diazinon	2						2
Dimethoate			1				1
Malathion	1					1	2
Malathion oxygen analog				1			1
Methamidophos	1						1
Methyl parathion	2	1	3				6
Mevinphos, alpha			1				1
Mevinphos, beta		1	3				4
Omethoate			1				1
Parathion	1						1
TDE, p,p'-		1					1
Toxaphene						1	1
Parsnip (3)							
Passion fruit (23)							
Phosmet	1	2	3				6
Pea, Chinese (694)							
Acephate	25	3	10	1			39
BHC, alpha		2					2
BHC, beta	1	1					2
BHC, delta	2						2
Chlorothalonil			1	1		1	3
Chlorpyrifos		1	1				2
DCPA	3						3
Diazinon	1	2	6				9
Dieldrin	3						3
Dimethoate	28	11	25	11	13	6	94
Endosulfan I	8	5	5	1			19
Endosulfan II	4	4	8	1			17
Endosulfan sulfate	9	5	4				18
Ethion	8				1	1	10
Lindane	2						2
Malathion	13	3	9	2	2	4	33
Methamidophos	52	6	10	2			70
Pea, Chinese (continued)							
Methomyl						1	1
Methyl parathion	1	3	2				6
Monocrotophos		3					3
Mevinphos, alpha	1						1
Omethoate	39	23	33	3	2	6	106
Parathion	2		2				4
Permethrin	3	1					4
Quintozene	1			1			2
Pea, English (113)							
Carbaryl			2	1			3
Chlorothalonil			1				1
Dimethoate	1				1		2
Endosulfan I	2						2
Endosulfan II	1	1					2
Endosulfan sulfate	1						1
Malathion	3		4				7
Methamidophos	8		2			1	11
Monocrotophos			1				1
Omethoate	2	3	3				8
Quintozene	2		2				4
Pea, sugar snap (10)							
Diphenyl 2-ethylhexyl phosphate	1						1
Methamidophos	1						1
Peach (50)							
Azinphos-methyl			2				2
Captan	2		4			1	7
Captafol				1			1
Carbaryl			4	2	4	3	13
Chlorpyrifos	1						1
Dicloran	1	1	2	1	3	2	10
Diphenyl 2-ethylhexyl phosphate						1	1
Endosulfan I	2						2
Endosulfan II	2						2
Endosulfan sulfate	2						2
Iprodione			1				1
Malathion			1		1		2
Omethoate	1	1	1				3
Parathion	2	1	1				4
Phosmet			1				1
Pirimicarb		2					2
Peanut (1)							
Malathion					1		1
Chlorpyrifos			1				1
Pear (24)							
Azinphos-methyl		2	4	1	1		8
Captan			3	3	1		7
Dicloran		1					1
Parathion	4						4
Phosmet			1				1
Thiabendazole			3				3
Pear, apple (2)							
Pear, bosc (1)							
Azinphos-methyl			1				1
Pecan, shelled (1)							
Pepino (2)							
Primiphos-methyl			1				1
Pepper, Anaheim (203)							
Acephat	3	1	5	2			11
Azinphos-methyl	1	2					3
BHC, alpha			1				1
BHC, beta	1						1
BHC, delta		1					1
Captan		1					1
Carbaryl			5				5

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Pepper, Anaheim (continued)							
Chlorothalonil	2						2
Chlorpyrifos	6	6	11				23
Chlorthiophos		1	1				2
Dimethoate	5	2	4				11
Diazinon	1						1
Endosulfan I	14	3					17
Endosulfan II	7	6	4				17
Endosulfan sulfate	11	4	1				16
Ethion	1		8	3	1		13
Lindane		1					1
Methamidophos	40	23	58	10	1	2	134
Methyl parathion	5	1	1				7
Monocrotophos	2	2	2	1			7
Omethoate	16	9	22	2	1		50
Parathion	1	1	1				3
Permethrin		4	5				9
Quintozene			1				1
Pepper, bell (1084)							
Acephate	32	35	146	24	7	4	247
Azinphos-methyl	1	1	1				3
BHC	1						1
BHC, alpha	1						1
BHC, beta	1						1
Captan		2				1	3
Carbaryl	4	8	27	5	1		45
Chlorothalonil	5	4				1	10
Chlorpyrifos	131	99	140	2			372
Chlorthiophos	2	3					5
DCPA	1	3	2	1			7
Diazinon		1	1				2
Dicloran	2		1				3
Dimethoate	41	14	17	1	1		74
Endosulfan I	76	8	3	1			88
Endosulfan II	67	13	6				85
Endosulfan sulfate	55						55
Ethion	31	31	32	4	3		101
Folpet			2				2
Gardona		1	1				2
Heptachlor	1	1					2
Iprodione	1	1	7	2			11
Malathion	6		5			1	12
Methamidophos	208	157	357	31		2	755
Methyl parathion	1	1					2
Monocrotophos	1						1
Omethoate	106	83	137	9	1		336
Parathion	10	2	1				13
Permethrin	19	15	6				45
Procymidone	2		2				5
Quintozene	8	2	2				12
Toxaphene			2				2
Vinclozolin		1	6				7
Pepper, bell, dehyd. (5)							
Acephate			1				1
Chlorpyrifos			2		1		3
Ethion			2				2
Methamidophos			1	1	2		4
Pepper, Caloro (5)							
Methamidophos		1	2	1			4
Omethoate				1			1
Pepper, Caribe (124)							
Acephate	1	2	1	1			5
Azinphos-methyl	1	2	2				5
Captan					1		1
Carbaryl				1			1
Chlorothalonil	1	1					2
Chlorpyrifos	6	3	5				14
Diazinon	1			1			2
Dimethoate	3	1	2				6
Endosulfan I	8	1	2				11
Endosulfan II	6	3	2				11
Endosulfan sulfate	10	1					11
Ethion	1	2	4	1			8
Pepper, Caribe (continued)							
Malathion			3				3
Methamidophos	22	9	18	5	2		56
Monocrotophos		1	2				3
Omethoate	9	4	16	2	1		32
Permethrin		3	7				10
Pepper, cayenne (4)							
Dimethoate	1						1
Methamidophos		1			1		2
Methyl parathion		1					1
Omethoate	1		1				2
Pepper, cubanell (7)							
Acephate		1	1				2
Chlorpyrifos	1		1	1			3
Methamidophos		1	4	2			7
Omethoate			4	1			5
Pepper, Fresno (7)							
Chlorpyrifos	1	1					2
Endosulfan I	1						1
Endosulfan II	1						1
Ethion	1						1
Methamidophos	3		1				4
Monocrotophos	1	1					2
Omethoate			1	2	1		4
Pepper, jalapeno (239)							
Acephate	2	1	5	1	1		10
Azinphos-methyl	2	1	5				8
BHC, alpha			1				1
BHC, beta	1						1
BHC, delta			1				1
Chlorothalonil	1		1		1		3
Chlorpyrifos	19	10	16				45
Diazinon	3	1	1				5
Dicloran	1						1
Dimethoate	5	1	5				11
Endosulfan I	4	2	1				7
Endosulfan II	3	1	3				7
Endosulfan sulfate	5	1	1				7
Ethion	4	5	13	1	4		27
Lindane		1					1
Malathion	1	3	3				7
Methamidophos	31	20	61	21	8	7	148
Methyl parathion	5	1		1			7
Monocrotophos	3	1	3				7
Omethoate	11	15	38	12	5	1	82
Parathion	3						3
Permethrin	1	1	12				14
Quintozene	3						3
Toxaphene				2		1	3
Pepper, misc. (15)							
Chlorpyrifos	1	1					2
Chlorthiophos		1					1
DCPA	1						1
Endosulfan I	1						1
Endosulfan II	1						1
Endosulfan sulfate	1						1
Methamidophos	2		5	1	1		9
Monocrotophos		1					1
Omethoate	1						1
Pepper, pasilla (67)							
BHC, alpha			1				1
BHC, beta	1						1
BHC, delta	1						1
Chlorpyrifos	5		2				7
Dimethoate	1		2				3
Lindane	1						1
Methamidophos	16	7	9	3	1		36
Methyl parathion	2						2
Monocrotophos		1					1
Omethoate	2	5	7	2			16
Permethrin			1				1

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Pepper, pasilla (continued)							
Parathion	1		1				2
Pepper, pickled (2)							
Methamidophos	1		1				2
Pepper, poblano (4)							
Dimethoate			1				1
Endosulfan I	1						1
Endosulfan II		1					1
Endosulfan sulfate	1						1
Ethion			1				1
Malathion			1				1
Methamidophos	1						1
Omethoate		1	2				3
Permethrin		1	2				3
Pepper, serano (274)							
Acephate	1	3	4	2			10
Azinphos-methyl	1	1	9	4	1		16
BHC, alpha	1	1	1				3
BHC, beta	2						2
BHC, delta	2						2
Chlorothalonil	1	2	1				4
Chlorpyrifos	7	3	9				19
Chlorthiophos		1	1				2
Diazinon			1				1
Dimethoate	4	3	5	1			13
Endosulfan I	3	3	1				7
Endosulfan II	3	1	1				5
Endosulfan sulfate	5						5
Ethion	12	4	19	5	1	1	43
Lindane	2						2
Malathion	4	1	4				9
Methamidophos	47	24	69	31	5	5	181
Methyl parathion	4	1	1				6
Monocrotophos		1	7	3			11
Omethoate	12	10	31	6	6	3	68
Parathion	3						3
Permethrin	3		9	1			12
Quintozene	2	1					3
Pepper, whole spice (2)							
Pepper, yellow (25)							
Chlorpyrifos	1						1
Dimethoate		1	1				2
Ethion	1						1
Methamidophos	2	3	4	1	1		11
Omethoate	3		1	1	1		6
Permethrin			2	1			3
Quintozene		1		1			2
Persimmon (2)							
Pineapple (23)							
Carbaryl			6	6	3	1	16
Pineapple juice conc. (1)							
Plantain (1)							
Plum (35)							
Dicloran	2	4	4	1	2	3	16
Dimethoate	1						1
Omethoate	1						1
Potato (35)							
DDE, p,p'-	13	6	2				21
Demeton-S-sulfone			1				1
Disulfoton sulfone	1						1
Thiabendazole					1		1
Toxaphene			1				1
Potato, Japanese-Yamaimo (1)							
Potato skins (1)							
Potato, sweet (5)							
Dicloran			1	2	1		4
Prune (1)							
Diazinon	1						1
Purslane (3)							
Methamidophos					1	2	3
Radicchio (7)							
Acephate	1						1
Methamidophos	1						1
Radish (184)							
DCPA	15		3	1	1	1	21
DDE		1					1
DDE, p,p'-	33	4					37
Dicloran		1	5	1			7
Dieldrin	2						2
Endosulfan I		1			1		2
Endosulfan II	1		1				2
Endosulfan sulfate	1		1				2
Linuron		1					1
Methamidophos			1	1			2
Mevinphos, beta		1					1
Omethoate	4	2	1	1			8
Radish, Italian (4)							
DCPA	1						1
DDE, p,p'-		1	1				2
Endosulfan I			1				1
Endosulfan II		1					1
Endosulfan sulfate	1						1
Malathion			1				1
Radish, white (3)							
Dicloran			1				1
Raisin (4)							
Diazinon			1				1
Rapini (22)							
DCPA	3	2					5
Dicloran	1						1
Endosulfan I	1						1
Endosulfan II	1						1
Mevinphos, alpha				1			1
Mevinphos, beta			1				1
Raspberry (87)							
Captan	2	1	5	15	4	8	45
Carbaryl			1	2	1	1	5
Dichlorfluanid			1				1
Dicofol			2				2
Folpet					1		1
Iprodione	2		6	1			9
Pirimiphos-methyl		1					1
Procymidone					1		1
Vinclozolin	1						1
Rhubarb (3)							
Rosemary, whole (2)							
Pirimiphos-methyl			1	1			2
Rutabaga (2)							
Dicloran				1			1
Shallot (1)							
Soft drink, carb. cola (2)							
Spinach (176)							
Acephate	2	1	3			1	7
Captan			4	1			5

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a] 0.05	0.10	0.50	1.0	2.0	>2.0	Total
Spinach (continued)							
Chlorothalonil	1		1	1		1	4
Chlorpyrifos		1					1
DCPA	2	2	1				5
DDE	1						1
DDE, p,p'-	10	4	7				21
DDT	1						1
DDT, p,p'-	2	1	2				5
Demeton-S-sulfone		1		1			2
Diazinon	1		1				2
Dieldrin	1						1
Dicloran	4	2	4			2	12
Dimethoate	2	3					5
Disulfoton sulfone		1					1
Endosulfan I	22	7	3	1		2	35
Endosulfan II	11	7	10	3			31
Endosulfan sulfate	7	5	16	3			37
Folpet	1		1	1			3
Hydroxy diazinon					1		1
Methomyl			5	4	1		10
Methamidophos	5	1	5	3	1		15
Methyl parathion	2						2
Mevinphos			1				1
Mevinphos, alpha	2	3					5
Mevinphos, beta	3	3	5				11
Omethoate	1		4				5
Parathion	3		2				5
Pentachloroaniline			2				2
Permethrin		1	9	12	1		23
Quintozene			1	1			2
Squash, acorn (74)							
BHC, beta	1						1
Chlorothalonil		1					1
Chlorpyrifos	1	1					2
Dieldrin	4	3					7
Dicloran		3	2	3	1		9
Endosulfan I	1						1
Endosulfan II	1						1
Endosulfan sulfate	1						1
Endrin	5						5
Methamidophos	3	2					5
Omethoate			1				1
Quintozene	2						2
Squash, banana (13)							
Methamidophos	2						2
Squash, bitter melon (10)							
Chlorpyrifos	1						1
Endosulfan I	1						1
Endosulfan II	1						1
Endosulfan sulfate		1					1
Methamidophos	1						1
Omethoate	1						1
Squash, butternut (16)							
Dieldrin	2						2
Methamidophos	1						1
Omethoate	1						1
Squash, cabocha (48)							
Captan		1					1
Chlorpyrifos	1						1
Dieldrin	16						16
Endosulfan I	2						2
Endosulfan II	1						1
Endosulfan sulfate	2						2
Endrin	9						9
Heptachlor epoxide	1						1
Methamidophos	7						7
Omethoate			1				1
Squash, Chinese (4)							
Dimethoate	1						1
Omethoate	1						1
Squash, crookneck (8)							
Dieldrin	2	1					3
Endrin			1				1
Squash, Italian (1113)							
Acephate	3	1	2				6
Captan			1				1
Chlorothalonil	4	5	3	2			14
Chlorpyrifos	6	1					7
Diazinon	4	2					7
Dieldrin	125	1					126
DDE, p,p'-	19						19
Dimethoate	7		5				12
Endosulfan I	189	39	10				238
Endosulfan II	196	8	3				207
Endosulfan sulfate	194	2					196
Endrin	130	8	1				139
Ethion	2	3					5
Heptachlor epoxide	5						5
Hydroxy diazinon			1				1
Lindane	1						1
Methamidophos	36	4	6	1			47
Methyl parathion	1	1					2
Monocrotophos		1					1
Omethoate	18	10	6				34
Pentachloroaniline	4						4
Quintozene	4						4
Thiabendazole	1		3				4
Vinclozolin			1				1
Squash, misc. (23)							
Endosulfan I	2						2
Endosulfan II	1						1
Endosulfan sulfate	1						1
Methamidophos	2						2
Omethoate		1					1
Squash, singua (8)							
Methamidophos	2		2				4
Squash, spaghetti (20)							
Acephate		1					1
Methamidophos	1						1
Squash, summer (79)							
Acephate	1						1
Chlorpyrifos	1						1
Dieldrin	1						1
Dimethoate	1						1
Endosulfan I	5	1					6
Endosulfan II	5						5
Endosulfan sulfate	5						5
Endrin	1						1
Methamidophos	1						1
Omethoate	1						1
Squash, yellow (157)							
BHC	1						1
Chlorothalonil			2	1			3
DDE, p,p'-	2	1	1				4
Diazinon	1						1
Dieldrin	24						24
Dimethoate	2						2
Endosulfan I	24	8	2				34
Endosulfan II	25						25
Endosulfan sulfate	25						25
Endrin	20	1					21
Heptachlor	1						1
Heptachlor epoxide	1						1
Methamidophos	5	2					7
Omethoate	4	1					5
Parathion	1						1
Spiralina (2)							
Starch, mung bean (1)							

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Strawberry (342)							
Acephate	1	1					2
Captan	4	4	23	19	19	49	118
Captafol					1	2	3
Carbaryl		1	1	3	4	1	10
Chlorothalonil	1						1
Cyhexatin		10	49	27	26	26	138
DDE, p,p'-	2						2
Dichlorfluanid					3	2	5
Dicofol			2	2			4
Dieldrin	1						1
Endosulfan I	5	4	8				17
Endosulfan II	2	4	10				16
Endosulfan sulfate	10	4	3				17
Endrin	1						1
Ethion			1				1
Folpet	2	2	13	15	10	8	50
Malathion			3	3	1		7
Malathion oxygen analog				1			1
Methamidophos	2	1	1				4
Methyl parathion	1		1				2
Mevinphos, alpha	3	1	6	1	1		12
Mevinphos, beta	5	3	1	1			10
Parathion	1						1
Permethrin	1						1
Procymidone	1						1
Vinclozolin	10	13	61	48	43	17	192
Sugar, glaze/icing mix (1)							
Sunflower root (1)							
Swamp cabbage (1)							
Swiss chard (18)							
Acephate			1				1
Carbaryl						2	2
Chlorothalonil						2	2
DCPA	1	1					2
Diazinon	1						1
Methamidophos		1					1
Mevinphos, alpha	2		2				4
Mevinphos, beta			1				1
Omethoate			1				1
Tamarillo (13)							
Acephate	1	1	1				3
Methamidophos	1						1
Taro (1)							
Tarragon (4)							
Pirimiphos-methyl						2	2
Tea, orange peel (1)							
Tomatillo (58)							
BHC			1	1			2
BHC, alpha			1				1
Chlorothalonil		1					1
Chlorpyrifos			1				1
DDT				1			1
DDT, p,p'-	1						1
Demeton-S-sulfone	1						1
Dimethoate	1						1
Disulfoton sulfone	1						1
Endosulfan I	1						1
Endosulfan II	1						1
Endosulfan sulfate			1				1
Methamidophos	9	2	4				15
Methyl parathion	1						1
Monocrotophos		1					1
Omethoate	2	1	1				4
Parathion	1						1
Permethrin	3	3	2				8
Toxaphene				1			1
Tomatillo sauce (1)							
Tomato (2506)							
Acephate	4						4
Azinphos-methyl			1				1
BHC, alpha	15	4					19
BHC, beta	8						8
BHC, delta	8						8
Carbaryl	3	4	2				9
Carbophenothion			1				1
Chlorothalonil	47	14	44	5	2		112
Chlorpyrifos	762	134	46				942
Chlorthiophos	2		1				3
DCPA			1				1
Diazinon	4						4
Dicloran	14	6	9	8	1	3	41
Dimethoate	31	4	6				41
Diphenyl 2-ethylhexyl phosphate	5	8	12	1			26
Endosulfan I	14	3					17
Endosulfan II	13	3					16
Endosulfan sulfate	11	1					12
Ethion	61	25	15				101
Fenvalerate	4	2	1	1			8
Folpet			1				1
Lindane	12						12
Malathion	3	1					4
Methamidophos	1059	177	70	3			1309
Methyl parathion	3		1				4
Monocrotophos		2					2
Omethoate	114	38	27				179
Parathion	7	3					10
Permethrin	140	28	7		1		176
Quintozene	3						3
Toxaphene		1					1
Vinclozolin		1	1				2
Tomato, canned (8)							
Methamidophos	2	1					3
Tomato juice (8)							
Methamidophos	4	2	1				7
Tomato, cherry (513)							
Acephate	4	5	3				12
Azinphos-methyl			2				2
BHC, alpha	3		1				4
BHC, beta	2						2
BHC, delta	1						1
Captan		1		2	1	1	5
Captafol		1		1			2
Carbaryl		1	3	1			5
Chlorothalonil	28	18	47	8	4	2	107
Chlorpyrifos	42	11	10				63
Chlorthiophos	1		2				3
Cypermethrin		1	1				2
DCPA	1						1
DDE, p,p'-	1						1
Diazinon	1	1					2
Dicloran	2	1	3	2			8
Dimethoate	3	2					5
Diphenyl 2-ethylhexyl phosphate	1	2	1				4
Endosulfan I	10	2					12
Endosulfan II	7	2	1				10
Endosulfan sulfate	9						9
Ethion	2	1	1				4
Fenvalerate	5	3	6				14
Lindane	2						2
Malathion	5	2					7
Methamidophos	101	37	34	3			175
Methyl parathion	7	1					8
Omethoate	14	5	7				26
Parathion	2	2					4
Permethrin	43	23	14				80
Quintozene	1						1
Toxaphene			2				2

Produce item (No. of samples examined) and pesticide	No. of findings in various ranges[a]						Total
	0.05	0.10	0.50	1.0	2.0	>2.0	
Tomato, paste (2)							
Tomato, romano (102)							
BHC, alpha		1					1
BHC, beta	1						1
BHC, delta	1						1
Chlorothalonil	2	1	2	1			6
Chlorpyrifos	7	1	1				9
Diphenyl 2-ethylhexyl phosphate			1				1
Endosulfan I	1						1
Endosulfan II	1						1
Endosulfan sulfate	1						1
Lindane	1						1
Malathion	2			1			3
Methamidophos	42	5	3				50
Omethoate	4	3					7
Parathion	1						1
Permethrin	5	1					6
Quintozene	1						1
Tomato sauce (10)							
Methamidophos	1						1
Tomato, stewed (2)							
Methamidophos	1						1
Turnip (39)							
DCPA	4	3	5				12
DDE	2						2
DDE, p,p'-	8	1					9
Mevinphos, alpha				1			1
Mevinphos, beta			1				1
Permethrin	1	1					1
Turnip, daikon (17)							
DCPA	2						2
Turnip greens (69)							
DCPA	13	3	19	2			37
DDE		1					1
DDE, p,p'-	21	4					25
Dimethoate		2					2
Endosulfan I	3		1	1	2		7
Endosulfan II	1	2	2	2			7
Endosulfan sulfate	2		1	1	1	2	7
Mevinphos, alpha		1	6	5			12
Mevinphos, beta	1	2	13	3	1		20
Omethoate	1	1					2
Permethrin			3				3
Water chesnuts (1)							
Water cress (2)							
Watermelon flavored ice (1)							
Wheat (3)							
Malathion			1			1	2
Wheat, durham (3)							
Wheat, red (12)							
Wine (88)							
Dimethoate	1	1					2
Omethoate	2						2
Ethyl carbamate	39	8	9				56
Witloof (6)							
Dimethoate	1	1					2
Omethoate		2					2

[a] The count given in a column covers a range that starts at a value greater than the previous column heading (in ppm) and includes the current column heading.

[b] Values underlined are indicative of residue levels found to be in excess of the tolerance level set by EPA or where no tolerance level has been previously established for that crop.

Ion Abundance Criteria for Gas Chromatographic/Mass Spectrometric Environmental Analysis

J. R. DONNELLY
Lockheed-EMSCO, 1050 E Flamingo Rd, Las Vegas, NV 89119
G. W. SOVOCOOL and R. K. MITCHUM
U.S. Environmental Protection Agency, PO Box 93478, Las Vegas, NV 89193

Mass and intensity calibration of gas chromatograph/mass spectrometer (GC/MS) responses is an important quality assurance issue for chemical analysis. Ion abundance calibration with decafluorotriphenylphosphine (DFTPP) was applied in 1975 to standardize quadrupole spectra to resemble the ion abundances that were obtainable from magnetic sector mass spectrometers. Modern quadrupole mass spectrometers provide significantly greater high-mass sensitivity than allowed under the 1975 study. Thus, those recommendations were reevaluated with 2 approaches. First, an interlaboratory study was conducted using 15 different gas chromatography/mass spectrometry (GC/MS) systems. Second, the U.S. Environmental Protection Agency Contract Laboratory Program (EPA-CLP) quality assurance data base was searched and over 6500 DFTPP tune results were plotted and evaluated. Based on these approaches, updated ion abundance criteria recommendations have been developed, which contemporary instruments can meet, and which meet data quality objectives regarding identification and quantitative analysis of analytes.

Mass and intensity calibration of mass spectrometer responses has been an important quality assurance issue since the introduction of the technique to chemical analysis. Early efforts by scientists at lower mass ranges included use of an air leak and background peaks that might be due in part at least to hydrocarbons. Development of a reliable calibrant for higher masses may have begun with the Phillips Research Grade hydrocarbons of the early 1940s. In January 1944, the National Bureau of Standards (NBS) released hydrocarbon standards for mass calibration, and in July 1944, the American Petroleum Institute (API) entered into a cooperative program with NBS (1). Use of fluorinated organic calibrants—e.g., perfluorokerosene (PFK) and perfluorotributylamine (PFTBA)—has been widespread for over 25 years (2, 3). These calibrants have discrete peaks at regular intervals over a wide mass range, are relatively volatile, and are very stable, so high-molecular-weight compounds or mixtures can be utilized (4, 5). These relatively volatile compounds are readily introduced into and removed from the mass spectrometer, and, because of the negative mass defect of fluorine, the exact masses involved are lower than those of most hydrocarbon-based analytes, rendering mass separation (at medium or high resolution) of the calibration peak from analytes relatively simple (6–9). Other calibrants for electron ionization and/or chemical ionization have been proposed and applied, for example, perfluoroalkylphospholonitrilates (10), perfluorotriheptyltriazines (11), and polyperfluoropropylene oxide (Fomblin-L) (12).

The capabilities of mass spectrometers have developed greatly over the approximately 40 years of their relatively routine application to chemical problems, starting with the need of oil companies to produce large amounts of high-octane gasoline during World War II (1, 13). After popularization of low-cost quadrupole gas chromatograph/mass spectrometers (GC/MS) in the early 1970s (14), ion abundance calibration was considered (15) to standardize quadrupole spectra to resemble the ion abundances obtainable from magnetic-sector mass spectrometers. Magnetic deflection and time-of-flight spectra comprised most of those found in readily available mass spectral libraries. However, criteria based on the performance of early quadrupole instruments may not be fully applicable today, particularly since quadrupole MS, a more recent (and perhaps more complex) (16) development than the magnetic-sector concept, had not reached maturity by the mid-1970s.

This early (1975) effort (15) to develop ion abundance calibration for GC/MS analysis used decafluorotriphenylphosphine (DFTPP) as the proposed calibrant which was tested on 15 systems—11 early quadrupoles of a single brand and model (Finnigan 1015), and 4 magnetic-sector systems. Proposed criteria were based on average spectral features from the pooled results on DFTPP. This calibrant was chosen for its stability and its spectrum of reasonably abundant ions over the comparatively wide mass range (51–443 daltons) needed for GC/MS monitoring of environmental contaminants.

A careful review of the experimental results from the original 15 determinations showed that 11 individual data sets (spectra) failed to meet at least one of the proposed criteria. This failure of the original data to pass the criteria derived from those data pointed out the danger of basing conclusions on averages of entries divorced from the data sets. Additionally, more than 10 years of environmental monitoring experience coupled with documented problems in application (17–21) of those criteria—particularly high/low mass relative sensitivity—suggested that further study and data analysis would be justified for the calibration of quadrupole MS ion abundances to resemble typical data from magnetic-sector instrumentation. Since some classes of environmentally significant compounds monitored by GC/MS possess molecular weights well over 200 daltons, instrument sensitivity at higher masses is an important consideration for environmental monitoring efforts performed for the U.S. Environmental Protection Agency (EPA). However, a result of meeting criteria established in the 1975 study is less high mass sensitivity than that readily obtained on modern instruments.

The chemistry of the ions formed in the mass spectrometer from DFTPP has been described, using MS/MS on a VG Model ZAB-3F (19, 20). Substantial differences in some ion abundance ratios, especially 198/442, were observed by using the ZAB-3F and varying concentration, source temperature, and accelerating voltage (flight time) (19, 20). Many such variations can be explained on the basis of concentration changes in the source from use of the capillary GC inlet system. A typical DFTPP mass spectrum is shown in Figure 1. Many standard solutions are contaminated with the DFTPP air oxidation product. The DFTPP oxide mass spectrum was not found in the EPA/NBS mass spectral library; its spectrum contains some peaks common to those of the DFTPP mass spectrum. Therefore, this DFTPP oxide was prepared (using H_2O_2) and its mass spectrum was obtained and interpreted (Figure 2).

Received February 18, 1987. Accepted July 27, 1987.

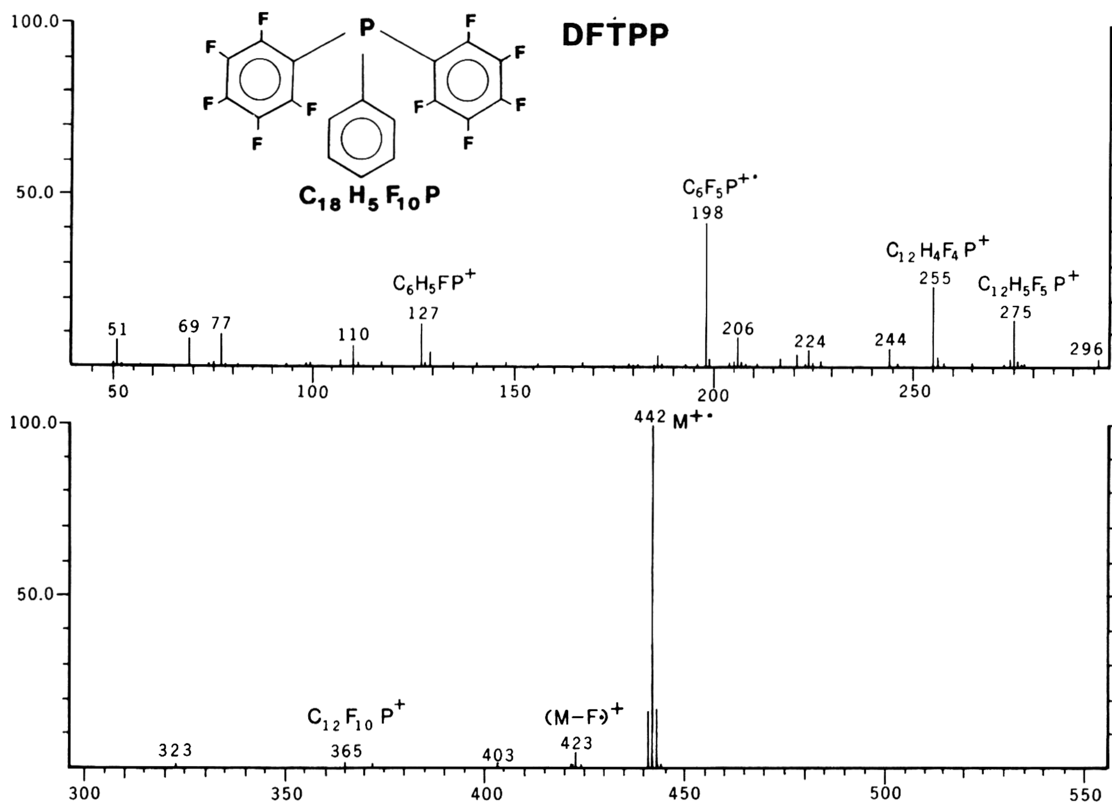


Figure 1. Typical DFTPP mass spectrum.

Figure 2. Mass spectrum of DFTPP oxide, prepared using H_2O_2.

Table 1. Ion abundance ratios from 1986 interlaboratory study

	442	198/442	199/198	443/442	441	51/198	51/442	127/198	127/442	275/198	275/442	365/198	365/442
EXTREL ELQ-400	95	106	6.2	17.3	11	44	46	45	47	15	15	1.6	1.7
EXTREL ELQ-400	100	73	7.9	19.6	13	37	27	43	32	21	15-18	1.1	.8
FINNIGAN 705 ION TRAP	100	13-19	NR	15.0-16.6	7-10	56-140	11-18	55-103	10-14	97-232	19-30	0-1.4	0-2.3
FINNIGAN 705 ION TRAP	100	52	8.0	15.4	11	64	33	26	14	26	14	2.6	1.3
FINNIGAN 1015 (PROBE)	100	42	6.5	NR	NR	53	22	66	27	8	3	2.0	.8
FINNIGAN 4023	74	135	6.3	17.9	11	44	60	46	62	24	30	2.2	3.0
FINNIGAN 4023	100	43-93	6.2	17.0-18.0	18	18-46	8-43	30-47	13-44	22-33	14-21	4.4	1.9-2.2
FINNIGAN 4500	43	229	6.9	19.6	8	41	97	41	97	20	47	1.3	2.9
FINNIGAN-MAT 8230	100	32-81	6.2-7.2	19.2-27.5	11-19	9-25	3-11	15-32	8-32	21-64	12-22	1.5-10.1	.5-5.4
FINNIGAN-MAT 8230 (PROBE)	100	40-42	6.0-6.6	18.6-20.2	11-12	17-18	7	30-32	12-13	37-39	15-16	3.0-4.7	1.3-1.9
HP 5970-B MSD	100	39	7.0	20.4	14	35	14	27	11	28	11	4.1	1.6
HP 5985	67-70	144-150	7.0	20.0-21.0	9	9	13	28	42-44	20	67-70	1.9	2.6-2.9
HP 5987A	66-68	148-152	6.5-7.0	8.1-20.5	7	15	23-29	50	73-78	18	26-32	1.5	2.2-24
HP 5996	100	34-78	5.1-7.0	18.2-19.7	11-15	26-33	11-23	19-28	8-22	19-31	9-22	3.6-6.5	1.6-3.6
KRATOS MS-50	100	24-32	3.2-6.9	17.8-19.6	9-11	NR	NR	39	7-13	33-54	8-11	1.6-2.8	.4-.9
NERMAG R10-10C	95-100	56-100	4.6-6.8	20.9-23.1	11-14	19-27	11-28	11-26	11-26	9-20	6-9	1.6-4.8	.9-2.9
NICOLET FTMS-2000 (PROBE)	100	80	6.1	18.5	21	28	23	22	17	15	12	2.0	1.6
VARIAN CH-5	99	101	8.6	25.6	21	NR	NR	38	38	25	25	1.5	1.5
VG-ZAB-3F	100	42-73	6.2-8.3	17.4-20.7	8-11	45-55	23-33	50	20-31	33-36	15-20	4.1-6.0	2.0-3.1
VG 12-250	100	62-98	6.1-6.3	17.3-17.7	14-15	38-52	23-51	42-48	26-47	19-22	13-19	1.7-2.3	1.4-1.7
VG TS-250	100	44	6.8	20.5	13	34	15	45	20	41	18	5.7	2.5

NR = NOT REPORTED

Experimental

An interlaboratory study of DFTPP spectra was performed using modern instruments operating in conformance with the EPA Contract Laboratory Program (CLP) requirements: 50 ng injected, 1 s/scan, 30 m fused silica GC column (J and W DB-5, or equivalent). A reference spectrum was also obtained on a Finnigan Model 1015 mass spectrometer since the recommendations of the 1975 study were based in large part on the performance of that instrument. The participants were asked to tune their GC/MS systems according to manufacturers' recommended procedures, for unit resolution throughout the mass range with PFK or PFTBA (51–503 daltons for PFTBA). The results, summarized in Table 1 and Figure 3, were used in the development of an updated set of criteria (Table 2) which were met by all GC/MS instruments. In addition to GC/MS determinations, for which DFTPP was initially selected as a calibrant, probe mass spectra were also measured on 3 systems (quadrupole, magnetic sector, and Fourier Transform) for comparison.

The Finnigan Model 4510 spectrum, one Extrel Model ELQ-400 spectrum, and one Finnigan Model 4023 spectrum were obtained under experimental conditions chosen to meet the criteria suggested in the 1975 study.

The EPA-CLP quality assurance data base was also searched, and results from more than 6500 DFTPP GC/MS tunes were plotted using the IBM Model 3090 computer and Statistical Analysis System (SAS) software (Figure 4). These data, which were contractually required to meet the criteria suggested in the 1975 study, are obviously a subset of possible tunes. Nonetheless, they are informative because they show where data tend to lie within the acceptance windows. For example, 51/198 and 442/198 ratios showed broad data dispersion within the acceptance windows. Data for some other criteria (68/69, 70/69, 199/198, 275/198, 365/198, 443/442) showed approximately Gaussian distributions, allowing one

Table 2. Recommended DFTPP ion abundance criteria

m/z	Recommendation	1975 Criteria (15)
51	15–75% of m/z 198	30–60% of m/z 198
127	15–60% of m/z 198	40–60% of m/z 198
275	10–60% of m/z 198	10–30% of m/z 198
198 or 442	base peak	198 must be base; 442 > 40% of m/z 198
68	≤2% of m/z 69	<2% of m/z 69
70	≤2% of m/z 69	<2% of m/z 69
197	≤1% of m/z 198	<1% of m/z 198
199	4.5–9% of m/z 198	5–9% of m/z 198
443	15–24% of m/z 442	17–23% of m/z 442
442	≥40% of m/z 198 if 198 is base peak	
198	≥30% of m/z 442 if 442 is base peak	
365	≥0.5% of m/z 198	>1% of m/z 198
441	delete criterion	present, <443

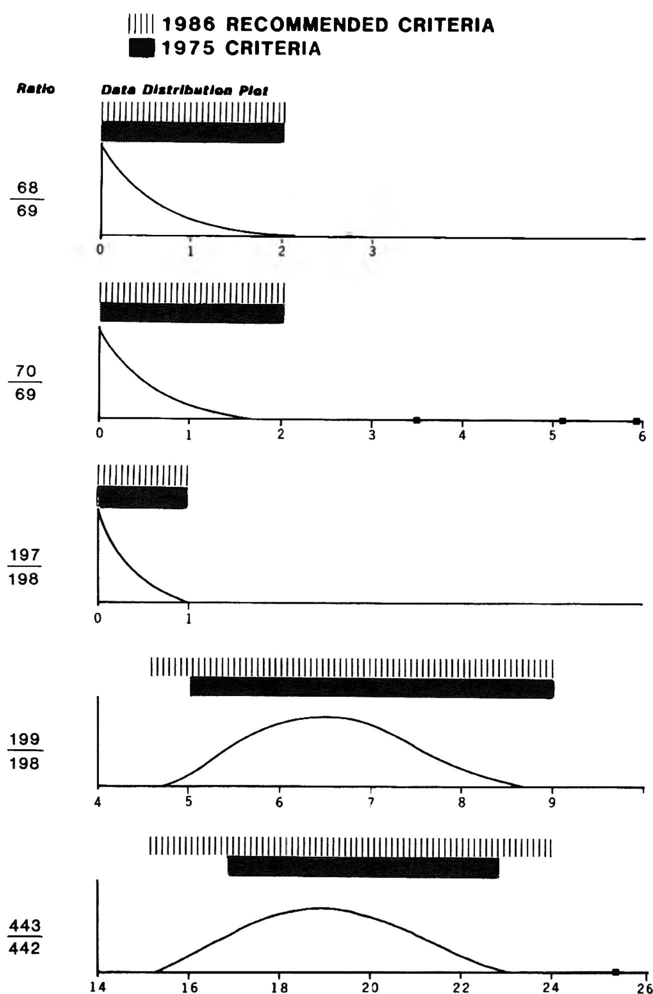


Figure 3. 1986 interlaboratory study of DFTPP spectra, obtained in accordance with EPA-PCL criteria.

to set updated criteria (acceptance windows) reflecting actual mass spectrometer performance.

Discussion

Excellent agreement was obtained between results from this interlaboratory survey and from distributions plotted using CLP data, extending Gaussian curves smoothly outside criteria acceptance windows where necessary to complete the curves. Experimental results and theoretical isotopic abundances showed that some acceptance windows should be changed slightly (Figures 3 and 4). The theoretical ratios for 199/198, $[C_6F_5P]^{+}$; 443/442, $[C_{18}H_5F_{10}P]^{+}$; and 70/69 (previously assumed to be $[CF_3]^+$, but predominantly $[PF_2]^+$) are 6.73, 20.3, and 1.12%, respectively. Some experimental isotopic abundance ratios (i.e., 199/198 and 443/442) tended to be slightly lower than theoretical, probably due to typical zero settings, which are chosen to conserve data system disk storage space. For example, 443/442 experimental values averaged 18–19%. The simple (from ^{13}C and ^{2}H) theoretical 443/442 ratio is 20.3%, but experimental values will reflect perturbations due to approximately 10–15% $[M - H]^+$ which contributes, via $[^{12}C_{17}{}^{13}C_1H_4F_{10}P]^+$, ca 2–3% to the observed intensity at m/z 442, and ca 0.2–0.3% to the observed m/z 443 signal, via $[^{12}C_{16}{}^{13}C_2H_4F_{10}P]^+$. This effect brings the experimental 443/442 ratio to 20.0–20.1% for 15% and 10% $[M - H]^+$, respectively. The individual cases of high 443/442 ratios may be due to poor high mass resolution and/or $[M + H]^+$ contribution, in part from the presence of calibrant in the source (PFTBA, in particular). A factor of 20 increase in $[M + H]^+$ has been observed in the ammonia CI mass spectra of certain compounds upon introduction of a small amount of PFTBA into the ion source (21). Similarily, the broad 69/198 curve in the CLP data and in certain data from the interlaboratory study might be due to $[CF_3]^+$ contribution from residual PFTBA or PFK calibrant. Some data relevant to m/z 68/69 and 70/69 were inconclusive. Often, no peaks were observed at m/z 68 or 70. Although m/z 70 has a theoretical abundance of 1.12% relative to m/z 69, contribution to m/z 69 from $[PF_2]^+$ would have no observable m/z 70 isotope. Recent data (20,22) indicate that $[PF_2]^+$ is the major contributor to response at m/z 69. No peak is expected at m/z 68. Regardless of which composition is the major contributor in an experimental spectrum to the observed signal at m/z 69, the earlier criteria that both m/z 68 and 70 not exceed 2% of the abundance at m/z 69 seem appropriate and justified as a resolution check, on the basis of the available data.

Results of this study suggest that broader criteria are necessary for the ion abundance of m/z 365. A lower abundance limit of 0.5% is recommended. This is coincidentally the reporting threshold for some data systems. The 1975 abundance criterion for m/z 441 ($441 < 443$) was met by most systems, but the ratio varied noticeably across different systems. No theoretical "correct" abundance ratio exists since the ratio is due to a reaction (loss of H), not a natural isotopic abundance. High mass resolution can be adequately verified by the 443/442 ratio. Finally, both the CLP data base and the interlaboratory study data indicate that criteria windows for m/z 51, 127, and 275 should be widened. No theoretical (or practical) "correct" relative abundances exist for these ions. Recommended criteria for these ions reflect observed data trends and are consistent with results of the interlaboratory study.

Conclusion

This survey indicates that current instrumentation generally provides better high mass sensitivity than that previously surveyed, and that new ion abundance criteria consistent with this improvement can be justified. We propose the criteria shown in Table 2, which reflect sensitivity, resolution, and mass accuracy checks at the low, middle, and upper mass regions of interest for environmental monitoring by GC/MS. Modern instruments can meet these criteria, whereas the 1975 criteria (for example, the 127/198, 275/198, and 442/198 ratios) were often not met in either this study or the earlier work used to develop the 1975 criteria. In fact, 11 data sets presented in the earlier study did not meet at least one of those suggested criteria derived from an averaging of the results for each parameter. Data generated under these new tuning recommendations meet environmental monitoring identification and quantitative analysis requirements using GC/MS software programs.

Acknowledgments

The authors gratefully acknowledge participation in this study by David Anthony, Diane Cho, Gerd Dielmann, Brady Englestead, Clinton Graden, Clyde Hedin, James Johnson, Tammy Jones, James Kinsinger, Jackson Lay, Warren Niederhut, Linda Perrin, Steven Pyle, David Speis, Yves Tondeur, and David Wang-Iverson. Helpful discussions by Yves Tondeur, Seymour Meyerson, Henry Fales, and Quirinus Grindstaff are gratefully acknowledged.

Although the research described in this article has been funded wholly or in part by EPA Contract No. 68-03-3249

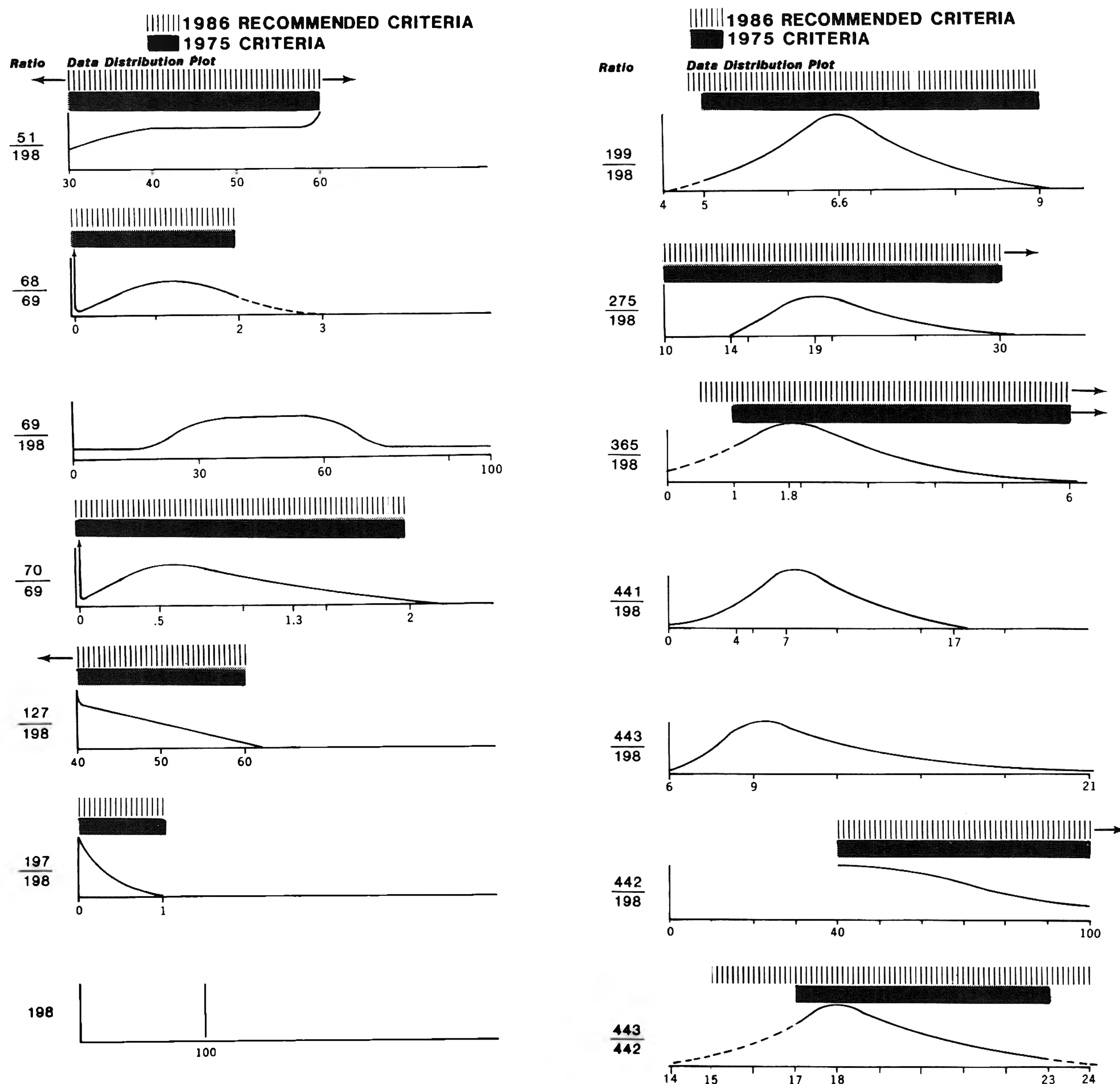


Figure 4. Plots of DFTPP GC/MS tunes from EPA-CLP quality assurance data base.

to Lockheed Engineering and Management Services Company, Inc., it has not been subject to the agency's review and therefore does not necessarily reflect the views of the agency, and no official endorsement should be inferred.

References

(1) Meyerson, S. (1986) *Org. Mass Spectrom* **21,** 197
(2) Beynon, J. H. (1960) *Mass Spectrometry and Its Applications to Organic Chemistry,* Elsevier, Amsterdam, Netherlands, p. 414
(3) Beynon, J. H. (1963) in *Advances in Mass Spectrometry,* Vol. 2, R. M. Elliott (Ed.), Pergamon, London, UK, p. 216
(4) Mohler, F. L., Bloom, E. G., Lengel, J. H., & Wise, C. E. (1949) *J. Am. Chem. Soc.* **71,** 337
(5) Bradt, P., & Mohler, F. L. (1955) *Anal. Chem.* **27,** 875
(6) Beynon, J. H., Saunders, R. A., & Williams, A. E. (1968) *The Mass Spectra of Organic Molecules,* Elsevier, Amsterdam, Netherlands, pp. 7, 381
(7) Desiderio, D. M. (1971) in *Mass Spectrometry: Techniques and Applications,* G. W. A. Milne (Ed.), Wiley, New York, NY, p. 32
(8) Watson, J. T. (1976) *Introduction to Mass Spectrometry,* Raven, New York, NY, pp. 75, 88
(9) Biemann, K. (1962) *Mass Spectrometry: Organic Chemical Applications,* McGraw-Hill, New York, NY, p. 38
(10) Fales, H. M. (1966) *Anal. Chem.* **38,** 1058
(11) Aczel, T. (1968) *Anal. Chem.* **40,** 1917
(12) Ligon, W. V. (1978) *Anal. Chem.* **50,** 1228
(13) Wiley, H. F. (1984) in *Retrospective Lectures,* R. E. Finnigan (Ed.), Thirty-Second Annual Conference on Mass Spectrometry and Allied Topics, American Society for Mass Spectrometry, San Antonio, TX, p. 102
(14) Finnigan, R. E. (1984) in *Retrospective Lectures,* R. E. Finnigan (Ed.), Thirty-Second Annual Conference on Mass Spectrometry and Allied Topics, American Society for Mass Spectrometry, San Antonio, TX, p. 117
(15) Eichelberger, J. W., Harris, L. E., & Budde, W. L. (1975) *Anal. Chem.* **47,** 995
(16) Dawson, P. H. (1986) *Mass Spectrom. Rev.* **5,** 1
(17) McGuire, J. M., Craig, J. S., & Thornton, C. B. (1983) in *Proceedings of the 31st Annual Conference on Mass Spectrometry and Allied Topics,* American Society for Mass Spectrometry, Boston, MA, p. 232
(18) Grindstaff, Q. C., Ternes, T. N., Almond, M. F., & Kearns, M. (1986) in *Proceedings of the 34th Annual Conference on Mass Spectrometry and Allied Topics,* American Society for Mass Spectrometry, Cincinnati, OH, p. 208
(19) Donnelly, J. R., Tondeur, Y., Sovocool, G. W., & Mitchum,

R. K. (1986) in *Proceedings of the 34th Annual Conference on Mass Spectrometry and Allied Topics,* American Society for Mass Spectrometry, Cincinnati, OH, p. 203

(20) Tondeur, Y., Niederhut, W. J., Campana, J. E., Mitchum, R. K., Sovocool, G. W., & Donnelly, J. R. (1988) *Biomed. Environ. Mass Spectrom.* (in press)

(21) Foltz, R. L. (1983) in *Proceedings of the 34th Annual Conference on Mass Spectrometry and Allied Topics,* American Society for Mass Spectrometry, Cincinnati, OH, p. 454

(22) Cody, R. B., Kinsinger, J. A., & Weil, D. A. (1987) in *Proceedings of the 35th Annual Conference on Mass Spectrometry and Allied Topics,* American Society for Mass Spectrometry, Denver, CO, p. 104

TECHNICAL COMMUNICATIONS

Direct Gas Chromatographic Analysis of Trichlorfon for Its Decomposition Products

STEPHEN C. SLAHCK
Mobay Corp., Agricultural Chemicals Division, PO Box 4913, Hawthorne Rd, Kansas City, MO 64120-0013

The analysis of trichlorfon by direct gas chromatography is discussed. A procedure is described for the determination of impurities in trichlorfon by using a fused silica capillary column and cold on-column injection. Attempts at quantitating trichlorfon using this system were unsuccessful due to problems with irreproducibility of the peak area. Although no derivatization was performed, there was no decomposition of the analyte as confirmed by mass spectrometry.

Trichlorfon (Dylox®), dimethyl (2,2,2-trichloro-1-hydroxyethyl)phosphonate, is a popular, effective insecticide for the control of a variety of pests that infest vegetables, fruit and field crops, and ornamentals. By far, most analytical methods determine trichlorfon by gas chromatography (GC). Trichlorfon is a thermally labile compound that is readily decomposed to dichlorvos (2,2-dichloroethenyl dimethyl phosphate) under GC conditions (1, 2), as well as to chloral and dimethyl phosphonate (3, 4) as shown in Figure 1. Occasionally, chromatographers have not recognized the thermal lability of trichlorfon and have reported trichlorfon and dichlorvos retention times in a method as being coincident. This appears to be the case with Watts and Storherr (5), who reported trichlorfon and dichlorvos to have the same relative retention times on 4 different GC columns. Recognizing the instability of trichlorfon, many researchers have developed derivative methods for GC analyses. Reactions which have been used include acylation (6, 7), methylation (7, 8), and silylation (3, 8, 9). Bowman and Dame (2) discussed the applicability of a silylation procedure for the analysis of soluble powder formulations.

The advent of fused silica open-tubular (FSOT) capillary technology has led to the availability of GC columns with highly inert surfaces; this has made analyses that were previously only possible using derivatization amenable to GC. The cold, on-column injector has provided a similar improvement by eliminating the flash pyrolysis associated with conventional injection systems. Using these improvements in capillary chromatography, an attempt was made to assay trichlorfon and provide information concerning its decomposition products.

METHOD

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5890 equipped with flame ionization detector and cold, on-column injector. *Operating conditions:* attenuation, 2^2; detector, 250°C; flow rates, air 400 mL/min, helium column ca 1 mL/min, helium make-up 19 mL/min, hydrogen 45 mL/min; range, 2^3; recorder chart speed, 30 cm/h; recorder range, 1 mV; column oven temperature program: initial hold time 0 min, initial temperature 40°C, rate of heating 10°/min, final temperature 280°C, final hold time 5 min.

(b) *Chromatographic column.*—Dimethyl silicone fluid-coated, 12 m × 0.2 mm id, FSOT capillary (Hewlett-Packard Cat. No. 19091-60010).

(c) *Syringe.*—≤10 μL, equipped with 180 × 0.17 mm id fused silica needle.

(d) *Reference standards.*—Mobay Corp., Agricultural Chemicals Division, PO Box 4913, Hawthorne Rd, Kansas City, MO 64120-0013.

Determination

Preparation of standard solutions.—Accurately weigh 200–300 mg of each standard into separate 100 mL volumetric flasks, dilute to volume with acetone, and mix thoroughly. Pipet 10 mL aliquot of each stock solution into separate 100 mL volumetric flasks. Dilute contents of each flask to volume with acetone and mix thoroughly. Inject 1 μL portions of each solution into cold, on-column injector, using syringe equipped with fused silica needle; immediately start temperature program. Inject each standard in duplicate. Peak areas should agree within ±1% of their average.

Preparation of samples.—Accurately weigh amount of sample representing 250 mg trichlorfon into 100 mL volumetric flask, dilute to volume with acetone, and mix thoroughly. Inject 1 μL portion under the same conditions as the standards. Inject samples in duplicate. Peak areas should agree within ±1% of their average.

Results and Discussion

A simple method was sought for the assay of trichlorfon and evaluation of its volatile decomposition products: trichloroacetaldehyde (chloral), dichlorvos, and dimethyl phosphonate. These compounds are not typically found as measurable impurities in freshly formulated trichlorfon. In traditional gas chromatography, the thermal lability and low UV absorptivity of trichlorfon and its impurities dictate that trichlorfon be derivatized to a stable adduct; when injected under conventional GC conditions, the derivative remains intact for the determination of impurities or decomposes to species different from the analyte of interest.

A direct-injection gas chromatographic method was examined which would resolve these impurities and provide a sufficiently inert system to avoid decomposition, thus eliminating the need for derivatization. A fused silica capillary column and a cold, on-column injector provided significantly

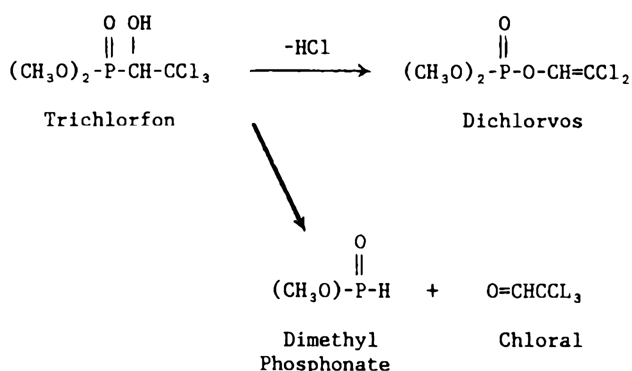
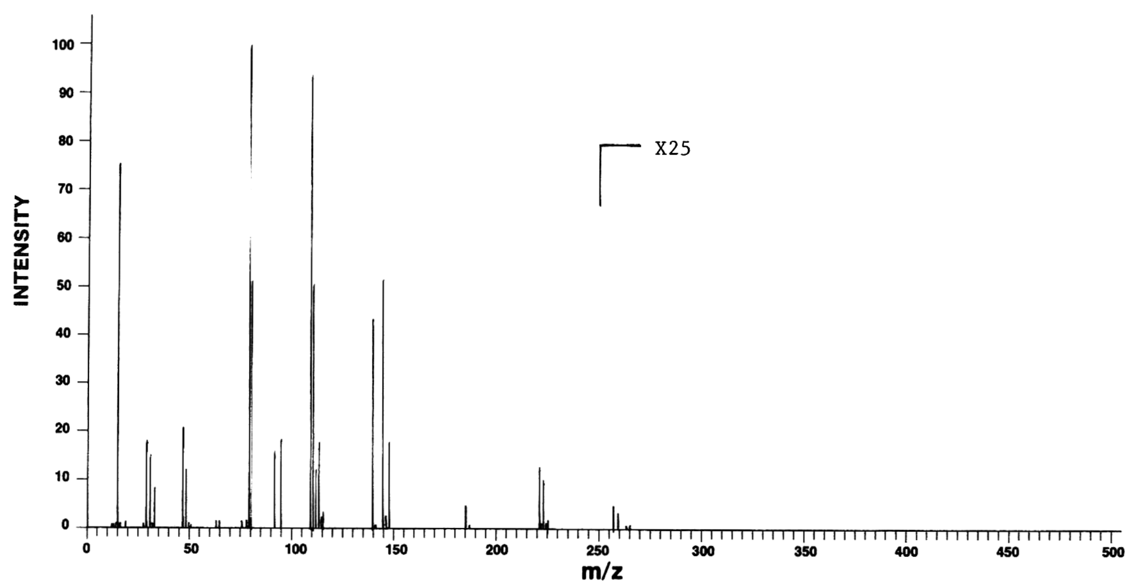


Figure 1. Decomposition routes of trichlorfon.

Received April 17, 1987. Accepted July 20, 1987.
Dylox is a registered trademark of Bayer AG, Leverkusen.



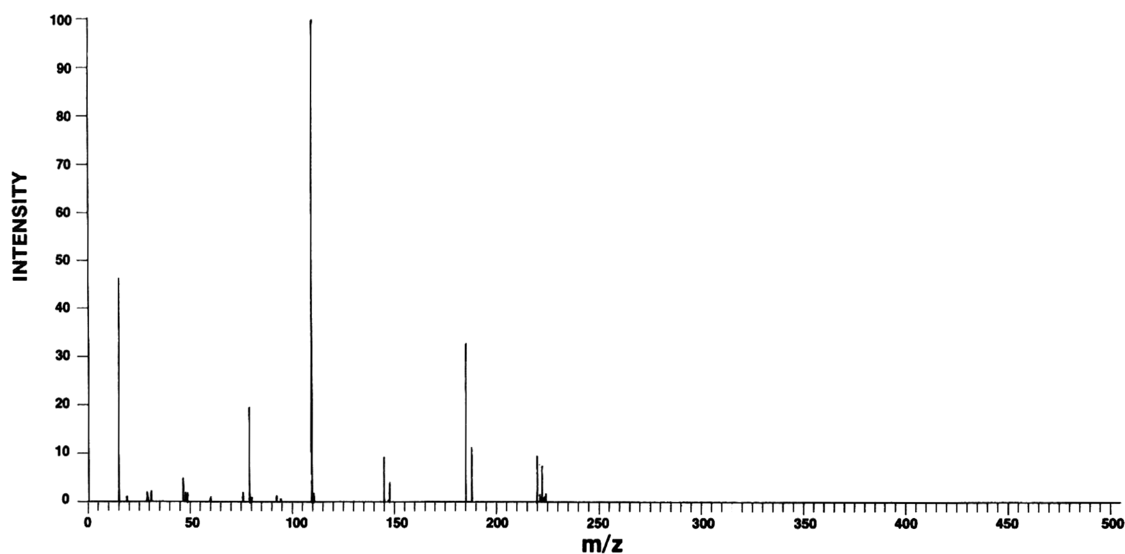


Figure 2. Mass spectral comparison of trichlorfon and dichlorvos after capillary chromatography.

more inert conditions and provided the needed separation, sensitivity, and specificity.

Initially, a 0.2% (w/v) acetone solution of primary standard trichlorfon (purity 99.8%) was injected into the direct-injection gas chromatographic system. This resulted in the elution of only one peak after the solvent front with a retention time of 8.4 min. Subsequent injections of reference standard solutions of trichloroacetaldehyde, dimethyl phosphonate, and dichlorvos indicated that each standard component was resolved from trichlorfon (especially dichlorvos). The retention times of trichloroacetaldehyde, dichlorvos, and dimethyl phosphonate (Figure 2) reference standards were 1.0, 1.7, and 4.9 min, respectively. These impurities were not detected in the primary standard at sensitivities of 0.08, 0.01, and 0.01%, respectively. The poorer sensitivity for trichloroacetaldehyde is due to its volatility. It is more difficult to resolve adequately from the solvent front.

Repetitive injections of the primary standard solution were made. No evidence of decomposition, i.e., peaks at the retention times of the impurity standards, was detected. Mass spectrometry was performed under identical direct-injection gas chromatographic conditions to confirm that the peak which eluted from the standard injection was indeed trichlorfon and was apparently free of decomposition. An examination of this peak on a Finnigan Model 4500 quadrapole mass spectrometer system provided the trichlorfon spectrum shown in Figure 2. The standard chromatographic peak was evaluated throughout for traces of dichlorvos, the major decomposition product normally observed during conventional GC analyses, by manually searching for a trace mass spectrum of dichlorvos. None was detected.

As shown in Figure 2, the low resolution electron ionization spectrum of trichlorfon does not yield a parent peak. Its fragmentation pathway progresses initially through a chlorine radical loss to give the 221 m/z fragment and related isotopic peaks. Dichlorvos does give a strong parent at 220 m/z which

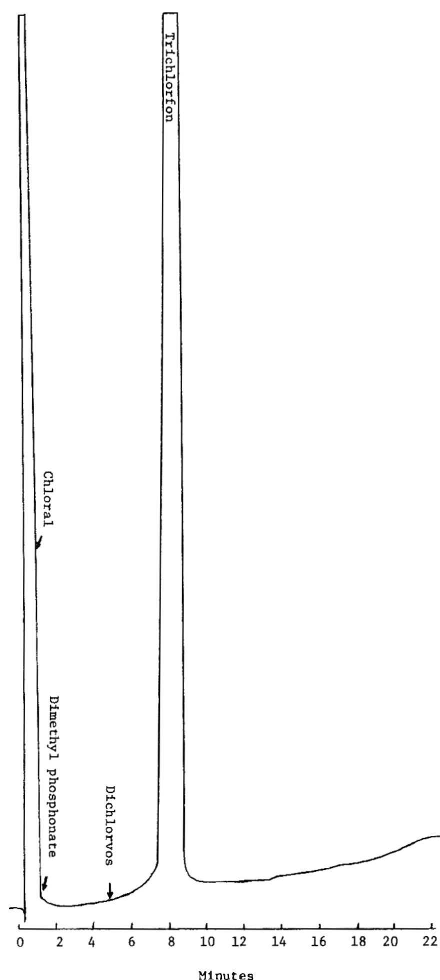


Figure 3. Direct GC chromatogram of trichlorfon primary standard.

is readily discernible from trichlorfon. Trichlorfon showed a unique fragment at 139 m/z which apparently represented either $(CH_3O)_2$-P(O)-CH-OH]$^{+\cdot}$ or $(CH_3O)_2$-P(O)-O-CH_2]$^{+\cdot}$. An examination of the mass spectra of the direct-injection capillary peaks showed only those typical of trichlorfon.

Since the direct-injection gas chromatographic technique provides a peak which represents only trichlorfon, an assay method seemed possible. However, a major obstacle surfaced immediately during method development. Replicate injections of 100 ng trichlorfon primary standard gave nonreproducible peak areas and peak heights, with and without an internal standard. Examination of data system interpretations of the peaks by using a graphics terminal showed no indications of errors. However, measurements of peaks from repetitive injections varied by ±5% relative to their average. This variance was a decade larger than expected.

Numerous empirical parameters were examined in an attempt to alleviate this situation. However, neither varying the trichlorfon concentration and the injection size nor examining other capillary coatings (e.g., OV-225) improved the results. Research on a wide-bore capillary column also provided no significant improvement. Insufficient resources were available to examine recent commercially released, thick-film coatings which might provide adequate results. With unsatisfactory reproducibility of the primary standard measurements, no further research toward the assay of trichlorfon was performed.

Although an assay method for trichlorfon using the direct injection GC technique could not be developed, this technology does provide the inert conditions necessary to examine volatile decomposition products in trichlorfon without the need for performing derivatizations; no measurable amounts of decomposition products form during the chromatography. A typical GC chromatogram with known decomposition product retention times identified is shown in Figure 3.

This in turn indirectly provides an overall indication of sample purity. A combination of the cold, on-column injection technique with commercially available thick-film, wide bore, fused silica capillary columns may provide the conditions necessary for reproducibility of injections. This would provide a simple and efficient method for the assay of trichlorfon.

References

(1) Flora, T. (1972) *Acta Chim. (Budapest)* **72**, 11–22
(2) Bowman, P. B., & Dame, P. W. (1974) *J. Assoc. Off. Anal. Chem.* **57**, 1128–1131
(3) Akhtar, M. H. (1982) *J. Agric. Food Chem.* **30**, 552–554
(4) Devine, J. M. (1973) *J. Agric. Food Chem.* **21**, 1095–1098
(5) Watts, R. R., & Storherr, R. W. (1969) *J. Assoc. Off. Anal. Chem.* **52**, 513–521
(6) Vilceanu, R., Schulz, P., Draghici, R., & Soimu, P. (1973) *J. Chromatogr.* **82**, 285–290
(7) Look, M., & White, L. R. (1981) *J Agric. Food Chem.* **29**, 673–674.
(8) Zitko, V., & Sergeant, D. B. (1977) *Fish. Mar. Serv. Res. Dev. Technol.* 714
(9) Sergeant, D. B., & Zitko, V. (1979) *Fish. Mar. Serv. Res. Dev. Technol.* 886.

Semiquantitative Enzyme-Linked Immunosorbent Assay of Soy Protein in Meat Products: Summary of Collaborative Study

JON E. McNEAL[1]
U.S. Department of Agriculture, Food Safety and Inspection Service, Science, Chemistry Division, Washington, DC 20250

An enzyme-linked immunosorbent assay (ELISA) for the determination of soy protein in meat products (1) was subjected to collaborative studies in 1984 and 1985 (2, 3). The results of the 1985 study were reported for 5 meat products containing different known levels of one of 5 different commercial soy ingredients submitted to 26 laboratories in 10 European countries. Sample 7 was distributed as a blind duplicate pair; collaborators also received blank samples. An evaluation of the data (Table 1) shows that the procedure provides more than an identification of soy, and is, in fact, a quantitative method if the nature of the added soy protein is known and especially if a sample of the specific soy is available for calibration. Because this is not always possible, the original authors have agreed to designate the method "semiquantitative," and they continue to study improvements in the quantitative aspects of the methodology as well as the use of commercial kits to perform the method. The method recommended by the Associate Referee has been adopted official first action.

Table 1. Analysis of variance of collaborative data for ELISA of soy protein in meat products[a]

Statistic	Sample 2	3	5	6	7[b]
Mean, %	1.85	2.75	1.71	2.17	4.24
SD					
Repeatability	0.39	0.32	0.37	0.31	0.45
Reproducibility	0.71	0.60	0.54	0.67	1.22
CV, %					
Repeatability	21.2	11.8	21.4	14.3	10.7
Reproducibility	38.4	21.8	31.6	30.9	28.8
% Laboratory/total variance	70.2	70.5	54.1	78.8	86.1
Av. CV_0 15.9%					
Av. CV_x 30.3%					
S_0 range 0.31–0.45					
S_x range 0.54–1.22					

[a] Data recalculated from 1985 collaborative study (3).
[b] 4 of 31 observations eliminated by Cochran test (laboratory Q, 3 results; laboratory M, 1 result).

Summary of Method

The meat sample is reduced to an acetone-dried powder, which is solubilized in a hot, concentrated solution of urea in aqueous buffer. The solution is cooled, diluted, and treated with a known excess of diluted soy protein antiserum.

The soy protein in the sample, acting as an antigen, interacts with some of the corresponding antibodies. The unreacted antibody is trapped on an immunosorbent, the inside surface of a well in a plastic microtitration plate that has been sensitized with an immobilized standard soy antigen. The captured antibody is a rabbit serum globulin, which is determined after adding a second antibody (goat anti-rabbit) to which an enzyme has been covalently attached (conjugate). Each interaction is followed by a washing step to remove any nonimmobilized species. The captured enzyme (alkaline phosphatase) is determined by adding *p*-nitrophenyl phosphate as a chromogenic substrate. The optical density after incubation is measured at 405–410 nm. By preparing a semilogarithmic plot of the optical densities of a series of solutions of a standard reference soy product against their concentrations, a calibration curve is obtained to which sample optical densities can be compared.

Recommendation

The Associate Referee, C. H. S. Hitchcock, recommended that the method be adopted official first action with an applicability statement to note that the method is semiquantitative, but may be quantitative when the nature of the added soy protein is known, especially when a sample is available for calibration.

Submitted for publication July 27, 1987.
The study published as ref. 2 was presented by R. Wood at the 97th AOAC Annual International Meeting, Oct. 3–6, 1983, at Washington, DC.
The recommendation of the Associate Referee, C. H. S. Hitchcock, 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 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) **71**, January/February issue.
[1] J. E. McNeal is the AOAC General Referee on Meat, Poultry, and Meat and Poultry Products.

References

(1) Hitchcock, C. H. S., Bailey, F. J., Crimes, A. A., Dean, D. A. G., & Davies, P. (1981) *J. Sci. Food Agric.* **32**, 157–165
(2) Crimes, A. A., Hitchcock, C. H. S., & Wood, R. (1984) *J. Assoc. Publ. Analysts* **22**, 59–78
(3) Olsman, W. J., Dobbelaere, S., & Hitchcock, C. H. S. (1985) *J. Sci. Food Agric.* **36**, 499–507

Development of Porcine Rapid Identification Method (PRIME) by Modified Agar-Gel Immunodiffusion

MARK E. CUTRUFELLI, RICHARD P. MAGEAU, BERNARD SCHWAB, and RALPH W. JOHNSTON[1]
U.S. Department of Agriculture, Food Safety and Inspection Service, Microbiology Division, Medical Microbiology Branch, Beltsville, MD 20705

A porcine rapid identification method (PRIME) has been developed for detection of pork in a wide variety of meat products. The test is an adaptation of previously developed field screening immunodiffusion tests for beef and poultry detection. The PRIME test was demonstrated to be specific, sensitive, and accurate in the analysis of samples in the laboratory and in a commercial meat processing establishment.

A porcine rapid identification method (PRIME) has been developed as a field screen for detection of pork tissue substitution or adulteration in any raw whole, ground, or formulated meat product. The test employs agar-gel immunodiffusion plates with a printed template for accurate placement of stabile freeze-dried reagent paper discs and sample discs saturated in meat tissue fluid. Within 18–24 h at room temperature, fusion of a sample immunoprecipitin line with a reference band formed in the agar between reference antigen and antibody discs indicates presence of pork in the sample. This basic procedure was established and described in previous publications on the ORBIT (1) and PROFIT (2) tests for respective identification of beef and poultry. Subsequent to a collaborative study (3), the method was adopted official first action by AOAC (4). This paper reports an adaptation of the method, requiring suitable reagent modifications, for detection of pork in raw meat products.

Experimental

Reagent Modifications

Prepare reference antiporcine antibody discs by impregnating blank filter paper discs (BBL No. 31039, Becton, Dickinson and Co., PO Box 243, Cockeysville, MD 21030) with 40 μL goat antiporcine albumin (Granite Diagnostics, Inc., PO Box 908, Burlington, NC 27215). Prepare porcine reference antigen discs by impregnating additional blank paper discs with 40 μL porcine serum albumin Fraction V (No. A-2764, Sigma) at 0.2% concentration in phosphate-buffered saline, pH 7.2. Let both sets of discs absorb their reagents and freeze-dry overnight as previously described (1). Prepare immunodiffusion plates as previously described (1), substituting fluorescein dye (No. F6377, Sigma) at final concentration of 1:60 000 in agar to distinguish PRIME from ORBIT and PROFIT plates.

Reaction Characteristics

Specificity for the PRIME test was determined by reacting blank paper sample discs saturated in homologous (porcine) and heterologous species meat tissue fluids against reference antibody discs. Sensitivity, as applied to ground meat mixtures, was assessed by testing prepared sample composites of known amounts of pork adulterant tissue added to ground red meat base tissue. Three replicates were tested at each adulterant level (1–22% by weight). Presence of a visible sample immunoprecipitin band that completely fused with the reference band was held as evidence of detection at a given percentage level of adulterant.

Received February 12, 1987. Accepted July 8, 1987.
[1] Microbiology Division, Washington, DC 20250.

Shelf Stability

Longevity of reagent discs was evaluated by storing some prepared antiporcine antibody discs and porcine reference antigen discs in glass vials with screw caps under conditions of room temperature and refrigeration (4°C). These discs were tested periodically to note any loss of immunoprecipitin band intensity.

Sample Analysis

To determine the accuracy and reliability of the PRIME test, 63 meat samples (Table 1) of a wide variety were first analyzed using the AOAC method (4) with reagent modifications for porcine detection described above. Twenty additional unknown samples (Table 1) were then analyzed in a blind field screen at a local processing plant in Washington, DC. Species origin of all 83 samples was confirmed by Ouchterlony agar-gel immunodiffusion techique (5).

Results and Discussion

In specificity determinations using reference antibody discs prepared with goat antiporcine serum, the following PRIME

Table 1. Results of PRIME laboratory and field trials on wide variety of meat product samples

Product	Species composition[a]	No. of samples	No. of samples with pork	Positive samples
Laboratory trial:				
Frank emulsion	bovine, pig, (chicken)	2	2	2
Frank emulsion	bovine, pig, chicken	2	2	2
Frank emulsion	bovine	6	0	0
Frank emulsion	bovine, (pig)	2	2	2
Frank emulsion	bovine, pig	2	2	2
Frank emulsion	chicken	4	0	0
Bologna emulsion	bovine	2	0	0
Bologna emulsion	bovine, pig	2	2	2
Pork sausage	pig	6	6	6
Pork sausage	pig, (bovine)	4	4	4
Minced treat	bovine, pig, (chicken)	2	2	2
German sausage	bovine, (pig)	2	2	2
Beef pattie mix	bovine, (pig)	2	2	2
Ground pork	pig, (bovine)	6	6	6
Ground pork	pig	3	3	3
Ground beef	bovine, (chicken)	2	0	0
Ground beef	bovine, (pig & poultry)	2	2	2
Ground beef	bovine	2	0	0
Whole beef	bovine	1	0	0
Whole pork	pig	9	9	9
Total		63	46	46
Field trial:				
Pork sausage	pig	4	4	4
Italian sausage	pig	8	8	8
Ground pork	pig	4	4	4
Ground beef	bovine	2	0	0
Beef patties	bovine	2	0	0
Total		20	16	16

[a] Identity of species in all samples was confirmed by Ouchterlony agar-gel immunodiffusion techique (5) using antispecies sera and extracts of authentic reference tissue. Species given in parentheses represent known adulterant tissue present in the test samples for the laboratory trial.

test reactions occurred for whole and ground tissue samples of known species origin: pig (+), horse (−), bovine (−), sheep (−), deer (−), chicken (−), turkey (−), red kangaroo (*Macropus rufus*) (−). These results demonstrate that PRIME test cross-reactivity problems should not arise if suitably reacting antiporcine serum is used for antibody disc preparation.

Sensitivity studies indicated that adulterant pork was detectable at the 5% and 3% levels in beef and sheep tissue bases, respectively (data not shown). Immunoprecipitin bands were, as expected, very weak at the endpoints. These sensitivity levels were considered highly adequate for the intended use of the PRIME test as a screening procedure.

Tests of shelf stability revealed that prepared PRIME reagent antigen and antibody discs stored for 1 year at 4°C produced immunoprecipitin bands of intensity equal to that of freshly prepared reagent discs. Antibody discs stored at room temperature produced lines of markedly decreased intensity after 6 months. Therefore, it is recommended that reference reagent discs be stored in the refrigerator to maximize shelf life.

Results of the laboratory and field trial analyses of samples are shown in Table 1. Of 83 total samples analyzed, 62 contained porcine proteins and gave positive PRIME reactions. Twenty-one samples, devoid of porcine proteins, gave negative reactions. The absence of any false positive or false negative reactions demonstrates the accuracy and reliability of the PRIME test. However, since the test is intended as a screening procedure, it is recommended that positive results always be confirmed by using the traditional Ouchterlony immunodiffusion technique (5) or by using isoelectric focusing (6), especially when legal action is considered for violative results.

The PRIME test, as well as its predecessors, the ORBIT and PROFIT tests, are commercially available in kit form at this time. The tests are used internationally by the U.S. Department of Agriculture, other national governments, and commercial laboratories to assure accurate labeling of meat products.

References

(1) Mageau, R. P., Cutrufelli, M. E., Schwab, B., & Johnston, R. W. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 949–954
(2) Cutrufelli, M. E., Mageau, R. P., Schwab, B., & Johnston, R. W. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 483–487
(3) Cutrufelli, M. E., Mageau, R. P., Schwab, B., & Johnston, R. W. (1987) *J. Assoc. Off. Anal. Chem.* **70**, 230–233
(4) "Changes in Methods" (1987) *J. Assoc. Off. Anal. Chem.* **70**, 389–390, sec. **24.C01-24.C06**
(5) Fugate, H. G., & Penn, S. R. (1971) *J. Assoc. Off. Anal. Chem.* **54**, 1152–1156
(6) Hamilton, W. D. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 119–122

ABSTRACTS: AOAC EUROPE IV/ASFILAB SEMINAR

Evolution of Vitamin Determinations in Food and Feed

ULRICH MANZ
F. Hoffmann-La Roche & Co., Ltd, 4002 Basle, Switzerland

Vitamins can be analyzed by biological, microbiological, and physicochemical methods. Biological methods are closely associated with the discovery of particular vitamins. These methods, time-consuming and expensive, are now used for basic research such as testing bioactivities of vitamins and vitamers. Microbiological methods make use of bacteria growing only in the presence of the vitamin to be tested. They are applied for water-soluble vitamins and their derivatives. However, bacteria growth is also influenced by other ingredients present in premixes and feeds (antibiotics, etc.). Physicochemical methods (LC, GC) are presently the most widely used procedures for determining vitamins; in the last 40 years, such methods have undergone enormous development and evaluation. Their precision and specificity have increased as well as their sensitivity. It is possible now to determine vitamins at extremely low concentrations in, for example, plasma or tissue, or to handle samples as small as a few milligrams. Some examples are vitamin K_1 in plasma and vitamin E and its homologs and vitamin A in feedstuff. However, the determination of small amounts of vitamin A in feedstuff is still a problem due to the variation of the vitamin distribution in the samples.

Fluorimetric Detection of Vitamins: Application of Fluorescence to the Analysis of Vitamins A, E, B_1, B_2, B_6, and C

J. P. ANTALICK and J. G. FAUGERE
Laboratoire Municipal de Bordeaux, France

In the field of human and animal nutrition, the control of vitamins has always been difficult because of the complexity of the medium and the low concentrations present. For some vitamins, liquid chromatography (LC) is the preferred method of analysis. Fluorimetric detection, because of its specificity and sensitivity, allows in many cases a simplification of the methods by elimination of the purification steps. Retinol, tocopherols, riboflavine, and pyridoxine, because they are naturally fluorescent, may be detected directly. Thiamine and ascorbic acid must undergo pre- or post-column derivatization. Thiamine may be transformed in Thiochrome by a post-column reaction with potassium ferricyanide. Ascorbic acid may be analyzed after oxidation by orthophenylene diamine in dehydroascorbic acid in a pre-column reaction. The best results are obtained with hydrosoluble vitamins. With fat-soluble vitamins, the increase in sensitivity is, perhaps, less important, but the fluorescence measurement simplifies the chromatograms.

The Quantitative Determination of Cyanocobalamin in Pharmaceutical Preparations by Liquid Chromatography

JAAP P. DE KLEIJN and CHRIS C. JANSEN
Organon Int. b.v., Oss, The Netherlands

In pharmaceutical vitamin products, cyanocobalamin (vitamin B_{12}) is mostly present in very small amounts. Determination of the cyanocobalamin potency is usually performed by microbiological procedures. Since microbiological methods have wide confidence intervals and long analysis times, we investigated the possibilities for a chemical determination, based on LC. The application of solid phase extraction procedures simplifies the sample preparation. Cyanocobalamin (CN-Cbl) is extracted from a tablet or oily preparation by 0.05M NaH_2PO_4, and subsequently adsorbed on C-18 material and then eluted with a methanol-0.05M NaH_2PO_4 (50 + 50) solution. Dyes can be removed by the use of a strong anion exchange column. A derivative of CN-Cbl is used as an internal standard to correct for volume changes and losses during the extraction procedure. Chromatography is performed on a conventional C-18 column using a 15–50% methanol gradient in 0.01M NaH_2PO_4 in 15 min. CN-Cbl is detected at 550 nm. The method is very accurate (mean recovery, 100.1%) and precise (RSD, 1.8%). Chromatographic and microbiological results are in excellent agreement.

Progress in Determination of Group B Vitamins

M. BOURGEAY-CAUSSE, M. GUILLAUMONT, and M. LECLERCQ
CERNIP-Institut Pasteur de Lyon et du Sud-Est France

Analytical methods for assay of group B vitamins in foods have progressed considerably in the past 10 years or so. An increasing number of LC and GC methods now tend to supersede traditional (microbiological or chemical) methods. Technically, LC methods are less homogeneous than microbiological methods and allow measurement of only one chemical compound, but they are more rapid and allow measurement of several components. All microbiological methods listed in the past 40 years measure biological activity, but the drawback is that they are time-consuming and only a few laboratories can perform these tests. It proved easy to correlate microbiological with biological methods in the animal model, since both methods measure biological activity. It is more difficult to correlate LC analysis with these methods. Each method has its own specific problems, but all methods must address sample preparation, which remains of prime importance. Thiamine is often given as a supplement to the diet and this raises the issue of the vitamin status of the French population. Thiamine will serve as an example for a study of the various problems associated with the development of the methods.

Presented at the AOAC Europe IV/ASFILAB (Association des Responsables de la Qualité et Fiablité Analytique) Seminar, October 20–21, 1987, Neuilly-sur-Seine, France.

Modern Methods for Determination of the Fat-Soluble Vitamins

LAWRENCE ROSNER
Aqualab, Inc., Chicago, IL 60607

The application of liquid chromatography has improved the specificity and sensitivity of many of the vitamin assays. It has likewise shortened the time required for some of the analyses. The methodology for each of the fat-soluble vitamins will be discussed. This will include preparation of samples, a description of chromatography conditions, and problems in measurement. In addition, data will be presented showing comparisons between results obtained from LC methods and those from conventional non-LC methods. Generally, the LC results are lower, due to greater specificity in measurement of true vitamin content.

New Approaches in Vitamin Analysis: Objectives and Strategy

PAUL M. FINGLAS, DAVID A. T. SOUTHGATE, and MICHAEL R. A. MORGAN
AFRC Institute of Food Research, Norwich Laboratory, Colney Ln, Norwich, Norfolk NR4 7UA, UK

Vitamin analyses form an essential component of the assessment of the nutritional value of foods and diets and the widespread adoption of nutritional labeling has created a substantial demand for vitamin analysis. A review of the suitability of current methods for the study of the effects of thermal processing on vitamins has identified the need for new approaches. The major issue that emerged was the lack of information on the performance characteristics of most vitamin methods in respect of precision, accuracy, repeatability, reproducibility, and overall robustness. There was also little evidence of quality assurance programs in vitamin analysis. These create a need for standard reference material for vitamins in foods which will require major research programs. Specific and less time-consuming procedures with defined methodological characteristics are required for vitamin analyses in general and especially for studies of the kinetics of vitamin degradation during processing and the optimization of processing conditions. Such methods would also have important applications in nutritional research on human nutrient requirements and the bioavailability of vitamins. The central objectives for future vitamins methodology will be outlined and discussed in relation to these applications.

The Biospecific Determination of Biotin and Pantothenate in Food

PAUL M. FINGLAS, DAVID A. T. SOUTHGATE, and MICHAEL R. A. MORGAN
AFRC Institute of Food Research, Norwich Laboratory, Colney Ln, Norwich, Norfolk NR4 7UA, UK

Microtitration plate analyses for biotin by a protein binding assay (PBA) and pantothenate by enzyme-linked immunosorbent assay (ELISA) have been developed for application to food. These assays have a number of advantages over conventional procedures for these vitamins. The analysis of biotin used a commercially available avidin-peroxidase conjugate and was validated for application to liver with a limit of detection of 10 pg biotin/well. The intra- and inter-assay CVs were 4.1% and 7.3%, respectively, and recoveries of vitamin added prior to extraction averaged 97.5%. Analysis of pooled lamb's liver samples gave a biotin content of 37 μg/100 g, which is in good agreement with that given in UK food tablets (41 μg/100 g), obtained by microbiological assay. Antisera against pantothenic acid was raised in rabbits and used in a double antibody, indirect ELISA. The assay was sensitive (detection limit = 0.5 ng/well) and specific for the vitamin. Vitamin recoveries averaged 94.3% and 91.9% for milk and potato samples, respectively, and intra- and inter-assay CVs were 10–20%. The ELISA values for pantothenate determined for 6 foods (milk, eggs, bread, potato, lettuce, and liver) compared favorably with those obtained by microbiological assay ($r > 0.99$) and those listed in food tables. Both assays have the potential for rapid, routine use with high sample processing and minimal equipment needs. They will have considerable benefits on assay time and cost when adopted by food analysts.

Simplification of the Sample Treatment for Vitamins A and E Determinations

CLAUDE F. BOURGEOIS
Produits ROCHE-Fontenay s/Bois France

Automated determination of fat-soluble vitamins in foods and feeds by modern methods is hampered by initial processing of sample. Traditional methods comprise the following steps: sample weighing, basic hydrolysis with KOH in hot alcohol solution, liquid/liquid partition of unsaponifiable with organic solvent, etc.This processing is cumbersome because large amounts of hazardous and expensive organic solvents must be used. Emulsions frequently occur. Two proposed approaches are as follows: (a) To replace liquid/liquid partition by liquid/solid: An aliquot of hydrolysis solution is layered onto a small kieselguhr column operated at normal pressure. After the aliquot is adsorbed, isooctane is poured onto the column to eluate retinol and tocopherol. Fatty samples created problems, most likely due to formation of large micelles that obstruct kieselguhr pores, so aliquots are no longer adsorbed and percolated. Use of methanol–ethanol–n-butanol (4 − 3 + 1) in the hydrolysis solution solved this problem. n-Butanol is a linear chain alcohol that reduces the size of micelles. Liquid/solid extraction was shown to be quantitative and reproducible. (b) To replace alcoholic KOH hydrolysis by DMSO tetraalkyl-ammonium hydroxide: Saponification proceeds rapidly at room temperature in such systems. Small amounts of water and methanol increase reaction rate. Greater amounts inhibit reaction. Liquid/solid extraction can be used with this system too. A DMSO technique is being studied.

Vitamin Supplementation of Foodstuffs: Regulatory Aspects and Outlooks

DANIEL KLEIN
Centre de Recherche Branche Epicerie BSN

An evolution of the life environment in the industrialized countries results in a reduction of human energy needs. Therefore, individuals have to reduce their food intake. Consequently, the level of absorbed micronutrients (e.g., vitamins and minerals) has been reduced as well. To prevent the risk of nutritional deficiencies, it is necessary to supplement foodstuffs with various micronutrients. Most industrialized countries have created legislative recommendations relative to the prevention of the above-mentioned deficiencies. In France, it is necessary to include vitamins in the foods of particular dietetic purposes (for instance, foods of reduced caloric value) at the level equivalent to the corresponding traditional foods. On the other hand, the restoration of the vitamin level lost during various technological processes in food manufacturing and storage is effective. Relevant points of view of other European countries, compared with Codex recommendations, are presented and discussed.

AOAC Overview of Conventional and Rapid Methods for Microbiological Analysis of Foods

WALLACE H. ANDREWS
Division of Microbiology, Food and Drug Administration, Washington, DC 20204

Food microbiology has long been considered an offshoot of clinical microbiology. Recently, however, food microbiologists have made great strides in establishing their field as a dynamic and independent one. One measure of this progress is seen in the quality and quantity of various procedures validated by an AOAC collaborative study. The diverse nature of these investigations is evidenced by the successful completion of such studies as the in vitro and in vivo demonstration of the invasiveness of *Escherichia coli*; use of gene probes to detect enterotoxigenic strains of *E. coli*; rapid detection of *Salmonella* spp. by using enzyme linked immunosorbent assay, hydrophobic grid membrane filter, or gene probe procedures; chromatographic detection of sporeformers in canned foods; and improved methods for *Staphylococcus aureus, Closridium perfringens,* and *Vibrio cholerae.* There is every indication that this rapid development and validation of microbiological methods for food analysis will continue unabated. This lecture will, first, provide an overview of the development of AOAC microbiological methods for food analysis, and, second, make a prediction of future trends in this rapidly evolving area.

Use of Pattern Analysis Techniques for Lactic Bacteria Counting

PATRICE BRACQUART
Laboratoire de Biochimie Appliquée Université de Nancy, I-B.P. 239-54506, Vandoeuvre les Nancy, France

The 8601 Analysor of the Bioconcept Society is dedicated to fast enumeration of microorganisms in biological media. We are testing that equipment to count the lactic acid bacteria in yogurt. Yogurt is a good model because it contains many cells of 2 different morphological bacteria: *Streptococcus thermophilus* (coccus) and *Lactobacillus bulgaricus* (rod). Preparation of the samples is easy and fast. It is necessary to use a special diluent that permits a good layer of bacteria in homogeneous casein. The bacteria are stained by acridin orange, while the casein is green. We try to use a unique program of recognition for all commercial yogurts. Generally, the recognition is better than 80%. Some lactobacilli strains and older cultures are difficult to recognize because there are granulations in the lactobacilli cells. The Analyzor has also been tested for biometric measurements. For example, differences are found in the cell tails of *S. thermophilus* of different morphological clones.

New Aspects of Traditional Enrichment of *Salmonella*

MARTIN BUSSE
Bakteriologisches Institut, Suddeutsche Versuchs- und Forschungsanstalt fur Milchwirtschaft, Weihenstephan, D-8050 Freising, FRG

New techniques, for example, ELISA tests, will to some extent replace traditional enrichment methods of *Salmonella* determination. This does not imply that enrichment procedures will be abandoned completely. Unfortunately, very little is known on the course of events during enrichment. Usually, enrichment broth is inoculated with 10% pre-enrichment solution. However, better results are obtained if a smaller inoculum is used. This effect may be due to the fact that, with a high inoculum, growth is terminated after relatively few steps of division. Under such conditions, successful enrichment may be impossible. Experimental data are presented which corroborate this assumption. In experiments on the time course of multiplication of *Salmonella* and other Enterobacteria, it has been shown, however, that too high an inoculum is not the only source of erratic results of the enrichment procedure. For example, immediately after inoculation of the selective broth, the *Salmonella* count may decrease considerably. At low initial counts, this may lead to a failure of enrichment.

Epifluorescence Microscopy and Reflectance Colorimetry for Counting Bacteria and Somatic Cells in Raw Milk

REMY GRAPPIN
INRA, Station Expermentale Laitiere, 39800 Poligny, France

GARY H. RICHARDSON
Utah State University, Logan, UT 84322-8700

Total bacteria count (TBC) and somatic cell count (SCC) are 2 important criteria of milk quality associated with quality of dairy products. TBC indicates the hygienic conditions in which milk has been produced and stored; SCC is related to mastitis, which causes significant losses and chemical modifications of milk. TBC and SCC are routinely performed in dairy laboratories. Epifluorescence microscopy is a good alternative method for the standard plate count method (SPC) for TBC and the microscope method for SCC. The direct

epifluorescence filter technique (DEFT) is a simple, rapid method for TBC or SCC. On 167 raw milk samples, ranging from 7×10^3 to 6×10^6 CFU/mL, the residual $\log_{10}$ SD of the regression SPC vs DEFT was: sy, x = 0.245 CFU/mL and the repeatability CV of the DEFT was: CV_r = 16.8%. Similar figures were obtained with SCC. The Bacto-Scan (Foss-Electric, DK), a fully automated instrument, was found suitable for TBC measurement, with a limit of detection of 30×10^3 CFU/mL, CV_r = 5.9%, and sy, x = 0.236. With the increasing need of better control of the bioactivity of milk and the advance in electronics and robotics, a new fully automated instrument has been recently developed based on reflectance colorimetry. Preliminary investigations show that among other criteria, this instrument can measure bacterial activity in milk and detect abnormal milk, providing estimates of TBC and SCC.

Use of Impedance and Optical Density Instruments in Food Microbiology

HJORLEIFUR EINARSSON
SIK-The Swedish Food Institute, Box 5401, S 402 29 Goteborg, Sweden

During the last 10–15 years, instruments based on impedance measurements for detection and enumeration of microorganisms from various food have been available commercially. The results of the instrument readings can be presented either as "total-count" or as the number of specific bacteria, such as "coliforms." Because the changes in impedance are the results of cell metabolism, these instruments can be used to study microbial activity and are thereby useful for quality control of growth media, for studying activity of starter cultures, and for vitamin analyses. Impedance measurements are normally calibrated against traditional counting methods like pour-plate or surface spreading. However, the readings from the instruments can be used more directly too, i.e., to estimate shelf-life of foods or for quality control. Lately, instruments based on optical measurements have been adapted for use in the food microbiology laboratory. In contrast to the impedance instruments, these instruments must have transparent media. On the other hand, the media can have different pH and salt concentrations which are limiting factors for the impedance instruments. The instruments are based on optical measurements and therefore are more suitable where different combinations of pH, salt, and other ionic ingredients are to be tested. The main advantages of both methods are that they allow many samples to be treated simultaneously (200 to 500). Since the systems are computerized, the results can, in many cases, be obtained much faster than with conventional methods.

Some Studies on Methodology for Recuperation of *Listeria monocytogenes* from Foods with Special Reference to Soft Cheeses

LYNTON J. COX, ANNEMARIE RENAUD, and M. VAN SCHOTHORST
Microbiology Section, Nestec Ltd, Central Quality Assurance Laboratory, 55 Av. Nestle, CH-1800 Vevey, Switzerland

Traditional cultural procedures are essential even in modern methodologies, due to low sensitivities (e.g., ELISA, gene probes). Study of the dynamics of growth is necessary to optimize recovery. Dynamics of enrichment of *Listeria* have been studied in the presence and absence of food material and competitive flora in TSB-YE + antibiotics (FDA medium) at 30°C and 10°C. The lag phases in the absence of cheese were 6 h and 4 days at 30°C and 10°C, respectively. In the presence of pasteurized cheese, lag phases of 4 h and 2–3 days were observed at 30°C and 10°C, respectively. Final concentrations of log 7.0 were obtained after 24 h at 30°C and log 5.0 after 7 days at 10°C in the presence of cheese. Similar growth curves are obtained for group D streptococci (a major competitive organism) at 30°C in E-broth and in the isolation medium. At 10°C, the lag phase is longer for these organisms than for *Listeria*. Further results are presented for the dynamics of growth in cheese in the presence of high numbers of competing organisms, estimated using selective enrichment (post-enrichment), non-selective enrichment, and 3 isolation media.

Evolution, Interests, and Limitations of Some Immunological Techniques and Food Microbiology

CECILE A. LAHELLEC
Poultry Research Centre, Ministry of Agriculture, Veterinary Service, PO Box 9, 22440 Ploufragan, France

From the last decade, immunological techniques, which were already used in different fields of research, especially in clinical laboratories, have also been more and more applied in food microbiology. Some reasons may explain that evolution, i.e., changes in food technology and necessity to control the industrial production more closely, but it must be noted that the conception of food microbiology has also changed, aiming more and more toward toxins investigations or only examining simultaneously a large number of samples. The applicability of immunological techniques in food microbiology must not be underestimated. Their specificity and sensitivity and the possibilities of automation are such that those techniques will probably develop extensively and be more and more helpful. Nevertheless, one must keep in mind that "immunology" and "miracle" techniques are not synonyms. The problem, inherent in the nature of the food products themselves, for the detection of some microorganisms is that, in many cases, it remains necessary to maintain the enrichment phase(s). Furthermore, in some cases, much remains to be done for their universal acceptance. Anyway, immunological techniques, even if they cannot solve all the problems by themselves, surely hold great promise.

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, 1111 N 19th St, Suite 210, Arlington, VA 22209, 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").

1/88

กำหนดส่ง

The JOURN ... ATION OF OFFICIAL A ... STS

EDITORIAL PROFILE

The *Journal of the AOAC* is the ... ie United States and more
of the Association of Officie ...
(AOAC). Published for analytica ... is a principal forum for ex-
and use analytical methodolog ... ng methods researchers in
scribers in their day-to-day wo ... regulated industries.
composition and contamination, ... has an average paid circula-
cosmetics, agricultural and hou ... ers and a referenced reader-
analysis, and environmental ... urchasers and specifiers of
analytical chemists, biologists ... supplies, testing apparatus,
chemists, toxicologists, specti ... ed products and services.
forensic and other scientists i ... *nal* because it is profession-
tive, and top management posi ...

ADVERTISING UNITS AND RA...

Size	1X		
Full Page non-bleed, 7 × 10" bleed, 8½ × 11¼"	$75...		
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.

...und by any condition other than
...er advertising materials.
...ct to prior approval by the Edi-
...e *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
1111 North 19th Street, Suite 210
Arlington, Virginia 22209
(703) 522-3032

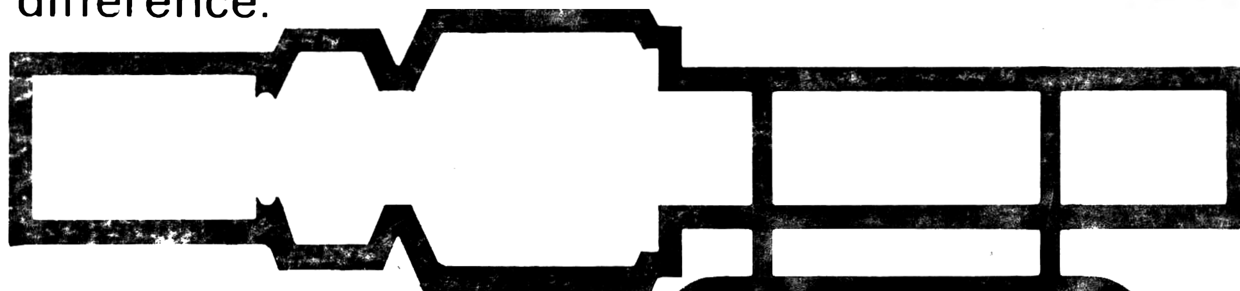
Each one is our special concern

CUSTOM PACKED HPLC COLUMNS

Custom packing HPLC columns has become our specialty. Any length, several ID's (including 3.2mm) and almost any commercially available packing material may be specified. We'll supply the column others won't.

With each column, you will receive the original test chromatogram plus a vial of the test mixture. Our advanced technology and computer testing is your assurance of a quality product.

When custom packing and testing is your special concern, we make the difference.



For further information contact:

ALLTECH ASSOCIATES, INC.
2051 Waukegan Road
Deerfield, Illinois 60015
312/948-8600

ALLTECH ASSOCIATES

CIRCLE 2 ON READER SERVICE CARD