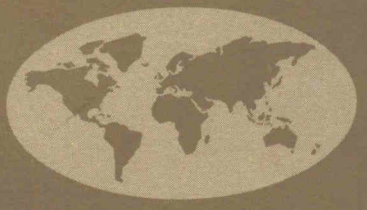
July/August 1992 Volume 75 Number 4



JOURNAL OF AOAC INTERNATIONAL

The Scientific Association Dedicated to Analytical Excellence

AOAC INTERNATIONAL

JAINEE 75(4) 591-772 (1992)
ISSN 1060-3271

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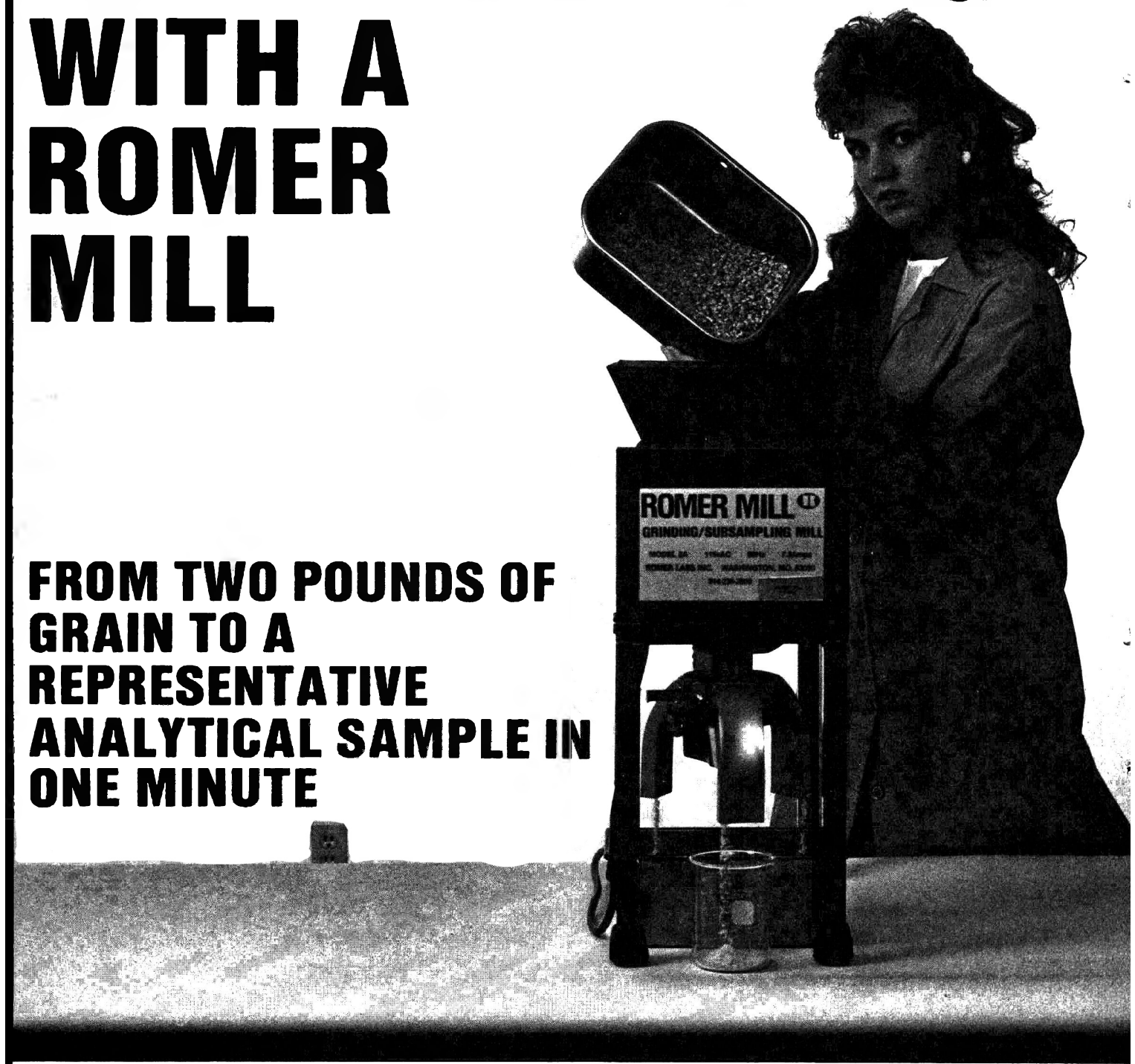

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A Peer-Reviewed Journal Publishing Basic and Applied Research in the Analytical Sciences Related to Foods, Drugs, Agriculture, and the Environment

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The *Journal of AOAC International* (ISSN 1060–3271) is published bimonthly by AOAC International, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. **Subscriptions** for Vol. 75 (1992) are: Members, $110.00 in U.S., $145.00 outside U.S.; Nonmembers, $160.00 in U.S., $190.00 outside U.S. Second class postage paid at Arlington, VA, and additional mailing offices. Abstracted or indexed in AGRICOLA, Analytical Abstracts, Biological Abstracts, Cambridge Scientific Abstracts, Chemical Abstracts, Current Advances In Ecological Sciences, Current Contents, and others. Printed in USA.
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For Your Information

Meetings

August 30–September 3, 1992: 106th AOAC International Annual Meeting and Exposition. Cincinnati, OH. Contact: AOAC Meetings Dept, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

August 29–30, 1992: AOAC Short Courses, Cincinnati, OH. Topics: QA, Lab Waste, How to Testify as an Expert Witness. Contact: AOAC Meetings Dept, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

August 30 and September 4, 1992: AOAC Board of Directors Meeting, Cincinnati, OH. Contact: Nora Petty, AOAC, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, telephone 703/522-3032.

September 3–4, 1992: AOAC Short Courses, Cincinnati, OH. Topics: Microbiological QA, Statistics, Improving Technical Writing Skills. Contact: AOAC Meetings Dept, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

October 8, 1992: MidAtlantic USA AOAC Regional Section Meeting. Contact: David B. MacLean, 6422 Alloway Ct, Springfield, VA 22152, telephone 703/451-1578.

October 12–17, 1992: AOAC Short Courses, St. Louis, MO. Topics: QA, Microbiological QA, Lab Waste, How to Testify as an Expert Witness, Statistics, Safety, Improving Technical Writing Skills. Contact: AOAC Meetings Dept, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

November 11, 1992: East European AOAC Subsection Meeting, Bratislava, Czechoslovakia. Contact: Margreet Lauwaars, PO Box 153, 6720 AD Bennekom, The Netherlands, telephone 31 8389 18725.

November 17–20, 1992: Central AOAC Regional Section Meeting, Kalamzaoo, MI. Contact: Sungsoo Lee, Kellogg Co., 235 Porter St, PO Box 3423, Battle Creek, MI 49016-3423, telephone 616/961-2823.

February 1–4, 1993: Southeast USA AOAC Regional Section Meeting, Atlanta, GA. Contact: Jan Hobson, Griffith

Corp., Rocky Ford Rd, PO Box 1847, Valdosta, GA 31603-8635, telephone 912/242-8635.

March 29–30, 1993: Europe AOAC Regional Section Meeting, Barcelona, Spain. Contact: J. Sabater, Laboratorio Dr. J. Sabater Tobella, Calle de Londres 6, 08029 Barcelona, Spain, telephone 34/3-410-9343.

July 25–29, 1993: 107th AOAC International Annual Meeting and Exposition, Washington, DC. Contact: AOAC Meetings Dept, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

Changes in 1992 AOAC International Annual Meeting

This year, for the first time, AOAC International will mail badges and tickets to everyone pre-registered for the annual meeting by the August 3 pre-registration deadline. These attendees will pick up their badge holders and meeting programs at the Pre-Registration Desk in Cincinnati, OH, and will be on their way in minutes.

This streamlined advance registration process will result in shorter lines and faster processing of on-site registrations, and is just one of several changes to make the upcoming 106th AOAC International Annual Meeting, August 30–September 3, 1992, the best ever.

Badges will be coded so registration personnel can identify members at a glance. If you forget your badge and tickets, replacements will be printed on-site, but the advantages of the "Speed Station" processing will be lost. AOAC Director of Administration and Meetings Marge Ridgell suggests, "Maybe we should include a bright red reminder string for attendees to tie on their index fingers—or maybe an orange neon sticker attached to the badge and tickets saying, 'Bring us to Cincinnati!'"

Event tickets will continue to be sold on-site on a space available basis, and on-site registrations will be handled as in past years.

The Opening Session will have a new look this year! The Wiley Award Address, formerly presented at the Opening Session, will now be the featured presentation of the Wiley Award Symposium. The Keynote Address and the President's Address will continue to highlight the Opening Session, which will also include all award presentations and a short acceptance speech by the 1992 Wiley Award Winner.

The revised schedule calls for the Opening Session to end at 10:00 a.m. on Monday, at which time the exhibit hall will open. No other programs or meetings will be scheduled before 1:00 p.m. on Monday, giving attendees 3 hours in which to visit the exhibit hall. AOAC Meetings, Symposia, and Educational Programs Committee Chairman Sam Page described this change as advantageous for everyone. "For a long time, attendees have asked for more time to spend on the exhibit floor without feeling they really should be in at least two other places at once," he laughed. "This also answers long-standing exhibitor requests for a chance at the attendees' undivided attention. The committee feels this will be a welcome change for everyone."

Also new to the exhibit hall this year is the Harvey W. Wiley Award Winner's Booth. This display will feature the accomplishments of the 1992 Wiley Award Winner, including photos and memorabilia, and this year's winner will be in the booth to greet attendees from the end of the Opening Session until approximately 11:30 a.m.

The AOAC Statistics Committee will also staff a booth to provide attendees an opportunity to follow-up on Sunday's Official Methods Board statistics training. Hands-on experience and one-on-one tutoring will be available in the exhibit hall.

Another first for the 1992 AOAC International Annual Meeting is the use of a convention center for the majority of the meeting programs and events. Prior to this year, the meeting, exhibits, short courses, and workshops have been self-contained in hotels. This year, most of these will be held in the Cincinnati Convention Center, which is connected to the headquarter's hotel, the Westin Cincinnati, and most major downtown hotels, by the skywalk for convenient access rain or shine.

One major advantage of meeting in a convention center is that meeting events will be concentrated in one wing rather than spread out over a large hotel or resort property. Attendees can move from place to place more quickly and see and hear more of the technical program.

Social Program Scheduled for Cincinnati

"The Central Regional Section is excited to have the 1992 AOAC International Annual Meeting in Cincinnati," exclaims the Regional Section President Marjeanne Morrison of O.M. Scott.

The AOAC Central Regional Section includes Cincinnati, and its section members look forward to welcoming their colleagues to this Queen of the River Cities for the 106th AOAC International Annual Meeting, August 30–September 3, 1992. "We'll roll out the red carpet with some of our typical Midwest hospitality," says Morrison. "Our members plan to turn out en masse."

The President's Reception on Sunday evening begins the AOAC festivities. The global character of AOAC International will be reflected in the theme, "AOAC Around the World." This kick-off event will be held at the Westin Cincinnati, headquarter's hotel for the meeting, located in Cincinnati's historic Fountain Square.

Both refreshments and decor will take on an international flavor as the Association salutes colleagues from near and far. Members and guests have always enjoyed this opportunity to mix and mingle and share news and views. This year, attendees will have the opportunity to test their "International IQ" in AOAC's Global Quest Contest and will be eligible for a prize drawing.

During the week of the meeting, Cincinnati will offer an enticing diversity of entertainment from dawn to dusk. On

Tuesday evening, it's cruising down the Ohio River aboard the AOAC International Dixieland Riverboat Cruise. Featuring the Admiral's feast, this evening of dining aboard promises good fun and good food under the stars. Buses will depart for the dock at Covington Landing from the headquarter's hotel. Early buses can be caught to explore the attractions of Covington Landing before the "All Aboard!" sounds, or attendees can relax and take a later bus and arrive in time to be "played aboard" with a Dixie melody.

Daytime entertainment begins with the traditional Guest Welcome Coffee on Monday morning, August 31, at 9:30 a.m., in the Cincinnati Convention Center. Tour guides will be on hand to provide additional details about the scheduled daytime tours and to answer questions about the River City's history, and all it has to offer.

Following the Guest Welcome Coffee, a city tour will give an overview of Cincinnati—the parks, the monuments, the attractions, and the diverse neighborhoods that make this such an interesting and entertaining city to see and explore. Attendees can stop for lunch and taste the famous Cincinnati chili or one of the other local favorites.

On Tuesday, attendees can visit the best of the old and the new at the Museum Center at Union Terminal. This nationally acclaimed landmark, a monument to Art Deco architecture, is now revitalized as the home of the Cincinnati Historical Society, the Cincinnati Museum of Natural History, the Children's Discovery Center, and the Robert D. Lindner Family Omnimax Theater, one of a small number in North America. The tour also features world-class exhibits combined with fascinating historic spaces like the Rookwood Tea Room and the Newsreel Theater. The original Union Terminal, erected in the 1930s and best known for its rotunda and enormous mosaic tile murals, showcases the architectural drama for which this age is famous.

Wednesday offers an opportunity to experience the history and hospitality of the beautiful Kentucky Bluegrass region with a visit to the Kentucky Horse Park. Located in an area world-renowned for majestic horse farms, white-fenced countryside and million-dollar horses, the Kentucky Horse Park is everything you would expect it to be. The park is dedicated to all breeds of horses, many of which will be in the Parade of Breeds. This is your chance to pet and photograph these magnificent animals. More history can be experienced with a stroll through historic barns and the prestigious Hall of Champions and a visit to the exciting exhibits and displays that honor equine breeds and achievements from all around the world. Attendees can tour the picturesque grounds by tram, horse-drawn omnibus, or elegantly appointed carriage, and watch the farrier and the harness maker at work.

Besides the daytime tours, which offer three unique aspects of the Cincinnati area, you can find other attractions as well. The 1992 AOAC International Annual Meeting also spans two sports seasons—football and baseball. It's exhibition season for the Cincinnati Bengals, and with baseball at its peak, it's a good time to catch the Cincinnati Reds in action.

"Cincinnati has a lot to offer," says Central Regional Section President Morrison. "It's a warm and friendly city, open and safe even at night, and everything downtown is accessible with the skywalk." AOAC guests and families will find much to see and enjoy in this exciting city on the banks of the Ohio.

AOAC International Annual Meeting Symposia

The 106th AOAC International Annual Meeting in Cincinnati, OH, scheduled for August 30–September 3, 1992, is certain to be exciting and informative. The meeting features a scientific program that includes topics on the cutting edge in analytical chemistry. Meeting attendees may choose from six symposia, each designed to address rapidly changing issues in scientific practice and technology. Each symposium is described here; each promises to be thought-provoking.

■ ***The Harvey W. Wiley Award Symposium on Fumonisins: Occurrence, Distribution, Production, Analysis Chemistry, and Mode of Action*** (Part 1, Monday, August 31, 1:00–4:30 p.m.; Part 2, Tuesday, September 1, 8:30 a.m.–12:15 p.m.). The discovery of the fumonisins in 1988 and the subsequent finding that carcinogenicity, or at least the promotion of carcinogenicity, is attributed to these compounds established them as important mycotoxins that occur in feeds and foods. Also, the establishment of fumonisins as causes of leukoencephalomalacia in equine species and pulmonary edema in swine adds to the significance of these mycotoxins. The ubiquity of the producing organisms, primarily *Fusarium moniliforme* and *F. proliferatum*, and the frequent finding of fumonisins in corn are a food and feed safety concern to producers, manufacturers, and consumers.

Because of the recent discovery of these mycotoxins and the need for rapid, worldwide dissemination of information by experts from around the world, this Wiley Award Symposium was organized by Cochairmen Glenn A. Bennett and John L. Richard, U.S. Department of Agriculture. P. Frank Ross, the 1992 Wiley Award Winner and an expert on the occurrence and analysis of the fumonisins, will deliver his keynote address, "What Are We Going to Do with this Dead Horse?," at this symposium. Additionally, this symposium brings together international speakers who convey the latest information on the occurrence,

distribution, production, analysis, and mode of action of the fumonisins. Information to be presented should be of interest to the mycologist, toxicologist, plant pathologist, analytical chemist, veterinarian, medical researcher, and regulator. The symposium is sponsored by Corn Refiners Association, Inc.

■ ***Current Topics in Lipid Analysis: Labeling Considerations*** (Part 1, Wednesday, September 2, 1992, 1:30–4:30 p.m.; Part 2, Thursday, September 3, 1992, 8:30 a.m.–12:00 noon) will feature 13 speakers from government, industry, and academia. This symposium will focus on issues and procedures in lipid labeling. The symposium will consist of papers on "the schizophrenic food label," labeling of meat and poultry, labeling regulations, European labeling issues, simplification of labeling, analytical issues, appropriate and simplified methods, sample preparation, and quality control.

Among the speakers attending the symposium will be representatives from FDA, USDA, The Procter & Gamble Company, Health and Welfare Canada, Kellogg, Kraft General Foods, Hazleton Laboratories, Trinity College Medical School, and the University of Texas Health Science Center. The symposium is organized by Cochairmen Joyce Beare-Rogers, Health and Welfare Canada, and Richard McDonald, U.S. Food and Drug Administration. The symposium is sponsored by Gerber Products Company, Silliker Laboratories, The Procter & Gamble Company, and Archer Daniels Midland Company.

■ ***Process Sensors and Control*** (Part 1, Monday, August 31, 1:00–4:45 p.m.; Part 2, Tuesday, September 1, 1:30–5:00 p.m.) will feature 10 examples of process control methods. Presentations in Part 1 will include "Applications of FTIR in the Food Processing Industry," "Near Line FTIR Analysis and Validation," "Development of a Noninvasive Method for Measuring the Crude Lipid Content in Whole Fish and Fish Products," "Application of NIR to Sugar Analysis: Comparison with Traditional Methods," and "Nonglass pH Electrode for Food and Beverages." Featured presentations in Part 2 will be "Application of NMR to the Analysis of Fat and Moisture in Foods," "NMR Analysis of Solid Fat Content in Edible Oils," "Micromachined Sensors for Process Analysis of Foods and Pharmaceuticals," "Application of Sensor Arrays and Neurol Networks to Odor Sniffing," and "Application of Fuzzy Logic to Process Control."

Chairman Donald E. Carpenter, Kraft General Foods, has organized exciting and informed speakers from industry and academia to present these topics. The symposium is sponsored by Kraft General Foods, Dow Chemical Company, and Heinz, U.S.A.

■ ***Milk: Antibiotics and Other Contaminants*** (Part 1, Tuesday, September 1, 8:30 a.m.–12:00 noon; Part 2, Tuesday, September 1, 1:30–4:30 p.m.) will have speakers from four countries, including six government scientists and four academicians. Chairman Joseph A. Settepani, U.S. Food and Drug Administration, has carefully selected topics featuring novel techniques or presenting continuing research. The symposium will begin with a discussion on using immunoassay and liquid chromatography for the analysis of matrix solid phase dispersion extracts. An approach to analyzing two of the most difficult classes of animal drugs (aminoglycosides and the β-lactam antibiotics) in milk will be presented. An application of on-column concentration of residues for ampicillin in milk will be discussed. The results of several years research on determinative and confirmatory procedures for the analysis of β-lactams in meat and milk under a cooperative agreement with FDA will be presented.

For Your Information

New applications scheduled for presentation include the analysis of tetracyclines in milk; the application of reflectance colorimetry to determine milk quality; and the latest data on the theory that milk from recently mastitic cows contains substances that interfere with several commercially available milk residue screening assays. Results of a recent study on the nature and depletion of residues in milk from sulfamethazine-treated lactating cattle and data obtained while conducting an evaluation of two commercially available milk residue screening assays for chloramphenicol in milk will be presented. The final presentation will provide valuable insights into current regulatory policies and monitoring programs in the European Economic Community.

■ ***Forensic Methods and Product Tampering*** (Part 1, Wednesday, September 2, 8:30–11:30 a.m.; Part 2, Thursday, September 3, 8:30 a.m.–12:00 noon). Cochairmen Robert Bianchi, U.S. Drug Enforcement Administration, and Fredrick Fricke and Karen A. Wolnik, U.S. Food and Drug Administration, have organized an exciting and topical symposium, peppered with a wealth of anecdotal information in several disciplines, not only drug tampering cases, but FBI and FDA cases as well. The symposium will begin with an historical overview of product tampering, illustrated with actual case studies. Regulatory and criminal considerations of actual tampering cases will be reviewed. Toolmark examinations of legitimate, counterfeit, and altered solid dosage forms, such as tablets and capsules, will be covered, as will the role of the Federal Bureau of Investigation's laboratories in examining suspect products. Technical considerations in conducting examinations of products suspected of tampering will also be discussed. Case examples will be used to illustrate various analytical techniques employed to detect tampering. The symposium is sponsored by American

For Your Information

Cyanamid Company, Agricultural Research Division.

■ ***Microbiology Update: Old Friends, New Enemies, Part 1. Listeria in Foods: Current Topics in Epidemiology, Ecology, Detection, and Control*** (Wednesday, September 2, 1:30–4:30 p.m.). Intensive research efforts of the past several years have produced an impressive body of knowledge concerning the important foodborne pathogen *Listeria monocytogenes*. An understanding of the factors affecting the growth and survival of the organism in foods, improved methods for recovery and detection, and increased emphasis on food processing plant sanitation collectively have allowed the industry to gain a significant measure of control over *Listeria monocytogenes*. However, continued diligence is necessary to ensure a food supply free of the health risk associated with this serious pathogen, and important research continues in many areas.

Sponsored by SmithKline Beecham Animal Health, Organon Teknika Corporation, and Westreco, Inc., and organized by Cochairmen Mark A. Mozola, Silliker Laboratories, and Michael H. Brodsky, Ontario Ministry of Health, this symposium will address several topics in the field of *Listeria* research, presenting up-to-date information of interest and importance to food microbiologists and others concerned with this subject. Specific topics to be addressed include the epidemiology of foodborne listeriosis, considerations in establishing *Listeria* tolerance limits for foods, advancements in methods of isolation and detection of *Listeria*, and food processing environmental control in managing *Listeria monocytogenes*.

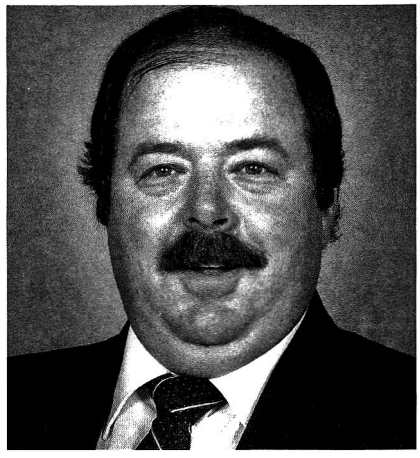
■ ***Microbiology Update: Old Friends, New Enemies, Part 2. Airborne Microorganisms of Significance and Special Interest*** (Thursday, September 3, 8:30–11:30 a.m.). Airborne microorganisms can have a major impact on the health of individuals in the workplace, home, and hospital environment. The most significant effects associated with bioaerosols, which occur in office buildings and houses, are the allergies that result from exposure. It is estimated that 20 million people in the United States suffer from allergies, many of which are caused by microbes. Institutional exposure to potentially hazardous microorganisms, such as in hospitals, has been linked to respiratory disease. These hospital associated diseases have been shown to be transmitted by the aerosol route.

Cochairmen Alfred P. Dufour, U.S. Environmental Protection Agency, and Michael H. Brodsky, Ontario Ministry of Health, have developed this symposium, which will address some of the common problems related to sampling bioaerosols, detecting microbes in aerosol samples, and linking illnesses to microbe source. Current aerosol sampling practices cause severe physiological and structural stress on microorganisms during the capture process. The resulting microbial injury may be partially overcome through the use of collection fluids and detection media that promote their resuscitation and recovery. The evaluation of air samplers and collection fluids will be discussed with respect to their efficiency for recovering microbes from indoor air environments. Their detection, however, is difficult because of the lack of growth media that recovers a broad spectrum of species in bioaerosol samples. Evidence will be presented to show that modified growth conditions promote the recovery of fungi not detected using traditional methods. Legionnaire's Disease is caused by a bacterium called *Legionella pneumophila*, which is usually isolated from water environments. There is good evidence, however, that the major route of transmission may be through aerosols. The acquisition of Legionnaire's Disease via aerosols will be discussed. The

symposium is sponsored by SmithKline Beecham Animal Health, Organon Teknika Corporation, and Westreco, Inc.

For further information and registration for the 106th AOAC International Annual Meeting, contact the AOAC Meetings Department, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032, fax 703/522-5468.

P. Frank Ross to Receive Harvey W. Wiley Award



P. Frank Ross, a leading specialist in analytical veterinary toxicology with the U.S. Department of Agriculture's Animal and Plant Health Inspection Service, National Veterinary Services Laboratories (NVSL) in Ames, IA, has been named to receive the 1992 Harvey W. Wiley Award in recognition of achievements in his field. Ross will receive this most prestigious of AOAC awards at the opening session of the 106th AOAC International Annual Meeting in Cincinnati, OH, on August 31, 1992.

Ross' career has been devoted to the field of analytical veterinary toxicology. He has been at NVSL since 1977, and is one of their leading scientists in the analysis of biological material for chemical agents as well as their specialist in chemical toxicologic analysis. He is responsible for the operation of the analytical portion of NVSL's diagnostic toxicology laboratory that handles complex analytical work necessary to support national animal disease eradication and enforcement work, cases of suspected poisoning in domestic animals and wildlife, and cases with unusual scope or circumstances. NVSL also handles samples submitted by other federal government agencies, U.S. state veterinary diagnos-

tic laboratories, foreign diagnostic laboratories, and international agricultural organizations, when specialized technical expertise or advanced analytical instruments is required.

Recently, Ross has been involved in pioneer work being done in fumonisin mycotoxins. This rapidly evolving field is of great importance to both human and animal health.

Devoted to fostering communication among diagnostic and analytical toxicologists, he facilitates an exchange network for diagnostic and check samples, analytical standards, methods, and ideas. In 1980, he was the driving force behind the formation of, and continues to coordinate, the Veterinary Analytical Toxicology Group within the American Association of Veterinary Laboratory Diagnosticians. This group helps bridge the communication gap between analysts and veterinary clinicians involved in veterinary toxicology. Ross has also served the NVSL's Safety and Health Council, the National Animal Health Monitoring System, and the U.S. Animal Health Association Committee on Environmental Residues, and is a member of the American Academy of Veterinary and Comparative Toxicology and the Society of Toxicology. As a result of his dedication, leadership ability, and technical expertise, the veterinary analytical toxicology network of laboratories in the United States is now an efficiently functioning group that can effectively and quickly respond to problems.

Ross is very active in AOAC International. Through his General Refereeship and collaborative studies, he has contributed to improvement in AOAC methodology. His work as a General Referee was recognized with the 1987 General Referee of the Year Award. Also, four Associate Referees under his General Refereeship have received awards for their collaborative studies. Currently a member of AOAC's Board of Directors, he has also served on the Committee on Fellows, the Symposium and Program Committee, the Constitution and Bylaws Committee, which he chaired in 1989–1990, and was chairman of the Task Force on Test Kits. He was one of the primary players in the formation of the Midwest Regional Section of AOAC International where he plans and conducts the annual Veterinary Analytical Toxicology Workshop that regularly attracts scientists from Canada and Europe.

Ross has authored or co-authored over 60 scientific publications and contributed to various books and manuals.

Ross received his Bachelors Degree from the University of Missouri, Columbia, MO, and his Masters Degree from Iowa State University, Ames, IA.

His work has earned him a reputation as an expert and a leader in his field. AOAC International is honored to name P. Frank Ross as the recipient of the 1992 Harvey W. Wiley Award.

Scott G. Coates Named to Manage AOAC Research Institute

Scott G. Coates was named in April to manage the AOAC Research Institute Test Kit Performance Testing Program. He brings 11 years of industrial management experience in test kit development, manufacture, and distribution to the Institute. Formerly an Operations Manager for BioClinical Systems, Inc., Coates is very sensitive to the needs and concerns of the test kit industry.

Coates is also experienced in the regulatory considerations of the test kit industry. While at BioClinical Systems, he participated in the development of the currently evolving *In Vitro Diagnostic Manufacturing Guideline*, presently working its way through the U.S. Food and Drug Administration. As a former test kit manufacturer, he is keenly aware of the impact of regulatory guidelines and statutes on the industry.

Trained as a microbiologist at the University of Maryland, Coates is currently planning to earn a Masters Degree in Technology Management at the University. Coates' field of concentration in the program is biotechnology management. The Technology Management program is a new curriculum designed to train scientists and engineers in the most current management techniques.

Methods Adopted First Action

As directed by the Board of Directors, the Official Methods Board is responsible for consideration of methods for first action approval. The following methods were adopted first action at the Official Methods Board meeting May 28–30, 1992, in St John's, NF, and became official at that time. These methods will be published in the fourth supplement (1993) to the 15th edition (1990) of *Official Methods of Analysis*.

■ *Foods I*: Crude Protein in Meat and Meat Products, Combustion Method.

Phenolic Antioxidants in Oils, Fats, and Butter Oil, Liquid Chromatographic Method.

■ *Foods II*: Total Dietary Fiber, Enzymatic-Gravimetric Method.

■ *Pesticide Residues and Related Topics*: Diquat and Paraquat Residues in Potatoes, Liquid Chromatographic Method.

■ *Microbiology and Extraneous Materials*: *Listeria* sp., Biochemical Identification Method (MICRO-ID Listeria).

Listeria sp., Biochemical Identification Method (Vitek GPI and GNI).

■ *Feeds, Fertilizers, and Related Topics*: Cholinesterase in Blood, pH Method.

ADVANCE REGISTRATION FORM

AOAC INTERNATIONAL

Return to: AOAC International-J, 1970 Chain Bridge Road, Dept. 0742, McLean, VA 22109-0742

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Advance Registration Deadline: postmarked by August 3, 1992

❑ **Yes!** I want to be there for the 106th Annual AOAC International Meeting & Exposition, at The Sabin Convention Center in Cincinnati, Ohio, August 31 – September 3, 1992.

Please submit a separate form for each registrant. Print or type.

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Telephone No: Daytime ( ) _____

Fax No:( ) _____

Is this your first AOAC International meeting? ❑ Yes ❑ No Will you attend the Business Meeting/Luncheon on Thursday, September 3, 1992? ❑ Yes ❑ No

Name of Guest/Spouse _____ Nickname for badge _____

(Guests/Spouses may not attend Meeting functions unless they have paid the guest registration fee.)

❑ If you have any special needs, check here and include your home phone number, as well as a phone number during the day.

I. Meeting Registration Fees

Full Meeting	Members*	Non-members
Advance	❑ $205	❑ $270
On-Site	❑ $230	❑ $295
One Day		
Check Day (s) attending: ❑ Mon ❑ Tues ❑ Weds ❑ Thurs		
Advance	❑ $145	❑ $155
On-Site	❑ $160	❑ $170
Exhibits Only	❑ $10	❑ $10
Spouse/Guest Registration	❑ $15	❑ $15

Registration Fee Enclosed $ _____ (510)

(Note: For special student rates, call the Meetings Department at +1 (703) 522-3032.)

Workshop on Antibiotics and Drugs in Feed†	❑ $70	❑ $80
Workshop on Juice and and Flavor Composition†	❑ $125	❑ $150
Workshop on Quality Assurance of Benchtop Mass Spectrometric Data†	❑ $125	❑ $150

† *Only meeting registrants are eligible to register for these workshops.*

Social Event	Per Person	Amount Enclosed
Dixieland Riverboat Cruise	❑ $ 40	$_____
Special Tours		
Monday: Cincinnati City Tour	❑ $19	$_____
Tuesday: New Union Terminal Museum Tour	❑ $ 32	$_____
Wednesday: Kentucky Horse Farm	❑ $ 32	$_____

Total Fee Enclosed for Social Events/Tours $ _____(570)

Subtotal I: Meeting Registration + Workshops + Social Events + Tours Fees $_____

II. Short Course Fees

	Members*	Non-members
One Course	❑ $495	❑ $560
Two or more, each	❑ $445	❑ $510
Special introductory fee for **Improving Your Scientific Writing Skills.** Available to AOAC International members only*.	❑ $350	

❑ Lab Waste Disposal $_____ (533)
❑ Analytical QA $_____ (530)
❑ Micro QA $_____ (531)
❑ How to Testify $_____ (534)
❑ Scientific Writing Skills $_____ (535)
❑ Statistics $_____ (532)

Subtotal II: Short Course Fees $_____

*AOAC International Member Number VM-_____. To be eligible for the member fees, you must include your Individual Member ID number or complete the membership application on this form. Member discounts are intended for individual members and are not transferable.

III. For AOAC International Membership

❑ I would like to become an AOAC International member and take advantage of the member discount. I am enclosing the $65 AOAC International membership fee along with meeting fees.

Education (Specify subjects and levels of degrees) _____

Present position (title & brief description) _____

❑ private industry ❑ government agency ❑ academia ❑ other

I hereby apply for membership in AOAC International, and if accepted, agree to abide by its rules and bylaws. **Subtotal III: New Member Dues $** _____

Signature _____ Date _____

GRAND TOTAL ENCLOSED ..... **$** _____ *Payment or credit card information must accompany this form.*

❑ I am enclosing a check payable to AOAC International. *(US funds drawn on US banks only, please)* Charge my ❑ VISA ❑ MasterCard ❑ Diners Club

Card Number _____ Expires _____ Signature _____

PROGRAM PREVIEW

The 106th Annual AOAC International Meeting & Exposition

Highlighting Chemical and Biological Analysis in the International Context

Cincinnati Convention Center, Cincinnati, Ohio ■ August 31-September 3, 1992

Profile of an AOAC International Meeting Attendee

This is *the* meeting for the analytical scientist and scientist/administrator. Are you a chemist, microbiologist, or biochemist? Does your work deal with foods, beverages, pesticides, additives, contaminants, toxins, vitamins, pharmaceuticals, fertilizers, feeds, soil, water, disinfectants, cosmetics, forensic substances, or related materials? If so, this is the meeting for you — four intensive days designed to help bring you up to date on some of the latest developments in your profession.

You'll have the chance to attend outstanding and informative symposia and other educational sessions, and also to meet and network with colleagues from across the nation and the world to share ideas, opinions and solutions. ■

It's Easy to Register

1. *Complete the Registration Form*
2. *Attach a check or credit card information*
3. ***Mail to: AOAC International-J 1970 Chain Bridge Rd., Dept. 0742 McLean, VA 22109-0742***
   FAX to: +1 (703) 522-5468, or
   Phone to: +1 (703) 522-3032.
   (Note: If you have not registered by August 3, please do not mail, fax or phone your registration, as it cannot be processed. Instead, please plan to register on-site in Cincinnati.)

Dear Fellow Scientists,

We here at AOAC International have chosen to focus the 106th Annual AOAC International Meeting & Exposition on "Chemical and Biological Analysis in the International Context." After all, the past few months have brought tremendous change in the world — we've seen the dissolution of several countries and the rise of numerous new ones. And of course, we have adopted a new name, AOAC International, reflecting the increased globalization of our membership.

This year's meeting promises to be an analytical science "summit," beginning with our keynote speaker, Antonio Silva Mendes, who is a European Communities official at the Directorate General, Office of Internal Market and Industrial Affairs.

As always, our planned technical program focuses on "cutting edge" science, bringing you up-to-date in your scientific area and helping you do your job better. We're offering such exciting symposia as:

- Fumonisin Toxins
- Process Sensors and Control
- Milk: Antibiotic Residues and Other Contaminants
- Forensic Methods/Product Tampering
- Current Topics in Lipid Analysis — with an Emphasis on Labeling
- Microbiology: Food and Environmental Updates

Look for a few innovations in this year's meeting. We're offering an extra workshop: Juice and Flavor Composition, Quality Assurance of Benchtop Mass Spectrometric Data and Antibiotics and Drugs in Feed. Plus, the Regulatory Roundtable is expanded to a full day, with two sessions. In keeping with our international theme, the Roundtable is devoted to EEC regulations and the North American trade zone regulatory considerations.

Other features include more than 200 poster presentations on dozens of topics in key chemical and microbiological analytical areas, short courses, the Open Forum and the laboratory instrumentation and services exposition.

So, make plans to join us in Cincinnati, Ohio, the "Queen City of the West" and the crossroads of America. Its varied ethnic heritage, Midwestern friendliness and cosmopolitan flair combine to make Cincinnati special. Hope to see you there!

Sincerely,

Ed Elkins

Edgar Elkins
President

SCHEDULE AT-A-GLANCE

Saturday August 29

Short Course
How to Testify
9:00 am – 5:00 pm

Short Course
Waste Disposal
9:00 am – 5:00 pm

Short Course
Analytical QA
9:00 am – 5:00 pm

Workshop
Juice & Flavor Composition
10:00 am

Methods Volunteer Education Session
Managing the Methods Validation Process: Statistical Guidelines
3:00 pm – 5:30 pm

President's Reception
6:30 pm
AOAC Around the World

Sunday August 30

Workshop
Antibiotics & Drugs in Feeds 8:30 am

Workshop
Juice & Flavor Composition 8:30 am

Workshop
QA of Benchtop Mass Spectrometric Data
8:30 am

Short Course
How to Testify
9:00 am – 5:00 pm

Short Course
Waste Disposal
9:00 am – 5:00 pm

Short Course
Analytical QA
9:00 am – 5:00 pm

Poster Session
Foods II
1:00 pm

Wiley Award Symposium
Fumonisins, Part I
1:00 pm

Symposium
Process Sensors & Control, Part I
1:00 pm

Poster Session
Drugs & Related Topics, Part I
3:00 pm

Workshop
The Modern Laboratory
7:00 pm

Monday August 31

Opening Session
8:30 am

Guest Welcome Coffee
9:30 am

Exhibit Hall Opens
10:00 am

Tour
Cincinnati City Tour
Departs 10:30 am

Poster Session
Pesticides, Part II
1:00 pm

Symposium
Process Sensors & Control, Part II
1:30 pm

Symposium
Milk, Part II
1:30 pm

Poster Session
Feeds, Fertilizers & Related Topics
3:00 pm

Dixieland Riverboat Cruise
6:00 pm

Tuesday September 1

Poster Session
Pesticides, Part I
8:30 am

Wiley Award Symposium
Fumonisins, Part II
8:30 am

Symposium
Milk, Part I
8:30 am

Poster Session
Environmental Quality
10:30 am

Tour
Union Terminal Museum Tour
Departs 10:30 am

Poster Session
Foods I, Part II
1:00 pm

Regulatory Roundtable
Part II
EEC Regulations
1:30 pm

Symposium
Nutrition Labeling of Lipids, Part I
1:30 pm

Symposium
Microbiology Update, Part I, Listeria Update
1:30 pm

Poster Session
Drugs & Related Topics, Part II 3:00 pm

AOAC Open Forum
7:30 pm

Wednesday September 2

Poster Session
Microbiology & Extraneous Materials
8:30 am

Regulatory Roundtable
Part I
North American Trade Zone Regulations
8:30 am

Symposium
Forensic Methods & Product Tampering, Part I
8:30 am

Poster Session
Foods I, Part I
10:30 am

Tour
Kentucky Horse Farm
Departs 9:30 am

Poster Session
Foods I, Part II
1:00 pm

Business Meeting
Includes Lunch
12 noon

Thursday September 3

Symposium
Nutrition Labeling of Lipids, Part II
8:30 am

Symposium
Forensic Methods & Product Tampering, Part II, 8:30 am

Symposium
Microbiology Update, Part II, Airborne Microorganisms
8:30 am

Short Course
Microbiological QA,
9:00 am – 5:00 pm

Short Course
Statistics for Methodology
9:00 am – 5:00 pm

Short Course
Improving Technical Writing Skills
9:00 am – 5:00 pm

Friday September 4

Short Course
Microbiological QA,
9:00 am – 5:00 pm

Short Course
Statistics for Methodology
9:00 am – 5:00 pm

Short Course
Improving Technical Writing Skills
9:00 am – 5:00 pm

Lunch 12:00 noon

Evening

AOAC INTERNATIONAL

Thanks to our Sponsors!

The following organizations, as sponsors or co-sponsors, have provided generous financial support for the 106th Annual AOAC International Meeting (as of April 10, 1992).

- American Cyanamid Company, Agricultural Research Division
- Archer Daniels Midland Company
- Bristol-Myers Squibb Pharmaceutical Research Institute
- Campbell Soup Co.
- CIBA-GEIGY Corporation – Agricultural Division
- The Coca-Cola Company
- Corn Refiners Association, Inc.
- DCA Food Industries Inc.
- Dow Chemical U.S.A.
- Dupont Agricultural Products
- Eli Lilly and Company
- Galbraith Laboratories, Inc.
- GENE-TRAK Systems
- General Mills, Inc.
- Gerber Products Company
- Hazleton Laboratories, Madison, WI
- HEINZ, U.S.A.
- Hershey Foods Corporation
- ICI Americas Inc.
- Kellogg Company
- Kraft General Foods
- Miles, Inc. Agriculture Division
- Mead Johnson Nutritional Group (a Bristol-Myers Squibb Company)
- Moorman Manufacturing Co.
- NESTLE, Vevey
- Ocean Spray Cranberries, Inc.
- Organon Teknika Corporation
- The Procter & Gamble Company
- Silliker Laboratories Group, Inc.
- SmithKline Beecham Animal Health
- Solvay Duphar B.V., The Netherlands
- Westreco, Inc.

Keynote Speaker—

Antonio Mendes to Speak at Opening Session

The Opening Session will feature Antonio Silva Mendes, a European Communities official at the Directorate General Office of Internal Market and Industrial Affairs. There, he is in charge of external relations in the conformity assessment field, in particular the mutual recognition agreements and the cooperation and technical assistance programs. He will speak on "International Perspectives on Certification and Accreditation."

Previously, he was Director at the Faro Regional Delegation of the Ministry of Industry and Energy, Secretary of the Portuguese Standard Committee for Quality Assurance and head of the Portuguese delegation to the ISO Technical Committee. He received an M.S. degree in Quality from the Technology University of Compiegne (France) and a Mechanical Engineer degree from the Lisbon University. ■

Cutting Edge Science—

Diverse Schedule of Technical Activities Planned

The technical program has been specially developed to assist analytical science professionals like yourself stay on the cutting edge of the constantly evolving technology of the nineties.

Choose from the following variety of events:

Wiley Award Symposium

Fumonisins: Occurrence, Distribution, Production, Analysis Chemistry and Mode of Action.

Featuring the Harvey W. Wiley Award address by P. Frank Ross, of the US Department of Agriculture, Agriculture Research Service, entitled "What Are We Going to Do With This Dead Horse?"

Other Symposia

Keep yourself and your lab up-to-date on technological changes with AOAC International symposia:

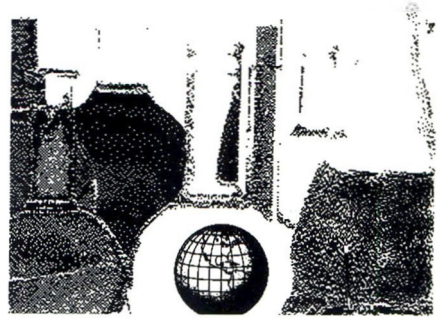
- Process Sensors and Control
- Milk: Antibiotic Residues and Other Contaminants
- Forensic Methods/Product Tampering
- Current Topics in Lipid Analysis — With an Emphasis on Labeling
- Microbiology Update: Listeria in Foods and Airborne Microorganisms

Poster Presentations

Gain first-hand knowledge and learn from the experience of other analytical scientists, some of whom have confronted and solved the very problem you may be working on right now!

Take time to look over these presentations by specialists working in the following areas: various foods and beverages, dietary fiber, nutrients, filth and extraneous materials, antioxidants, food additives, natural toxins, food microbiology, drugs, cosmetics, drug and device related microbiology, environmental quality, water, disinfectants, metals and other elements, radioactivity, feeds, drugs in feeds, fertilizers, soils, pesticide formulations and residues, veterinary analytical toxicology, tobacco, forensic methods, and related subjects.

Presenters will be on hand to answer your questions. ■



General Information

Registration

Register by August 3 for a savings of $25 over the on-site registration fee.

The full meeting registration fee admits you to all technical programs except the short courses and the three Workshops. The workshops have separate, additional fees. To register for a workshop you must be registered for the meeting. The full meeting fee includes the President's Reception and the Business Meeting Luncheon. See Registration Form for fees.

Student rate registrations are available. For information, contact the AOAC Meetings Department at +1 (703) 522-3032.

Refunds

AOAC will refund the full meeting registration fee if written notification is received in the AOAC International office by August 17, 1992. A $35 service fee will be charged for cancellations received after that date.

Accommodations

The meeting headquarters hotel is the Westin Hotel Cincinnati. The daily rate for AOAC meeting attendees is $72.00 per night, single or double, plus tax. Major credit cards are accepted, including VISA, MasterCard, American Express and Diners.

To make your room reservation, call the Westin Hotel Cincinnati at +1 (513) 621-7700. To receive the special rate, mention that you are registering for the AOAC meeting.

Alternate accommodations are available at the Hyatt Regency Cincinnati where the special AOAC rate is $78.00 per night, single or double, plus tax. Call +1 (513) 579-1234 for reservations, and mention AOAC to receive the discounted rate.

Make your reservations as soon as possible to be assured of a room. And, be sure to note your confirmation number.

Special Airfares and Car Rental Rates

Delta Airlines is the official airline of the 106th Annual AOAC International Meeting and Exposition. Attendees will receive substantial discounts on travel to Cincinnati. Fare information is available by calling Delta Airlines at (800) 241-6760; ask for File number R0133.

Alamo Rent-A-Car is offering special rates for AOAC meeting attendees. For details, call (800) 732-3232 and mention ID #254576, Rate Code G3.

Remember, using the services of our official hotel and carriers saves you— and AOAC International—money!

Popular, Progressive and Participatory—

Three Intensive Workshops Offered

Workshop on Antibiotics and Drugs in Feeds

Back by popular demand! Features presentations which include a review of the progress made since 1989 when AOAC first inaugurated this popular workshop. Plus, the workshop offers lots of opportunity to informally discuss antibiotic and drug methods.

The workshop fee is $70 for AOAC members and $80 for non-members. Only 1992 AOAC annual meeting attendees are eligible to register for this workshop.

Hands-On Workshop on Juice and Flavor Composition

With 14 training stations, you're assured of an opportunity to perform various kinds of chemical analyses or view simulations of techniques for the determination of isotopic compositions. Register early, as the number of participants must be limited to ensure the "hands-on" aspects of this workshop.

The workshop fee is $125 for AOAC members and $150 for non-members. Only 1992 AOAC annual meeting attendees are eligible to register for this workshop.

Workshop on Quality Assurance of Benchtop Mass Spectrometric Data

This workshop is centered on "benchtop" applications. You learn the importance of careful consideration of criteria for confirmation of analyte identity and quantitation especially regarding their use for regulatory and other applications with legal considerations. Data comparability among the several types of commercially available benchtop instruments will also be discussed.

The workshop fee is $125 for AOAC members and $150 for non-members. Only 1992 AOAC annual meeting attendees are eligible to register for this workshop. ■

Open Forum Addresses Attendees Problems and Solutions

Take advantage of this opportunity to network with a group whose sum total of analytical experience adds up to several hundred years! Have a tricky question, a problem or a tough situation? You may find the answer right here! Put it before the group and you'll be sure to get lots of useful suggestions and advice. The subject matter is directed by your needs, and you can be sure the pace will be fast and the exchange candid. ■

Analytical Equipment and Services Exposition— The Latest Technology

Want to see — and touch — what's new in laboratory equipment and services? Then you'll definitely want to visit this expo. The following companies will demonstrate their newest products. And, in response to your many requests, we've cleared a two-hour block of time on Monday morning just for visiting the exhibits.

- ABC Laboratories
- ABC Research Corporation
- Acculab, Inc.
- Agri-Diagnostics
- Airco Gases
- Alltech Associates, Inc.
- Bio-Control Systems, Inc.
- Bio-Rad Laboratories
- Bio-Tek Instruments, Inc.
- bioMerieux Vitek, Inc.
- Burdick & Jackson
- CEM Corporation
- Data Specifics
- Dionex
- EM Science
- Eppendorf North America, Inc.
- Fisons Instruments
- Foss Food Technology
- GENE-TRAK Systems Corporation
- GFS Chemicals, Inc.
- Hach Company
- Hazleton Laboratories
- International Biotechnologies Incorpoated–Kodak
- Idetek, Inc.
- International Bioproducts
- International Diagnostic Systems Corp.
- JT Baker, Inc.
- J & W Scientific
- LDC Analytical
- Lancaster Laboratories, Inc.
- Leco Corporation
- 3M Microbiology Products
- Medallion Laboratories
- Michelson Laboratories, Inc.
- MIDI
- Millipore Corporation
- NIST
- Neogen Corporation
- Nicolet Instruments Corporation
- Northwest Analytical, Inc.
- Organon Teknika
- Orion Research, Inc.
- Perkin Elmer Co.
- Perstorp Analytical, Inc.
- Pickering Laboratories, Inc.
- Q Laboratories, Inc.
- R-Tech
- Radiometer America, Inc.
- Ralston Analytical Laboratories
- Robot Coupe USA, Inc.
- Romer Laboratories, Inc.
- S.G.E. Incorporated
- Savant Instruments
- Silliker Laboratories
- Southern Testing & Research Laboratories, Inc.
- Spex Industries
- Statking Consulting, Inc.
- Supelco, Inc.
- Telecation, Inc.
- Teledyne Hastings
- Thermedetec, Inc.
- Thermo Jarrell Ash Corporation
- UIC, Inc.
- U.S. Pharmacopeia
- Unipath Co. – Oxoid Division
- Waters Chromatography
- Webb Technical Group, Inc.
- Wheaton Coated Products
- Woodson Tenent Laboratories, Inc.
- YSI Incorporated

Addresses Real Issues. . .Real Problems—

AOAC Adds Two New Courses to '92 Short Course Program

Make the most of your time and travel dollars...attend one or more of AOAC International's two-day short courses, either immediately before or immediately after the annual meeting.

These intensive two-day sessions have been developed for the analytical science professional like yourself — the courses address real issues and problems faced daily in the workplace. Taught by knowledgeable and experienced experts, these courses will help you keep your edge in scientific and management skills — no easy task in this time of rapid change.

These courses will help hone your skills, improve your job performance and gain practical experience. Plus, your lab benefits through lower costs, higher yield, greater efficiency and increased reliability.

To register, simply choose the courses that fit your needs and complete the appropriate section of the meeting registration form.

Cancellations received less than 10 working days prior to a course will be subject to a fee of $50.00. No refunds will be give for cancellations received after a course has started. AOAC International reserves the right to cancel any course as necessary.

Registration Fees for Courses: For one person taking one course, fees are $495 for AOAC International members (except "Improving Your Technical Writing Skills", which is $350 for members), $560 for non-members. For more than one person and/or more than one course, fees are $445 per course for AOAC International members, $510 for non-members.

The discounted rate is available when submitting more than one registration *in one payment.* (The full amount will be charged if for any reason cancellations reduce your organization's attendance to one person taking one course.) ■

NEW!

Improving Your Technical Writing Skills

September 3 - 4, 1992

A Special Offer — Exclusively for AOAC Members! Take Improving Your Technical Writing Skills at the low introductory rate of $350! Sign up today!

This course can take the agony out of writing a technical paper! It is a MUST for EVERY scientist.

You'll learn how to get your paper published by the journal of your choice with a minimum of revisions... And leave with helpful hints, checklists and memory aids. Send samples of your writing to the course director in advance of the course and you'll find a written critique waiting for you in the classroom. Plus, the course director will provide follow-up at no additional cost.

Program covers:

- Identifying your audience
- Selecting the journal
- Organizing your material
- How to get started
- Building from an outline
- First draft
- The need for revision
- Traditional sections of a scientific paper
- What is the author's obligation to the reader
- How much detail is enough
- Text, tables and figures
- Should negative results be reported?
- Who should be referenced
- Who should be acknowledged

NEW!

How To Testify as an Expert Witness

August 29 - 30, 1992

Every participant is videotaped and receives a copy of the tape!

For regulators, forensic scientists and anyone else who may be called upon to testify. This course will benefit those with years of testifying experience as well as those who have never taken the witness stand.

You'll gain the basic information, opportunity for immediate practice with feedback, and the take-home resources you need to become an effective expert witness. The program includes lecture, role-playing with critique and videotaping of each participant in cross-examination exercises, plus useful tips on how to use all this information effectively.

Program covers:

- The anatomy of a case
- Legal and ethical responsibilities as an expert witness
- Qualifications
- Appearance and demeanor
- Assessing the audience
- Communication techniques
- Direct examination
- Demonstrative evidence
- Cross examination

Laboratory Waste Disposal, Environmental Compliance and Safety

August 29 - 30, 1992

Protect yourself and your lab from regulatory non-compliance action and yourself and your employees from health hazards.

For anyone involved in the management or execution of laboratory processes, this course will cover the US EPA requirements for generators of hazardous waste, OSHA regulation for exposure of employees to health hazards, and the components of good laboratory waste management and hygiene programs, including record keeping, compliance reporting and training.

Program covers:

- Hazard communication
  - Labels, tags, signs, MSDs
  - Substance inventory
  - Information sources
  - Employee information and training
  - Hazcom exercise
- Basic toxicology
- EPA regulations and inspections
- RCRA training plans
- Use of spill control equipment
- Problem session
- Rule 120 for RCRA generators
- Laboratory hygiene plan
- Elements of a lab health and safety program
- DOT regulations for shippers of hazardous waste
- Use of monitoring equipment and exercise
- Respiratory protection
- Fire protection and control

Quality Assurance for Analytical Labs

August 29 - 30, 1992

Free AOAC International reference manual included: Quality Assurance Principles for Analytical Labs! Newly revised in 1991 and valued at $60!
A good QA program results in better analyses at lower cost.

For laboratory managers, supervisors, quality assurance coordinators, and others responsible for development and supervision of quality assurance programs who want to learn how and why to plan, design, implement and manage a lab QA program.

Program covers:

- Introduction and definitions
- Basic statistics
- Control charting/proficiency testing
- Sampling and QA
- Analytical methods and QA
- Records and reporting
- Equipment and supplies management
- Organizing and planning for QA
- Writing a QA manual
- Auditing for QA
- Computers and information management systems in QA

. . . **Courses** *(continued on following page)*

Courses *(continued)*

Quality Assurance for Microbiological Labs

September 3 - 4, 1992

Planning and administering a good quality assurance (QA) program will build confidence in your micro lab.

For industrial, regulatory and academic microbiology analysts, supervisors and managers who want to learn how to plan and administer a quality assurance program in the context of the microbiology lab setting.

Program covers:

- Design and implementation of a QA program
- Quality control of culture media and reagents
- Quality control of laboratory equipment
- Quality control of analysis
- Quality management of analytical performance
- Measurement controls and statistics in the micro lab
- Proficiency testing and check sample programs
- Lab audits, accreditation and certification

Statistics for Methodology Development

September 3 - 4, 1992

Produce better, more reliable and verifiable methods through the use of statistics.

Anyone involved in developing, validating or using methods in an analytical laboratory will benefit by becoming more confident and effective in the use of statistics as a lab tool and in using the services of statisticians. You'll learn how to evaluate, control and predict variability and have the opportunity for lots of hands-on computer practice.

Program covers:

- The collaborative study process and its role in methods validation
- Defining, developing, evaluating and using the analytical measurements process
- Descriptive statistics — measure
- Distributions
- Linear regression
- Control charts
- Analysis of variance/experimental design
- Interlaboratory studies: protocols, minimization of variation, and special analytical tools
- Hands-on computer assisted workshop in calculating and applying statistics to case studies and collaborative study simulation

Expanded Regulatory Roundtable Zeroes in on International Regulation

Don't miss this popular AOAC meeting feature which has been expanded to a full day, with two sessions. Each session features a select panel who will begin with formal presentations and follow with open discussion and a question and answer period. One will be moderated by Alex Williams, the former Government Chemist of England, and will focus on European Economic Community (EEC) Regulations. The other session will be devoted to discussion of North American trade zone regulatory considerations and will be headed by Bobbi Dresser, FDA Office of International Affairs. ■

Methods Volunteer Education Focuses on Statistical Guidelines for Managing Methods Validation ...Features Hands-On Practice

Managing the Methods Validation Process: Statistical Guidelines

Do you currently participate in the AOAC methods validation process? Have you considered doing so? You are invited to attend this methods volunteer education session to learn how to make participation easier and more effective. Learn about the available tools—the harmonized collaborative study guidelines and their corollary statistics worksheets—and how to use them. Computers will be available during the week for hands-on practice. ■

Come to Cincinnati— The Queen City of the West

The AOAC International meeting will be held at the Sabin Convention Center, a unique building designed for convenience and accessibility. Most notably, it is integrated into the famous Skywalk, a covered or enclosed pedestrian system which connects virtually all major downtown facilities into one compact element. Your hotel, the meeting site, dozens of superb shops and fine downtown restaurants are all linked, each no more than 10 minutes walk from the other.

Cincinnati prides itself on offering something for everyone...from sports fans to art buffs to music lovers. It combines a varied ethnic heritage, Midwestern friendliness and cosmopolitan flair, with a variety of attractions to choose from. Enjoy a horse-drawn carriage ride...meander down the Ohio River in a spectacular river boat...visit the world famous Cincinnati Zoo or the highly rated Cincinnati Art Museum...sample some Cincinnati-style chili.

You'll find Cincinnati lively and diverse. No matter how you spend your free time, Cincinnati is sure to please!■

Social Program

An integral part of any meeting is the social program. Social events expand your learning and network opportunities and provide the change of pace that allows you to make the most of your attendance. All work and no play is neither fun nor efficient!

Be sure to sign up well in advance for special events and tours. This will help us plan better for your comfort and enjoyment...not to mention guaranteeing a place on the bus!■

President's Reception has International Theme

The President's Reception, co-sponsored by **Solvay-Duphar B.V.; the Netherlands** and **General Mills, Inc.,** will be held Sunday evening, August 30.

Kicking off the meeting with an international flair we'll sample food and drink from around the world. All attendees and guests are invited to this gala reception to be held at the Westin Hotel Cincinnati, the meeting headquarters hotel.■

The Guest Program Begins Monday Morning with Welcome Reception

Your accompanying guests are invited to get acquainted over a continental breakfast served at 9:30 am, Monday, August 31. Representatives from Party Planners will be on hand to talk about the many exciting things to do in the Cincinnati area, and to describe the scheduled tours.■

Climb Aboard for Old-Fashioned Fun on the Dixieland Riverboat Cruise

Be sure to sign up for the Riverboat Cruise on Tuesday, September 1st. The $40 per person cost includes dinner (cash bar) and spunky Dixieland band entertainment.

You'll spend a balmy summer evening cruising along the Ohio River, on a majestic side-wheeler, the Funliner, the largest boat in the Cincinnati area. It promises to be an evening of genial company, good food and lively entertainment. Space is limited however, so be sure to sign up early.■

See the Best of Cincinnati on one of these Exciting Tours

Cincinnati City Tour

The tour, scheduled for Monday, August 31st, leaves at 10:30 am, returns at 2:30 pm and costs $19.00 per person. No meals are included.

This tour takes you through the highlights of various landmarks that make the "Queen City" so special. You'll visit the charming narrow streets atop Mt. Adams and view the best sights of the Ohio River, Northern Kentucky and Cincinnati. Stops include a visit to the Krohn Conservatory, one of the largest greenhouses in the country, and a visit to Covington's charming Mainstrasse Village for a bit of German heritage. A stop will also be made at the entertaining Covington's Landing where guests may explore on their own and get some lunch.

New Union Terminal Museum Tour

The tour, scheduled for Tuesday, September 1st, leaves at 10:30 am and returns at 2:30 pm. The cost is $ 32.00 per person. You're on your own for lunch.

Guests will be picked up at the Convention Center to begin their journey to the Union Terminal "Museum Center" for a three hour guided tour. Imagine stepping through a crack in a glacier and going back 19,000 years to Ice Age Cincinnati. Giant prehistoric animals — one-ton ground sloths, seven-foot-tall bison and 600- pound beavers roam the harsh landscape. This football field sized exhibit and others in the Cincinnati Museum of Natural History tell the story of the Ohio Valley's ecology, geology and climatology in a lively, interactive way.

This wonderful journey will also take guests for a stroll along the public landing as it was in the late 1850's; explore the stern-wheel, two-deck packet boat and visit the shops and offices of that period, all accurately reproduced.

Kentucky Horse Farm Tour

The tour, scheduled for Wednesday, September 2nd, leaves at 9:30 am, returns at 4:30 pm and costs $32.00 per person. Lunch is not included.

Experience the history and hospitality of the Kentucky horse breeding community. Enjoy this unique opportunity to see a working horse farm, visit the barns, watch the farrier and harness maker at work. A special feature of this tour is the Parade of Breeds, which includes not only a chance to see (and pet) the horses but also a brief history of each breed. Then relax with a tour of the grounds by tram, horse-drawn omnibus, or elegantly appointed carriage. ■

Symposia and Roundtable Sponsors

We would like to express our appreciation to the organizations providing financial support for the following programs:

Fumonisins: Occurrence, Distribution, Production, Analysis Chemistry, and Mode of Action
Corn Refiners Association, Inc.

Microbiology Update: Old Friends, New Enemies
SmithKline Beecham Animal Health, Organon Teknika Corporation, and **Westreco, Inc.**

Nutrition Labeling of Lipids
Silliker Laboratories Group, Inc., Archer Daniels Midland Company and **Gerber Products Company;** travel for international speakers sponsored by **The Procter & Gamble Company**

Process Sensors and Control
Dow Chemical Company, HEINZ, U.S.A., and **Kraft General Foods**

Regulatory Roundtable on EEC Regulations
The Coca-Cola Company

Regulatory Roundtable on North American Trade Zone Regulations
Dupont Agricultural Products and **CIBA-GEIGY Corporation–Agricultural Division**

None of the funds from any corporate sponsor are used by AOAC International to pay expenses of U.S. federal employees participating in this Annual Meeting.

SUNDAY

WORKSHOP

Antibiotics and Drugs in Feeds

Sunday, August 30, 8:30 am-4:00 pm

Chairman: **Mary Lee Hasselberger,** Nebraska Department of Agriculture

Sponsored Cooperatively by: **AOAC International, Association of American Feed Control Officials,** and the **US Food and Drug Administration**

Introductory Remarks: **Mary Lee Hasselberger**

Drugs in Feeds

Moderator: **Audrey Gardner,** New York Agricultural Experiment Station

Progress Reports on Analytes Presented at the 1989 and 1991 Workshops: Instrumental Methods:

Carbadox. **Alicia Henk,** Pfizer, Inc.
Monensin. **Mark Coleman,** Lilly Research Laboratory
Sulfamethazine. **Robert Smallidge,** Office of the Indiana State Chemist
Lasalocid. **Alexander MacDonald,** Hoffman-LaRoche, Inc.
Oxytetracycline, Report on HPLC/Bioassay Interlaboratory Study. **Mary Lee Hasselberger,** Nebraska Department of Agriculture
Decoquinate. **Dorothy Hoskins,** Hess & Clark, Inc.

Statistical Analysis of Data from Routine Bench Work and Intralaboratory Validation. **Dan Mowrey,** Lilly Research Laboratory

Validation of HPLC Data Systems. **Thomas P. Layloff,** US Food and Drug Administration

Replication of Drug Results in Feeds Ground in Several Different Mills. **Mary Lee Hasselberger,** Nebraska Department of Agriculture

Antibiotics in Feeds

Moderator: **Denise Riley Moore,** Woodson-Tenent Laboratories

Apramycin, General Discussion. **Candice Herrick, Hussein Ragheb,** Office of the Indiana State Chemist, and **John Lamb,** Eli Lilly and Company

Agar Well Microbiological Method for the Assay of Virginiamycin in Type A and Type B Medicated Articles. **James A. Miller, Mary Ann Pfannenstiel,** and **David Gottschall,** SmithKline Beecham Animal Health

Use of Bioautography in a Diagnostic Laboratory to Differentiate Antibiotics in Animal Feed. **Wynne Landgraf,** National Veterinary Services Laboratories

Progress Report on Analytes Presented at the 1989 and 1991 Workshops: Microbiological Methods:

Tylosin. **Mark Coleman,** Lilly Research Laboratory
Bacitracin. **Anil Desai,** A.L. Laboratories, Inc.
CTC. **Mary Lee Hasselberger,** Nebraska Department of Agriculture
Neomycin. **Gerald Stahl,** Upjohn Company

General Discussion Session

WORKSHOP

Quality Assurance of Benchtop Mass Spectrometric Data

Sunday, August 30, 8:30 am - 4:00 pm

This workshop will emphasize "benchtop" applications. The ease of operation of mass spectrometers permits their use by analysts without extensive experience in mass spectrometry. Their use for regulatory and other applications with legal considerations necessitates careful consideration of criteria for confirmation of analyte identity and quantitation. Data Comparability among the several types of commercially available benchtop instruments will also be discussed.

TECHNICAL PROGRAM DETAILS

WORKSHOP

Juice and Flavor Composition

Saturday, August 29, 10:00 am - 6:00 pm
Sunday, August 30, 8:30 am - 4:00 pm

Chairman: **Allan R. Brause,** Analytical Chemical Services of Columbia, Inc.

This workshop will include 14 training stations where participants will have the opportunity to perform chemical analyses or view simulations of techniques for the determination of isotopic compositions. Early registration is advised, as the number of participants must be limited to permit the "hands-on" aspects of this workshop. The following stations will be offered:

Component	Test	Instrument	Instructor
Sugars	HPLC	Waters CHA Column	**Carla Barry,** Agriculture Canada
Organic Acids	HPLC	Dual Column C18	**Elia Coppola,** Ocean Spray Cranberries, Inc.
Anthocyanins	HPLC	C18 Column	**Ron Wrolstadt,** Oregon State University
d,l Malic Acid	HPLC	Hamilton PRP	**Kodjo Adadevoh,** Analytical Chemical Services of Columbia, Inc.
Pulpwash	Petrus	UV, Vis and Fluorimeter	**Paul Cancalon,** Florida Department of Citrus
Benzoate/Sorbate Preservatives	HPLC	Poly LC Column	**Linda Klein,** Gerber Products Company
Metals	AAS	Perkin Elmer 3100	**Karen Daniels,** ABC Research
Isotope (CSIRA) (OSIRA)	GC/MS	Simulation MS	**Ken Winters,** Coastal Science Laboratories, Inc.
Sugars	SNIF/NMR	Simulation NMR	**Gilles Martin,** Eurofins
Pattern Recognition	Computer	IBM-PC	**Sam Page,** US Food and Drug Administration
Polyphenols	HPLC	C18 Gradient	**Sandford Kirksey,** The Procter & Gamble Company
Invert Sugars	HPLC/PAD	Waters/Bio-Rad	**Nicholas Low,** University of Saskatchewan
Flavors	GC/MS	Perkin Elmer Q Mass	**Gary Konnagan,** Alex Fries Brothers

Managing the Methods Validation Process: Statistical Guidelines

Sunday, August 30, 3:00 pm - 5:30 pm

Do you currently participate in the AOAC methods validation process? Have you considered doing so? You are invited to attend this methods volunteer education session to learn how to make participation easier and more effective. Learn about the available tools — the harmonized collaborative study guidelines and their corollary statistics worksheets — and how to use them. Computers will be available during the week for hands-on practice.

MONDAY

Opening Session

Monday morning, August 31, 8:30 - 10:00 am

The Opening Session marks the official beginning of the 106th Annual AOAC International Meeting. The following is a tentative schedule of events.

Opening Remarks: Edgar R. Elkins, President

Presentation of the Harvey W. Wiley Award to P. Frank Ross: Edgar R. Elkins

The **Address of the 1992 Harvey W. Wiley Award Winner,** *What Are We Going to Do with this Dead Horse?*, will be given in the **Harvey W. Wiley Award Symposium on Fumonisins: Occurrence, Distribution, Production, Analysis Chemistry, and Mode of Action,** Monday afternoon, 1:00-4:30 pm

Presentation of Other Awards: Edgar R. Elkins
- Collaborative Study of the Year
- General Referee of the Year
- Associate Referee of the Year Awards
- Fellows of the AOAC
- Special Awards
- Employee Service Awards
- AOAC International Scholarship Announcement

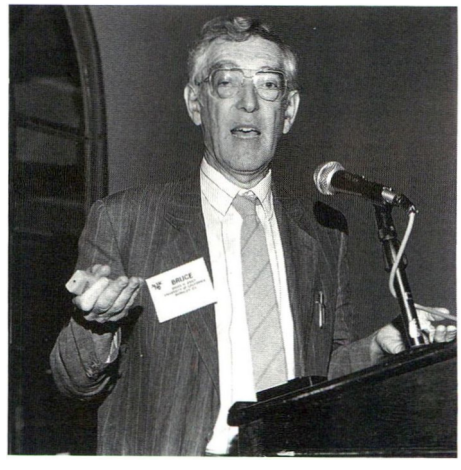
Introduction of the President's Address: Henry B.S. Conacher, President-elect

President's Address: Edgar R. Elkins

Keynote Address: Antonio Silva Mendes, European Communities, Directorate General Office of Internal Market and Industrial Affairs

Award of the Presidential Plaque to Edgar R. Elkins: H. Michael Wehr

Opening of Exhibition



SYMPOSIUM

Harvey W. Wiley Award Symposium

Fumonisins: Occurrence, Distribution, Production, Analysis Chemistry, and Mode of Action, Part I

Monday, August 31, 1:00-4:30 pm

Chairman: **Glenn A. Bennett,** US Department of Agriculture

Cosponsored by **Corn Refiners Association, Inc.**

1:00 Introduction. **Glenn A. Bennett,** US Department of Agriculture

1:15 Keynote Address: What Are We Going to Do with this Dead Horse? **P. Frank Ross,** 1992 Harvey W. Wiley Award Recipient, US Department of Agriculture

2:00 BREAK

2:30 Biotic and Abiotic Factors in Fumonisin Production and Accumulation. **Joseph Le Bars,** Institute National de la Recherche Agronomic

3:00 Production of Fumonisins in Liquid Culture. **David Miller,** Agriculture Canada

3:30 Fumonisin Production by *Fusarium* species on Solid Substrates. **Paul E. Nelson,** Pennsylvania State University

4:00 Examination of European Isolates of *Fusarium* for Production of Fumonisins. **Angelo Visconti,** Instituto Tossine e Micotossine

TECHNICAL POSTER SESSION

Foods II

Monday, August 31, 1:00-2:30 pm

Poster Session Coordinator: **Elia Coppola,** Ocean Spray Cranberries, Inc.

Sponsored by **Hershey Foods Corporation**

Topics include: Alcoholic Beverages; Cereal and Cereal Products; Dietary Fiber; Fruits and Fruit Products; Nonalcoholic Beverages; Processed Vegetable Products; Sugars and Sugar Products; Vitamins and Other Nutrients; General Topics

Alcoholic Beverages

Determination of Procymidone in Wine: Comparison of Data Between Liquid/Liquid Extraction-LC and Solid Phase Extraction-GC Methods. **Rafael Sarmiento, Sumer Dugar,** and **Mike Ethridge,** US Bureau of Alcohol, Tobacco and Firearms

Determination of Ethyl Carbamate in Alcoholic Beverages and Foods by Gas Chromatography with Mass Selective Detection: Collaborative Study. **Benjamin J. Canas,** US Food and Drug Administration

Cereal and Cereal Products

Combining Complementary, Rapid Methods of Fat Analysis to Meet Nutritional Labeling Requirements for the Baking Industry. **Bradford A. Burns,** Nabisco Foods Group, Inc.

Application of High Performance Liquid Chromatography (HPLC) to Nutritional Labelling of Carbohydrates. **John Morawski, Art Sims,** and **Barbara Kenney,** Millipore Corporation, Waters Division

AOAC Fat Acidity Analysis: Potentiometric Titration in a Medium without Toluene. **Ralph H. Lane,** University of Alabama

General Referee Report: Cereals and Cereal Products. **Ralph W. Lane,** University of Alabama

Dietary Fiber

Determination of Total Dietary Fiber in Products with Little or No Starch, Nonenzymatic-gravimetric Method: Collaborative Study. **Betty W. Li,** US Department of Agriculture

A Cultivar x Location x Storage Study on Dietary Fibre in Flesh and Skin of Potatoes. **W.J. Mullin, M. Wolynetz,** Agriculture Canada

Determination of Total, Soluble, and Insoluble Dietary Fiber in Foods. **Sungsoo C. Lee,** Kellogg Company

Determination of Soluble Dietary Fiber: Collaborative Study. **Leon Prosky,** US Food and Drug Administration

General Referee Report: Dietary Fiber. **Leon Prosky,** US Food and Drug Administration

Fruits and Fruit Products

Composition of Strawberry Juice. **Dana A. Krueger, Jeanne Maciel, Elisabeth Shifrin,** and **Song Lin,** Krueger Food Laboratories

General Referee Report: Fruits and Fruit Products. **Frederick E. Boland,** US Food and Drug Administration

Nonalcoholic Beverages

Heavy Metals Speciation in Tea Infusion using LC/AAS Method. **Kiyoshi Matsuo, Kunio Okano,** and **Tetsuhisa Goto,** Japanese National Research Institute of Vegetables, Ornamental Plants, and Tea

Determination of Volatile Poisons in Beverages using Static Headspace Gas Chromatography with a Mass Spectrometric Detector. **Kevin J. Mulligan,** US Food and Drug Administration

Processed Vegetable Products

General Referee Report: Processed Vegetable Products. **Thomas R. Mulvaney,** US Food and Drug Administration

Sugars and Sugar Products

Production of Oligosaccharides during Sucrose Inversion. **Paul F. Cancalon,** Florida Department of Citrus

SNIF-NMR Method (Site Specific Deuterium Nuclear Magnetic Resonance) for Detecting Beet Sugar in Fruit Juices. **Gilles G. Martin** and **Claude G. Guillou,** Eurofins

General Referee Report: Sugars and Sugar Products. **Margaret A. Clarke,** Sugar Processing Research Institute, Inc.

Vitamins and Other Nutrients

Automated Immunoanalysis for Measurement of Lipoprotein Cholesterol. **Marsha Gray, Anne Plant, William MacCrehan** and **Willie May,** National Institute of Standards and Technology

Determination of Cholesterol in Foods by p-Nitrobenzoate Derivatization and HPLC. **Thomas W. Hamill, Edna R. Young** and **Abdel-Gawad M. Soliman,** US Food and Drug Administration

HPLC Determination of Thiamine, Riboflavin and Pyridoxine in Medical Foods. **G. William Chase, William O. Landen, Jr.,** and **Abdel-Gawad M. Soliman,** US Food and Drug Administration

Measurement of Vitamin A and Beta-Carotene by HPLC for Nutritional Labeling. **Art Sims, John Morawski** and **Barbara Kenney,** Millipore Corporation, Waters Division

Determination of Vanillin and Ethyl Vanillin in Vanilla Extracts by HPLC. **Sidney Kahan,** Kahansultants, Inc.

Rapid and Precise Method for the Determination of All-trans Vitamin A Palmitate and Cis-isomers in Infant Formulae and Adult Nutritional Supplements. **Leon T. Dupuis, Joseph L. LeBoeuf** and **Timothy Kearsley,** Wyeth Ltd./Ltee

Rapid and Precise Method for the Determination of Thiamine in Infant Formulae and Adult Nutritional Supplements by HPLC and Fluorometric Detection. **Leon T. Dupuis, Joseph L. LeBoeuf** and **Timothy Kearsley,** Wyeth Ltd./Ltee

Microbiological Assay for Biotin in Multivitamins. **Ken Baker, Allan Gennis** and **Anthony Cundell,** Lederle Laboratories

Applications of a New, Moderately Polar High Temperature Fused Silica GC Capillary Column for Analysis of Mono, Di, Tri-Glycerides, Cholesterol, Sterols, and Other Lipid Fractions. **Marc Dinnauer,** J&W Scientific

Determination of Thiamine and Riboflavin in Infant Formulas and Enterals by HPLC using UV and Fluorescence Detectors in Series. **Matthew G. Sliva, Kathleen A. Pfender, Astor E. Green** and **Janice R. Saucerman,** Bristol-Myers Squibb Company

Determination of Cholesterol in Prepared Foods by Direct Injection. **Sam Al-Hasani, Jan Hlavac** and **Mark Carpenter,** ConAgra Frozen Foods

Rapid Assay of Vitamin A in Concentrated Oils, Powders and Premixes by Reverse Phase HPLC. **Michael Hinsberg,** BASF Corporation

SYMPOSIUM

Process Sensors and Control, Part I

Monday, August 31, 1:00-4:45 pm

Chairman: **Donald E. Carpenter,** Kraft General Foods

Cosponsored by **Kraft General Foods, Dow Chemical Company** and **HEINZ, U.S.A.**

1:30 Introduction. **Donald E. Carpenter,** Kraft General Foods

1:45 Applications of FTIR in the Food Processing Industry. **William J. McShane,** Kraft General Foods Technology Center

2:15 Near Line FTIR Analysis and Validation. **James Redzak,** Colgate Palmolive

2:45 BREAK

3:15 Development of a Noninvasive Method for Measuring the Crude Lipid Content in Whole Fish and Fish Products. **Anna G. Cavinato,** D2 Development, and Barbara A. Rasco, University of Washington

3:45 Application of NIR to Sugar Analysis: Comparison with Traditional Methods. **Margaret Clarke, Cynthia McDonald-Lewis** and **Enrique R. Arias,** Sugar Processing Research Institute, Inc.

4:15 Nonglass pH Electrode for Food and Beverages. **Michael Yitref,** Uniloc

TECHNICAL POSTER SESSION

Drugs and Related Topics, Part I

Monday, August 31, 3:00-4:30 pm

Poster Session Coordinator: **Philippe Leroux,** PHL Consultant

Topics include: Cosmetics; Drugs; Forensic Sciences; General Topics

Sponsored by **Bristol-Myers Squibb Pharmaceutical Research Institute**

Cosmetics

Comparison of a New Contact Membrane Technique to Conventional Direct Inoculations for Evaluating Adequacy of Preservation in Cosmetics. **Tony T. Tran, M. Shurbaji** and **L.B. Koopman,** US Food and Drug Administration

Rapid Method for the Determination of Nitrosating Agents in Cosmetic Products by Chemiluminescence Detection of Nitric Oxide. **Hardy J. Chou** and **Donald C. Havery,** US Food and Drug Administration

Drugs

Microwave Digestion of Pharmaceuticals for Inductively Coupled Plasma Spectrometric Detection. **Brenda S. Sheppard, Cindy Gasten, Barbara Barnes** and **Karen Wolnick,** US Food and Drug Administration

Derivative Spectrophotometry as a Stability-Indicating Method for Determination of Bupivacaine Hydrochloride. **Maria I.R.M. Santoro** and **Eduardo B. Govato,** Universidade de Sao Paulo

Determination of Steroid Hormones in Pharmaceutical Preparations by HPLC. **Maria I.R.M. Santoro, Erika R.M. Hackmann** and **Salete A. Benetton,** Universidade de Sao Paulo

Identification of Methyl Palmitate in Nitrofurantoin Capsules by GC/MS. **Ross D. Kirchhoefer, Ruby L. Brown, Jim F. Brower** and **Judy Young,** US Food and Drug Administration

Quantitation of Floctafenine by HPLC and Spectrophotometric Methods. **Kamla M. Emara, A. Horria** and **Y. Bekenaz,** Assiut University

Detection of Methamphetamine Enantiomers by Liquid Chromatography with Fluorescence. **Yu-Pen Chen, Mei-Chich Hsu** and **Chun-Sheng Chien,** Taiwan National Laboratories of Foods and Drugs

New Spectrophotometric Method for the Estimation of Certain Benzodiazepine Drugs. **Kamla M. Emara** and **Nadia M. Mahfouz,** Assiut University

Analysis of Cromolyn Sodium in Bulk Drug Substance and Dosage Forms. **Linda L. Ng,** US Food and Drug Administration

General Referee Report: Drugs IV. **Linda L. Ng,** US Food and Drug Administration

Forensic Sciences

Identification of Steroids by TLC/FTIR Microscope. **Sritana C. Yasui,** US Food and Drug Administration

Screening for 50 Basic Drugs Utilizing Robotics, HPLC, and GC-MSD in Milk, Colas and Vegetable Oil. **Anthony W. Smallwood,** US Food and Drug Administration

Applications of Particle Beam and Thermospray LC/MS in the Investigation of Product Tampering. **Rick A. Flurer, Mantai Z. Mesmer** and **R. Duane Satzger,** US Food and Drug Administration

General Topics

Use of TGA and DSC to Ensure the Authenticity of Drug Formulations. **John W. Robinson,** US Food and Drug Administration

Purge and Trap GC/MS Determination of Benzene in Denture Adhesives. **Thomas L. Barry** and **Glenn Petzinger,** US Food and Drug Administration

Dissolution Profiles of Sustained Release Niacin Formulations. **Ross D. Kirchhoefer** and **Sharon Hipp,** US Food and Drug Administration

Impurities in Niacin by HPLC. **Ross D. Kirchhoefer,** US Food and Drug Administration

Identification of Quinolinic Acid in Niacin by GC/MS. **Ross D. Kirchhoefer,** US Food and Drug Administration

Concerning the Optimization of HPLC Chiral Resolutions. **Charlotte A. Brunner, Hae-Young Ahn, Gerald K. Shiu** and **Thomas D. Doyle,** US Food and Drug Administration

Glycoalkaloids Content in Wild Tubers (*Solanum cardiophyllium, S. ebrenbergii*). **Jose L. Ibave, Martin Villalobor** and **Elsa Romero,** University of Chihuahua

TUESDAY

SYMPOSIUM

Harvey W. Wiley Award Symposium

Fumonisins: Occurrence, Distribution, Production, Analysis Chemistry, and Mode of Action, Part II

Tuesday, September 1, 8:30 am - 12:15 pm

Chairman: **John L. Richard,** US Department of Agriculture

Cosponsored by **Corn Refiners Association, Inc.**

8:30 Assessment of Fumonisins in Human Foods by Immunochemical Methods. **James J. Pestka,** Michigan State University

9:00 High Performance Liquid Chromatography Methods of Analysis for Fumonisins. **Glenn A. Bennett,** US Department of Agriculture

9:30 Labelled Fumonisins: Production of Stable Isotopes and Their use in Analytical Methods. **Ronald D. Plattner,** US Department of Agriculture

10:00 BREAK

10:30 Stability and Problems in Determination of Fumonisins in Foods. **Peter M. Scott** and **Guillaume A. Lawrence,** Health and Welfare Canada

11:00 Cytotoxicity of Fumonisins in Avian Lymphocytes. **Mary A. Dombrink-Kurtzman,** US Department of Agriculture

11:30 Fumonisins and Sphingolipid Biosynthesis. **Ron Riley,** Richard B. Russel Research Center

12:00 Summation

SYMPOSIUM

Milk: Antibiotics and Other Contaminants, Part I

Tuesday, September 1, 8:30 am - 12:00 noon

Chairman: **Joseph A. Settepani,** US Food and Drug Administration

8:30 Opening Remarks. **Joseph A. Settepani,** US Food and Drug Administration

8:35 Preparation of Milk for Immunoassay and HPLC Screening using Matrix Solid Phase Dispersion. **Steven A. Barker,** Louisiana State University

9:00 Analysis of Penicillin and Streptomycin in Milk by HPLC. **James** D. **MacNeil,** Agriculture Canada

9:30 Determination of Ampicillin in Milk using Automated HPLC Cleanup. **William A. Moats,** US Department of Agriculture

10:00 **BREAK**

10:30 Multiresidue Analysis of Some Beta-Lactam Antibiotics in Bovine Milk by HPLC and Confirmation by LC/MS. **Krystyna L. Tyczkowska,** North Carolina State University

11:00 Analysis of Tetracycline Antibiotics in Milk by HPLC and TLC/MS. **Hisao Oka,** Aichi Prefectural Insitute of Public Health

TECHNICAL POSTER SESSION

Pesticides, Part I

Tuesday, September 1, 8:30-10:00 am

Poster Session Coordinator: **Leon D. Sawyer,** US Food and Drug Administration

Topics include: CIPAC Studies; Multiresidue Methods; Organophosphorus Insecticide Formulations; Organophosphorus Pesticides; Radioactivity

Sponsored by **ICI Americas, Inc.**

CIPAC Studies

General Referee Report: CIPAC Studies. **Alan R. Hanks,** Office of the Indiana State Chemist and Seed Commissioner

Multiresidue Methods

Rapid, Automated Liquid/Liquid Extraction of Priority Pollutants from Aqueous Matrices. **Loren Schrier, Kevin P. Kelly** and **Leemer Cernohlavek,** ABC Laboratories

Application of On-line SFE and GPC Cleanup to Analysis of Trace Level Compounds in Food and Agricultural Products. **Jim J. Stunkel, Said Saim** and **David L. Stalling,** ABC Laboratories

Comparison of Headspace, Steam Distillation, and Purge and Trap Techniques for the Capillary Gas Chromatographic Analysis of Halogenated Volatiles in Foods. **B. Denis Page** and **Gladys M. Lacroix,** Health and Welfare Canada

Monitoring of Pesticide Residues on Vegetables and Fruits by using Multiresidue Analysis Methods. **Sue-Sun Wong, Li-Ji Wu** and **G.C. Li,** Taiwan Agricultural Chemicals and Toxic Substances Research Institute

Multiresidue Determination of Pesticides and Industrial Chemicals in Seafood Products using a Solid Phase Extraction Cleanup and GLC Detection. **Frank J. Schenck, Roberta Wagner** and **Michael K. Hennessy,** US Food and Drug Administration

Analysis of Nitrogen-, Phosphorus- and Chlorine-Containing Pesticides in River and Drinking Waters by Gas Chromatography/Mass Spectrometry GC/MS. **Francis P. Scanlan** and **David Benanou,** Compagnie Generale des Eaux - Anjou Recherche

Criteria for Adapting Immunoassays to the Analysis of Pesticide Residues in Agricultural Commodities. **Scott W. Jourdan, Adele M. Scutellaro, Mary C. Hayes** and **David P. Herzog,** Ohmicron Corporation

Pesticide Residues in Milk by Enzyme Immunoassay. **Bruce S. Ferguson, Titan S. Fan** and **Rodney J. Bushway,** ImmunoSystems, Inc.

General Referee Report: Multiresidue Methods. **Leon D. Sawyer,** US Food and Drug Administration

Organophosphorus Insecticide Formulations

General Referee Report: Organophosphorus Insecticide Formulations. **William R. Betker,** Miles Inc.

Organophosphorus Pesticides

Determination of Bromofenofos in Milk by High-Performance Liquid Chromatography with Electrochemical Detection. **Kazue Takeba, Takeshi Itoh, Masao Matsumoto,** Tokyo Metropolitan Research Laboratory of Public Health, and **Hiroyuki Nakazawa,** Japanese National Institute of Public Health

Analysis of Organophosphates, Methoprene and Pyrethroid Residues in Wheat and Barley Foods using Laboratory Immunoassay Kits. **Simone Edward, Amanda Hill,** CSIRO Division of Plant Industry, **David Kelsey,** ImmunoSystems, Inc., and **John Skerritt,** CSIRO Division of Plant Industry

Radioactivity

Determination of Iodine-131 at Low-levels in Milk: Collaborative Study. **Edmond J. Baratta,** US Food and Drug Administration, and **David G. Easterly,** US Environmental Protection Agency

General Referee Report: Radioactivity. **Edmond J. Baratta,** US Food and Drug Administration

TECHNICAL POSTER SESSION

Environmental Quality

Tuesday, September 1, 10:30 am-12:00 noon

Poster Session Coordinator: **Verdel K. Dawson,** US Fish and Wildlife Service

Topics include: Cooperative Studies; Herbicides; Inorganics in Water; Organics in Water; General Topics

Sponsored by **Eli Lilly and Company**

Cooperative Studies

Polymeric Reagent Characterized by Specific Dual Detectability on HPLC as an Electrophile for Derivatizing Amines in Environmental Samples. **Weh S. Wu, Kazik Jedrzejczak** and **Virindas S. Gaind,** Ontario Ministry of Labour

General Referee Report: Cooperative Studies. **Joseph R. Donnelly,** Lockheed Engineering & Sciences Company

Herbicides

Determination of the Ethanesulfonic Acid Metabolite of Alachlor in Water by High-Performance Liquid Chromatography. **Rodney J. Bushway, Brian Perkins, David Baker, Carol Macomber, Titan Fan** and **Bruce Ferguson,** University of Maine

Inorganics in Water

Determination of Dissolved Hexavalent Chromium in Drinking Water, Ground Water, and Industrial Wastewater Effluents by Ion Chromatography: Collaborative Study. **Kenneth W. Edgell,** Bionetics Corporation, **James E. Longbottom,** US Environmental Protection Agency, and **Robert J. Joyce,** Dionex Corporation

Determination of Inorganic Anions in Water by Ion Chromatography: Collaborative Study. **Kenneth W. Edgell,** Bionetics Corporation, **James E. Longbottom** and **John D. Pfaff,** US Environmental Protection Agency

Organics in Water

Explosives Residues Analysis in Ground and Surface Waters using SDB Disks. **Craig Markell,** 3M Company, **Gabe LeBrun** and **Pat Rethwill,** PACE, Inc.

Quantitation of the Urea Herbicides, Diuron and Chlortoluron, in Water by Enzyme Immunoassay. **Karen Larkin, Titan Fan, David Kelsey** and **Bruce Ferguson,** ImmunoSystems, Inc., **Nanju Lee** and **John Skerritt,** CSIRO Division of Plant Industry

Measurement of Isoproturon in Water by Enzyme Immunoassay. **Jonathan J. Matt, Titan S. Fan, David E. Kelsey** and **Bruce S. Ferguson,** ImmunoSystems, Inc.

Measurement of Chlorpyrifos in Irrigation Drainage Water by Enzyme Immunoassay. **John Skerritt, Amanda Hill, Kathleen Bowmer,** CSIRO Division of Plant Industry, **Wolfgang Korth, Martin Thomas,** CSIRO Division of Water Resources, **Titan Fan** and **Bruce Ferguson,** ImmunoSystems, Inc.

Application of Semipermeable Membrane Devices to Monitoring of Aquatic Environments for Contamination by Polycyclic Aromatic Hydrocarbons. **Jon A. Lebo, Jim L. Zajicek, James N. Huckins, Jimmie D. Petty, Paul H. Peterman** and **Robert W. Gale,** US Fish and Wildlife Service

Fungi from Petroleum Contaminated Soil and Utilization of Petroleum Product as C-source by these Mycoflora. **Sarwat Parvez,** King Saud University

General Topics

Evaluation of Outlier Detection Procedures for Data from Interlaboratory Method Validation Studies. **Florence A. Fulk, James E. Longbottom** and **Paul W. Britton,** US Environmental Protection Agency

Design of Reference Materials to Meet the Quality Requirements of Environmental Analysis. **Robert E. Thompson, Zora E. Bunn, Cynthia S. Smith** and **Patricia E. Beyer,** ManTech Environmental Technology, Inc.

Petro Risc™: An Immunoassay for the Rapid, On-site Screening for Gasoline and Diesel in Soil. **James P. Mapes, Karen D. McKenzie, Shannon P. Arrowood, William B. Studabaker, Randy L. Allen, Wayne B. Manning** and **Stephen B. Friedman,** EnSys, Inc.

Quality Assurance for Environmental Analysis of PCBs by High Resolution Mass Spectrometry. **Joseph R. Donnelly, Andrew H. Grange** and **G. Wayne Sovocool,** Lockheed Engineering & Sciences Company

TECHNICAL POSTER SESSION

Pesticides, Part II

Tuesday, September 1, 1:00-2:30 pm

Poster Session Coordinators: **Harvey Newsome,** Health and Welfare Canada, and **Saidul Zafar Qureshi,** Aligarh Muslim University

Topics include: Disinfectants; Metals and Other Elements; Organohalogen Pesticides; Organonitrogen Pesticides; General Topics

Sponsored by **Miles, Inc. Agriculture Division**

Disinfectants

Capillary Gas Chromatographic Determination of Glutaraldehyde and Phenol in Disinfectants. **James W. Danielson** and **Richard Thompson,** US Food and Drug Administration

Metals and Other Elements

Multielement Scheme for the Determination of 23 Elements in a Single Sample Digest Solution. **Ronald W. Marts,** US Food and Drug Administration

Determination of Chromium and Molybdenum in Medical Foods by Graphite Furnace Atomic Absorption Spectrophotometry. **Edwin C. Phifer, Edna R. Young, Ronald R. Eitenmiller** and **Abdel-Gawad M. Soliman,** US Food and Drug Administration

Determination of Lead Released from Ceramic Ware into 4% Acetic Acid using Electrothermal Atomization-Atomic Absorption Spectrophotometry. **Susan C. Hight,** US Food and Drug Administration

Lead Release from Ceramic Mugs with Lead Containing Glazes using Coffee and Acetic Acid. **William R. Mindak, William H. Lamont, William C. Cunningham** and **Stephen G. Capar,** US Food and Drug Administration

Detection of Lead and Other Elements in Ceramic Glazes and Housewares by Radioisotope-Induced X-Ray Emission. **David L. Anderson, William C. Cunningham** and **Tyler R. Lindstrom,** US Food and Drug Administration

Reduction of Lead Levels in Canned Foods. **Lester M. Crawford** and **Edgar R. Elkins,** National Food Processors Association

Analysis of Pb in Ca Supplements by Graphite Furnace Atomic Absorption Spectrophotometry. **Paul H. Siitonen, Stephen G. Capar** and **Harold C. Thompson, Jr.,** US Food and Drug Administration

Comparison of Two Rapid Tests for Detection of Lead in Ceramic Ware. **Scott P. Dolan, Stephen G. Capar, William C. Cunningham, Richard M. Jacobs** and **Randall J. Plunkett,** US Food and Drug Administration

General Referee Report: Metals and Other Elements. **Stephen G. Capar,** US Food and Drug Administration

Organohalogen Pesticides

Application of a Pattern Recognition Technique to Polychlorinated Biphenyls Found in the Serum of Residents of New Bedford, Massachusetts. **Virlyn W. Burse, Samuel P. Caudill, Margaret P. Korver, Patricia C. McClure** and **Susan L. Head,** Centers for Disease Control

General Referee Report: Organohalogen Pesticides. **Bernadette M. McMahon,** US Food and Drug Administration

Organonitrogen Pesticides

Determination of Paraquat and Diquat in Crops using Ion-Pairing HPLC. **Tina M.P. Chichila** and **Dalia M. Gilvydis,** US Food and Drug Administration

Determination of Aldicarb in Citrus Juice by Enzyme Immunoassay. **Jeanne A. Itak, Michele Y. Selisker, Robert G. Sandberg** and **David P. Herzog,** Ohmicron Corporation

Determination of Captan in Peaches and Apple Juice by a Magnetic Particle-based Immunoassay. **Jeanne A. Itak, Michele Y. Selisker,** Ohmicron Corporation, **James R. Fleeker,** North Dakota State University, **Joseph X. Dautlick** and **David P. Herzog,** Ohmicron Corporation

Determination of Herbicides in Agricultural Samples by Immunoassay. **Donna A. Fitzpatrick, Dennis R. Stocker, James H. Rittenburg** and **G. David Grothaus,** Agri-Diagnostics Associates

Capillary Gas Chromatographic Determination of Thiabendazole in Citrus Juices and Apple Juices. **Mitsuo Oishi, Kazuo Onishi, Motohiro Nishijima,** and **Itsu Kano,** Tokyo Metropolitan Research Laboratory of Public Health, **Hiroyuki Nakazawa,** National Institute of Public Health

Determination of Glyphosate and its Metabolite in Total Diet Food Samples using HPLC with Post Column Derivatization. **Ronald G. Luchtefeld,** US Food and Drug Administration

General Topics

Method for Determination and Confirmation of Cis-9-Tricosene in Technical Material by Capillary GC and GC/MS. **Jinren Ko, Jack Nguyen** and **Jim Burleson,** Zoecon Corporation

Use of Immunoassays to Detect Crop Protection Chemicals: Applications in Soil Analysis and Worker Safety. **Robin R. Charlton, Charles W. Carlson, Laure H. Kenyon, Theodore H. Carski, William F. Smith, Wayne J. Steele, II, Frederick A. Liberatore, Kai S. Leung** and **Catherine S. Valteris,** E.I. du Pont de Nemours and Company

Microgram Spectrophotometric Determination of Aromatic Dinitro Compounds in Aprotic Solvents. **Saidul Zafar Qureshi, Nafisur Rahman** and **Seema Haque,** Aligarh Muslim University

High Purity Analytical Grade Solvents from Recovered Materials using Spinning Band Distillation. **Jim J. Stunkel** and **Kevin P. Kelly,** ABC Laboratories

Analysis of Amphetamines in Various Food Matrices Following o-Phthalaldehyde Derivatization and HPLC with UV Fluorescence, and Electrochemical Detection in Series. **Lorrie Lin,** US Food and Drug Administration

SYMPOSIUM

Process Sensors and Control, Part II

Tuesday, September 1, 1:30-5:00 pm

Chairman: **Donald E. Carpenter,** Kraft General Foods

Cosponsored by **HEINZ, U.S.A., Kraft General Foods,** and **Dow Chemical Company**

1:30 Application of NMR to the Analysis of Fat and Moisture in Foods. TBA

2:00 NMR Analysis of Solid Fat Content in Edible Oils. TBA

2:30 Micromachined Sensors for Process Analysis of Foods and Pharmaceuticals. **Marc Madou,** Teknekron Sensor Development Corporation

3:00 BREAK

3:30 Application of Sensor Arrays and Neurol Networks to Odor Sniffing. **Joseph Stetter,** Transducer Research, Inc.

4:00 Application of Fuzzy Logic to Process Control. TBA

4:30 Concluding Remarks. **Donald E. Carpenter,** Kraft General Foods

SYMPOSIUM

Milk: Antibiotics and Other Contaminants, Part II

Tuesday, September 1, 1:30-4:30 pm

Chairman: **Joseph A. Settepani,** US Food and Drug Administration

1:30 Analysis of Milk for Antibiotics, Total Coliform, and Sporeforming Microbial Activities and Enzymatic Milk Quality Parameters using Reflectance Colorimetry. **Gary H. Richardson,** Utah State University

2:00 Antibiotic Residue Screening Tests: How Well Do They Perform on Individual Animal Milk Samples? **James S. Cullor,** University of California

2:30 Depletion of Residues from Milk of Cows Orally and Intravenously Dosed with Sulfamethazine. **Guy Paulson,** US Department of Agriculture

3:00 BREAK

3:30 Evaluation of Screening Assays for Residues of Chloramphenicol in Milk. **Laura A. Adam,** US Food and Drug Administration

4:00 Antibiotics and Sulfonamides in Milk: Significance, Evaluation, MRLs, and Concepts of Detection from an International Point of View. **Walther H. Heeschen,** Federal Dairy Research Center

4:30 Closing Remarks. **Joseph A. Settepani,** US Food and Drug Administration

TECHNICAL POSTER SESSION

Feeds, Fertilizers and Related Materials

Tuesday, September 1, 3:00-4:30 pm

Poster Session Coordinator: **Peter F. Kane,** Office of the Indiana State Chemist

Topics include: Antibiotics in Feeds; Drugs in Feeds; Feeds; Fertilizers and Agricultural Liming Materials; Nutrients in Soils; Tobacco; Veterinary Analytical Toxicology

Sponsored **by Eli Lilly and Company**

Antibiotics in Feeds

Simultaneous Determination of Three Polyether Antibiotics in Feed using High-Performance Liquid Chromatography with Fluorescence Detection. **Hirohiko Asukabe, Hideaki Murata, Ken-ichi Harada** and **Makoto Suzuki,** Meijo University, **Yoshitomo Ikai** and **Hisao Oka,** Aichi Prefectural Institute of Public Health

Validation of the Particle Concentration Fluorescence Immunoassay of Tylosin. **Alan L. Wicker, Daniel J. Sweeney, Daniel H. Mowrey** and **Mark R. Coleman,** Eli Lilly and Company, **Deborah K. Morris** and **Catherine L. Brockus** International Diagnostics Systems Corporation

Determination of Monensin by TLC in Feeds: Collaborative Study. **Wynne Landgraf** and **P. Frank Ross,** US Department of Agriculture

Extraction of Antibiotics from Feed and Tissues using SPE Cartridges. **Dean Rood,** J&W Scientific

Validation of the Monensin HPLC Assay for Feeds. **Mark R. Coleman, John W. Moran, Thomas D. Macy** and **J. Matt Rodewald,** Lilly Research Laboratories

General Referee Report: Antibiotics in Feeds. **Hussein S. Ragheb,** Purdue University

Drugs in Feeds

Determination of Ractopamine Hydrochloride in Animal Feeds by High-Performance Liquid Chromatography with Electrochemical Detection. **Thomas D. Macy, Jerry J. Lewis** and **Mark R. Coleman,** Lilly Research Laboratories

Use of Temperature Modulated HPLC for the Analysis of Sulfadimethoxine and Ormetoprim in Multiple Biological Matrices. **Nicholas P. Milner, James M. Gifford** and **George Weiss,** Hoffmann-La Roche Inc.

General Referee Report: Drugs in Feeds. **Robert L. Smallidge,** Office of the Indiana State Chemist and Seed Commissioner

Feeds

Analysis of Thiamine in Rodent Feed by High Performance Liquid Chromatography Utilizing Post Column Derivatization and Fluorescence Detection. **Theresa Gehring, Willie M. Cooper, Claude L. Holder** and **Harold C. Thompson, Jr.,** US Food and Drug Administration

Fertilizers and Agricultural Liming Materials

General Referee Report: Fertilizers and Agricultural Liming Materials. **Peter F. Kane,** Office of the Indiana State Chemist

Nutrients in Soils

General Referee Report: Nutrients in Soils. **Thomas L. Jensen,** Nebraska State Department of Agriculture

Tobacco

Enzyme Immunoassay for the Measurement of Nicotine in Tobacco Extracts. **Jonathan J. Matt, Carol A. Macomber, Titan S. Fan, David E. Kelsey** and **Bruce S. Ferguson,** ImmunoSystems, Incorporated

Quantitative Determination of KABAT® (Methoprene) in Tobacco Samples using a Competitive Enzyme-Linked Immunosorbent Assay. **Deborah Dixon-Holland, Michael Pallmer, Barbara Christensen, Chari Seiffert, Donald Farnum** and **James Harness,** Neogen Corporation

Veterinary Analytical Toxicology

Phenylpropanolamine (PPA) Analysis for Pharmacokinetic Studies by Solid State Extraction from Biological Samples, HPLC Separation and Fluorescent Derivative Detection. **Marvin J. Bleiberg** and **S. Ellis,** US Food and Drug Administration

Rapid Test for the Semiquantitative Determination of Nitrate in Forages: Intralaboratory Study. **Anant V. Jain,** University of Georgia

Use of Liquid Nitrogen for Incorporation of Gas Plant Coal Tar Residues into Laboratory Rodent Feed for Toxicological Evaluation. **Harold C. Thompson, Jr., Dan M. Nestorick** and **Larry G. Rushing,** US Food and Drug Administration

WEDNESDAY

Regulatory Roundtable, Part I

Wednesday, September 2, 8:30-11:30 am

Focus: **North American Trade Zone Regulations**

Moderator: **Bobbi Dresser,** US Food and Drug Administration

Cosponsored by **CIBA-GEIGY Corporation - Agricultural Division** and **Dupont Agricultural Products**

This concept was introduced at the 1985 Annual Meeting to further encourage communication among state, national, and international governments and industries on matters of regulatory concern.

The invited panelists will each briefly address the issue of North American trade zone regulations and discuss the complex factors involved in regulation. An open discussion will follow.

SYMPOSIUM

Forensic Methods and Product Tampering, Part I

Wednesday, September 2, 8:30-11:30 am

Co-Chairmen: **Robert Bianchi,** US Drug Enforcement Administration, **Fredrick Fricke,** and **Karen A. Wolnik,** US Food and Drug Administration

Sponsored by **American Cyanamid Company, Agricultural Research Division**

8:30 Introduction

8:35 Keynote address: Historical Overview of Product Tampering. **Richard C. Swanson,** US Food and Drug Administration

9:30 **BREAK**

10:00 Investigative Response to Consumer Product Tampering. **Kenneth Nimmich,** Federal Bureau of Investigation

10:30 Prospects for Tamper Evidency in Packaging. **Hugh Lockhart,** Michigan State University

11:00 Application of Ion Chromatography in Forensic Cases. **Lisa A. Kane,** US Food and Drug Administration

TECHNICAL POSTER SESSION

Microbiology and Extraneous Materials

Wednesday, September 2, 8:30-10:00 am

Poster Session Coordinator: **Russell S. Flowers,** Silliker Laboratories Group, Inc.

Topics include: Drug- and Device-Related Microbiology; Filth and Extraneous Materials; Food Microbiology (Dairy); Food Microbiology (Nondairy); General Topics

Sponsored by **GENE-TRAK Systems**

Drug- and Device-Related Microbiology

Validation of an Analytical Enzyme Linked Immunosorbent Assay (ELISA) for the Determination of bST in Milk. **Stuart F. Easley, Maurice E. Scheetz, II, Billy J. Barker, Daniel H. Mowrey, Wendell C. Smith, Mark A. Vise, Alan L. Wicker** and **Mark R. Coleman,** Lilly Research Laboratories

Rapid Screening for the Presence of Toxic Materials in Foods by Means of Luminescent Microbial Bioassay. **Kevin J. Mulligan** and **Martin P. Votel,** US Food and Drug Administration

Comparison of Agar Diffusion Assays Measured by Caliper versus an Image Analyzer. **Ken Baker, Allan Gennis** and **Anthony Cundell,** Lederle Laboratories

Filth and Extraneous Materials

Extraction of Light Filth from Cheeses: Collaborative Study. **Marvin J. Nakashima,** US Food and Drug Administration

Food Adulteration and Traditional Methods in Subcontinent. **M.M. Zahid Shah Taimuri** and **Kishwar Shabina,** Karachi Metropolitan Corporation

Light Filth in Condimental Hot Sauces: Collaborative Study. **John R. Bryce,** US Food and Drug Administration

Light Filth in Bean Paste: Collaborative Study. **Larry E. Glaze** and **John R. Bryce,** US Food and Drug Administration

General Referee Report: Filth and Extraneous Materials. **Jack L. Boese,** US Food and Drug Administration

Food Microbiology (Dairy)

Listeria-Tek Elisa Method for the Detection of *Listeria monocytogenes* in Dairy Products, Seafoods, and Meats: Collaborative Study. **Michael S. Curiale, Wendy Lepper,** Silliker Laboratories Group, Inc., and **Barbara Robison,** Organon Teknika

DNA Hybridization Method for the Detection of *Listeria* in Dairy Products, Seafoods, and Meats: Collaborative Study. **Michael S. Curiale, Terri Sons, Luanne Fanning, Wendy Lepper, Dawn McIver,** Silliker Laboratories Group, Inc., **Susan Garramone,** GENE-TRAK Systems, Inc. and **Mark A. Mozola,** Silliker Laboratories Group, Inc.

Six-Hour Preenrichment for the Recovery of *Salmonella* spp. from Low Moisture Dairy Foods. **Thomas S. Hammack, Felicia B. Satchell, Wallace H. Andrews, R. Miguel Amaguana, Lynda B. Koopman, Geraldine Allen June, Patricia S. Sherrod** and **Verneal R. Bruce,** US Food and Drug Administration

Analysis of Total Microbial Numbers in Raw and Pasteurized Milk using Reflectance Colorimetry: Collaborative Study. **Gary H. Richardson, T.C. James Yuan, Barry E. Stokes** and **Donald V. Sisson,** Utah State University and Wescor, Inc.

Food Microbiology (Nondairy)

Oxoid *Salmonella* Rapid Test Method for the Detection of Motile *Salmonella* in Foods. **Karl F. Eckner** and **Michael S. Curiale,** Silliker Laboratories Group, Inc.

Elevated-Temperature, Colorimetric, Monoclonal, Enzyme-linked Immunosorbent Assay for Rapid Screening of *Salmonella* in Foods: Collaborative Study. **Karl F. Eckner, Wendy A. Dustman,** Silliker Laboratories Group, Inc., **Barbara J. Robison,** Organon Teknika Corporation, and **Russell S. Flowers,** Silliker Laboratories Group, Inc.

Rapid Assay for E. coli 0157:H7. **George S. Golumbeski, John Priest** and **Shauna Navarro,** Promega Corporation

General Referee Report: Food Microbiology (Nondairy). **Wallace H. Andrews,** US Food and Drug Administration

General Topics

Implementing Quality Management (QA/QC) in the Analytical Laboratory. **Mary G. Schultz, William L. Brown,** ABC Research Corporation

Salmonella Immunoenzymatic Detection: A New Test. **Agnes Roux, Denis Kelsch, Sophie Pollet, Marie-Laure Sorin** and **Xavier Drouet,** TRANSIA-DIFFCHAMB

Listeria Immunoenzymatic Detection: A New Test. **Marie-Laure Sorin** and **Xavier Drouet,** TRANSIA-DIFFCHAMB

TECHNICAL POSTER SESSION

Foods I, Part I

Wednesday, September 2, 10:30 am to 12:00 noon

Poster Session Coordinator: **John Gilbert,** Ministry of Agriculture, Fisheries and Food

Topics include: Antioxidants; Dairy Chemistry; Flavors; Food Additives; Meat, Poultry, and Meat and Poultry Products; General Topics

Sponsored by **Ocean Spray Cranberries, Inc.**

Antioxidants

Evaluation of Antioxidative Activity of Natural Antioxidants by HPLC-Chemiluminescence Measurement of Fatty Acid Hydroperoxides. **George C. Yang** and **Parvin Yusaei,** US Food and Drug Administration

Dairy Chemistry

Performance Evaluation of the Babcock and Modified Mojonnier Methods in a Group of Labs over Time: 1988 through 1991. **David M. Barbano, Joanna M. Lynch,** Cornell University, **Patrick A. Healy,** Market Administrators Office, and **J. Richard Fleming,** Texas Milk Market

Performance Evaluation of Dry Calibration Milk Powders for Raw Milk Testing with Mid-Infrared Analyzers. **David M. Barbano, Joanna M. Lynch,** Cornell University, and **J. Richard Fleming,** Texas Milk Market

General Referee Report: Dairy Chemistry. **Robert L. Bradley,** University of Wisconsin--Madison

Flavors

Composition of Vanilla Extract. **Dana A. Krueger, Jeanne Maciel** and **Song Lin,** Krueger Food Laboratories, Inc.

Determination of Coumarin as an Adulterant in Vanilla Flavoring Products by HPLC. **Richard D. Thompson** and **Terry J. Hoffman,** US Food and Drug Administration

Food Additives

Rapid Aspartame Determination using Proven Biosensor Technology. **Jackie Winzeler, Jay Johnson** and **Rob Spokane,** Yellow Springs Instrument Company

Liquid Chromatography of Polyamines in Foods using On-Column Fluorescence Derivatization and Column-Switching Techniques. **Koichi Saito, Masakazu Horie, Yoshikazu Tokumaru,** Saitama Institute of Public Health, and **Hiroyuki Nakazawa,** National Institute of Public Health

Plastics Packaging, Aids to Polymerization: Polertine Food Contaminants. **John Gilbert, Laurence Castle** and **Peter Fordham,** Ministry of Agriculture, Fisheries, and Food

Determination of N-Nitrosodibenzylamine in Hams Processed in Elastic Rubber Nettings. **W. Fiddler, J.W. Pensabene** and **R.A. Gates,** US Department of Agriculture

New Phase for the HPLC Analysis of Acids, Bases, Zwitterions and Neutral Compounds in a Variety of Food Products. **Terry Reid, Elwood Doughty** and **Tracy Ascah,** SUPELCO, Inc.

Application of the Particle Beam Interface to HPLC-TEA for Chemiluminescence Detection of Non-Volatile N-Nitrosamines. **Stanley M. Billedeau, Thomas M. Heinze,** US Food and Drug Administration, **Jon J. Wilkes,** Vestec Corporation, and **Harold C. Thompson, Jr.,** US Food and Drug Administration

Meat, Poultry, and Meat and Poultry Products

Z.K. Shah Method for Rapid Fat Extraction. **M.M. Zahid Shah Taimuri** and **Kishwar Shabina,** Karachi Metropolitan Corporation

Combustion Method for Crude Protein in Meat and Meat Products: Collaborative Study. **Marcia King-Brink** and **Joseph G. Sebranek,** Iowa State University

Evaluation of a Simple and Rapid Colorimetric Method for Cholesterol Determination in some Dairy and Meat Products. **C. Mike Kuo, John R. Schultz,** KRUG Life Sciences and **Richard L. Sauer,** NASA Johnson Space Center

GC Headspace Determination of Methyl Bromide in Selected Foods, using ECD and HECD. **James L. Daft,** US Food and Drug Administration

Considerations for the Optimum Supercritical Fluid Extraction (SFE) of Fat from Food Products. **Joseph Levy, Athos Rosselli** and **Lori Dolata,** Suprex Corporation

Species Contamination in Raw and Cooked Meats. **Y. Peggy Hsieh, Sue H. Ho** and **Betsy B. Woodward,** Florida Department of Agriculture and Consumer Services

Evaluation of the Infratec Food and Feed Analyzer for At-line Process Control in Meat Processing Plants. **Hans Berg** and **Kurt Kolar,** Swedish Meat Research Insitute

General Topics

Mono-Dispersed Polystyrene DVB Resins for HPLC Analysis of Foods. **Elwood Doughty** and **Terry Reid,** SUPELCO, Inc.

Regulatory Roundtable, Part II

Wednesday, September 2, 1:30-4:30 pm

Focus: **EEC Regulations**

Moderator: **Alex Williams**

Sponsored by **The Coca-Cola Company**

This concept was introduced at the 1985 Annual Meeting to further encourage communication among state, national, and international governments and industries on matters of regulatory concern.

The invited panelists, **Antonio Silva Mendes,** European Communities, Directorate General, Office of Internal Market and Industrial Affairs; **Roger Wood,** Ministry of Agriculture, Fisheries and Food, UK; **Maire Walsh,** State Laboratory, Ireland; **Hugo Van Buuren,** Keuringsdienst van Waren Maastricht, Holland; and **J. Schoenermark,** European Committee for Standardization, Brussels, will each briefly address the issue of EEC regulations and discuss the complex factors involved in regulation. An open discussion will follow.

SYMPOSIUM

Nutrition Labeling of Lipids, Part I

Wednesday, September 2, 1:30-4:30 pm

Co-Chairmen: **Richard E. McDonald,** US Food and Drug Administration, and **Joyce Beare-Rogers,** Health and Welfare Canada

Cosponsored by **Silliker Laboratories Group, Inc., Gerber Products Company, The Procter & Gamble Company,** and **Archer Daniels Midland Company**

1:30 The Schizophrenic Food Label. **Sanford Miller,** University of Texas Health Science Center at San Antonio

2:00 Nutrition Labeling of Meat and Poultry. **David Soderberg** and **Richard Ellis,** US Department of Agriculture

2:30 Update on Nutrition Labeling Regulations. **John Vanderveen,** US Food and Drug Administration

3:00 **BREAK**

3:30 Nutrition Labeling of Lipids: Issues from a European Perspective. **Michael Gibney,** Trinity College Medical School

4:00 Simplification of Lipid Labeling. **Joyce Beare-Rogers,** Health and Welfare Canada

SYMPOSIUM

Microbiology Update: Part I

Listeria in Foods: Current Topics in Epidemiology, Ecology, Detection and Control

Wednesday, September 2, 1:30-4:30 pm

Co-Chairmen: **Mark A. Mozola,** Silliker Laboratories Group, Inc., and **Michael H. Brodsky,** Ontario Ministry of Health

Cosponsored by **SmithKline Beecham Animal Health, Organon Teknika Corporation,** and **Westreco, Inc.**

1:30 Opening Remarks. **Mark A. Mozola,** Silliker Laboratories Group, Inc., and **Michael H. Brodsky,** Ontario Ministry of Health

1:40 Epidemiology of Foodborne Listeriosis. **Anne Schuchat,** Centers for Disease Control

2:00 Considerations in Establishing *Listeria* Tolerance Limits for Foods. **J. Stan Bailey,** US Department of Agriculture

2:40 **BREAK**

3:00 Advancements in Methods of Isolation and Detection of *Listeria* in Foods. **Michael Curiale,** Silliker Laboratories Group

3:40 Role and Impact of Food Processing Environmental Control in Managing *Listeria monocytogenes*. **Michael Cirigliano,** T.J. Lipton Company

TECHNICAL POSTER SESSION

Foods I, Part II

Wednesday, September 2, 1:00-2:30 pm

Poster Session Coordinator: **Mary Trucksess,** US Food and Drug Administration

Topics include: Plant Toxins; Mycotoxins; Seafood Products; General Topics

Plant Toxins

General Referee Report: Plant Toxins. **Samuel W. Page,** US Food and Drug Administration

Mycotoxins

Determination of Deoxynivalenol in 1991 U.S. Winter and Spring Wheat by High Performance Thin Layer Chromatography. **Cecilia Fernandez, Michael E. Stack** and **Robert M. Eppley,** US Food and Drug Administration

Occurrence of Ochratoxin A in Sorghum and Animal Feed in Mexico. **Juan Carlos Medina Bravo, Joel Munoz Sanchez** and **Miguel Romero,** Investigacion Aplicada, S.A. de C.V.

Survey of European Cereals for the Presence of Fumonisins B_1 and B_2. **Alain Pittet** and **Dominique Tornare,** Nestec Ltd.

Efficacy of Ammonia Treatment on Reducing Aflatoxin Levels in Corn. **Douglas L. Park,** University of Arizona, **Amaury Martinez,** Universidad Central de Venezuela, and **Cong Ying Weng,** University of Arizona

Neogen Automated Residue Monitor (ARM™): A Fully Automated ELISA Workstation for Mycotoxin Analyses. **James Harness, Douglas Leatherman, Richard Rieck, Rudy Haidle** and **Albert Grzybowski,** Neogen Corporation

Comparison of DON in Four Commodities by TLC, GC, HPLC, and Neogen ELISA Methods. **Karen S. Harlin, Patricia A. Grant, Gerald L.K. Bargren, Gary O. Bordson** and **Gavin L. Meerdink,** University of Illinois-Urbana

Immunoaffinity Isolation of Fumonisin B_1 and Application to Analysis in Corn. **Thomsen J. Hansen, Kevin F. Donahue** and Paul L. Skipper, Vicam

Sero-hematological Alterations in Broiler Chicks on Feed Modified with *Fusarium proliferatum* Culture Material or Fumonisin B_1 and Moniliformin. **Tariq Javed,** University of Illinois, **John L. Richard, Glenn A. Bennett** and **Mary A. Dombrink-Kurtzman,** US Department of Agriculture, **Louise M. Cote** and **William B. Buck,** University of Illinois

Embryopathic and Embryocidal Effects of Purified Fumonisin B_1 or *Fusarium proliferatum* Culture Extract on Chicken Embryos. **Tariq Javed,** University of Illinois, **John L. Richard, Glenn A. Bennett, Mary A. Dombrink-Kurtzman,** US Department of Agriculture, **Ken W. Koelkebeck, Louise M. Cote, Robert W. Leeper,** and **William B. Buck,** University of Illinois

Comparative Pathologic Changes in Broiler Chicks on Feed Amended with a *Fusarium proliferatum* Culture or Purified Fumonisin B_1 and Moniliformin. **Tariq Javed, Ralph M. Bunte,** University of Illinois, **Glenn A. Bennett, John L. Richard, Mary A. Dombrink-Kurtzman,** US Department of Agriculture, **Louise M. Cote** and **William B. Buck,** University of Illinois

Fumonisin Production by *Fusarium moniliforme*: Comparison of Analyses by ELISA and HPLC. **Lilian Marovatsanga, Juan Azcona-Olivera** and **James J. Pestka,** Michigan State University

Simultaneous Detection of Aflatoxins, Zearalenone and Fumonisins by Multi-Analyte Immunoblot Assay. **Mohamed Abouzied** and **James J. Pestka,** Michigan State University

Performance of Two Immunochemical Assays in the Analysis of Peanuts for Aflatoxin at 37 Field Laboratories. **Joe W. Dorner, Paul D. Blankenship** and **Richard J. Cole,** US Department of Agriculture

General Referee Report: Mycotoxins. **Peter M. Scott,** Health and Welfare Canada

Seafood Products

Liquid Chromatographic Method for Analysis of the Therapeutant Sarafin® (sarafloxacin hydrochloride) in Channel Catfish Fillets. **Jeffery R. Meinertz, Verdel K. Dawson, Mark W. Tubergen, Bea Cheng** and **William H. Gingerich,** US Fish and Wildlife Service

Liquid Chromatographic Determination of the Elimination Rates of the Therapeutant Sarafin® (sarafloxacin hydrochloride) from Channel Catfish after Treatment by Oral Gavage. **Verdel K. Dawson, Jeffery R. Meinertz** and **William H. Gingerich,** US Fish and Wildlife Service

Liquid Chromatographic Analysis of Benzocaine in Blood Plasma of Fish. **John L. Allen,** US Fish and Wildlife Service

Thin Layer Chromatographic Determination of Okadaic Acid in Fish Tissue and *Provocentrum concavum* Cultures. **E. Scott Rigsby** and **Douglas L. Park,** University of Arizona

Validation of the Solid-Phase Immunobead Assay (Ciguatect™) for Toxins Associated with Ciguatera Poisoning. **Douglas L. Park** and **Sam M. Rua, Jr.,** University of Arizona

Determination of Residual Florfenicol in Yellow Tail Fish by High Performance Liquid Chromatography. **Tomoko Nagata, Masanobu Saeki,** Public Health Laboratory of Chiba Prefecture, **Hisao Oka,** Aichi Prefectural Institute of Public Health, and **Hiroyuki Nakazawa,** National Institute of Public Health

Simultaneous Determination of Quinoline Derivatives in Fish and Meat by High Performance Liquid Chromatography. **Masakazu Horie, Koichi Saito, Yoji Tokumaru, Norihide Nose,** Saitama Prefectural Institute of Public Health, and **Hiroyuki Nakazawa,** National Institute of Public Health

Development of Reference Materials for Paralytic Shellfish Poison: Intercomparison of Methods. **Hans P. van Egmond,** National Institute of Public Health and Environmental Protection

Method for the Estimation and Confirmation of Oxytetracycline in Salmon Tissue. **Michael W. Gilgan, B. Garth Burns** and **Jeffrey van de Riet,** Fisheries and Oceans Canada

Determination of Chloramphenicol Residues in Shrimp by Gas Chromatography: Interlaboratory Study. **Robert K. Munns, David C. Holland, Guy R. Stehly, Steven M. Plakas, Jose E. Roybal, Joseph M. Storey** and **Austin R. Long,** US Food and Drug Administration

General Referee Report: Seafood Toxins and Products. **James M. Hungerford,** Fisheries and Oceans Canada

General Topics

Adsorption Characteristics of Selected Analytes on Synthetic Membrane Filters used in HPLC. **Marvin Carlson** and **Richard D. Thompson,** US Food and Drug Administration

Implementation and Benefits of a Laboratory Information Management System (LIMS) at Stouffer Foods. **Cynthia A. DeSarno,** Laboratory MicroSystems, Inc.

TECHNICAL POSTER SESSION

Drugs and Related Topics, Part II

Wednesday, September 2, 3:00-4:30 pm

Poster Session Coordinator: **James D. MacNeil,** Agriculture Canada

Topics include: Drug Residues in Animal Tissues

Sponsored by **Bristol-Myers Squibb Pharmaceutical Research Institute**

Drug Residues in Animal Tissues

Determination of Clenbuterol in Bovine, Ovine and Swine Tissues by Electron Impact Gas Chromatography Mass Spectrometry. **Roger T. Wilson, Joseph M. Groneck, Kathleen P. Holland** and **Carolyn Henry,** US Department of Agriculture

Modified Method for the Determination of Ivermectin Residues in Animal Tissues. **Craig D.C. Salisbury,** Agriculture Canada

Determination of Tilmicosin and Tylosin Residues in Animal Tissues by Reversed-Phase Liquid Chromatography. **Wayne Chan, Geoff C. Gerhardt** and **Craig D.C. Salisbury,** Agriculture Canada

Determination of Streptomycin Residues in Animal Tissues by Reversed Phase Liquid Chromatography. **Geoff C. Gerhardt, Craig D.C. Salisbury** and **James D. MacNeil,** Agriculture Canada

Detection of Residues in Butter, Skim Milk Powder and Other Dairy Products Prepared from Milk Fortified with Sulfamethazine, Sulfadimethoxine, Tetracycline, Gentamycin, and Chloramphenicol. **Carla P. Barry, Chesley Randall** and **Wayne Modler,** Agriculture Canada

Analytical Monitoring Protocol of Dairy Products for Residues of Chloramphenicol. **Carla P. Barry** and **Guy Dupont,** Agriculture Canada

Analytical Monitoring Protocol of Egg and Egg Products for Residues of Chloramphenicol. **Carla P. Barry** and **Guy Dupont,** Agriculture Canada

Identification of Residual Tetracyclines in Meat by TLC/FAB MS with a Sample Condensation Technique. **Hisao Oka, Yoshitomo Ikai, Junko Hayakawa,** Aichi Prefectural Institute of Public Health, **Katsuyoshi Masuda, Ken-ichi Harada, Makoto Suzuki,** Meijo University, **Valerie Martz** and **James D. MacNeil,** Agriculture Canada

Current Overview of Feed Additives and Veterinary Drugs and Their Residual Analysis in Japan. **Hiroyuki Nakazawa, Masahiko Fujita,** National Institute of Public Health, **Hisao Oka,** Aichi Prefectural Institute of Public Health, **Tomoko Nagata,** Public Health Laboratory of Chiba Prefecture, **Kazue Takeba,** Tokyo Metropolitan Research Laboratory of Public Health, **Masakazu Horie, Koichi Saito,** Saitama Prefectural Institute of Public Health, and **Mitsuo Oishi,** Tokyo Metropolitan Research Laboratory of Public Health

Determination of Monensin Residue in Tissues by HPLC. **John W. Moran, Jackie S. McQuade** and **Mark R. Coleman,** Eli Lilly and Company

Determination of Apramycin in Swine Kidney Tissue by High Performance Liquid Chromatography with Fluorescence Detection. **Daniel J. Sweeney, Alvin L. Donoho** and **Mark R. Coleman,** Eli Lilly and Company

Confirmation of Sulfonamide Residues in Bovine Milk by GC/MS. **Valerie B. Reeves,** US Food and Drug Administration

Automated Turbidimetric Microtiter Plate Assay for Nystatin. **Anthony M. Cundell, Allan Gennis** and **Anil Sawant,** Lederle Laboratories

Microbial Assay for Nystatin: Collaborative Study. **Ken Baker** and **Anthony Cundell,** Lederle Laboratories

Reference Materials and Methods for Anabolic Agents and Veterinary Drugs: An Overview of Developments within the European Community. **Leendert A. van Ginkel, Rainer W. Stephany, Paul W. Zoontjes, Hennie J. van Rossum, Paul L.W.J. Schwillens** and **Andre Spaan,** National Institute of Public Health and Environmental Protection

Depletion of Injected Procaine and Benzathine Penicillin G from Tissues of Finished Beef Steers. **Gary O. Korsrud, Joe O. Boison,** Agriculture Canada, **Mark G. Papich,** University of Saskatchewan, **William D.G. Yates, James D. MacNeil,** Agriculture Canada, **Eugene D. Janzen, John J. McKinnon,** University of Saskatchewan, **Donald A. Landry, Gerard Lambert, Man Sen Yong** and **Leonard Ritter,** Health and Welfare Canada

Determination of the Anti-Coccidial Drug Halofuginone in Eggs by Liquid Chromatography. **David C. Holland, Robert K. Munns, Jose E. Roybal, Jeffrey A. Hurlbut** and **Austin R. Long,** US Food and Drug Administration

Gas Chromatographic Analysis of Gentamicin and Neomycin Residues in Bovine Kidney. **Susan B. Clark, Carolyn A. Geisler** and **Jeffrey A. Hurlbut,** US Food and Drug Administration

Enzyme Immunoassay for Detection of the Poultry Coccidiostat Halofuginone. **Loyd D. Rowe, Ross C. Beier, Marcel H. Elissalde** and **Larry H. Stanker,** US Department of Agriculture

Development of a Monoclonal-based ELISA for Salinomycin. **Marcel H. Elissalde, Ross C. Beier, Loyd D. Rowe** and **Larry H. Stanker,** US Department of Agriculture

Application of Immunoassays for Residue Analysis in Foods. **Larry H. Stanker, Marcel H. Elissalde, Loyd D. Rowe** and **Ross C. Beier,** US Department of Agriculture

Liquid Chromatographic Determination of Multiple Sulfonamide Residues in Bovine Milk: Method Trial. **Michael D. Smedley,** US Food and Drug Administration

Beta-Agonist Drug Residues in Bovine Liver: Multiresidue Screening Method. **G. Giacomini, E. Bastiani, R. Jaforte, F. Balaben** and **Maurizio Paleologo Oriundi,** Genego SpA

General Referee Report: Drug Residues in Animal Tissues. **Charlie J. Barnes,** US Food and Drug Administration

AOAC Open Forum

Wednesday Evening, September 2, 8:30 - 10:00 pm

Hershel F. Morris, Presiding

Once again, AOAC International will open the floor to all for discussion of problems of mutual interest. This is an opportunity to share your concerns and your successes and to learn from your colleagues.

THURSDAY

SYMPOSIUM

Nutrition Labeling of Lipids, Part II

Thursday, September 3, 8:30 am - 12:00 noon

Co-Chairmen: **Richard E. McDonald,** US Food and Drug Administration, and **Joyce Beare-Rogers,** Health and Welfare Canada

Cosponsored by **Gerber Products Company, Silliker Laboratories Group, Inc., The Procter & Gamble Company,** and **Archer Daniels Midland Company**

8:30 Nutrition Labeling of Lipids: Analytical Issues and Opportunities. **Gary R. Beecher,** US Department of Agriculture

8:50 Lipid Methods Appropriate for Nutrition Labeling. **Alan Sheppard,** US Food and Drug Administration

9:10 Analysis of Fat in Low Fat Cereal Products. **Jerry Ngeh-Ngwainbi,** Kellogg Company

9:30 Comparison of Various Methods to Extract Fat. **Bryan L. Madison, Sonya A. Wilson,** and **Adrian C. Smith,** The Procter & Gamble Company

9:50 **BREAK**

10:20 Simplifying the AOAC Method for Determination of Cholesterol in Multicomponent Foods. **Raymond H. Thompson,** US Department of Agriculture

10:40 Implementing an Automated Sample Preparation System for Cholesterol Analysis of Foods. **John H. Johnson** and **Ida C. Tsui,** Kraft General Foods

11:00 Lipid Analysis in a Contract Testing Environment: Method Applicability and Quality Control. **Mary K. Krogul, Randall Smith** and **Wayne Ellefson,** Hazleton Laboratories

11:20 Application of IR and NMR Methods to Analysis of Lipids. **Richard E. McDonald, Magdi M. Mossoba** and **Eugene P. Mazzola,** US Food and Drug Administration

SYMPOSIUM

Forensic Methods and Product Tampering, Part II

Thursday, September 3, 8:30-12:00 noon

Co-Chairmen: **Robert Bianchi,** US Drug Enforcement Administration, **Fredrick Fricke** and **Karen A. Wolnik,** US Food and Drug Administration

Sponsored by **American Cyanamid Company, Agricultural Research Division**

8:30 Introduction

8:35 Keynote address: Building the Tampering Case in Regulatory and Criminal Settings. **James T. O'Reilly,** The Procter & Gamble Company and University of Cincinnati College of Law

9:30 **BREAK**

10:00 Examination of Counterfeit Solid Pharmaceutical Preparations. **Edward Franzosa,** US Drug Enforcement Administration

10:30 Examples of Forensic Analysis using FTIR. **Daniel J. Brown,** US Food and Drug Administration

11:00 Detecting Drug Fraud in the Pharmaceutical Industry: Developments in the FDA's Thermal and Spectroscopic Fingerprinting Techniques. **Michael Schoeck,** US Food and Drug Administration

SYMPOSIUM

Microbiology Update: Part II

Airborne Microorganisms of Significance and Special Interest

Thursday, September 3, 8:30-11:30 am

Co-Chairmen: **Alfred P. Dufour,** US Environmental Protection Agency, and **Michael H. Brodsky,** Ontario Ministry of Health

Cosponsored by **SmithKline Beecham Animal Health, Organon Teknika Corporation** and **Westreco, Inc.**

8:30 Opening and Introductions

8:45 Assessment of Microbiological Aerosols in Indoor Air Environments. **Alfred P. Dufour,** US Environmental Protection Agency

9:15 Aeromycology and Indoor Air Quality. **Sidney Crow,** Georgia State University

9:45 **BREAK**

10:15 Acquisition of Legionnaires' Disease through Aerosols. **Joseph F. Plouffe,** Ohio State University

10:45 Evaluation of Air Samplers and Collection Fluids in the Recovery of Bacteria from Indoor Air Environments. **Pasquale V. Scarpino,** University of Cincinnati

11:15 Review and Questions

New Products

Air Sample Bags

SKC sample bags, used for personal and area monitoring, are fabricated from PVF (Tedlar) film, a material that is inert to a wide range of chemicals. Tedlar is manufactured without plasticizers. SKC sample bags can be used for collecting air containing common solvents, hydrocarbons, chlorinated solvents, and many other classes of chemicals. Tedlar film retains its tensile strength, toughness, and fatigue resistance over a wide temperature range, assuring SKC sample bags a long service life. SKC sample bags can be used in any climate from very cold to extremely hot. The tight, smooth surface of the Tedlar film prevents the permeation of gases and has very low surface adsorption, assuring negligible sample loss and composition change. SKC Inc.
Circle No. 338 on reader service card.

UV/Vis Dual Wavelength Detector

An advanced and reliable UV-Vis detector, the SpectraSYSTEM UV2000, provides high sensitivity detection. This detector performs quantitative single- or dual-wavelength operation for routine or trace analyses. On-the-fly scanning, without stopping the flow, and the "Develop File" software automatically analyze a separation's most favorable wavelength for sensitivity, selectivity, and quantitation. All flow cells are front-mounted and pre-aligned. Spectra-Physics Analytical.
Circle No. 339 on reader service card.

Maximum Residue Limits for Pesticides in Foods

To better serve its member nations, institutions, and individuals, the FAO/WHO Food Standards Program (Codex Alimentarius) has developed a data system on Maximum Residue Limits (MRLs) for pesticides in foods. This data system provides information on pesticides, commodities, and pesticide/commodity combinations. This program requires MS-DOS 3.3, or higher; floppy disk driver, 3.5 or 5.25 in.; and hard disk space with 15 MB free. The system has notable advantages for users: information on all Codex MRLs for pesticides adopted or under discussion according to the Codex procedure; speed in obtaining information on MRLs for a single commodity or a single pesticide and related characteristics; obtaining of selected printout; facility to compare Codex and national MRLs for governments and institutions; and data updated annually. FAO.
Circle No. 340 on reader service card.

Seismic Secondary Containment Shelving

The seismic secondary containment shelving, which is patented, was designed for use by laboratories, hospitals, household hazardous waste programs, or businesses using hazardous materials. There is a continuous solid lip framing the shelves to prevent articles from falling off. Beneath each shelf is a sump to catch any spills or leaks, with a drain and cap for drainage should a leak occur. The shelves themselves are suspended from a sub-roof frame assembly, with allthread rods to allow for total adjustability to hold any size containers. The allthread rods also provide seismic protection to items stored on the shelves. Unified Safety Corp.
Circle No. 341 on reader service card.

UniSlide Power Stand for Stereo Microscopes

UniSlide power stands are used to remotely raise and lower a stereo microscope. This helps the viewer refocus the microscope. When used with a foot switch, the viewer can control the position of the microscope and have both hands free to manipulate or dissect the object being viewed. The power stand consists of a stable base and rigid vertical UniSlide assembly. The UniSlide is driven by a DC gear motor and variable speed motor control. Velmex, Inc.
Circle No. 342 on reader service card.

Benchtop Preparative LC System

The Kiloprep 100 benchtop LC system is capable of scaling up normal phase separations from TLC or flash chromatography. The Kiloprep 100 (KP 100) is an isocratic system that can be optimized for use with 40 or 100 mm (2, 3, or 4 in.) columns and offers a flow rate range of 50–500 mL/min with a maximum operating pressure of up to 2000 psig. Its easy-to-use manual controls provide users with an ideal instrument for rapid, high resolution purification of multigram quantities of product. All valves are actuated from front panel switches with LEDs that indicate the selected flow path. the KP 100 also includes a recycle valve and a 3 headed pump designed to provide users with a rugged, yet reliable pumping system for consistent operation and easy service. Biotage, Inc.
Circle No. 343 on reader service card.

Bio-Columns Biocompatible Plastic Size Exclusion HPLC Columns

Bio-Columns plastic HPLC columns protect and preserve the biological activity of proteins, peptides, and nucleic acids. Completely manufactured from PEEK, an extremely rigid and strong plastic, the columns have no metal components that can contaminate the sample and cause biological deactivation. The Bio-Columns HPLC columns can withstand pressures to 5000 psi, salt solutions to 3M concentration, and detergents such as SDS and Triton X-100. Packed with Bio-Sil SEC 125, 250, or 400 silica gels, they can be used with 100% acetonitrile or in a pH range of 2–8, and can

The JOURNAL OF AOAC INTERNATIONAL

EDITORIAL PROFILE

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

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

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separate compounds in the 3000 to 1 million dalton range. Bio-Rad Laboratories.
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RAM FLAT Compactor for Low Volume Applications

The RAM FLAT compactor, the Model 55SC, is an economically priced unit designed for low volume drug crushing, as well as for compacting material within 55 gallon drums. The Model 55SC's streamlined design uses a single lever control to lower and raise the compaction head. Yet, the heavy duty NFPA standard 4 in. cylinder and 2-stage gear pump, produces a powerful 40 000 lb of compaction force. S & G Enterprises, Inc.
Circle No. 345 on reader service card.

E. coli Pulser Apparatus

The *E. coli* Pulser apparatus is a single electroporation instrument that provides the conditions specific for the electrotransformation of *Escherichia coli*. These pulse conditions, first published in 1988, have been verified in numerous reports since that time. The *E. coli* Pulser apparatus generates these conditions, and only these conditions, resulting in an economical device that provides the highest efficiencies possible for the transformation of *E. coli*. The compact instrument may be used with either the 0.2 cm or the 0.1 cm electroporation cuvettes. It delivers the optimal 5 millisecond pulse to high-resistance *E. coli* samples, and allows handy quick-selection of the 2 most frequently used voltage settings. The *E. coli* Pulser apparatus is equipped with solid state electronics and microprocessor control. Bio-Rad Laboratories.
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Process Analzyer Automates Molybdenum Testing

Available in low and high range models, Hach's economical MO42 molybdenum analyzer is a low maintenance, microprocessor-controlled analyzer designed for continuous, yet accurate analysis of molybdenum. The low range model will provide results in the 0–3 mg/L range. The high range model monitors samples in the 0–10 mg/L range. Both analyzers provide continuous output proportional to sample concentration. Hach Co.
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Advertise in The Referee

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The Referee is the monthly newsletter of AOAC International, received by association members as part of their membership benefits. With more than 3,500 members of AOAC, readers include analytical chemists, microbiologists and other biologists, biochemists, toxicologists, spectroscopists, forensic and other scientists in laboratory, administrative and top management positions. ***The Referee*** is their source for information regarding AOAC's worldwide methods development program, collaborative studies, meetings, symposia, regional sections, methods concerns, publications, and employment needs and opportunities.

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1. trim size 8½″ x 11″
2. printing process: offset
3. colors available: black only
4. screens not to exceed 133 lines
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6. full page bleed: 8¾″ x 11¼″
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Discounts available through combined advertising with the *AOAC Journal!*

Books in Brief

Successful Management of the Analytical Laboratory. By Oscar I. Milner. Published by Lewis Publishers, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1992. 176 pp. Price: U.S. $39.95/Outside U.S. $48.00. ISBN 0-87371-438-5.

Successful Management of the Analytical Laboratory provides a comprehensive discussion of the problems that face analytical laboratory managers and presents proven techniques for improving the operation and performance of analytical laboratories. A wide range of topics are covered, including functions of various laboratory types (including a discussion of legal proceedings that involve defending laboratory data), staffing an organization, motivation, management and development of personnel, personal relations and communication, sample handling, workload optimization, equipment selection and justification, budgeting and cost control (including methods for calculating the dollar return on investments in capital equipment), and information management systems. The book emphasizes measures that managers can take to ensure quality performance in both the laboratory and its personnel while maintaining the overall cost effectiveness of the operation.

Supercritical Fluid Technology: Theoretical and Applied Approaches in Analytical Chemistry. Edited by Frank V. Bright and Mary Ellen P. McNally. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1992. 384 pp. Price: U.S. and Canada $86.95. ISBN 0-8412-2220-7.

This new volume presents the current state of supercritical fluid technology and provides a link between researchers studying fundamental aspects of the science and those involved in the application of supercritical fluid technology to difficult chemical problems. It includes discussions of the fundamental aspects of solvation in supercritical fluid media and presents applications in spectroscopy, chromatography, extraction, and

Instructions to Authors

Scope of Articles and Review Process

The *Journal of AOAC International* 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 desktop publisher; *(4)* desktop publisher sends page proofs to author for final approval.

General Information

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 International, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA, submit typewritten original plus 3 photocopies (1 side only, white bond, 8½ × 11 in. [21½ × 28 cm] of complete manuscript in order as follows—1. Title page; 2. Abstract; 3. Text (introduction, method or experimental, results and/or discussion, acknowledgements, 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. Once a manuscript has been accepted for publication, authors will receive instructions for submitting the final version of their accepted manuscript to AOAC on diskette. AOAC accepts MS-DOS-based files from most word processing packages or ASCII text files on MS-DOS-formatted diskettes. (DO NOT SEND DISKETTE WITH ORIGINAL MANUSCRIPT SUBMISSION.)

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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

(1) 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

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

OFFICIAL METHODS REFERENCE

(1) *Official Methods of Analysis* (1990) 15th 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(s), 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.

8. **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").

1/92

computer modeling. It also offers a discussion on regulatory agencies' role in the implementation of supercritical fluid technology.

Analytical Chemistry Refresher Manual. By John Kenkel. Published by Lewis Publishers, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1992. 400 pp. Price: U.S. $65.00/Outside U.S. $78.00. ISBN 0-87371-398-2.

Analytical Chemistry Refresher Manual provides a comprehensive refresher in techniques and methodology of modern analytical chemistry. Topics include sampling and sample preparation, solution preparation, and discussions of wet and instrumental methods of analysis; spectrometric techniques of UV, vis, and IR spectroscopy; NNMR, mass spectrometry, and atomic spectrometry techniques; analytical separations, including liquid-liquid extraction, liquid-solid extraction, instrumental and non-instrumental chromatography, and electrophoresis; and basic theory and instrument design concepts of gas chromatography and high-performance liquid chromatography. The manual also covers automation, potentiometric and voltammetric techniques, and the detection and accounting of laboratory errors.

Instrumentation in Analytical Chemistry: 1988–1991. Edited by Louise Voress. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1992. 478 pp. Price: U.S. & Export $44.95. ISBN 0-8412-2191-X.

An anthology of 50 articles that first appeared in *Analytical Chemistry*, this volume provides an excellent overview of the entire field of analytical instrumentation and features state-of-the-art developments. This fourth volume in the series focuses on robotics, computers, and laboratory data management; atomic and molecular spectroscopy, electroanalytical chemistry, and chemical sensors; separations; mass spectroscopy, and surface analysis.

The Analysis of Drugs of Abuse. Edited by Terry A. Gough. Published by John Wiley & Sons, Inc., 1 Wiley Dr., Somerset, NJ 08875-1272, 1991. 648 pp. Price $90.00/£90.00. ISBN 0-4719-2267-6.

This volume, devoted to the analysis of drugs that are commonly misused, provides a comprehensive source of up-to-date information. Detailed individual chapters are written by experts in the field describing various analytical techniques and applications. A special feature of the book is its emphasis on the complementary roles of chemist, law enforcer, and the law maker in combatting drug smuggling and the need for collaboration.

Chromatography, 5th Edition: Fundamentals and Applications of Chromatography and Related Differential Migration Methods. Edited by E. Heftmann. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1992. 552 pp. Price: U.S. $179.50/Dfl. 350.00. ISBN 0-444-88236-7 (Fundamentals and Techniques), 630 pp. Price U.S. $333.50/Dfl. 650.00. ISBN 0-444-88237-5 (Applications).

These are completely new books, organized according to the successful plan of the previous 4 editions. While avoiding repetition of material covered in the previous editions, the authors have succeeded in presenting a coherent and comprehensive picture of the state of each topic. Part A (Fundamentals and Techniques) covers the theory and fundamentals of such methods such as column and planar chromatography, countercurrent chromatography, field-flow fractionation, and electrophoresis. Affinity chromatography and supercritical fluid chromatography are covered for the first time. Part B (Applications) presents various applications of these methods New developments in the analysis and separation of inorganic compounds, amino acids, peptides, proteins, lipids, carbohydrates, nucleic acids, their constituents and analogs, porphyrins, phenols, drugs, and pesticides are reviewed and summarized. Important topics such as environmental analysis and the determination of synthetic polymers and fossil fuels, are covered for the first time.

Laboratory Accreditation and Data Certification: A Guide For Successful Laboratories. By Carla H.Dempsey and James D. Petty. Published by Lewis Publishers, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1991. 240 pp. Price: U.S. $65.00/Outside U.S. $78.00. ISBN 0-87371-291-9.

This book provides descriptions of current laboratory accreditation schemes and explains why these schemes fall short of assuring data purchasers that the data produced from accredited laboratories are always quality products. The book then presents a system for laboratory accreditation in conjunction with data certification that assure data purchases their data are useful for the purposes for which they are intended. Simple quality assurance and quality control techniques, in addition to concepts of total quality management, are described and then applied to the environmental laboratory industry. This "System for Success" was developed from real problems and real solutions within the industry and represents an integration of proven techniques that offer a better way to ensure quality laboratory data is obtained.

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DISCUSSIONS IN ANALYTICAL CHEMISTRY

Regulatory Mass Spectrometry for Pesticide Residue Analysis: Past, Present, and Future

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The Past

Thirty years ago, mass spectrometers were thinly spread throughout the scientific world, with the highest concentrations of active research occurring in the United Kingdom, Germany, and the United States. It was no mere accident that these locations were the active nuclei and served to crystallize the future role of mass spectrometry (MS) as a popular applied science. The early pioneers were master instrument builders from various universities. Primarily because of their vision and insight, the compact commercial instruments were born and so were several new instrument companies. Monopoly, however, was not the order of the day, and fierce competition coupled with vision created a healthy atmosphere of consumer choice.

The early development of MS expounded the carbonium ion chemistry governing the extensive fragmentation of molecules under electron ionization (EI) at 70 eV. This fingerprinting capability has been the cornerstone of identification and structural elucidation because of the ability to relate the various fragment ions to the molecular structure. The science of MS might well have remained firmly in the domain of the academic researchers for gas-phase studies but for the growing appetite of a new breed of inspired chemists—the analytical chemist. Their contribution to MS was in recognition of this powerful analytical technique and application of the technology to problem solving. Parallel, and perhaps in synchronous harmony with instrumentation development, chemical technology had improved the global standard of living through improved food science.

Since the publication of Rachel Carson's *Silent Spring* in 1962 (1), a plethora of public health protection laws have been passed by numerous countries. Such intensity in the legislative arena has ultimately focused on the much increased role of analytical chemistry in nutrition as well as the protection of the public health from toxic residues.

Scrutiny has intensified primarily on the safety of the food supply from harmful residues. Five main avenues for the introduction of chemicals into the food chain have been categorized: (*1*) the use of insecticides, fungicides, and miticides to curb infestations and increase crop yields, which has resulted in residues above the legal tolerance level; (*2*) the illegal use of pesticides not yet registered for use on certain crops; (*3*) the persistence of many agrochemicals and their metabolites in the environment, which serves as an indirect route to transport in the ecosystem; (*4*) the accidental contamination of the food supply with chemicals designated and approved for industrial applications only; and (*5*) the deliberate contamination of the food supply with a poison to receive press coverage and scare the public.

The emphasis on regulatory pesticide analysis quickly resulted in the use of MS for *confirmation of presence*. Although gas chromatography with element-sensitive detectors could easily achieve detection to the high part-per-billion level, confirmation of presence involved the higher level technology of combined gas chromatography/mass spectrometry (GC/MS).

The impact of GC/MS and liquid chromatography/mass spectrometry (LC/MS) in the area of food sciences has been the subject of a number of comprehensive reviews (2–5). These reviews have all indicated a strong trend toward the application of MS to trace toxic substances in the food chain. However, caution is needed in the final determination of the trace contaminant's exact origin—ubiquitous contaminant, sample impurity, or artifact of the sample workup. Most reports in the literature on the application of MS have dealt with confirmation of suspected pesticides in the food supply. However, the incidences of unknown analytical responses pose a potential health hazard, particularly if they are found to contain phosphorus, nitrogen, halogen, or sulfur. In these cases, MS has been used to identify the encountered compounds, most of which are found to be metabolites of the pesticides applied.

The Present

Modern analytical chemistry has, therefore, become dependent upon a number of MS techniques that have quickly replaced more conventional approaches. Two main reasons for these directional changes have emerged. First, the exponential pace of technological developments in the field of analytical instrumentation has provided a cornucopia of selections and capabilities within the field of MS, e.g., magnetic, quadrupole, ion traps, bench tops, and MS engines. Second, the increasing awareness of the importance of trace-level analysis has

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spawned a new growing family of associated hybrid techniques especially developed to solve particular critical problems, e.g., chemical ionization (CI), atmospheric pressure ionization, fast atom bombardment, thermospray, and electrospray.

This array of MS techniques available to the mass spectrometrist and their successful deployment has resulted in a unique research situation. Solutions to various food chemical problems have provided valuable insight into the fundamental theories and mechanistic pathways governing the processes involved. Additionally, the analytical approaches adopted have established new experimental avenues permitting extrapolation to other critical situations. The wealth of data generated by this intense scientific inquiry into trace analysis has stimulated the research chemist to seek alternative methods to detect even lower levels, literally pushing the threshold of detection to the femtogram level.

Current trends have indicated that this increased reliance on MS is notable because of the ability to provide detection and confirmation of presence, often in a single analysis. The unique role that MS plays in fortifying scientific evidence can be regarded as a fundamental requirement to proof of presence. The beneficiaries of this process of providing verified experimental data are the toxicologists, because they play important roles in setting "safe levels" as well as probing the basic mechanisms of toxicity. Without the availability of MS, the basic mechanisms of toxicity would be difficult to study. With the close cooperation of these 2 basic sciences, giant strides can be made in the protection of the public health.

Evolving Elements for Confirmation of Presence

The experimental process of confirmation of trace levels has been placed on MS because of its strengths in reproducibility, repeatability, specificity, and limit of detection. Scientists are not exempt from the mundane rigors of supporting their findings. In a court of law, the standard of proof required in a criminal case is "beyond reasonable doubt"; in a civil court, "the preponderance of evidence" must support the conclusion. Because methods of confirmation by MS techniques are often developed on an *ad hoc* basis to deal with an emergency situation, they cannot always be validated by interlaboratory testing. What has emerged over the past decade, however, is a set of criteria generally recognized as scientifically sound.

The highest level of confirmation that can be provided by MS is the exact correlation between the full mass spectral scans of a reference standard and the sample, where analysis is performed within the same working day. Usually, EI spectra contain sufficient structurally related fragment ions to permit absolute identification. Under such conditions, the relative abundance ratios should fall, experimentally, within 5%. Quite often, however, the presence of background ions may interfere severely with exact comparisons. This practice of direct comparisons represents the highest level of specificity obtainable by MS.

In the field of trace-level analysis, however, the opportunity to use full mass spectral scans to confirm the presence of a substance is often impractical. However, the multiple ion monitoring (MID) technique makes full use of sensitivity of detection; it allows the analysis to ignore potential interferences and concentrate on ions belonging to the compound under investigation. It is in this particular sphere that the evolving criteria for confirmation have received the most attention, with the focus being on the exact number of ions to be monitored to provide proof of presence. This examination of criteria for confirmation has been complicated because of the myriad of MS techniques available for analyzing food samples. It was argued more than a decade ago that a minimum of 3 structurally related ions would be necessary to provide proof of presence (6). This assumption was based on a statistical approach using an extensive mass spectral data base as a model of a universal repository containing all possible organic compounds. Without paying attention to relative abundance ratios, 3 ions were required to eliminate compounds with similar fragment ion selections from consideration. To improve the criteria for confirmation, the relative abundance ratios were required to be within 5% when compared with a reference standard recorded under similar conditions. Evolution of new techniques, particularly soft ionization methods, has prompted a reinvestigation of supporting evidence for confirmation because little or no fragmentation is observed.

Over the past decade, a number of reviews of the application of MS to trace levels have been published (2–5). These reviews have provided specific case histories illustrating the power of various MS techniques and their hybrids to provide identification and confirmation. More recently, a review of evolving criteria for confirmation at residue levels in food and drugs has attempted to address the general consensus derived from experimental case histories of what constitutes proof of presence (7).

Although there is great variation in the MS methods applied to residue analysis, the underlying criterion that at least 3 structurally significant ions are necessary for confirmation has been established experimentally. However, most members of the discipline have elected to go beyond this minimum criterion whenever possible. In the case of soft ionization methods established by LC/MS, the lack of specificity has been overcome by the use of LC/tandem MS (MS/MS). The use of product ion spectra derived from protonated-molecule ions has become a practical solution to analyzing many thermally labile compounds. There was no debate, however, over the key principle that MS has provided the necessary science to examine trace levels in the food supply as well as to provide research technologies intended to quantify toxicity, specifically carcinogenesis.

The Future

Instrument Development

With regard to new instruments, the ion-trap mass spectrometer would seem to have a bright future, based on preliminary studies on selected residues (8). The ability of this relatively low-cost detector to obtain full mass spectral scans on picogram levels of pesticide residues injected on megabore or capillary columns has attracted a great deal of attention. However, several major difficulties must be resolved before widespread acceptance occurs: (*1*) The sample extracts may degrade

the first few meters of the chromatographic stationary phase. Experiments indicate that precolumns and guard columns may alleviate this problem. In any event, sample cleanup ultimately may be the final solution to the problem. (*2*) The construction of a comprehensive pesticide standard reference data base will be necessary to confirm the presence of a particular residue. (*3*) The identification of unknown analytical responses will require more sophisticated software development.

Revising the Analytical Approach

Collision experiments via MS/MS have been practiced with increasing frequency and are expected to eventually replace the conventional approaches to confirmation through product ions derived from molecule ions. For the near future, MS applications in food science can be predicted with reasonable reliability. In organic chemistry, soft ionization methods have prompted the use of MS/MS to increase the specificity required for proof of presence. The relationship between product ion spectra and precursor ions is gradually overtaking the conventional EI and CI approaches to confirmation of presence. The next quantum jump in technology will come with commercial instruments such as the ion trap, in which experiments involving MS/MS on high-molecular-weight compounds will yield a pyramid of related product ions. Structural elucidation approaches will change drastically from the classical use of EI fragmentation patterns to the use of MS/MS. In fact, transition has already occurred in a number of research laboratories and should evolve rapidly into everyday problem solving by the end of the century.

Improvements in Software

MS will probably make its largest contribution to the science of pesticide residue analysis in the area of software development. Although computerized mass spectrometers exist, much operator interaction is still required to help isolate the eluting compound of interest and then proceed with identification. Computer programs are not yet comparable to human intelligence in interpreting chromatograms. For instance, even with window applications, there is no simple way to identify all chlorinated compounds present, although chlorine clusters are easily recognized because they are 2 mass units apart and generally lose 35 or 36 mass units. This theory can be extrapolated to various generic classes of compounds such as organophosphates, organonitrogens, ureas, and carbamates. It is hoped that in the next generation of ion-trap mass spectrometers, the software programmers will incorporate expert systems that will routinely be able to pinpoint certain generic classes when requested. In addition, normal sample matrix constituents should be maintained on a separate data base to allow simple subtraction from a incurred residue to reveal the pesticide residues.

Impact of Biotechnology

Most of the problems in analyzing food samples involve low-molecular-weight compounds. The criteria for the confirmation outlined above have been chosen with this molecular-weight range in mind. However, the next generation of pesticides derived by biotechnology will involve compounds of much higher molecular weight. With increasing molecular weight, the criteria for confirmation will be revised to increase the number of ions required.

Yes, MS has traveled its very own marathon since the science first graced a university campus in the early 1930s.

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SPECIAL REPORTS

Analytical Use of Linear Regression. Part I: Regression Procedures for Calibration and Quantitation

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Although commonly used by analysts, linear regression requires careful attention to 5 fundamental assumptions. This paper summarizes these assumptions and describes the effects of deviations from these assumptions and various approaches to correct for such deviations. Specific cases are defined where classical unweighted or weighted linear regression methods are still applicable in calibration experiments, even though the independent x-variable contains random error. Another topic of this paper is the proper construction of confidence intervals, not only for the relatively easy case of predicting a response value given an analyte concentration (or amount) value but also for the theoretically more difficult case of predicting a concentration (or amount) value given a response value. Various approaches based on single-use discrimination intervals (SDI), multiple-use discrimination intervals (MDI), and unlimited-use discrimination intervals (UDI) are discussed for the latter case. This discussion examines both the theory and analytical applicability of each approach. A simplified procedure based on calculated g-values is introduced for guidance on whether or not to use the SDI approximation. The lengths of the MDIs and UDIs are compared for several test data sets, and the modified Scheffé UDI approach is found to be advantageous because of its relative simplicity and short intervals. Its use is recommended when neither of the described SDI approaches is appropriate, i.e., when the comparison of several predicted analyte concentration (or amount) values obtained from the same calibration curve is desired.

Linear regression could be the most used and abused statistical method. A common mistake is to blindly force a "classical" regression fit on any set of data with a presumed linear relationship. Whether this or some other type of fit should be used is governed by how well the assumptions for the particular regression method are met by the actual data.

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This article is the first of a 2-part series exploring the use of linear regression by analytical chemists. Part I focuses on using linear regression when it can be shown or assumed that all of the random error in a data set can be assigned to the dependent (or y-) variable. Those cases where the application of classical regression is still allowable even though the independent (or x-) variable is subject to error are noted and discussed. This use is of particular importance to analytical calibration experiments. The use of the obtained regression line for quantitating "unknowns" *and* placing error limits about these predicted values is also discussed. Part II examines the situation where error exists in both variables and must be accounted for during the statistical analysis.

This work had its origin in our research group's many frustrated attempts to fit straight lines to real data and, more importantly, use them in a conscientious manner. Although some information does exist in the analytical chemistry literature, it tends to be deficient in documentation, explanation of the approaches suggested, and discussion of alternative approaches. The statistical literature, on the other hand, contains considerable information on this subject. However, much of the statistical information is present in relatively obscure (to most analytical chemists) publications. Therefore, our objectives were to survey the relevant literatures on linear regression, to provide a thorough treatment that includes pertinent references, consistent nomenclature, and related mathematical formulae, and to show by theory and practical example those approaches best suited to typical problems in analytical chemistry.

Assumptions

This section describes how the assumptions behind linear regression methods regulate their proper use. Therefore, the assumptions for "classical" regression are presented, and then the effects of deviations from these assumptions and methods that correct for these effects are noted.

1. Linear Model

The first assumption requires a straight-line relationship between the dependent and independent variables.

$$Y_i = a + bX_i + \varepsilon_i \qquad (1)$$

For typical analytical applications, Y_i in Equation 1 is an observed analytical response, X_i is the weight or concentration of analyte present, and ε_i is the random error or residual term for the i-th data pair. Also, a represents the intercept and b the slope obtained by applying a least squares fit to the data. If a linear relation does not exist initially, data can often be transformed to obtain linearity. Common approaches are log-log and semilog transformations. It should be noted that if a data transformation is used, the data should be kept in the transformed metric through all calculations of standard errors and confidence intervals. Only at the end of the calculation should the results be transformed back into the original metric (1).

Linearity is often checked by using either the product-moment correlation coefficient, r, or the coefficient of determination, R^2. The coefficient of determination gives a measure of the portion of the total variability in a data set that is explained by a particular model. In the present case, the model is a straight line with variability in y only. For this case, the product-moment correlation coefficient is equivalent to the square root of R^2; however, this relation does not hold for nonlinear models. Even though r is widely used in an attempt to quantitate linearity, or "goodness of fit," to a linear model, it gives no information that is not already provided by the slope and its standard error. In fact, r is simply a scaled version of the slope, i.e., the slope estimate multiplied by a factor to keep r always between −1 and +1 (2, 3).

$$r = b\left[\frac{S_{xx}}{S_{yy}}\right]^{1/2} = \frac{S_{xy}}{(S_{xx}\,S_{yy})^{1/2}} \qquad (2)$$

This expression for r introduces shorthand notation for the corrected sums of squares terms that will be used throughout the remainder of this article. For the summations within this shorthand notation, i ranges from 1 to n.

$$S_{xx} = \sum (X_i - \bar{X})^2 = \sum X_i^2 - \frac{(\sum X_i)^2}{n} \qquad (3a)$$

$$S_{yy} = \sum (Y_i - \bar{Y})^2 = \sum Y_i^2 - \frac{(\sum Y_i)^2}{n} \qquad (3b)$$

$$S_{xy} = \sum (X_i - \bar{X})(Y_i - \bar{Y}) = \sum X_i Y_i - \frac{\sum X_i \sum Y_i}{n} \qquad (3c)$$

By itself, the correlation coefficient gives only a relative idea of the linearity inherent in a particular data set. This ability is extremely mismatched with the task for which many researchers use it, i.e., the task of quantitating linearity. As a caution, it has been reported that a value for r close to 1 can be misleading when used for this purpose (1, 4, 5). Data sets in which nonlinear deviation is concentrated in 1 or 2 areas of a graph (e.g., at the upper and lower ends) and data sets that are subject to slight but overall persistent curvature are prime examples of this problem. The latter problem is illustrated in Figure 1.

In an effort to extend the usefulness of the correlation coefficient, significance tests have been developed for it.

$$t = r\left[\frac{n-2}{1-r^2}\right]^{1/2} \qquad (4)$$

This 2-tailed t-test with n − 2 degrees of freedom (1, 3, 5–7) may be used to determine whether r is statistically significant. If r is deemed significant, one may say that a significant amount of linear association exists between y and x, i.e., the regression is significant. But, as pointed out by Fisher in his 1915 work (8), the above test is identical to a significance test on the slope estimate.

So, as previously stated, the correlation coefficient does not really provide anything new. It is better used for estimating the amount of correlation that exists between 2 populations of bivariate normal distribution (6), *not* for quantitating linearity.

2. Error in Y Only

The independent variable must either be free of error or its level of error must be insignificant compared with the level of error in the dependent variable. This assumption determines the form of the sum of squared errors equation that is minimized to obtain formulas for estimating the slope and intercept (i.e., a least-squares approach is employed).

$$S = \sum \varepsilon_i^2 = \sum (Y_i - \hat{Y}_i)^2 = \sum (Y_i - a - bX_i)^2 \qquad (5)$$

If this assumption is not fulfilled, terms for errors in x must be included in Equation 5. This greatly complicates the matter of obtaining a formula for the slope, a topic that is discussed in Part II.

In many instances when calibration is performed, significant error does exist in the independent variable due to the lack of precision and accuracy with which standards are made or obtained. Three basic cases commonly arise in practice from this problem (9). The first case occurs when the values for y and x are obtained by measurement.

$$Y_i = \eta_i + \varepsilon_i \quad \text{and} \quad X_i = \xi_i + \delta_i \qquad (6)$$

where Y_i and X_i are the measured values of the dependent and independent variables, respectively. Their true values are η_i and ξ_i, and their respective errors are ε_i and δ_i. If the obtained Y_i values are regressed against the obtained X_i values, the resulting slope is biased (6, 9).

$$b' = \frac{b\,\sigma_x^2}{\sigma_x^2 + \sigma_\delta^2} \qquad (7)$$

In this expression, b' is the obtained slope, b is the slope that would be obtained if x had been measured without error, σ_x^2 is the variance over all the obtained X_i values, and σ_δ^2 is the vari-

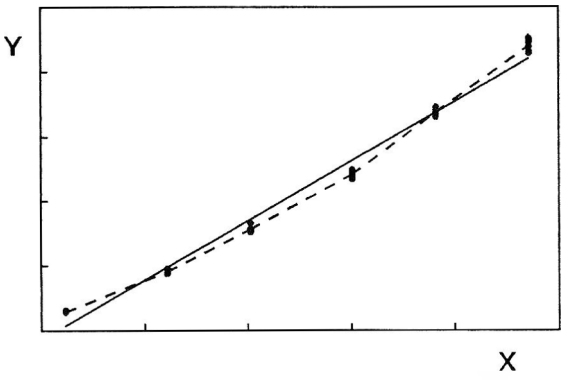

Figure 1. Failure of correlation coefficient to detect nonlinearity; for graphed data r = 0.993; solid line represents linear fit.

ance of the measurement errors of x. Equation 7 shows that the slope will often be underestimated in this case (that in turn will cause the intercept to be overestimated). In this uncontrolled case, the ξ_i values are fixed while the X_i values are variable. Also, via Equation 6, the X_i and δ_i are not independent.

In the second case, rather than measure x, its values are brought to approximately X_i. This is what is commonly done in calibration experiments. Standards are prepared with values for their analyte concentrations obtained either from a reference value or from a gravimetric or volumetric procedure combined with dilution to the final values. If this process is unbiased, the concentrations will vary only in a random manner from the preselected X_i. In other words, the X_i values are fixed and the ξ_i values are now variable. The errors, δ_i (and ε_i as well), are now independent of the regressed X_i. Note that it is assumed that only random errors are present (i.e., systematic errors are absent). For this case, Berkson (9) has shown that the obtained slope estimate is unbiased. The crucial point of his argument is that the expectations $E[\ \Sigma\delta_i\ (X_i - \overline{X}\ )\ ]$ and $E[\ \Sigma\varepsilon_i\ (X_i - \overline{X}\ )\ ]$ are 0. This can be shown because the covariance of 2 random variables that are independent of one another is 0 (7). Although the X_i values here are fixed and not random, by definition $E[\ X_i - \overline{X}\ ] = 0$; therefore, the statements hold. Berkson states, "Since in the repeated experiments, the average of which determines the expectation, the X_i are fixed, the average of the slopes b and the means a are determined by the average value of Y_i for fixed X_i." He then concludes that "the regression has as its expectation the true law." This could be rephrased by stating that the error in x is averaged out and its effects unbiasedly transferred to the y-axis. This situation is commonly referred to as the "fixed-x," "controlled-x," or simply "Berkson" case. Further discussion has been presented by Kendall (10, 11), Lindley (12), Scheffé (13), Brownlee (7), and Mandel (14).

The third case occurs if, after setting the x-values, their values are measured with some technique to obtain the X_i. This reverts the mathematics back into a situation akin to the first case. The slope estimate will again tend to be underestimated according to an expression similar to Equation 7. According to Berkson (9), this bias can be made negligible if the X_i values are selected to cover a wide range.

In summary, Berkson's second case provides a theoretical construct that allows the use of "classical" regression in most calibration experiments even though a basic assumption is violated and the x-errors are not accounted for in the regression model. An important point is that the x-errors present must be random so that if the true values (that in this case are variable) were averaged, their means would be, in the long run, the controlled values, X_i. If, as in the first and third cases, the X_i values are variable because of measurement error, the resulting slope will tend to be biased low. In these latter instances, one of the approaches in Part II should be used.

3. Random and Homogeneous Error

The statistical error must not reside only in the dependent variable but also be random and have a homogeneous (or homoscedastic) variance.

$$E\ [\varepsilon_i] = 0 \quad \text{and} \quad V\ [\varepsilon_i] = \sigma^2 \tag{8}$$

Homogeneous means that σ^2 from Equation 8 must be constant over the entire range of x employed. The assumption of constant error variance is best tested by examining a residuals plot, with several repeats on y for each x. Figures 2 and 3 illustrate cases with homogeneous and heterogeneous variances, respectively, along with concomitant confidence intervals.

If, as often happens in calibration experiments, the error variance is not constant over the calibration range, 1 of 2 remedies may be employed. The first remedy is to transform the data into a metric where the error variance is constant (3, 6, 15). Common transformations include logarithmic, square root, and angular. Note here that the caveat cited under Assumption 1 with regard to keeping the data in the transformed metric until the final calculation also applies to this case.

The other remedy for nonhomogeneous (or heteroscedastic) variance is to employ variance-weighted regression. This can be covered theoretically by rewriting the right-hand portion of Equation 8 (6, 16, 17).

$$V\ [\varepsilon_i] = \tau_i\ \sigma^2 = \sigma_i^2 \tag{9}$$

Very often σ_i^2 increases with X_i and Y_i, as shown in Figure 3, and τ_i can thus be thought of as a definite function of X_i or Y_i. However, the relation can be unpredictable, and the τ_i values are then not known. This latter case presents a practical problem, because weighted regression methods require known values or at least very good estimates for either the τ_i or the σ_i^2 values. If these values are not known they must be estimated by taking several replicates of Y_i for each X_i. Three or 4 replicates are not sufficient to properly estimate a variance; at least 8–10 are usually required (1, 18–20). Although this can be a severe constraint, it also has the advantage of providing a form of "robust" regression. This is because the most common way to perform weighted regression is simply to use reciprocal vari-

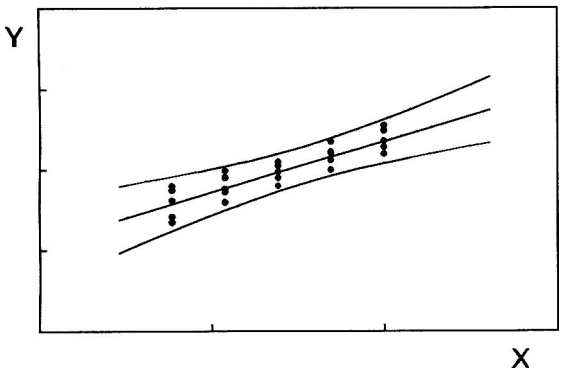

Figure 2. Example of homogeneous error variance.

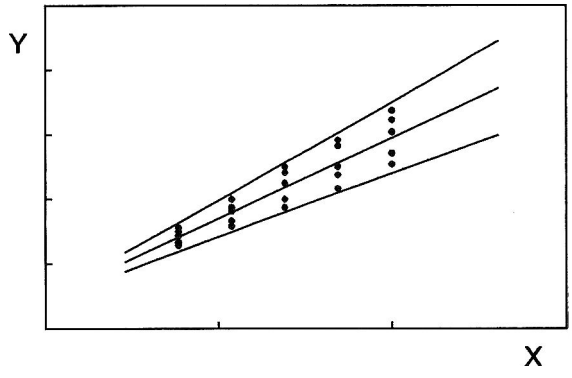

Figure 3. Example of nonhomogeneous error variance.

ances for weights, i.e., $w_i = 1/s_i^2$, where s_i^2 is the experimental estimate of σ_i^2. This relationship ensures that, if replication is employed, outlier values of Y_i will be given low weights.

The use of y-variance weights affects the classical model by including the weights in the error sum of squares.

$$S_w = \sum w_i \varepsilon_i^2 = \sum w_i (Y_i - \hat{Y}_i)^2 \qquad (10)$$

Contrary to the effect of adding in x-errors, this adjustment causes no severe impediments to obtaining formulas for the slope and intercept. More sophisticated methods for treating this problem exist (17, 19, 21–23). These frequently use some type of variance modeling but do more to complicate the situation than can be explained in this paper. The literature indicates that the use of weighting factors to correct for heteroscedasticity usually has a minimal effect on experimentally obtained slopes and intercepts. The main effect is to yield more reliable confidence intervals by allowing their widths to vary across the calibration range according to variation in individual error variances (5, 17).

4. Uncorrelated Errors

The next assumption states that no residual term is correlated with another.

$$COV\,[\varepsilon_i, \varepsilon_j] = 0 \quad \text{for } i \neq j \qquad (11)$$

Equation 11, in conjunction with Equations 1 and 8, yields expressions for the expected values and variances of Y_i.

$$E\,[Y_i] = a + bX_i \quad \text{and} \quad V\,[Y_i] = \sigma^2 \qquad (12)$$

This is the form for nonweighted regression only.

5. Normally Distributed Error

The last assumption states that the residuals are distributed normally with a mean of 0 and a variance of σ^2.

$$\varepsilon_i \sim N\,(0, \sigma^2) \qquad (13)$$

This assumption, combined with Assumption 4, means that ε_i and ε_j are not only uncorrelated but are also necessarily independent.

Equations for Parameters and Their Errors

Formulas for the slope and intercept of the y-error regression model are easily derived from the assumptions discussed above. This is done by minimizing the particular error sum of squares (nonweighted or weighted) with respect to the parameters of the model, a and b. This results in a pair of normal equations that may be solved simultaneously to yield expressions for a and b. For the nonweighted case, the result is (with subscript i's omitted throughout):

$$a = \frac{\sum Y}{n} - \frac{b\sum X}{n} = \bar{Y} - b\bar{X} \qquad (14a)$$

$$b = \frac{\sum XY - \sum X \sum Y / n}{\sum X^2 - (\sum X)^2 / n} \qquad (14b)$$

$$= \frac{\sum (X - \bar{X})(Y - \bar{Y})}{\sum (X - \bar{X})^2} = \frac{S_{xy}}{S_{xx}} \qquad (14c)$$

The results for the y-variance weighted case are very similar.

$$a = \frac{\sum wY}{\sum w} - \frac{b\sum wX}{\sum w} = \bar{Y}_w - b\bar{X}_w \qquad (15a)$$

$$b = \frac{\sum wXY - \sum wX \sum wY / \sum w}{\sum wX^2 - (\sum wX)^2 / \sum w} \qquad (15b)$$

$$= \frac{\sum w\,(X-\bar{X})\,(Y-\bar{Y})}{\sum w\,(X-\bar{X})^2} = \frac{S_{wxy}}{S_{wxx}} \qquad (15c)$$

The variance of the regression, σ^2, is estimated in the nonweighted case by dividing the error sum of squares (Equation 5) by its degrees of freedom, yielding the usual mean square error type of expression.

$$s_{yx}^2 = \sum \frac{\varepsilon_i^2}{df} = \frac{S}{n-2} \qquad (16a)$$

$$= \frac{S_{yy} - bS_{xy}}{n-2} = \frac{S_{yy} - b^2 S_{xx}}{n-2} \qquad (16b)$$

This variable is represented by several different symbols in the literature, e.g., s^2, s_y^2, s_{yx}^2, $s_{y/x}^2$. The choice of s_{yx}^2 differentiates it from s_i^2 and s_{yi}^2, the individual variances of y at separate levels of x. This is important because further examination of the equations for s_{yx}^2 reveals that it is actually a pooled variance of y over the range of x-values.

For the weighted case, the size of s_{wyx}^2 is directly affected by the relative size of the weights and, thus, is not usually an estimate of σ^2. However, it is needed for calculating estimates for the variances of the intercept and slope.

$$s_{wyx}^2 = \frac{\sum w_i \varepsilon_i^2}{df} = \frac{S_{wyy} - bS_{wxy}}{n-2} \qquad (17)$$

Once a s_{wyx}^2 value is calculated, expressions for the variances of the intercept and slope can be formulated. Note that the standard errors of the intercept and slope are equivalent to their standard deviations (i.e., the square roots of their variances). For the nonweighted case, the variances are as follows:

$$s_a^2 = s_{yx}^2 \left[\frac{1}{n} + \frac{\bar{X}^2}{S_{xx}}\right] = \frac{s_{yx}^2 \sum X^2}{n\,S_{xx}} \qquad (18a)$$

$$s_b^2 = \frac{s_{yx}^2}{S_{xx}} \qquad (18b)$$

The expressions for the weighted case are very similar.

$$s_a^2 = s_{wyx}^2 \left[\frac{1}{\sum w} + \frac{\bar{X}_w^2}{S_{wxx}}\right] = \frac{s_{wyx}^2 \sum wX^2}{\sum w\,S_{wxx}} \qquad (19a)$$

$$s_b^2 = \frac{s_{wyx}^2}{S_{wxx}} \qquad (19b)$$

The weighted average of x is calculated as $\bar{X}_w = \Sigma wX / \Sigma w$.

Prediction Intervals

Estimation of the parameters and their associated errors is not the end-all of linear regression. Usually, these results are used to "predict" values of one of the data variables based on a value for the other variable. If these values are within the range of the calibration data, it is termed "interpolation." If they are not, it is termed "extrapolation." Extrapolation is strongly discouraged because it cannot always be correctly assumed that the model on which the calibration is based will hold outside the limits of the actual data. This caveat applies to both the linearity of the model as well as its error structure.

For a reported value to have any interpretable merit, an estimate of its uncertainty must be reported. In this section, the focus is on estimating uncertainties for the case of predicting values of y based on chosen values of x. Confidence intervals obtained from this process are commonly called "prediction intervals." The prediction equation comes from Equation 12.

$$\hat{Y}_i = a + bX_i \qquad (20)$$

A variance for the predicted $\hat{Y}_i$ can be obtained by using a propagation of errors approach. However, Equation 20 cannot be used directly, because the variances of a and b are not independent. In fact, they are strongly correlated (1, 14, 24). Substituting for a via Equation 14a gives a useful equation from which to work.

$$\hat{Y}_i = \bar{Y} + b\,(X_i - \bar{X}) \qquad (21)$$

Because the variances of $\bar{Y}$ and b are independent (2), errors can be propagated through this equation directly.

$$V[\hat{Y}_i] = V[\bar{Y}] + (X_i - \bar{X})^2\,V[b] \qquad (22a)$$

$$= \frac{s_{yx}^2}{n} + \frac{s_{yx}^2\,(X_i - \bar{X})^2}{S_{xx}} \qquad (22b)$$

$$= s_{yx}^2 \left[\frac{1}{n} + \frac{(X_i - \bar{X})^2}{S_{xx}}\right] \qquad (22c)$$

According to theory, this formula applies only to values of y predicted from values of x that were included in the calibration data set. This variance is often referred to as the "true mean" value.

To build a prediction interval about a $\hat{Y}_i$ value, a 2-tailed t-value is needed that is based on a chosen confidence coefficient and the appropriate degrees of freedom. A 95% prediction interval requires a t-value with a confidence coefficient of 0.975 (significance coefficient of 0.025) and n – 2 degrees of freedom. The upper and lower limits of the prediction interval (*P.I.*) are then obtained by using the following equation:

$$P.I. = \hat{Y}_i \pm t_\nu^{\alpha/2}\,s_{\hat{Y}_i} = \hat{Y}_i \pm t_\nu^{\alpha/2}\,(V[\hat{Y}_i])^{1/2} \qquad (23)$$

For values of y, $\hat{Y}_o$, predicted from "new" values of x, X_o, an extra component of y-variance must be added to the calibration variance of Equation 22 to obtain the variance of $\hat{Y}_o$ (1, 2).

$$V[\hat{Y}_o] = s_{yx}^2 + V[\hat{Y}_i] \quad (24a)$$

$$= s_{yx}^2 \left[1 + \frac{1}{n} + \frac{(X_o - \bar{X})^2}{S_{xx}}\right] \quad (24b)$$

Prediction intervals about $\hat{Y}_o$ values are constructed in the same manner as is expressed in Equation 23.

$$P.I. = \hat{Y}_o \pm t_\nu^{\alpha/2} s_{\hat{Y}_o} = \hat{Y}_o \pm t_\nu^{\alpha/2} (V[\hat{Y}_o])^{1/2} \quad (25)$$

The relative dominance of the terms in the brackets that are multiplied by s_{yx}^2 in Equations 22 and 24 decreases from left to right. Thus, except at the extreme ends of the calibration curve, Equation 22 can be grossly approximated by $s_{\hat{Y}_i}^2 \approx s_{yx}^2/n$, and Equation 24 can be similarly approximated as $s_{\hat{Y}_o}^2 \approx s_{yx}^2$. The following conclusions can be made from the derived expressions: Equation 22 gives a "standard error of the mean" type variance, one that is estimated from several replicates of y (the replication comes from the pooling process for s_{yx}^2 noted above). Equation 24, on the other hand, gives the variance of a single unreplicated value in addition to the calibration variance.

For y-variance weighted regression, these expressions require only slight modification. For example, Equation 24 may be rewritten as follows:

$$(s_{\hat{Y}_o}^2)_w = s_{wyx}^2 \left[\frac{1}{w_o} + \frac{1}{\sum w} + \frac{(X_o - \bar{X}_w)^2}{S_{wxx}}\right] \quad (26)$$

Here, w_o is a weight chosen so that it is appropriate to the value of $\hat{Y}_o$.

Both the calculation and use of these intervals will now be demonstrated with real measurement data for total volatile sulfur fluxes from soils at 2 field sampling sites, one near Ames, IA, and the other at Celeryville, OH. A full discussion of these field data has already been published (25). Therefore, a summary of the results obtained from applying a semilogarithmic linear regression to the gaseous sulfur flux vs temperature data (i.e., ln F vs T) is presented here solely for illustrative purposes. A comparison of flux values from the 2 sites is made by constructing a prediction interval for each site's data for a temperature of 30°C (= X_o). The results are summarized in Table 1.

By using these results with Equation 25, upper and lower 95% prediction interval limits of 3.077 and 2.195, respectively, are obtained for the Iowa site. For the Ohio site, the upper and lower limits are 4.786 and 3.523, respectively. Note, however, that these limits are still in the semilogarithmic metric. To get back to the linear metric, the inverse natural logarithms of these limits and the predicted $\hat{Y}_o$ values must be taken. The results of doing this are shown in the bottom 2 rows of Table 1.

Two observations are made from these example results. First, the predicted fluxes from the 2 sites are shown to be significantly different at the 95% confidence level because the prediction intervals do not overlap. Second, these intervals are not symmetric about the predicted flux values after they are transformed into the linear metric. This is because of the nonlinear equation that was used to model the data. An improper analysis of these data may have yielded symmetric limits, but a proper one does not.

Discrimination Intervals

Drawing confidence bands for values of y (i.e., prediction intervals) is frequently very useful. However, when an analytical chemist performs a calibration experiment, the ultimate objectives are prediction of a quantitative value for x based on a reading of y (e.g., an instrumental response) for some "real" sample and subsequent determination of confidence bands about this x-value. To distinguish the results of this case from those of the y-prediction case, the error limits obtained for x are commonly called a "discrimination interval."

On the basis of a y-value (Y_o), or the mean of several repeats of y for the same sample, a predicted value for x ($\hat{X}_o$) can easily be obtained by rearranging either Equation 20 or 21.

$$\hat{X}_o = \frac{Y_o - a}{b} = \bar{X} + \frac{Y_o - \bar{Y}}{b} \quad (27)$$

A more difficult task is to establish a confidence band about the value for $\hat{X}_o$. A simple propagation of errors approach akin to that discussed above for the y-prediction case will not suffice because it results in an infinite variance for $\hat{X}_o$ (26). The reason is that the equation for $\hat{X}_o$ is a nonlinear combination of variables that are each normally distributed (27). A mean and variance for the distribution of this nonlinear combination of normal distributions cannot be defined exactly. If the combination was linear, then $\hat{X}_o$ would itself be normally distributed and its mean and variance would be finite. Note that because a and b are not statistically independent, this only works for the rightmost portion of Equation 27. An alternative idea is the inverse regression approach, i.e., regress x vs y instead of y vs x. The idea is to force the errors into the variable about which predictions are to be made and confidence bands are to be drawn. Eisenhart (28) argued that this approach yields meaningless results and that the "fitting should be done in terms of the deviations that actually represent error." The inverse regression approach is also rejected on the grounds that the $\hat{X}_o$ obtained is an inconsistent estimator (26). The resulting conundrum is commonly known in the statistical literature as the "calibration problem" (1, 26, 28–30).

Single-Use Discrimination Intervals

Although there is no wholly satisfactory answer to this "calibration problem," an approach has been devised that works for most calibration experiments (1, 6, 14, 24). Rather than attempt to calculate the mean and variance of $\hat{X}_o$, this approach directly derives confidence limits for $\hat{X}_o$ on the condition that

the calibration slope can be bounded away from 0. First, a prediction interval is constructed about the Y_o (or $\bar{Y}_o$) response value via Equation 25. Intersecting the limits of this interval with the Y_o value and dropping perpendiculars down to the x-axis yields upper and lower limits (X_U and X_L, respectively) for the desired discrimination interval. For this approach, the best estimate of $\hat{X}_o$ is obtained via Equation 27. Figure 4 shows the potential for mischief inherent in this approach. Unless the slope is sufficiently steep and the prediction band sufficiently narrow, the discrimination interval will be infinitely wide.

To express this approach in mathematical form, we start by constructing a prediction interval about X_L (starting about X_U yields an identical result) by using Equation 25.

$$P.I. = Y_L \pm ts_{Y_L} = a + bX_L \pm ts_{yx}\left[1 + \frac{1}{n} + \frac{(X_L - \bar{X})^2}{S_{xx}}\right]^{1/2} \quad (28)$$

Here, t is a 2-tailed value with n – 2 degrees of freedom ($t_\nu^{\alpha/2}$). From Figure 5, we see that if the value for Y_L is taken and the height of this interval added to it, Y_o is obtained.

$$Y_o = a + b\hat{X}_o \quad (29a)$$

$$= a + bX_L + ts_{yx}\left[1 + \frac{1}{n} + \frac{(X_L - \bar{X})^2}{S_{xx}}\right]^{1/2} \quad (29b)$$

Solving for X_L yields the following quadratic equation:

$$X_L^2(1-g) + 2X_L(g\bar{X} - \hat{X}_o) \quad (30)$$

$$+ \hat{X}_o^2 - g\bar{X}^2 - gS_{xx}\left(1 + \frac{1}{n}\right) = 0$$

Here, g is used to greatly simplify the expression.

$$g = \frac{t^2 s_{yx}^2}{b^2 S_{xx}} \quad (31)$$

Solving Equation 30 via the quadratic formula and incorporating the result for X_U yields:

$$\{X_U, X_L\} = \frac{\hat{X}_o - g\bar{X}}{1-g} \pm \frac{ts_{yx}}{b(1-g)} \quad (32)$$

$$\times\left[\left(1 + \frac{1}{n}\right)(1-g) + \frac{(\hat{X}_o - \bar{X})^2}{S_{xx}}\right]^{1/2}$$

The first term on the right-hand side of Equation 32 may be rewritten as follows:

$$\frac{\hat{X}_o - g\bar{X}}{1-g} = \hat{X}_o + \frac{g(\hat{X}_o - \bar{X})}{1-g} \quad (33a)$$

Table 1. Regression statistics and prediction intervals at 30°C for total volatile sulfur flux (ng S/m^2–min) field data from 2 sites

Parameter	Ames, IA	Celeryville, OH
n	16	37
a	–0.253401	0.458518
b	0.096321	0.123193
s_{yx}^2	0.036567	0.093993
$\bar{X}$	35.500	30.581
S_{xx}	322.000	2028.757
$\hat{Y}_o$	2.636235	4.154298
$t_\nu^{\alpha/2}$	2.145	2.0315
Predicted flux	13.961	63.707
Upper limit	21.700	119.765
Lower limit	8.981	33.888

$$= \bar{X} + \frac{\hat{X}_o - \bar{X}}{1-g} \quad (33b)$$

From Equation 33a it is apparent that the interval is not entirely symmetrical about the predicted value $\hat{X}_o$. This is due to the non-normality of its distribution, as discussed above. Even though Equation 33a includes a subtraction from $\hat{X}_o$ in the numerator, the influence of the denominator always counterbalances this and makes $(\hat{X}_o - g\bar{X})/(1-g) > \hat{X}_o$ in all observed instances.

If m replicate measurements are made of the Y_o value, the "1" term within the brackets in Equation 32 may be replaced by 1/m.

$$\{X_U, X_L\} = \frac{\hat{X}_o - g\bar{X}}{1-g} \pm \frac{ts_{yx}}{b(1-g)} \quad (34)$$

$$\times\left[\left(\frac{1}{m} + \frac{1}{n}\right)(1-g) + \frac{(\hat{X}_o - \bar{X})^2}{S_{xx}}\right]^{1/2}$$

If enough replicates are done to make m significant in comparison to n, the value for the regression variance may be updated by pooling it together with the new measurements (27).

$$(s'_{yx})^2 = \frac{\sum(Y_i - \hat{Y}_i)^2 + \sum(Y_{o,j} - \bar{Y}_{o,j})^2}{(n-2) + (m-1)} \quad (35a)$$

$$= \frac{(n-2)s_{yx}^2 + \sum(Y_o - \bar{Y}_o)^2}{n+m-3} \quad (35b)$$

Note that the value for t will now have n + m − 3 degrees of freedom. In Equations 35a and 35b, the summations for i range again from 1 to n, and the summations for j range from 1 to m.

As noted above, the limits of this discrimination interval may be undefined. The point at which this happens is controlled by the g-value. If g > 1, the limits are infinite. The expression for g can be rewritten to clarify its meaning.

$$g = \frac{t^2 s_{yx}^2}{b^2 S_{xx}} = \left(\frac{t s_b}{b}\right)^2 \tag{36}$$

Therefore, we see that g is a measure of the statistical significance of the slope value. If the slope is not statistically significant, the limits for the discrimination interval will be undefined and, therefore, not calculable. This satisfies the stated condition that the slope must be bounded away from 0.

Further use can be made of the g-value for deriving an approximation to Equation 34. If g is sufficiently small, i.e., less than ca 0.1, Equation 34 can be approximated by assuming that g is insignificant compared with 1 (g << 1).

$$\{X_U, X_L\} \approx \hat{X}_o \pm \frac{t s_{yx}}{b} \tag{37}$$

$$\times \left[\frac{1}{m} + \frac{1}{n} + \frac{(\hat{X}_o - \bar{X})^2}{S_{xx}}\right]^{1/2}$$

This symmetrical interval, which is comparable with a prediction interval, allows an expression to be written for the variance of the predicted $\hat{X}_o$.

$$s_{\hat{X}_o}^2 \approx \left(\frac{s_{yx}}{b}\right)^2 \tag{38}$$

$$\times \left[\frac{1}{m} + \frac{1}{n} + \frac{(\hat{X}_o - \bar{X})^2}{S_{xx}}\right]$$

This result is exactly the same as the one produced by Bennett and Franklin (27). They assumed that the distribution of $\hat{X}_o$ is approximately normal, allowing them to propagate errors directly through the right-most portion of Equation 27.

Inferences similar to those made above for prediction intervals following Equation 24 may also be made here. If m = 1 or is at least << n (as is usually the case) and $\hat{X}_o$ is not at the extremes of the calibration range, then Equation 38 can be grossly approximated as follows:

$$s_{\hat{X}_o}^2 \approx \frac{s_{yx}^2}{mb^2} \rightarrow \left(\frac{s_{yx}}{b}\right)^2 \text{ for } m = 1 \tag{39}$$

Thus, the variance for a predicted x-value can be grossly approximated by taking the y-calibration variance and scaling it by the slope value (akin to a proportionality constant).

Figure 6 gives a closer look at the g-value and how it affects the approximation in Equation 37. In this illustration, the percent errors of the lengths of discrimination intervals (Equation 37 as compared to Equation 34) are expressed as a function of their respective values of g. The data for this graph came from a variety of linear regression results produced in our laboratory. The data are summarized in Table 2. Applying linear regression to these data yields a slope of 59.537 and an intercept of –0.198. The standard deviations of the slope and intercept are 0.077 and 0.631, respectively. There is a good linear relationship between the percent errors and the values of g. No theoretical reason is known for this result, and no previous mention of it has been made in the literature. Using this type of graph and/or the concomitant data, one can decide whether or not to use the approximation based on the calculated g-value and the level of error associated with it.

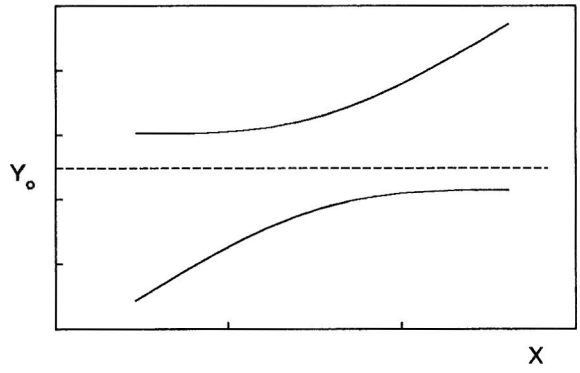

Figure 4. Example of infinite error limits.

Multiple-Use Discrimination Intervals

The preceding descriptions regarding the construction of discrimination intervals (and prediction intervals as well) have used a 2-tailed value of t with n − 2 degrees of freedom and significance level α. These procedures apply only to the construction of 1 such interval for a particular calibration curve (1, 14, 31–33). If 2 such intervals are constructed using the same calibration curve and *t*-value, the simultaneous (or joint) confidence of the 2 intervals is no longer 1 − α, but is instead somewhat greater than 1 − 2α (34). In fact, for k such intervals, the joint confidence will be on the order of 1 − *k*α (34–36).

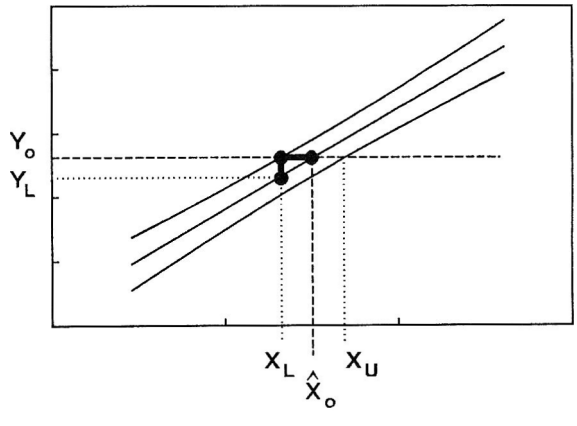

Figure 5. Construction of a discrimination interval.

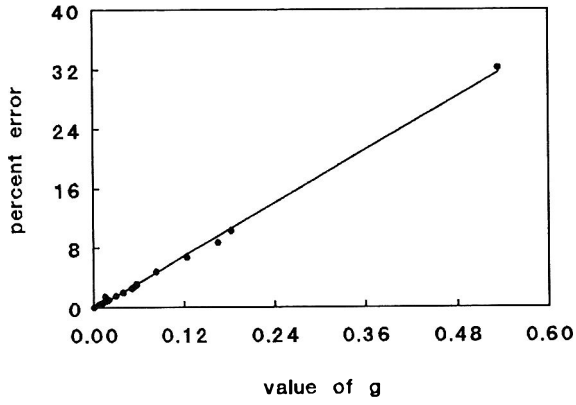

Figure 6. Relationship of percent error of discrimination interval length and value of g.

Practically, one can construct several discrimination intervals about predicted x-values using the same calibration curve and value of t, as analytical chemists usually do. However, comparisons among these x-values and their intervals assuming a confidence level of $1 - \alpha$ are not valid because the confidence level has shrunk to around $1 - k\alpha$ (k being the number of intervals in a particular comparison).

The approaches to preparing intervals with constant joint confidence levels are directly related to the multiple comparisons of means (or separation of means) procedures used in conjunction with ANOVA (analysis of variance). In the following section, the 3 basic approaches to multiple-use discrimination intervals (MDI) are discussed for the case when the number of intervals is known beforehand (32, 33).

Bonferroni t-intervals.—For this approach, 2-tailed values of t with $n - 2$ degrees of freedom are used as before. However, the significance coefficient will be $\alpha/2k$ instead of the usual $\alpha/2$. As noted above, k is the number of simultaneous intervals that will be constructed. The difficulty here is that ordinary *t*-tables do not include *t*-values for the $\alpha/2k$ significance coefficients that are commonly required. The following relationship is helpful in some instances:

$$t_{n-2}^{\alpha/2k} = (F_{1,n-2}^{\alpha/k})^{1/2} \qquad (40)$$

Tabulated values for t with significance $\alpha/2k$ do exist (32, 33); however, these tables are somewhat limited. One may interpolate within these or ordinary t-tables, or one may use the following expression:

$$t_{\nu}^{\alpha} \approx z_{\alpha}\left[1 - \frac{z_{\alpha}^2 + 1}{4\nu}\right]^{-1} \qquad (41)$$

In this equation, z_α is the upper α (1-tailed) point of the standard normal distribution. For large ν, this appears to be a good approximation; for small ν ($\nu << 10$), it is suitable provided $\nu > k$; for intermediate values of ν (≈ 7–10), k should not be too much larger than ν.

Using a *t*-value with a significance of $\alpha/2k$ instead of $\alpha/2$ ensures that the simultaneous confidence level for the k intervals will be $\geq 1 - \alpha$, i.e., the Bonferroni coefficient $t_{\nu}^{\alpha/2k}$ yields a somewhat conservative interval. For $k = 1$, this coefficient is the same as the nonsimultaneous *t*-value. All that is required to use this approach is to find or calculate a value for $t_{\nu}^{\alpha/2k}$ and substitute it for $t_{\nu}^{\alpha/2}$ in the expression for g, then calculate the discrimination limits via Equation 34, again substituting in the new *t*-value. Actually, more general forms for these expressions may be written.

Table 2. Percent error of approximate discrimination interval lengths as a function of g-value[a]

File name	g	% error
RIP_ONE	0.000002	0.000171
BILL_BR2	0.000317	0.016032
BILL_TWO	0.000407	0.020500
BILL_ONE	0.000685	0.034378
BILL_BR1	0.000776	0.038867
MAN_ONE	0.000793	0.039736
TWELVE	0.006234	0.319771
ELEVEN	0.009164	0.464376
CV_SOIL	0.012599	0.644029
RIP_TWO	0.014664	1.462780
NASA14	0.015836	0.833465
NASA45	0.017902	0.899421
NASA24	0.019820	1.002482
NASA15	0.029252	1.510134
NASA34	0.038884	1.965733
NASA35	0.049857	2.536557
NASA25	0.052516	2.697765
MAN_TWO	0.053678	2.743418
AM_SOIL	0.056318	3.086680
NASA12	0.056914	3.046405
NASA21	0.056914	3.016184
NASA13	0.082516	4.760074
NASA23	0.123626	6.659880
MACTEST	0.165188	8.709136
SYX_SA	0.182641	10.29060
ANDSN	0.533595	32.19081

[a] Linear regression statistics for % error vs g: n = 26, a = –0.198, b = 59.537, s_a = 0.631, and s_b = 0.077.

$$\{X_U, X_L\} = \frac{\hat{X}_o - g\bar{X}}{1-g} \pm \frac{K\, s_{yx}}{b\,(1-g)} \qquad (42)$$

$$\times \left[\left(\frac{1}{m} + \frac{1}{n}\right)(1-g) + \frac{(\hat{X}_o - \bar{X})^2}{S_{xx}}\right]^{1/2}$$

$$\text{and } g = \frac{K^2 s_{yx}^2}{b^2 S_{xx}} \qquad (43)$$

For the nonsimultaneous approach, $K = t_{\nu}^{\alpha/2}$; for the Bonferroni approach, $K = t_{\nu}^{\alpha/2k}$; and for the approaches noted below, K will take on other forms. If the newly defined g is sufficiently small, an approximation similar to that in Equation 37 may again be used.

$$\{X_U, X_L\} \approx \hat{X}_o \pm \frac{Ks_{yx}}{b} \times \left[\frac{1}{m} + \frac{1}{n} + \frac{(\hat{X}_o - \bar{X})^2}{S_{xx}}\right]^{1/2} \quad (44)$$

Scheffé's S-method.—For this approach, $K = (kF^{\alpha}_{k,n-2})^{1/2}$ will be used, where everything is as defined before. This approach also yields somewhat conservative limits, i.e., the simultaneous confidence level $\geq 1 - \alpha$. Also, as before, this coefficient reduces to the nonsimultaneous one $\sqrt{F^{\alpha}_{1,\nu}} = t^{\alpha/2}_{\nu}$ for k = 1. The advantage to this approach is that the F in the coefficient is the 1-tailed value widely tabulated in statistical publications.

Maximum modulus t-intervals.—In this approach, $K = u^{\alpha}_{k,n-2}$ will be used. The coefficient is the upper tail α point of the distribution of the maximum absolute value of k Student t_ν-variables. The obtained intervals are again somewhat conservative. Also, the coefficient does reduce to $t^{\alpha/2}_{\nu}$ for k = 1. The tables for u are somewhat scarce, but the common one (32, 33) is more useful than the one for the Bonferroni coefficient.

Comparison of limited-k approaches.—A comparison of these approaches is summarized in Table 3. This table gives values for the coefficients assuming a confidence level of 95% and varying values for k and n.

From Table 3 it is seen that, in agreement with published theory (32), $u^{\alpha} < t^{\alpha/2k} < (kF^{\alpha})^{1/2}$ for all usual cases. Disconcertingly, this order is the exact opposite of the availability of the coefficients. Also, it is apparent that intervals based on these coefficients become quite large as k increases when compared with the nonsimultaneous approach. The principle used by statisticians when choosing among intervals is to select the shortest one from the theoretically sound choices. On the basis of this criterion alone, the maximum modulus approach would be chosen.

Although weighted regression has not been mentioned since the end of the section on prediction intervals, note that those principles also apply to all the equations presented here concerning discrimination intervals. For example, Equation 42 may be rewritten for the y-weighted regression case as follows:

$$\{X_U, X_L\} = \frac{\hat{X}_o - g\bar{X}_w}{1-g} \pm \frac{K\, s_{wyx}}{b\,(1-g)} \times \left[\left(\frac{1}{w_o} + \frac{1}{\sum w}\right)(1-g) + \frac{(\hat{X}_o - \bar{X}_w)^2}{S_{wxx}}\right]^{1/2} \quad (45)$$

Here, $g = K^2 s^2_{wyx}/b^2 S_{wxx}$, and, as before, w_o is a weight appropriate to the value of Y_o.

Unlimited-Use Discrimination Intervals

This section describes 3 approaches for dealing with the case where k is either unknown beforehand or possibly large enough to make the procedures discussed in the preceding section impractical. The following unlimited-use discrimination intervals (UDIs) approaches yield results that may be used as often as is wished to construct intervals from a single calibration curve. However, they have the disadvantage of increased complexity because each UDI requires either 2 or 3 statistical coefficients, whereas each MDI required only 1 such coefficient.

Bonferroni tolerance intervals.—The first UDI approach has been described in the statistical literature (33–35) and applied in the analytical literature (18, 36). It uses the Bonferroni inequality (on which the Bonferroni *t*-intervals discussed above are also based) to build a tolerance interval. First, a confidence band is placed about the regression line by employing the Working-Hotelling band (37) for the entire line (confidence = $1 - \alpha/2$).

$$a + bX \pm (2F^{\alpha/2}_{2,\nu})^{1/2} s_{yx} \left[\frac{1}{n} + \frac{(X - \bar{X})^2}{S_{xx}}\right]^{1/2} \quad (46)$$

Note that the required F is a 2-tailed value. This interval is depicted in Figure 7 by the band around the regression line.

Next, a confidence interval is constructed about the Y_o (or $\bar{Y}_o$) value. For this, a value is needed for σ, which is bounded above using the following expression.

$$\sigma \leq s_{yx} \left[\frac{n-2}{\chi^2_{\alpha/2,\nu}}\right]^{1/2} \quad (47)$$

Here, χ^2 is the *lower* $\alpha/2$ percentile point with n – 2 degrees of freedom; the probability of this statement is also $1 - \alpha/2$. This upper bound for σ is now used to construct the Y-interval.

$$\{Y^U_o, Y^L_o\} = Y_o \pm z_{\gamma/2}\sigma \quad (48)$$

In this expression, $z_{\gamma/2}$ is the upper 2-tailed point of the standard normal distribution, and γ may or may not be chosen to equal α. This second interval is depicted in Figure 7 by the horizontal band around Y_o starting at the y-axis and extending through the Working-Hotelling band about the regression line. The Y_o interval is somewhat exaggerated in size in this figure for clarity. Upper and lower tolerance limits for $\hat{X}_o$ are obtained by dropping the intersections of the Y_o interval with the regression band to the x-axis.

Table 3. Comparison of coefficients for multiple-use discrimination intervals (MDI)

k	n	$t^{\alpha/2}_{\nu}$	$t^{\alpha/2k}_{\nu}$	$(kF_{k,\nu}{}^{\alpha})^{1/2}$	$u^{\alpha}_{k,\nu}$
1	5	2.571	2.57	2.571	2.571
2	5	2.571	3.17	3.402	3.091
2	10	2.228	2.64	2.865	2.609
5	10	2.228	3.17	4.078	3.103
10	10	2.228	3.58	5.457	3.468
20	10	2.228	4.01	6.853	3.823

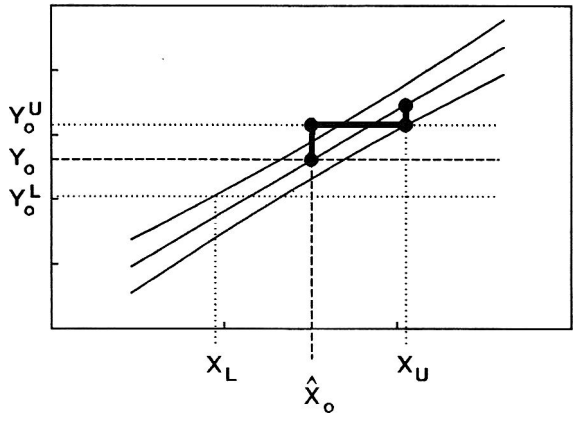

Figure 7. Construction of an unlimited-use discrimination interval.

The interval expressed in Equation 48 may be shortened by taking replicates of Y_o (18, 33, 36). If m replicates are employed, Equation 48 may be reexpressed as follows:

$$\{Y_o^U, Y_o^L\} = \bar{Y}_o \pm \frac{z_{\alpha/2}\sigma}{\sqrt{m}} \tag{49}$$

The Bonferroni inequality implies that the probability that Equations 46 and 47 both hold is at least $1 - \alpha$ (again, this makes a conservative interval). By comparing this situation with the one at the start of the section on discrimination intervals, we see that the previous comments about the necessity of a steep slope along with a narrow regression band are even more important here because of the added band about Y_o.

Assuming a positive slope and using Equations 46, 47, and 49 with reference to Figure 7, the expression for the upper limit on $\hat{X}_o$ can be formulated as follows:

$$a + bX_U - (2F_{2,\nu}^{\alpha/2})^{1/2} s_{yx} \left[\frac{1}{n} + \frac{(X_U - \bar{X})^2}{S_{xx}}\right]^{1/2} \tag{50}$$

$$= a + b\hat{X}_o + z_{\alpha/2} s_{yx} \left[\frac{n-2}{\chi^2_{\alpha/2,\nu} m}\right]^{1/2}$$

The expression for the lower limit is similar.

$$a + bX_L + (2F_{2,\nu}^{\alpha/2})^{1/2} s_{yx} \left[\frac{1}{n} + \frac{(X_L - \bar{X})^2}{S_{xx}}\right]^{1/2} \tag{51}$$

$$= a + b\hat{X}_o - z_{\alpha/2} s_{yx} \left[\frac{n-2}{\chi^2_{\alpha/2,\nu} m}\right]^{1/2}$$

A pragmatic solution for this combination of equations will now be presented. This solution gives the same results as the one published by Lieberman et al. (34) but is handier and more lucid. First, some shorthand notation will be adopted.

$$H = 2F_{2,\nu}^{\alpha/2} \frac{s_{yx}^2}{b^2} \tag{52a}$$

$$g = \frac{H}{S_{xx}} \tag{52b}$$

$$L = \frac{s_{yx}}{b\sqrt{m}} z_{\alpha/2} \left[\frac{n-2}{\chi^2_{\alpha/2,\nu}}\right]^{1/2} \tag{52c}$$

Note that the expression for g in Equation 52b is of the same form as in Equation 43. Using this notation, solving Equations 50 and 51 for X_U and X_L, respectively, and combining the solutions yield the following result:

$$\{X_U, X_L\} = \frac{\hat{X}_o - g\bar{X} \pm L}{1-g} \pm \frac{H^{1/2}}{1-g} \tag{53}$$

$$\times \left[\frac{1-g}{n} + \frac{(\hat{X}_o - \bar{X} \pm L)^2}{S_{xx}}\right]^{1/2}$$

There are 3 ± signs in this expression. For positive slopes, X_U is calculated by using the plus sign each time, and X_L is calculated using the minus sign each time. For negative slopes, the first and third ± signs are flipped, leaving the second as is, i.e., X_U is calculated using the sequence (−,+,−) and X_L is calculated using the sequence (+,−,+). This is illustrated in the following expression:

$$\{X_U, X_L\} = \frac{\hat{X}_o - g\bar{X} \mp L}{1-g} \pm \frac{H^{1/2}}{1-g} \tag{54}$$

$$\times \left[\frac{1-g}{n} + \frac{(\hat{X}_o - \bar{X} \mp L)^2}{S_{xx}}\right]^{1/2}$$

Although this case is more complicated than the MDI cases previously described, the solution is quite similar; only here 2 extra terms are included that take care of the band about Y_o. Again, the requirement that $g < 1$ applies, otherwise Equations 53 and 54 will be undefined.

Scheffé intervals.—The second UDI approach is that of Scheffé (38). To date, the complete form for these intervals has not been applied in the analytical literature. Scheffé's approach is described as "interval estimation" in the original derivation article (38). By constraining the probability area of his discrimination intervals to the range bounded by the calibration data, Scheffé defined a coefficient, c, which yields intervals claimed to be generally shorter than the Bonferroni UDIs described above. Once c is known, Scheffé's intervals may be drawn using expressions similar to those in Equation 52 along with

Equation 53 (or Equation 54 for negative slopes). The following expressions illustrate this:

$$H = 2\,F^{\alpha}_{2,\nu}\,\frac{c^2 s^2_{yx}}{b^2} \qquad (55a)$$

$$g = \frac{H}{S_{xx}} \qquad (55b)$$

$$L = \frac{c\,s_{yx}}{b\sqrt{m}}\,z_{\nu_2}\left[\frac{n-2}{{}_{\alpha}\chi^2_{\nu}}\right]^{1/2} \qquad (55c)$$

Note that for the Scheffé intervals, F and χ^2 are 1-tailed values, whereas the Bonferroni UDIs require 2-tailed values for these statistics. This by itself shortens the Scheffé intervals in comparison. Practically, the use of Scheffé's tables for c requires calculation of a value for his quantity s_2, which we have been unable to calculate. For the purpose of comparing types of intervals, however, a value of c = 1 will suffice because the majority of the tabulated values are within 2–5% of c = 1.

Modified Scheffé intervals.—The third UDI approach is based on a modification of Scheffé's method. It has been described in both the analytical (23) and statistical (39) literatures. This approach is different because a simple *t*-interval is constructed about the Y_o (or $\bar{Y}_o$) value using the standard deviation of the regression, s_{yx}. The proponents of this approach argue against the need to put a bound on σ by using its experimental estimate, s_{yx}, in conjunction with a χ^2-value. The intervals for this approach may be constructed by modifying the expressions in Equation 52 as shown in Equation 56, and then using them in either Equation 53 or 54.

$$H = 2\,F^{\alpha}_{2,\nu}\,\frac{s^2_{yx}}{b^2} \qquad (56a)$$

$$g = \frac{H}{S_{xx}} \qquad (56b)$$

$$L = \frac{t^{\nu_2}_{\nu}\,s_{yx}}{b\sqrt{m}} \qquad (56c)$$

This approach is recommended over the other 2 UDI approaches and the 3 MDI approaches because of its simplicity. The required coefficients are by far the simplest and most readily available.

Comparison of Discrimination Interval Lengths

A summary of the comparison of lengths of the various discrimination intervals discussed above is presented in Tables 4–7. The example comparisons were generated by using a variety of data sets. Each table assumes a different value for k. Because SDI and UDI lengths do not change with k, the lengths of these intervals are shown in Table 4 only.

Several observations may be made from these tables. The most obvious is that SDI lengths are always significantly shorter than corresponding MDI and UDI lengths. Another elementary observation is that the MDI lengths increase with the values for k. The Scheffé MDIs lengthen much more rapidly with increasing k than do the corresponding Bonferroni and maximum modulus MDIs. For k > 5, the Scheffé MDIs are so long that they are useless in comparison to the other MDIs. The Bonferroni and maximum modulus MDIs are comparable in length, although, as noted above, the maximum modulus intervals are uniformally shorter.

In most instances, the relative UDI lengths can be expressed as modified Scheffé < Scheffé < Bonferroni. The exception is for large n, for which the relative lengths become Scheffé < Bonferroni < modified Scheffé. This reversal is probably due to the much increased values of χ^2 at large n. The Scheffé and Bonferroni UDIs employ this statistic, whereas the modified Scheffé UDIs do not.

For k = 2 and k = 5, the shortest MDIs are shorter than the shortest UDIs. For k = 10 and k = 20, this situation is reversed and the MDIs grow longer. Even for k = 5, the shorter UDIs are quite comparable in length to the corresponding MDIs. Thus, the only clear advantage of the MDIs is for instances when k is known ahead of time to be less than 5. The UDIs are more generally useful than the MDIs because they are of comparable or shorter length (except for very small k) and because their independence of k renders them much more flexible. Modified Scheffé UDIs are particularly useful because of their relative shortness and their simplicity.

A last observation from Tables 4–7 is that replicating Y_o (i.e., going from m = 1 to m = 3) yields significantly shorter intervals in all instances. This agrees with common statistical sense, which argues for replication whenever possible.

Conclusion

One major goal of this study was to summarize the theory that allows the use of classical linear regression formulations when the assumption that all error must reside in the dependent variable is violated. The use of classical linear regression was shown to be permissible within the framework of the controlled-x theory developed by Berkson (9) and subsequently expanded upon by others. This result is of immense importance to those analytical chemists who must calibrate their analytical methods with standards that are not, as a rule, inerrant. For cases when error in the independent variable must be accounted for separately from error in the dependent variable, the reader is referred to Part II.

In discussing the assumption of a linear relation between the dependent and independent variables, the inadequacy of using the correlation coefficient as a quantitative measure of that linearity was noted. Despite previous warnings about this unwarranted application of the correlation coefficient, it remains a common *faux pas* among analytical chemists. Visual observa-

Table 4. Comparison of discrimination interval lengths for k = 5

File name	m	SDI	B-MDI[a]	S-MDI	M-MDI	B-UDI	S-UDI	M-UDI
BILL_BR1	3	90.9	126.8	163.9	125.2	156.7	141.9	131.0
BILL_BR1	1	148.2	206.8	267.1	204.1	231.5	210.7	191.8
BILL_BR2	3	58.1	81.0	104.6	79.9	99.9	90.5	85.5
BILL_ONE	3	208.9	276.5	357.8	276.6	248.3	238.3	251.4
BILL_TWO	3	161.0	213.0	275.7	211.6	191.0	183.4	193.5
ELEVEN	3	145.5	203.8	264.8	201.1	260.4	234.3	216.7
TWELVE	3	119.3	166.9	216.5	164.7	211.6	190.8	176.5
AM_SOIL	3	5.74	8.31	11.24	8.16	12.30	10.66	9.19
CV_SOIL	3	6.37	8.60	11.22	8.55	9.90	9.19	9.13
GVALUE	3	0.0145	0.0196	0.0254	0.0195	0.0220	0.0204	0.0197
NASA12	3	69.6	97.1	131.4	96.3	125.7	112.6	108.6
NASA13	3	84.6	119.7	166.0	118.6	161.8	142.7	137.3
NASA14	3	34.6	47.3	61.8	46.9	55.2	50.7	48.8
NASA15	3	47.4	65.1	86.0	64.6	78.3	71.4	68.6
NASA21	3	82.1	114.6	155.1	113.6	148.5	133.1	128.4
SYX_SA	3	19.5	30.7	51.1	30.1	53.9	42.3	39.2
SYX_SA	1	31.0	48.6	79.5	47.6	87.8	68.1	61.4

[a] B-MDI = Bonferroni MDI, S-MDI = Scheffé MDI, M-MDI = maximum modulus MDI, B-UDI = Bonferroni UDI, S-UDI = Scheffé UDI, and M-UDI = modified Scheffé UDI.

tion of the data, along with its least squares fit, is a more effective means of detecting nonlinearity than reliance on a value of r close to ±1. Also in the discussion of the linearity assumption, it was noted that various transformations may be employed to linearize data, and that using these transformations in a proper manner results in asymmetrical confidence intervals.

Another major objective of this study was to provide a comprehensive guide to properly construct various types of confidence intervals using linear regression parameters. These intervals were divided into 2 categories, prediction intervals and discrimination intervals. Prediction intervals are constructed for values of the dependent variable based upon chosen values of the independent variable. These intervals are useful in instances when one wishes to model data for one variable based on values of another variable. If more than one independent variable is needed for a model, multiple regression techniques are necessary. This paper focused on linear regression only.

Considerable attention was also devoted to the second category of confidence intervals. Discrimination intervals are extremely useful to analytical chemists who employ calibration. This category was subdivided into 3 subsections: SDI, MDI, and UDI. All these intervals are the outgrowth of the attention paid by statisticians to what they call the calibration problem. This problem arises from the difficulties inherent in constructing confidence intervals for values of the independent variable based on measured values of the dependent variable. The relatively large number of these types of intervals is a result of the dissatisfaction of statisticians with the comprehensive merits of any one of them.

Many analytical chemists are already familiar with the simplest solution to the calibration problem, i.e., the SDI approach, because it is often the one presented in analytical textbooks. In practice, this approach may be further simplified via approximation. The success of this approximation depends on the

Table 5. Comparison of discrimination intervals for k = 2

File name	m	B-MDI[a]	S-MDI	M-MDI
BILL_BR1	3	106.6	116.2	106.0
BILL_BR1	1	173.9	189.4	172.8
BILL_BR2	3	68.1	74.2	67.7
BILL_ONE	3	239.1	261.7	238.0
BILL_TWO	3	184.2	201.7	183.4
ELEVEN	3	170.9	186.4	169.9
TWELVE	3	140.1	152.7	139.3
AM_SOIL	3	6.68	7.34	6.64
CV_SOIL	3	7.36	8.06	7.34
GVALUE	3	0.0168	0.0183	0.0167

[a] B-MDI = Bonferroni MDI, S-MDI = Scheffé MDI, and M-MDI = maximum modulus MDI.

Table 6. Comparison of discrimination intervals for k = 10

File name	m	B-MDI[a]	S-MDI	M-MDI
BILL_BR1	3	142.8	217.9	139.2
BILL_BR1	1	232.8	355.3	227.0
BILL_BR2	3	91.1	139.1	88.9
BILL_ONE	3	302.2	463.1	299.6
BILL_TWO	3	232.8	356.7	230.9
ELEVEN	3	230.0	356.3	224.1
TWELVE	3	188.2	290.1	183.5
AM_SOIL	3	9.21	15.94	8.98
CV_SOIL	3	9.49	14.87	9.41
GVALUE	3	0.0217	0.0333	0.0215

[a] B-MDI = Bonferroni MDI, S-MDI = Scheffé MDI, and M-MDI = maximum modulus MDI.

Table 7. Comparison of discrimination intervals for k = 20

File name	m	B-MDI[a]	S-MDI	M-MDI
BILL_BR1	3	158.4	296.3	152.9
BILL_BR1	1	258.2	483.1	249.3
BILL_BR2	3	101.1	188.8	97.6
BILL_ONE	3	326.3	612.0	323.6
BILL_TWO	3	251.4	471.1	249.3
ELEVEN	3	255.7	495.8	246.7
TWELVE	3	209.1	400.1	201.8
AM_SOIL	3	10.38	28.20	9.96
CV_SOIL	3	10.34	20.43	10.25
GVALUE	3	0.0237	0.0448	0.0234

[a] B-MDI = Bonferroni MDI, S-MDI = Scheffé MDI, and M-MDI = maximum modulus MDI.

value of g, as shown. Furthermore, the error in using this approximation was shown to be linearly related to the value of g, a result that has not been previously mentioned in the literature.

Although the SDI approach is useful to analytical chemists, its actual applicability is somewhat limited. In theory, it is not permissible to construct more than one SDI from a particular linear regression data set and subsequently compare these intervals. As discussed, the reason behind this is that the joint confidence level of the intervals is less than the individual confidence levels on which the intervals were based. If comparisons are to be made among results from the same data set, either separate regressions must be performed for each result or the MDI or UDI approaches must be employed.

The theory for the MDI and UDI approaches was discussed, and their lengths were compared by using a variety of data sets. Except for small k, UDI lengths are comparable to or shorter than MDI lengths. Our conclusion is that the UDI approaches are generally more useful than the MDI approaches. The modified Scheffé UDI approach was found to be particularly useful because of its relative simplicity and the relative shortness of its intervals.

Although the adoption of the MDI and UDI approaches by analytical chemists is strongly encouraged, obstacles to their use by the mainstream of the community do exist. A major obstacle is that knowledge of these approaches has not been widely disseminated. Only a small number of publications on this subject have appeared in the chemical literature. Another obstacle is the relative complexity of the formulas resulting from these approaches. In particular, MDIs and UDIs require the use of various statistical coefficients for which tables are not readily available. These obstacles could be largely overcome if software packages were developed and made available that include both the necessary coefficients and calculations.

For all the intervals constructed as examples in this paper, coefficients based on significance levels, α and γ, of 0.05 were chosen. As noted, the resulting intervals all have confidence levels of 95%. These choices appear to be somewhat standard within the literature. Of course, shorter intervals may be constructed by raising the significance levels and consequently lowering the confidence levels. However, doing so must be balanced against the consequences of a particular interval not bracketing the "true" value.

Although our desire was to prepare a comprehensive guide regarding the uses of linear regression by analytical chemists, at the end we recognized that many subjects need further consideration by both statisticians and practicing analysts. Chief among these is the use of weighted linear regression. A comprehensive and practical guide to the heteroscedastic variance case does not presently exist. A few simple guidelines for this case were given here, but a more thorough treatment is badly needed. Three other subjects are natural outgrowths of our study to date. First is the extension of the various confidence intervals included in this work to cases where nonlinear regression is necessary. Second is the extension of the principles discussed in this work to the calculation of *practical* detection limits. Third is the extension of these principles to the use of standard additions, which, unfortunately, involves extrapolation of calibration data.

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SPECIAL REPORTS

Analytical Use of Linear Regression. Part II: Statistical Error in Both Variables

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This paper critically evaluates the literature techniques that attempt to incorporate the existence of both x- and y- errors into linear regression statistics. This evaluation focuses on the relative theoretical and practical merits of 4 techniques: (*1*) the effective variance approach, (*2*) the constant variance ratio approach, (*3*) the York approach, and (*4*) the Williamson approach. The practical use of these different approaches is illustrated with the aid of actual results from an interlaboratory comparison study. On the basis of our comparative evaluation, the constant variance ratio approach is sound, yet simple, and is strongly recommended as long as the constant ratio criterion can be satisfied. However, the Williamson approach is applicable in more situations and is also highly recommended because of its theoretical virtues, mathematical consistencies, and practical applications. The other 2 methods are not recommended for use because of reasons that are summarized in this paper. The lack of available software remains an impediment to widespread use of many of the described statistical procedures.

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Part I focused on the application of linear regression to analytical chemistry problems that could be assumed to be devoid of error in the independent variable (x). Part II examines the case where error is present in x and, therefore, must be taken into account. The presence of error in x greatly complicates the process of obtaining a valid expression for the slope of a least squares regression model. Various statistical approaches for attacking this latter case will be discussed in this paper, with attention directed toward their practical use.

The statistical techniques evaluated here have already been presented in the literature. However, this material is widely scattered throughout the statistical and analytical publications. The analytical branch, in particular, often contains faulty and misleading information. As a result, knowledge of how to treat the error-in-x case in a statistically valid manner is extremely lacking among analytical chemists. The purpose of this paper is to critically examine the pertinent statistical techniques for their relative theoretical and practical merits.

It should be noted that the usual titles applied to the variables of the x- and y-axes (i.e., independent and dependent, respectively) have no real application to the present case. Furthermore, predicting a value of one variable given a chosen or

measured value of the other variable is not usually of concern in the problems relevant to the methods of this paper. Rather than focusing on predictions from a calibration curve, problems where error exists in both axes are usually solved by using the obtained regression parameters to make a comparison of the variables represented by the axes.

Statement of the Problem

In mathematical terms, the difference between this case and the cases discussed in Part I is that here the variability in the x-values must be accounted for in addition to the variability in the y-values when the expression for the weighted error sum of squares is written:

$$S_w = \sum [w_{X_i} (X_i - \hat{X}_i)^2 + w_{Y_i} (Y_i - \hat{Y}_i)^2] \quad (1a)$$

$$= \sum [\frac{(X_i - \hat{X}_i)^2}{s^2_{X_i}} + \frac{(Y_i - \hat{Y}_i)^2}{s^2_{Y_i}}] \quad (1b)$$

This expression apparently has not been used to derive formulas for intercepts and slopes. Rather, all researchers to date have employed the basic approach of Deming (1) and York (2). In these works, Equation 1 has been reduced to an expression involving only y-residuals by intelligently choosing the weighting factors.

$$S_w = \sum W_i (Y_i - \hat{Y}_i)^2 = \sum W_i (Y_i - a - bX_i)^2 \quad (2)$$

$$W_i = \frac{1}{s^2_{Y_i} + b^2 s^2_{X_i}} \quad (3a)$$

$$= \frac{w_{Y_i} w_{X_i}}{b^2 w_{Y_i} + w_{X_i}} \quad (3b)$$

All subsequent workers have started with this preceding set of expressions. When Equation 2 is minimized with respect to a and b, as is done in the nonweighted and y-variance weighted cases, difficulties quickly arise due to the dependence of the weights, W_i, on the slope, b. Because of these difficulties, workers have either avoided the problem altogether or devised various ways to circumvent it. These various approaches are investigated in the discussion below.

Effective Variance Approach

This approach, courtesy of Irvin and Quickenden (3) among others, uses the expressions for intercept and slope from the y-variance weighted case (i.e., Equation 15 in Part I). The W_i from Equation 3 of this paper is substituted for the previously used w_i in Equation 15 of Part I. The result is the following intercept and slope expressions (subscript i's omitted throughout).

$$a = \frac{\sum WY - b\sum WX}{\sum W} \quad (4)$$

$$b = \frac{\sum WXY - \sum WX \ \sum WY / \ \sum W}{\sum WX^2 - (\sum WX)^2 / \ \sum W} \quad (5)$$

This approach relies on an initial estimate for b (often obtained by first applying nonweighted regression) to calculate the weights of Equation 3. The W_i values are then used to calculate a new estimate of b via Equation 5, which is then substituted into Equation 3 for new W_i values. This iterative process is repeated until b converges to some chosen number of decimal places, after which a is calculated.

The problem with this approach is that it does not give an estimate for b that is invariant upon switching axes, i.e., the slope obtained when y is regressed against x does not equal the reciprocal of that obtained when x is regressed against y. This result gives the false impression that 2 different regressions exist for a single data set. Actually, this inconsistency is not surprising because the formula for b is not correctly derived from Equation 2. Because of Equation 3, Equation 2 requires that the derivation include the dependence of the W_i on b. It may be argued that the effective variance approach yields a simple approximation to a more rigorous solution. However, previous work in the literature (4) has shown that this is not the case. The effective variance method does not consistently give results any closer to results from more rigorous methods (which are described below) than the results from nonweighted and/or y-variance weighted regressions. Therefore, this method is not recommended for use.

Constant Variance Ratio Approach

Mandel (5), Waakers et al. (6), and Anderson (7) describe the constant variance ratio approach that requires that the ratio of the variances of Y_i and X_i be constant for all values of Y_i and X_i.

$$\lambda = \frac{V[Y_i]}{V[X_i]} = (\frac{s_{Y_i}}{s_{X_i}})^2 \quad (6)$$

It should be noted that λ is sometimes defined in the literature as the reciprocal of Equation 6. The main advantage of the constant variance ratio approach is 2-fold. First, no weighting is necessary beyond Equation 6. Second, no iteration is re-

quired for the expression of the slope. The concomitant equations for the slope and intercept are as follows:

$$b = \frac{S_{yy} - \lambda S_{xx} + [(S_{yy} - \lambda S_{xx})^2 + 4\lambda S_{xy}^2]^{1/2}}{2 S_{xy}} \quad (7)$$

$$a = \frac{\sum Y - b \sum X}{n} \quad (8)$$

The corrected sum of squares terms are equivalent to those of Equation 3 in Part I. In contrast to the effective variance method, this shortcut method does give an estimate of the slope that is invariant upon switching axes and is, therefore, recommended when Equation 6 for constant variance ratio can be satisfied.

This approach is potentially very useful for the comparison of 2 different analytical methods. For this application, the 2 methods will often each exhibit their own fairly constant variability over some defined range of analyte concentration or amount and, therefore, fulfill Equation 6. Another frequent case that will satisfy Equation 6 is when the variabilities of the 2 methods both change in the same way over the defined range (i.e., the variabilities change in a constant proportion). For some other cases, a constant $\lambda = 1$ may apply. The application envisioned is the comparison of 2 sets of samples when the same measurement method is used.

If this or other approaches of investigating possible differences between 2 analytical methods are used, it is necessary to show statistically whether or not the obtained slope is significantly different from one. To accomplish this, the uncertainty in the obtained slope value must be estimated. Creasy (8) and Anderson (7) give the following expression for a confidence interval (CI) about b:

$$CI = \lambda^{1/2} \tan \left[\arctan \left(\frac{b}{\lambda^{1/2}}\right) \pm \frac{\arcsin R}{2}\right] \quad (9)$$

$$R^2 = \frac{4t^2 \lambda (S_{xx} S_{yy} - S_{xy}^2)}{[(S_{yy} - \lambda S_{xx})^2 + 4\lambda S_{xy}^2] (n-2)} \quad (10)$$

Equation 9 yields upper and lower confidence limits for the slope. The symbols are all as defined above. For this expression, t is a 1-tailed value (t_ν^α), and all trigonometric arguments are in radians. No similar expression exists to date for an uncertainty interval about the intercept value, although Creasy's paper may supply a starting point for derivation of such an interval.

To illustrate the practical use of this method, the data in Table 1 will be used. This table shows results for 2 laboratories from an interlaboratory comparison study that actually included a total of 5 laboratories. For this example, 2 of these laboratories, which exhibit roughly comparable precisions, have been chosen (Laboratory 1 and Laboratory 4 in Table 1).

Table 1. Interlaboratory comparison data for Laboratories 1 and 4

Laboratory 1 value	SD	Laboratory 4 value	SD
16.7	8.9	24.6	14.0
9.6	8.9	16.4	8.0
177.3	22.4	181.7	3.6
130.8	16.4	145.8	11.7
136.6	18.3	123.7	15.6
111.5	15.4	100.5	10.2
77.6	10.5	78.5	6.6
75.7	11.7	59.3	21.6
31.2	4.4	36.2	5.8
138.9	19.0	130.0	7.7
131.0	18.8	121.9	26.2
89.7	12.5	83.5	14.8
37.5	7.5	42.9	9.7
33.0	6.2	42.3	15.5
23.4	6.5	28.3	9.9
93.8	21.2	98.9	7.8
34.7	8.3	57.9	17.3
45.8	8.0	47.1	13.3
82.7	9.2	110.0	19.4
110.3	12.3	113.6	12.5
93.8	11.8	108.3	16.9
81.5	9.9	95.5	24.4
91.5	9.9	112.6	48.7
59.9	9.7	95.0	28.6
19.1	4.9	30.6	10.4

The standard deviation values from each laboratory have been averaged. This results in an average standard deviation of 11.7 for Laboratory 1 and 15.2 for Laboratory 4. If Laboratory 1 is chosen to represent x and Laboratory 4 chosen to represent y, then $\lambda = (15.2/11.7)^2 = 1.688$. The corrected sum of squares terms may be acquired either from a special computer program for this method or from a classical least squares program. The results are $S_{xx} = 49594.5416$, $S_{yy} = 44640.7896$, and $S_{xy} = 45170.1356$. Putting these values into Equations 7 and 8 gives b = 0.9368 and a = 10.9479. To calculate a 95% confidence interval for the slope, the above values are used along with a 1-tailed *t*-value of 1.714; upper and lower limits are 1.0383 and 0.8423, respectively. Because the interval includes a slope of 1, one can conclude with a confidence level of 95% that Laboratories 1 and 4 gave equivalent results for the samples analyzed.

York Approach

Prior treatments of a completely general approach to the problem of error in x as well as in y have been presented by York (2), McIntyre et al. (9), Williamson (10), and, most recently, Thompson (11). In this section, York's approach will be followed for the most part. The McIntyre et al. approach is very similar to York's but contains some errors in its derivations. In the next section, Williamson's approach will be more closely examined.

By using Equation 3 and minimizing Equation 2 with respect to the parameters a and b, York (2) obtained a "least squares cubic" expression for the slope.

$$b^3 \sum W^2 s_X^2 U^2 - 2b^2 \sum W^2 s_X^2 UV \qquad (11)$$

$$- b\ [\ \sum WU^2 - \sum W^2 s_X^2 V^2\ ] + \sum WUV = 0$$

In this expression, the subscript i's have been omitted for W, s_X, U and V. Also, $U_i = X_i - \bar{X}_W$ and $V_i = Y_i - \bar{Y}_W$, where the weighted averages are $\bar{X}_W = \Sigma WX / \Sigma W$ and $\bar{Y}_W = \Sigma WY / \Sigma W$. Equation 11 is not, of course, a true cubic equation in b, because the W_i terms (and indirectly the U_i and V_i terms) include a further dependence on b. York's approach to solving Equation 11 involves treating it as a cubic equation by using an initial estimate of b (via nonweighted regression or "best guess") in Equation 3 to calculate initial estimates of the W_i values. The "cubic" is then solved using these W_i values. The resultant new estimate of b is again used to calculate new W_i values, which are again used in Equation 11. As in the effective variance method, this iterative process is repeated until b converges. The last set of W_i values is then used with the final b to estimate the intercept.

$$a = \bar{Y}_W - b\bar{X}_W = \frac{\sum WY - b\sum WX}{\sum W} \qquad (12)$$

York's method for solving Equation 11 involves first rewriting it.

$$b^3 - 3\alpha b^2 + 3\beta b - \gamma = 0 \qquad (13)$$

$$\alpha = \frac{2\sum W^2 s_X^2 UV}{3\sum W^2 s_X^2 U^2} \qquad (14)$$

$$\beta = \frac{\sum W^2 s_X^2 V^2 - \sum WU^2}{3\sum W^2 s_X^2 U^2} \qquad (15)$$

$$\gamma = \frac{-\sum WUV}{\sum W^2 s_X^2 U^2} \qquad (16)$$

York then gives 3 real roots for Equation 13.

$$b_{j+1} = \alpha + 2\,(\alpha^2 - \beta)^{1/2} \cos\left(\frac{\phi + 2\pi j}{3}\right),\ j = 0, 1, 2 \qquad (17)$$

$$\phi = \arccos\left[\frac{\alpha^3 - \frac{3}{2}\alpha\beta + \frac{1}{2}\gamma}{(\alpha^2 - \beta)^{3/2}}\right] \qquad (18)$$

Again, all trigonometric arguments are in radians. York states that the required root is always b_3 (j = 2), and our experience agrees with this. As with the constant variance ratio method, York's method yields a slope estimate that is invariant upon switching axes.

The problem with York's method is, as also noted by Williamson (10), that the iteration process is somewhat slow to converge to a solution, and in many instances completely fails to converge. This will be illustrated by using the data in Table 2. These data are from the same interlaboratory comparison study used in the example for the constant variance ratio. Here, data from 2 laboratories with noncomparable precisions have been chosen (Laboratory 3 and Laboratory 5 in Table 2). Results from applying York's method to this data starting with different initial estimates of b (b_o) are summarized in Table 3. Ten iterations were used for each successful result. From Table 3, we can see that York's method fails to converge for a range of b_o values roughly centered about 0 and extending close to the target final slope value. Also note that even when York's method does converge, the final slope value is slightly different for different b_o values. This latter result is due to the method's slowness to converge. For example, a b_o value of 5.5 requires 14 iterations to converge to the target slope value of 5.926, while $b_o = 10$ requires 17 iterations to converge to the target slope.

Our results for other data sets verify that York's method very frequently fails to converge for b_o values close to 0. As the above example illustrated, it sometimes fails for a fairly large range of b_o values that extend close to the target slope. For a few data sets, York's method fails to converge no matter what value for b_o is chosen. If York's method is to be used, then b_o values that are sure to exceed the final slope value should be chosen. Also, a large number of iterations should be employed, e.g., 20 or more.

An alternative to York's method for solving the least squares cubic equation is the Newton-Raphson method. This method is itself an iterative procedure for solving polynomials and is described in many mathematical textbooks. The further complication here is that nested iterations are required. Also, Newton-Raphson's method will find 3 different roots for a cubic equation, depending on where the search is started. In summary, although York's approach is completely general and invariant upon switching axes, problems do exist with its practical use.

For the variances of the parameters, a certain amount of confusion and/or disagreement also exists. Let us first examine York's original expressions (2).

$$V[b] = \frac{\sum W_i (bU_i - V_i)^2}{(n-2)\sum W_i U_i^2} \qquad (19)$$

$$V[a] = V[b]\,\frac{\sum W_i X_i^2}{\sum W_i} \qquad (20)$$

Table 2. Interlaboratory comparison data for Laboratories 3 and 5

Laboratory 3 value	SD	Laboratory 5 value	SD
44.3	22.1	36.9	7.4
27.7	11.1	24.6	4.9
181.5	10.0	781.3	156.3
147.2	5.5	626.9	125.4
101.8	13.3	531.9	106.4
102.9	5.5	432.2	86.4
70.8	4.4	172.7	34.5
28.8	8.9	130.5	26.1
44.3	6.6	54.3	10.9
120.1	23.8	559.0	111.8
86.3	8.9	524.2	104.8
63.1	5.5	183.7	36.7
56.5	6.6	64.4	12.9
64.2	5.5	63.5	12.7
42.1	5.5	42.5	8.5
93.0	7.7	217.6	43.5
63.1	4.4	127.4	25.5
31.6	11.1	70.7	14.1
125.1	7.7	473.0	94.6
100.2	24.9	488.5	97.7
99.1	23.8	465.7	93.1
75.3	4.4	210.1	42.0
66.4	6.6	484.2	96.8
95.2	11.1	209.0	41.8
44.3	7.7	45.9	9.2

On first inspection, these appear to be newly derived for this regression approach. Further investigation, however, reveals that these expressions are equivalent to those from the y-variance weighted regression case. This becomes apparent if w_i is substituted for W_i in the numerator of $V\,[b]$ in Equation 19.

$$\sum w_i\,(bU_i - V_i)^2 = b^2 \sum w_i\,(X_i - \bar{X}_w)^2 \qquad (21)$$

$$+ \sum w_i\,(Y_i - \bar{Y}_w)^2 - 2b \sum w_i\,(X_i - \bar{X}_w)\,(Y_i - \bar{Y}_w)$$

$$= b^2\,S_{wxx} + S_{wyy} - 2b\,S_{wxy} \qquad (22)$$

$$= S_{wyy} - b\,S_{wxy} \qquad (23)$$

The result of Equation 21 shows that the numerator of the expression for the variance of the slope in Equation 19 is equivalent to the numerator of s^2_{wyx}, as shown in Equation 17 of the Part I paper. The denominator of s^2_{wyx}, as shown in Part I, is $n - 2$. This latter term is included in the denominator of $V\,[b]$ in the above Equation 19. The expression for the slope variance of the y-variance weighted regression case was formed by dividing s^2_{wyx} by S_{wxx} (Equation 19 in Part I). The denominator ΣWU^2 in Equation 19 above can be shown to be equivalent to S_{wxx} in Part I by a process similar to that used in Equations 21–23. This line of reasoning confirms that York's slope variance

Table 3. Nonconvergence problem with York's method: Each final slope value was acquired with 10 iterations of York's least squares cubic equation using York's solution method

b_o	Final b
−10	5.932
−6	5.927
−5	Failed
−1	Failed
0	Failed
1	Failed
5	Failed
5.1	Failed
5.3	Failed
5.4	Failed
5.5	5.924
5.7	5.925
5.9	5.926
6	5.927
7	5.929
10	5.932

is equivalent to that from the y-variance weighted regression case. A similar analysis shows that York's expression for the variance of the intercept is also equivalent to the expression from y-variance weighted regression.

Recently, Thompson (11) gave similar expressions that look more familiar.

$$V\,[b] = \frac{1}{S_{wxx}} \qquad (24)$$

$$V\,[a] = \frac{\sum w_i X_i^{\,2}}{\sum w_i S_{wxx}} \qquad (25)$$

Note that if these expressions are multiplied by s^2_{wyx}, the results are again exactly equivalent to the expressions of Equation 19 in Part I. In fact, Williamson (10) stated that it is common practice to include the s^2_{wyx} factor [which he refers to as S/(n − 2): see Equations 16 and 17 of the Part I paper] *only* when S/(n − 2) exceeds unity. Williamson also gives his own expressions for slope and intercept variances, which will be discussed below.

After the appearance of Williamson's paper, York published another paper (12) that focused on adding the effects of correlated errors into his approach. When York discussed the slope and intercept variances, he claimed to use "the usual method of partial differentiation" and then gave "reasonable approximate values" that did not include the S/(n − 2) factor this time.

$$V\,[b] = \frac{1}{\sum Z_i\,U_i^{\,2}} \rightarrow \frac{1}{S_{wxx}} \qquad (26)$$

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Table 4. Comparison of York and Williamson methods in fulfilling invariancy requirements: Each set of results was acquired using 20 iterations of the respective methods

	x = Laboratory 3 y = Laboratory 5	x = Laboratory 5 y = Laboratory 3
	York	
b_o	6	0.2
final b	5.926344	0.168738
1/b	0.168738	5.926343
s_b	0.716915	0.021064
$V[b]/b^2$	0.014634	0.015583
	Williamson	
b_o	6	0.2
final b	5.926343	0.168738
1/b	0.168738	5.926343
s_b	0.638652	0.018184
$V[b]/b^2$	0.011613	0.011613

In this expression, the Z_i values represent weighting factors that include correlation effects. In summary, there is both agreement and confusion concerning the proper form for these approximate variance expressions.

Williamson Approach

Williamson (10) started with the same expressions as York for the weighted sum of squares and the weighting factors (i.e., Equations 2 and 3). However, instead of using a "cubic" equation he created a "linear" equation for an exact solution.

$$b\sum W_i z_i U_i = \sum W_i z_i V_i \qquad (27)$$

$$z_i = W_i (s_{Y_i}^2 U_i + b\, s_{X_i}^2 V_i) \qquad (28)$$

Williamson reasoned that because the denominators of the W_i include b, the equation that York wrote as a cubic could be construed as a polynomial of any chosen degree in b. By writing it in the form of a linear equation, its iteration and solution are much easier. Also, as stated by Williamson and confirmed by our experience, the linear equation converges more rapidly and much more often than York's system of equations. For example, again using the data in Table 2, Williamson's method converges to a final slope value of 5.926 in 3 iterations for $b_o = 5.5$ and in 5 iterations for $b_o = 10$. Williamson's method works fine with b_o at or around 0.

The linear equation is invariant upon switching axes as well. Williamson's method has not failed to converge to date in our tests, although Kalantar has indicated that it can fail under certain extremely unrealistic conditions (13). Williamson's choice of expression for the intercept agrees completely with York. Thus, the conclusion is that York should be commended for starting the hunt for the solution to the oft-neglected problem of handling x-errors when regressing data. However, Williamson's method is preferable and is recommended for actual application.

In addition to his work on the expression for b, Williamson also gives expressions for the variances of the slope and intercept.

$$V[b] = Q^2 \sum W_i^2 (s_{Y_i}^2 U_i^2 + s_{X_i}^2 V_i^2) \qquad (29)$$

$$V[a] = \frac{1}{\sum W_i} + 2(\bar{X}_W + 2\bar{z})\,\bar{z}Q + (\bar{X}_W + 2\bar{z})^2 V[b] \qquad (30)$$

$$Q^{-1} = \sum W_i \left[\frac{U_i V_i}{b} + 4 z_i' (z_i - U_i)\right] \qquad (31)$$

$$\bar{z} = \frac{\sum W_i z_i}{\sum W_i} \text{ and } z_i' = z_i - \bar{z} \qquad (32)$$

These expressions are much more complex than either York's or Thompson's variance expressions, but are more in line with what one would expect from differentiating the complex slope expressions of this case.

Williamson, at this point, has another invariancy requirement in addition to the usual one concerning the slope. He stated that the variance of the slope should also be invariant upon switching the axes. By this he meant that the value of the quantity $V[b]/b^2$ should be constant when x and y designations are exchanged. Williamson showed data to support the contention that his expression for the slope variance is completely consistent with this idea, although York's expression is not.

Our own experience, as illustrated in Table 4, agrees with Williamson. The results in this table are again based on the data in Table 2. Twenty iterations were used to obtain each set of results in Table 4. The results show that for Williamson's method, $V[b]/b^2$ is the same whether Laboratory 3 or Laboratory 5 is assigned to x. For York's method, $V[b]/b^2$ changes when the axes are switched.

Conclusion

This paper demonstrates that incorporating errors of the dependent variable into the least squares linear regression model greatly complicates the derivation of expressions for the slope and intercept. This problem has caused statisticians and chemists to develop several approaches for its amelioration. A number of these approaches were examined in this paper for their relative theoretical and practical merits.

The effective variance approach is not rigorously derived and, not surprisingly, gives poor results. The obtained slope is

not invariant upon switching the variables of the x- and y-axes. Also, previous work in the literature showed that the obtained slope is not consistently any closer to the result from more rigorous approaches than slopes obtained from nonweighted or y-variance weighted regression. The effective variance method is, therefore, not recommended.

The constant variance ratio approach works well if the constant λ criterion can be met. This method does give a slope that is invariant upon switching axes and provides a computationally simple method for calculating slopes and intercepts. The constant λ criterion is well-matched with the scenario of comparing analytical methods. A useful expression for a confidence interval about the slope has been presented in the literature. However, no similar expression for a confidence interval about the intercept has yet appeared. On balance, the constant variance ratio approach is sound, yet simple, and its use is thus strongly recommended when applicable.

York provided a completely general and rigorous approach based on the work of Deming. The obtained slope is invariant upon switching axes. However, the iterative solution method is slow to converge and often fails to do so. York provided expressions for variances of both the slope and intercept. These expressions were shown to be equivalent to those of the y-variance weighted regression approach discussed in the Part I paper. Rigorous expressions for the slope and intercept in the x- and y-variance weighted case are much more complex than the expressions in the y-variance weighted case. Consequently, it is logical that rigorous expressions for the slope and intercept variances should also be more complex in the x- and y-variance weighted case than in the y-variance weighted case. York's variance expressions do not follow this logic and are, therefore, suspect. Furthermore, Williamson showed that York's slope variance expression does not meet his invariancy criterion. In summary, York's work broke ground for other researchers, but his methods are not generally useful in practice.

Williamson's approach is the most generally useful of those surveyed in our study. It is invariant upon switching axes. Its iterative solution method rapidly converges and has never failed to converge in the our testing. Also, Williamson provided expressions for variances of the slope and intercept. These expressions are much more complex than York's and, therefore, more in-line with what is expected for the x- and y-variance weighted case. The slope variance expression does meet Williamson's own invariancy criterion. In summary, Williamson's approach is highly recommended based on both its theoretical and practical merits.

A more recent paper by Thompson (11) espoused an approach that appears similar to the approaches of York and Williamson. However, Thompson uses a different minimization procedure to obtain slope and intercept values. His expressions for slope and intercept variances are very similar to York's (and to those from the y-variance weighted case) and, therefore, fall under the same suspicion as do York's. At the present time, we have not examined Thompson's approach sufficiently to judge its potential merits.

A problem with implementing any of the approaches discussed is the lack of available software. User-friendly packages for performing these statistical procedures that have been thoroughly tested *and* documented are needed.

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CHEMICAL CONTAMINANTS MONITORING

Trace Metals in Edible Tissues of Livestock and Poultry

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Data from a random-sampling study are presented for trace metals in edible tissues of livestock (bovine including bull, steer, cow, heifer, calf; ovine including mature sheep and lambs; porcine including market hogs, boar/stag, and sow) and poultry (including young and mature chicken, young turkey, and duck). Tissue homogenates were ashed, and residual materials were dissolved in hydrochloric acid for analysis by atomic absorption spectroscopy. Statistical summaries of data are provided for the trace metals lead, cadmium, cobalt, copper, iron, manganese, nickel, and zinc. The heavy metals of toxicological concern, lead and cadmium, are emphasized in this study. Lead and cadmium were rarely detected in muscle (0.2–0.5% positive among 2314 animals sampled). Lead was also infrequently detected in liver (1.8% positive) and kidney (2.4% positive). Nearly 46% of livers analyzed were positive for cadmium, and approximately 78% of kidney samples were positive for cadmium. No regulatory limits are established in the United States for the trace metals reported in this study, although restrictions on the use of kidneys from mature poultry as human food have been established because of concern about potential cadmium levels. Kidneys from this study, more frequently than livers, bore cadmium levels that exceeded the regulatory limits of other countries or organizations. Regulatory implications of the data are discussed.

Metals in biological systems include environmental contaminants and essential cofactors of enzyme systems; many of these cofactors have limited toxicity to plants and animals (1–3). Either class of metals could be acutely toxic at certain exposure concentrations (4, 5), and the heavy metals lead and cadmium can cause long-term irreversible health effects (6, 7). The concentrations of trace metals in meats are of concern to the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA), which is responsible for ensuring that the U.S. meat supply in commerce is wholesome and unadulterated under authority of the Federal Meat Inspection Act, the Federal Poultry Products Inspection Act, and the Federal Food, Drug, and Cosmetic Act (FFDCA). These statutes grant FSIS the authority to collect samples of edible tissues from animals presented for slaughter and to test for compliance. However, authority is granted by FFDCA to the U.S. Food and Drug Administration (FDA) to set limits for residues of animal drugs and environmental contaminants in edible tissues of livestock and poultry and to conduct follow-up (on-farm) investigations whenever residue problems are identified by FSIS. The general term "regulatory limits" in the context of this paper includes various specific terms for limits such as "tolerance" and "action level," which may trigger different regulatory actions. No regulatory limits are currently established in the United States for metals other than arsenic in animal tissues. The nonessential metal arsenic has been continuously monitored by FSIS since the 1970s, and these data will be presented in a future publication.

Monitoring data are presented for trace metals in tissues of randomly sampled healthy animals, with the regulatory focus on the nonessential metals cadmium and lead. Data for the essential metals cobalt, copper, iron, manganese, nickel, and zinc are presented as normal baseline levels. Concern for toxicity of essential metals to humans is more often directed at industrial contamination (mines, smelters, refineries) or supplement misuse, rather than normal dietary exposure (2).

To address growing concern about possible human dietary exposure to excessive concentrations of heavy metals, FSIS conducted a special exploratory program to determine the occurrence and concentration of trace metals in tissues of healthy livestock and poultry randomly selected from those presented for slaughter in 1985–1986. In addition, suspect animals were tested as necessary under inspector-initiated (targeted) sampling.

METHOD

Muscle, liver, and kidney specimens were collected at slaughter from 2314 animals from 14 production classes (17–328 animals per class) in 1985 or 1986 by FSIS inspectors using a random national sampling plan in which each produc-

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Table 1. Lowest reliable quantitations and recovery data from representative samples fortified with trace metals

Metal	Lowest reliable quantitation, ppm	Standard fortification, ppm[a]	Mean rec., %[b]
Cadmium	0.025	3.33	95.1 ± 3.1
Cobalt	0.15	0.67	102.4 ± 3.1
Copper	0.09	1.33	101.1 ± 8.3
Iron	0.12	32.00	98.2 ± 2.6
Lead	0.5	3.33	90.7 ± 3.8
Manganese	0.055	1.33	105.9 ± 2.4
Nickel	0.15	0.67	101.0 ± 5.5
Zinc	0.018	32.00	101.4 ± 2.5

[a] A blank tissue specimen was fortified with trace metals as a positive control for each analysis.
[b] The mean ± 1 standard deviation was determined for 100 of the fortified blank specimens analyzed in 1985–1986.

tion class was sampled for 1 calendar year. Poultry tissue samples were combined from 6 birds to provide an adequate quantity of pooled tissue for analysis. Tissue specimens were frozen and shipped to an FSIS laboratory for analysis by FSIS Method No. 5.010 (8). Because of occasional inadvertent accidents in specimen handling, the actual specimen numbers for muscle, liver, and kidney were not equal for all production classes.

In the laboratory, each tissue specimen was chopped, homogenized, and stored frozen until analysis. One sample each of tissue blank and fortified tissue blank was included with each specimen set as negative and positive controls. The homogenate was mixed with magnesium nitrate solution (6.67%) and dried ca 6 h at 90–95°C. The dry sample was completely ashed in a muffle furnace at 500–550°C. A second treatment in the muffle furnace removed any traces of organic residue in the ash. The ash was dissolved in hydrochloric acid for analysis by atomic absorption (AA). Results are expressed in parts per million based on fresh weight of tissue. Method validation data, including the lowest reliable quantitations (LRQs) for each analyte and recovery data from representative fortified tissue blanks, are presented in Table 1. Results were summarized by using Statistical Analysis System (SAS) software to determine mean, standard deviation, and range of positive specimens (those exceeding the limit of quantification) and percentages of all specimens for each production class. Data were identified and summarized by SAS for specimens that contained metal concentrations that exceeded certain regulatory limits for cadmium and lead proposed by the Codex Committee on Food Additives and Contaminants or by other countries (Table 2).

Results and Discussion

For all specimens analyzed, lead and cadmium, respectively, were detected in 0.2 and 0.5% of muscle, 1.8 and 45.9% of liver, and 2.4 and 78.2% of kidney. Lead and cadmium, respectively, were detected in muscle for each production class at 0–1.3 and 0–1.8%, in liver at 0–14.7 and 9.3–95%, and in kidney at 0–14.7 and 27.9–100%. Cobalt and nickel, respec-

Table 2. Regulatory limits for lead and cadmium

Country or organization (reference)	Limits (ppm) in edible tissues[a]			Production class
	Muscle	Liver	Kidney	
		Lead		
Canada (10–12)	NA[b]	2.0	2.0	All species[c]
New Zealand (17)	2.0	NA	NA	All species
The Netherlands (14–16)	0.05	1.0	1.5	Cattle
	0.5	0.4	1.0	Swine
	0.05	NA	NA	Poultry
		Cadmium		
Codex (9)	NA	2.0	2.0	All species
Australia (13)	0.2	1.25	2.5	All species
Canada (10–12)	NA	1.0	1.0	All species
The Netherlands (14–16)	0.05	1.0	3.0	Cattle, swine
	0.05	NA	NA	Poultry

[a] The general term "regulatory limits" includes the specific terms tolerance, action level, maximum residue limit, provisional limit, and others.
[b] NA reflects a tissue for which no limit has been established.
[c] All species reflects edible tissues of all livestock and poultry.

Table 3. Trace metals in muscle, 1985–1986 study

Trace metal	Production class	No. specimens	Positive specimens[a]					P95[b]
			No.	Percent	Range	Mean	SD	
Lead	Calf	327	1	0.3	0.62–0.62	0.62	—	—
	Heifer/steer	285	1	0.4	0.52–0.52	0.52	—	—
	Bull/cow	93	1	1.1	0.58–0.58	0.58	—	—
	Lamb	163	1	0.6	0.77–0.77	0.77	—	—
	Mature sheep	34	0	0.0	—	—	—	—
	Market hog	318	1	0.3	1.2–1.2	1.2	—	—
	Boar/sow	279	0	0.0	—	—	—	—
	Young chicken	311	0	0.0	—	—	—	—
	Mature chicken	306	0	0.0	—	—	—	—
	Young turkey	60	0	0.0	—	—	—	—
	Duck	100	0	0.0	—	—	—	—
Cadmium	Calf	327	0	0.0	—	—	—	—
	Heifer/steer	289	1	0.3	0.13–0.13	0.13	—	—
	Bull/cow	95	1	1.1	0.12–0.12	0.12	—	—
	Lamb	165	3	1.8	0.11–0.27	0.20	0.08	—
	Mature sheep	34	0	0.0	—	—	—	—
	Market hog	326	2	0.6	0.11–0.32	0.21	0.15	—
	Boar/sow	281	1	0.4	0.13–0.13	0.13	—	—
	Young chicken	312	0	0.0	—	—	—	—
	Mature chicken	308	3	1.0	0.10–0.14	0.12	0.02	—
	Young turkey	61	1	1.6	0.28–0.28	0.28	—	—
	Duck	99	0	0.0	—	—	—	—
Cobalt	Calf	327	5	1.5	0.20–0.32	0.23	0.05	—
	Heifer/steer	287	4	1.4	0.21–7.0	1.92	3.39	—
	Bull/cow	95	2	2.1	0.21–0.21	0.21	0.00	—
	Lamb	165	1	0.6	0.21–0.21	0.21	—	—
	Mature sheep	34	2	5.9	0.21–0.22	0.21	0.01	0.21
	Market hog	324	10	3.1	0.20–0.25	0.21	0.02	—
	Boar/sow	280	2	0.7	0.20–0.20	0.20	0.00	—
	Young chicken	311	9	2.9	0.20–0.23	0.21	0.01	—
	Mature chicken	308	3	1.0	0.17–0.22	0.20	0.03	—
	Young turkey	61	2	3.3	0.20–0.20	0.20	0.00	—
	Duck	99	4	4.0	0.20–0.22	0.21	0.01	—
Zinc	Calf	327	327	100.0	13.0–69.0	32.8	10.5	54.0
	Heifer/steer	289	289	100.0	1.6–84.0	41.7	7.95	51.0
	Bull/cow	95	95	100.0	24.0–90.0	49.0	10.7	71.0
	Lamb	165	165	100.0	17.0–168.0	34.1	13.4	49.0
	Mature sheep	34	34	100.0	18.0–55.0	33.0	8.60	49.0
	Market hog	326	324	99.4	6.5–58.0	24.0	9.62	44.0
	Boar/sow	281	281	100.0	5.0–340.0	33.8	22.0	59.0
	Young chicken	312	312	100.0	0.64–31.0	9.21	5.17	18.0
	Mature chicken	308	308	100.0	0.76–30.0	11.0	6.24	23.0
	Young turkey	61	61	100.0	6.9–36.0	21.9	8.55	33.0
	Duck	99	99	100.0	9.9–35.0	21.3	7.55	34.0
Copper	Calf	327	327	100.0	0.40–78.0	1.56	4.32	2.3
	Heifer/steer	289	289	100.0	0.22–28.0	1.77	1.59	2.1
	Bull/cow	95	95	100.0	0.47–3.1	1.41	0.45	1.9
	Lamb	165	165	100.0	0.47–5.6	1.47	0.72	2.8
	Mature sheep	34	34	100.0	0.57–31.0	2.32	5.15	6.2
	Market hog	326	326	100.0	0.37–69.0	1.16	4.17	1.3
	Boar/sow	281	281	100.0	0.42–11.0	0.93	0.71	1.4
	Young chicken	312	311	99.7	0.12–6.6	0.44	0.40	0.66

Table 3. ***Continued***

Trace metal	Production class	No. specimens	Positive specimens[a]					P95[b]
			No.	Percent	Range	Mean	SD	
	Mature chicken	308	308	100.0	0.23–2.5	0.67	0.32	1.2
	Young turkey	61	61	100.0	0.28–1.6	0.83	0.33	1.4
	Duck	99	99	100.0	0.99–6.7	3.03	1.36	6.0
Iron	Calf	327	327	100.0	0.80–50.0	14.3	7.30	32
	Heifer/steer	289	289	100.0	4.4–78.0	35.1	6.69	42
	Bull/cow	95	95	100.0	16.0–56.0	35.4	7.82	46
	Lamb	165	165	100.0	9.0–56.0	20.5	7.37	30
	Mature sheep	34	34	100.0	7.0–79.0	25.9	11.3	34
	Market hog	326	324	99.4	0.13–86.0	10.2	5.29	15
	Boar/sow	281	281	100.0	7.4–183.0	16.5	11.2	25
	Young chicken	312	312	100.0	2.0–38.0	5.47	3.52	9.0
	Mature chicken	308	308	100.0	3.0–75.0	9.61	6.75	17
	Young turkey	61	61	100.0	3.1–19.0	9.81	4.02	16.0
	Duck	99	99	100.0	10.0–59.0	24.2	10.0	41
Maganese	Calf	327	325	99.4	0.06–5.0	0.19	0.29	0.30
	Heifer/steer	287	284	99.0	0.06–4.0	0.29	0.38	0.31
	Bull/cow	95	95	100.0	0.06–0.79	0.18	0.08	0.26
	Lamb	165	164	99.4	0.06–0.60	0.20	0.09	0.34
	Mature sheep	34	33	97.1	0.07–0.63	0.21	0.11	0.53
	Market hog	324	322	99.4	0.06–1.5	0.13	0.08	0.18
	Boar/sow	280	270	96.4	0.06–3.0	0.14	0.19	0.19
	Young chicken	311	304	97.7	0.06–0.28	0.13	0.03	0.18
	Mature chicken	308	308	100.0	0.06–4.2	0.19	0.26	0.25
	Young turkey	61	61	100.0	0.08–2.4	0.21	0.29	0.24
	Duck	99	99	100.0	0.10–6.7	0.30	0.69	0.31
Nickel	Calf	327	8	2.4	0.20–0.45	0.30	0.09	—
	Heifer/steer	289	6	2.1	0.20–0.60	0.27	0.16	—
	Bull/cow	95	4	4.2	0.20–40.0	10.2	19.9	—
	Lamb	165	2	1.2	0.20–0.26	0.23	0.04	—
	Mature sheep	34	1	2.9	0.25–0.25	0.25	—	—
	Market hog	326	16	4.9	0.20–8.0	0.82	1.95	—
	Boar/sow	281	10	3.6	0.20–0.38	0.24	0.05	—
	Young chicken	312	13	4.2	0.20–0.44	0.26	0.08	—
	Mature chicken	308	11	3.6	0.20–0.30	0.23	0.03	—
	Young turkey	61	4	6.6	0.20–0.29	0.23	0.04	0.20
	Duck	99	6	6.1	0.20–1.3	0.52	0.49	0.21

[a] Concn > LRQ.
[b] 95th percentile for ail specimens (only P95 > LRQ shown).

tively, were detected in muscle for each production class at 0–5.9 and 1.2–17.6%, in liver at 9.3–59.0 and 22.6–46.7%, and in kidney at 4.4–59.5 and 34.3–75.4%. Nearly all specimens were positive for the remaining essential metals in this study (copper, iron, manganese, and zinc) at 96.2–100% in muscle, 99.5–100% in liver, and 99.3–100% in kidney.

Summary statistics are presented for trace metal positives in muscle, liver, and kidney (Tables 3–5). Note that for the production classes in which only one positive specimen was detected, the minimum, maximum, and mean values are identical and the standard deviation and 95th percentile are not presented. Several samples of liver or kidney contained high levels of iron that were recorded as ">999" in the FSIS data base because of limitations of the field length.

The Codex Committee on Food Additives and Contaminants is considering a draft guideline level of 2 ppm for cadmium in liver, kidney, and offal products; the Committee has proposed no guideline at this time for lead or other trace metals in fresh meats (9). Canada (10–12), Australia (13), The Netherlands (14–16), and New Zealand (17) have established regulatory limits for lead and/or cadmium in livestock and poultry tissues. Agriculture Canada may initiate on-farm inspections and feed analysis to assess potential problems for animal and human health when action levels are exceeded (10–12). The

Table 4. Trace metals in liver, 1985–1986 study

Trace metal	Production class	No. specimens	Positive specimens[a]					P95[b]
			No.	Percent	Range	Mean	SD	
Lead	Calf	327	16	4.9	0.52–1.5	0.84	0.30	—
	Heifer/steer	285	1	0.4	1.0–1.0	1.00	—	—
	Bull/cow	93	0	0.0	—	—	—	—
	Lamb	162	1	0.6	0.71–0.71	0.71	—	—
	Mature sheep	34	5	14.7	0.54–0.75	0.61	0.08	0.60
	Market hog	318	10	3.1	0.50–1.8	0.81	0.38	—
	Boar/sow	280	4	1.4	0.54–0.91	0.70	0.17	—
	Young chicken	312	0	0.0	—	—	—	—
	Mature chicken	307	1	0.3	0.54–0.54	0.54	—	—
	Young turkey	59	0	0.0	—	—	—	—
	Duck	111	3	2.7	0.51–0.81	0.62	0.17	—
Cadmium	Calf	327	37	11.3	0.10–1.0	0.19	0.16	0.16
	Heifer/steer	289	105	36.3	0.10–17.0	0.30	1.65	0.19
	Bull/cow	95	85	89.5	0.10–0.91	0.24	0.14	0.49
	Lamb	164	38	23.2	0.10–0.28	0.14	0.05	0.17
	Mature sheep	34	28	82.4	0.10–0.55	0.24	0.15	0.54
	Market hog	326	97	29.8	0.10–0.56	0.14	0.06	0.19
	Boar/sow	282	205	72.7	0.10–1.4	0.21	0.14	0.39
	Young chicken	313	29	9.3	0.10–0.37	0.13	0.05	0.12
	Mature chicken	309	284	91.9	0.10–131.0	0.71	7.76	0.44
	Young turkey	60	57	95.0	0.11–0.73	0.27	0.12	0.45
	Duck	111	73	65.8	0.10–0.44	0.15	0.06	0.24
Cobalt	Calf	327	107	32.7	0.20–0.46	0.27	0.06	0.33
	Heifer/steer	287	91	31.7	0.20–2.4	0.25	0.23	0.25
	Bull/cow	95	54	56.8	0.20–28.0	1.15	4.76	0.30
	Lamb	164	40	24.4	0.20–0.27	0.22	0.02	0.23
	Mature sheep	34	12	35.3	0.20–0.36	0.24	0.04	0.25
	Market hog	324	58	17.9	0.20–0.28	0.22	0.02	0.22
	Boar/sow	281	82	29.2	0.20–0.41	0.22	0.03	0.23
	Young chicken	312	29	9.3	0.20–0.28	0.22	0.02	0.21
	Mature chicken	309	51	16.5	0.20–0.27	0.22	0.02	0.23
	Young turkey	60	16	26.7	0.20–0.30	0.22	0.02	0.22
	Duck	111	17	15.3	0.20–0.26	0.22	0.02	0.22
Zinc	Calf	327	327	100.0	2.7–378.0	103.0	72.9	250.0
	Heifer/steer	289	289	100.0	15.0–85.0	38.2	9.39	55.0
	Bull/cow	95	95	100.0	5.1–300.0	53.4	35.9	117.0
	Lamb	164	164	100.0	23.0–75.0	39.2	8.50	53.0
	Mature sheep	34	34	100.0	26.0–162.0	47.8	24.5	103.0
	Market hog	326	326	100.0	0.24–466.0	66.9	30.6	105.0
	Boar/sow	282	282	100.0	0.71–273.0	63.7	30.8	121.0
	Young chicken	313	313	100.0	2.7–83.0	30.6	6.58	39.0
	Mature chicken	309	309	100.0	4.8–167.0	50.5	16.5	80.0
	Young turkey	60	60	100.0	22.0–44.0	32.2	4.44	41.0
	Duck	111	111	100.0	24.0–87.0	58.0	11.6	75.0
Copper	Calf	327	327	100.0	1.7–525.0	138.0	112.0	397.0
	Heifer/steer	289	289	100.0	1.3–193.0	46.1	30.8	106.0
	Bull/cow	95	95	100.0	1.3–260.0	43.7	52.1	171.0
	Lamb	164	164	100.0	3.9–565.0	89.8	72.1	218.0
	Mature sheep	34	34	100.0	4.3–529.0	131.4	110.0	415.0
	Market hog	326	326	100.0	1.1–249.0	11.1	19.3	31.0
	Boar/sow	282	282	100.0	0.78–421.0	18.3	39.9	62.0
	Young chicken	313	313	100.0	0.40–19.0	4.42	1.29	5.5

Table 4. ***Continued***

Trace metal	Production class	No. specimens	Positive specimens[a]					P95[b]
			No.	Percent	Range	Mean	SD	
	Mature chicken	309	309	100.0	2.5–22.0	4.60	1.27	5.8
	Young turkey	60	60	100.0	4.1–41.0	7.14	5.04	14.0
	Duck	111	111	100.0	1.1–129.0	66.7	22.1	103.0
Iron	Calf	327	327	100.0	0.66– >999.0	68.1	108.0	246.0
	Heifer/steer	289	288	99.7	29.0–244.0	54.5	19.4	79.0
	Bull/cow	95	95	100.0	27.0–399.0	77.0	61.2	183.0
	Lamb	164	164	100.0	29.0–212.0	59.6	28.5	109.0
	Mature sheep	34	34	100.0	35.0–320.0	100.0	66.9	285.0
	Market hog	326	326	100.0	0.12–630.0	191.0	79.4	323.0
	Boar/sow	282	282	100.0	14.0– >999.0	363.0	183.0	700.0
	Young chicken	313	313	100.0	3.0–214.0	99.3	28.2	148.0
	Mature chicken	309	309	100.0	23.0–910.0	129.0	72.2	231.0
	Young turkey	60	60	100.0	90.0–218.0	137.0	33.0	212.0
	Duck	111	111	100.0	46.0–425.0	163.0	76.3	314.0
Maganese	Calf	327	326	99.7	0.20–3.7	1.93	0.63	3.1
	Heifer/steer	287	287	100.0	0.16–27.0	2.89	1.52	3.6
	Bull/cow	95	95	100.0	1.4–24.0	2.86	2.25	3.5
	Lamb	164	164	100.0	1.2–37.0	3.66	3.42	5.4
	Mature sheep	34	34	100.0	1.3–4.3	2.68	0.68	3.9
	Market hog	324	324	100.0	0.12–414.0	4.20	22.9	3.8
	Boar/sow	281	281	100.0	0.70–3.8	2.37	0.57	3.3
	Young chicken	312	311	99.7	1.7–414.0	4.15	23.3	3.5
	Mature chicken	309	309	100.0	0.06–41.0	3.43	2.22	4.4
	Young turkey	60	60	100.0	2.3–3.9	3.24	0.31	3.7
	Duck	111	111	100.0	0.50–600.0	10.4	56.5	6.5
Nickel	Calf	327	85	26.0	0.20–1.3	0.29	0.15	0.32
	Heifer/steer	289	77	26.6	0.20–0.57	0.27	0.09	0.30
	Bull/cow	95	28	29.5	0.20–0.46	0.23	0.05	0.25
	Lamb	164	43	26.2	0.20–0.44	0.25	0.06	0.29
	Mature sheep	34	9	26.5	0.20–0.32	0.24	0.04	0.27
	Market hog	326	127	39.0	0.20–239.0	2.14	21.2	0.33
	Boar/sow	282	86	30.5	0.20–0.57	0.26	0.07	0.28
	Young chicken	313	111	35.5	0.20–1.1	0.28	0.13	0.34
	Mature chicken	309	91	29.4	0.20–0.81	0.27	0.11	0.30
	Young turkey	60	28	46.7	0.20–0.42	0.26	0.05	0.32
	Duck	111	35	31.5	0.20–0.71	0.25	0.09	0.28

[a] Concn > LRQ.
[b] 95th percentile for all specimens (only P95 > LRQ shown).

Netherlands has established provisional legal limits and action levels that also trigger investigations of farms producing animals with tissues exceeding the regulatory limits for metals (14–16).

FSIS data are presented for lead and cadmium in liver and kidney exceeding the regulatory limits proposed by Codex and established by Canada (Table 6). Only 1 kidney specimen, which contained 2.2 ppm lead, among the 2314 livers and kidneys sampled from the animals in the FSIS study exceeded the 2 ppm Canadian action level for lead in liver and kidney. Four liver specimens exceeded the 2 ppm Codex limit for cadmium, and 6 liver specimens exceeded the 1 ppm Canadian action level. Kidneys, more frequently than livers, contained cadmium levels that exceeded Codex or Canadian limits. Fifty-five kidney specimens exceeded the 2 ppm Codex limit for cadmium, and 215 kidney specimens exceeded the 1 ppm Canadian action level. Only 1 specimen pooled from young chicken kidneys contained cadmium at a level exceeding the limit. This level (414 ppm) far exceeds the maximum level in mature chicken specimens (3.6 ppm). Lead and cadmium are

Table 5. Trace metals in kidney, 1985–1986 study

Trace metal	Production class	No. specimens	Positive specimens[a]					P95[b]
			No.	Percent	Range	Mean	SD	
Lead	Calf	328	13	4.0	0.53–1.7	0.87	0.43	—
	Heifer/steer	283	5	1.8	0.50–0.76	0.58	0.10	—
	Bull/cow	93	2	2.2	0.69–0.72	0.70	0.02	—
	Lamb	160	6	3.8	0.54–0.77	0.64	0.09	—
	Mature sheep	34	5	14.7	0.50–0.88	0.60	0.16	0.60
	Market hog	312	5	1.6	0.51–1.7	0.97	0.54	—
	Boar/sow	279	6	2.2	0.51–0.77	0.62	0.13	—
	Young chicken	311	5	1.6	0.50–2.2	0.93	0.72	—
	Mature chicken	304	5	1.6	0.50–0.94	0.63	0.19	—
	Young turkey	60	0	0.0	—	—	—	—
	Duck	110	7	6.4	0.52–0.91	0.66	0.15	0.53
Cadmium	Calf	328	140	42.7	0.10–8.1	0.36	0.76	0.42
	Heifer/steer	288	281	97.6	0.10–9.6	0.38	0.62	0.84
	Bull/cow	95	95	100.0	0.13–32.0	1.52	3.37	3.8
	Lamb	162	85	52.5	0.10–0.61	0.18	0.10	0.28
	Mature sheep	34	30	88.2	0.12–3.4	0.83	0.85	3.0
	Market hog	321	301	93.8	0.10–1.9	0.30	0.24	0.76
	Boar/sow	281	277	98.6	0.10–4.4	0.65	0.56	1.5
	Young chicken	312	87	27.9	0.10–414.0	4.92	44.4	0.19
	Mature chicken	306	303	99.0	0.10–3.6	1.03	0.70	2.5
	Young turkey	61	61	100.0	0.13–1.5	0.56	0.28	0.97
	Duck	111	109	98.2	0.11–1.1	0.25	0.14	0.45
Cobalt	Calf	328	70	21.3	0.20–0.49	0.28	0.06	0.34
	Heifer/steer	286	31	10.8	0.20–1.3	0.25	0.20	0.21
	Bull/cow	95	9	9.5	0.20–0.28	0.22	0.03	0.21
	Lamb	162	25	15.4	0.20–0.26	0.22	0.02	0.22
	Mature sheep	34	7	20.6	0.20–0.27	0.23	0.03	0.26
	Market hog	318	34	10.7	0.19–0.29	0.23	0.02	0.23
	Boar/sow	280	15	5.4	0.20–0.36	0.22	0.04	0.20
	Young chicken	311	47	15.1	0.20–0.34	0.22	0.03	0.22
	Mature chicken	306	43	14.1	0.20–0.28	0.22	0.02	0.22
	Young turkey	61	5	8.2	0.21–0.24	0.22	0.01	0.21
	Duck	111	66	59.5	0.20–0.33	0.24	0.03	0.27
Zinc	Calf	328	328	100.0	2.1–247.0	27.4	19.9	51.0
	Heifer/steer	288	288	100.0	5.9–34.0	20.1	3.57	27.0
	Bull/cow	95	95	100.0	11.0–59.0	21.0	7.01	37.0
	Lamb	162	162	100.0	10.0–54.0	24.5	5.41	34.0
	Mature sheep	34	34	100.0	3.0–48.0	22.2	7.54	43.0
	Market hog	321	321	100.0	0.24–58.0	25.0	5.39	33.0
	Boar/sow	281	281	100.0	15.0–61.0	24.7	6.09	34.0
	Young chicken	312	312	100.0	11.0–33.0	21.3	2.84	25.0
	Mature chicken	306	306	100.0	2.6–232.0	26.2	13.1	32.0
	Young turkey	61	61	100.0	16.0–23.0	19.7	1.30	21.0
	Duck	111	111	100.0	12.0–67.0	22.2	5.14	25.0
Copper	Calf	328	328	100.0	1.7–164.0	6.34	14.9	8.0
	Heifer/steer	288	288	100.0	1.4–49.0	4.65	2.70	5.5
	Bull/cow	95	95	100.0	2.1–403.0	8.15	41.0	5.1
	Lamb	162	162	100.0	0.81–108.0	5.39	9.77	7.0
	Mature sheep	34	34	100.0	2.6–10.0	3.95	1.41	7.7
	Market hog	321	321	100.0	1.5–53.0	6.65	3.51	11.0
	Boar/sow	281	281	100.0	2.1–25.0	6.73	3.31	12.0
	Young chicken	312	312	100.0	1.4–9.4	2.81	0.67	3.8

Table 5. ***Continued***

Trace metal	Production class	No. specimens	Positive specimens[a]					P95[b]
			No.	Percent	Range	Mean	SD	
	Mature chicken	306	306	100.0	2.1–12.0	3.07	0.86	4.1
	Young turkey	61	61	100.0	3.1–5.2	3.68	0.39	4.7
	Duck	111	111	100.0	3.4–61.0	5.90	6.69	6.7
Iron	Calf	328	328	100.0	0.13–750.0	35.4	55.2	70.0
	Heifer/steer	288	288	100.0	4.4–133.0	55.8	13.8	78.0
	Bull/cow	95	95	100.0	25.0–222.0	62.4	29.4	106.0
	Lamb	162	162	100.0	20.0–338.0	45.6	39.4	90.0
	Mature sheep	34	34	100.0	23.0–590.0	77.0	103.0	253.0
	Market hog	320	320	100.0	0.61–314.0	46.5	22.4	69.0
	Boar/sow	281	281	100.0	24.0–>999.0	79.0	68.0	131.0
	Young chicken	312	312	100.0	3.0–166.0	50.6	10.9	63.0
	Mature chicken	306	306	100.0	5.0–177.0	59.4	17.7	84.0
	Young turkey	61	61	100.0	41.0–88.0	59.2	10.2	76.0
	Duck	111	111	100.0	36.0–160.0	50.6	13.2	65.0
Maganese	Calf	328	328	100.0	0.21–3.5	0.66	0.35	1.2
	Heifer/steer	286	286	100.0	0.22–2.0	1.13	0.18	1.4
	Bull/cow	95	95	100.0	0.57–1.4	0.99	0.17	1.3
	Lamb	162	162	100.0	0.15–13.0	1.13	1.01	1.4
	Mature sheep	34	34	100.0	0.58–1.5	0.93	0.21	1.4
	Market hog	318	318	100.0	0.13–12.0	1.40	0.65	1.7
	Boar/sow	280	279	99.6	0.11–2.9	1.22	0.31	1.7
	Young chicken	311	311	100.0	0.20–3.0	2.03	0.25	2.4
	Mature chicken	306	304	99.3	0.51–4.4	2.29	0.34	2.8
	Young turkey	61	61	100.0	1.90–3.0	2.37	0.26	2.8
	Duck	111	111	100.0	0.23–6.0	2.54	0.52	2.9
Nickel	Calf	328	142	43.3	0.15–1.7	0.35	0.23	0.55
	Heifer/steer	288	126	43.8	0.20–1.4	0.29	0.15	0.39
	Bull/cow	95	41	43.2	0.20–0.51	0.28	0.09	0.42
	Lamb	162	81	50.0	0.20–2.3	0.36	0.27	0.54
	Mature sheep	34	17	50.0	0.20–2.3	0.47	0.54	1.3
	Market hog	320	186	58.1	0.20–45.0	0.57	3.29	0.50
	Boar/sow	281	104	37.0	0.20–0.80	0.29	0.11	0.37
	Young chicken	312	163	52.2	0.20–5.2	0.35	0.43	0.44
	Mature chicken	306	105	34.3	0.20–1.5	0.36	0.22	0.49
	Young turkey	61	46	75.4	0.20–2.3	0.38	0.41	0.68
	Duck	111	45	40.5	0.20–3.1	0.36	0.44	0.47

[a] Concn > LRQ.
[b] 95th percentile for all specimens (only P95 > LRQ shown).

unlikely to pose human health hazards to the U.S. consumer from dietary exposure to meat and poultry products.

Direct comparison of FSIS data for lead and cadmium levels in animal tissues with published data is not presented because the limits of quantification for the various analytical methods differ by as much as an order of magnitude. The median values in the published studies were often below the quantification limit of the FSIS method for lead and cadmium. [Since the 1985–1986 special study, the LRQs of the FSIS method for trace metals have been lowered to 0.05 ppm lead and 0.002 ppm cadmium as a result of the incorporation of inductively coupled plasma (ICP) optical emission spectrometry.]

Summary statistics for lead and cadmium concentrations in tissues from nonrandom sampling of animals in 1970–1979 are compared with data from the random monitoring study conducted in 1985–1986 (Table 7). The magnitude and distribution of positives for cadmium and lead in animal tissues are similar in both data sets. The incidence of heavy metals in edible tissues of livestock and poultry apparently is not increasing overall; however, the toxicity of cadmium and lead continues

Table 6. FSIS data for specimens that exceed regulatory limits established by Codex and Canada

Metal	Tissue	Species	No. exceeding	Minimum concn exceeding, ppm	Maximum concn exceeding, ppm
Lead	Kidney	Mature chicken	1[a]	2.2	2.2
Cadmium	Liver	Heifer	1[b,c]	17.0	17.0
		Sow	1[c]	1.4	1.4
		Mature chicken	3[b]	2.9	131.0
		Mature chicken	4[c]	1.4	131.0
	Kidney	Calf	3[b]	2.2	8.1
		Calf	7[c]	1.1	8.1
		Heifer	2[b]	2.3	9.6
		Heifer/steer	8[c]	1.1	9.6
		Cow	15[b]	2.1	32.0
		Bull/cow	40[c]	1.1	32.0
		Market hog	6[c]	1.1	1.9
		Sow	7[b]	2.5	4.4
		Boar/sow	36[c]	1.1	4.4
		Young chicken	1[b,c]	414.0	414.0
		Mature chicken	27[b]	2.1	3.6
		Mature chicken	113[c]	1.1	3.6
		Young turkey	3[c]	1.1	1.5
		Duck	1[c]	1.1	1.1

[a] No. of specimens from the 1985–1986 FSIS study exceeding 2.0 ppm Canadian action level for lead in liver and kidney.
[b] No. of specimens from the 1985–1986 FSIS study exceeding 2.0 ppm Codex maximum residue limit for cadmium in liver and kidney plus offal.
[c] No. of specimens from the 1985–1986 FSIS study exceeding 1.0 ppm Canadian action level for cadmium in liver and kidney.

to focus the attention of regulators on exposure to heavy metals in the diets of livestock and poultry, as well as humans.

Regulatory Implications

The results of this study and other published studies (18–21) indicate that the risk of human exposure to trace metals of toxicological concern in meat and poultry is low. FAO/WHO (20) does not list lead in fresh meats as a contributor in the human diet and lists cadmium in liver, kidney, and offal as contributing less than 8% of the estimated human dietary intake of cadmium. More than 60% of dietary lead is a result of exposure to beverages such as fruit juices stored in lead-soldered cans, and approximately 30% is from cereals, fruits, and vegetables (20). More than 80% of dietary cadmium exposure is from more common items in the diet (cereals and vegetables) that contain low burdens of cadmium rather than animal organs that can accumulate cadmium (20).

The role of the regulatory community is broadened when regional or global pollution problems have an impact on lead and cadmium concentrations in air, water, soil, and food. Some exposure of animals may result from accumulation of heavy metals on forage and feed crops exposed to local industry (mines, smelters, refineries) and vehicle exhaust (2, 20). Lead and cadmium contamination of feed items grown on agricultural land treated with phosphate fertilizers or municipal sewage sludge has been demonstrated (21). However, the most common sources of lead poisoning in animals include ingestion at dump sites of lead-based paints and lead-containing waste, such as batteries, putty, asphalt products, lead shot, leaded gasoline, and spent oil (3, 21, 22). Human poisoning by lead- and cadmium-contaminated meats is unlikely to occur by current environmental exposure of livestock and poultry, as evidenced in the Canadian national monitoring studies (10–12) and this U.S. study. Nevertheless, the goal of minimizing exposure to heavy metals seems prudent.

No regulatory limits are currently established for metals other than arsenic in tissues of livestock and poultry slaughtered in the United States. The need for limits on the essential metals of low toxicity is questionable. FSIS has requested that FDA determine appropriate limits for cadmium and lead present in animal tissues as a result of environmental exposure to heavy metals under the authority of the Federal Food, Drug, and Cosmetic Act. The focus of future deliberations for FSIS is the appropriate regulatory response to heavy metal residues that exceed any proposed limits. Canada and The Netherlands both may conduct on-farm investigations when lead levels exceeding the limits are detected in animal tissue. The intent of the on-farm investigation is to identify the source of lead and prevent future poisonings.

Lead poisoning incidents in Canada have resulted in liver and kidney levels of approximately 3–340 ppm (22). Tissues of the poisoned animals from this study did not enter the human food chain because the animals were condemned for poor health. Regulatory concern about lead and cadmium focuses on exposures to humans at levels less than the toxic dose for ani-

Table 7. Comparison of 90th percentiles for lead and cadmium in edible tissues collected from targeted sampling in 1970–1979 surveillance programs and from random sampling in monitoring programs in 1985–1986[a]

		90th percentiles (ppm metals)					
		Muscle		Liver		Kidney	
Metal	Species	1970s	1980s	1970s	1980s	1970s	1980s
Lead	Calf	0.90	0.58	1.10	1.20	1.00	1.30
	Heifer	<0.50	<0.50	<0.50	<0.50	0.90	0.50
	Steer	0.70	0.52	0.80	1.00	0.90	0.76
	Cow	<0.50	<0.50	<0.50	<0.50	1.00	0.69
	Lamb	<0.50	1.20	0.60	0.71	0.70	0.75
	Market hog	0.80	0.62	0.50	1.00	0.50	1.70
	Young chicken	<0.50	<0.50	<0.50	<0.50	0.90	2.20
	Mature chicken	0.60	<0.50	0.80	0.54	0.60	0.94
	Duck	<0.50	0.77	0.80	0.81	0.90	0.83
Cadmium	Calf	0.10	0.32	0.25	0.28	0.50	0.92
	Heifer	<0.025	<0.025	0.40	0.37	1.00	0.72
	Steer	0.20	0.13	0.40	0.22	0.75	0.73
	Cow	0.10	0.12	0.70	0.40	3.50	3.60
	Lamb	0.10	0.14	0.10	0.22	0.30	0.34
	Market hog	0.10	0.32	0.20	0.22	0.50	0.78
	Young chicken	<0.025	<0.025	0.20	0.19	0.20	0.33
	Mature chicken	<0.025	<0.025	0.50	0.49	2.00	2.10
	Duck	<0.025	<0.025	0.40	0.30	0.60	0.40

[a] The numbers of animals sampled in the 1970s were as follows: 17–36 calves, 144–163 steer, 2192 cows for lead and 16 cows for cadmium, 133–155 heifers, 471 lambs for lead and 17 lambs for cadmium, 374 market hogs for lead and 201 market hogs for cadmium, 260–262 young chickens for lead and 81–83 for cadmium, 41–43 mature chickens, and 261 ducks.

mals (9, 23). No guidelines are currently available to FSIS inspectors concerning release for slaughter of other exposed animals in an affected group that may not have signs of toxicosis but may contain lead residues in liver and kidney. Establishment of regulatory limits would help to ensure that the U.S. consumer would not be exposed to meat and poultry products adulterated with heavy metals of toxicological concern.

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DIETARY FIBER

Comparison of Different Methods for Determination of Dietary Fiber

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Several methods are available for determination of dietary fiber. To increase insight into the relative merits of these methods, the acid-detergent fiber (ADF) and neutral-detergent fiber (NDF) methods of Van Soest et al., the Hellendoorn method, the method of Prosky et al. (AOAC method), and the Englyst method were compared with respect to the amount and the nonstarch polysaccharide (NSP) composition of dietary fiber determined in 4 food products. Our results show that the ADF/NDF detergent methods are inaccurate for the determination of cellulose, hemicellulose, and lignin, and that the NDF and Hellendoorn methods are less-suited for the determination of insoluble dietary fiber. There is a discrepancy between the amount and the NSP composition of the dietary fiber determined by the AOAC and Englyst methods. This is because of either overestimation of the amount of dietary fiber in the AOAC method (coprecipitation of oligosaccharides or Maillard reaction products) or to underestimation of the amount of dietary fiber in the Englyst method (loss of polysaccharides during hydrolysis or derivatization), or both. Differences in isolation methods lead to differences in amounts of soluble and insoluble dietary fiber found by different methods. For both the Englyst and the AOAC methods, calculation of the amount of soluble dietary fiber from the difference between total and insoluble dietary fiber is preferable, because large errors may occur in determining the soluble portion. We have shown that although different methods can yield comparable dietary fiber values, the NSP composition can vary greatly. Therefore, we recommend using those methods that determine specifically well-defined components of the dietary fiber. The chromatographic Englyst method is preferred for this reason, and because it gives insight into the type of polysaccharides present.

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For several years, dietary fiber has received considerable attention because of its possible beneficial effects for human health. In 1974, Trowell defined dietary fiber as "that part of plant material in our diet which is resistant to digestion by secretions of the human digestive tract" (1). As this definition did not include polysaccharides present in some food additives, e.g., gums and pectins, Trowell et al. extended the definition to include "all the polysaccharides and lignin that are undigested by endogenous secretions of the human digestive tract" (2).

Since this definition, definitions of dietary fiber have been the subject of much discussion (3–11), paralleled by discussion about suitable methods for determination of dietary fiber. The main problem was that the definitions of dietary fiber were more or less based on its physiological properties, which cannot be determined chemically. At present, there is still confusion as to the type of components that should be included in the term "dietary fiber" and hence about the most appropriate method of analysis.

Several methods had previously been developed for the determination of crude fiber, unavailable carbohydrates, or dietary fiber. The first known measurements of fiber were performed by Einhof in 1806 (12, 13). Since then, a number of gravimetric, colorimetric, and chromatographic methods have been developed. The Weende method determined only crude fiber and was adopted by AOAC in 1887 (14). In the period from 1963 to 1981, the acid-detergent fiber (ADF) and the neutral-detergent fiber (NDF) methods for determination of hemicellulose, cellulose, and lignin were developed and improved (15–20). In 1969, Southgate introduced an enzymatic method for determination of soluble and insoluble dietary fiber (21–23), and Hellendoorn et al. reported an enzymatic gravimetric method for insoluble dietary fiber in 1975 (24).

A gas chromatographic (GC) method for determination of soluble and insoluble dietary fiber was developed (25–27), as

was a method with stepwise acid hydrolysis and GC and/or colorimetric quantification of total, insoluble, and soluble nonstarch polysaccharides (NSP) (8, 9, 28–32). In 1983, Asp et al. reported a gravimetric method for determining total, insoluble, and soluble dietary fiber (33). The determination of total dietary fiber was given official final action by AOAC in 1986 (34–37). In 1988, Prosky et al. (38) presented an enzymatic gravimetric method for determining insoluble, soluble, and total dietary fiber in food based on the method of Asp et al.

Methods are usually compared with respect to the amount of dietary fiber determined. However, this comparison gives no information about the type of dietary fiber that is determined by these methods. The purpose of our study was to improve insight into the relative merits of methods for determination of dietary fiber with respect to the amount and composition of the dietary fiber determined. We compared the gravimetric methods of Van Soest et al. (ADF, NDF), Hellendoorn et al., and Prosky et al. (AOAC) and the chromatographic Englyst method with respect to the amount of dietary fiber and the composition of its NSP.

The Hellendoorn method was selected because it was developed at our laboratories and was used widely until a few years ago. The ADF and NDF methods were selected because they have been very popular for several decades and are still used in many laboratories. The AOAC and the Englyst methods were selected because they are believed to be important methods for determination of dietary fiber at this time.

Four food products were chosen as representatives of fiber-containing foods: whole-wheat meal, dried apples, untoasted soy bran, and toasted soy bran. Whole-wheat meal and dried apples were chosen because these products are important sources of dietary fiber. The 2 samples of soy bran were chosen because these products are very rich in dietary fiber.

Experimental

Materials

Whole-wheat meal and dried apples were bought in local stores. Untoasted and toasted soy bran were supplied by a Dutch manufacturer. All samples were milled at 1 mm and stored at –25°C. All reagents used were analytical grade.

Methods

ADF was determined according to Van Soest and Wine (19). A 1 g sample was hydrolyzed with 0.5M sulfuric acid for 1 h at 100°C. After filtration, the residue was washed with hot water, acetone, and petroleum ether and then dried and weighed. A correction was made for the amount of ash in the residue, and the amount of ADF was calculated. The lignin was solubilized in potassium permanganate, and values for the amount of lignin and cellulose were obtained.

NDF was determined according to Van Soest and Wine (18) with the modification of Pikaar et al. (39). The sample was boiled in water for 1 min at 100°C to gelatinize the starch. After cooling, the starch in the sample was hydrolyzed with pancreatin for 1 h at 37°C, and the sample was treated 1 h at 100°C with NDF reagent pH 7 (30 g sodium lauryl sulfate, 18.61 g sodium hydrogen–EDTA, 6.81 g sodium tetraborate, 4.56 g disodium hydrogen phosphate, and 10 mL 2-ethoxyethanol in 800 mL distilled water). After filtration, the residue was washed with hot water, acetone, and petroleum ether and then dried and weighed. A correction was made for the amount of ash in the residue, and the amount of NDF was calculated.

Dietary fiber was determined according to Hellendoorn et al. (24) by boiling 0.5 g sample in water for 1 h to gelatinize the starch. After cooling, the sample was incubated with pepsin and hydrochloric acid for 18 h at pH 1 and 40°C to hydrolyze the protein. The sample was neutralized and then incubated with pancreatin and sodium lauryl sulfate for 1 h at pH 7 and 40°C to hydrolyze the starch. The pH was adjusted to 4.5, and the sample was filtered and washed with water and acetone. The residue was dried and weighed. No corrections were made for the amount of ash and for residual protein.

Dietary fiber was determined by the AOAC method according to Prosky et al. (38). A 0.5–1 g sample was incubated at pH 6.0 for 15 min at 100°C with α-amylase (Termamyl; NOVO Biolabs, Copenhagen, Denmark) and allowed to cool. The pH was adjusted to 7.5, and the sample was incubated with protease for 30 min at 60°C to hydrolyze the protein. After cooling the sample, the pH was adjusted to 4.5 and the sample was incubated with amyloglucosidase for 30 min at 60°C to hydrolyze the starch dextrins. From this point, the methods for total dietary fiber and soluble/insoluble dietary fiber proceed differently.

For determination of total dietary fiber, the soluble portion was precipitated overnight with 80% ethanol (v/v) at room temperature. After filtration, the residue was washed successively with ethanol and acetone, dried, and weighed. A correction was made for ash and protein, and the amount of total dietary fiber was calculated.

For separate determination of insoluble and soluble fiber, the samples were filtered and the residue was washed successively with ethanol and acetone, dried, and weighed. A correction was made for ash and protein, and the amount of insoluble dietary fiber was calculated. The soluble fiber in the filtrate was precipitated overnight with 80% ethanol (v/v), collected by filtration, and washed with ethanol and acetone. The residue was dried and weighed; a correction was made for ash and protein, and the amount of soluble dietary fiber was calculated.

Dietary fiber was determined according to Englyst by a condensed version of the method of Englyst and Cummings (32), which was supplied to participants of the Dietary Fibre Collaborative Trial, Part IV [joint United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF)/EC Trial] organized by R. Wood in 1990. In this method, a 300 mg sample was boiled in methyl sulfoxide for 30 min to disperse the resistant starch. Then, the sample was incubated with α-amylase (Termamyl) for 10 min at 100°C, cooled to 50°C, and incubated with pancreatin and pullulanase at pH 5.2 for 30 min at 50°C and then for 10 min at 100°C to hydrolyze the starch and protein.

For determination of total dietary fiber, the soluble portion was precipitated with 80% ethanol (v/v) for 0.5 h at 0°C and then centrifuged. The residue was washed with ethanol, dried

Table 1. Amounts of cellulose, hemicellulose, uronic acids, and lignin in 4 products (g/100 g d.m.)[a]

Product	Cellulose	Hemicellulose	Uronic acids	Lignin
Whole-wheat meal	1.9	7.9	0.3	1.0
Dried apples	5.4	3.1	3.2	0.4
Untoasted soy bran	42.6	18.2	11.3	1.9
Toasted soy bran	30.1	12.6	6.8	1.5

[a] Cellulose and lignin were determined according to the ADF procedure. Hemicellulose was calculated as the difference between NDF and ADF residues. Uronic acids were determined colorimetrically according to the Englyst procedure.

with acetone, and subjected to stepwise acid hydrolysis. To disperse the cellulose, the residue was treated with 12M sulfuric acid for 1 h at 35°C. To hydrolyze the NSP, the solution was diluted with water to 2M sulfuric acid and incubated for 1 h at 100°C. Neutral sugars in the hydrolysate were determined by GC after derivatization to alditol acetates. The neutral sugars were calibrated and calculated as described in the MAFF/EC trial method. Inositol was used as internal standard. The amount of uronic acids in the hydrolysate was determined spectrophotometrically by the dimethylphenol method (32).

For determination of insoluble dietary fiber, precipitation with ethanol was replaced by treatment with pH 7 phosphate buffer for 30 min at 100°C. Then, the determination proceeded as described above. The amount of soluble dietary fiber in the supernatant was determined after buffer treatment and centrifugation in the method for insoluble dietary fiber. The soluble fiber in the supernatant was precipitated by 80% ethanol (v/v) for 30 min at 0°C. The method continued as described above for determination of total dietary fiber.

For determination of the NSP composition of dietary fiber residues isolated in the gravimetric ADF, NDF, Hellendoorn, and AOAC methods, the residues were hydrolyzed and derivatized as described for the Englyst method.

Cellulose was measured by the method of Updegraff (40). Lignin and hemicelluloses were removed by extraction with an acetic acid–nitric acid reagent. The remaining cellulose was then dissolved in 67% sulfuric acid, anthrone reagent was added, and the cellulose was determined colorimetrically.

The GC determinations were performed on a Carlo Erba HRGC 5300 mega series chromatograph equipped with an autosampler, a flame ionization detector, and an integration computer. A Supelco SP-2330 wide-bore capillary column (30 m × 0.75 mm) was used. GC conditions were as follows: injection temperature, 270°C; column temperature, 220°C; detector temperature, 270°C; carrier gas, hydrogen; column pressure, 0.35 kPa; split flow, 15 mL/min; and injection volume, 1 μL.

Statistics

All analyses were performed in duplicate unless stated otherwise. The statistical significance of the results was determined by analysis of variance with a 2-tailed Student's *t*-test. A significance level of 5% was used.

Results and Discussion

As stated above, to obtain insight into the relative merits of methods for determination of dietary fiber, the gravimetric methods of Van Soest et al. (ADF and NDF), Hellendoorn et al., and Prosky et al. (AOAC) and the chromatographic Englyst method were compared with respect to the amount of dietary fiber and the NSP composition of the dietary fiber found in 4 food products.

For insight into dietary fiber composition, the amounts of cellulose, hemicellulose, uronic acids, and lignin were determined in whole-wheat meal, dried apples, untoasted soy bran, and toasted soy bran. Cellulose and lignin were determined according to the ADF method. Hemicellulose was calculated as the difference between NDF and ADF residue. Uronic acids were determined colorimetrically according to the Englyst method. The results are shown in Table 1.

The dietary fiber of whole-wheat meal consists largely of hemicellulose and contains almost no uronic acids. The dietary fiber of dried apples consists mainly of cellulose, uronic acids, and hemicellulose. High levels of cellulose, hemicellulose, and uronic acids and a small amount of lignin were found in the 2 samples of soy bran. Because there is a substantial difference in the composition of untoasted and toasted soy bran, it is assumed that the source of these products is different.

Table 2 shows the amounts of dietary fiber in whole-wheat meal, dried apples, untoasted soy bran, and toasted soy bran as determined by the ADF, NDF, Hellendoorn, AOAC, and Englyst methods. The AOAC and Englyst methods determine total, insoluble, and soluble dietary fiber. For each product, dietary fiber values with different superscripts are significantly different ($P < 0.05$).

Tables 3–6 list the NSP composition of the dietary fiber residues as isolated by the ADF, NDF, Hellendoorn, and AOAC methods, as well as the amount of NSP measured by the Englyst method, for whole-wheat meal, dried apples, untoasted soy bran, and toasted soy bran, respectively. The methods do not differ significantly for every product tested. Overall statistical analysis of data obtained by the NDF, ADF, Hellendoorn, AOAC-total, AOAC-insoluble, AOAC-soluble, Englyst-total, Englyst-insoluble, and Englyst-soluble methods, however, showed the following significant differences in NSP composition. The amounts of uronic acids, rhamnose, and arabinose are different for all methods. The amounts of fucose, xylose, galactose, and glucose are similar for the NDF and Hellendoorn methods. The amounts of xylose and mannose are similar for the NDF and AOAC-insoluble methods. The amounts of xylose are similar for the NDF and Englyst-total, Hellendoorn and AOAC-insoluble, Hellendoorn and Englyst-total, Hellendoorn and Englyst-insoluble, and AOAC-insoluble and Englyst-insoluble methods. The amounts of galactose and glucose

Table 2. Dietary fiber content in 4 products determined by 5 different methods (mean and standard deviation in g/100 g d.m.)[a]

Dietary fiber method	Whole-wheat meal	Dried apples	Untoasted soy bran	Toasted soy bran
NDF	$10.8 \pm 0.2^{b,f}$	$8.9 \pm 0.6^{b,f}$	$62.6 \pm 1.5^{b,f}$	$44.1 \pm 0.5^{b,f}$
ADF	2.9 ± 0.2^{c}	5.8 ± 0.1^{c}	44.5 ± 0.4^{c}	31.5 ± 0.2^{c}
Hellendoorn	$9.3 \pm 0.3^{b,f}$	$9.1 \pm 0.1^{b,f}$	$66.5 \pm 1.2^{b,f}$	$46.8 \pm 0.1^{b,f}$
AOAC-total	12.0 ± 0.4^{d}	13.0 ± 0.5^{d}	76.1 ± 2.2^{d}	53.9 ± 2.3^{d}
AOAC-insoluble	10.1 ± 0.6^{b}	9.8 ± 0.1^{b}	69.3 ± 1.2^{b}	49.0 ± 0.8^{b}
AOAC-soluble	1.1 ± 0.4^{e}	2.8 ± 0.6^{e}	8.7 ± 0.6^{e}	6.4 ± 0.5^{e}
Englyst-total	8.9 ± 0.1^{f}	9.9 ± 0.5^{f}	63.7 ± 0.8^{f}	44.8 ± 0.2^{f}
Englyst-insoluble	7.2 ± 0.1^{g}	7.2 ± 0.1^{g}	53.8 ± 0.7^{g}	38.2 ± 0.5^{g}
Englyst-soluble	3.3 ± 0.1^{h}	4.5 ± 0.6^{h}	9.6 ± 0.5^{e}	7.6 ± 0.2^{e}

[a] Mean of 4 determinations.
[b–h] For each product, dietary fiber values with different superscripts are significantly different ($P < 0.05$).

are similar for the AOAC-total and the Englyst-total methods. The total amount of NSP is similar for the NDF and Englyst-insoluble and the Hellendoorn and Englyst-insoluble methods.

We conclude that, although different methods for determination of dietary fiber can give comparable values, the NSP composition of dietary fiber residues can differ greatly. Thus, it can be risky to use gravimetric methods for determination of dietary fiber without knowing which components are actually measured.

ADF and NDF Methods

Cellulose and lignin are considered to be the major constituents of ADF. The amount of cellulose can be determined in the ADF residue after lignin is removed with potassium permanganate (19). Tables 3–6 show that the ADF residue contains not only glucose but also other polysaccharidic components (mainly xylose and uronic acids; average amount 19%). Although noncrystalline regions of cellulose may include small amounts of sugars other than glucose, the amounts of non-glucose polysaccharidic components found in the ADF residues indicate the presence of noncellulose polysaccharides. According to Englyst et al., the ADF residue may contain up to 10% noncellulose polysaccharides (8). Tables 3–6 also show that, when ADF is treated with permanganate to remove lignin, small but significant amounts of NSP are also removed in most products (average 7%). The ADF residue from which the lignin has been removed (ADF-lignin) still contains some noncellulose polysaccharides. These results indicate that the ADF method is not suited for accurate determinations of the amounts of cellulose and lignin.

Table 3. NSP composition of dietary fiber in whole-wheat meal isolated by different methods and analyzed by Englyst gas chromatographic method (g/100 g d.m.)

Constituent	NDF	ADF	ADF-lignin	Hellendoorn	AOAC-total
Uronic acids	0.24	0.05	0.08	0.28	0.47
Rhamnose	0.08	0.03	0.03	0.09	0.15
Fucose	0.00	0.00	0.00	0.00	0.00
Arabinose	1.77	0.00	0.00	1.38	2.37
Xylose	3.21	0.15	0.14	2.70	4.10
Mannose	0.13	0.06	0.05	0.14	0.35
Galactose	0.13	0.00	0.00	0.14	0.38
Glucose	2.29	1.49	1.33	2.18	2.57
Total NSP	7.85	1.79	1.62	6.90	10.37

Constituent	AOAC-insoluble	AOAC-soluble	Englyst-total	Englyst-insoluble	Englyst-soluble
Uronic acids	0.31	0.10	0.31	0.25	0.08
Rhamnose	0.09	0.05	0.13	0.13	0.12
Fucose	0.00	0.00	0.00	0.00	0.00
Arabinose	1.93	0.29	2.07	1.63	0.45
Xylose	3.52	0.44	3.54	2.84	0.68
Mannose	0.14	0.14	0.19	0.14	0.09
Galactose	0.15	0.14	0.43	0.15	0.28
Glucose	2.23	0.10	2.29	2.04	1.64
Total NSP	8.38	1.25	8.95	7.16	3.34

Table 4. NSP composition of dietary fiber in dried apples isolated by different methods and analyzed by Englyst gas chromatographic method (g/100 g d.m.)

Constituent	NDF	ADF	ADF-lignin	Hellendoorn	AOAC-total
Uronic acids	0.47	0.19	0.21	1.34	3.89
Rhamnose	0.15	0.04	0.04	0.18	0.31
Fucose	0.15	0.00	0.00	0.14	0.17
Arabinose	0.47	0.00	0.00	0.21	0.88
Xylose	0.93	0.24	0.15	0.97	1.04
Mannose	0.21	0.18	0.13	0.24	0.47
Galactose	0.53	0.04	0.01	0.54	0.82
Glucose	5.09	4.84	4.28	5.06	4.92
Total NSP	8.00	5.51	4.81	8.66	12.50
Constituent	**AOAC-insoluble**	**AOAC-soluble**	**Englyst-total**	**Englyst-insoluble**	**Englyst-soluble**
Uronic acids	1.00	2.42	3.20	0.64	2.45
Rhamnose	0.17	0.10	0.25	0.21	0.17
Fucose	0.15	0.01	0.13	0.11	0.00
Arabinose	0.67	0.18	0.74	0.46	0.32
Xylose	1.00	0.07	0.76	0.75	0.10
Mannose	0.24	0.18	0.22	0.21	0.21
Galactose	0.63	0.17	0.72	0.49	0.28
Glucose	4.71	0.08	3.92	4.37	0.99
Total NSP	8.55	3.21	9.93	7.23	4.49

Table 7 shows the amount of cellulose in the 4 products determined by the ADF method, as calculated from the amount of glucose in the ADF residue and as determined by the Updegraff method. Except for the dried apples, there is good agreement between the amount of glucose in the ADF residue and the results of the Updegraff method. In the ADF method, the amount of cellulose is overestimated; for all products, there is a discrepancy between the amount of cellulose determined by the ADF method and the amount calculated from the glucose in the ADF residue or determined by the Updegraff method ($P < 0.05$).

NDF is considered to be composed of hemicellulose, cellulose, and lignin. The amount of hemicellulose can be calculated as the difference between NDF and ADF (18). Tables 3–6 show that the NDF residue contains considerable amounts of uronic acids. The ADF residue also contains uronic acids [generally not to the same extent as the NDF residue ($P < 0.05$)] and xylose. Therefore, we find the NDF/ADF methods inaccurate for the determination of the amount of hemicellulose.

NDF and Hellendoorn Methods

The NDF and Hellendoorn methods are believed to measure insoluble dietary fiber (20, 22, 24, 41, 42). Table 2 shows that there is no difference in dietary fiber contents determined by the NDF, Hellendoorn, and AOAC-insoluble methods for all products examined. Neither were differences observed among the amounts of dietary fiber determined by the NDF, Hellendoorn, and Englyst-total methods. The NSP composition given in Tables 3–6 shows that smaller amounts of NSP are determined by the NDF and Hellendoorn methods than by the AOAC-insoluble and Englyst-total methods ($P < 0.05$). For most products, a higher amount of glucose is determined by the NDF and Hellendoorn methods than by the AOAC-insoluble and Englyst-total methods ($P < 0.05$). In addition, the NSP composition of the NDF and Hellendoorn residues is different; the NDF residue usually contains more arabinose and less uronic acids and rhamnose than the Hellendoorn residue ($P < 0.05$).

According to Selvendran and Dupont, the main problems encountered with the NDF method are losses of lignin and detergent-soluble components and contamination of the NDF residue with (modified) starch, leading to overestimation of NDF (43). Englyst et al. also mention incomplete starch removal by the NDF method (8). Although the problem of incomplete starch removal is partly overcome in the modified detergent methods (8, 39, 43), our results show higher glucose values in the NDF residue, indicating incomplete starch removal. We conclude that, although the amount of NDF compares well with the amount of insoluble dietary fiber determined in the AOAC method, it is not only the insoluble dietary fiber that is determined.

Hellendoorn claims to measure the insoluble indigestible residue (24, 42). Incomplete disruption of tissue structures, however, can lead to incomplete degradation of starch and protein in the Hellendoorn method (43, 44), and no correction is made for ash and residual protein. The higher glucose values found in the Hellendoorn residue compared with the AOAC-insoluble residue (Tables 3–6) point to incomplete starch removal. Furthermore, during the hydrolysis of protein with pepsin–hydrochloric acid, some hydrolysis of polysaccharides may occur (45). This means that, although the amount of dietary fiber determined by the Hellendoorn method compares well with the amount determined by the AOAC-insoluble

Table 5. NSP composition of dietary fiber in untoasted soy bran isolated by different methods and analyzed by Englyst gas chromatographic method (g/100 g d.m.)

Constituent	NDF	ADF	ADF-lignin	Hellendoorn	AOAC-total
Uronic acids	2.79	3.10	2.37	6.35	10.49
Rhamnose	1.00	0.44	0.43	1.22	1.55
Fucose	0.19	0.00	0.00	0.21	0.34
Arabinose	3.43	0.01	0.07	2.46	4.59
Xylose	7.17	3.63	3.34	7.29	7.19
Mannose	4.93	1.31	1.02	3.56	8.12
Galactose	1.33	0.06	0.05	1.22	2.95
Glucose	28.34	31.95	30.74	28.35	25.17
Total NSP	49.16	40.49	38.01	50.67	60.38

Constituent	AOAC-insoluble	AOAC-soluble	Englyst-total	Englyst-insoluble	Englyst-soluble
Uronic acids	7.82	2.84	11.31	5.49	4.72
Rhamnose	1.38	0.19	1.31	1.06	0.37
Fucose	0.34	0.02	0.34	0.28	0.06
Arabinose	4.36	0.19	4.77	4.18	0.56
Xylose	7.04	0.06	7.30	7.30	0.17
Mannose	5.18	2.84	7.63	5.72	1.87
Galactose	1.98	1.02	3.04	1.95	1.17
Glucose	24.89	0.12	27.98	27.81	0.67
Total NSP	52.98	7.26	63.68	53.78	9.58

method, the composition of the Hellendoorn residue is less representative of insoluble dietary fiber than the composition of the AOAC-insoluble residue.

AOAC and Englyst Methods

The AOAC method is considered to include NSP, lignin, and resistant starch in the dietary fiber, whereas the Englyst method includes only NSP (32, 38). Table 2 shows that the amounts of dietary fiber determined by the AOAC-total method are indeed higher than those determined by the Englyst-total method ($P < 0.05$). The discrepancy, however, seems too large to be explained by the amounts of lignin and/or resistant starch only.

Reistad and Frøhlich (46) have found that the Asp method gives dietary fiber values that are 1.1 to 1.4 times higher than those obtained with the method of Englyst. This difference could not be explained by the amount of lignin present. Deelstra et al. (47) and Mongeau and Brassard (48) also conclude that the difference between the amounts of dietary fiber determined by the AOAC and Englyst methods cannot be explained by lignin only. Coprecipitation of oligosaccharides and Maillard reaction products can be a problem in methods in which the ethanol-insoluble residue is isolated (such as the AOAC method), especially for products rich in starch or protein (43, 49).

The total composition of the 2 samples of soy bran was determined (protein, starch, fat, dietary fiber, and ash). The amount of components not included in either of these determinations (free sugars, oligosaccharides, and phenolic compounds) was estimated to be 3–4%. When the dietary fiber values determined by the AOAC method were used, the total composition slightly exceeded 100% (107% and 105% for untoasted and toasted soy bran, respectively). When the dietary fiber values determined by the Englyst method were used, the total composition was less than 100% (96% for both samples). Although the total composition of only 2 samples was investigated, the results suggest that the AOAC method slightly overestimates the dietary fiber content. On the basis of just the amount of NSP determined in the Englyst method, the amount of dietary fiber is slightly underestimated, partly because resistant starch and lignin are not included in this method.

Tables 3–6 show that the AOAC-total residue usually contains larger amounts of uronic acids, rhamnose, fucose, arabinose, xylose, mannose, and total NSP and similar amounts of galactose and glucose compared with residues obtained with the Englyst method ($P < 0.05$). Reistad and Frøhlich reported that the Asp method gave higher dietary fiber values than the Englyst method (46). However, the NSP content of the residue of the gravimetric Asp method (determined according to Englyst) was equal to the NSP content determined by the Englyst method. The NSP compositions determined by the 2 methods differed. Reistad and Frøhlich conclude that the difference in dietary fiber values determined by the 2 methods methods is caused by components other than NSP, or by an underestimation of the NSP content in the Englyst method (46). A loss of NSP in the Englyst method could occur during hydrolysis or derivatization of the polysaccharides. In our experiments, the results were corrected for the estimated losses during hydrolysis and for possible losses during derivatization as described in the MAFF/EC trial method. It is not clear, however, to what extent these corrections cover the actual losses. Selvendran et al. (50) report that no acid hydrolysis method

Table 6. NSP composition of dietary fiber in toasted soy bran isolated by different methods and analyzed by Englyst gas chromatographic method (g/100 g d.m.)

Constituent	NDF	ADF	ADF-lignin	Hellendoorn	AOAC-total
Uronic acids	2.24	2.11	1.52	4.10	7.80
Rhamnose	0.79	0.25	0.24	0.86	1.11
Fucose	0.16	0.00	0.00	0.17	0.27
Arabinose	2.25	0.02	0.04	1.77	3.32
Xylose	5.20	2.57	2.55	5.24	4.93
Mannose	2.26	0.80	0.72	2.09	4.07
Galactose	0.98	0.08	0.07	1.16	2.57
Glucose	22.09	22.39	22.76	22.39	19.64
Total NSP	35.97	28.22	27.90	37.78	43.70
Constituent	AOAC-insoluble	AOAC-soluble	Englyst-total	Englyst-insoluble	Englyst-soluble
Uronic acids	5.26	2.42	7.42	3.92	3.43
Rhamnose	0.97	0.17	0.90	0.71	0.30
Fucose	0.27	0.02	0.27	0.24	0.04
Arabinose	2.96	0.23	3.45	2.90	0.61
Xylose	4.85	0.07	5.27	5.25	0.12
Mannose	2.12	1.66	3.77	2.60	1.23
Galactose	1.83	0.66	2.47	1.74	1.22
Glucose	19.02	0.12	21.26	20.80	0.64
Total NSP	37.26	5.38	44.83	38.16	7.59

will cleave all sugar–sugar linkages and give a quantitative yield of each monosaccharide; at the same time all hydrolysis methods cause some degradation of mono- or polysaccharides. Moreover, uronic acids linked to neutral sugars yield acid-resistant aldobiuronic acids, and the tendency of pectins and pectic acids to precipitate in acid can result in incomplete hydrolysis of the associated neutral sugars (50).

Precipitation of oligosaccharides can cause the amount of NSP in the AOAC method to be overestimated. Marlett and Navis (49) have compared the AOAC and Theander methods. They indicate that, because the AOAC method does not call for the removal of oligosccharides, they can coprecipitate with 80% ethanol or can lead to an increase in lignin content through the formation of Maillard reaction products (49). In the AOAC method, coprecipitation of phosphate is another problem. Errors could be large, especially in products with a low dietary fiber content. This problem has been partly overcome by using dilute phosphate buffer, but according to Selvendran et al. the method needs further improvement, particularly for products low in dietary fiber (50).

Our results indicate that either some NSP is lost in the Englyst method or the amount of NSP is overestimated in the AOAC method, or both.

The sum of soluble and insoluble dietary fiber determined by the AOAC method compares well with the amount of total dietary fiber found by the method. For the Englyst method this also holds for both samples of soy bran, but for the wholewheat meal and the dried apples the sum of soluble and insoluble dietary fiber is significantly higher than the total amount of dietary fiber ($P < 0.05$). In his method, Englyst calculates the amount of soluble dietary fiber as the difference between total and insoluble dietary fiber (32). In our experiments, we actually determined the soluble portion to compare the results with the AOAC method, in which soluble dietary fiber has to be determined (38).

The cause of the discrepancy between the Englyst-total, the Englyst-insoluble, and the Englyst-soluble methods could be the different environment from which the soluble dietary fiber is precipitated. It can be seen from the NSP composition that the difference between the sum of soluble and insoluble dietary fiber and total dietary fiber is mainly caused by glucose. Therefore, either all of the cellulose is not determined in the Englyst-total method or the soluble dietary fiber is contaminated with oligosaccharides. The first possibility is not very likely because no difference between the sum of soluble plus insoluble dietary fiber and total dietary fiber was found for the 2 soy bran products, which contain the highest levels of cellulose. Englyst et al. suggest that incomplete precipitation of soluble dietary fiber can be avoided by calculating soluble fiber as the difference between total and insoluble dietary fiber (8). Our results, however, suggest that an overestimation of the amount of soluble dietary fiber is the reason for calculating it as the difference between total and insoluble dietary fiber.

The AOAC method can be improved if the amount of soluble dietary fiber is calculated rather than determined. Large errors may occur in the determination of the often small amounts of soluble dietary fiber.

Distributions of NSP over insoluble and soluble dietary fiber determined by the AOAC and the Englyst methods differed (Tables 3–6). Generally, in the AOAC method, a larger

Table 7. Cellulose determined by ADF method, amount of glucose in ADF residue, and cellulose determined by Updegraff method in 4 products (g/100 g d.m.)

Product	Cellulose determined by ADF method	Cellulose as glucose in ADF residue	Cellulose determined by Updegraff method
Whole-wheat meal	1.9	1.5	1.5
Dried apples	5.4	4.9	0.7
Untoasted soy bran	42.6	30.7	27.2
Toasted soy bran	30.1	22.7	20.1

part of the dietary fiber is found to be insoluble dietary fiber, whereas in the Englyst method, a larger part of the dietary fiber is found to be soluble dietary fiber ($P < 0.05$). Tables 3–6 show that there are also some differences in the compositions of insoluble and soluble dietary fiber as determined by the AOAC and Englyst methods ($P < 0.05$).

Reistad and Frøhlich have found that differences in the amount of soluble and insoluble dietary fiber are due to differences in the isolation procedures (46). Marlett et al. (51) reported that the amount of soluble dietary fiber varies from method to method and that methyl sulfoxide treatment (as used in the Englyst method) does not affect the distribution between soluble and insoluble dietary fiber. According to Englyst and Cummings, the reason for the higher proportion of soluble dietary fiber obtained by the Englyst method is due to the pH used for extraction (52). Our results show that differences in reaction conditions under which soluble and insoluble dietary fiber are separated have great influence on the amounts of insoluble and soluble dietary fiber measured.

Conclusions

We have shown that, although different methods for analysis of dietary fiber can give comparable values, the NSP composition of dietary fiber residues can differ greatly. For this reason, it can be risky to use a gravimetric method for analysis of dietary fiber without knowing which components are actually measured.

From the results presented here, we conclude that the ADF and NDF methods are not suited for accurate determinations of cellulose, hemicellulose, and lignin. The NDF and Hellendoorn methods are less-suited for the determination of insoluble dietary fiber.

Our results indicate that the dietary fiber content is slightly overestimated when determined by the AOAC method and slightly underestimated when determined by the Englyst method. Nonetheless, we prefer the Englyst method because it determines well-defined components of dietary fiber. Englyst's GC determination has advantages over the colorimetric determination because estimation of neutral sugars by colorimetric methods is not very reliable due to interferences and different color yields of the different sugars. Moreover, GC determination of the neutral sugars yields insight into the type of polysaccharides present.

For determination of dietary fiber, modern methods like the AOAC and Englyst methods should be preferred to older methods like the ADF/NDF and Hellendoorn methods. Both the AOAC method and the colorimetric Englyst method are well-suited for routine analysis of dietary fiber because the methods are relatively rapid and no sophisticated equipment is needed.

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DISINFECTANTS

Hard Surface Carrier Test for Efficacy Testing of Disinfectants: Collaborative Study

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A collaborative study was undertaken to evaluate a new disinfectant efficacy method called the hard surface carrier test. This new method is a qualitative carrier test that uses disposable glass carriers and standardized bacterial cultures. Ten laboratories tested 6 disinfectant-type formulations, which included positive and negative controls, against 3 microorganisms. No significant differences were found among the 10 laboratories for tests with *Pseudomonas aeruginosa* and *Salmonella choleraesuis*, but a small but statistically significant difference was present among laboratories for *Staphylococcus aureus*. The majority of data for this organism showed very good agreement; however, several tests exhibited slightly higher positive responses, which resulted in this overall difference. This difference was not considered significant within the scope and precision of this method or when compared with results for the other 2 organisms. The initial estimates of pure intralaboratory variance, determined from mean squares from analysis of variance results, were 1.009, 0.295, and 1.553 for *P. aeruginosa*, *S. aureus*, and *S. choleraesuis*, respectively. The experimental error for *S. aureus* was 3–5 times smaller than for the other 2 organisms, which helps explain the statistical significance of the results observed with this organism. Final estimates of intralaboratory variance obtained after dropping nonsignificant terms from the models were 0.90, 0.30, and 0.58 for *P. aeruginosa*, *S. aureus*, and *S. choleraesuis*, respectively. Small but statistically significant differences were noted in the formulation means for *P. aeruginosa* and *S. aureus* but not for *S. choleraesuis*. The results of this study suggested a performance standard of ≤2 positive carriers out of 60 tested for *S. aureus* and *S. choleraesuis*, and ≤3 positive carriers out of 60 tested for *P. aeruginosa*. This standard was derived from an analysis of the data by calculating an expected count of positive carriers and a 95% upper confidence limit for a set of 60 carriers. The method has been adopted first action by AOAC International.

In 1953, AOAC adopted the use-dilution method (UDM) to confirm germicidal activity of disinfectant dilutions obtained with the phenol coefficient test (1, 2). This test has since been expanded from the original 10 carriers to 60 carriers, and *Pseudomonas aeruginosa* has joined *Staphyloccocus aureus* and *Salmonella choleraesuis* as a test organism for hospital disinfectants (2). Today, the U.S. Environmental Protection Agency (EPA) requires that the UDM be used to generate efficacy data to support disinfectant registration (3).

The UDM has been criticized for inconsistent results and lack of reproducibility. One report stated that the disinfectant claims for 22% of the hospital disinfectants registered with EPA could not be reproduced (4). In 1985, an 18 laboratory collaborative study was conducted under the direction of the University of North Carolina (UNC) using 6 EPA registered disinfectants. "Extreme variability of the test results among laboratories testing identical products" was detected (5). In 1986, UNC ran another collaborative study incorporating 32

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changes into the method. Again, variability was seen, especially with *P. aeruginosa* and *S. aureus*. These studies were run with stainless steel cylinders and without control of the inoculum level. It was decided that the development of a new reproducible method was necessary (6).

The UDM has several identified deficiencies that may account for test variability (7–9): (*1*) the inconsistent surfaces of the stainless steel penicylinders that carry the bacteria into the disinfectant; (*2*) an inconsistent inoculum level; and (*3*) a difficult-to-follow, vague, and ambiguous written method.

The surfaces of 4 types of carriers were examined by scanning electron microscopy (SEM): 2 types of stainless steel (Fisher and S & L), glass, and porcelain. The surfaces of the stainless steel penicylinders and porcelain carriers showed grooves and pitting, sometimes severe. (Although grooves and pitting are inherent with porcelain carriers because of the nature of the material, the pitting and cracks found in some stainless steel cylinders were surprising.) These areas provide places for bacteria to reside and be protected from disinfectants. In contrast, the surfaces of the glass carriers were very smooth (7). It was found that polishing the surface of the stainless steel penicylinders reduced pitting and grooves. Use of the polished penicylinders provided more consistent UDM test results (8).

The UDM is one of the few official antimicrobial methods that does not require a standardized bacterial inoculum. Consequently, it is very unlikely that disinfectants would be similarly challenged in replicated tests. This may lead to variable end results. Some attempts to standardize test culture counts were made and were somewhat successful (9).

As currently written, the UDM is vague and ambiguous and lacks specific information, for example, the number of carriers removed from the culture at one time; the number of carriers placed in a given Petri plate; and identification of critical control points that are essential to proper execution of the method. This ambiguity often leads to subjective interpretation of the method. Consequently, a laboratory may develop its own version of the test that may differ significantly from those of other laboratories.

The hard surface carrier test is similar to the UDM in that it is a qualitative test using bacteria dried onto a hard surface carrier. Moreover, it presents the same challenge to the disinfectant as does the UDM (J. Bauer, L & F Products, unpublished data). However, it differs significantly in that disposable glass carriers have replaced the stainless steel penicylinders used in the UDM, and the challenge cultures are prepared differently and are standardized. Also, compared to the UDM, the hard surface carrier test is a more detailed method that leaves little room for subjective interpretation.

Collaborative Study

Ten laboratories that routinely test disinfectants for efficacy participated in the collaborative study. This group included 3 state laboratories, 1 federal laboratory, 2 independent laboratories, and 4 disinfectant manufacturers. One microbiologist from each participating laboratory was designated as the "test person" and was solely responsible for conducting all testing. This analyst was instructed to maintain a separate notebook for this study in which to keep detailed notes about the test and to record results. The following 6 disinfectant-type formulations, which included a positive and a negative control, a marginal formulation, and a blind duplicate, were used in the study:

Disinfectant 1.—*n*-Alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) dimethylbenzylammonium chlorides, 4.50%; *n*-alkyl (68% C_{12}, 32% C_{14}) dimethylethylbenzylammonium chlorides, 4.50%; and inert ingredients, 91.00%. The use-dilution was 1:128.

Disinfectant 2.—Disinfectant cleaner formula without active ingredient germicides and tetrasodium ethylenediaminetetraacetate. Inactive detergents were retained. The use-dilution was 1:64; negative control.

Disinfectant 3.—Sodiumxylene sulfonate, 10.8%; triethanolaminedodecylbenzene sulfonate, 6.3%; orthophenyl phenol, 5.7%; trisodium ethylenediaminetetraacetate, 3.0%; paratertiary-amylphenol, 1.8%; and inert ingredients, 72.4%. The use-dilution was 1:100.

Disinfectant 4.—Polyethoxypolypropoxypolyethoxy ethanoliodine complex, 11.30%; nonylphenoxypoly (ethyleneoxy) ethanol-iodine complex, 10.80%; and inert ingredients, 77.90%. The use-dilution was 1:512; marginal product. (Disinfectant 4 was diluted 1:2 in distilled water before it was sent to the test laboratories. The test laboratories were instructed to dilute it 1:256. The actual dilution, therefore, was 1:512.)

Disinfectant 5.—Orthophenyl phenol, 11.75%; orthobenzylparachlorophenol, 7.82%; tetrasodium ethylenediaminetetraacetate, 2.96%; sodium dodecylbenzenesulfonate, 3.17%; isopropyl alcohol, 2.60%; essential oils, 0.30%; and inert ingredients, 71.40%. The use-dilution was 1:128; positive control. (The EPA registered use-dilution is 1:256.)

Disinfectant 6.—Same as Disinfectant 1. Disinfectants 1 and 6 were distributed as blind duplicate samples.

The formulations were tested in the order listed. All test laboratories were asked to test each of the 6 formulations against *P. aeruginosa* before starting tests on *S. aureus*. Not until testing against *S. aureus* was complete was *S. choleraesuis* to be started. Sixty carriers were tested for each organism against each formulation.

The method was sent to the test laboratories several months in advance of the test formulation samples. Laboratory personnel were asked to review the method, obtain all specified materials, and practice the culture preparation techniques. Formulations were sent to the laboratories in amber, high-density polyethylene bottles, in 100–300 mL samples. Dilutions of the formulations were prepared by the test laboratories.

Comments on the method from the laboratories were generally positive. One collaborator stated that the increased manipulation during culture preparation could present a greater chance for culture contamination.

Statistical Analyses

Statistical analyses were performed at Brigham Young University's Center for Statistical Research. The laboratories mailed the test results directly to the center.

As with any analysis of a carefully developed and well-designed research project, the final and best interpretation can only be made in conjunction with expert(s) in the subject matter. The microbiologists and the statisticians carefully worked together to interpret and understand the results.

It seems appropriate to begin with a brief discussion of the nature of the data to clarify the analysis to be reported. In their simplest form, the data are binomial with an unknown p (fraction of positive carriers), because a single carrier was inoculated and examined for microbial growth. If growth was observed, the carrier was counted as positive; if no growth was noted, it was counted as negative. Thus, the data could have been recorded and analyzed as zeros and ones. For purposes of simplicity and convenience, the data were tallied as the number of positives out of 60 carriers. The resulting counts are actually frequencies, which could as easily have been represented as percentages or proportions.

Realizing that the data are, in essence, proportions, and that Disinfectants 1 and 6 are the same (and, thus, provide for an estimate of the intralaboratory error), it is not appropriate to consider the usual categorical data analysis. The analyses obtainable from CATMOD in SAS (10), or similar statistical computer programs, make no allowance for the estimation of an intralaboratory error. (CATMOD is a component of SAS/STAT® Software Integrated Procedures. CATMOD analyzes data that can be represented by a contingency table. It fits linear and log-linear models to functions of response frequencies by weighted-least-squares or maximum likelihood, and performs analysis of variance, logistic regression, and repeated measurements analysis.) Such analyses are correct only if one believes that such an error does not exist. However, there is the possibility of such an error, and it is commonly known that such errors do exist (11).

The problem is obviated by using a weighted analysis of variance (12), where the weights are the reciprocal of the variance of the estimated count in each cell of the 2-way laboratory × product table. This is easily accomplished using PROC GLM in SAS. (PROC GLM is a component of SAS/STAT Software Integrated Procedures. PROC GLM performs analysis of variance, analysis of covariance, reported measures analysis, multiple regression, and polynomial regression.) Disinfectants 1 and 6 were the same, so this analysis allows an estimate of the intralaboratory variance without making any assumption on the laboratory × formulation interaction. If there had been no replication between Disinfectants 1 and 6, the analysis could have been done in CATMOD as long as the populations were properly defined. When there is no replication, the CATMOD analysis and the weighted analysis of variance are the same. Because some of the cells have zero counts, and, thus, an estimated proportion of zero, a small positive number was added to each cell to facilitate the computations. Following recommendations of Grizzle et al. (12) and Berkson (13), 0.5 was added to the zero cells.

Using the data from the 10 laboratories, we also attempted to establish a performance standard.

Hard Surface Carrier Test Methods

(Applicable to testing disinfectants miscible with H_2O to determine effectiveness of given bactericidal concentration using standard test strains under controlled conditions. Test results may not necessarily reflect a product's efficacy on a variety of inanimate surfaces or within specified environments. These microbiological methods are very sensitive and technique-oriented. Exact adherence to the method with identified critical control points, good microbiological techniques, good laboratory practices, and quality control are required for proficiency and for validity of results. It is essential that the glass carriers employed in these tests are discarded after one use. Do not reuse carriers.)

Caution: Potential biohazard. Bacteria used in this study, *Salmonella choleraesuis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, are potentially pathogenic microorganisms. Please follow Biosafety Level 2 practices and techniques when handling these microorganisms. For more information see "CDC/NIH Biosafety in Microbiological and Biomedical Laboratories" 2nd Edition, May 1988, U.S. Department of Health and Human Services (available from the U.S. Government Printing Office, Washington, DC 20402).

991.47 Testing Disinfectants Against *Salmonella choleraesuis*—Hard Surface Carrier Test Method

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Method Performance:
See Table **991.47** for method performance data.

A. Reagents

(**a**) *Culture media.—(1) Nutrient broth.*—Dissolve 5 g beef extract (1 lb jar only, stored at 4°, Difco, PO Box 1058, Detroit, MI 48232), 5 g NaCl (biological grade), and 10 g peptone (Anatone, peptic hydrolysate of pork tissue, American Laboratories, Inc., 4410 S 102nd St, Omaha, NE 68127) in 1 L H_2O, and boil 20 min. Do not use squeeze-tube packed beef extract. Stir beef extract in jar thoroughly before weighing portion. Cool to room temperature, and adjust to pH 7.0 ± 0.2. Filter through paper (Whatman 2V, 32 cm, Cat. No. 1202320, or equivalent). Place 10 mL portions in 20 × 150 mm test tubes, and autoclave 15 min at 121°. Use this broth for daily transfers of test cultures.

(2) Bacto Synthetic Broth AOAC.—Contains 0.05 g L-cystine, 0.37 g DL-methionine, 0.4 g L-arginine, 0.3 g DL-histidine, 0.85 g L-lysine, 0.21 g L-tyrosine, 0.5 g DL-threonine, 1.0 g DL-valine, 0.8 g L-leucine, 0.44 g DL-isoleucine, 0.06 g aminoacetic acid, 0.61 g DL-serine, 0.43 g DL-alanine, 1.3 g L-glutamic acid, 0.45 g L-aspartic acid, 0.26 g DL-phenylalanine, 0.05 g DL-tryptophane, 0.05 g L-proline, 3.0 g NaCl, 0.2 g KCl, 0.05 g $MgSO_4$, 1.5 g KH_2PO_4, 4.0 g Na_2HPO_4, 0.01 g nicotinamide, and 0.01 g thiamine HCl/L. (Mixture may be obtained from Difco and prepared according to manufacturer's directions.) Dispense in 10 mL portions into 20 × 150 mm test tubes,

Table 991.47. Method performance for 991.47, testing disinfectants against *Salmonella choleraesuis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, hard surface carrier test method

Organism	Disinfectant[a] A	B	C
S. choleraesuis			
p[b]	0.0258	0.0066	0
60 × p	1.55	0.4	0
UCL	4	2	0
CC	97.8	99.2	100
S. aureus			
p	0.0208	0.0383	0.0083
60 × p	1.25	2.3	0.5
UCL	3	5	2
CC	96.1	97.0	98.6
P. aeruginosa			
p	0.0425	0.015	0.0133
60 × p	2.55	0.9	0.8
UCL	5	3	2
CC	95.4	98.7	95.3

[a] A, dimethylbenzylammonium chloride 4.5% and *n*-alkyldimethylbenzylbenzylammonium chloride 4.5%, use dilution 1:128; B, orthophenyl phenol 5.7%, use dilution 1:100; and C, orthophenyl phenol 11.75% and orthobenzylparachlorophenol 7.28%, use dilution 1:128 (twice the recommended use concentration).

[b] p = Fraction of positive carriers, 60 × p = expected number of positives, UCL = upper confidence limit of expected positive carriers out of 60, and CC = confidence coefficient.

and autoclave 15 min at 121°. Cool; pH must be 7.1 ± 0.1. Add 0.1 mL of 10% filter-sterilized glucose solution, **A(e)**, to each 10 mL tube prior to inoculation. Use this broth for daily transfers of test cultures.

(3) Letheen broth.—Contains 10.0 g peptic digest of animal tissue, 5.0 g beef extract, 0.7 g lecithin, 5.0 g Polysorbate 80, and 5.0 g NaCl/L. (Mixture may be obtained from Difco or BBL and prepared according to manufacturer's directions.) Dispense in 10 mL portions into 20 × 150 mm test tubes, and autoclave 15 min at 121°. pH must be 7.0 ± 0.2. Use for subcultures when testing quaternary ammonium, phenolic, and pine oil-type disinfectants. For other disinfectants, such as halogens, add 0.1% sodium thiosulfate, and for glutaraldehyde, add 0.25% sodium bisulfite to medium before subculture. Other neutralizers may also be used for chemicals not identified herein.

(4) Trypticase soy agar.—Contains 15.0 g pancreatic digest of casein, 5.0 g papaic digest of soybean meal, 5.0 g NaCl, and 15.0 g agar/L. Pour plate agar. (Mixture may be obtained from BBL and prepared according to manufacturer's directions.) Store in bottles, flasks, or tubes, such as 500 mL Erlenmeyer flasks or 20 × 150 mm test tubes. Sterilize 15 min at 121°. Place in 45° H_2O bath until agar is 45°. pH must be 7.3 ± 0.2. Pour in 20 mL portions into 15 × 100 mm sterile Petri dishes (TSA plates) for spread plate method.

(5) Nutrient agar.—To nutrient broth, **A(a)**(*1*), before autoclaving, add 1.5% Bacto agar (Difco, or equivalent), and boil until dissolved. Cool; pH must be 7.2 ± 0.2. Place 10 mL portions into 20 × 150 mm test tubes, autoclave 15 min at 121°, and slant.

(6) MacConkey's agar.—Contains 17.0 g pancreatic digest of gelatin, 1.5 g pancreatic digest of casein, 1.5 g peptic digest of animal tissue, 10.0 g lactose, 1.5 g bile salts, 5.0 g NaCl, 0.03 g neutral red, 0.001 g crystal violet, and 13.5 g agar/L. (Mixture may be obtained from BBL or other manufacturer and prepared according to manufacturer's directions.) pH must be 7.4 ± 0.2. Dispense into 15 × 100 mm sterile Petri dishes.

(b) *Test organism.*—Use *Salmonella choleraesuis*, ATCC No. 10708. Replace every 6 months directly from ATCC. Reconstitute in nutrient broth, **A(a)**(*1*), and streak onto nutrient agar slants, **A(a)**(*5*). Incubate both 18–24 h at 37°. Store working stock slants at 2–5°. Examine broth for contamination by inoculating trypticase soy agar (TSA), **A(a)**(*4*), and MacConkey's agar, **A(a)**(*6*). Incubate overnight at 37°. Examine plates for purity and colonial morphology (flat, round, and opaque, 1–3 mm in diameter). Perform gram stain from TSA. Gram stain should show gram-negative rods fitting morphology in Bergeys Manual. If not, discard broth. If uncontaminated, continue storage of working slants up to 30 days at 2–5°. Select well-isolated colonies from TSA and inoculate nutrient agar slants. Incubate 18–24 h at 37°. Store agar slant stock cultures at 2–5°. Additional biochemical tests may be performed as needed to confirm bacterium genus and species. After 30 days, prepare fresh nutrient agar working slant by subculturing from a stock culture slant. From working agar slants, make at least 3 and no more than 15 consecutive daily transfers using 4 mm id loop, or 10 µL pipet, in synthetic broth, **A(a)**(*2*), incubating 18–24 h at 37° before preparing agar lawn. Do not store broth cultures over weekend before preparing agar lawn; use freshly grown cultures only. (If only 1 daily transfer has been missed, it is not necessary to repeat the 3 consecutive transfers.)

(c) *Phosphate buffer stock solution.*—0.25M. Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust pH to 7.2 with 1N NaOH, and dilute to 1 L.

(d) *Phosphate buffer dilution water (PBDW).*—Add 1.25 mL 0.25M phosphate buffer stock solution, **A(c)**, to 1 L H_2O and dispense for 1:10 dilutions. Autoclave 20 min at 121°.

(e) *Filter-sterilized glucose solution, 10% (w/v).*—Biological grade, 10% glucose, filtered through 0.45 µm membrane. Aseptically place into sterile glass flask, and store at 2–5°.

(f) *Sterile distilled water.*—See **955.14A(d)**.

B. Apparatus

(a) *Glassware.*—Disposable serological (glass or plastic) or volumetric pipets; 1 L volumetric flask for germicide dilution; 20 × 150 mm and 25 × 150 mm borosilicate glass tubes;

Pasteur pipets; 150, 200, and 400 or 500 mL beakers. Clean glassware with detergent in hot water, rinse with hot water, and then deionized water. Sterilize clean glassware with dry heat at 170° 1 h for loose glassware, 2 h for glassware in metal containers, or steam at 121° for 20 min (with dry cycle for loose glassware). Before using this method and when cleaning agents change, perform APHA test for inhibitory residues on glassware using the test organisms after 3 broth transfers (APHA, *Standard Methods for the Examination of Water and Waste Water* (1985) 16th Ed., 834–835).

(**b**) *Petri dishes.*—15 or 20 × 100 mm glass (Pyrex) or plastic disposable Petri dishes. [**Note**: For use in drying carriers, place 2 layers (Whatman No. 2) 9 cm paper in each dish, place dishes in wire basket or other open container, and sterilize 2 h at 170°.] Clean and sterilize glass Petri dishes as in **B(a)**.

(**c**) *Water bath.*—Constant temperature, capable of maintaining 20 ± 0.5°, and holding ≥20 hole test tube rack for 25 × 150 mm test tubes.

(**d**) *Test tube racks.*—Any convenient style, for holding ≥20 test tubes, 20 × 150 mm and 25 × 150 mm.

(**e**) *Inoculating loop.*—For broth culture transfer. 95% platinum, 3.5% rhodium alloy, 18 or 19 gauge, 4 mm id loop with 75 mm shank (Baxter Scientific Products, or equivalent), or disposable 100 mm loops (Nunc, Cat. No. 2-54437, Baxter Scientific Products, or equivalent).

(**f**) *Wire hook.*—For carrier transfer. Make 3–5 mm bend (ca 60°) at end of suitable platinum or platinum alloy wire, No. 23 B & S gauge, in appropriate holder (Matthey-Bishop Inc., 1401 King Rd, Westchester, PA 19830, or equivalent).

(**g**) *Thermometer.*—0–50° range, subdivided to 0.1°, with manufacturer's certification of traceability to National Institute of Standards and Technology (NIST). Use for calibration of thermometers used to monitor laboratory equipment. A semiannual check against an NIST certified thermometer must be performed and recorded.

(**h**) *Carriers.*—Disposable fire-polished borosilicate glass carriers, 9 ± 1 mm length, 6 ± 1 mm id, 8 ± 1 mm od (Bellco Glass, PO Box B, 340 Edrudo Rd, Vineland, NJ 08360, Order No. 2090-S0012, or equivalent). Discard visibly flawed carriers (with scratches, cracks, and large chips). Carriers with barely perceptible chips are acceptable. Do not reuse carriers.

(**i**) *Dacron swab.*—Sterile, individually wrapped (Baxter Scientific Products, Cat. No. A5005-1, or equivalent).

(**j**) *Sterile absorbent pads with dispenser.*—47 mm diameter, cellulose fiber pads (Millipore Corp., Bedford, MA 01730, Cat. No. AP10047SI, or equivalent).

(**k**) *Sterile filtering system.*—47 mm diameter reusable sterile filtering system (Sterifil Aseptic System, Millipore Corp., Bedford, MA 01730, Cat. No. XX1104700, or equivalent).

(**l**) *Sonicator.*—Any sonicator that does not reduce bacterial viability after 10 min (Branson Ultrasonic Cleaner P-220, or equivalent). Validate by placing broth culture into sonicator for 10 min. Bacterial plate count must equal an unexposed control.

(**m**) *Spectrometer.*—Instrument to measure absorbance or percent transmittance at 580 nm.

(**n**) *pH meter.*—Calibrate daily or as necessary, using 2-point calibration with commercial buffer solutions (Baxter Scientific Products, or equivalent). If pH of sample is expected to be in alkaline range, calibrate pH meter with pH 7 and pH 10 buffer solutions. If pH of sample is expected to be in acid range, calibrate pH meter with pH 4 and pH 7 buffer solutions.

(**o**) *Sterility validation.*—Check sterility of pre-sterilized articles or apparatus using appropriate media that supports both bacterial and fungal growth.

C. Operating Technique

(**a**) *Carrier preparation.*—Place ca 200 carriers into 400 mL beaker, and add H_2O to cover. Decant water, and add EtOH. Decant EtOH, and rinse 3 times with H_2O. Place 24 carriers each into 25 × 150 mm test tube; cover with H_2O. Autoclave 20 min at 121°. Cool and store at room temperature.

(**b**) *Culture preparation.*—From stock nutrient agar slant, inoculate tube(s) of synthetic broth with sterile loop. Make at least 3 consecutive daily transfers, using inoculating loop or 10 µL pipet, incubating at 37°. Prior to testing, add 0.1 mL of 24 ± 4 h culture, with sterile pipet, to each of 5 tempered TSA plates, and spread culture onto plate to make a lawn. Incubate 24 ± 4 h at 37°. To each plate, add 10 mL synthetic broth. With sterile dacron swab, gently swab surface of plate to remove growth. Suspend cells from swab in synthetic broth. With pipet, collect synthetic broth containing suspended bacterial cells, and pool in sterile glass flask. Pass suspension, under vacuum, through 47 mm sterile absorbent pads using sterile filtration unit. Collect suspension in sterile flask. Sonicate 5 min, and let stand for 10 min. Water level in sonicator must be at same level as suspension in flask. Flask must contact bottom of sonicator. Use suspension only on day it is prepared. Refrigerate suspension when not in use.

(**c**) *Development of standard curve.*—Dilute suspended culture in synthetic broth: 1:5, 1:10, 1:20, and 1:25. Determine bacterial count of each dilution, with TSA, in duplicate, employing standard plate count procedures using surface streak or pour plates. Determine percent transmittance of each dilution. Repeat this 2 times with 2 fresh suspensions so each dilution is in triplicate. Determine plate count average for each dilution, and convert to $\log_{10}$. Plot percent transmittance vs $\log_{10}$ of bacterial count on graph paper. Reconfirm for each newly purchased ATCC culture.

(**d**) *Culture standardization.*—Prepare culture as in **C(b)**. Determine percent transmittance. Adjust culture with synthetic broth to 5–10 × 10^9 bacteria/mL. [**Note**: It may be necessary to adjust this culture above or below this level if suspension is outside 0.5–2.0 × 10^6 bacteria (or colony forming units)/dried carrier.]

(**e**) *Carrier inoculation.*—Carrier inoculation is a critical, technique sensitive procedure. Aseptically decant sterile H_2O from prepared glass carriers, and remove any H_2O remaining on bottom with sterile pipet or by draining. Using sterile pipet, add 24 mL standardized culture (1 mL for each of the 24 carriers). Carriers must be completely submerged. Cap tubes or bottles, and keep at room temperature 15 min. Using flamed, then cooled wire hook, remove carriers from suspension. Tap carri-

ers several times against side of tube to remove excess droplets of culture medium. Remove no more than 5 carriers/wire hook each time from suspension. Repeat 1–5 carrier transfer procedures until ≤12 carriers are placed into sterile Petri dish with filter paper. Use this suspension only once to inoculate 24 carriers; then discard. Use fresh suspension for next 24 carriers. With sterile wire hook, tip each carrier to 60°, and rotate carrier 360° to blot off excess medium at ring base. Move each carrier to dry section of paper and stand upright, avoiding contact between carriers. Gently probe inside of each carrier with sterile dacron swab to remove excess liquid on inside of ring. Use 1 dacron swab/12 carriers; then discard swab. Cover dishes and transport to incubator, keeping carriers upright. Discard carriers that fall over. If >1 carrier falls over, repeat procedure. Dry in incubator (without humidification system) 40 min at 37°. The 11th cylinder in each plate is extra, if needed, and the 12th cylinder is used for bacterial enumeration.

To determine carrier load, place carrier into 10 mL letheen broth and sonicate 10 min. Count in duplicate using standard plate count procedures, serially diluting in PBDW, and employing TSA for surface streak or pour plate. Average bacterial counts must be 0.5–2.0 × 10^6/dried carrier. Using carriers with fewer counts invalidates test. If carrier counts are above this range and disinfectant gives ≥2 positives/60 tested, retest to confirm results. Use carriers with counts falling within specified range in the retest.

(**f**) *Germicide preparation.*—Prepare germicide test solution in sterile volumetric flask with sterile H_2O unless otherwise stated or directed on sample container label. For dilution of sample, use >1.0 mL of sample v/v for liquid products; use w/v for dry products, weighed to 2 decimal places. Prepare ≥1000 mL. Prepare solutions <3 h prior to use to ensure stability. Dispense 10 mL portions of germicide test solution into 20 tubes, 25 × 150 mm. Place tubes in 20 ± 0.5° water bath, ≥10 min, until contents reach bath temperature.

(**g**) *Carrier exposure.*—Transfer is a critical, technique-sensitive procedure. Using 2 alternately flamed, then cooled hooks, add 1 contaminated dried carrier to 1 germicide tube every 30 s, without touching either inside wall of tube with contaminated carrier or hook while placing carrier in tube or removing hook. Do not swirl or shake tubes after adding carrier. Start timer when 1st carrier is placed into solution. At exactly 10 min, begin extracting carriers every 30 s, in order exposed, again alternating 2 flamed, then cooled wire hooks. False positives can result from transfer of live organisms to sides of tube due to aerosol formation. Shake carrier against side of tube to remove excess germicide. Place each carrier into tube of letheen broth, or other appropriate growth medium with appropriate neutralizer. Shake well, and incubate all subculture tubes 48–54 h at 37°. Observe for growth or no growth, as determined by presence or absence of turbidity. Confirm growth of test organism in positive tubes by subculture or gram stain. Subculture to selective media, or biochemical test may be performed to confirm bacterium genus and species. If tubes are contaminated, test is considered invalid and must be repeated.

(**h**) *Neutralization confirmation.*—Absence of residual effects of test germicide in subculture medium must be assured. Randomly select one negative tube for each 10 tubes tested, and to each tube add appropriate amount of 18–24 h test strain broth culture in diluted PBDW to deliver 5–100 cells/tube. Confirm number of cells added by pour plate method (inoculate 15 × 100 mm sterile Petri dishes and overlay with 15–20 mL trypticase soy agar or use spread plate technique with glass rod). Prepare in duplicate. Incubate 48–54 h at 37°, count colonies on plates to determine inoculum size (5–100 colonies/plate for valid results), and examine tubes for growth. Growth in all tubes indicates effective germicide neutralization. Absence of growth in ≥1 tubes indicates residual germicide and invalidates test results. If same lot of disinfectant is tested repeatedly, only 1 neutralization confirmation is necessary.

Ref.: JAOAC **75**, July/August issue (1992)

991.48 Testing Disinfectants Against *Staphylococcus aureus*—Hard Surface Carrier Test Method

First Action 1991

Method Performance

See Table **991.47** for method performance data.

A. Reagents

Use reagents specified in **991.47** and in addition:

(**a**) *Mannitol salts agar.*—Contains 5.0 g pancreatic digest of casein, 5.0 g peptic digest of animal tissue, 1.0 g beef extract, 75.0 g NaCl, 10.0 g D-mannitol, 0.025 g phenol red, and 15.0 g agar/L. (Mixture may be obtained from BBL or other manufacturer and prepared according to manufacturer's directions.) pH must be 7.4 ± 0.2. Dispense into 15 × 100 mm sterile Petri dishes.

(b) *Test organism.*—Use *Staphylococcus aureus*, ATCC No. 6538. Obtain semiannually, directly from ATCC. Reconstitute in nutrient broth, **991.47A(a)**(*1*), and streak onto nutrient agar slants, **991.47A(a)**(*5*). Incubate both 18–24 h at 37°. Store slants at 2–5°. Examine broth for contamination by inoculating TSA plates, **991.47A(a)**(*4*), and mannitol salts agar, **A(a)**. Incubate overnight at 37°. Examine plates for purity and colonial morphology (round, shiny, and yellow, 1–2 mm in diameter). If colonies are not uniformly yellow and shiny, pick a colony that is, and streak onto TSA. Repeat if necessary. If still unsuccessful, obtain new culture from ATCC. Perform gram stain from TSA. Gram stain should show gram-positive cocci. If not, discard broth. If uncontaminated, continue storage of slant 30 days at 2–5°. Additional biochemical tests may be performed as needed to confirm bacterium genus and species. After 30 days, transfer to fresh nutrient agar slant. Proceed as in **991.47A**(b), for daily transfers.

B. Apparatus

See **991.47**.

C. Operating Technique

Proceed as in **991.47**, except change test organism, and change culture density and carrier recovery to 1–5 × 10^9 bacteria/mL and 1–5 × 10^6 bacteria/dried carrier, respectively. Using

carriers with fewer counts invalidates test. If carrier counts are above this range and disinfectant gives ≥2 positives/60 tested, retest to confirm results. Use carriers with counts falling within specified range in the retest.

Ref.: JAOAC **75**, July/August issue (1992)

991.49 Testing Disinfectants Against *Pseudomonas aeruginosa*—Hard Surface Carrier Test Method

First Action 1991

Method Performance:

See Table **991.47** for method performance data.

A. Reagents

Use reagents specified in **991.47** and, in addition:

(a) *CTA medium.*—Contains 0.5 g L-cystine, 20.0 g pancreatic digest of casein, 2.5 g agar, 5.0 g NaCl, 0.5 g Na_2S, and 0.017 g phenol red/L. (Mixture may be obtained from BBL and prepared according to manufacturer's directions.) Dispense 10 mL portions into 20 × 150 mm test tubes, and autoclave 15 min at 121°. pH must be 7.3 ± 0.2.

(b) *Pseudosel agar.*—Contains 20.0 g pancreatic digest of gelatin, 1.4 g MgCl, 10.0 g K_2SO_4, 13.6 g agar, and 0.3 g cetrimide/L. (Mixture may be obtained from BBL or other manufacturer and prepared according to manufacturer's directions.) pH must be 7.2 ± 0.2. Dispense into 15 × 100 mm sterile Petri dishes.

(c) *Test organism.*—Use *Pseudomonas aeruginosa*, ATCC No. 15442. Obtain semiannually, directly from ATCC. Reconstitute in nutrient broth, **991.47A(a)**(*1*), and inoculate CTA tube, **(a)**. Incubate both 18–24 h at 37°. Store tube at 2–5°. Examine broth for contamination by inoculating TSA plates, **991.47A(a)**(*4*), and Pseudosel agar, **(b)**. Incubate overnight at 37°. Examine plates for purity and colonial morphology (flat, greenish-yellow, and opaque, 2–4 mm in diameter). Perform gram stain from TSA. Gram stain should show gram-negative rods. If not, discard broth. If uncontaminated, continue storage of CTA tube 30 days at 2–5°. Additional biochemical tests may be performed as needed to confirm bacterium genus and species. After 30 days, transfer to fresh CTA. Proceed as in **991.47A(b)** for daily transfers, except use nutrient broth instead of synthetic broth. Take care not to disturb pellicle by tilting tube to retract pellicle before inserting loop or pipet. When removing growth from agar and to dilute suspension when generating standard curve data, suspend cells in nutrient broth.

B. Apparatus

See **991.47**.

C. Operating Technique

Proceed as in **991.47**, except change test organism, and change culture density and carrier recovery to $1–5 \times 10^9$ bacteria/mL and $1–5 \times 10^6$ bacteria/dried carrier, respectively. Using carriers with fewer counts invalidates test. If carrier counts are above this range and disinfectant gives ≥3 positives/60 tested, retest to confirm results. Use carriers with counts falling within specified range in the retest.

Ref.: JAOAC **75**, July/August issue (1992)

Results and Discussion

The results from each of the 10 participating laboratories for testing 6 formulations against *P. aeruginosa*, *S. aureus*, and *S. choleraesuis* are shown in Table 1. Laboratory B showed 1 positive carrier out of 60 tested (+1/60) for Disinfectant 1 and +18/60 for Disinfectant 6 for *P. aeruginosa*. This led to a rather large estimate of intralaboratory error, which in and of itself is no problem except that such a value as +18/60 was judged to be an outlier. A repeat with a new sample of Disinfectant 6 produced +0/60. Therefore, although the 18 value was retained in Table 1, it was omitted from subsequent analyses. Laboratory G found +11/60 for testing formulation 3 against *S. aureus* (Table 1). A repeat of the sample produced similar results. These results are unexplained. However, this laboratory produced the expected responses with the other 5 formulations against *S. aureus* as well as the expected results for all 6 formulations against *P. aeruginosa* and *S. choleraesuis*. Disinfectant 2 was a negative control. A high number of positive carriers were expected and found. These results were not included in the statistical analyses of the data.

Table 2 gives the results from the weighted analyses of variance of the data in Table 1. The results are not the same for all organisms. No statistical differences in positive carrier counts due to laboratories were found for *P. aeruginosa* and *S. choleraesuis*, but laboratories were statistically different for *S. aureus*. The formulation × laboratory interaction was nonsignificant for *P. aeruginosa* and *S. choleraesuis*, so whatever differences might exist among the formulations are the same and are consistent across all 10 laboratories for these 2 organisms. The formulation × laboratory interaction is significant only for *S. aureus*. However, this may be due to the apparent ability of all laboratories to show better agreement on Disinfectants 1 and 6 for this organism than for the other 2. Note that the intralaboratory mean square is only 0.295 for *S. aureus*, but it is 1.009 and 1.553 for *P. aeruginosa* and *S. choleraesuis*, respectively (Table 2).

Least square means for laboratories and formulations are shown in Tables 3 and 4. Although the significant interaction between laboratories and formulations for *S. aureus* usually would indicate that one should not interpret the laboratory means (because they are not consistent for all formulations), we suggest an examination of Table 3. Laboratories D, E, and G tend to have larger means for *S. aureus* than do the other 7 laboratories. The precision of the other 7 laboratories was very tight. Small variations in positive results from Laboratories D, E, and G, therefore, produced a statistical difference. This is due to a single value for Laboratories E and G and a tendency for all values in Laboratory D to be slightly higher than in the other laboratories. (See *S. aureus*, Table 1). The data support the view that for *S. aureus* there is a statistically significant difference among the laboratories. The statistical difference

Table 1. Number of positive carriers out of 60 replicates for 10 laboratories testing 6 formulations against 3 microorganisms

	Formulation					
Laboratory	1[a]	6[a]	2	3	4	5
	P. aeruginosa					
A	1	3	27	1	4	1
B	1	18[b]	60	3	4	0
C	6	0	56	1	0	2
D	0	3	44	1	7	0
E	3	4	41	0	2	2
F	0	1	57	1	3	1
G	2	3	33	1	7	1
H	0	1	34	0	7	0
I	1	1	50	1	1	1
J	1	2	46	0	2	0
	S. aureus					
A	1	2	60	3	1	0
B	1	1	60	0	0	2
C	2	2	60	1	3	0
D	2	3	60	5	3	2
E	3	2	55	1	6	0
F	0	0	60	0	2	0
G	0	2	60	11	1	0
H	0	1	60	0	0	0
I	2	0	60	2	2	1
J	1	0	60	0	2	0
	S. choleraesuis					
A	3	1	14	1	0	0
B	1	1	13	1	2	0
C	4	0	60	0	2	0
D	1	0	60	0	1	0
E	1	7	48	2	2	0
F	0	1	56	0	1	0
G	1	0	45	0	3	0
H	2	0	15	0	5	0
I	6	0	28	0	0	0
J	1	1	46	0	2	0

[a] Blind duplicate samples.
[b] A repeat at this laboratory with a new sample of disinfectant 6 produced +0/60. Original value, 18, omitted from statistical analysis.

within the *S. aureus* data is not significant when compared with data for the other 2 organisms, however. This statistical difference, therefore, appears not to be biologically significant within the scope and precision of this method. Table 3 shows that the laboratory means are not all that different for the 3 organisms.

There were differences among formulations for *P. aeruginosa* and *S. aureus* but not *S. choleraesuis*. All formulations appeared to be uniformly effective against *S. choleraesuis*.

Table 2. Weighted analysis of variance of data from Table 1

Source	DF	MS	*F*	*p*-value
	P. aeruginosa			
Formulation	3	6.319	8.26	0.014
Laboratory	9	1.060	1.05	0.472
Formulation × laboratory	27	0.981	0.97	0.557
Error	9	1.009	—	—
	S. aureus			
Formulation	3	3.949	13.38	0.0008
Laboratory	9	2.357	7.99	0.0016
Formulation × laboratory	27	0.959	3.25	0.0274
Error	10	0.295	—	—
	S. choleraesuis			
Formulation	3	3.054	1.97	0.1830
Laboratory	9	0.566	0.36	0.9279
Formulation × laboratory	27	0.341	0.22	0.9992
Error	10	1.553	—	—

This was not surprising. *S. choleraesuis* has been shown to be the most sensitive of the 3 organisms to disinfectants (6). The formulations were designed to show different levels of activity. Therefore, one would expect to see these differences among the more resistant organisms. The differences will be somewhat obscured with the more sensitive *S. choleraesuis*.

To obtain a better estimate of the relative contribution to the total variance due to formulation, laboratories, and intralaboratory sources, we reanalyzed the *P. aeruginosa* and *S. choleraesuis* data because the laboratory × formulation interaction was found to be nonsignificant. The weighted analyses of variance summarized in Table 5 show that only negligible differences in terms of significance are evident when compared with results in Table 2 for the same microorganisms. Because the laboratory mean squares are smaller than the corresponding intralaboratory mean squares in Table 5, variance components cannot be estimated from these analyses, unless one assumes the variance due to laboratories is zero for these 2 organisms. Table 6 contains estimates of variance components for *P. aeruginosa* and *S. choleraesuis* based on this assumption and for *S. aureus* based on the results in Table 2. These were computed by hand because we are not aware of any program that calculates variance components from a weighted analysis of variance. These computations assume that laboratory is a random factor, which is the same assumption made by Cole et al. (6). By its very nature, the weighted analysis of variance leads to nonorthogonal sums of squares and, hence, the equivalent of an unbalanced design. The variance components for *S. aureus* are estimated following the method outlined by Bowen (W.M. Bowen, unpublished M.S. thesis, Brigham Young University, Department of Statistics, Provo, UT).

Table 3. Least square means and standard errors for 3 organisms and 10 laboratories[a]

Laboratory	*P. aeruginosa*		*S. aureus*		*S. choleraesuis*	
A	1.88	(0.64)	1.46	(0.30)	0.88	(0.51)
B	2.13	(0.71)	1.00	(0.25)	1.13	(0.62)
C	1.12	(0.49)	1.63	(0.31)	0.98	(0.57)
D	2.34	(0.71)	3.10	(0.44)	0.67	(0.47)
E	1.98	(0.61)	2.47	(0.38)	1.58	(0.71)
F	1.42	(0.57)	0.88	(0.24)	0.67	(0.47)
G	2.85	(0.77)	3.33	(0.45)	1.17	(0.64)
H	2.17	(0.69)	0.54	(0.18)	1.70	(0.76)
I	1.00	(0.47)	1.45	(0.31)	0.62	(0.43)
J	1.08	(0.48)	0.92	(0.25)	1.00	(0.58)
Min.	1.00		0.54		0.62	
Max.	2.85		3.33		1.58	

[a] Means are counts per 60 carriers. Standard errors are in parentheses.

It should be pointed out, however, that formulation is a fixed factor and its values in Table 6 are not true variance components. They have been calculated and included for comparative purposes.

The results in Table 6 indicate that for *P. aeruginosa* and *S. choleraesuis* over 90% of the variance in positive carriers out of 60 is due to intralaboratory sources, with a lower and probably nonsignificant amount attributable to formulation differences. On the other hand, the *S. aureus* results show 16% of the variance due to intralaboratory sources, with approximately equal amounts assignable to differences among formulations, among laboratories, and to a laboratory × formulation interaction.

Because the interactions were found to be nonsignificant for *P. aeruginosa* and *S. choleraesuis*, the means for these 2 organisms should be reestimated. Although there is also no laboratory effect for these 2 organisms, the laboratory term is left in the analysis to provide a unique estimate for each laboratory. These new means are shown in Tables 7 and 8. The *S. aureus* means are the same as in Tables 3 and 4.

Disinfectant 2 was a negative control; that is, a high number of positive results were expected. The active germicidal ingredients of this formulation were left out. All that remained were surfactants, fragrance, and dye. The results from this formulation, therefore, were not included in the statistical analyses. However, some microbial kill was observed, as seen by no growth in the subculture tubes. This kill was seen only with the gram-negative organisms, not against *S. aureus*, a gram-positive organism. In fact, 9 laboratories showed +60/60 against *S. aureus* with this formulation. The 10th laboratory had +55/60. We concluded that something other than the germicide in this formulation was antimicrobial, specific for gram-negative organisms. Wash-off of the organisms from the carrier cannot be considered because enough organisms would have been carried over in the liquid to produce growth in the subculture tubes.

Table 4. Least square means and standard errors for 3 organisms and 4 formulations[a]

Disinfectant	*P. aeruginosa*		*S. aureus*		*S. choleraesuis*	
1 & 6	1.39	(0.27)	1.26	(0.13)	1.00	(0.27)
3	1.05	(0.32)	2.50	(0.26)	0.75	(0.34)
4	3.75	(0.59)	2.10	(0.24)	1.90	(0.53)
5	1.00	(0.31)	0.85	(0.16)	0.50	(0.28)

[a] Means are counts per 60 carriers. Standard errors are in parentheses.

Table 5. Weighted analysis of variance for *P. aeruginosa* and *S. choleraesuis*, assuming the laboratory × formulation interaction is zero

Source	DF	MS	*F*	*p*-value
		P. aeruginosa		
Formulation	3	2.53	2.56	0.070
Laboratory	9	0.56	0.57	0.816
Error	36	0.99	—	—
		S. choleraesuis		
Formulation	3	1.43	2.13	0.113
Laboratory	9	0.23	0.34	0.956
Error	37	0.67	—	—

Using the data from the 10 laboratories for Disinfectants 1, 3, 5, and 6, a performance standard was determined. The data for Disinfectant 4 were not used because it was a marginal formulation. These data made it possible to (*1*) estimate the probability of an individual carrier being positive, (*2*) calculate the expected number of positive carriers out of 60, and (*3*) determine a "95%" upper confidence limit on the expected number of positive carriers out of 60. The Poisson distribution was approximated to be binomial, and this was used in constructing the confidence limit. These results are shown in Table 9 for *P. aeruginosa*, *S. aureus*, and *S. choleraesuis*.

The discrete nature of the binomial distribution (and also the Poisson distribution) makes it virtually impossible to obtain exact 95% limits. The reported limits are wider than they would be if the confidence coefficient could be fixed at exactly 0.95. Again, because the data are discrete the confidence limits have been rounded up to the nearest integer. A performance standard should be no smaller than the expected number of positive carriers and no larger than the upper confidence limit.

Because the upper confidence limits are larger than they should be, for the 2 reasons above, we recommend a performance standard that is no less than the rounded expected value. On the basis of results from this study, a value of ≤ 2 positive carriers out of 60 tested for *S. aureus* and *S. choleraesuis* and ≤ 3 positive carriers out of 60 tested for *P. aeruginosa* were selected as the preliminary performance standards.

Conclusions and Recommendations

(*1*) On the basis of the data presented here, the hard surface carrier test should be adopted first action by AOAC International.

Table 6. Variance components estimates based on the statistically significant effects in each model for 3 organisms

Source	*P. aeruginosa*	%	*S. aureus*	%	*S. choleraesuis*	%
Formulation	0.101[a]	10	0.424[a]	24	0.042[a]	7
Interlaboratory	NA[b,c]	NA[a]	0.566	31	NA[a]	NA[a]
Interaction	NA[b]	NA[a]	0.526	29	NA[a]	NA[a]
Intralaboratory	0.902	90	0.295	16	0.582	93

[a] Not true variance component because formulation is not a random effect.
[b] Cannot be estimated because these sources are assumed to be nonexistent.
[c] NA = not applicable.

(*2*) We suggest a performance standard of ≤2 positive carriers out of 60 tested for *S. aureus* and *S. choleraesuis* and ≤3 positive carriers out of 60 tested for *P. aeruginosa*.

(*3*) We have shown that the hard surface carrier test produces consistent and reproducible results when the disinfectant-type formulations are diluted in distilled water and are tested in the absence of organic soil. Effectiveness in hard water and in the presence of soil is desirable for disinfectants marketed today. Therefore, we recommend that (*1*) a study be done to investigate the efficacy of disinfectant-type formulations diluted in hard water and in the presence of soil, and (*2*) a final performance standard for an effective hard surface disinfectant be established based on a 95% upper confidence limit rather than the expected value, which was chosen in this study as a conservative standard. This standard should be calculated from a large (>6) sample size of disinfectant-type formulations.

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Table 7. Least square means and standard errors for 3 organisms and 10 laboratories where there is assumed to be no interaction between laboratory and formulation for *P. aeruginosa* and *S. choleraesuis*[a]

Laboratory	*P. aeruginosa*		*S. aureus*		*S. choleraesuis*	
A	1.60	(0.53)	1.46	(0.30)	0.78	(0.32)
B	1.37	(0.54)	1.00	(0.25)	0.99	(0.35)
C	0.71	(0.42)	1.63	(0.31)	0.84	(0.32)
D	1.17	(0.44)	3.10	(0.44)	0.66	(0.29)
E	1.59	(0.53)	2.47	(0.38)	1.34	(0.40)
F	1.07	(0.44)	0.88	(0.24)	0.66	(0.29)
G	1.97	(0.58)	3.33	(0.45)	0.77	(0.31)
H	0.96	(0.39)	0.54	(0.18)	0.86	(0.32)
I	1.01	(0.44)	1.45	(0.31)	0.62	(0.29)
J	1.06	(0.41)	0.92	(0.25)	0.87	(0.32)
Overall	1.16	(0.15)	NA[b]		0.80	(0.10)
Min.	0.71		0.54		0.62	
Max.	1.97		3.33		1.34	

[a] Means are counts per 60 carriers. Standard errors are in parentheses.
[b] NA = not applicable.

Table 8. Least square means and standard errors for 3 organisms and 4 formulations where there is assumed to be no interaction between laboratory and formulation for *P. aeruginosa* and *S. choleraesuis*[a]

Disinfectant	*P. aeruginosa*		*S. aureus*		*S. choleraesuis*	
1 & 6	1.20	(0.24)	1.26	(0.13)	0.94	(0.17)
3	0.83	(0.29)	2.50	(0.26)	0.66	(0.20)
4	2.17	(0.45)	2.10	(0.24)	1.25	(0.29)
5	0.80	(0.28)	0.85	(0.16)	0.50	(0.18)

[a] Means are counts per 60 carriers. Standard errors are in parentheses.

Table 9. Fraction of positive carriers (p), expected number of positives (60 × p), upper confidence limit (UCL) of expected positive carriers out of 60, and confidence coefficients (CC) for 3 disinfectants against 3 microorganisms

Disinfectant	p	60 × p	UCL	CC
		P. aeruginosa		
1 & 6	0.0425	2.55	5	95.4
3	0.015	0.9	3	98.7
5	0.0133	0.8	2	95.3
		S. aureus		
1 & 6	0.0208	1.25	3	96.1
3	0.0383	2.3	5	97.0
5	0.0083	0.5	2	98.6
		S. choleraesuis		
1 & 6	0.0258	1.55	4	97.8
3	0.0066	0.4	2	99.2
5	0	0	0	100.0

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DRUG RESIDUES IN ANIMAL TISSUES

Determination of Malachite Green and Its Leuco Form in Water

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Liquid chromatographic (LC) analysis can detect malachite green residues in water at less than 10 μg/L. Water samples were concentrated on disposable diol columns, eluted with 0.05M *p*-toluenesulfonic acid in methanol, and determined by reversed-phase LC. When combined with a lead oxide postcolumn reactor, the LC method can simultaneously determine both leuco and chromatic forms of malachite green. Recoveries averaged 95.4% for the chromatic form and 57.3% for the leuco form of malachite green oxalate and leuco malachite green in spiked pond water samples. Recoveries of the carbinol form of malachite green (an equilibrium product of the dye in water) from spiked tap water samples averaged 98.6%. Recoveries of leuco malachite green were low and pH-dependent.

Malachite green has been used to treat fish for external fungal and protozoan infections since 1933 (1–4), but it was never registered for use on food fish (5). Malachite green belongs to the triphenylmethane class of dyes, some of which are animal carcinogens (6). Meyer and Jorgenson (7) demonstrated that malachite green caused significant developmental abnormalities when administered to eggs of rainbow trout (*Oncorynchus mykiss*) and to pregnant New Zealand white rabbits (*Oryctolagus cuniculus*).

Malachite green and crystal violet, another triphenylmethane dye, are reduced through biotransformation to their leuco (colorless) forms in animals (8–10). The leuco form of malachite green (Figure 1) is a precursor of the chemical during production and could be a contaminant of the commercially prepared dye. The carbinol form of malachite green (Figure 1), also colorless, is an acid-base equilibrium product of malachite green in solution. Although never tested for carcinogenicity, the leuco and carbinol forms of malachite green are structurally similar to classical aromatic amine carcinogens.

Concern about the health risks of both leuco and chromatic malachite green required development of a method to monitor possible residues. At present, the chromatic and leuco forms of other dyes can be determined simultaneously by liquid chromatography (LC) only with electrochemical detection (11, 12). Bauer et al. (13) determined leuco and chromatic malachite green in a sample by splitting the sample and oxidizing half with PbO_2. The amount of leuco malachite green in the sample was determined by the difference in the malachite green between the unaltered and oxidized subsamples. Allen and Meinertz (14) recently reported a method in which the leuco and chromatic forms of malachite green are separated by LC; after postcolumn oxidation of the leuco malachite green, both forms are detected by visible spectrophotometry. The method of postcolumn oxidation after extraction was included in the development of our procedure to detect malachite green residues in water.

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Use of trade names does not imply U.S. Government endorsement of commercial products.

Experimental

Apparatus

(a) *Solid-phase extraction column.*—Baker 10 Diol columns (3 mL size) obtained from J.T. Baker Chemical Co., Phillipsburg, NJ.

(b) *Liquid chromatograph.*—Beckman Model 110A pump (Beckman Instruments Inc., Fullerton, CA) equipped with Model 210 injection valve and 50 μL fixed loop injector to deliver analyte to μBondapak C_{18} column, 300 × 3.9 mm id (Waters, Milford, MA), particle size 10 μm. Detector: Waters Lambda-Max, Model 481 spectrophotometer operated at 618 nm. Chromatographic data from injections were collected and analyzed with System Gold chromatographic software (Beckman Instruments, Inc., Arlington Heights, IL). Isocratic, filtered mobile phase (flow rate 2.0 mL/min): methanol–aqueous acetate buffer (85 + 15) (0.05M sodium acetate plus 0.1M glacial acetic acid in LC-grade water).

(c) *Postcolumn reactor.*—Stainless steel tube, 32 × 4 mm id, packed with 10% PbO_2 suspended in Celite 545 (PbO_2 is dry-mixed with Celite to give uniform mixture), and capped with 2 μm frits. As reactor is being packed with PbO_2 in Celite, it is gently tapped to prevent formation of voids in reactor. Postcolumn reactor is placed in line between LC column and spectrophotometric detector.

Reagents

All chemicals were reagent grade.

(a) *Malachite green oxalate.*—Cat. No. 1264 (Eastman Kodak Co., Rochester, NY).

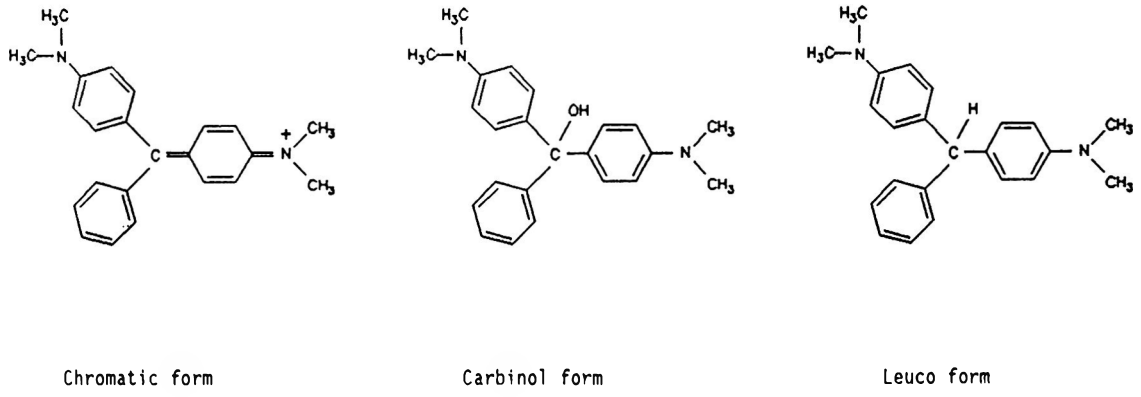

Figure 1. Structures of the chromatic, carbinol, and leuco forms of malachite green.

(b) *Leuco malachite green.*—Cat. No. 3620 (Eastman Kodak Co.).

(c) *Malachite green carbinol base.*—Cat. No. 22,910-5 (Aldrich Chemical Co., Inc., Milwaukee, WI).

(d) *Anhydrous acetic acid, sodium salt.*—Cat. No. 24,124-5 (Aldrich Chemical Co.).

(e) *Solvents.*—Glacial acetic acid, methanol, and water were LC grade (J.T. Baker Chemical Co.).

(f) *p-Toluenesulfonic acid monohydrate (98%).*—Cat. No. 16,199-3 (Aldrich Chemical Co.).

(g) *Lead dioxide.*—Cat. No. 5727, ACS grade (Mallinckrodt, Inc., St. Louis, MO).

(h) *Celite 545.*—Cat. No. C-212 (Fisher Scientific, Pittsburgh, PA).

(i) *Malachite green oxalate and leuco malachite green stock solutions.*—1 mg/mL in methanol. Standard solutions of malachite green oxalate and leuco malachite green at 0.5, 1.0, and 2.0 µg/mL were prepared fresh daily in (85 + 15) mobile phase.

Water Extraction

Diol columns are preconditioned by eluting with 2 mL methanol followed by 5 mL deionized water. Water samples are adjusted to pH 6.0 with 1.0M HCl or 1.0M NaOH, and 50 mL of each sample is eluted through diol column at flow rate of <4 mL/min. Malachite green residues are eluted from column with two 1.0 mL portions of 0.05M *p*-toluenesulfonic acid in methanol. Sample volume is adjusted to 2.0 mL with methanol and injected into LC column.

Recovery Study

The effect of pH on the recovery of malachite green residues through the method was determined by using deionized water samples adjusted to pH 6.0, 7.0, and 9.0 with 1.0M NaOH and 1.0M KH_2PO_4. Well water samples were pH 8.0. Three replicates of deionized water at each pH and 6 of well water were spiked with leuco malachite green and malachite green oxalate (chromatic form) at 50.0 µg/L each, concentrated on the diol columns, and analyzed by the LC procedure.

The minimum detection limit was determined by analyzing 7 replicate samples of well water, pH 8, each spiked with 25.0 µg/L leuco malachite green and malachite green as specified by the U.S. Environmental Protection Agency (EPA) procedure (15). The concentration of 25 µg/L was estimated to be the limit of detection from previous chromatograms.

Three replicate samples of water from a pond that contained fish were spiked at 25.0, 50.0, and 100 µg/L each of leuco malachite green and malachite green oxalate. The samples were adjusted to pH 6, and leuco and chromatic malachite green were determined by using LC with the diol column extraction method. Three well water samples (pH 8.0) were spiked with malachite green carbinol at 50.0 µg/L, concentrated on the diol column, extracted, and analyzed by the LC procedure. The conversion of recovered malachite green to malachite green carbinol was calculated as follows:

$$\text{Mgr-to-carbinol conversion factor} = \text{MW carbinol (346.48)}/[\text{MW Mgr oxalate (927.02)}/2] = 0.85866$$

Results and Discussion

Solid-phase extraction of malachite green from water was investigated by using C_{18}, phenyl, octyl, and diol extraction columns. The most satisfactory recoveries were obtained with the diol columns. The mean recoveries for chromatic malachite green in deionized or well water were 98.3% at pH 6.0, 91.7% at pH 7.0, 98.3% at pH 8.0, and 99.3% at pH 9.0; mean recoveries of leuco malachite green at the respective pH levels were 61.2, 50.1, 48.3, and 39.1% (Figure 2). Although the recovery of chromatic malachite green was apparently not affected by changes in pH, the recovery of leuco malachite green decreased as the pH increased. The extraction of leuco malachite green from water by the diol column decreased not only at the higher pH values but also at pH values less than 6.0; at these lower pH values, the malachite green becomes protonated and its retention in the column is reduced. Therefore, water samples should be adjusted to pH 6.0 before extraction to obtain the optimum recovery of malachite green residues. The minimum

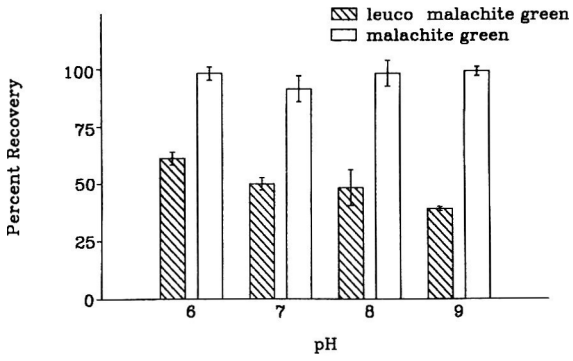

Figure 2. Mean percent recoveries and standard deviations of leuco and chromatic malachite green from waters of various pH levels (n = 3).

detection limits, as determined by the EPA method (15) for samples spiked with both forms of malachite green at 25 µg/L, were 2.83 µg/L for the chromatic form and 2.01 µg/L for the leuco form.

Recoveries of malachite green residues (chromatic, leuco, and total) from pond water spiked with both the chromatic and leuco forms of malachite green at 25.0, 50.0, and 100 µg/L are shown in Table 1. The concentration of the spike did not appear to influence the recovery of malachite green residues in pond water. No interference in the analyses were noted from the pond water samples (Figure 3).

Malachite green carbinol is an acid-base equilibrium product of malachite green in water. An average of 98.6% was recovered from well water samples spiked with malachite green carbinol at 50 µg/L (Table 2). The acidic condition of the diol column extraction converted the carbinol form of malachite green to the chromatic form, after which the equivalents of carbinol were calculated.

Table 1. Mean percent recoveries and standard deviations (in parentheses) of malachite green residues from pond water spiked at concentrations of 25.0, 50.0, and 100 µg/L each of leuco malachite green (Leuco) and malachite green (Mgr) (n = 3).

	Mean rec., %		
Chemical form	25 µg/L[a]	50 µg/L	100 µg/L
Leuco	58.5	55.1 (4.7)	62.5 (0.89)
Mgr	96.2	93.0 (3.5)	97.0 (2.3)
Leuco + Mgr	77.4	74.0 (3.8)	79.8 (0.85)

[a] Only 2 samples were analyzed at 25 µg/L, and no standard deviation was calculated.

Our method allows for the simple and rapid determination of malachite green residues in water. Extraction of both the chromatic and leuco forms of malachite green on the diol column and their subsequent elution with *p*-toluenesulfonic acid take about 20 min. LC determination with the postcolumn oxidation reactor (14) and conventional UV-vis detector takes about 17 min/sample, after standards have been run. The method shows good reproducibility and simultaneously detects the chromatic, leuco, and carbinol forms of malachite green at concentrations of less than 10 µg/L. Although the recovery of leuco malachite green is low, the reproducibility and the high sensitivity of the method allow the leuco form to be detected at very low concentrations. The low recovery of leuco malachite green may be due to the use of a polar solid-phase extraction column, as well as possible photolysis of the leuco product.

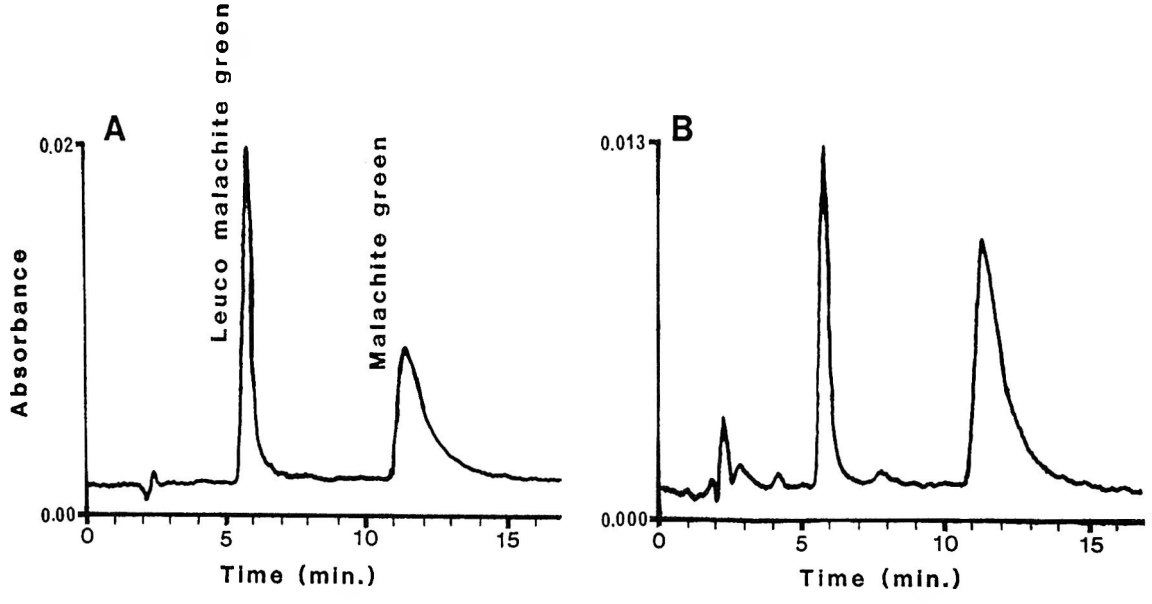

Figure 3. Chromatograms of 25 ng each of leuco malachite green and malachite green oxalate standards (A) and an extract of pond water spiked with leuco malachite green and malachite green oxalate (B).

Table 2. Mean recovery and standard deviation (in parentheses) of malachite green carbinol from well water spiked with 50 μg/L carbinol (n = 3)

		Carbinol	
Sample No.	Mgr rec., μg/L[a]	Concn rec., μg/L	Rec., %
1	57.2	49.1	98.2
2	59.1	50.7	101
3	56.3	48.4	96.7
Mean rec., %			98.6 (2.2)

[a] The residue recovered as malachite green was converted to the equivalent of malachite green carbinol by multiplying the malachite green residues by 0.85866.

Roybal et al. (11), in determining crystal violet and leuco crystal violet, achieved good selectivity and sensitivity with a liquid chromatograph and an electrochemical detector. However, the UV-vis detector used in our method is generally more available, and detection in the visible spectrum also increases the specificity of the analysis.

We were able to simultaneously determine both the parent dye and its leuco form at a single wavelength by placing the reaction chamber containing PbO_2 between the LC column and the spectrophotometric detector. Bauer et al. (13) also used PbO_2 oxidation to determine the chromatic and leuco forms of malachite green in fish tissue, but not simultaneously. Data on the presence and amount of leuco malachite green were still determined by the difference in concentrations obtained from 2 injections. In our system, more than 600 dye samples have been injected into the reaction chamber with no noticeable impairment in the conversion of the leuco form to the chromatic form. However, the PbO_2 will eventually be depleted, and the reaction chamber should be checked periodically to ensure that it is operating efficiently (14).

Acknowledgments

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Determination of Leucogentian Violet in Chicken Fat by Liquid Chromatography with UV Detection

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UV detection is proposed as an alternative to electrochemical detection for the determination of leucogentian violet (LGV) in chicken fat by liquid chromatography. UV detection was combined with a previously reported extraction technique and new chromatographic conditions to yield a method that was evaluated at the target level of 10 ppb. Baseline separation of LGV from matrix interferences was achieved with a C_{18} bonded silica column and a mobile phase of methanol–pH 4 ammonium acetate (90 + 10). Average recovery for samples fortified at 5, 10, and 20 ppb was 76.4%, with a coefficient of variation (CV) of 4.8%. Fat from chickens raised on feed medicated with 30 ppm gentian violet was found to contain 17.6 ppb LGV, with a CV of 7.9%, after a 3-day withdrawal period. The detection limit for the system used was estimated to be 0.4 ng, and the limit of quantitation for the method was estimated to be 2 ppb.

The triphenylmethane dye gentian violet (GV) has been used as a mold inhibitor in poultry feed for some time. There is now a concern over health risks associated with consumption of poultry raised on GV-containing feed (1, 2). The disposition of GV and its polar metabolites has been studied in various chicken tissues by autoradiography (2) and in liver and muscle by liquid chromatography with electrochemical detection (LC/ECD) (3). Nonpolar residues of GV can also be found after a metabolic reduction to leucogentian violet (LGV). Prompted by health concerns, the U.S. Food and Drug Administration (FDA) has pursued the development of regulatory methods capable of determining LGV residues in fat at the 10 ppb level. Recently, the LC/ECD technique has been applied to the determination of LGV for regulatory use (4).

The method reported here provides the analyst with a more accessible alternative to ECD. LGV has a UV chromophore at 265 nm with adequate absorptivity for use in a determinative procedure. (The reduced form of GV is colorless, and, thus, is not amenable to detection at a visible wavelength.) UV detection was combined with reversed-phase LC and applied to fat samples extracted as described by Munns et al. (4). The feasibility of this approach has been tested, and although the detection limit is not as low as that reported for the LC/ECD method, it is still possible to measure LGV at an estimated limit of 2 ppb. The results of an internal validation of this method, performed at the FDA's Center for Veterinary Medicine, Division of Veterinary Medical Research, are included.

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Tissue that has been stored at –20°C or below is sliced into small pieces with a knife and then thoroughly blended with an immersion blender. From this point forward, tissue is extracted as described by Munns et al. (4). In the last step, acetonitrile is evaporated to about 1 mL, and 800 µL water is added. The solution is then diluted to 2.0 mL with acetonitrile.

For the validation data reported here, 100 µL or 5% of the extract was injected on-column. A reversed-phase column packing consisting of octadecylsilane groups bonded to silica was used with a mobile phase of methanol–pH 4 ammonium acetate buffer (90 + 10). Peak heights were measured manually by using the strip chart output and a detector attenuation of 0.01 AUFS. Quantitation was based on a standard curve prepared from LGV injections of 1.25–10 ng, equivalent to a range of 2.5–20 ppb. The chromatographic procedure in this method uses a C_{18} column rather than a cyano column, but this still results in baseline separation of LGV from other UV chromophores and from demethylated GV metabolites.

METHOD

The apparatus and reagents as described by Munns et al. (4) are listed here for convenience and are highlighted by asterisks. The procedural steps are not repeated but can be found in Reference 4 under *Extraction.*

Apparatus

(a) *Liquid chromatograph.*—Series 410 LC pump, ISS-100 autoinjector with 200 µL loop, and LC-235 diode array UV detector with accompanying GP-100 recorder (Perkin-Elmer Corp., Norwalk, CT 06856), or equivalent. This detector monitors a 5 nm window in the UV spectrum and was used to determine the wavelength of maximum absorbance for LGV (265 nm).

(b) *LC column.*—DuPont Zorbax ODS column, 150 × 4.6 mm, 5 μm particles, used at ambient temperature. (Data shown here were acquired with this column. It is no longer made by DuPont, but a similar product is available from MAC-MOD Inc., Chadds Ford, PA.) The column was preceded by a 20 mm guard column (LC-18-DB, Supelco, Inc., Bellefonte, PA 16823). A Supelco LC-18-DB column, 150 × 4.6 mm, 5 μm particles, was evaluated and can also be used as an analytical column if the mobile phase is adjusted to methanol–pH 4 ammonium acetate buffer (86 + 14).

(c) *Polypropylene beaker.*—1 L disposable (Cat. No. B2722-1LA, Baxter Healthcare Corp., Scientific Products Division, McGaw Park, IL 60085).

(d) *Immersion blender.*—Kitchenmate immersion blender (Quantum Marketing, Columbus, OH). Alternative to Hobart blender.

(e) **Beaker.*—Phillips, 125 mL (Cat. No. 1080, Corning Glass Works, Corning, NY 14831).

(f) *Syringe.*—100 μL (Cat. No. 710, Hamilton Co., Reno, NV 89510).

(g) *Funnel.*—75 mm diameter (Pyrex, Cat. No. 618075, Corning).

(h) **Filters.*—Whatman glass fiber, 934-AH, 11 cm (Cat. No. 1827-110, Whatman, Inc., Clifton, NJ 07014); Millipore disposable 0.45 μm polytetrafluoroethylene (PTFE) membrane (Cat. No. SLSR 025 NB, Millipore Corp., Bedford, MA 01730).

(i) **Separatory funnel.*—125 mL (Pyrex, Cat. No. 6402, Corning).

(j) **Flask.*—Pear-shape, 100 mL, 24/40 *T* (No. K-608700, Kontes Co., Vineland, NJ 08360).

(k) *Dispensers.*—Repipet, 50 mL capacity, adjusted to 25 mL (Cat. No. 3050A, LabIndustries, Berkeley, CA 94710); 10 mL tilting dispensor (Cat. No. 759301-0010, Kontes).

(l) *Volumetric pipet.*—5 mL (Cat. No. 761000-005, Kontes).

(m) **Centrifuge tubes.*—15 mL, graduated (Cat. No. 45153-15, Kimble Division, Owens-Illinois, Inc., Toledo, OH 43666).

(n) **Rotary evaporator.*—Buchi Model R-110 with cold trap (Brinkmann Instruments, Inc., Westbury, NY 11590).

(o) **Evaporator.*—N-Evap Model 111 (Organomation Associates, Inc., South Berlin, MA 01549-0159).

(p) *Volumetric tubes.*—2 mL, Class A (Pyrex, Cat. No. 56402, Corning).

(q) *Volumetric flasks.*—5 and 100 mL, Class A (Kimax, Cat. No. 280145, 28014100, Kimble).

(r) *Pipettors.*—Eppendorf 100–1000 μL adjustable, with disposable pipet tips (Brinkmann).

Reagents and Solutions

(ACS grade, except where specified otherwise.)

(a) *Methylene chloride.*

(b) **Sodium citrate.*—Trisodium citrate. Use to prepare saturated solution containing ca 130 g/100 mL.

(c) *Hydrochloric acid.*—1N. Combine 41 mL with 459 mL water.

Table 1. Quantities for preparation and injection of standards

	Combine (volume in μL)			Inject 100 μL		
Flask	Stock (*2*)	ACN	H_2O	ng/μL	ng	equiv. ppb
1	500	2500	2000	0.1	10	20
2	250	2750	2000	0.05	5	10
3	125	2825	2000	0.025	2.5	5
4	62	2938	2000	0.0124	1.24	2.5

(d) *Water.*—Prepared with Barnstead Organopure system (Barnstead Co., Boston, MA 02132), or equivalent LC/UV grade.

(e) *Aqueous buffer.*—(*1*) Ammonium acetate, 50mM: Weigh 3.87 g ammonium acetate, transfer to 1 L volumetric flask, and dilute to volume with water. (*2*) Acetic acid, 1M: Measure 57.5 mL glacial acetic acid into 1 L volumetric flask, and dilute to volume with organopure water. (*3*) Combine 710 mL of (*1*) with 100 mL of (*2*) to give ca pH 4, and filter through nylon filter with 0.45 μm pores.

(f) *Standard.*—Leucocrystal violet (Cat. No. L-5760, Sigma Chemical Co., St. Louis, MO 63178).

(g) *Stock solutions.*—(*1*) Accurately weigh 10 mg LGV standard, transfer to 100 mL volumetric flask, dilute to volume with LC grade methanol, and sonicate (100 ng/μL). (*2*) Transfer 1 mL to another 100 mL volumetric flask and dilute to volume with LC grade acetonitrile (1 ng/μL). These solutions are stable in the refrigerator (ca 4°C) for 3 months.

(h) *Injection standards.*—To four 5 mL volumetric flasks, add first the acetonitrile, then the stock solution (*2*), and then 2 mL water. Dilute to 5 mL with acetonitrile. (There is a negative volume of mixing for this solvent pair.) These standards can be prepared weekly, if stored at room temperature. Use quantities shown in Table 1.

Sample Preparation

Store tissues at –20°C or below, and prepare by slicing them into small pieces with knife. Blend thoroughly while still cold in polypropylene beaker with immersion blender. (It is convenient to blend 100 g at one time and store at –80°C until ready to extract.)

Extraction

Weigh 10 g samples of blended fat for extraction. Prepare fortified controls by adding standard stock solution (*2*) from 100 μL syringe directly onto cold fat (1 μL added to 1 g tissue = 1 ppb). Let samples sit for 5 min after fortification. Working at steady pace, follow Reference 4 from this point forward, except for final step. Note that fat that has been blended with immersion blender melts to produce yellow oil, whereas oil does not separate from fat ground in Hobart food chopper (4). After acetonitrile solution is reduced to ca 1 mL, mix by vortexing and transfer to 2 mL volumetric tube. Add 800 μL water, and dilute to 2 mL (±0.02) with acetonitrile. Filter through recommended 0.45 μm PTFE filter before injection on LC.

Liquid Chromatography

Use mobile phase of methanol–pH 4 ammonium acetate buffer (90 + 10) at flow rate of 1.0 mL/min. Different C_{18} column may require minor adjustment of solvent proportions. Program autoinjector to inject 100 µL (5% of extract). Retention time of LGV is 8–9 min with Zorbax column, and 7–8 min with Supelco column, depending on ambient temperature. Inject all standards before and after extracts. Adjust attenuation so that peak for high level standard (20 ppb) is 80–90% full scale. If samples larger than this are expected, set detector to higher attenuation and prepare standard curve to cover higher concentration range with at least 3 calibration points. Integrator can be used to measure peaks drawn off scale, provided base line is checked for accuracy. Alternatively, high level samples can be diluted by known factor to bring them into calibration range upon reinjection.

Calculations

The procedure measures nanograms injected on-column. Convert to ppb (ng/g) in fat sample as follows:

(**a**) Apply linear regression, using all standards and without including origin or blanks. Calculate standard curve of form $y = mx + b$ (peak height = slope × ng + y intercept).

(**b**) Determine ng injected from standard curve applied as in (**a**) to peak height measured for each sample.

(**c**) Because 5% of sample is injected, ppb = (measured ng × F)/(0.05 × g tissue).

(**d**) Percent recovery in fortified tissue = 100 × (measured ng × F)/(0.05 × total LGV added).

Dilution factor F is used when high level samples are diluted (e.g., F = 2 for 1 + 1 dilution). If no dilution was made, F = 1.

Notes

(**a**) Extracts remain stable at ambient temperatures for several days.

(**b**) Quick and dramatic loss of LGV response occurred when extracts were stored below 0°C in freezer.

(**c**) To avoid cross-contamination of glassware, centrifuge tubes, volumetric flasks, beakers, and separatory funnels should be rinsed with 1N HCl, distilled water, and methanol after detergent washing.

(**d**) During rotary evaporation step, solutions should not be allowed to go to dryness, as LGV may be adsorbed to glassware or converted to GV.

(**e**) Solutions should not be exposed to metal ions (4).

Results and Discussion

The results of a validation study of this method are presented in Tables 2 and 3. The study consisted of 5 replicates each of control tissue fortified at 5, 10, and 20 ppb and 5 tissue samples from dosed chickens. The dosed chickens received feed containing GV at 30 ppm for 6.5 weeks and were sacrificed 72 h after withdrawal from this feed. The extractions were performed on 5 different days, and blank controls were analyzed on Days 1, 3, 4, and 5. No LGV signals were observed in the blank controls. Samples for Days 4 and 5 were extracted on consecutive days and were analyzed together on Day 5. Standard curves were linear (r ranged from 0.9993 to 1.000), and y intercepts were nearly zero (<0.3% FSD negative offset

Table 2. Recovery of LGV from fortified control fat

Nominal ppb[a]	Day	Found, ppb	Rec., %
5	1	3.93	80.2
	3	3.78	77.6
	4	3.56	71.1
	5	4.05	80.2
	5	3.78	75.6
Mean			76.9
SD			3.79
CV, %			4.9
10	1	7.36	76.3
	3	7.98	78.6
	4	7.99	78.8
	4	6.99	69.7
	5	7.34	74.3
Mean			75.5
SD			3.75
CV, %			5.0
20	1	15.4	76.5
	3	15.8	77.9
	4	15.3	75.9
	4	14.4	71.3
	5	16.7	82.7
Mean			76.9
SD			4.10
CV, %			5.3
All fortified samples			
Mean			76.4
SD			3.66
CV, %			4.8

[a] Fat samples were not exactly 10 g fat, so the ppb levels listed in this column are only nominal values. However, the exact ppb levels from each fortified sample were used to calculate recoveries.

Table 3. Determination of LGV residue in dosed tissues

Day	Retention time, min	Found, ng	Found, ppb
2	8.55	7.94	15.6
2	8.49	8.99	18.2
3	8.87	9.90	19.4
3	8.98	8.85	17.7
5	8.96	8.96	17.3
Mean			17.6
SD			1.39
CV, %			7.9

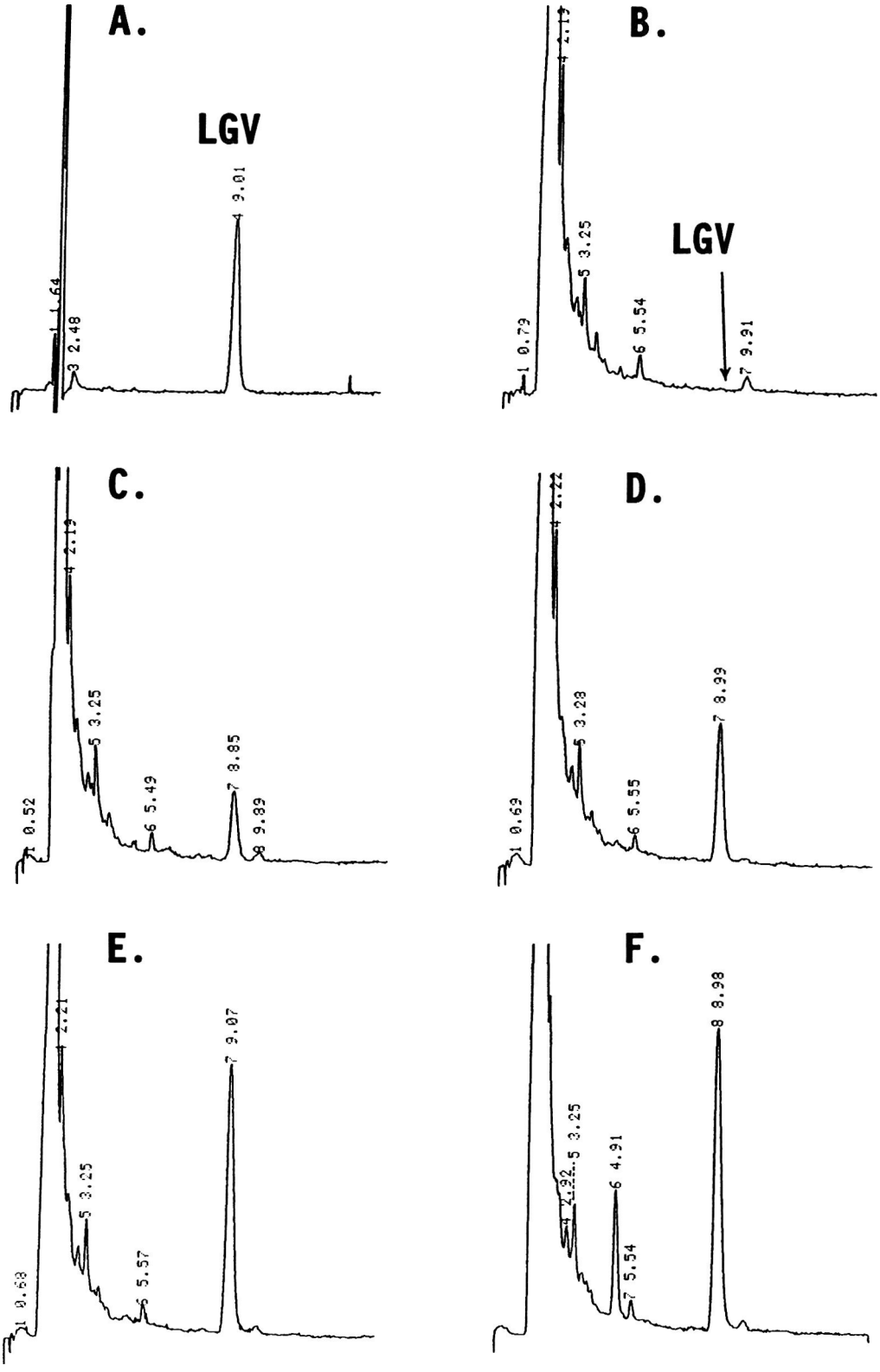

Figure 1. UV determination of leucogentian violet in chicken fat: A, 5 ng leucogentian violet, equivalent to 10 ppb; B, blank control; C, 5 ppb fortified control; D, 10 ppb fortified control; E, 20 ppb fortified control; and F, incurred residues. All chromatograms were acquired at a detector attenuation of 0.01 AUFS.

at origin). Recovery of about 75% of fortified LGV was consistently obtained.

Retention time was sensitive to ambient temperature. Retention times sometimes varied from one day to another (Table 3). On some occasions, retention time drifted during a set of analyses. For this reason, a column heater may be necessary when environmental conditions are not well controlled. Nevertheless, it was possible to identify the LGV peak by tracking parallel changes in matrix peak retention times. The acetonitrile–water proportion of injection solvents also affected retention time. This source of variability was minimized by use of the 2 mL volumetric tubes.

Figure 1A shows a typical chromatogram from the LGV standard. Analyses of blank controls show that no matrix interference elutes at the LGV retention time (Figure 1B). The method is, thus, in accord with FDA requirements that interferences be <10% of the target concentration (10 ppb). A small matrix peak eluted fully resolved from LGV about 1 min later. Figures 1C, D, and E show chromatograms from the analysis of controls fortified at 5, 10, and 20 ppb, respectively; Figure 1F shows LGV recovered from dosed chicken tissue. The metabolite leucomethyl violet, resulting from reduction of GV and loss of one methyl group, appeared in the chromatogram at 4.91 min. This peak can provide corroborative evidence that LGV residues are present. It may also be observed as an impurity in the LGV standard, at <1% relative abundance.

Estimation of the limit of quantitation (LOQ) was based on the apparent peak height of noise signals in blank controls. When the LOQ was defined as the mean (in ng) plus 10 times the standard deviation of these measurements, a value of 0.85 ng was obtained. This is equivalent to an LOQ of 1.7 ppb (rounded to 2 ppb) if 5% of the extract from 10 g fat is injected. When the limit of detection was defined as equal to the mean (in ng) plus 3 times the standard deviation in blanks, a value of about 0.4 ng was obtained. In repetitive injections of 0.5 ng standard on-column, the average ratio of signal height to peak-to-peak noise was about 5. The LC/ECD technique has lower detection limits (roughly 0.04 ng LGV), or about one order of magnitude lower than the limit for UV detection (5). The UV detector, therefore, required a larger injection volume than the LC/ECD system as described by Munns et al. (4) (100 µL compared to 20 µL).

The chromatography used here was developed during a study of LC/mass spectrometry (MS) using the particle beam interface. The particle beam mechanism is optimized with high organic solvent content and volatile buffers; therefore, a C_{18} column, 90% methanol, and an ammonium acetate buffer were used. The LC/MS system was also used to identify the leucomethyl violet metabolite peak by measuring its molecular weight and characteristic electron impact mass spectrum. The compatibility of UV detection with the chromatographic system as described by Munns et al. (4) was also evaluated. Analysis of a blank control showed that no UV matrix interferences were observed at LGV retention time. The larger injection volume (100 vs 20 µL) required a lower flow rate (0.8 mL/min) so that demethylated metabolites from a sample of dosed chicken fat could be resolved from LGV (see Figure 1D, Reference 4).

Acknowledgments

Dosed and control tissues were provided by David Wagner and Herbert Righter of the Animal Nutrition and Biology Branch, DVMR.

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Matrix Solid-Phase Dispersion Extraction and Liquid Chromatographic Determination of Ivermectin in Bovine Liver Tissue

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A method for the isolation and liquid chromatographic (LC) determination of the antiparasitic drug ivermectin in bovine liver is presented. Liver samples (0.5 g) are blended with 2 g C_{18} (octadecylsilyl-derivatized silica packing material). A column made from the C_{18}–liver matrix is washed with 3 mL hexane, then the ivermectin is eluted with methylene chloride–ethyl acetate (3 + 1). After purification by alumina solid-phase extraction, the ivermectin is derivatized and analyzed by LC with fluorescence detection. The overall recovery of ivermectin added to liver was 74.6%. The lowest level validated in tissue by the method was 10 ppb, and the limit of detection was 1 ppb. This method and a classical extraction method gave comparable results for a liver sample that contained incurred ivermectin residues. The method uses small volumes of solvents, has a limited number of sample manipulations, and does not require solvent partitioning or backwashing of extracts. These characteristics make this method attractive when compared to classical isolation procedures for ivermectin.

Ivermectin is a potent antiparasitic agent derived from the mycelia of *Streptomyces avermectilis*. It is a mixture of >80% 22,23-dihydroavermectin B_{1a} and <20% 22,23-dihydroavermectin B_{1b}. The structure of these compounds is shown in Figure 1. Ivermectin residues are depleted from the animal rather slowly. Tway et al. (1) found the unaltered drug in cattle liver tissue over a period of 28 days postadministration, and Alvinerie et al. (2) found detectable residues of ivermectin in cow's milk up to 3 weeks postadministration. Ivermectin is presently registered for use in beef cattle, reindeer, swine, and sheep. The tolerances are 15, 15, 20, and 30 ppb dihydroavermectin B_{1a}, respectively, and liver is the target tissue (3).

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Liquid chromatographic (LC) methods using UV detection have been reported for the determination of ivermectin in plasma (4–6) and milk (2), but UV detection is not sufficiently sensitive or specific enough to detect low levels of ivermectin in tissue. Tway et al. (1) used a fluorescent derivative to determine ivermectin in tissue at 10 ppb with a limit of detection of 1–2 ppb. Unfortunately, this method employs time-consuming multiple extraction and solvent partition cleanup steps.

Recently, Barker et al. (7) demonstrated that biological matrixes can be homogeneously dispersed with C_{18} (40 μm octadecylsilyl-derivatized silica), the resulting mixture packed into a column, and various residues selectively eluted from the column. This matrix solid-phase dispersion (MSPD) method provides a rapid alternative to conventional methods for tissue extractions. It eliminates the need for tedious homogenization and multiple solvent partition cleanup steps. The MSPD method has been successfully used for the isolation of sulfonamides, benzimidazoles, chlorsulfuron, chloramphenicol, furazolidone, and tetracyclines in milk as well as sulfonamides, benzimidazoles, organophosphates, and β-lactams from animal-derived matrixes (7–13).

Although the MSPD extraction of drug residues from muscle and milk provides a sufficiently clean extract for LC determination, the extraction of liver results in too many coextracted constituents. We report here the first application of MSPD combined with solid-phase extraction (SPE) for the isolation of spiked and incurred ivermectin residues from liver tissue followed by LC determination using the fluorescent derivatization method of Tway et al. (1).

Experimental

Reagents

(a) *Solvents.*—*N,N*-Dimethylformamide (DMF), Photrex reagent grade (J.T. Baker, Inc., Phillipsburg, NJ); hexane, distilled-in-glass, non-UV grade; all other organic solvents were

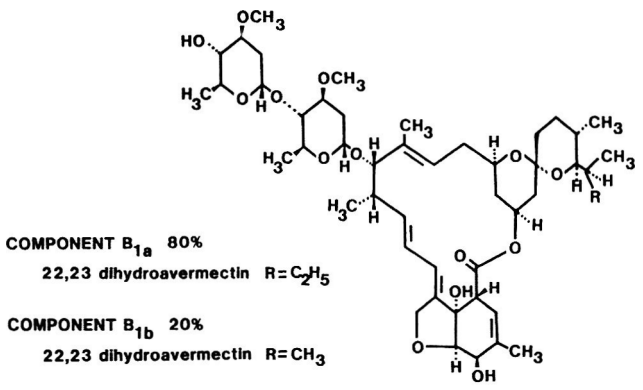

Figure 1. Structure of ivermectin.

distilled-in-glass, suitable for spectrophotometry, and LC grade (EM Science, Gibbstown, NJ).

(**b**) *Acetic anhydride.*—Analytical reagent grade (Mallinckrodt, Inc., Paris, KY).

(**c**) *1-Methylimidazole.*—99% pure (Aldrich Chemical Co., Milwaukee, WI).

(**d**) *Water.*—For LC analysis, distilled and deionized (Milli-Q Water System, Waters Corp., Milford, MA).

(**e**) *Ivermectin reference standard.*—1.38% (w/w) dihydroavermectin B_{1a} in glycerol formal, Lot No. L640,471-076P003 (Merck Sharpe and Dohme Research Laboratories, Rahway, NJ).

(**f**) *Working standard solution.*—500 ng/mL dihydroavermectin B_{1a} was prepared in methanol and stored at −20°C. Working standard solutions stored under these conditions are stable at least 4 years. Aliquots of the working standards were derivatized to obtain a standard curve with each set of samples. The derivative is not stable for long-term storage.

(**g**) *Column material.*—Bulk C_{18}, Bondesil, 40 μm, 18% load, endcapped, octadecylsilyl-derivatized silica (Analytichem International, Harbor City, CA).

(**h**) *Liver tissue.*—Control beef liver tissue was obtained from a local market and ground 3 times in a meat grinder. A beef liver sample containing incurred ivermectin residue was obtained from U.S. Department of Agriculture, Food Safety and Inspection Service, Athens, GA. Tissue samples were stored frozen at −20°C.

Apparatus

(**a**) *Syringe barrels.*—Used as extraction columns; empty reservoirs, 8 mL size, and frits (Analytichem International).

(**b**) *SPE cartridges.*—SepPak Alumina-B, No. 51820 (Waters Corp., Milford, MA). Supelclean LC–silica SPE cartridges, 3 mL size (No. 5-7010, Supelco, Inc., Bellefonte, PA).

(**c**) *Agate mortar and pestle.*—75 mm od (Thomas Scientific, Swedesboro, NJ).

(**d**) *SPE vacuum manifold.*—Supelco, Inc.

(**e**) *Adapters.*—Used to attach Alumina-B SepPak cartridges to manifold. The tapered ends of yellow (5–100 μL) disposable pipet tips were cut so that they would fit into the manifold. The cartridges were fitted into the large-diameter end of the tip.

(**f**) *Silylated conical centrifuge tubes.*—Used for derivatization reaction, 15 mL size, conical borosilicate glass centrifuge tubes were silylated with Sylon CT (Supelco, Inc.) following the procedure of Tway et al. (1). These tubes were then used for 2 months before the silylation was repeated. After each derivatization, tubes were soaked 2 h in methylene chloride, followed by hand washing with soap and water. (**Note**: If the tubes are machine washed, the silylation procedure will have to be repeated.)

(**g**) *LC system.*—Model 250 isocratic LC pump (Perkin-Elmer Corp., Norwalk, CT), loop injector with 50 or 100 μL loop (Rheodyne Corp., Cotati, CA), Model FS 970 fluorescence detector (ABI-Kratos, Inc., Ramsey, NJ). Column: 5 μm Econosil C_{18}, 250 × 4.6 mm (Alltech Associates, Deerfield, IL) and Newguard C_{18} guard column cartridge (Brownlee Labs, Santa Clara, CA). Operating conditions: mobile phase, methanol–water (95 + 5); flow, 2.0 mL/min; column temperature, 37°C; retention time of fluorescent product, ca 14 min. Detector parameters: excitation wavelength, 364 nm; excitation filter, Corning 7-54; emission filter, 418 nm cutoff filter; range, 0.2–1.0 μA. Injection volume, 100 μL for 10 ppb fortification level, 50 μL for higher levels.

Extraction Procedure

Weigh 2 g C_{18} packing into a mortar, and place liver sample composite (0.5 g) onto the C_{18}. For recovery studies, inject an appropriate volume of working standard solution (500 ng/mL) into the tissue using an LC syringe and allow tissue to set 5 min. Blend tissue with the C_{18} with an agate pestle until a homogeneous mixture is obtained. Transfer the resultant C_{18}–liver matrix to an empty syringe barrel (reservoir) fitted with a frit. Compress column using a glass syringe plunger, and place column on a vacuum manifold. Wash column with hexane (3 mL), using ca 2 in. Hg vacuum. When all hexane is eluted, increase vacuum to maximum for 5 s to remove any residual hexane. Remove column from the manifold and attach a methylene chloride-washed Alumina-B SPE cartridge below the C_{18}–tissue column. Attach the tandem columns to vacuum manifold, using an adapter. Elute tandem columns with three 2 mL aliquots of methylene chloride–ethyl acetate (3 + 1) using ca 2 in. Hg vacuum. Discard eluates. Remove and discard upper (C_{18}–liver) column. Draw air through alumina cartridge for 5 s. Attach an empty syringe barrel to top of alumina cartridge, and wash cartridge with 1 mL acetone using 2 in. Hg vacuum. Increase vacuum to maximum, drawing air through cartridge for 5 s. Remove cartridge from manifold and elute with three 2 mL aliquots of methanol, collecting eluates in a silylated centrifuge tube. (**Note**: The methanol elution is performed without vacuum. It may be necessary to initiate the flow by applying pressure to the column head with a pipet bulb.)

Fluorescent derivative formation of samples and standards.—To each of 5 empty silylated centrifuge tubes, using an LC syringe, add aliquots of working standard solution appropriate to bracket the residue level in the tissue. Evaporate the methanol in the silylated tubes containing the standards and sample (from the extraction) to *dryness* under nitrogen at 60°C.

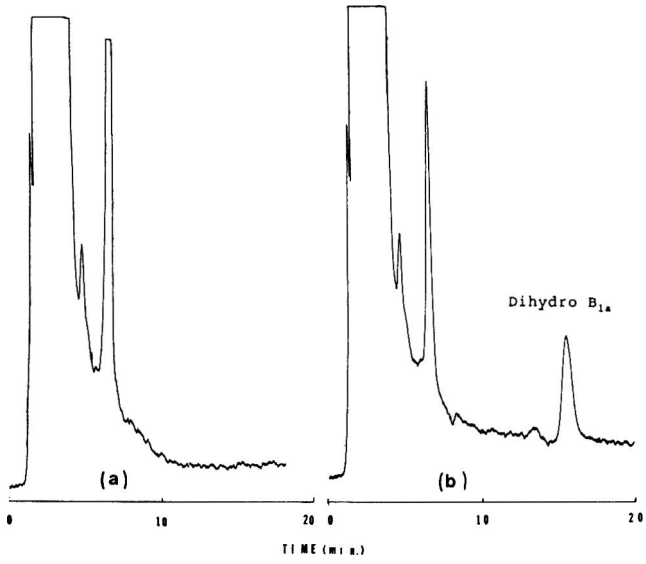

Figure 2. Typical liquid chromatograms of sample extracts: (a) control bovine liver sample; (b) bovine liver fortified with 20 ppb dihydroavermectin B_{1a}.

(**Note**: Any residual methanol or moisture will interfere with the derivatization.)

Using a 1.0 mL measuring pipet, add 0.1 mL freshly prepared mixture of DMF–acetic anhydride–1-methylimidazole (9 + 3 + 2) to each of the sample and standard tubes. Stopper tubes tightly with polypropylene stoppers, vortex briefly, and place in a 95°C oil bath for 1 h. (**Note**: Liquid in tubes should become dark and opaque. If liquid in any of the tubes is light colored or transparent, the derivatization reaction may have been inhibited by the presence of residual moisture, methanol, or tissue components. In this case, the extraction and derivatization will have to be repeated.)

Let tubes cool and add 1 mL chloroform. Meanwhile, prepare silica SPE cartridges by washing with ca 3 mL chloroform. Place silica cartridges on the vacuum manifold, and place 100 × 16 mm borosilicate culture tubes under the cartridges to collect the eluate. Set the vacuum at ca 2 in. Hg. Using a Pasteur pipet, transfer contents of centrifuge tubes to silica SPE cartridges. Wash each silylated centrifuge tube 3 times with 2 mL chloroform, adding chloroform to silica cartridges. Elute cartridges with an additional 2 mL chloroform. Evaporate chloroform eluate to dryness under nitrogen at 60°C. Dissolve residue in 0.5 mL methanol, and inject onto chromatograph.

The samples and standards are quantitated by peak-height measurements. Obtain a standard graph by plotting peak height (mm) vs standard concentration (ng/mL). The standard curve should be linear with a regression coefficient (r) of >0.98.

Table 1. Recoveries of ivermectin from bovine liver samples fortified with dihydroavermectin B_{1a}

Fortification level, ng/g	Rec., %[a]	% RSD
10	75.3	11.4
15	72.1	10.6
20	74.1	9.9
25	77.4	10.3
30	73.7	12.9
40	74.9	7.3

[a] Based on 5 determinations at each concentration.

Results

Representative liquid chromatograms of blank and fortified liver samples are shown in Figures 2(a) and (b), respectively. Table 1 shows the concentrations examined and the percentage ivermectin recovered from fortified liver samples. Table 2 compares the ivermectin residue recovered by MSPD vs a classical extraction method from a liver sample containing incurred residue.

A typical standard curve encompassing a range of 7.0–40 ng/mL ivermectin was linear (correlation coefficient, $r = 0.998$) with a slope of 3.11 and y-intercept of 3.18. The lowest level validated in tissue by this method was 10 ppb. At this level, an LC peak of ca 35 mm was generally observed, and reproducible recoveries were achieved. Discernable peaks were visible at ca 1 ppb ($S/N = 3$). The relative standard deviations (RSDs) of replicate standard injections ($n = 3$) for 10, 20, and 40 ng/mL standards were 6.1, 4.8, and 3.2%, respectively.

Discussion

The isolation of drug or chemical residues from tissue can be a time-consuming and laborious task. Traditional methods for the isolation of ivermectin from liver tissue can include multiple homogenization and centrifugation steps, followed by evaporation of large volumes of solvent, and further cleanup by hexane–acetonitrile and (acetonitrile + water)–hexane solvent partition steps (1).

The extraction of ivermectin from liver tissue using MSPD rapidly yields extracts with a minimum of interfering coextractants. When tissue is blended with the lipophilic C_{18}, the latter serves to disrupt the lipid bilayer of the cell membranes. The cellular components and any drug residues are essentially dispersed over a large surface area (1000 m^2 per 2 g C_{18}), thereby exposing the entire sample to the extraction process. Even though the volume of extracting solvents is small (6 mL), the process can be envisioned as an exhaustive extraction whereby a large volume of solvent is passed over an extremely thin layer of sample. Lipids were eluted from the C_{18}–liver complex with hexane while the ivermectin was retained.

Table 2. Incurred Ivermectin residues recovered from beef liver by 2 different methods

Method	No. of determinations	Drug residue, ppb[a]	% RSD
MSPD	6	13.0	9.2
Tway et al. (1)	3	12.8	2.8

[a] Ivermectin B_{1a}.

Methylene chloride only partially eluted ivermectin from the C_{18}–tissue complex, while ethyl acetate eluted too much tissue material along with the ivermectin. Ivermectin was eluted from the MSPD complex with a mixture of methylene chloride–ethyl acetate (3 + 1).

This eluate still contained tissue components that were evident when the solvent was evaporated. If certain unidentified tissue components are present above a certain threshold level, the subsequent derivatization reaction will be inhibited in direct proportion to the amount of tissue material present (F.J. Schenck, 1986, unpublished data). Cleanup of the MSPD eluate with commercially available alumina, silica, and Florisil SPE cartridges was attempted. The alumina SPE cartridge resulted in a significant cleanup of the MSPD eluate. This was evident because of the minimal residue remaining after the solvent was evaporated and because the number of extraneous peaks present when the ivermectin derivative was chromatographed was less than when Florisil or silica cartridges were used.

Ivermectin was tightly adsorbed to the basic alumina, and as much as 10 mL acetone would not elute any ivermectin. Conversely, as little as 1 mL acetone eluted pigmented residue material. The ivermectin was then quantitatively eluted with methanol.

LC with fluorescence detection, as described by Tway et al. (1), combined a high degree of specificity with the sensitivity required to detect low part-per-billion levels of ivermectin residue in liver tissue. Conversely, using LC with UV detection, the limit of detection was >100 ppb because of coextracted tissue components.

The MSPD method eliminates many of the problems associated with classical isolation techniques. The method uses small quantities of solvent and has a minimal number of steps. In contrast, classical methods for the isolation of ivermectin from biological matrixes such as liver require large volumes of extracting solvents (>100 mL), multiple extractions, and evaporation of large volumes of extracting solvents. Even though the RSDs on a liver sample containing incurred ivermectin residue were higher for the MSPD method, the savings in terms of time and solvent requirements make this method attractive when compared to classical isolation techniques.

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DRUG RESIDUES IN ANIMAL TISSUES

Matrix Solid-Phase Dispersion Extraction and Liquid Chromatographic Determination of Nicarbazin in Chicken Tissue

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This method outlines the necessary steps for the isolation and determination of the drug nicarbazin in chicken liver and muscle tissue. Tissue samples were blended with octadecylsilyl-derivatized silica packing material (C_{18}). A column made from the C_{18}–tissue matrix is first washed with hexane, and then the 4,4′-dinitrocarbanilide (DNC) portion of the nicarbazin complex is eluted with acetonitrile. After further cleanup using alumina cartridge chromatography, DNC is determined by reversed-phase liquid chromatography with UV detection at 340 nm. Recoveries based on DNC were 95.8 and 83.7% from liver and muscle tissues, respectively. This method and a classical ethyl acetate extraction method gave comparable results on 4 chicken liver and 3 muscle samples that contained incurred nicarbazin residues. C_{18} sorbents from different manufacturers as well as lipophilic sorbents other than C_{18} were also studied. The proposed extraction and cleanup procedure requires less than 30 mL solvent, fewer sample manipulations, and does not require solvent partitioning or backwashing of extracts. This combination of characteristics makes this method more attractive than classical isolation procedures for nicarbazin.

Nicarbazin, a 1 + 1 molar complex of 4,4′-dinitrocarbanilide (DNC) and 4,6-dimethyl-2-pyrimidinol (HPD), is used to prevent coccidiosis in chickens. The tolerance is 4.0 ppm in liver, kidney, skin, and muscle tissue (1). Because the HPD portion of the complex is excreted more rapidly than the DNC portion, all methods determine the DNC moiety in tissue (2). Current methods for the determination of nicarbazin in tissue use either homogenization with ethyl acetate (3, 4), with ethyl acetate–dimethyl sulfoxide (5), or chloroform–ethyl acetate–dimethyl sulfoxide (6). All of these methods use multiple, time-consuming organic solvent extractions of the residue from the tissue matrix.

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Recently, Barker et al. (7) demonstrated that biological matrixes can be homogenized with C_{18} sorbent (40 μm octadecylsilyl-derivatized silica), the resulting homogenate packed into a column, and various residues selectively eluted from the column. This method, called matrix solid-phase dispersion (MSPD), provides a rapid alternative to conventional methods of tissue extraction. It eliminates the need for time-consuming homogenization and multiple solvent partition cleanup steps. The MSPD method has been successfully used for the isolation of certain drugs from animal-derived matrixes and milk (8–13).

The method described here is based on the MSPD extraction of the DNC moiety of nicarbazin in chicken liver and muscle tissue, followed by solid-phase extraction (SPE) cleanup and liquid chromatographic determination with UV detection (LC/UV). We used this method to analyze liver and muscle tissues containing spiked and incurred nicarbazin residues. The ruggedness of the MSPD extraction procedure using C_{18} sorbents from different manufacturers as well as other lipophilic reversed-phase sorbents also has been studied and is reported here.

Experimental

Reagents and Expendable Materials

(a) *Solvents.*—Hexane was distilled in glass, non-UV grade; methanol and acetonitrile were distilled in glass, LC grade, suitable for spectrophotometry (EM Science, Gibbstown, NJ). *N,N*-Dimethylformamide (DMF) was ACS reagent grade (Fisher Scientific, Pittsburgh, PA).

(b) *Water.*—Deionized and filtered using a Milli-Q Water System (Waters Corp., Milford, MA).

(c) *Column material.*—Bulk C_{18}, Bondesil, 40 μm, 18% load, endcapped, octadecylsilyl-derivatized silica (Analytichem International, Harbor City, CA) was used throughout the study unless otherwise noted. Other column materials used

were obtained by cutting open SPE cartridges to obtain the sorbent. The cartridges used for this purpose were SepPak C_{18} (Waters Corp.), Supelclean LC_{18} and LC_8 (Supelco, Inc., Bellefonte, PA), and Bond Elut cyclohexyl (Analytichem International).

(**d**) *SPE cartridges.*—Alumina-B SepPak (Waters Corp.).

(**e**) *Analytical standard.*—Nicarbazin, Lot No. X45226 (Eli Lilly and Co., Indianapolis, IN). A stock nicarbazin solution (250 μg/mL) was prepared in DMF. An intermediate standard solution (50 μg/mL in DMF) and a working standard solution (2.55 μg/mL in methanol) were prepared by dilution of the stock solution. LC standard solutions (0.9, 0.6, 0.3, and 0.15 μg/mL) were prepared in methanol–water (75 + 25). All standard solutions were stored at 4°C.

Apparatus

(**a**) *Liquid chromatograph.*—Perkin-Elmer series 410 LC pump and ISS-100 autosampler, injection volume 50 μL (Perkin-Elmer Corp., Norwalk, CT), ABI-Kratos Model 383A UV-vis detector set at 340 nm (Kratos, Inc., Ramsey, NJ). Octadecylsilyl-derivatized silica column (3 μm, 15.0 cm × 4.6 mm id, Econosphere, Alltech Associates, Deerfield, IL); solvent flow rate, 0.8 mL/min; column temperature, ambient.

(**b**) *Syringe barrels.*—Used as extraction columns; empty reservoirs, 8 mL size, and frits (Analytichem International).

(**c**) *Adapters.*—Used to attach the alumina SepPak SPE cartridges to vacuum manifold; cut a portion of the tapered end of a yellow (5–100 μL) disposable pipet tip. The tapered end of the tip is fitted into the vacuum manifold, and the SepPak cartridges are inserted into the large end of the tip.

(**d**) *SPE vacuum manifold.*—Supelco, Inc.

(**e**) *Agate mortar and pestle.*—75 mm od (Thomas Scientific, Swedesboro, NJ).

Tissues

Control chicken liver and breast muscle tissues were obtained from a local market. Tissues containing incurred residues were obtained from the U.S. Food and Drug Administration, Division of Veterinary Medical Research, Beltsville, MD. Chickens were given feed containing 125 ppm (113 g/ton) nicarbazin. Drugs were withdrawn 24 and 36 h before sacrificing. Each tissue sample was ground 4 times with a meat grinder and stored at −20°C before analysis.

Extraction Procedure

Weigh 2 g C_{18} packing into mortar, and place tissue sample composite (0.5 g) on top of packing. For recovery studies, using an LC syringe, inject an appropriate volume of nicarbazin standard (250 or 50 μg/mL in DMF) into the tissue and allow to equilibrate 5 min. Blend tissue and C_{18} packing with a pestle until a homogeneous mixture is obtained. Transfer resultant C_{18}–liver matrix to an empty fritted syringe barrel (reservoir).

Compress column by using a glass syringe plunger, and place it on vacuum manifold. Wash column with 3 mL hexane, using ca 2 in. Hg vacuum to aid elution. When all hexane is eluted, increase vacuum to maximum for 5 s to remove any residual hexane. Elute column 2 times with 3 mL acetonitrile using 2 in. Hg vacuum, collecting eluate in a disposable borosilicate glass culture tube. Evaporate acetonitrile to dryness under nitrogen at 60°C and dissolve residue in 2 mL DMF. Attach an empty reservoir to an alumina cartridge and rinse cartridge with 5 mL DMF. Transfer residue dissolved in DMF to alumina cartridge and elute without applying vacuum, discarding eluate. (It may be necessary to initiate the flow by applying gentle pressure with a rubber bulb.) Rinse culture tube that contained residue 2 times with 2 mL DMF, and add rinsings to alumina cartridge, discarding eluate. When all DMF is eluted, place alumina cartridge on vacuum manifold and aspirate cartridge with full vacuum for 15 s. Adjust vacuum to ca 3 in. Hg and wash cartridge with 3 mL hexane. When all hexane is eluted, aspirate cartridge at full vacuum for an additional 5 min.

Remove alumina cartridge from vacuum manifold and elute cartridge twice with 3 mL methanol each time, collecting eluate in a 10 mL volumetric flask. After elution is complete, add 2.5 mL water to the flask, add methanol to volume, and mix.

Inject 50 μL of each standard and sample solution into the LC system. Obtain standard curve by plotting concentration of standards vs peak area.

Calculations for Dosed Tissue

Use the following equation:

$$\text{ppm nicarbazin} = (C \times V \times F_{DNC})/(W \times 0.7089)$$

where C = concentration of nicarbazin as determined from standard curve (μg/mL); V = final volume (10 mL); F_{DNC} = actual fraction of DNC in nicarbazin reference standard (typically 0.674–0.730); W = weight (g) of tissue extracted; and 0.7089 = theoretical fraction of DNC in nicarbazin reference standard.

Results and Discussion

The isolation of drug or chemical residues from tissue can be a time-consuming and laborious task. Traditional methods for the isolation of nicarbazin from poultry tissue include multiple homogenization and centrifugation steps, followed by evaporation of large volumes of solvent.

The extraction of the DNC moiety of nicarbazin from tissue using MSPD rapidly yields extracts with a minimum of interfering coextractants. When tissue is blended with C_{18} packing, the cell membranes are disrupted. The cellular components and any drug residues are essentially dispersed over a large surface area (1000 m^2/2 g C_{18}), thereby optimizing the sample extraction efficiency. Even though the volume of extracting solvents is small (6 mL), the process can be envisioned to be an exhaustive extraction because a large volume of solvent is passed over an extremely thin layer of highly dispersed sample. Lipids are eluted from the C_{18}–tissue complex with hexane. DNC is then eluted from the MSPD complex with acetonitrile. Because this eluate most likely still contains tissue components that could interfere with the LC determination, the alumina SPE cartridge

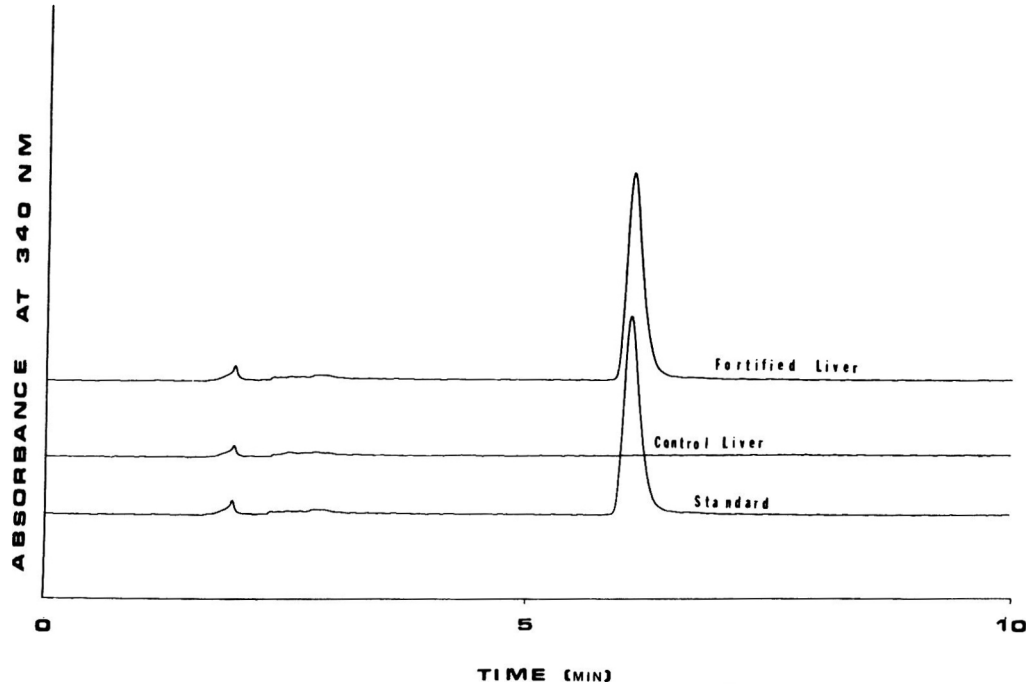

Figure 1. Chromatogram of 4,4′-dinitrocarbanilide in 0.4 μg/mL nicarbazin standard, control chicken liver, and fortified chicken liver containing 8 ppm nicarbazin.

cleanup described by Lewis et al. (4) is used to purify the MSPD eluate before LC analysis.

Representative chromatograms resulting from the analysis of blank and fortified liver samples are shown in Figure 1. DNC is eluted in about 6 min, with no interfering coeluted peaks from the tissue matrix. Table 1 shows the results of recovery studies performed on spiked chicken liver and muscle tissues. As a new extraction technology, MSPD has been widely applied to the extraction of drug residues from tissue and milk (7–13). All of these studies entail the recoveries of residues from fortified tissues. To demonstrate that the MSPD will efficiently extract incurred drug residues from tissues, 4 liver and 3 muscle tissue samples were extracted by the MSPD method and also by a classical extraction method (4). Table 2 shows that the 2 methods give comparable results.

The ruggedness of any method may depend on the consistency of the quality of the reagents used. Although the purity of solvents and reagents available for chemical analysis is fairly

Table 1. Recovery of nicarbazin from fortified chicken tissues using the MSPD method

Tissue	Fortification, ppm	Rec., %[a]	CV, %
Liver	2.0	95.4	4.7
	4.0	95.1	3.3
	6.0	96.2	4.9
	8.0	95.6	2.2
	10.0	96.9	2.8
	15.0	90.5	1.2
Muscle	1.0	91.0	2.7
	2.0	84.4	2.8
	4.0	81.4	5.9
	8.0	80.1	8.4
	10.0	81.7	4.0

[a] $n = 5$.

Table 2. Recoveries of incurred nicarbazin residues from chicken tissue samples by 2 different methods, MSPD and classical[a]

Tissue	Sample No.	Method	*n*	Nicarbazin, ppm	CV, %
Liver	1	MSPD	6	7.9	5.4
		Classical	3	6.8	4.5
	2	MSPD	6	4.7	4.4
		Classical	3	3.7	8.1
	3	MSPD	6	7.8	4.3
		Classical	3	6.4	3.9
	4	MSPD	4	16.2	2.1
		Classical	3	13.7	7.2
Muscle	5	MSPD	5	1.6	4.8
		Classical	3	1.4	3.7
	6	MSPD	5	2.4	8.8
		Classical	3	2.4	3.6
	7	MSPD	5	1.6	2.8
		Classical	5	1.2	10.6

[a] Method of Lewis et al. (4).

Table 3. Recoveries of nicarbazin residues from chicken liver tissue spiked at 4.0 ppm using the MSPD method with various LC sorbents

Sorbent	Manufacturer	*n*	Rec., %	CV, %
C_{18}	Analytichem	5	95.1	3.3
C_{18}	Waters	3	90.8	6.1
C_{18}	Supelco	3	92.5	3.2
C_8	Supelco	3	94.4	2.4
Cyclohexyl	Analytichem	3	92.9	2.0

uniform, the properties of the C_{18} material available from various manufacturers varies greatly. Such variables as the degree of endcapping and the use of proprietary manufacturing processes result in great variations among manufacturers. Also, variations from lot-to-lot from the same manufacturer may occur. In an effort to test the ruggedness of the MSPD extraction procedure, C_{18} obtained from 3 different manufacturers plus 2 other lipophilic sorbents, C_8 and cyclohexyl, were employed in the method. Table 3 shows that the recoveries of nicarbazin residues from liver tissue were the same regardless of the sorbent used.

The MSPD method eliminates many of the problems associated with classical isolation techniques. The method uses small quantities of solvent and has a minimal number of steps. In contrast, classical methods for the isolation of nicarbazin from tissue matrixes require large volumes of solvents (over 200 mL) and multiple extractions. The savings of time and quantity of solvent make this method attractive when compared to classical isolation techniques.

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DRUGS IN FEEDS

Liquid Chromatographic Assay of Dimetridazole, Ipronidazole, and Ronidazole in Feeds and Premixes

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A rapid method has been developed for the determination of dimetridazole (DMZ), ipronidazole (IPZ), and ronidazole (RNZ) in turkey feeds, swine feeds, and premix. The compounds are extracted from samples with warm methanol, the extract is purified over a short alumina column, and an aliquot of the eluate is analyzed by reversed-phase liquid chromatography and UV detection at 309 nm. Alumina cleanup of premixes is not essential, although the resulting chromatograms are cleaner. Recoveries of DMZ from feed formulations ranged from 97 to 103% at the 10.0, 50.0, and 100.0 ppm levels, with a standard deviation (SD) of 0.62–3.2%. Recoveries of IPZ ranged from 94.4 to 101.2% at the 5.0, 50.0, and 100.0 ppm levels (SD, 0.42–4.4%). RNZ recoveries ranged from 95 to 100.7% at the 6.0, 60.0, and 120.0 ppm levels (SD, 1.2–5.33%).

Ronidazole (RNZ), dimetridazole (DMZ), and ipronidazole (IPZ) are antimicrobial or antiprotozoal drugs approved by the Italian Ministry of Health as coccidiostats and for prevention and treatment of histomoniasis and trichomoniasis in poultry. However, their use in swine feeds has not been approved. These drugs are also useful as growth enhancers, although they have been found to be carcinogenic in some laboratory animals (1) and mutagenic in bacteria (2). For these reasons, it is often necessary to determine them in medicated and unmedicated feeds for turkey and swine.

The colorimetric method (3, 4) currently used to detect RNZ in feeds is subject to interference from other drugs such as furazolidone, nihydrazone, DMZ, and IPZ. The absorption chromatography procedure currently used does not remove all interferences. RNZ in feeds has also been determined electrochemically (5).

Various methods based on polarography (6, 7), gas chromatography (8), and spectrophotometry (9) have been reported for detection of DMZ and IPZ in medicated feeds. Liquid chromatographic (LC) methods were mainly used to determine residues in tissues of treated animals (10–13). Some analysts applied the LC technique to feeds, but found that interfering compounds or supports caused difficulties (14–17). Roybal et al. (18) described an LC method for the determination of DMZ and IPZ in swine feed. The drugs were extracted with either methanol or methylene chloride, subjected to an acid-base cleanup, separated on a C_{18} column, and detected at 320 nm.

The aim of this work was to develop an LC method for the simultaneous determination of RNZ, DMZ, and IPZ in poultry and swine feeds and premixes at concentration levels of 5–150 ppm without interferences.

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METHOD

Apparatus

(a) *Liquid chromatograph.*—Model 510 solvent delivery system, Model 740 Data Module, Model 490 programmable multiwavelength detector, U6K injector (Waters Associates, Milford, MA). Chromatographic conditions: flow rate, 1.0 mL/min; column temperature, 30°C; detection, 309 nm.

(b) *LC column.*—Stainless steel, 150 × 4.6 mm id, packed with Supercosil LC-18-DB (deactivated for basic compounds), 10 μm pores (Supelco Inc., Bellefonte, PA).

(c) *Precolumn.*—Supelguard column, 2 cm cartridge filled with 5 μm Supelcosil LC-18-DB packing (10 μm pores).

(d) *Cleanup column.*—Borosilicate glass, 200 × 10 mm id, fitted with sintered-glass disc and Teflon stopcock.

(e) *Membrane filters.*—0.45 μm Millipore.

Reagents

(a) *Methanol.*—Analytical reagent grade, and LC grade.

(b) *Water.*—LC grade.

(c) *Phosphoric acid.*—85% (w/v); analytical reagent grade.

(d) *Aluminum oxide.*—90 basic for column chromatography (70–230 mesh ASTM, Merck & Co., Inc., Rahway, NJ). Heat alumina 3 h at 600°C, cool, and store in vacuum desiccator. Weigh 100 g dried alumina into 250 mL Erlenmeyer flask,

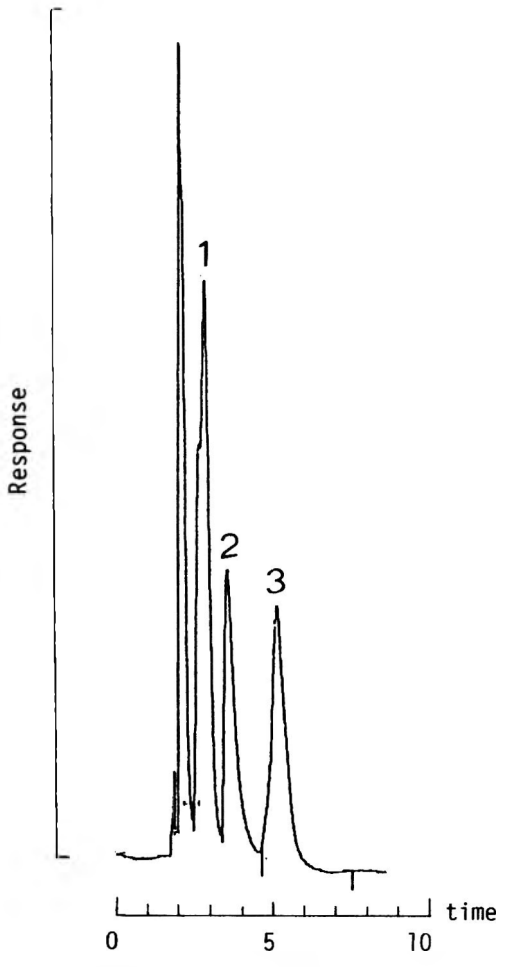

Figure 1. Chromatograms of reference standard mixture containing 10, 6.0, and 5.0 μg/mL, respectively, of (1) ronidazole, (2) dimetridazole, and (3) ipronidazole.

pipet 2.0 mL water into flask, and stopper. Heat 5 min on steam bath and vigorously shake warm flask until powder is free-flowing. Cool, and let stand overnight.

(e) *LC mobile phase.*—LC grade methanol–0.4% phosphoric acid (55 + 45).

(f) *Standard solutions.*—Prepare individual stock solutions containing, respectively, RNZ, DMZ, and IPZ (Merck) at 100, 60, and 50 μg/mL by dissolving 10, 6, and 5 mg in 100 mL methanol. Prepare working standard solutions by diluting 10.0 mL of each stock solution to 100 mL with LC grade methanol.

Sample Preparation

(a) *Complete feeds.*—Grind coarse or pelleted feed to pass 20-mesh sieve, weigh 10.0 g sample into 250 mL conical flask, and add 60–70 mL analytical reagent grade methanol. Reflux 30 min on steam bath at 60°C. Periodically swirl flask to ensure complete extraction. Cool to room temperature, transfer to 100 mL Erlenmeyer flask, rinse flask twice with 5 mL portions of the methanol, and dilute to volume. Let particulates settle, and filter through Whatman No. 1 filter paper. Prepare cleanup column by dry-packing 10 g alumina, and add 30–35 mL sample extract. Collect eluate and filter through 0.45 μm Millipore filter.

(b) *Feed premix.*—Proceed as for complete feeds but excluding cleanup.

Calibration Curve

Inject separate 10 μL aliquots of DMZ, IPZ, and RNZ solutions containing 0.5, 2.5, 5.0, 10.0, 50.0, 100.0, or 200.0 μg of each/mL methanol. Measure peak areas and calculate linear regression and coefficient of determination (r_2, slope and y-intercept) vs concentration.

Determination and Calculations

Quantify DMZ, IPZ, and RNZ by comparing peak area ratios of sample extracts with standard solution of ca same concentration, using identical injection volumes (10 μL). Inject standard; then, inject sample in duplicate, followed by standard injection. Repeat determination if peak areas of identical injections are not the same (difference >1% of area counts).

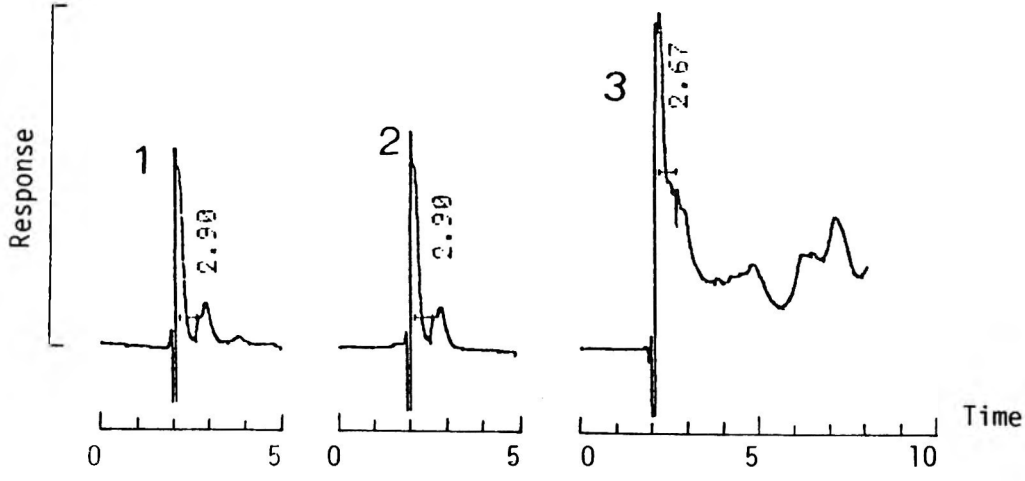

Figure 2. LC chromatograms of (1) ronidazole reference standard (0.6 μg/mL), (2) medicated feed extract (6.0 ppm) after alumina cleanup, and (3) without alumina cleanup.

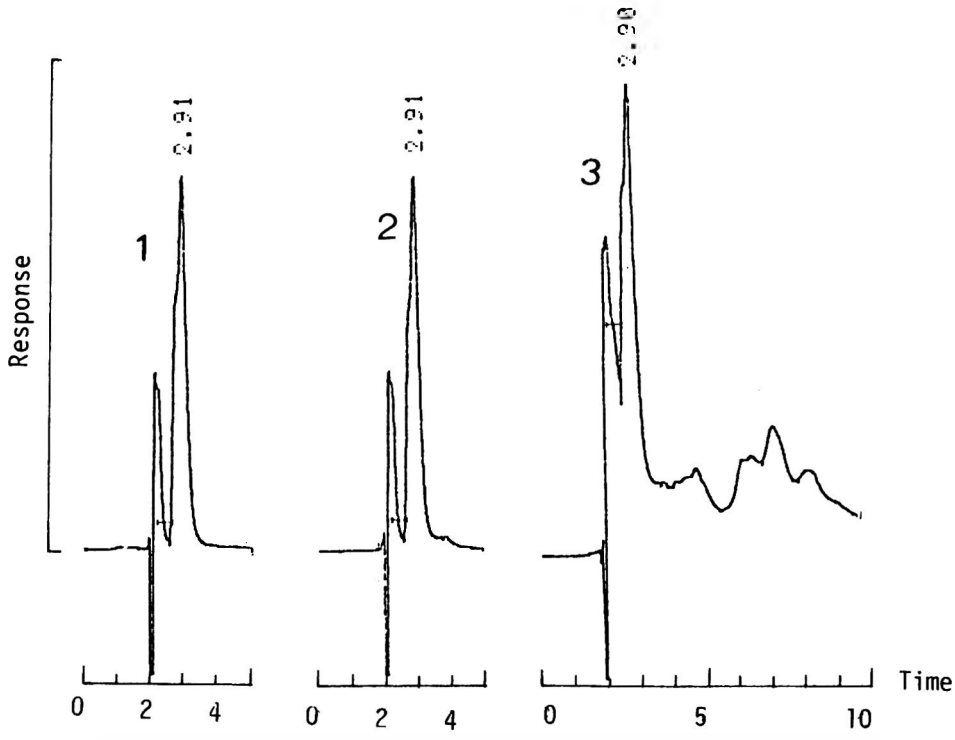

Figure 3. LC chromatograms of (1) ronidazole reference standard (6.0 μg/mL), (2) medicated feed extract (60 ppm) after alumina cleanup, and (3) without alumina cleanup.

Calculate concentrations of DMZ, IPZ, and RNZ by following formula:

$$\text{Concn, ppm} = (Hu \times C \times F \times 1000)/(Hs \times Pc \times 1000)$$

where Hu and Hs = peak areas of sample and standard, respectively; C = concentration of standard (μg/mL); Pc = sample weight (g); and F = diluting factor.

Results and Discussion

The aim of this work was to develop a simple and reproducible LC method for the routine determination of DMZ, IPZ, and RNZ in feeds and premixes. The drugs are chemically similar and used in poultry feed to prevent and treat the same microbial infection. Therefore, simultaneous detection would be useful.

DMZ, IPZ, and RNZ reference standards used in different concentrations were well separated and completely recovered by this method, with standard deviations of 0.3–5.3%.

The method was tested in turkey feeds containing 40% corn, 30% wheat, 20% soybean, 3% fish meal, 6.75% meat meal, and 0.25% vitamin–mineral premix; in swine feeds containing 45% corn, 30% barley, 15% roasted soybean, 5% beet molasses, 4.5% each of calcium carbonate, sodium bicarbonate, and sodium chloride, and 0.25% vitamin–mineral premix; and in poultry premix.

The drugs were incorporated at concentration levels of 10, 50, and 100 ppm DMZ; 5.0, 50, and 100 ppm IPZ; and 6, 60, and 120 ppm RNZ by adding them to the feed base in methanol, shaking for 30 min, and then removing the solvent under reduced pressure.

Figure 1 depicts a typical chromatogram of a standard mixture of the 3 nitroimidazole compounds containing RNZ at 10 μg/mL, DMZ at 6 μg/mL, and IPZ at 5 μg/mL. Figures 2 and 3 show chromatograms of RNZ reference standard (0.6 and 6 μg/mL) and turkey medicated feed extract (6 and 60 ppm) with and without alumina cleanup. Similar chromato-

Table 1. Dimetridazole recoveries from 3 feed types fortified at different levels

	Dimetridazole levels[a]		
Feed	10.0 ppm	50.0 ppm	100.0 ppm
Turkey feed	10.3 ± 0.32	51.5 ± 0.43	100.4 ± 0.87
Swine feed	9.7 ± 0.23	50.4 ± 0.56	98.7 ± 0.94
Poultry premix	9.9 ± 0.18	47.7 ± 0.31	97.9 ± 0.72
Av. rec., %	99.7	99.8	99.0

[a] The values represent the mean of 3 determinations ± standard deviation.

Table 2. Ipronidazole recoveries from 3 feed types fortified at different levels

Feed	Ipronidazole levels[a]		
	5.0 ppm	50.0 ppm	100.0 ppm
Turkey feed	4.7 ± 0.22	47.7 ± 0.63	98.5 ± 0.42
Swine feed	4.8 ± 0.19	48.8 ± 0.42	100.9 ± 0.57
Poultry premix	5.1 ± 0.18	50.3 ± 0.57	99.7 ± 0.87
Av. rec., %	97.4	97.8	99.7

[a] The values represent the mean of 3 determinations ± standard deviation.

Table 3. Ronidazole recoveries from 3 feed types fortified at different levels

Feed	Ronidazole levels[a]		
	6.0 ppm	60.0 ppm	120.0 ppm
Turkey feed	6.2 ± 0.21	60.4 ± 1.5	116.5 ± 3.27
Swine feed	5.7 ± 0.24	58.7 ± 1.2	117.7 ± 5.81
Poultry premix	5.8 ± 0.32	59.6 ± 0.7	118.7 ± 2.13
Av. rec., %	98.2	99.3	98.0

[a] The values represent the mean of 3 determinations ± standard deviation.

grams were obtained for IPZ and DMZ. Tables 1–3 summarize the recovery studies obtained with turkey and swine feeds and premixes containing nitroimidazole compounds at 3 different concentration levels. The total mean recoveries were 99.5% for DMZ, 98.3% for IPZ, and 98.5% for RNZ.

In conclusion, this method appears to be a reliable means of identification, determination, and confirmation of the presence of imidazole compounds in feeds and premixes.

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ELEMENTAL ANALYSIS

Determination of Lanthanum, Europium, and Ytterbium in Food Samples by Electrothermal Atomic Absorption Spectrometry

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A method had been developed for the determination of lanthanum, europium, and ytterbium in food samples by using electrothermal atomic absorption spectrometry with a graphite furnace lined with tungsten foil. The proposed method provides higher sensitivity than inductively coupled plasma/atomic emission spectrometry (ICP/AES); the characteristic masses are 8.1×10^{-9} g for lanthanum, 3.9×10^{-11} g for europium, and 4.27×10^{-12} g for ytterbium. The precision, accuracy, and interferences of the method were also investigated. The method can be used for the analysis of trace amounts of lanthanum, europium, and ytterbium in various foods without predissociation of the matrixes of the digested solutions. The results obtained by the method are in good agreement with those from ICP/AES.

The rare earth elements are widely used in industry and have received considerable attention because of their application in some fertilizers. The content of the rare earth elements in foods has gained increasing interest during recent years. Atomic absorption spectrometry (AAS) has been used to determine lanthanum, europium, and ytterbium in geological samples, but this method presents some problems because of the formation of carbides during the ashing and atomization steps, which decreases the sensitivity and produces the strong memory effect. To overcome these problems, a pyrolytically coated graphite tube has been used (1–5). Attempts have also been made to use a precoated graphite tube with salt of tantalum, zirconium, or tungsten (6–8). Other methods have used a tantalum liner inserted inside the graphite furnace (9–16). However, the main disadvantage of these atomizers is their short usable lifetime.

This paper describes the determination of lanthanum, europium, and ytterbium in food samples by electrothermal AAS using a graphite tube lined with tungsten foil. The proposed method offers the advantages of selectivity, high sensitivity, and a wide range of determination without predissociation of the matrixes of the digested solutions.

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Experimental

Apparatus

(a) *Atomic absorption spectrometer.*—Perkin Elmer 5000, with a Zeeman background correction and fitted with HGA 500 graphite furnace, AS 40 autosampler, and PR 100 printer (Perkin Elmer Corp., Norwalk, CT). Argon is the sheath gas. Operating conditions are summarized in Table 1.

(b) *Graphite tube lined with tungsten foil.*—Lining of tungsten metal was prepared from metal foil of 99.96% purity and 0.1 mm thickness. A 12 × 15 mm rectangular strip was used. The metal lining of tungsten foil was made by forming a tungsten strip around a glass rod whose diameter was slightly less than that of the tube, and the lining was inserted into the center of a new pyrolytically coated graphite tube. Then, a metal rod was inserted into the tube and firmly rolled inside the tube to attach the tungsten foil smoothly to the inner lining of the graphite tube. To prevent the distortion of the foil on heating, the tube lined with foil was pretreated in the HGA 500; it was subjected twice to 2500°C high-temperature for 5 s in a current of argon. The lifetime of the tungsten–surface atomizer was ca 130 firings.

(c) *Glassware.*—Soak 24 h in HNO_3 (5%, v/v) and rinse with deionized water before each use.

Reagents

(a) *Lanthanum oxide stock standard solution.*—1 mg/mL. Dissolve 0.1000 g La_2O_3 (previously dried at 850°C) in 10 mL concentrated hydrochloric acid by gentle heating. Dilute to 100 mL with water. Working solutions were prepared by diluting appropriate aliquots of the stock standard solution.

(b) *Europium oxide stock standard solution.*—1 mg/mL. Weight 0.1000 g Eu_2O_3 (previously dried at 850°C) and proceed as described for lanthanum oxide stock standard solution.

(c) *Ytterbium oxide stock standard solution.*—1 mg/mL. Weight 0.1000 g Yb_2O_3 (previously dried at 850°C) and proceed as described for lanthanum oxide stock standard solution.

Table 1. Instrument conditions for determination of lanthanum, europium, and ytterbium

Parameter	Lanthanum	Europium	Ytterbium
Wavelength, nm	550.1	459.4	398.8
Slit width, nm	0.4	0.7	0.7
Lamp current, mA	30	20	14
Background correction	On	On	On
Injection volume, µL	20	20	20
Drying step			
Temperature, °C	120	120	120
Ramp, s	5	5	5
Hold time, s	10	10	10
Charring step			
Temperature, °C	1300	1050	1050
Ramp, s	5	2	2
Hold time, s	10	10	10
Atomization step			
Temperature, °C	2450	2300	2300
Ramp, s	0	0	0
Hold time, s	4	3	3
Cleaning step			
Temperature, °C	2500	2400	2400
Ramp, s	1	1	1
Hold time, s	3	3	3

(d) *Hydrochloric acid.*—Ultrapure grade (Shanghai Co., Shanghai, China).

(e) *Nitric acid.*—Ultrapure grade (Shanghai).

(f) *Deionized water.*—Deionized twice-distilled water.

Preparation of Samples

Food samples such as rice, wheat, corn, milk powder, vegetables, and tea, which were obtained from the local markets, were oven-dried and ground. An accurately weighed sample (ca 5–10 g) was placed in a porcelain crucible and covered. The crucible was heated 1 h on a hot plate, several drops of nitric acid were then added to aid charring, and heating was continued. The temperature was increased gradually to avoid sputtering of the sample. After charring was complete, the crucible was heated 30 min in a muffle furnace at 400°C; the temperature was increased to 600°C for 6 h. The crucible was removed and cooled, and then 0.5 mL concentrated nitric acid was added; the crucible was again heated 30 min in a muffle furnace at 400°C, and the temperature was increased to 600°C for 6 h. The sample ash was treated with 0.5 mL hydrochloric acid and 0.5 mL water (warming may be necessary). The clear solution was transferred quantitatively into a 5 mL calibrated flask and diluted to the mark with water. In this ashing method, the preparation of a reagent blank was necessary.

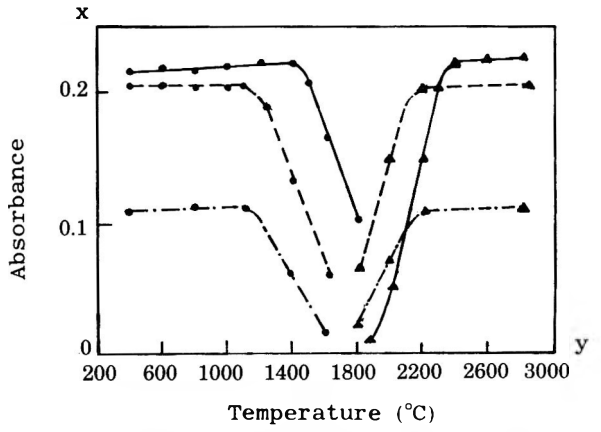

Figure 1. Ashing and atomization curves: •, ashing; ▲, atomization; —, lanthanum (4.0×10^{-7} g); - - -, europium (1.0×10^{-9} g); and - · -, ytterbium (2.0×10^{-10} g).

Procedure

Aliquots of the treated solutions (20 µL) were injected into the graphite furnace lined with tungsten foil by means of the autosampler. Measurements of lanthanum, europium, and ytterbium were made by using the atomizer conditions given in Table 1. Maximum power heating, with the internal argon gas flow off, was used for atomization to achieve maximum sensitivity. Readings on the spectrometer were taken by using the peak-height mode. A calibration graph was constructed, and lanthanum, europium, and ytterbium levels of unknown samples were calculated from the graph.

Results and Discussion

Optimum Atomization Conditions

To optimize the ashing and atomization temperatures, ashing and atomization curves were constructed for an aqueous solution containing 10 µg/mL lanthanum, 0.05 µg/mL europium, and 0.01 µg/mL ytterbium; results are shown in Figure 1. In this study, the optimum ashing temperatures were 1300°C for lanthanum and 1050°C for both europium and ytterbium. The dip in the ashing curve must be related to the volatilization of these elements. The optimum atomization temperatures were 2450°C for lanthanum and 2300°C for both europium and ytterbium.

The ashing times for the samples in hydrochloric acid matrix were 10 s. The atomization times of the 3 elements were maintained for only 3–4 s, because longer atomization times did not effectively reduce the peak height of the blank solution, but did reduce the usable life of the graphite tube.

Effect of Hydrochloric Acid Concentration

The effect of the hydrochloric acid concentration on absorbance was examined in the 0–8.0% range; results are shown in Figure 2. Constant absorbance readings of lanthanum and ytterbium were obtained in the 1.0–8.0% and 2.0–8.0% ranges, respectively. The absorbance of europium did not produce any

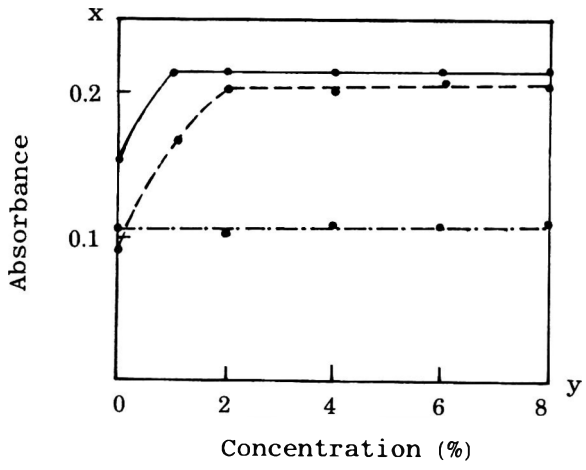

Figure 2. Effect of HCl concentration on absorbance: —, lanthanum (4.0×10^{-7} g); - - -, europium (1.0×10^{-9} g); and - · -, ytterbium (2.0×10^{-10} g).

change in the 0–8.0% range. In this work, hydrochloric acid concentration was 2.0%, v/v.

Calibration Curves

To obtain calibration curves, standard solutions containing 0–10 μg/mL lanthanum, 0–0.1 μg/mL europium, and 0–0.02 μg/mL ytterbium with the optimum amount of hydrochloric acid were subjected to the furnace program. The correlation coefficients of the calibration curves of lanthanum, europium, and ytterbium for absorbance vs concentration were 0.9991, 0.9986, and 0.9994, respectively.

Sensitivity and Detection Limit

Sensitivity can be conveniently measured in terms of a "characteristic mass." In this work, characteristic masses of lanthanum, europium, and ytterbium were 8.1×10^{-9} g, 3.9×10^{-11} g, and 4.27×10^{-12} g, respectively, which were about the same as those found in the literature (9). The detection limit, the lowest concentration level that can be determined to be statistically different from a blank, is defined as 3 times the within-batch standard deviation of a single blank determination, corresponding to a 99% confidence level. In this study, the detection limits of lanthanum, europium, and ytterbium were 7.85×10^{-9} g, 3.78×10^{-11} g, and 4.04×10^{-12} g, respectively.

Accuracy of Method

To study the accuracy of the method, recoveries of standard additions of lanthanum, europium, and ytterbium to samples were investigated by the entire sample preparation procedure. Each addition was performed in duplicate. The results obtained gave recoveries ranging from 82 to 103% for lanthanum, 79 to 107% for europium, and 80 to 103% for ytterbium (see Table 2).

Precision of Method

The precision (relative standard deviation) of the method was obtained for replicate analysis of one sample during the same run; results are shown in Table 3. The within-batch precision of the method, obtained for 10 replicates of 3 samples with different concentrations of lanthanum, europium, and ytterbium, varied over the range of 5.9 to 9.9% for lanthanum, 3.2 to 10.2% for europium, and 2.5 to 9.0% for ytterbium.

Interference Study

For interference studies, different amounts of other ions were added to the test solution containing 10 μg/mL lanthanum, 0.05 μg/mL europium, or 0.01 μg/mL ytterbium, and the elements were determined by using the proposed procedure. The absorbance data were compared to the value obtained for

Table 2. Recovery of lanthanum, europium, and ytterbium from food samples

Element	No.	Sample	Present, μg/mL[a]	Added, μg/mL	Found, μg/mL	Rec., %
Lanthanum	1	Rice	1.55	2.5	3.90	94.0
	2	Grain	1.60	2.5	3.65	82.0
	3	Milk powder	2.10	2.5	4.30	88.0
	4	Wheat	2.30	2.5	4.40	84.0
	5	Corn	1.85	3.0	4.95	103.0
Europium	6	Rice	0.048	0.100	0.135	87.0
	7	Grain	0.044	0.100	0.125	81.0
	8	Milk powder	0.054	0.100	0.133	79.0
	9	Wheat	0.035	0.100	0.128	93.0
	10	Tea	0.032	0.075	0.112	107.0
Ytterbium	11	Rice	0.017	0.030	0.048	103.0
	12	Grain	0.012	0.030	0.037	83.0
	13	Milk powder	0.024	0.030	0.048	80.0
	14	Wheat	0.019	0.030	0.046	90.0
	15	Tomato	0.016	0.030	0.040	80.0

[a] Tenfold preconcentrations in original samples.

Table 3. Precision of the determination of lanthanum, europium, and ytterbium

Element	Sample	Concn, μg/mL	*N*	SD	RSD, %
Lanthanum	Rice	2.0	8	0.00237	9.9
	Wheat	4.0	9	0.00374	8.8
	Tea	20.0	8	0.0127	5.9
Europium	Rice	0.025	12	0.0046	10.2
	Milk powder	0.050	12	0.0070	6.5
	Wheat	0.075	11	0.0052	3.2
Ytterbium	Rice	0.0025	11	0.00379	9.0
	Wheat	0.010	11	0.00989	5.2
	Tomato	0.020	11	0.0103	2.5

pure standard solutions. Results are shown in Table 4, which shows that all tested elements did not interfere with the determination of lanthanum, europium, and ytterbium. Interference caused by other rare earth elements was also investigated and appeared negligible. This illustrates that the selectivity of the method is very good.

Memory Effect

Lanthanum, europium, or ytterbium in the presence of carbon are considered to form a nonvolatile carbide that may result in a memory effect (9). This memory effect was reduced when a pyrolytically coated graphite tube lined with tungsten foil was used. A 20 μL aliquot of 10 μg/mL lanthanum, 0.10 μg/mL europium, or 0.03 μg/mL ytterbium injected into a graphite tube lined with tungsten foil showed a return to background level without blank firing. However, a 20 μL aliquot of the same concentration of element injected into an unlined graphite tube showed significant memory even after 5 blank firings. This result shows that the atomizer of tungsten surface can completely remove the memory effect in the determination of lanthanum, europium, and ytterbium.

Comparison of the Methods

A comparison of the results obtained by the proposed method with those obtained using inductively coupled plasma/atomic emission spectrometry (ICP/AES) (17) is shown in Table 5. Results show that there is no significant difference between the 2 methods ($P > 0.05$).

Table 4. Effect of foreign ions on the determination of lanthanum, europium, and ytterbium

			Relative absorbance		
Ion	Added as	Amount added, ppm	Lanthanum	Europium	Ytterbium
None			1.00	1.00	1.00
Ca^{2+}	$CaCO_3$	500	0.97	1.02	0.94
Mg^{2+}	$MgCO_3$	200	0.97	1.05	0.95
K^+	KCl	500	0.89	0.94	0.91
Na^+	NaCl	500	1.05	0.96	0.94
Cu^{2+}	$Cu(NO_3)_2$	10	1.00	1.02	1.05
Fe^{3+}	$Fe(NO_3)_3$	100	0.89	1.03	0.91
Zn^{2+}	$Zn(NO_3)_2$	10	0.97	0.96	1.07
Pb^{2+}	$Pb(NO_3)_2$	10	0.98	1.04	1.04
Cr^{6+}	$K_2Cr_2O_7$	10	0.91	1.10	1.01
Cd^{2+}	$Cd(NO_3)_2$	10	0.90	1.04	0.93
Mn^{2+}	$MnSO_4$	10	0.99	0.97	1.00
Al^{3+}	$Al(NO_3)_3$	5	0.93	0.93	0.92
Sn^{4+}	$SnCl_4$	5	0.94	1.02	0.95
Si^{2+}	Na_2SiO_2	100	1.09	0.94	1.10
Se^{4+}	$Se(NO_3)_4$	2	1.00	0.90	1.00
Cl^-	NaCl	300	1.05	0.96	0.94
NO_3^-	$Fe(NO_3)_3$	80	0.89	1.03	0.91
SO_4^{2-}	$MnSO_4$	100	0.99	0.97	1.00

Table 5. Comparison of concentrations determined by 2 methods

	Proposed method, μg/mL			ICP/AES method, μg/mL		
Sample No.	Lanthanum	Europium[a]	Ytterbium[a]	Lanthanum	Europium	Ytterbium
1	0.155	0.187	0.092	0.142	0.198	0.090
2	0.210	0.107	0.053	0.221	0.098	0.051
3	0.230	0.047	0.023	0.213	0.051	0.026
4	0.285	0.027	0.015	0.298	0.025	0.010
5	0.110	0.011	0.005	0.123	0.011	0.007
6	0.185	0.023	0.004	0.213	0.024	0.003
7	0.195	0.089	0.048	0.198	0.101	0.039
8	0.085	0.049	0.051	0.104	0.052	0.049
9	0.063	0.104	0.097	0.078	0.099	0.071
10	0.050	0.024	0.011	0.052	0.021	0.008

[a] Results of synthetic samples.

Conclusion

A sensitive, selective method for the determination of lanthanum, europium, and ytterbium in foods by AAS using a graphite tube lined with tungsten foil is described. The atomization of these elements from the tungsten surface gives high sensitivity and negligible memory effect. The proposed method provides accurate and precise results for the determination of lanthanum, europium, and ytterbium in food samples. The validity of the method was shown by comparison of results obtained with those of the ICP/AES method.

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ELEMENTAL ANALYSIS

Direct Determination of Individual Organic Fluorine Compounds by Aluminum Monofluoride Molecular Absorption Spectrometry

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Recovery of organic fluorine by direct aluminum monofluoride molecular absorption spectrophotometry, following injection of samples into the graphite furnace and use of sodium fluoride standards, varies from 0 to 100% from compound to compound depending on the compound's volatility and the mechanism of thermal decomposition. This problem has now been overcome by employing standards made from the very same fluorochemical that is present in the sample. This approach is applicable to samples containing only 1 known fluorochemical. Some industrial applications of this technique that meet the needs of quality control, industrial hygiene policies, and certain U.S. Food and Drug Administration regulations are described.

The aluminum monofluoride molecular absorption spectrometry (AlF MAS) method developed originally by Tsunoda et al. (1) is one of the most rapid and sensitive methods for microdetermination of fluorine. This technique, involving direct injection of the sample into the graphite furnace, has been employed by Chiba et al. (2) and Fujimori et al. (3) for the determination of total fluorine (organic plus inorganic F^-) in human blood plasma and serum. However, the preceding procedures are not adaptable to automated sampling.

Venkateswarlu et al. (4) improved upon the above methods and described a simpler procedure that is amenable to automation and that saves considerable amounts of time and labor. Further, Venkateswarlu et al. demonstrated that direct injection of the sample into the furnace, a procedure described by other workers (2, 3), does not give quantitative results for total fluorine in serum samples from plant workers exposed to diverse fluorochemicals, and that prior conversion of organic fluorine to inorganic fluoride is necessary for obtaining accurate results. The sodium biphenyl technique reported by Venkateswarlu (5) was used to accomplish such a conversion.

These observations pertaining to poor recoveries of total and organic fluorine from blood samples with the direct-injection technique should not, however, deter one from taking advantage of the very same technique for the rapid determination of organic fluorine in other situations. For example, if one has a sample containing only 1 known fluorochemical and standards for calibration can be prepared with the very same fluorochemical, an accurate and rapid determination of the fluorochemical in the sample should be possible. This report contains data in support of the concept that, although a sample containing diverse unknown fluorochemicals cannot always be accurately analyzed for total fluorine by the direct-injection procedure, samples containing a single known fluorochemical can be so analyzed accurately by employing a standard calibration curve obtained with the fluorochemical involved (and not with inorganic fluoride).

Because of its simplicity, rapidity, accuracy, and sensitivity, the AlF MAS method is highly suitable for industrial analytical laboratories dealing with organic fluorochemicals. Some pertinent examples are provided in this report.

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Experimental

Reagents

Fluoride stock solutions were prepared by dissolving the appropriate amount of sodium fluoride (Mallinckrodt, Paris, KY) in distilled, deionized water. Perfluorooctanoic acid, ammonium perfluorooctanoate, (*N*-methylperfluorooctanesulfonamido)ethanol, (*N*-ethylperfluorooctanesulfonamido)ethanol, FC-905, FC-860, FC-391, FC-326, and FX-229 were obtained from 3M and were used as received. Vinylidine difluoride–hexafluoropropylene copolymer (Fluorel) was also obtained from 3M as the neat gum stock. The copolymer solution was prepared by dissolving the polymer in methanol and then diluting the solution with 10% methanol in water. Stock aqueous solutions of each fluorochemical were prepared and then diluted to an appropriate working concentration before analysis. A stock solution of the copolymer exclusively in methyl ethyl ketone (MEK) was also prepared.

The aluminum matrix Solution I contained 0.1M aluminum nitrate (Mallinckrodt), 0.05M nickel nitrate (Mallinckrodt), and 0.05M strontium nitrate (Aldrich, Milwaukee, WI). Suitable volumes of this stock solution were added to the samples to provide an aluminum ion concentration of 0.01M in the final sample solution injected into the atomic absorption furnace.

Table 1. Furnace programmings

Step	Procedure A	Procedure B
Injection I	25 µL sample plus matrix	25 µL matrix (no sample)
Drying I	150°C, 20 s ramp, 20 s hold	120°C, 20 s ramp, 5 s hold
Stop	—	Cool furnace to room temperature
Injection II	—	25 µL sample
Drying II	—	150°C, 25 s ramp, 10 s hold
Ashing	700°C, 10 s ramp, 10 s hold, 19 s baseline correction	Same as Procedure A
Atomization	2200°C, 0 s ramp, 10 s hold, stop flow, read	Same as Procedure A
Final step	2600°C, 1 s ramp, 3 s hold	Same as Procedure A

Aluminum matrix Solution II was prepared by diluting aluminum matrix Solution I with an equal volume of water.

Apparatus

The Perkin-Elmer Model 4000 atomic absorption spectrophotometer was outfitted with a background corrector, platinum lamp (Cat. No. 303-6051), Model HGA-400 furnace programmer, standard graphite furnace (Cat. No. 290-1633), and autosampler (Cat. No. AS-40, Perkin Elmer Corp., Norwalk, CT).

Procedure A (1 Injection)

Samples were prepared by diluting stock solutions with distilled, deionized water to bring the fluoride concentration to 0.1–1.0 ppm.

An aliquot of aluminum matrix Solution I (100 µL) was added to 900 µL of the diluted sample, and 25 µL of the mixture was injected into the graphite furnace. The AlF signal at 227.5 nm was recorded. Details of the furnace programming are shown in Table 1. The absorbances of the sample solutions were converted to parts per million fluorine by reference to the calibration curve obtained with the standards prepared with the same fluorochemical that was present in the sample.

Slopes of these organic fluorine compound calibration curves, as well as of inorganic fluoride calibration curves, vary slightly from day to day and with each new furnace. Usually, we replace the furnace after about 100 injections.

Procedure B (2 Injections)

Aluminum matrix Solution II (25 µL) was injected into the furnace and dried at 120°C. This was followed by injection of 25 µL sample solutions, ashing, atomization, and recording the AlF signal at 227.5 nm. Details of furnace programming are shown in Table 1.

Results and Discussion

Direct-injection AlF MAS is a quick and convenient method for the determination of fluoride in complex matrixes. It has the advantages of simplicity and speed and is readily automated. It does not require the labor-intensive steps involved in the oxygen-bomb and oxygen-flask combustion techniques. However, there is a caveat in this technique. Some fluorochemicals are notoriously volatile. During the 150°C drying and 700°C ashing cycles, some organic fluorine compounds or their thermal decomposition fragments may be partially or totally lost because of volatilization. Because the injected volume is small relative to the surface area of the graphite furnace, and because the furnace is also being flushed with argon at the rate of 300 mL/min, such losses are further enhanced.

In light of these possible problems, we postulated that the "recovery" of fluorine by the direct-injection procedure from various organic-fluorine-containing samples will be highly dependent upon the volatility of the sample, the degree of fluorination, and the mechanism of thermal decomposition of the fluorochemical. Recovery should decrease as the volatility of the sample increases and should also vary from sample to sample if the fluorochemical differs between samples. To test this hypothesis, a selected number of fluorochemicals were analyzed over a 0–1.0 µg F/mL range by direct injection, and the recoveries were compared to those obtained using sodium fluoride standards. The average recoveries for a number of fluorochemicals are summarized in Table 2.

The recoveries of perfluorocarboxylates and perfluorosulfonamido alcohols are shown in Figure 1. The perfluorooctanoates showed low recoveries of fluoride content compared to sodium fluoride. The ammonium salt had a

Table 2. Recoveries of fluorine from various fluorochemicals

Compound name	Structure	NaF standards av. rec., %
(*N*-Methylperfluorooctanesulfonamido)ethanol	$C_8F_{17}SO_2N(CH_3)CH_2CH_2OH$	10.9
(*N*-Ethylperfluorooctanesulfonamido)ethanol	$C_8F_{17}SO_2N(C_2H_5)CH_2CH_2OH$	11.0
Perfluorooctanoic acid	$C_7F_{15}COOH$	25.9
Ammonium perfluorooctanoate	$C_7F_{15}COONH_4$	28.7
Fluorel	$(CH_2F_2)_n[CF_2CF(CF_3)]_m$	86.8

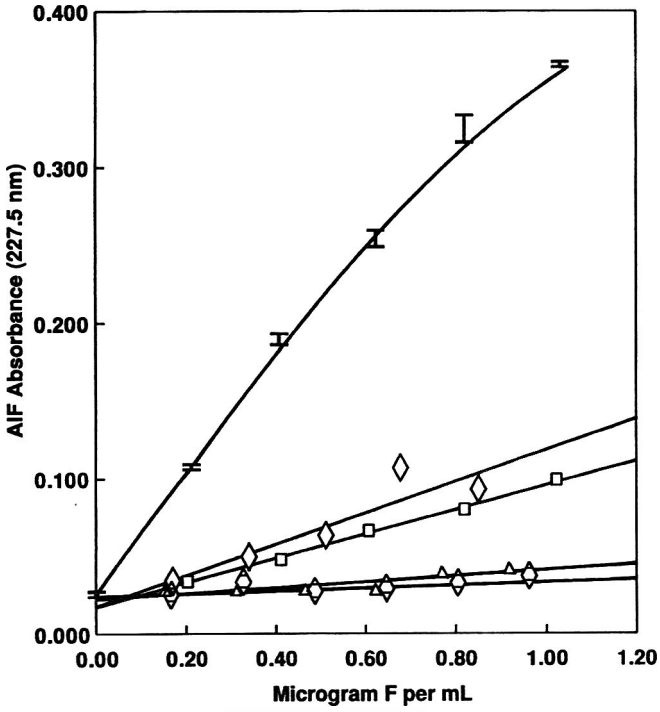

Figure 1. Fluorine recovery of perfluorinated carboxylates and sulfonamido alcohols using Procedure A: (◊) ammonium perfluorooctanoate (r^2 = 0.9216); (□) perfluorooctanoic acid (r^2 = 0.9978); (△) (*N*-methylperfluorooctanesulfonamido)ethanol (r^2 = 0.8875); (▽) (*N*-ethylperfluorooctanesulfonamido)ethanol (r^2 = 0.7216); (l) sodium fluoride calibration curve showing error terms (r^2 = 0.9950). Values are the averages of 5 replicate determinations.

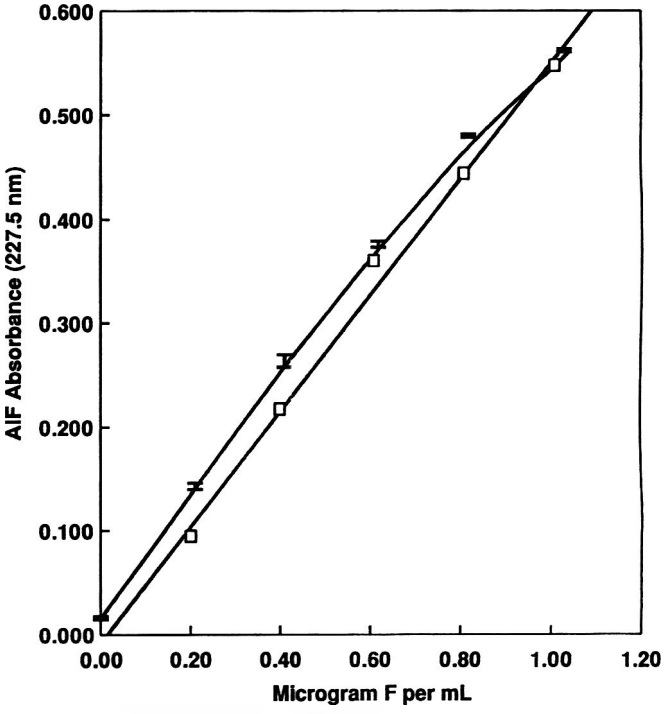

Figure 2. Fluorine recovery of fluoropolymers using Procedure A: (□) Fluorel (r^2 = 0.9966); (l) sodium fluoride calibration curve showing error terms (r^2 = 0.9975). Values are the averages of 4 replicate determinations.

slightly higher recovery, probably due to the slightly higher temperature required for its decomposition to ammonia and perfluorooctanoic acid before volatilization. Response factors, relative to sodium fluoride, were reproducible for each of the samples. A quantitative recovery would be expected if the sample were nonvolatile, as in the case of potassium perfluorooctane sulfonate (5). The response linearity was also good. The correlation coefficient of the ammonium salt is decreased by a single point (0.68 ppm fluoride), which decreases the coefficient from 0.9999.

More volatile fluorochemicals were analyzed, and the results are also shown in Figure 1. Both samples were more volatile than the perfluorocarboxylates, and the resulting recoveries were very poor relative to recovery of sodium fluoride. Neither of the (*N*-alkylperfluorooctanesulfonamido)ethanol samples gave a significant AlF signal when analyzed by the previously mentioned routine. However, the shallow response slopes were reproducible, as in the case with the carboxylates, but with significantly lower correlation coefficients. In general, for samples that give relatively low absorption values, the signal-to-noise ratio can be increased significantly by increasing the amount of sample injected. Likewise, strong signals can be attenuated by injecting less sample.

A sample of a fluoropolymer was analyzed for its fluoride content by both the standard Schöniger oxygen-flask technique and by the direct-injection technique. Sextuplet analyses by the oxygen-flask technique produced a fluoride content of 65.6 ± 0.9%. This value was used in calculating the fluorine content of stock fluoroelastomer solution and the standards prepared therefrom for obtaining the calibration curve. Because the fluoropolymer was a linear chain of vinylidine difluoride and hexafluoropropylene monomers and of approximately 45 000 Daltons, its volatility should be low enough to prevent evaporative losses during the drying step. The results of the direct-injection analysis of the fluoropolymer gum are shown in Figure 2. Over the entire concentration range studied, the recovery was nearly 90%, and the correlation coefficient was excellent. This is most likely due to the nonvolatility of the sample. However, because the chemistry of the fluoropolymer is different from that of the perfluorochemicals, a difference in reaction kinetics with the aluminum ion matrix cannot be ruled out as having no effect.

Thermogravimetric analysis data (Figure 3) lends support, in a broad sense, to our interpretations associating decreasing fluorine recoveries with increasing volatility of the compounds. Again, it must be recognized that the recoveries are also influenced by the differences in the patterns of thermal decomposition of the individual compounds and reaction kinetics with the aluminum matrix.

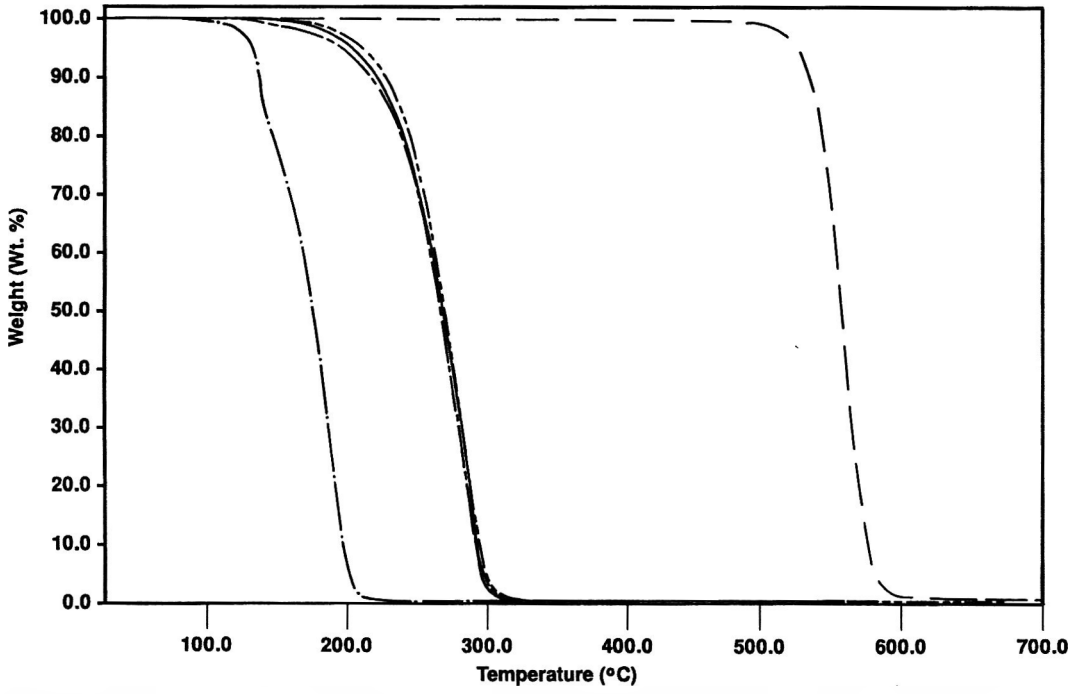

Figure 3. Data from thermogravimetric analysis of selected fluorochemicals: (– · –) $C_7F_{15}COOH$; (— · —) $C_8F_{17}SO_2N(CH_3)CH_2CH_2OH$; (—) $C_8F_{17}SO_2N(C_2H_5)CH_2CH_2OH$; (— · · —) $C_7F_{15}COONH_4$; (— —) Fluorel.

Figure 4 shows calibration curves obtained with a series of complex fluorochemical-containing formulations. The considerable variations in the slopes demonstrate that differences in sample volatility and the functionality of the fluorochemical have a profound effect on fluorine recovery. Typical recoveries of 3 of the above fluorochemicals at 1 μg/mL concentration using inorganic fluoride standards as well as standards made with the respective fluorochemicals are shown in Table 3. The results establish that it is possible to determine individual organic fluorine compounds by the direct-injection AlF MAS method, provided the standards are prepared with the fluorochemical and not with inorganic fluoride.

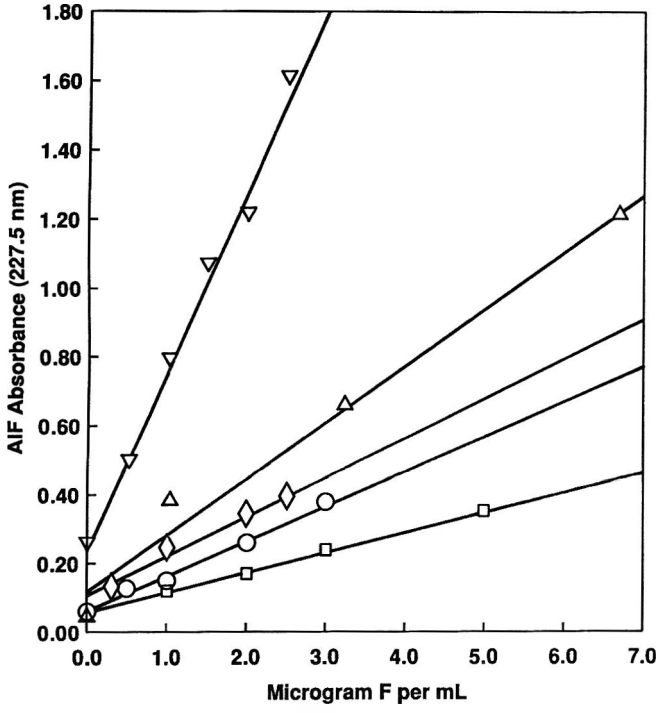

Figure 4. Fluorine response curves for commercial fluorochemical-containing polymers using Procedure B: (▽) FC-391 (r^2 = 0.9956); (△) FX-229 (r^2 = 0.9900); (◊) FC-860 (r^2 = 0.9932); (○) FC-326 (r^2 = 0.9961); (□) FC-905 (r^2 = 0.9985).

The various calibration curves shown in this paper were used in our laboratory for determining the respective fluorochemicals in several diverse materials and in a variety of situations. Typical samples that were analyzed were (*1*) respirators and air filters (to monitor a fluorochemical level in the worker's breathing zone and in the ambient air); (*2*) the level of a fluorochemical in a certain formulation (quality control); and (*3*) water, acetic acid, and heptane extracts of materials into each of which a known fluorochemical was incorporated (U.S. Food and Drug Administration (FDA)-related samples).

An established practice is to add small amounts of fluorocarbon elastomers, such as Fluorel (3M), to polyolefin resins to improve their processibility and to avoid melt fracture during extrusion of the resins (6). Polyethylene film is one of the products obtained in this way. Because such film could be used in the food industry, we were required by FDA to submit information on the levels of the fluorocarbon elastomer that could be extracted during storage and processing of food. This information was obtained by fluorine analysis of extracts of the polyethylene film. These extractions were performed with water, 3% acetic acid, and heptane under specified conditions (7, 8). Premixing of the aqueous aluminum matrix solution and heptane extracts possibly containing highly hydrophobic fluoroelastomer could result in precipitation of the fluoroelastomer.

Table 3. Comparison of recoveries of fluorine in selected fluorochemicals by Procedure B using sodium fluoride and fluorochemical standards

Fluorochemical[a]	NaF standards, µg F found (mean ± SD)	n^b	Fluorochemical standards, µg F found (mean ± SD)	n^b	µg F expected
FC-326	0.43 ± 0.027	4	0.99 ± 0.046	4	1.0
FC-860	0.22 ± 0.029	5	1.03 ± 0.144	5	1.0
FC-905	0.29 ± 0.042	5	1.02 ± 0.123	5	1.0

[a] 3M fluorochemical formulations.
[b] Number of samples analyzed.

Such premixing, then, could lead to false low results. The single-injection procedure (A) accordingly was replaced by 2 separate injections of the aluminum matrix solution and of the extract with an intervening drying (no ashing) step (Procedure B). Water and acetic acid extracts were also analyzed by the same procedure. A typical calibration curve obtained in these studies is shown in Figure 5. The fluoroelastomer standards were made in MEK, in which the polymer is extremely soluble.

Reliability and limitations of the fluorine analytical method in the part-per-billion range were established by analysis of heptane solutions spiked with the fluoroelastomer in MEK to provide levels of 10–50 ppb F in the hexane–MEK mixture (Table 4). Taking into account various sources of error and the day-to-day variations in response of the instrument (caused by changes in the energy of the lamp and the progressive changes in the physical condition and performance of the carbon-rod furnace under several successive firings), placing the reliability of the overall analytical procedure at any better than 20 ±10 ppb would not be safe. Heptane extracts (of the fluoroelastomer-incorporated film) that were found to contain no detectable levels of fluorine were spiked with the fluoroelastomer (in MEK) to a level of 20 ppb and injected into the furnace. Similar spiking of the water and acetic acid extracts (which also contained no fluorine) was attempted but was unsuccessful. The fluoroelastomer that is extremely hydrophobic was instantly thrown out of solution on spiking. As an alternative, we spiked the water and acetic acid extracts with a solution of potassium perfluorooctanesulfonate, which is a stable perfluorochemical and had yielded 100% recovery of fluorine following injection into the graphite furnace (4). The results of recovery in all 3 cases are very satisfactory, considering the very low levels of determination (Table 5).

Determination of fluorine in other samples (e.g., air filters, respirators, formulations) was considerably less difficult because of the much higher levels of fluorine involved. For example, the organic fluorochemical in the air filters was extracted overnight at room temperature with dimethoxyethane by using a mechanical shaker. Standards of the fluorochemical were also prepared in the same extractant. Suitable aliquots of the extracts and the standards were injected into the furnace as in Procedure B.

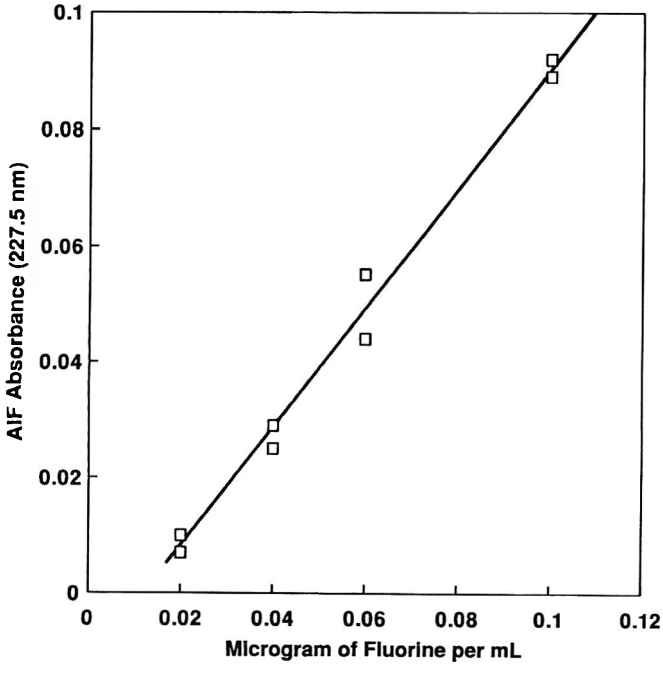

Figure 5. Fluorine calibration curve for Fluorel, in the part-per-billion fluorine range using Procedure B (r^2 = 0.9945).

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Table 4. Limit of sensitivity of the AlF MAS method, Procedure B[a]

Fluorine, ppb expected	Fluorine, ppb found
10	2, 5, 7, 10
20	16, 21, 22, 22
30	30, 30, 32, 33, 35
40	36, 37, 38
50	46, 52

[a] Analysis of hexane solutions containing low levels of the fluoroelastomer.

Table 5. Recovery of organic fluorine added to various extracts of low-density polyethylene film containing Fluorel, Procedure B

	Extract	Fluorine added[a] as	F^-, ppb found (mean ± SD)	n^b	Rec. of added fluorine, %
A	Heptane	Fluorel	18.5 ± 1.0	4	92.5
B	Acetic acid, 3%	Potassium perfluorooctanesulfonate	18.3 ± 2.0	3	91.5
C	Water	Potassium perfluorooctanesulfonate	19.8 ± 1.9	4	99.0

[a] 20 ppb.
[b] Number of samples analyzed.

sions. We also would like to acknowledge help from Terry Kruger, who did the thermogravimetric analysis.

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FRUIT AND FRUIT PRODUCTS

Differential Determination of Furfural and Hydroxymethylfurfural by Derivative Spectrophotometry

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Derivative spectrophotometric methods have been developed for determining furfural and hydroxymethylfurfural and for resolving their binary mixtures on the basis of the UV spectra and the Winkler reaction. The latter method permits the simultaneous determination of both compounds in orange and grapefruit juices without previous separations.

Nonenzymic browning is considered one of the major causes of quality loss in citrus and grapefruit juices (1). High accumulation of furaldehydes indicates that the Maillard type of reaction and acid-catalyzed sugar degradation might be a potential source of browning in stored canned grapefruit juices. 5-(Hydroxymethyl)-2-furaldehyde (HMF) is the principal decomposition product of the acid-catalyzed hydrolysis of glucose and fructose, and furfural is the principal product of the hydrolysis of pentose (2).

Furfural, which has been shown to be virtually absent in freshly processed citrus juice, increases with storage temperature (1). Both furfural and HMF may be related to the darkening of juice and are also useful indicators of temperature abuse or storage time in diverse foods such as juice (3, 4), honey (5, 6), milk (7), and wine (8). Consequently, the analytical control of those compounds is of considerable importance to the food industry.

Spectrophotometric techniques are used for determining HMF and furfural in foods. Dinsmore and Nagy (9) described a colorimetric method for determining furfural in citrus juices on the basis of the reaction with aniline. HMF has been determined by using the Winkler reaction (10) or the reaction with thiobarbituric acid (11); however, these methods determine total furaldehydes. Recently, Montilla Gómez et al. (8) described the use of thiosemicarbazide for determining furfural and HMF in Malaga wines. However, the color reactions used are not specific, and binary mixtures of HMF and furfural cannot be determined without previous separation because of spectral overlapping. Derivative spectrophotometry offers greater selectivity than normal spectrophotometry (12), and mixtures of compounds with highly overlapped spectra have been resolved by this approach.

In this paper, the first–fourth derivative UV spectra of HMF and furfural are used for resolving their binary mixtures. Also, first-derivative spectra of the color reactions of HMF or furfural with barbituric acid and *p*-toluidine (the Winkler reaction) have been used for resolving the mixtures of the furaldehydes. In addition, because of the advantageous nature of derivative spectrophotometry for reducing the background, both aldehydes can be determined in orange and grapefruit juices without pretreatment of the samples.

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Experimental

Apparatus and Reagents

All chemicals used were reagent grade.

(a) *Spectrophotometer.*—Beckman DU-50 spectrophotometer connected to an IBM PC-286 XT computer equipped with Beckman Data Leader software (Beckman Instruments, Inc., Fullerton, CA 92634). Olivetti DM-282 printer was used for all absorbance measurements and treatment of data.

(b) *p-Toluidine solution.*—Prepare 10% solution (w/v) by dissolving 10 g *p*-toluidine (Sigma Chemical Co., St. Louis, MO 63178) in 10 mL acetic acid and diluting to 100 mL with isopropyl alcohol.

(c) *Barbituric acid solutions.*—Dissolve 0.5 g barbituric acid (E. Merck, Darmstadt, Germany) in 100 mL water.

(d) *HMF standard solutions.*—Prepare 0.01% solution (w/v) by dissolving 0.01 g reagent (Sigma Chemical Co.) in 100 mL water.

(e) *Furfural standard solutions.*—Prepare 0.01% solution (w/v) by dissolving 0.01 g reagent (Sigma Chemical Co.) in 100 mL water.

Determination of HMF and Furfural

By UV absorption spectra.—Samples were prepared in 25 mL volumetric flasks containing ≤12 μg HMF or ≤11 μg furfural or the binary mixtures per mL; 5 mL pH 2.8 buffer solution was added, and the solutions were diluted to volume with water. Absorption spectra of the samples were recorded between 350 and 200 nm against deionized water at scan speed

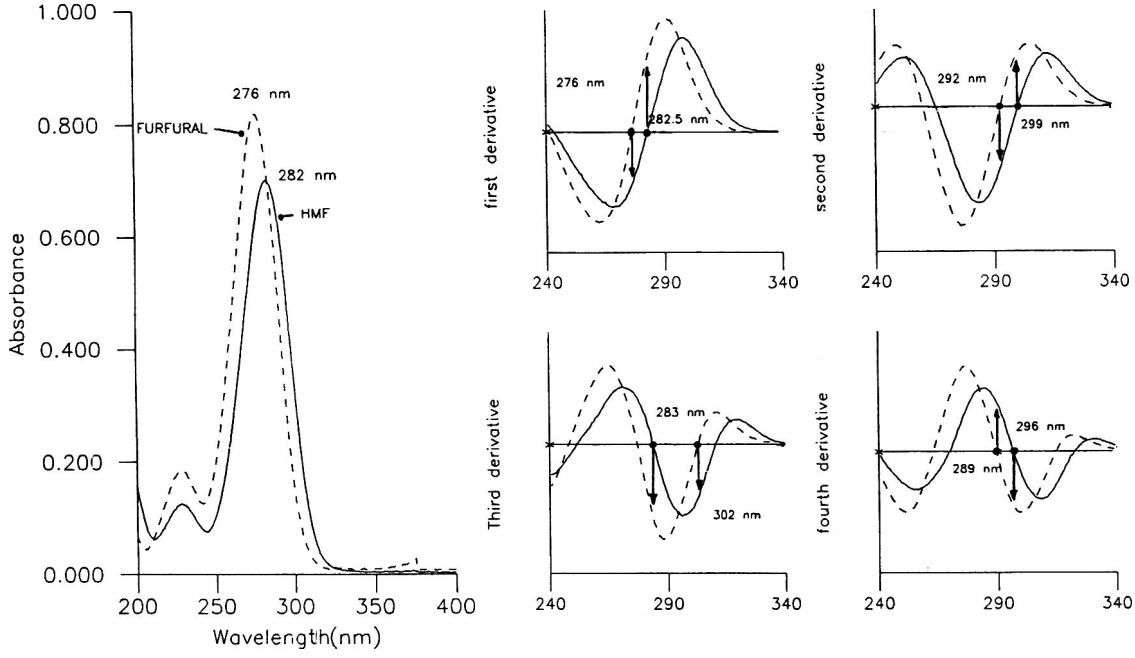

Figure 1. (a) Absorption spectra of HMF and furfural at pH 2.8; (b) first-derivative spectra ($\Delta\lambda$ = 12 nm); (c) second-derivative spectra ($\Delta\lambda$ = 40 nm); (d) third-derivative spectra ($\Delta\lambda$ = 60 nm); and (e) fourth-derivative spectra ($\Delta\lambda$ = 60 nm).

of 750 nm/min. The first–fourth order derivative UV spectra were calculated with 12, 40, 40, and 60 nm $\Delta\lambda$ values, respectively. On the basis of these derivative spectra and by using the 0-crossing measurements, the following determinations can be realized: (*1*) HMF content is determined by measuring at $^1D_{276}$, $^2D_{292.5}$, $^3D_{302.5}$, or $^4D_{289.5}$; and (*2*) furfural content is determined by measuring at $^1D_{282.5}$, $^2D_{299.5}$, $^3D_{283.5}$, or $^4D_{296.5}$.

By the Winkler reaction.—Samples were prepared in 25 mL volumetric flasks containing ≤10 μg HMF or ≤4 μg furfural or their binary mixtures per mL; 15 mL *p*-toluidine solution and 3 mL barbituric acid solution were added, and the solutions were diluted to volume with water. Samples were thermostated at 18°C, and the absorption spectra were recorded 5 min later, between 700 and 450 nm, against deionized water at scan speed 750 nm/min. First derivative spectra were calculated with $\Delta\lambda$ = 40 nm. HMF content was determined by measuring at $^1D_{582}$, and the furfural content was determined at $^1D_{549}$ by using the 0-crossing points.

In orange juices.—Samples were prepared in 25 mL calibrated flasks to contain 3 mL orange juice spiked with different amounts of aldehyde, and the determination according to the Winkler reaction was followed.

In grapefruit juices.—Samples were prepared in 25 mL calibrated flasks to contain 5 mL grapefruit juice spiked with different amounts of aldehyde, and the determination according to the Winkler reaction was followed, except that the absorption spectra were recorded against a blank containing only red grapefruit juice.

Table 1. Statistical data for calibration graphs

Regression equation	Correlation coef. r^2 (n = 8)
$^1D_{276}$ = – 0.0002 – 0.0028 C[a]	0.9998
$^1D_{282.5}$ = 0.0003 + 0.0042 C[b]	0.9994
$^2D_{292.5}$ = – 0.0002 – 0.0042 C[a]	0.9998
$^2D_{299.5}$ = 0.0001 + 0.0019 C[b]	0.9996
$^3D_{302.5}$ = – 0.0002 – 0.0026 C[a]	0.9998
$^3D_{283.5}$ = – 0.0002 – 0.0041 C[b]	0.9996
$^4D_{289.5}$ = 0.0002 + 0.0023 C[a]	0.9998
$^4D_{296.5}$ = – 0.0001 – 0.0029 C[b]	0.9994

[a] C = μg/mL HMF.
[b] C = μg/mL furfural.

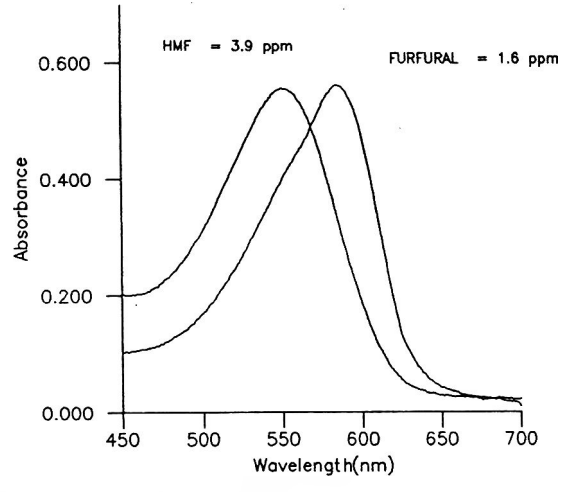

Figure 2. Absorption spectra of colors obtained with HMF and furfural by the Winkler reaction.

Table 2. Determination of HMF and furfural in synthetic mixtures by diverse orders of derivative spectra

Theoretical, μg/mL		Rec., %							
		HMF				Furfural			
HMF	Furfural	1_D	2_D	3_D	4_D	1_D	2_D	3_D	4_D
3.68	2.50	101	91	95	93	101	97	98	102
9.24	2.50	104	102	104	101	93	88	91	99
9.24	3.50	102	101	103	101	95	90	92	103
2.50	10.00	93	96	92	113	104	101	101	101
6.18	5.40	93	89	94	93	107	103	104	106
10.30	5.40	96	93	99	94	100	100	100	100
4.12	9.00	95	92	90	92	103	102	101	101
16.48	5.40	91	96	104	97	85	81	81	83
Av.		97	95	98	98	99	95	96	99
RSD, %		2.0	2.0	2.0	2.5	2.5	2.5	2.6	2.4

Results and Discussion

UV Derivative Spectrophotometry

HMF and furfural aqueous solutions showed overlapping UV spectra at all pH values; therefore, the use of the derivative spectra can resolve this problem satisfactorily.

First, we tested the influence of pH on the absorption spectra of HMF and furfural solutions and found that the spectra remain unchanged between pH 2 and 11. A pH of 2.8 was selected to attempt the quantitative determination of the binary mixtures.

Figure 1 shows the absorption spectra of the HMF and furfural at pH 2.8 and their first–fourth order derivative spectra. The absorption spectra are very overlapped; however, different calibration graphs can be obtained with all derivative UV spectra by using the 0-crossing points as measurement signals.

The optimization of the derivative spectra was based on the influence of the $\Delta\lambda$ used in the differentiation of the absorption spectra of the HMF and furfural solutions, respectively. Different $\Delta\lambda$ values were tested in all order-derivative spectra. In the first-derivative mode, when the $\Delta\lambda$ value increased from 4 to 40 nm, the signal amplitude decreased slightly, and $\Delta\lambda = 12$ nm was considered to be the optimum. In the higher derivative modes, the signal amplitudes increased with $\Delta\lambda$, and $\Delta\lambda$ = 40 nm for second- and third-derivative spectra and $\Delta\lambda = 60$ nm for fourth-derivative spectra were considered optimal.

Ranges of concentrations for HMF and furfural were used to obtain calibration graphs for both compounds in all order-derivative modes and by using the respective 0-crossing points. The most interesting statistical data, summarized in Table 1, show that all derivative orders studied are suitable for determining HMF and furfural up to 12 and 11 μg/mL, respectively.

HMF and furfural were determined simultaneously in synthetic mixtures by measuring the signals at the selected wavelengths in each order-derivative mode. Table 2 presents the results of the determinations for different mixtures; satisfactory results were obtained, with a mean recovery of 97%.

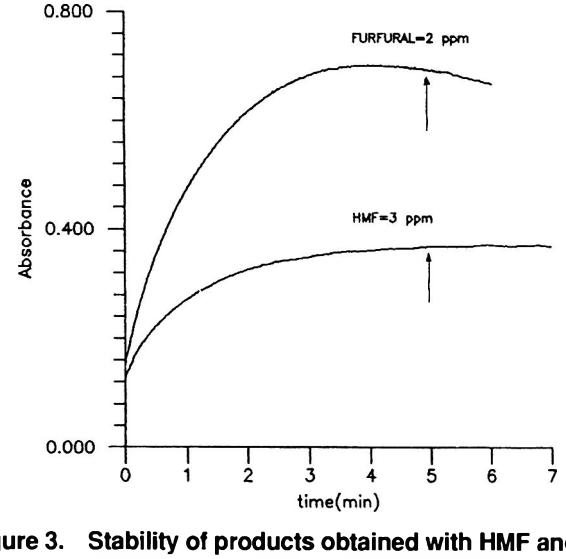

Figure 3. Stability of products obtained with HMF and furfural by the Winkler reaction.

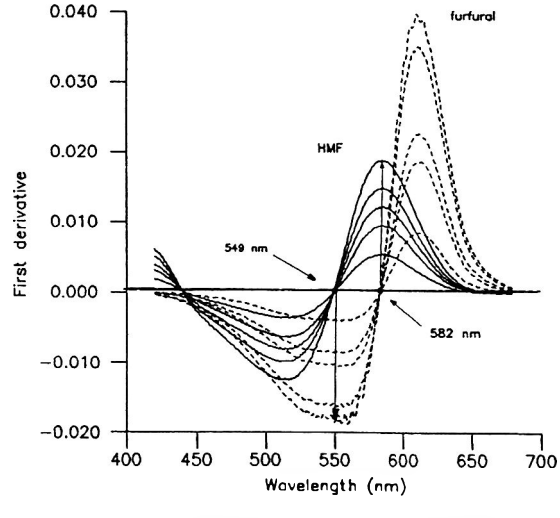

Figure 4. First-derivative spectra of the colored products obtained with HMF and furfural by the Winkler reaction.

Table 3. Statistical data for calibration graphs by application of the Winkler reaction

Parameter	HMF	Furfural
Regression equation	$^1D_{582} = 0.0001 + 0.0020\ C$	$^1D_{549} = -0.0001 - 0.0044\ C$
Correlation coefficient r^2 ($n = 8$)	0.9986	0.9980
Concn range, μg/mL	0.1–10	0.07–4

Table 4. Determination of HMF and furfural in synthetic mixtures by first-derivative spectra and the Winkler reaction

Theoretical, μg/mL		Found, μg/mL		Rec., %	
HMF	Furfural	HMF	Furfural	HMF	Furfural
2.60	0.61	2.70	0.49	104	80
3.90	0.61	4.05	0.51	104	84
5.20	0.61	5.60	0.51	107	84
1.30	1.22	1.60	1.02	123	84
1.30	1.83	1.40	1.76	107	96
1.30	2.44	1.60	2.51	123	103
Av.				111	88
RSD, %				3	4

Winkler Reaction

The use of the Winkler reaction for determining HMF is well known. However, the reaction between barbituric acid, *p*-toluidine, and HMF is not specific; other furaldehydes also react, giving similar color, as in the case of furfural. The absorption spectra of the colors obtained with HMF and furfural are shown in Figure 2; a strong overlap can be observed. Because of the advantageous nature of derivative spectrophotometry for the detection and location of the wavelengths of poorly-resolved components, the quantitative determination of the HMF and furfural mixtures was possible.

A severe disadvantage of the Winkler reaction is the instability of the color obtained. Strict control of the reaction time and temperature is necessary. When the samples are kept at 18°C, the stability is adequate for obtaining reproducible ab-

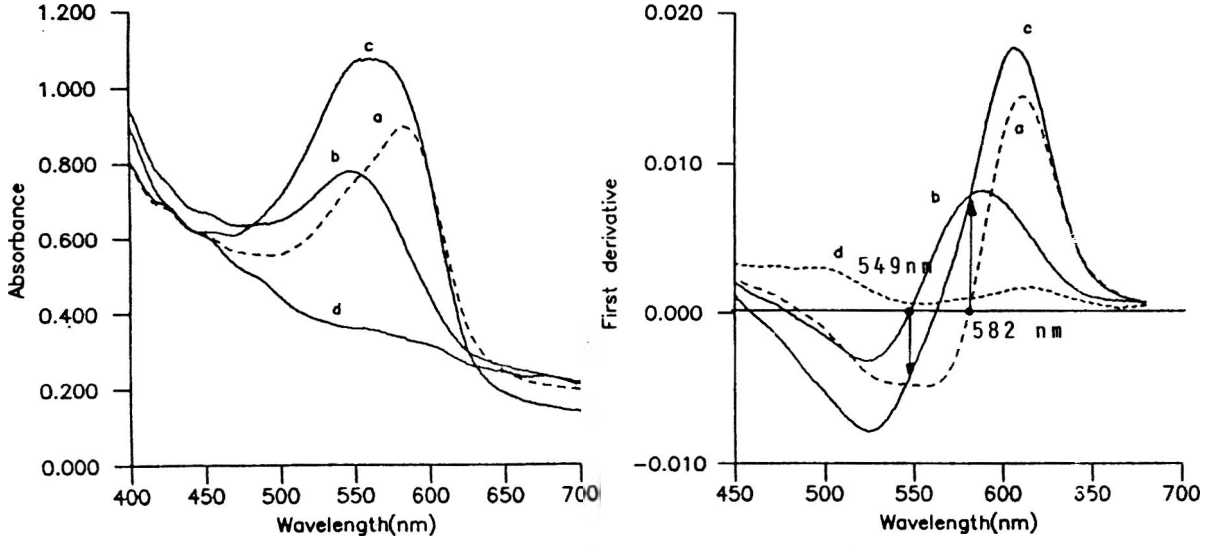

Figure 5. Absorption and first-derivative absorption spectra of the product obtained by the Winkler reaction over orange juice solutions (3:25 dilution) spiked with (a) 1 ppm furfural, (b) 3.4 ppm HMF, (c) 1 ppm furfural + 3.4 ppm HMF, and (d) orange juice without spiking.

Table 5. Determination of HMF and furfural in orange juice by first-derivative spectra and the Winkler reaction

Added to juice, μg/mL[a]		Found in juice, μg/mL[a]			
HMF	Furfural	HMF	Rec., %	Furfural	Rec., %
—	—	4.42	—	0.97	—
—	—	3.41	—	1.16	—
—	8.30	4.16	—	9.33	101
—	11.67	4.58	—	12.23	97
—	16.67	4.58	—	16.70	95
14.17	—	19.92	106	1.16	—
28.33	—	33.11	100	0.90	—
42.50	—	45.49	96	1.16	—
14.17	8.33	20.08	108	8.92	96
28.33	8.33	34.59	106	8.75	94
42.50	8.33	47.50	100	8.58	97
56.33	8.33	61.17	100	8.58	97
14.17	16.67	18.92	100	18.00	102
21.08	16.67	27.33	107	18.00	102
8.33	16.67	13.67	102	19.00	105

[a] A 3 mL portion of orange juice was diluted to 25 mL before absorbance was measured.

sorption spectra if the measurements are made in 3–6 min, as can be inferred from Figure 3. We have chosen 5 min as optimum time for scanning the absorption spectra.

First-derivative spectra of HMF and furfural allowed the measurement of their binary mixtures. The influence of the $\Delta\lambda$ was optimized and $\Delta\lambda$ = 40 nm was considered suitable. A range of concentrations of HMF and furfural were recorded, as shown in Figure 4, and calibration graphs were obtained (in the 0-crossing points) at $^1D_{582}$ and $^1D_{549}$, respectively.

The statistical data obtained from calibration graphs are summarized in Table 3, and the results obtained in the resolution of the synthetic binary mixtures are summarized in Table 4. Mean recoveries of 111 and 85% for HMF and furfural, respectively, were obtained.

Applications

The proposed method based on the Winkler reaction has been tested in orange and grapefruit juices by analyzing different samples containing HMF and furfural or samples spiked with the furaldehydes.

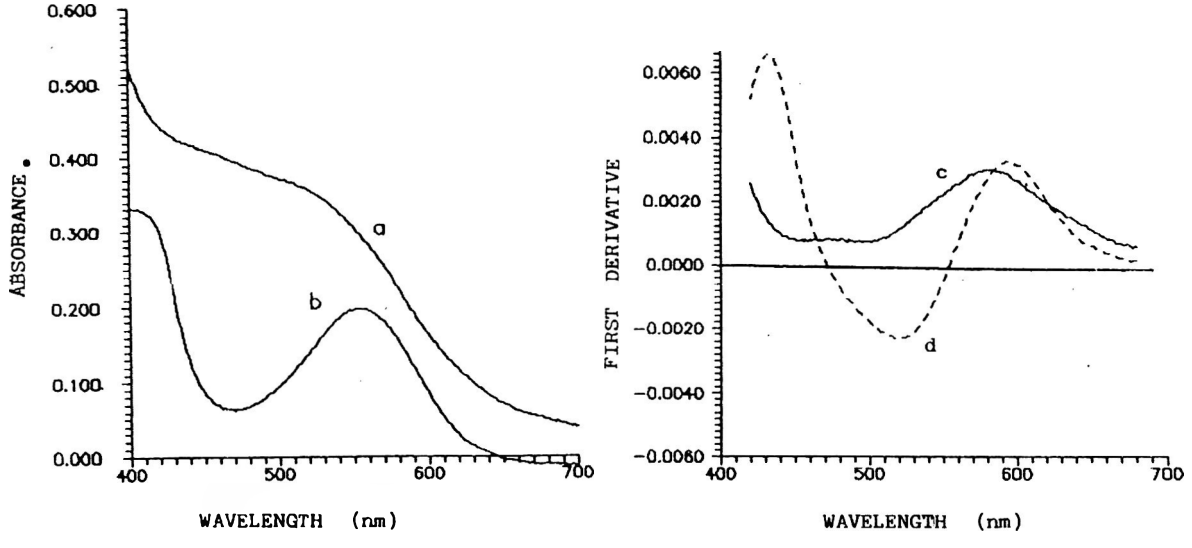

Figure 6. Absorption (a) and first-derivative absorption (c) spectra of grapefruit juice (5:25 dilution) measured against water; absorption (b) and first-derivative absorption (d) spectra of grapefruit juice (5:25 dilution) obtained by the Winkler reaction measured against grapefruit juice.

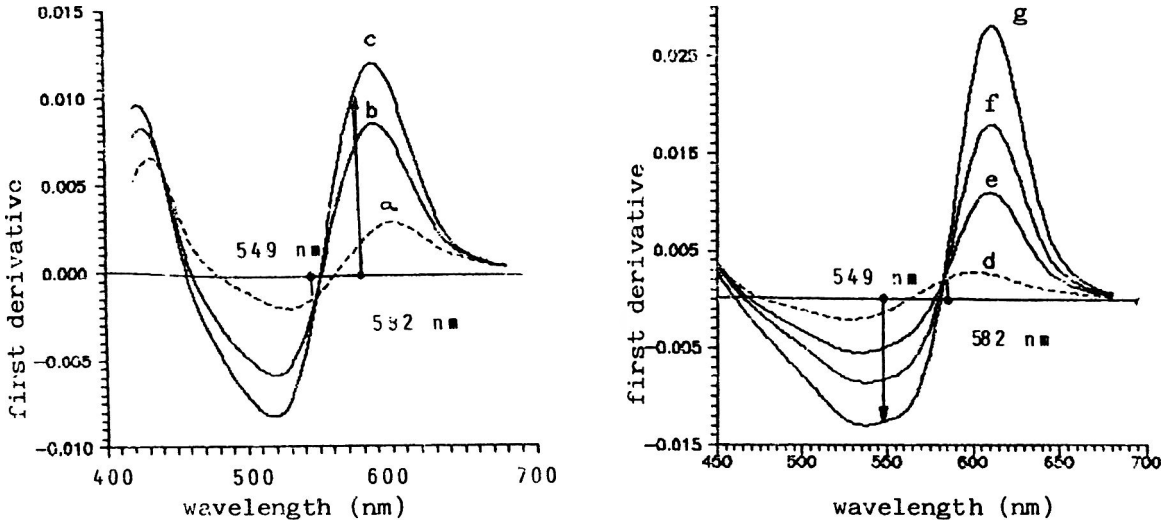

Figure 7. First-order derivative spectra of grapefruit juice solution (5:25 dilution) obtained by the Winkler reaction: (a) and (d), sample without spiking; (b) and (c), sample spiked with 3.38 and 5.08 ppm HMF, respectively; (e), (f), and (g), sample spiked with 1, 2, and 3 ppm furfural, respectively.

The determination in orange juice is very simple because the turbid background of juice is easily eliminated by using the first-derivative spectrum. Figure 5 shows the turbid background of a sample of HMF- and furfural-free orange juice and the same orange juice spiked with HMF, furfural, and a mixture of both aldehydes when 0-order derivative spectra were used. Thus, if first-order derivative UV spectra are used, the turbid background is negligible at wavelengths used for determining HMF and furfural, as can be deduced from Figure 5, whereas the mixture of the furaldehydes can be resolved.

Results obtained by the method of standard additions are summarized in Table 5. Mean values of 4.87 μg/mL (RSD = 0.26%; n = 15) and 0.96 μg/mL (RSD = 0.14%; n = 15) were found for HMF and furfural, respectively.

In the case of the grapefruit juices, the colored background of the samples is not eliminated by using the first-derivative spectrum (Figure 6). To obviate this problem, the samples were measured against a blank containing the same amount of the juice, but the reagents of the Winkler reaction were not added. The standard addition method was applied for each component individually (Figure 7); the results obtained are summarized in Table 6. The mean contents of HMF and furfural found in the original juice were 4.22 μg/mL (RSD = 0.52%; n = 9) and 1.44 μg/mL (RSD = 0.15%; n = 9), respectively.

Conclusions

The proposed method allows the simultaneous determination of HMF and furfural in orange and grapefruit juices without previous separations. The proposed method also minimizes the disadvantages of the Winkler method.

Acknowledgment

The authors gratefully acknowledge financial support from the DGCICYT project PB88-0431.

Table 6. Determination of HMF and furfural in grapefruit juices by first-derivative spectra and the Winkler reaction

Added to juice, μg/mL[a]		Found in juice, μg/mL[a]			
HMF	Furfural	HMF	Rec., %	Furfural	Rec., %
—	—	5.00	—	1.50	—
—	—	5.25	—	1.50	—
8.50	—	12.30	95	1.40	—
16.90	—	21.50	96	1.25	—
25.40	—	29.75	97	1.40	—
—	5.00	5.00	—	6.62	102
—	10.00	5.25	—	11.08	97
—	15.00	5.25	—	17.03	104
8.5	10.00	13.00	94	12.16	107

[a] A 5 mL portion of grapefruit juice was diluted to 25 mL before absorbance was measured.

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MICROBIOLOGICAL METHODS

Modified Colorimetric DNA Hybridization Method and Conventional Culture Method for Detection of *Salmonella* in Foods: Comparison of Methods

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A new, improved colorimetric DNA hybridization method for detection of *Salmonella* in food was developed. A new modified Colorimetric GENE-TRAK® *Salmonella* Assay (m-DNAH) is similar to the current Colorimetric GENE-TRAK *Salmonella* Assay (c-DNAH) except that the number of assay steps has been reduced, indicator dyes have been added to some reagents to facilitate the tracking of reagent additions, and the probe set has been modified so that subgenus V *Salmonella* can be detected. The new probe set detected 453/453 *Salmonella* strains representing at least 239 serovars and 50 somatic antigen groups, including 8 strains of subgenus V *Salmonella* that were not detected by the current probe set. In addition, a total of 225 non-salmonellae representing highly homologous organisms such as *Enterobacter agglomerans*, *Escherichia coli*, and *Citrobacter freundii* were all nonreactive with the modified probe set. Overall, a comparison of m-DNAH to the conventional *Bacteriological Analytical Manual*/AOAC culture method using 240 inoculated food samples and 80 naturally contaminated raw poultry samples showed that both methods were equally effective for detecting *Salmonella* in food products. The method has been adopted first action by AOAC International to replace method 990.13.

The Colorimetric GENE-TRAK® *Salmonella* Assay (c-DNAH) is a DNA probe-based diagnostic kit that permits rapid and accurate detection of *Salmonella* in food samples. The method, **990.13**, was introduced in 1988, collaboratively studied in 1989, and received official first action status in 1989 (1). The procedure uses nucleic acid probes to detect polynucleotide sequences that are uniquely conserved among *Salmonella* organisms. The assay format requires cultural enrichment of test samples, lysis of target organisms by alkaline reagents, hybridization of probes to target nucleic acids, capture of the probe-target complexes onto a polystyrene stick, and colorimetric detection. Overall, the method has proven to be at least equivalent to conventional methods for detecting *Salmonella* in foods. Furthermore, the method has been reported to offer significant specificity advantages when compared with commercially available immunoassay methods (2).

Extensive testing of the c-DNAH has shown that the method is broadly inclusive for *Salmonella* servovars. *Salmonella* belonging to subgenus V are the only sevovars known to be unreactive with the probe set used in the GENE-TRAK test (3). This limitation does not significantly affect the utility of the method for food testing; however, it would be desirable to include the capability to detect subgenus V *Salmonella* as part of the GENE-TRAK test.

The proposed modified Colorimetric GENE-TRAK *Salmonella* Assay (m-DNAH) uses many of the same reagents used in the current method, **990.13** (1). Indicator dyes have been added to the lysis reagent and probe solution. These dyes result in the formation of highly colored solutions, which conveniently confirm that each reagent has been added in the proper sequence. The neutralization reagent used in **990.13** has been eliminated. The probe solution used in the modified test is identical in composition to a combination of the existing neutralization reagent and probe solution except that the new solution contains an indicator dye and an additional probe sequence that is specific for subgenus V *Salmonella*. Finally, the format has been further simplified by reducing the number of required washing steps. Overall, these minor modifications simplify and shorten the test format and broaden the inclusivity of the c-DNAH. All other performance features are unaffected by any of the proposed modifications.

Experimental

Study Objective

The objective of the present study was to compare the proposed m-DNAH method to the conventional *Bacteriological Analytical Manual* (BAM)/AOAC method for detecting *Sal-*

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The recommendation was approved by the General Referee, the Committee Statistician, and the Committee on Microbiology and Extraneous Materials and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1992) *J. AOAC Int.* **75**, 223.

Table 1. Test products, test organisms, and inoculation levels used in comparison of modified DNAH method with method 990.13 and BAM/AOAC procedure

Test product	Organism	Level	MPN, cells/g
Chocolate	*S. senftenberg*	High	0.93
		Low	0.03
Nonfat dry milk	*S. bovismorbificans*	High	0.43
		Low	0.07
Soy flour	*S. malawi*	High	0.15
		Low	0.09
Dried egg	*S. typhimurium*	High	4.60
		Low	0.04

monella in inoculated and naturally contaminated food products. The study was further designed to demonstrate that the m-DNAH method is equivalent to method **990.13** (c-DNAH) in all aspects except that the modified method detects subgenus V *Salmonella*, whereas method **990.13** does not.

Evaluation of Inclusivity and Exclusivity of Modified Probe Set

The inclusivity (ability to detect all salmonellae) and exclusivity (nonreactivity with non-salmonellae) of the modified probe set was tested using an extensive panel of organisms. A total of 453 *Salmonella* strains representing at least 239 serovars and 50 somatic antigen subgroups were tested for inclusivity. A total of 225 non-salmonellae representing highly homologous organisms such as *Enterobacter agglomerans*, *Escherichia coli*, and *Citrobacter freundii* were used to evaluate exclusivity. Overnight cultures were grown in either GN broth (inclusivity) or TSB/YE (exclusivity) to a density of approximately 5×10^8 cells/mL. Test samples (500 µL) were then assayed as in method **990.13**, except that the probe solution was modified to include the subgenus V probe in addition to the probes used in the current probe solution.

Comparison of Sensitivity of Modified Probe vs Current Probe

The sensitivity (number of organisms in the enriched sample required for a positive assay response) of the new probe set was compared to that for the current probe set using 5 food types. The test products included raw pork, roast beef, peanut butter, dried egg, and soy flour. Preenrichment, selective enrichment, and postenrichment of each test product was conducted as described in method **990.13**. For each food type, a single set of selective enrichment broths was used to inoculate 7 sets of postenrichment cultures (GN broths). Following postenrichment, each set of GN cultures was then spiked with *Salmonella* organisms. The final concentration of *Salmonella* organisms in the 7 sets of postenrichment cultures ranged from 2×10^4 to 2×10^7 cells/mL. A different *Salmonella* strain was used to inoculate each of the sample lots. Raw pork, roast beef, peanut butter, dried egg, and soy flour were inoculated with *S. anatum*, *S. agona*, *S. cubana*, *S. infantis*, and *S. malawi*, respectively. Immediately following inoculation, the GN broths were assayed using both the current and the modified probe sets.

Evaluation of Modified Salmonella Test in a Food Study

Test samples.—The m-DNAH method was compared with the c-DNAH method (**990.13**) and the conventional BAM/AOAC procedure using 5 food types. The test products included nonfat dry milk, soy flour, milk chocolate, dried whole egg, and raw turkey. Inoculated samples were used for all products except raw turkey, which was naturally contaminated.

Test organisms.—Inoculated foods were prepared using a variety of *Salmonella* serovars. Table 1 summarizes test products, test organisms, and inoculation levels.

Preparation of inoculated foods.—Freeze-dried cell pellets of *Salmonella* were used to inoculate dried and semisolid food products. For dry products, "seed" foods were prepared by dry blending the freeze-dried cell pellets with 500 g of test product. Chocolate was inoculated by warming the test product to 55°C prior to inoculation to ensure proper distribution of inoculum. The "seeds" were held at room temperature for 10–12 days to allow stabilization of the levels of viable *Salmonella*. After stabilization, inoculation levels of the "seed" were determined using the most probable number (MPN) method based on AOAC culture methods. Each "seed" was then mixed with additional food product to obtain the desired inoculation level. Actual inoculation levels for the diluted "seeds" were confirmed by MPN determinations.

Analysis of inoculated food samples.—Twenty different subsamples of each of 4 foods were analyzed for *Salmonella* at each of 3 inoculation levels (high, low, and uninoculated) by the appropriate cultural method described in the BAM (4), the c-DNAH method (**990.13**), and the m-DNAH method. Twenty-five gram samples were preenriched as described in BAM. A common preenrichment broth was used for all 3 assay methods. After preenrichment, subsequent enrichment and assay steps for the DNA hybridization methods were conducted as in method **990.13** or as described for the modified DNA hybridization method. Samples that produced positive hybridization assay results were further analyzed to confirm the presence of *Salmonella* by streaking GN broths to xylose lysine deoxycholate (XLD), Hektoen enteric (HE), and bismuth sulfite (BS) agar plates. All subsequent steps in the identification of suspicious colonies were performed according to AOAC methods (5).

Analysis of naturally contaminated raw ground turkey.—Twenty different subsamples from each of 4 lots of raw ground turkey were tested. Contamination levels for each lot were determined by the MPN method.

Preenrichment of raw turkey samples was conducted in lactose broth for 24 h at 37°C. A common preenrichment culture was used to inoculate selective enrichment broths used for the BAM and DNA hybridization methods. Enrichment steps following preenrichment were conducted as described in BAM

Table 990.13. Method performance for *Salmonella* in foods, colorimetric deoxyribonucleic acid hybridization method

Food type	Level[a]	Agreement[b]	False-negative rate, % m-DNAH[c]	False-negative rate, % culture[d]
Chocolate	0.93	100	0	0
	0.03	95	0	4.2
Nonfat dry milk	0.43	100	0	0
	0.07	100	0	0
Soy flour	0.15	100	0	0
	0.09	100	0	0
Dried egg	4.60	100	0	0
	0.04	100	0	0
Turkey (Lot 1)	0.04	75	0	55.5
Turkey (Lot 2)	<0.03	75	0	27.8
Turkey (Lot 3)	0.04	70	31.6	0
Turkey (Lot 4)	0.09	65	33.3	75.0

[a] Inoculation or contamination level as determined by most probable number analysis (cells/g)
[b] Percentage of samples read identically by BAM/AOAC culture method (4) and modified DNA hybridization (m-DNAH) method.
[c] Calculated as number of samples found to be positive by BAM/AOAC culture method and negative by m-DNAH method divided by total number of negative samples by m-DNAH method (misclassified positives plus correctly classified negatives).
[d] Calculated as number of samples found to be positive by m-DNAH method and negative by BAM/AOAC culture method divided by total number of negative samples by BAM/AOAC culture method (misclassified positives plus correctly classified negatives).

for the BAM/AOAC method and as described in method **990.13** for the DNA hybridization methods. Samples tested by the hybridization methods were analyzed as in method **990.13** or as described for the modified DNA hybridization procedure. Postenrichment cultures of samples producing positive hybridization assay results were streaked to selective plating agars, and typical isolates were confirmed as described above (4).

990.13 *Salmonella* in Foods—Colorimetric Deoxyribonucleic Acid Hybridization Method

First Action 1991

Method is test procedure for presence of *Salmonella* in all foods. Because a certain percentage of false-positive reactions may be encountered, positive assays must be confirmed by standard culture methods [see (**H**), *Confirmation of Positive DNA Hybridization Results*]. (**Note**: DNA probes used in this assay are reactive with all *Salmonella* subgenera.)

Method Performance:

See Table **990.13** for method performance data.

A. Principle

Detection of *Salmonella* ribosomal RNA (rRNA) uses specific DNA probes. Following preenrichment, selective enrichment, and postenrichment of test samples, bacteria are lysed, and labeled *Salmonella*-specific DNA probes are added for solution phase hybridization. If *Salmonella* rRNA is present in test sample, fluorescein-labeled detector probe and polydeoxyadenylic acid (poly dA)-tailed capture probe will hybridize to target rRNA sequences. Polydeoxythymidylic acid (poly dT)-coated solid phase (dipstick) is then introduced into hybridization solution. Base pairing between poly dA and poly dT facilitates capture of probe–target hybrid nucleic acid molecules onto solid support. Unbound probe is removed by washing, and dipsticks are incubated in horseradish peroxidase–antifluorescein conjugate solution. Conjugate binds to fluorescein label present on hybridized detector probe. Unbound conjugate is washed away, and dipsticks are incubated in substrate–chromogen solution. Reaction of horseradish peroxidase with substrate converts chromogen to blue compound. Reaction is stopped with acid, which changes color of chromogen to yellow. Absorbance at 450 nm is measured. Absorbance in excess of threshold value indicates presence of *Salmonella* in test sample.

B. Apparatus

(**a**) *Photometer.*—To measure absorbance at 450 nm. Blank and sample wells to accommodate 12 × 75 mm test tubes containing solution volume of 1 mL.

(**b**) *Test tube racks.*—3 needed. Plastic, heat-resistant (65°), to accommodate at least 50 test tubes, 12 × 75 mm. Minimum 5 wells/row with 18 mm spacing between wells (measured between centers of wells).

(**c**) *Dipstick holders.*—Plastic device to hold 5 dipsticks in row with 18 mm spacing between dipsticks (center to center).

(**d**) *Wash basins.*—4 needed. Metal or plastic, heat-resistant (65°), 10 × 10 × 9 cm containers, with covers.

(**e**) *Test tubes.*—Glass, 12 × 75 mm.

(**f**) *Heating water bath.*—Capable of maintaining 65 ± 1.0°. Able to accommodate 1 test tube rack and 1 wash basin and water level of 5 cm.

(g) *Repeater pipet.*—Optional. With syringe-barrel tips, capable of accurately delivering aliquots of 0.1, 0.2, 0.25, and 0.75 mL. Alternatively, serological pipets may be used.

Items (a)–(d) are available from GENE-TRAK Systems, 31 New York Ave, Framingham, MA 01701 USA. Substitute materials from other sources must be tested for equivalence.

C. Reagents

(a) *Lysis solution.*—1 bottle (10 mL). Contains 0.75N NaOH and 0.05% thymolphthalein. (*Caution*: Corrosive; avoid contact with skin; if contact occurs, wash skin thoroughly with cold water.)

(b) *Probe solution.*—1 bottle (20 mL). Contains fluorescein-labeled, *Salmonella*-specific, synthetic oligonucleotide DNA probe and polydeoxyadenylic acid-tailed, *Salmonella*-specific, synthetic oligonucleotide DNA probe in 1.05M Tris, pH 7.5; 0.5mM disodium ethylenediaminetetraacetate; 0.05% bovine serum albumin; 0.005% NP-40 (nonionic detergent); 0.025% cresol red; and 0.05% sodium azide. Probes must exhibit specificity for *Salmonella* and lack of cross-reactivity with other Enterobacteriaceae and other bacteria. Specificity is determined by testing pure cultures of selected bacteria, grown in nonselective media to titer $\geq 10^7$/mL in the assay. Appropriate specificity test panels should include multiple representatives of all *Salmonella* subgenera and representatives of other groups of bacteria that may be present in foods, especially other Enterobacteriaceae.

(c) *Wash solution 20× concentrate.*—2 bottles (250 mL each). Contains 1.0M Tris, pH 7.5; 0.4M disodium ethylenediaminetetraacetate; 3.0M sodium chloride; and 0.2% Tween-20.

(d) *Enzyme conjugate 100× concentrate.*—1 vial (1 mL). Contains horseradish peroxidase–antifluorescein polyclonal antibody conjugate.

(e) *Substrate solution.*—1 bottle (56 mL). Standard horseradish peroxidase (HRP) detection reagent for use in ELISA and other HRP-based colorimetric assays. Contains urea peroxide.

(f) *Chromogen solution.*—1 bottle (28 mL). Standard HRP detection reagent for use in ELISA and other HRP-based colorimetric assays. Contains tetramethylbenzidine.

(g) *Stop solution.*—1 bottle (25 mL). Contains 4.0N H_2SO_4. (*Caution*: Corrosive; avoid contact with skin; if contact occurs, wash skin thoroughly with cold water.)

(h) *Dipsticks.*—2 containers (50 each). Polystyrene dipsticks, 8 cm (5 cm handle and 3 cm fin). Fin has 5 paddlelike protrusions coated with poly dT. Binding capacity of poly dT-coated dipsticks should exceed 250 ng poly dA. Dipsticks should be tested in combination with matrix of other reagents to ensure proper method sensitivity.

(i) *Positive control solution.*—1 bottle (5 mL). Contains formaldehyde-inactivated *Salmonella typhimurium* in concentrations sufficient to produce absorbance ≥1.0 when tested in assay. Actual cell concentration used may vary depending on strain of organism employed, media, and conditions used for its preparation.

(j) *Negative control solution.*—1 bottle (5 mL). Contains formaldehyde-inactivated *Citrobacter freundii* in concentrations sufficient to produce absorbance >0.15 in assay when stringency conditions of assay (hybridization and/or wash temperatures) are incorrect. Correctly performed assay should yield absorbance ≤0.15 for negative control. Actual cell concentration used may vary depending on strain of organism employed, media, and conditions used for its preparation.

(k) *Preenrichment medium.*—See **967.26A** or the *Bacteriological Analytical Manual*, 1984, 6th Ed., AOAC, Arlington, VA, Chapter 7, sec. C, with the following exception:

Raw meats and raw milk products.—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Cap jar securely, and blend 2 min at high speed (ca 20 000 rpm). Let stand 60 min at room temperature. Mix contents well by shaking and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 using sterile 1N NaOH or HCl; cap jar securely and mix contents well before determining final pH. Aseptically transfer contents to sterile, wide-mouth, screw-cap 500 mL jar. Loosen jar cap 1/4 turn and incubate 24 ± 2 h at 35°.

(l) *Selective enrichment media.*—(*1*) Tetrathionate broth, **967.25A(c)**. (*2*) Selenite cystine broth, **967.25A(b)(*1*)** or (*2*).

(m) *GN broth.*—20 g tryptose, 1 g dextrose, 2 g D-mannitol, 5 g Na citrate, 0.5 g Na desoxycholate, 1.5 g monopotassium phosphate, 4 g dipotassium phosphate, and 5 g NaCl. Dissolve ingredients in 1 L H_2O. Dispense 10 mL portions into 16 × 125 mm test tubes. Cap tubes loosely and autoclave 15 min at 121°. Final pH should be 7.0 ± 0.2 at 25°.

(n) *Diagnostic reagents.*—As necessary for culture confirmation of positive DNA hybridization assays. See **967.25B**.

Items (a)–(j) are available as Colorimetric GENE-TRAK *Salmonella* Assay (DNA Hybridization Test for Detection of *Salmonella*) from GENE-TRAK Systems. Store probe solution, enzyme conjugate 100× concentrate, substrate solution, chromogen solution, positive control solution, and negative control solution at 2–8°. Store all other solutions and dipsticks at room temperature (<30°). Reagent volumes given above are sufficient for 100 determinations.

D. General Instructions

Include 1 positive control and 1 negative control with each group of test samples.

Do not touch fin portion of dipstick with fingers; hold by handle only. Do not reuse dipsticks or wash solution.

Use separate pipets or tips for each sample and reagent to avoid cross-contamination. Exercise care not to contaminate substrate–chromogen mixture with enzyme conjugate.

Return reagents requiring refrigeration to 2–8° storage immediately after use. Refer to storage requirements on individual reagent bottle labels.

Treat all materials in contact with bacterial cultures as biohazardous material, and decontaminate by appropriate methods.

Caution: Probe solution contains 0.1% Na azide. Disposal of this reagent into sinks with copper or lead plumbing should

be followed immediately with large quantities of water to prevent potential formation of explosive residues.

E. Sample Preparation

(**a**) *Preenrichment.*—Preenrich sample in nonselective medium to initiate growth of salmonellae. Procedure will vary with product type and must be performed as indicated in **C(k)**.

(**b**) *Selective enrichment.*—Transfer 1 mL incubated preenrichment culture to tube containing 10 mL selenite cystine broth (prewarmed to 35°) and 1 mL to tube containing 10 mL tetrathionate broth (prewarmed to 35°) as in **967.26B(a)**. Incubate 6 h at 35° with the following exception:

Raw meats and raw milk products.—Incubate selenite cystine and tetrathionate broths for 18 ± 2 h at 35°.

(**c**) *Postenrichment.*—Remove selective enrichment cultures from incubation and mix by hand or with vortex mixer. Transfer 1 mL tetrathionate culture to tube containing 10 mL GN broth (prewarmed to 35°). Transfer 1 mL selenite cystine culture to separate tube containing 10 mL GN broth (prewarmed to 35°). Incubate GN broths 12–18 h at 35° with the exception of raw meats and raw milk products (see below). Return tetrathionate and selenite cystine tubes to 35° for incubation up to total of 24 ± 2 h.

Raw meats and raw milk products.—Incubate GN broths 6 h at 35°. Return tetrathionate and selenite cystine tubes to 35° for incubation up to total of 24 ± 2 h.

F. DNA Hybridization Assay

(*1*) Label sufficient number of 12 × 75 mm glass test tubes. Include tubes for 1 positive control and 1 negative control per assay. Place tubes in rack in rows of 5.

(*2*) Remove sample GN broths from 35° incubation. Vortex-mix or otherwise mix each culture. For each sample, pipet 0.25 mL from each of the 2 GN broths (one derived from tetrathionate, one from selenite cystine) into single tube. Record sample numbers on data sheet.

(*3*) Mix positive and negative control solutions. Pipet 0.5 mL positive control solution into positive control tube. Pipet 0.5 mL negative control solution into negative control tube. Return controls to 2–8° storage.

(*4*) Add 0.10 mL lysis solution to each tube. Shake rack of tubes by hand for 5 s. Incubate tubes 5 min at room temperature. (**Note**: After addition of lysis solution, resulting solution should have blue color. If any tubes are not blue, recheck that lysis solution has been added.)

(*5*) Add 0.20 mL probe solution to each tube. Shake rack of tubes by hand for 5 s. Cover tubes with Al foil. Place rack of tubes in 65° water bath and incubate 15 min. Return probe solution to 2–8° storage. (**Note**: After addition of probe solution, resulting solution should have red color. If any tubes are not red, recheck that probe solution has been added.)

(*6*) For each 25 tests performed, prepare 1.0 L 1× wash solution. Add 50 mL 20× wash solution to 950 mL distilled or deionized H_2O.

(*7*) Prepare wash basin containing 300 mL 1× wash solution. Cover basin and place in 65° water bath until needed.

(*8*) Place appropriate number of dipsticks into dipstick holders. Rinse dipsticks 2–3 min in 1× wash solution at room temperature. Remove excess solution by blotting to absorbent paper (touch tip of fin portion of dipstick to paper).

(*9*) Remove foil from sample tubes and place dipsticks into tubes. Incubate dipsticks in tubes in 65° water bath for 1 h.

(*10*) Set up second rack of 12 × 75 mm tubes and label appropriately.

(*11*) Prepare sufficient 1× enzyme conjugate by mixing 100× enzyme conjugate concentrate and 1× wash solution. Dispense 0.75 mL 1× enzyme conjugate into each empty tube. Return remaining 100× enzyme conjugate concentrate to 2–8° storage.

(*12*) Remove dipsticks from tubes. Wash dipsticks with gentle shaking for 1 min in 65° wash solution.

(*13*) Blot dipsticks on absorbent paper. Place dipsticks into second set of tubes containing enzyme conjugate. Incubate 20 min at room temperature.

(*14*) Set up a third rack of tubes and label appropriately. Include additional tube for blank.

(*15*) Prepare mixture of substrate–chromogen consisting of 2 parts substrate solution and 1 part chromogen solution. Dispense 0.75 mL substrate–chromogen solution into each empty tube. Return remaining substrate solution and chromogen solution to 2–8° storage.

(*16*) Prepare 2 basins, each containing 300 mL fresh 1× wash solution at room temperature.

(*17*) Remove dipsticks from enzyme conjugate tubes. Wash dipsticks sequentially with gentle shaking for 1 min each in fresh wash solution at room temperature.

(*18*) Blot dipsticks on absorbent paper. Place dipsticks into third set of tubes containing substrate–chromogen solution. Incubate 20 min at room temperature.

(*19*) Remove dipsticks from tubes and discard. Add 0.25 mL stop solution to each tube containing substrate–chromogen solution, including blank. Shake rack of tubes by hand to mix contents.

(*20*) Measure absorbance, *A*, at 450 nm. To read each tube, place reference tube in reference well on left side of photometer and sample tube in sample well on right side. Absorbance will be displayed digitally. Wait for reading to stabilize before recording result for each tube on data sheet.

(*a*) Determine negative control absorbance value by placing tube labeled "Blank" in reference well on left side of photometer. Place negative control tube in sample well on right side of photometer.

(*b*) Determine positive control absorbance value by placing tube labeled "Blank" in reference well on left side of photometer. Place positive control tube in sample well on right side of photometer.

(*c*) Determine test sample absorbance value by placing negative control tube in reference well on left side of photometer and test sample tube in sample well on right side.

G. Data Analysis

(*1*) *A* for negative control should be ≤0.15 (read against blank).

Table 2. Organisms tested to demonstrate inclusivity of modified colorimetric GENE-TRAK *Salmonella* assay

Salmonella subgenus	Serovars tested	Strains tested	Strains positive by assay
I	214	352	352
II	15	16	16
III	1	49	49
IV	3	4	4
V	6[a]	8	8
VI	unknown[b]	7	7
Unclassified	unknown[b]	17	17

[a] Subgenus V serovars tested included *S. balboa*, *S. bongor*, *S. brookfield*, *S. camdeni*, *S. malawi*, and *S. maregrosso*.
[b] Serotypes undetermined.

(2) *A* for positive control should be ≥1.00 (read against blank). If these results are not obtained, assay should be repeated.

Negative criterion.—Test sample is considered negative (nonreactive for presence of *Salmonella*) if *A* is ≤0.10 (read against negative control).

Positive criterion.—Test sample is considered positive (reactive for presence of *Salmonella*) if *A* is >0.10 (read against negative control).

H. Confirmation of Positive DNA Hybridization Results

Samples found positive by DNA hybridization assay must be confirmed by standard culture methods. Except in rare cases, assay can be confirmed from GN broths alone. However, tetrathionate broth and selenite cystine broth cultures should be retained (2–8°) for later evaluation in cases where assay is not confirmed from GN broths. For confirmation, streak cultures to HE, XLD, and BS plates described in **967.26B** and identify typical and suspicious colonies as in **967.26C**, **967.27**, and **967.28**.

Ref.: *JAOAC* **75**, July/August issue (1992)

Table 3. Organisms tested to demonstrate exclusivity of the modified colorimetric GENE-TRAK *Salmonella* assay

Genus	Species tested	Strains tested	Strains unreactive
Aeromonas	1	2	2
Citrobacter	4	27	27
Escherichia	1	46	46
Edwardsiella	3	3	3
Enterobacter	5	71	71
Hafnia	1	10	10
Klebsiella	2	35	35
Morganella	2	2	2
Proteus	1	3	3
Providencia	4	9	9
Serratia	4	9	9
Shigella	4	6	6
Yersinia	1	2	2

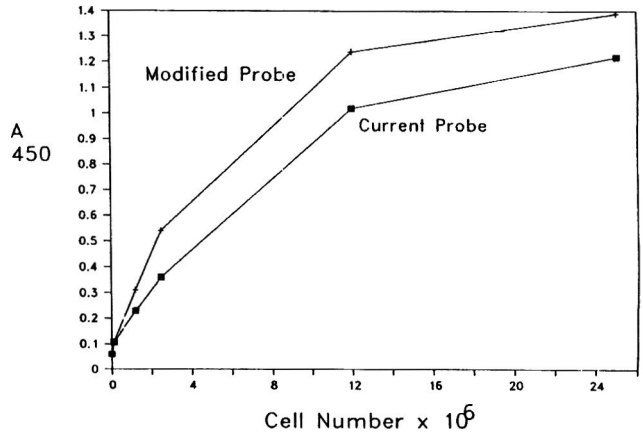

Figure 1. Sensitivity of probes used in modified and current (990.13) DNAH methods: detection of *S. anatum* in ground pork.

Results

Inclusivity and Exclusivity

The results of the inclusivity studies are summarized in Table 2. All organisms tested in the inclusivity panel were positive using the modified probe set. Assay signals (A_{450}) for all organisms ranged from 1.55 to 1.80. The average A_{450} signal of all strains tested was 1.70. This panel included subgenus V *Salmonella*, which cannot be detected using the current probe set.

The results of the exclusivity testing are summarized in Table 3. Assay signals for all test samples were negative using the modified probe set. The A_{450} values ranged from 0.03 to 0.07. The average signal was 0.04.

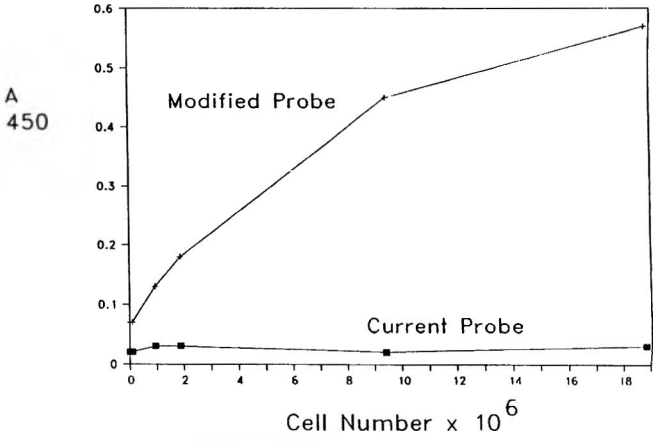

Figure 2. Sensitivity of probes used in modified and current (990.13) DNAH methods: detection of *S. malawi* in soy flour.

Table 4. Results for colorimetric DNAH methods and BAM/AOAC culture method for detection of *Salmonella* in inoculated foods

Product	Inoculation level, MPN, cells/g	Samples tested	m-DNAH positive[a]	c-DNAH positive[b]	BAM/AOAC positive[c]
Chocolate	0.93	20	20	20	20
	0.03	20	16	16	15
	uninoc.	20	1	1	0
Nonfat dry milk	0.43	20	20	20	20
	0.07	20	10	10	10
	uninoc.	20	0	0	0
Soy flour	0.15	20	15	0	15
	0.09	20	5	0	5
	uninoc.	20	0	0	0
Dried egg	4.6	20	20	20	20
	0.04	20	12	12	12
	uninoc.	20	0	0	0

[a] Samples positive by the modified DNAH method, including confirmation.
[b] Samples positive by the current DNAH method (**990.13**) including confirmation.
[c] Samples positive by the BAM/AOAC conventional culture method.

These experiments demonstrated that the inclusivity and exclusivity properties of the modified probe set are equivalent to those for the current probe set (3), except that the modified probe set detects subgenus V *Salmonella*, whereas the current probe set does not. All other specificity properties of the c-DNAH remain unchanged by the inclusion of the subgenus V-specific probe sequence.

Sensitivity

A comparison of the sensitivities of the current and modified probe sets showed that both probe sets were capable of detecting as few as 10^6 *Salmonella* cells spiked into postenrichment broth cultures. The sensitivity of the modified probe set was equivalent to that of the current probe set on the basis of experiments done with test samples spiked with *S. agona* in roast beef, *S. cubana* in peanut butter, *S. infantis* in dried egg, and *S. anatum* in raw pork. A representative sample showing the results obtained for the spiked pork samples in illustrated in Figure 1. Results for the other food types were essentially identical. In addition, tests using *S. malawi*, a subgenus V *Salmonella*, spiked into soy flour revealed that as few as 10^6 subgenus V *Salmonella* cells could be detected using the modified probe set. By comparison, the current probe failed to detect this organism even at higher levels of inoculation (Figure 2).

Inoculated Food Samples

The results of the comparisons between the hybridization assays and the conventional BAM/AOAC procedure for detecting *Salmonella* in 240 food samples are shown in Table 4. All 3 meth-

Table 5. Statistical analysis of data comparing colorimetric DNAH methods to conventional BAM/AOAC method for detection of *Salmonella* in inoculated foods[a]

		False-negative rate, %		
Product	Inoculation level, MPN, cells/g	m-DNAH[b]	c-DNAH[c]	BAM/AOAC
Chocolate	0.93	0	0	0
	0.03	0	0	4.2
Nonfat dry milk	0.43	0	0	0
	0.07	0	0	0
Soy flour	0.15	0	NA[d]	0
	0.09	0	NA[d]	0
Dried egg	4.60	0	0	0
	0.04	0	0	0

[a] False-negative rates were calculated by the method of Fleiss (6) in which the false-negative rate is defined as the number of false-negative results divided by the total number of negative samples (misclassified positives plus the number of correctly classified negatives).
[b] Modified DNAH method.
[c] Current DNAH method (**990.13**)
[d] NA = not applicable. Soy flour was inoculated with subgenus V *Salmonella* known not to react with c-DNAH probes.

Table 6. Comparison of BAM/AOAC culture method, modified (m-DNAH), and current (c-DNAH) colorimetric DNAH methods for detection of *Salmonella* in raw ground turkey

Method	Turkey lot	Contamination level, MPN, cells/g	DNAH positive	BAM/AOAC positive	Total positive[a] (X^2)[b]
Current DNAH	1	0.04	15	11	16 (1.5)
	2	<0.03	8	2	8 (4.2)
	3	0.04	1	7	7 (4.2)
	4	0.09	17	12	18 (2.3)
Modified DNAH	1	0.04	16	11	16 (3.2)
	2	<0.03	7	2	7 (3.2)
	3	0.04	1	7	7 (4.2)
	4	0.09	17	12	18 (2.3)

[a] Total positive represents total number of samples positive by at least one of the methods used.

[b] X^2 = (| samples positive by DNAH and negative by BAM/AOAC − samples positive by BAM/AOAC and negative by DNAH| −1)^2/(samples positive by DNAH and negative by BAM/AOAC + samples positive by BAM/AOAC and negative by DNAH). $X^2 > 3.84$ indicates a statistically significant difference at $P < 0.05$.

ods were in complete agreement for 3 of the 4 inoculated food types, with the exception of 2 samples of chocolate that were negative by the conventional BAM/AOAC procedure but were confirmed positive by both hybridization methods. The fourth food, soy flour, was inoculated with the subgenus V serovar *S. malawi*. The m-DNAH method was in complete agreement with the conventional BAM/AOAC method for all soy samples tested. By comparison, the c-DNAH method failed to detect any positive samples because of the inability of the probes used in the c-DNAH method to detect subgenus V *Salmonella*. Calculations of false-negative rates (6) by food type per inoculation level are given in Table 5.

Naturally Contaminated Raw Poultry Samples

The results of the comparisons between the hybridization assays and the conventional BAM/AOAC procedure for detecting *Salmonella* in 80 naturally contaminated raw poultry samples are summarized in Table 6. Both hybridization assays detected more positive samples than did the BAM/AOAC procedure for all but one lot of turkey analyzed. A pairwise statistical analysis of the results using McNemar's test (7) indicated that the observed differences between the hybridization methods and the BAM/AOAC method were statistically significant ($P < 0.05$) for 2 of the 4 lots when comparing c-DNAH and BAM/AOAC (1 in favor of c-DNAH, 1 in favor of BAM/AOAC) and for only 1 of the 4 lots when comparing m-DNAH and BAM/AOAC (in favor of BAM/AOAC). Furthermore, a similar analysis comparing the 2 hybridization methods revealed no significant difference between these 2 methods (data not shown).

Discussion

The results of this study demonstrate that the proposed m-DNAH method is equivalent to the conventional BAM/AOAC method, on the basis of experiments conducted using a variety of inoculated and naturally contaminated food products. In addition, the modified test detects *S. malawi* and other *Salmonella* belonging to subgenus V, which are not detected by the c-DNAH method. This improvement further expands the overall inclusivity of the method. All other specificity properties of the c-DNAH remain unchanged by the proposed modifications. Furthermore, the proposed format modifications, including the combination of neutralization reagent and probe solutions, the elimination of 1 wash step, and the addition of indicator dyes to the lysis reagent and the probe solution, shorten and simplify the test format.

Recommendation

On the basis of the results of this study, it is recommended that the modified colorimetric deoxyribonucleic acid hybridization method for *Salmonella* in foods be adopted first action to replace the current first action method **990.13**.

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MYCOTOXINS

Determination of Total Aflatoxin Levels in Peanut Butter by Enzyme-Linked Immunosorbent Assay: Collaborative Study

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Laboratories in Australia, Japan, Spain, the United Kingdom, and the United States participated in a collaborative study to evaluate a commercial enzyme-linked immunosorbent assay for the determination of total aflatoxin. Collaborators were sent 10 randomly numbered samples (5 blind duplicates) of roasted peanut butter. Two pairs were "blank" peanut butters to which aflatoxin B_1, B_2, G_1, and G_2 standards had been added. The other 3 pairs of peanut butters were 1 low aflatoxin level sample and 2 naturally contaminated samples. The assay is based on indirect competition. Test samples containing (free) aflatoxin, added to aflatoxin-coated microwells, compete for specific monoclonal rat anti-aflatoxin. As the concentration of aflatoxin in the test samples increases, the amount of rat anti-aflatoxin binding to the aflatoxin attached to the well decreases. After a wash step to remove unbound material, the amount of rat anti-aflatoxin bound to the well is determined by its reaction with peroxidase conjugated rabbit anti-rat globulin. Bound peroxidase activity is then determined by the addition of a substrate, whose color development is inversely proportional to the aflatoxin concentration and is measured by absorbance. Coefficients of variation (CV) for total aflatoxin concentrations, for mean levels of 9, 30, and 89 μg/kg, were between 28 and 37% for the low level and 2 naturally contaminated samples, which contained mainly aflatoxin B_1. CVs for the spiked samples were lower (24–25%) for mean levels of 11 and 20 μg/kg; recoveries were 84 and 89%, respectively. Ranges for relative standard deviations for repeatabilty and reproducibility were 9–30% and 25–37%, respectively. The method has been adopted first action by AOAC International.

Immunoassays have been developed for the analysis of several mycotoxins (1), including the aflatoxin group. Aflatoxins are the carcinogenic and teratogenic metabolites of *Aspergillus flavus* and *A. parasiticus*, and they can contaminate cereal crops, groundnuts, and their products (2). Because of the need to control aflatoxin levels in the food supply, many countries have passed legislation stating maximum tolerance levels for these toxins, which vary from 1 to 50 μg/kg (3). Monitoring for aflatoxin to ensure foodstuffs do not contain contamination above the permitted tolerance level requires initial, rapid screening of large numbers of food samples, and the use of enzyme-linked immunosorbent assays (ELISAs) offers significant advantages.

Interlaboratory studies are essential for the evaluation of commercial ELISAs and for critical assessment of their application to food contamination monitoring. Recently, an ELISA method for analysis of aflatoxin B_1 was collaboratively studied (4) and adopted first action by AOAC International for screening corn and peanut products, as a follow-up to the first action method, **989.06**, for the ELISA screening test for aflatoxin B_1 in cottonseed and mixed feeds. Another ELISA collaborative study by 12 laboratories using an antibody-coated porous polyethylene cup system to analyze aflatoxins B_1, B_2, and G_1 in corn, cottonseed, peanuts, and peanut butter (5) was adopted first action as a screening method for these 3 aflatoxins at levels ≥20 μg/kg in cottonseed and peanut butter and ≥30 μg/kg in corn and raw peanuts. In 1988, a 16-laboratory study was undertaken to evaluate 3 ELISAs commercially available in the United Kingdom to quantify aflatoxin levels in roasted peanut butter (6). At that time, the study indicated that the assays were acceptable for screening but not for use in enforcement actions. Possibly because the study was not designed according to AOAC collaborative study guidelines (7), high within- and between-laboratory variances resulted. Therefore, to validate the Biokits total aflatoxin ELISA kit, the AOAC collaborative

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study reported here was conducted for the analysis of aflatoxins in peanut butter.

Collaborative Study

The 13 collaborating laboratories were sent a Biokits total aflatoxin ELISA kit (8), 10 samples (approximately 12 g each) of roasted peanut butters contained in 20 mL wide-necked polyethylene bottles, a protocol, and data sheets. The 10 trial samples, randomly coded with a 3-digit number, included 2 samples of "blank" peanut butter [<1 μg/kg aflatoxin concentration, estimated by liquid chromatography (LC) (9)] to which aflatoxin standards had been added (Spikes 1 and 2), 1 sample of peanut butter with low-level aflatoxin concentration, and 2 samples of peanut butters naturally contaminated with aflatoxins B_1, B_2, G_1, and G_2. All 5 samples were sent as blind duplicates. Spike 1 contained aflatoxins B_1 (5 μg/kg), B_2 (2 μg/kg), G_1 (5 μg/kg), and G_2 (1 μg/kg). Spike 2 contained aflatoxins B_1 (10 μg/kg), B_2 (3 μg/kg), G_1 (7 μg/kg), and G_2 (2 μg/kg).

The 2 levels of naturally contaminated peanut butter were prepared by blending uncontaminated, retail-purchased peanut butter for 1–2 h with naturally contaminated smooth roasted peanut butter having an aflatoxin B_1 concentration of approximately 200 μg/kg (9). The blending procedure was previously demonstrated (10), through analysis of subsamples, to be sufficiently rigorous to produce homogeneous material. Spiked peanut butters were produced by adding aflatoxin standard solutions to uncontaminated peanut butter and, after solvent evaporation, blending as above.

Collaborators were asked to analyze a 10 g portion of each peanut butter sample by following exactly the procedure described in the protocol. Results reported by collaborators were to be the mean of triplicate assays of each sample.

991.45 Total Aflatoxin Levels in Peanut Butter—Enzyme-Linked Immunosorbent Assay Method (Biokits)

First Action 1991

[Quantitative for aflatoxin (B_1, B_2, G_1, and G_2) at ≥9 and ≤90 μg/kg in peanut butter]

Method Performance:

(*Note*: Aflatoxin concentrations are means after outlier removal.)

9 μg aflatoxin/kg peanut butter

$s_r = 2.6$; $s_R = 3.1$; $RSD_r = 30.3\%$; $RSD_R = 37.2\%$

30 μg aflatoxin/kg peanut butter

$s_r = 4.3$; $s_R = 9.7$; $RSD_r = 14.5\%$; $RSD_R = 32.8\%$

90 μg aflatoxin/kg peanut butter

$s_r = 19.0$; $s_R = 25.3$; $RSD_r = 21.4\%$; $RSD_R = 28.5\%$

A. Principle

Indirect competitive enzyme immunoassay is a specific test for total aflatoxin. Solvent extracts of peanut butter are added to plastic microwells precoated with aflatoxin, and specific monoclonal rat anti-aflatoxin is added to microwells. As concentration of aflatoxin increases in samples, amount of rat anti-aflatoxin binding to toxin attached to well decreases. Unbound material is removed by washing, and amount of rat anti-aflatoxin bound to well is determined by reaction with peroxidase conjugated rabbit anti-rat globulin. Bound peroxidase activity is determined by addition of substrate. Color development is inversely proportional to aflatoxin concentration in diluted extracts and is measured by absorbance.

B. Specificity

Specifications for aflatoxins in this assay are stated in terms of cross-reactivity, which is defined as ratio of mass of aflatoxin B_1 to mass of cross-reacting substance (at 50% inhibition of maximum binding), expressed as a percentage. Cross-reactivities are as follows: aflatoxin B_1, 100%; aflatoxin G_1, 100%; aflatoxin B_2, ca 60%; aflatoxin G_2, ca 60%; aflatoxin M_1, <0.1%; aflatoxin B_{2a}, <0.1%; and aflatoxin G_{2a}, <0.1%.

C. Precautions

Aflatoxins are toxic and carcinogenic. Use fume hood and wear gloves. Incinerate used gloves, etc. To decontaminate glassware, soak ≥30 min in 5% sodium hypochlorite. Adjust pH to 7.0–8.0 using 2M HCl, add 5% acetone, mix, and soak 30 min.

D. Apparatus

(**a**) *Fume hood.*—Flow rate ≥0.5 m/s linear velocity.

(**b**) *High-speed blender.*—Explosion proof, with 1 L stainless steel jar.

(**c**) *Graduated glass cylinders.*—1 L and 250 mL, class A.

(**d**) *Flasks.*—Conical flasks, 150 mL, ground glass jointed, stoppered.

(**e**) *Test tubes.*—Disposable, glass, 5–10 mL.

(**f**) *Gloves.*—Disposable, vinyl.

(**g**) *Micropipet.*—Calibrated pipet or automatic pipet, with disposable tips, capable of accurate delivery of 50 μL.

(h) *Repeating dispenser.*—Dispenser, with disposable tips, capable of accurate delivery of 50 and 100 μL.

(**i**) *Microtiter plates.*—Containing 375 μL flat bottom wells, 96 wells/plate.

(**j**) *Plate washer.*—Capable of repetitive filling and aspirating of a microtiter plate, as described in **D(i)** (Automatic system Nunc Immuno Wash, Gibco Ltd, or equivalent).

(**k**) *Plate reader.*—Spectrophotometer that will read microtiter wells, as described in **D(i)**, in strips, with 405–420 nm interference filter (Uniskan/multiskan microwell plate reader, Labsystems Ltd, or equivalent).

(**l**) *Filter paper.*—20–25 μm particle retention, qualitative grade (Whatman No. 4, or equivalent).

(**m**) *Wrapping film.*—Waterproof, flexible film (Parafilm M, or equivalent).

E. Reagents

(**a**) *Standard aflatoxin solutions.*—1 vial each (1.0 mL) 16, 40, 160, 400, and 1600 pg/mL aflatoxin B_1 in 0.01M phosphate

buffered saline (PBS) with 2% acetonitrile, 0.05% Tween 80 (wetting agent), and 0.05% sodium azide.

(**b**) *Negative control.*—1 bag (55 g) peanut meal, <1 μg/kg total aflatoxin.

(**c**) *Positive control concentrate.*—1 vial (1 mL) aflatoxin B_1 at 2 ng/mL in acetonitrile–H_2O (50 + 50).

(**d**) *Antigen.*—1 vial (6.0 mL) rat anti-aflatoxin in PBS with 0.1% bovine serum albumin (BSA), 0.05% Tween 80, and 0.05% sodium azide.

(**e**) *Conjugate.*—1 vial (11.5 mL) aflatoxin assay peroxidase conjugate in PBS with 0.1% BSA and 0.01% thiomersal.

(**f**) *ABTS concentrate.*—1 vial (0.65 mL) 1.5% 2,2′-azino-di(3-ethylbenzthiazoline)sulfonic acid.

(**g**) *ABTS diluent.*—1 vial (12 mL) citric acid (2.3%, w/v)/H_2O_2 (0.015%, w/v).

(**h**) *Wash solution concentrate.*—1 bottle (100 mL) Tris buffered saline, 0.05% Tween 80, and 0.01% thiomersal.

(**i**) *Aflatoxin assay diluent concentrate.*—1 vial (25 mL) 0.1% BSA in PBS, 0.05% Tween 80, and 0.25% sodium azide.

(**j**) *Stop solution.*—1 vial (6.0 mL) 1.5% NaF.

(**k**) *Microwell module.*—1 aflatoxin sensitized microwell module (96 assay wells). Keep dry during storage.

(**l**) *Aqueous acetonitrile solution.*—Acetonitrile–H_2O (50 + 50).

Items (**a**)–(**l**) are available as Biokits total aflatoxin ELISA kit (Cortecs Diagnostics Ltd, Deeside Industrial Park, Clwyd CH5 2NT, UK). Store kit at 2–8°C. After opening kit, use within 4 weeks and minimize exposure to room temperature. Kit is sufficient for 12 determinations, each in triplicate.

F. Preparation of Samples and Negative Control

Blend 10.0 g each peanut butter sample with 50 mL aqueous acetonitrile solution 2–3 min. Filter and dilute filtrate 1:25 in aflatoxin assay diluent concentrate (**i**)–H_2O (20 + 80). For negative control, follow same procedure, using 10.0 g aflatoxin-free peanut meal.

G. Preparation of Test Solutions

(**a**) Mix all vials by inversion before opening.

(**b**) Prepare working diluent solution by diluting aflatoxin assay diluent concentrate 1:5 with water.

(**c**) Prepare negative control solution by adding 0.2 mL aflatoxin-free peanut meal extract to 4.8 mL working diluent solution. Use this to dilute (1:25) the positive control concentrate (**c**) to produce the positive control solution.

(**d**) Prepare working ABTS solution by adding 0.5 mL ABTS concentrate to contents of ABTS diluent vial (12 mL).

(**e**) Prepare the working wash solution by diluting wash solution concentrate 1:10 with water.

H. Enzyme Immunoassay Procedure

Once assay has started, all steps should be completed without interruption.

(*1*) Pipet 50 μL each into wells; aflatoxin standard solutions (in duplicate), positive and negative control solutions (in triplicate), and diluted extract filtrate of unknowns (in triplicate). Pipet 50 μL working diluent solution into maximum binding well. Leave substrate blank well empty.

(*2*) Pipet 50 μL rat anti-aflatoxin solution into each well except substrate blank well, gently mix plate by side-to-side rocking, cover with film, and incubate 16–18 h at 2–6°C.

(*3*) Aspirate each well 5 times with working wash solution, and tap inverted plate on several layers of absorbent tissue to remove residual droplets of wash solution.

(*4*) Pipet 100 μL aflatoxin assay peroxidase conjugate into each well except substrate blank well, mix plate, and incubate 1 h at room temperature (19–23°C). Repeat washing sequence as in (*3*).

(*5*) Pipet 100 μL working ABTS solution into each well, mix plate, incubate at room temperature 30–40 min, and mix plate. Blank plate reader on air and measure absorbance of maximum binding well. If A is <1.4 units, continue incubation until this value is reached.

(*6*) Pipet 50 μL stop solution into each well, mix plate, blank plate reader on substrate blank well, and measure absorbance of each well, using 414 nm filter.

I. System Suitability

For optimum performance, assay should be performed between 19 and 23°C, which should result in maximum binding absorbance in range of 1.4–2.0 absorbance units. Ratio of mean absorbance of maximum binding well to 16 pg/mL standard wells should approximate 0.85 (range 0.70–0.95). This ratio indicates that assay components are performing within specifications.

Under some incubation conditions (e.g., above 23°C), maximum absorbance value will rise beyond scale of some microplate readers. This may reduce difference between maximum binding and 16 pg/mL standard mean absorbances, and results of the assay may be invalid. Assay should be repeated, adjusting substrate incubation time to obtain suitable maximum binding level and discrimination between maximum binding and 16 pg/mL standard.

Absorbance value of substrate blank should be <0.15, when read against air. Mean absorbance of negative control should be between that of maximum binding value and 16 pg/mL standard. Positive control solution response should be equivalent to sample containing 10 μg/kg total aflatoxin.

J. Calculation of Results

Use prelabeled semilog graph paper (x-axis log, 1–500; y-axis, 0–2) to draw calibration curve by plotting mean of the 2 absorbance values for each of the 5 standards vs concentration of standards. Calculate mean absorbance of each sample triplicate, and read aflatoxin concentration from curve to determine total aflatoxin concentration for each unknown sample.

CAS–1162–65–8 (aflatoxin B_1)
CAS–7220–81–7 (aflatoxin B_2)
CAS–1165–39–5 (aflatoxin G_1)
CAS–7241–98–7 (aflatoxin G_2)

Ref.: *JAOAC* **75**, May/June issue (1992)

Table 1. Collaborative results and statistical analysis of Biokits ELISA determination of total aflatoxin in peanut butters

Laboratory	Spike 1		Spike 2		Sample 1		Sample 2		Sample 3		Positive control
1	11	8	19	17	13	9	32	18	62	62	11
2	9	10	22	21	6	6	28	28	87	153	8
3[a]	150	24	29	40	10	102	36	23	7	88	7
4	9	14	18	24	9	16	32	23	110	120	14
5[a]	4	15	35	19	3	10	30	39	131	169	20
6	16	16	27	26	7	9	40	35	100	110	10
7	7	10	18	15	6	6	22	20	62	110	9
8	12	12	20	19	8	7	28	28	94	82	13
9	9[b]	21[b]	23[b]	39[b]	15	9	46	51	53	60	7
10	8	10	15	14	6	6	50[b]	15[b]	66	82	10
11	13	13	25	27	8	9	38	39	103	109	18
12	8	7	13	11	6	5	17	18	64	76	10
13	12	13	21	19	7	13	22	28	105	84	10
Mean	10.9		19.6		8.5		29.7		88.8		10.9
Spike level	13.0		22.0		—		—		—		—
SD	2.8		4.7		3.1		9.5		25.0		3.1
CV, %	25		24		37		32		28		28
r	4.4		5.1		7.2		12.0		53.2		—
R	7.8		13.4		8.8		27.3		70.8		—
s_r	1.6		1.8		2.6		4.3		19.0		—
s_R	2.8		4.8		3.1		9.7		25.3		—
RSD_r	14.5		9.2		30.3		14.5		21.4		—
RSD_R	25.7		24.4		37.2		32.8		28.5		—
n	20		20		22		20		22		11

[a] Data from this laboratory excluded from statistical treatment.
[b] Outlier by Cochran's test.

Results

Table 1 shows the results of the total aflatoxin concentration (in µg/kg) found in the peanut butter samples by the 13 collaborators. The samples are labeled 1–3, Spike 1, and Spike 2. Sample 1 was a roasted peanut butter with a low concentration of aflatoxin; Samples 2 and 3 were naturally contaminated roasted peanut butters. Table 1 shows the levels of aflatoxins added to "aflatoxin-free" peanut butter [<1 µg/kg total aflatoxin concentration, estimated by LC (9)] to prepare the 2 spike samples. Data for the positive control values are also included in Table 1, with statistical details of spike and sample mean values, standard deviations (SD), coefficients of variation (CV), repeatability values (r) with repeatability standard deviation (s_r) and relative standard deviation (RSD_r), and reproducibilty values (R) with reproductibility standard deviation (s_R) and relative standard deviation (RSD_R) after removal of outliers (7, 10). Data from Laboratories 3 and 5 were excluded from statistical treatment (see below). Numbers of data points (*n*) are also included.

Table 2 shows the overall precision (11) of the immunoassay data from each of the laboratories where unknown sample data have been reduced to a single percent CV.

Table 2. Overall precision of Biokits ELISA collaborative study of total aflatoxin in peanut butters

Laboratory	CV, %
1	13
2	13
4	26
6	7
7	18
8	7
9	44
10	12
11	16
12	8
13	12

Discussion

Data from 2 laboratories were excluded from the statistical treatment shown in Table 1. Data from Laboratory 3 were excluded after a problem with the laboratory plate reader was reported. Data from Laboratory 5 were excluded as unsatisfactory because 2 standard replicates, a control and 1 unknown point, had been rejected by the laboratory and because the shape of their standard curve differed grossly from that of the

other laboratories. Application of Cochran's test ($p \leq 0.05$) (7, 10) produced 3 pairs of outliers. There were no outliers shown by Dixon's (10) or Grubbs tests ($p \leq 0.05$) (7).

The precision of the assay is reflected by the CVs being between 24 and 37%. CVs were lower for the spiked samples than for the naturally contaminated samples. These latter samples have also been analyzed in a collaborative trial using an immunoaffinity column cleanup and LC quantitation (12), where mean levels for total aflatoxin concentration, uncorrected for recovery (53–61%), were 4, 15, and 38 µg/kg, compared with means of 9, 30, and 89 µg/kg in this ELISA trial. CVs in the earlier trial were 32–44%, somewhat higher than for the ELISA trial.

Estimated recovery levels for the 2 spikes were 84 and 89%. These levels are much higher than those in the immunoaffinity column collaborative trial (12).

For Samples 1–3, RSD_R decreased from 37 to 29% as the mean aflatoxin concentration increased. In the LC study, there was no discernible trend, but RSD_Rs were higher (33–45%). No trends, with increasing aflatoxin concentration, were observed for RSD_r in either study (12).

Overall, assay precision was assessed by reducing the data from each of the individual ELISA results for an unknown sample to a single percent CV. Such a measure of overall assay precision is now a commonly used quality assurance parameter in medical diagnostic immunoassays (11). The CV value is obviously partly affected by the reagents themselves (e.g., precision of plate manufacture) and partly by operator variation (e.g., imprecision in pipetting). As a guide (11), the CV value should be less than 25%; however, in a laboratory very familiar with a particular ELISA assay, it should be 5–15%. Ten of the 11 laboratories performed well (Table 2), with a CV range between 7 and 26% (mean 14%). Laboratory 9 reported a CV of 44%, and this assay was clearly outside acceptable limits. However, the data from this laboratory was included for statistical consideration, unlike the data for Laboratories 3 and 5, which were excluded because of equipment/assay failure.

The control value of Laboratory 11 was high, 18 µg/kg on a nominal value of 10.0 µg/kg, compared to the mean of the other 10 values, 10.2 µg/kg. However, this laboratory had good overall precision and showed good agreement with the mean trial data.

Four participants made comments regarding the analytical method and the results obtained. Two participants stated that they were very pleased with the performance of the kit, and one said it was "more handy to use" than several other ELISA kits for aflatoxin analysis. Another participant noted that the assay was easy and very rapid. Still another participant stated that, although the protocol was quite lengthy, it was easy to follow.

Recommendation

Statistical analysis has shown that results from 10 of 11 laboratories using this method had acceptable or good precision. We recommend that the Biokits total aflatoxin ELISA kit be adopted first action for the determination of aflatoxin in peanut butters where mean concentrations are between 9 and 90 µg/kg total aflatoxin.

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G.M. Wood, BFMIRA, Leatherhead, UK

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PESTICIDE FORMULATIONS

Liquid Chromatographic Method for Determination of Methamidophos in Technical Products and Pesticide Formulations: CIPAC Collaborative Study

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A reversed-phase liquid chromatographic (LC) method for the determination of technical methamidophos and 5 different formulated products was collaboratively studied in 18 laboratories. Samples were dissolved in water, separated by LC, and detected at 210 nm. Quantitation was done by peak area measurements. Relative standard deviations for repeatability and reproducibility ranged from 0.35 to 0.80 and from 1.14 to 1.60, respectively. The method has been approved first action by AOAC International as a CIPAC-AOAC method.

Methamidophos, phosphoramidothioic acid *O,S*-dimethyl ester, is an insecticide used against various chewing and sucking insects and spider mites. Formulations consist of emulsifiable and soluble concentrates.

A liquid chromatographic method for the determination of methamidiphos from technical material and formulated products was collaboratively studied by using a reversed-phase column, a mobile phase of acetonitrile and water, and UV detection at 210 nm. Methamidophos content was calculated from peak areas by using an external standard.

Collaborative Study

The method for determination of methamidophos was collaboratively studied under the direction of the German Pesticide Analytical Committee by Bayer AG, Dormagen, Germany. Samples of 75% technical concentrate and 20, 50, and 60% soluble liquid concentrate were sent to 21 laboratories around the world. Eighteen laboratories completed the analysis based on duplicate determinations of aqueous dilutions of the samples performed on each of 2 different days.

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Results of the study were presented at the 1990 CIPAC meeting in Gammarth, Tunesia.

The report has been evaluated by the General Referee, Committee Statistician, and Committee Safety Advisor and reviewed by the Committee on Pesticide Formulations and Disinfectants. The method has been approved first action by the Official Methods Board at their January 1992 meeting. Association actions will be published in "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76**, January/February issue.

[1] A.R. Hanks is the AOAC International General Referee on Pesticide Formulations: CIPAC Methods.

992.01 Methamidophos in Technical Products and Pesticide Formulations—LC Method

First Action 1992

CIPAC-AOAC Method

(Applicable to technical products and soluble liquid concentrate formulations.)

Method Performance:

Technical product, 75%
$s_r = 0.34$; $s_R = 0.75$; $RSD_r = 0.45\%$; $RSD_R = 1.00\%$
Soluble liquid concentrate (SL 600), 60%
$s_r = 0.29$; $s_R = 0.56$; $RSD_r = 0.59\%$; $RSD_R = 1.14\%$
Soluble liquid concentrate (VL 60), 60%
$s_r = 0.45$; $s_R = 0.80$; $RSD_r = 0.76\%$; $RSD_R = 1.34\%$
Soluble liquid concentrate (SL 400), 40%
$s_r = 0.27$; $s_R = 0.54$; $RSD_r = 0.80\%$; $RSD_R = 1.60\%$
Soluble liquid concentrate (SL 19.5), 20%
$s_r = 0.07$; $s_R = 0.23$; $RSD_r = 0.35\%$; $RSD_R = 1.14\%$

A. Principle

Technical materials and soluble liquid concentrate samples are dissolved in water, and methamidophos is separated by reversed-phase liquid chromatography (LC) using a water–acetonitrile eluant, detected at 210 nm, and quantitated by peak area measurement using external standard.

B. Apparatus

(a) *Liquid chromatograph.*—With 20 μL sample loop. Operating conditions: mobile phase flow rate, 1.5 mL/min; detector sensitivity, to obtain peak height of 80–90% full scale; col-

Table 1. Statistical analysis of collaborative results for LC determination of methamidophos in technical and formulated products (outliers removed)[a]

Product	No. Laboratories[b]	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	r_{95}	R_{95}
Technical	14 (18)	75.2	0.34	0.75	0.45	1.00	0.94	2.10
SL 600	15 (18)	49.3	0.29	0.56	0.59	1.14	0.80	1.60
SL 400	17 (18)	33.8	0.27	0.54	0.80	1.60	0.75	1.50
VL 60	16 (18)	59.6	0.45	0.80	0.76	1.34	1.30	2.20
SL 19.5	13 (17)	20.1	0.07	0.23	0.35	1.14	0.26	0.65

[a] Statistical analysis in accordance with ISO 5725 (uses the Dixon test for determining outliers).
[b] Laboratories reporting outliers not included in statistical analysis; total number of laboratories in study in parenthesis.

umn temperature, 35°C; methamidophos retention time, 3.2 min.

(b) *Detector.*—UV at 210 nm.

(c) *Electronic integrator.*—Preferred for determination of peak area measurements.

(d) *Chromatographic column.*—Stainless steel, 250 × 4.6 mm, packed with 5 µm C_8 material (LiChrospher 100RP 8, 5 µm, used in method development; at least 5 other column packings were successfully used in collaborative study).

(e) *Blender.*—With explosion-proof motor.

Caution: (*1*) See safety note on acetonitrile. (*2*) Methamidophos is highly toxic. Avoid oral, dermal, and inhalation exposure.

C. Reagents

(a) *Acetonitrile.*—LC grade.

(b) *Mobile phase.*—Water–acetonitrile (94 + 6, v/v). Mix 940 mL water and 60 mL acetonitrile. Degas.

(c) *Methamidophos.*—Analytical standard of known purity (Bayer AG, Dormagen, Germany; Mobay Corp., Kansas City, MO).

D. Preparation of Calibration Solution

Weigh (to nearest 0.1 mg) ca 0.25 g methamidophos into each of two 100 mL volumetric flasks. Dissolve in 50 mL H_2O and dilute to volume. Mix.

E. Preparation of Samples

Homogenize sample in blender. Weigh (to nearest 0.1 mg) sufficient sample to contain 0.25 g methamidophos into 100 mL volumetric flask, and dilute to volume with H_2O. It may be necessary to melt technical methamidophos before homogenizing in H_2O bath at <45°C. Analyze prepared samples within 24 h.

F. Determination

Inject 20 µL of 2 calibration solutions alternately until calibration factor varies by <1% for last 2 injections. Inject 20 µL of sample solution 2×. Repeat injection of 2 calibration solutions.

G. Calculations

Calculate methamidophos content using average calibration factor, f, for calibration solutions:

$$\text{Methamidophos, \%} = (H_w \times f)/w$$

where $f = (s \times P)/H_s$ and H_w = average peak area of 2 injections of sample solutions; w = sample mass (g); s = mass of methamidophos standard (g) (average of 2 solutions prepared); P = purity of methamidophos standard (%); H_s = average peak area for calibration solutions (2 injections before and 2 after sample solutions).

Ref.: *JAOAC* (1992) **75**, July/August issue

CAS-10265-92-6 (methamidophos)

Results and Recommendation

Results of the collaborative study were presented by H. Tengler and K.W. Kruger (Bayer AG, ZF-D Zentrale Analytik, Postfach 10 01 40, 4047 Dormagen 1, Germany) at the 34th meeting of the Collaborative International Pesticides Analytical Council (CIPAC), 1990, in Gammarth, Tunesia (CIPAC Reports 3577R and 3578M). Performance parameters were calculated according to ISO 5725.

Results and the limited number of outliers prove that the method is very rugged (Table 1). The collaborators used at least 6 different column packing materials, including C_{18}, in addition to the C_8 recommended, with no apparent influence on the outcome.

The General Referee recommends that the method be adopted first action as a CIPAC-AOAC method.

Acknowledgments

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W. Gau and K.W. Kruger, Bayer AG, Germany

PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Headspace Method for Rapid Determination of Methyl Bromide in Assorted Nut Samples

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A headspace method has been developed for the rapid, safe determination of methyl bromide (MeBr) in raw and processed nuts. Fifty grams of frozen nut meats is blended 3 min at high speed in 200–250 mL aqueous solution in a sealed 1 L blender cup fitted with a septum port on its cover. The cup is equilibrated 10 min in a 25°C water bath. Two 250 ±150 μL aliquots of the sample headspace gas are sequentially removed with a gas-tight syringe and injected into a dual column/dual detector gas chromatograph. One determination is made with a GS-Q wide-bore capillary column and a Hall electrolytic conductivity detector, the other with a packed column, 20% OV-101 on Chromosorb W(HP), and a ^{63}Ni electron capture detector. The average variation for the manual injections is about 5% for both column/detector systems. To prevent random emission of MeBr during the determination, dated standards, headspace wastes, and electron capture detection effluent are passed through liquid traps. The overall recovery of MeBr from fortified samples is about 40%; the coefficient of variation is 29%. Eighteen assorted nut samples were analyzed on both systems at a quantitation limit of <100 ng/g; no detectable MeBr was found.

Methyl bromide (MeBr), a toxic gaseous chemical (1) frequently used to fumigate nuts and other selected foodstuffs in storage or transit, generally leaves no unpleasant aftertaste in commodities treated at the recommended dosages. Fumigation with MeBr appears well-suited to raw foodstuffs such as nuts or spices that undergo relatively little processing before human consumption.

The toxicity of MeBr has been determined with test animals. In a teratogenic inhalation study (2), several rabbits exposed for 7 h daily to 70 ppm MeBr died on Day 9; mortality reached 96% on Day 27. Despite its toxicity, MeBr is thought to dissipate readily from stored, treated foodstuffs exposed to the open air. Thus, products made from the treated foodstuffs should pose no potential health problems to consumers.

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MeBr is also known to react with certain naturally occurring food chemicals, such as the amino acids, to form by-products of unknown toxicity. One reaction product, dimethyl sulfide, gives processed nuts, particularly the roasted products, an aftertaste similar to creamed corn or smoked oysters (3; H. Davis, Eagle Snacks, Inc., St. Louis, MO, personal communication). This dimethyl sulfide aftertaste comes from an MeBr reaction with methionine, a naturally occurring amino acid in the nuts. Today, many confectionery and food companies examine raw and bulk nuts for residues of both MeBr and dimethyl sulfide before the final processing (Davis, personal communication).

Like most halogenated fumigants or pesticides, MeBr is soluble in liquid fat or oil. Residual, intact MeBr could be present in treated, fat-containing commodities even after lengthy exposure or "off-gassing" periods. Consequently, dependable methods for determining trace levels of toxic MeBr in all kinds of food commodities, including fat-containing types, are needed for monitoring these foods for chemical contaminants.

Most methods for determining MeBr in domestic and exported/imported food commodities are limited to the nonfat foodstuffs such as flour products (4), cereal foodstuffs (5–8), citrus fruit (9), or spices (10, 11). However, a few can be used to analyze both nonfat and fatty foods (12–18; P. Hartsell, U.S. Department of Agriculture, personal communication). King et al. (9), for example, developed a headspace method for analyzing grapefruit by using a sealed blender cup and an aqueous extracting solution. They showed that the amount of MeBr partitioned into the sample phase increased when a small amount of oil was added to the sample during extraction. Ford (11) also used a sealed blender cup for analyzing pineapple and spices. DeVries et al. (14) analyzed 1 g of wheat, flour, cocoa, or peanuts in small vials. More recently, Page and Avon (16) developed a method for analyzing homogenized corn, wheat, flour, cocoa beans, cheese, spices, prunes, dates, and citrus fruits in 30 mL headspace vials. In developing this method, they tested foods that were actually fumigated and found that incurred residues were recovered. The samples were first homogenized in an ice–water slurry and then extracted with sodium sulfate in the vials. MeBr was determined with a gas chromatographic (GC) system programmed at subambient temperature.

These and other headspace methods (15, 17, 18; Hartsell, personal communication) currently used to check both domestic and exported/imported foodstuffs for MeBr are generally

based on the method of King et al. (9). However, Ford et al. (18) automated their headspace method for spices (11), pineapple, or nuts. Originally, they automated the injection step by using a portable XonTech GC system equipped with a motorized loop-type injector valve and an air sampling pump. The GC system automatically drew headspace from the blender cup through a connecting line and injector valve and then automatically injected 0.25 mL into a GS-Q wide bore capillary column connected to a tritium detector. Later, they improved the method further by adding a multiple sampling valve and a GC system equipped with ^{63}Ni electron capture detection (ECD). This improvement increased their ability to analyze large numbers of raw and processed samples, in which they have found a few incurred MeBr residues (18).

In the present study of the XonTech GC system, the low-voltage tritium ECD system appeared well-suited to making environmental determinations in the field. During the testing of assorted food types in our laboratory, however, the endogenous background interferences from certain foods such as raw fruit and onions adversely affected baseline stability, often obliterating the determination completely. As Ford's group discovered, ^{63}Ni ECD yielded a stable base line. Still, because the GC conditions used in our study were set for the rapid determination of MeBr (t_R, 2–3 min), occasional late-eluting background peaks from previous injections of nut samples interfered with the determination.

Therefore, to meet our analytical requirements for speed, versatility, and capacity to analyze several food types, the best aspects from some of the above methods (9, 15–18; Hartsell, personal communication) were combined into 1 simple, fast, and versatile method for determining MeBr in nuts (and assorted foods). Because analyst safety was a major consideration, we passed any effluent or waste containing MeBr through alcohol traps. Also, to make the method adaptable to other laboratories not having automated gas chromatographs or loop-type injection valves, we used relatively small, manually operated gas-tight syringes to make gaseous transfers and injections of headspace. These syringes are accurate; the needles have ports on the side instead of at the tip. In addition, Hall electrolytic conductivity detection (HECD) was used in the determinative step. MeBr is relatively more sensitive to HECD than to ^{63}Ni ECD. In our laboratories, HECD has been found to be about 3-fold more sensitive to MeBr than ECD when both detectors are set to the same sensitivity for 2 ng chloroform. Although endogenous interference from nut samples occurs only occasionally in determinations by ECD, HECD generally eliminates such effects completely. It also provides immediate confirmation of residue identity on a second column/detector system (19).

This report shows that although MeBr gas is partially soluble in aqueous solution and sample, yielding a mean headspace recovery of 40% for nuts, the method described here can still be used to screen assorted nut samples for nanogram-per-gram levels of incurred residues. It also shows that toxic MeBr can be handled conveniently and safely in the laboratory without hazard to the analyst or other laboratory personnel.

Experimental

Reagents

(a) *Solvents.*—Ethyl alcohol, deionized water.

(b) *Sodium sulfate.*—ACS grade; heat overnight at ca 180°C.

(c) *Extracting solution.*—Dissolve 284 g sodium sulfate in 4 L deionized water (w/v), stir, heat at ca 80°C, and purge overnight with 200 mL/min compressed breathing-quality air.

(d) *Methyl bromide (99%).*—Compressed (lecture bottle, stored under constant ventilation in hood) and tested against certified standard (Kin-Tek Laboratories, Inc.); gas density at 25°C, ca 3900 ng/μL.

Apparatus

(a) *Safety gear.*—Breathing air face mask (or gas respirator) and rubber gloves approved by National Institute of Occupational Safety and Health or Mine Health and Safety Administration.

(b) *Methyl bromide waste traps.*—1.5 L ethyl alcohol in 2 L Erlenmeyer flask, or larger; inlet, 1/8 in. tubing with fritted tip extended to bottom of flask; outlet, multichamber column condenser.

(c) *Table top fan.*—3-speed, induction, capable of circulating air in working area.

(d) *Blender cups.*—Eberbach, 1 L, stainless steel (s.s.) or aluminum alloy (No. 8520-60) with s.s. screw-on covers having O-ring seals and port for bulkhead fittings (Eberbach Corp.).

(e) *Bulkhead fittings.*—1/4 in., Swagelok No. SS-400-R1-4 with PTFE gaskets, fitted into screw-cover ports.

(f) *Silicone septa.*—9.5 mm diameter for Swagelok ports on blender covers; 9 mm diameter for GC inlets, Microsept F-138, or equivalent (Alltech Associates, Inc.).

(g) *Commercial blender.*—Waring, heavy duty, 2-speed, Waring EP1, or equivalent (Waring Products Corp.).

(h) *Water bath.*—Approximately 25°C, 6 in. immersion depth (Blue M Electric Co.).

(i) *Gas-sampling bags.*—2 L, laminated, aluminum-clad, fitted with silicone septa and twist-valve ports, or equivalent (Calibrated Instruments).

(j) *Gas-tight syringes.*—5, 10, and 25 μL with 5 cm fixed, open-tipped needles (26s); 500 μL with 5 cm removable side-port needle (22s); all equilibrated at ca 25°C (L. Carson and H. Hollifield, U.S. Food and Drug Administration, personal communication) (Alltech Associates, Inc.).

(k) *Lecture bottle regulator.*—2-stage, with low-volume needle-type outlet valve (Air Products and Chemicals, Inc.).

(l) *Gas-metering valve.*—Brooks or Porter, diaphragm, constant flow, capable of metering 200–400 mL/min flows.

(m) *Utility gases.*—Compressed, dried, breathing-quality air (or nitrogen, optional); 2 outlets, variable flow and constant flow, metered through Brooks or Porter valve after calibrating with bubble meter.

(n) *Timer.*—Stopwatch with alarm, or equivalent.

(o) *Tubing.*— PTFE, 1/8 in. od with assortment of 1/8 in. s.s. fittings, 2 mm id with rubber couplers, ca 6 mm od, 2.5 mm id.

(p) *Gas chromatograph.*—Dual column/dual detector, HECD-halogen mode and constant current ^{63}Ni ECD; both detectors adjusted to 50–70% full scale response for 3.05 ng MeBr (see operating conditions below). Other conditions: inlets, 200°C; oven, 100°C, isothermal.

GC Operating Conditions

(a) *HECD-halogen mode system.*—Column, 30 m × 0.5 mm id, GS-Q wide-bore capillary (J & W Scientific) with 1 m retention gap and 13 cm × 3.5 mm id inlet sleeve containing small plug of glass wool; helium carrier gas flow, 20 mL/min; hydrogen reactor gas flow, 50 mL/min, passed through purifier (AADCO, Rockville, MD); 1-propanol conductivity solvent flow, 0.4 mL/min; base temperature, 250°C; reactor, 900°C; range, 10; attenuation, 10×; MeBr t_R, ca 2.1 min on 1 mV strip chart recorder; chart speed, 0.5 cm/min.

(b) *ECD system.*—Column, 3.6 m × 4 mm id glass, packed with 20% OV-101 on 80–100 mesh Chromosorb W(HP); 5% CH_4/Ar carrier gas flow, 40 mL/min; detector, 350°C; MeBr t_R, ca 2.8 min on Hewlett-Packard 3390A integrating recorder; recorder settings: ZERO, 10; ATT 2↑, 1; CHT SPD, 0.5 cm/min; PK WD (RANGE), 0.01; THRSH, 0; AR REJ, 5; INTG No.8 TIME, 0.1 min; STOP TIME, 6 min.

Standard Preparation

Danger! Methyl bromide is toxic. Make all dilutions in well-ventilated hood. Wear positive-displacement breathing air mask (or filter-type gas respirator) and rubber gloves.

(a) *Pure methyl bromide.*—Label 2 L gas-sampling bag. Flush bag with breathing-quality air (or nitrogen) 3 times; empty completely, using vacuum. Close "twist" valve of bag (see C, Figure 1) while still connected to vacuum. Using 1/8 in. od PTFE tubing, 1/8 in. s.s. fittings, and small section of rubber tubing, connect outlet valve of lecture bottle (D) to alcohol trap (F) connected in turn to slight vacuum. With regulator outlet valve closed, open main valve on lecture bottle. Adjust regulator pressure to ca 10 psi. Slowly open outlet valve and gradually purge regulator and lines with MeBr through alcohol trap. Close outlet valve.

Disconnect tubing line from trap and connect it to labeled evacuated gas-sampling bag (B). Open twist valve of bag. Slowly open outlet valve and let MeBr vapor (not liquid) slowly fill bag to ca 2/3 full. (Note: If liquid enters bag, its rapid volatilization will fill bag too fast.) Close outlet valve; close valve to bag.

Close main valve on lecture bottle. Disconnect line from bag and reconnect it to alcohol trap. Reopen outlet valve. Let regulator and lines bleed through trap. Loosen regulator nut at lecture bottle. Let hood air pass through regulator and lines via trap. Re-tighten regulator nut at lecture bottle. Close regulator pressure and outlet valves. Disconnect line from trap. Store bag of pure MeBr (I) and lecture bottle (J) in hood.

(b) *Methyl bromide working standard.*—Label and flush second 2 L gas-sampling bag. Connect constant flow source

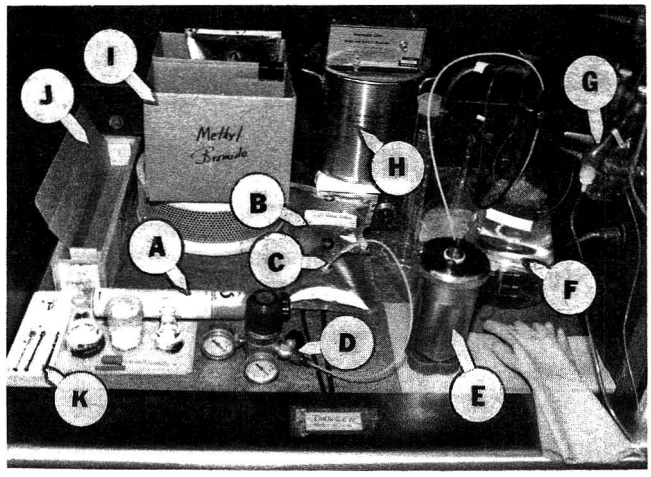

Figure 1. Equipment for methyl bromide (MeBr) headspace analysis (in hood): (A) lecture bottle of pure MeBr, (B) aluminum-clad gas-sampling bag, (C) twist valve (silicone septum is on back side of bag), (D) regulator outlet valve, (E) vented sample cup, (F) MeBr waste trap (alcohol), (G) breathing-air face mask, (H) vessel for making extracting solution, (I) storage for MeBr standards, (J) storage for lecture bottle, and (K) gas-tight syringes.

(200–400 mL/min) of breathing-quality air (or nitrogen) to second evacuated bag. Using stop watch, meter 1600 mL air into bag. Close bag valve and disconnect line; using gas-tight syringe, transfer 5 µL pure MeBr gas from first bag to second via septa on both bags. Let MeBr mix and diffuse 15 min. MeBr working concentration = (5 µL × 3900 ng/µL @25°C)/1600 mL = 12.19 ng/mL. Make this dilution fresh daily after slowly evacuating old working standard through alcohol trap.

Cap Headspace Measurement

Fill one or more blender cups with ca 25°C water; measure average volume of blender cup(s). Also, measure combined volume of reagent (200 mL) and 50 g sample in graduated cylinder. Sample headspace volume = cup volume – combined reagent–sample volume.

Sample Storage and Preparation

Store nut samples (whole packaged meats or in-shells) frozen (–60°C, if available; otherwise, –4°C) until analysis.

Determination

In well-ventilated work area, add 200 mL sodium sulfate solution and 50 g or less frozen shelled meat sample (whole, or chopped into 1/8–1/4 in. pieces) to Eberbach blender cup. Purge cup headspace with compressed breathing-quality air (from variable flow source); seal with screw cover. Blend 3 min. Place in 25°C water bath and equilibrate 10 min. Purge well-rinsed 500 µL gas-tight syringe with headspace 3–5 times and draw 250 ±150 µL headspace into syringe. Draw sample in needle back into syringe barrel immediately after removing syringe from cup port. Inject onto capillary/HECD GC system (wait 5 s before removing syringe from injection port). Repeat

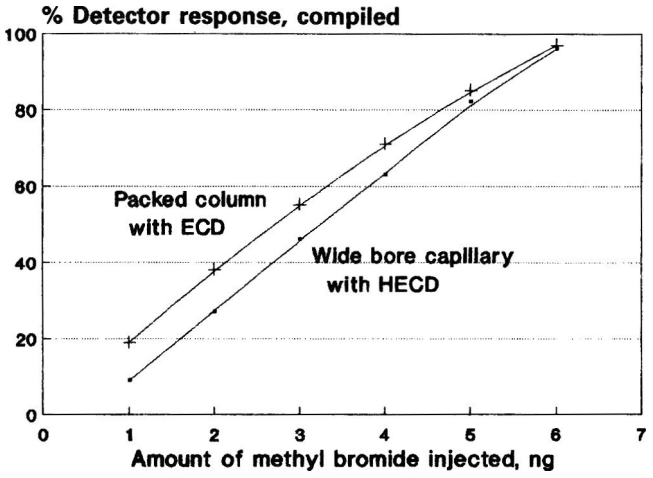

Figure 2. Standard curves for both GC determinative systems.

for second column/detector system. Make determination against one or more 250 µL (3.05 ng) injections of working standard from each system. Calculate amount of MeBr found in sample as follows:

$$(\text{ng/g or ppb}) = (A \times B)/(C \times D)$$

where A = ng MeBr found in 0.25 mL headspace gas; B = total headspace volume (e.g., 872 mL); C = 0.25 (factor to convert ng/0.25 mL to ng/mL); and D = sample weight (e.g., 50 g).

(a) *Fortification of samples*—Using gas-tight syringe, just before extracting, add 3–15 µL pure MeBr (234–1170 ng/g equivalent) to 50 g sample sealed in cup. Proceed with extraction and determination.

(b) *Sample disposal after determination.*—After determination, set sealed cup in hood. Remove 1/4 in. nut and septum from cover. Quickly insert end of 1/8 in. PTFE tubing (already connected to alcohol trap and vacuum source) through bulkhead fitting to ca halfway in cup (see E, Figure 1). Vent cup through trap (F) for 5 min before removing cover and disposing sample contents. Clean cup with hot water.

Results and Discussion

Once set up, the method is rapid, safe, and easy to operate. The analysis time per sample for a series of samples determined simultaneously is about 15 min. The relatively small transfers of gaseous MeBr and headspace with gas-tight syringes enhances the efficiency of sample fortifications and GC injections. The alcohol traps prevent traces of MeBr from entering the laboratory air or the outside atmosphere.

The aqueous sodium sulfate solution extracted the samples satisfactorily. Although 200 mL was used during most of this study, increasing the volume to 250 mL gave a smooth, slurry-type blend for all nut meats during extraction. The sodium sulfate, also used by Page and Avon (16) and Ford et al. (18), caused no mechanical problems such as freezing the blender cup's bearings after sitting overnight. The use of stronger extracting solutions, e.g., acetone or acetonitrile, could have enhanced the partitioning of MeBr residue from sample into the liquid phase; however, these stronger solutions could also bind the residue in solution, hindering further partitioning into the headspace.

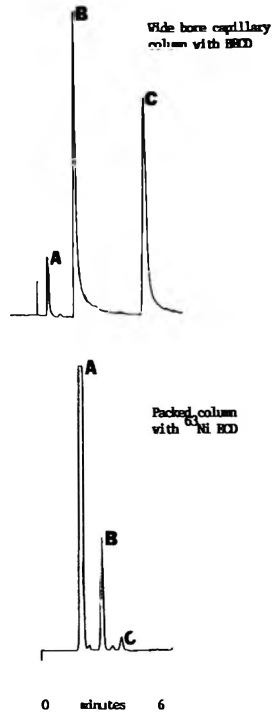

Figure 3. Gas chromatograms of fortified mixed nut sample from both GC systems: (A) air peak, (B) 2 ng MeBr, and (C) 0.65 ng methylene chloride background peak; fortification level, 468 ng/g.

Measuring the initial combined reagent–sample volume was difficult with some samples. Certain meats were bulky; they often floated on the reagent and made the meniscus hard to read. Also, the relative density of each sample was slightly different. Therefore, to determine the average headspace volume, a ground mixture of assorted nuts was used to find an average reagent–sample volume (248 mL). The resultant average headspace volume was 872 mL.

The percent variation for manual injections was determined by twice injecting a series of ten 250 µL portions of the working standard into each GC column/detector system. This variation was 8% with the wide-bore column/HECD system and 25 with the packed-column/ECD system. The overall variation for both systems was about 5%.

The linearity of GC response was determined by injecting successively increasing volumes (100–1000 µL) of the MeBr working standard into both GC systems. The comparatively narrow bore size and higher head pressure at the capillary inlet caused more variation to occur with this column than with the 4 mm diameter packed column; yet, the overall linearity of response for both systems was suitable up to a tested injection volume of 1 mL (see the standard curves in Figure 2). Also, any moisture still in the headspace after the equilibration step had little adverse effect on the determination. The nickel reaction tube of the HECD system was replaced about every 2 weeks

Table 1. Methyl bromide recoveries from 2 determinative (column/detector) systems

Commodity	Sample, g	Fort. level, ng/g	Rec.,%[a]
Pecans, packaged	50	390	27 : 33
Almonds, packaged	50	468	34 : 39
Almonds, unshelled	50	468	23 : 35
Brazil nuts, unshelled	50	468	22 : 27
Cashews, canned	50	468	33 : 38
Crunchy peanut butter	50	468	69 : 54
Filberts, unshelled	50	468	36 : 42
Mixed nuts, canned	50	468	54 : 55
Peanuts, unshelled	50	468	39 : 34
Peanuts, unshelled	50	468	25 : 32
Peanuts, Spanish	50	468	36 : 41
Walnuts, packaged	50	468	29 : 35
Almonds, packaged	15	520	47 : 46
Creamy peanut butter	15	520	42 : 41
Creamy peanut butter	15	520	42 : 50
Creamy peanut butter	15	520	56 : 45
Filberts, unshelled	15	520	34 : 32
Mixed nuts, canned	15	520	60 : 49
Peanuts, unshelled	15	520	25 : 32
Pecans, packaged	15	520	34 : 35
Peanuts, Spanish	15	520	35 : 44
Creamy peanut butter	50	546	53 : 53
Creamy peanut butter	50	546	43 : 42
Pecans, unshelled	50	546	19 : 28
Pistachios, unshelled	50	546	47 : 38
Walnuts, unshelled	50	546	22 : 31
Almonds, unshelled	10	780	28 : 36
Brazil nuts, unshelled	10	780	31 : 34
Cashews, canned	15	780	38 : 58
Crunchy peanut butter	15	780	71 : 55
Peanuts, unshelled	10	780	33 : 35
Peanuts, unshelled	10	780	27 : 35
Pistachios, unshelled	15	780	69 : 45
Walnuts, packaged	15	780	49 : 39
Pecans, unshelled	10	1170	69 : 43
Walnuts, unshelled	10	1170	52 : 41
Mean rec.			40 : 40

[a] Key = capillary/HECD : packed column/ECD.

during continuous analysis. Figure 3 shows gas chromatograms of a fortified sample.

Thirty-six spike recoveries were determined with both systems. Overall fortifications for this study ranged from 390 to 1170 ng/g; most samples were spiked at 468–780 ng/g (see Table 1). For the capillary/HECD system, the recoveries ranged from 19 to 71% [average, 40%; coefficient of variation (CV), 37%]. For the packed-column/ECD system, the recoveries ranged from 27 to 58% (average, 40%; CV, 20%). The overall CV for both systems was 29%.

The mean MeBr recovery of 40% from fortified samples appears low compared with traditional 60–90% recoveries obtained for other fumigants by other methods. Therefore, additional recoveries were determined from fortified mixtures of assorted nuts. The mean recovery for MeBr was lowest at the lower end of the spiking range (155 ng/g). It increased with the addition of larger amounts of MeBr to the sample and reagent blank, leveling off at about 600 ng/g (see Figure 4). Although lower than ideal, this 40% recovery is satisfactory compared with an 18% MeBr recovery obtained with a liquid extraction method (20) for fat-containing foods.

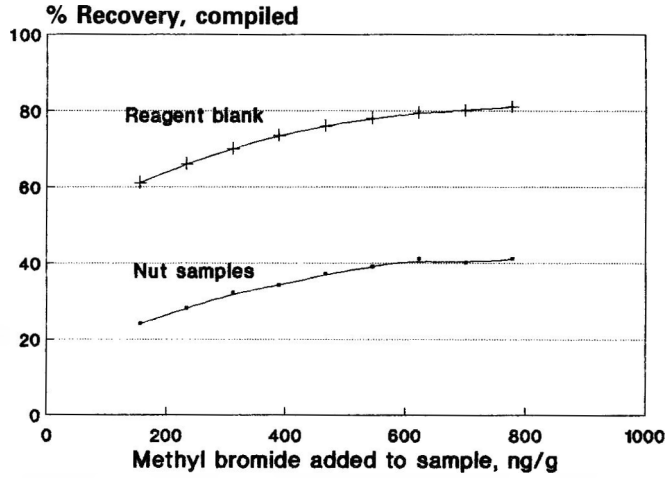

Figure 4. Recovery profiles for fortified reagent blanks and assorted nut mixtures.

Although the mean recovery from nuts and peanut butters is lower than the desired recovery of 80% or more, it is consistently low. That is, this trend toward low recovery is a known profile for this kind of sample with this method. Because the analyst knows this profile beforehand, the samples can be dependably screened for MeBr by using correction factors (18), if necessary.

It can be argued that the nut and peanut butter samples used in this study were not fortified independently before the analysis; therefore, the resultant recoveries do not show how an actual residue might be recovered from a sample. This argument is true to some extent. That is, in this study, the MeBr fortification was merely added to the headspace of the sample cup before sample extraction, and the amount remaining in the headspace after the extraction was determined. However, because MeBr is extremely volatile, securing accurately measured prefortified samples would be very difficult. Such fortifications would have to be kept sealed during all transfers to prevent losses, because even small losses would yield inaccurate results. By comparing the potential for error, the technique used here, even though somewhat the reverse of true fortification, probably yields relatively accurate recoveries. It is also much easier to perform. Furthermore, other laboratories use this and similar methods to determine actually incurred residues from treated foodstuffs (15, 16, 18; Hartsell, personal communication).

Compared to the CV reported for other methods, the 29% average CV found for this headspace method is satisfactory. For example, the overall CV for the recovery of liquid fumigants from certain liquid extraction methods is about 40% (21).

Moreover, the CV for 71 MeBr determinations from the same method is about 114%, much greater than the 29% found in this study.

To further test the capillary/HECD system for ruggedness and dependability, about 50 samples containing natural volatile substances (raw fruits and vegetables from the U.S. Food and Drug Administration's market basket studies, 22) were analyzed; no MeBr could be detected. Resultant chromatograms and recovery determinations were satisfactory. The results from these and other selected food samples now being studied in this laboratory will be reported later.

In conclusion, the method is rugged and dependable for screening assorted nuts and peanut butters and other food types as shown by King et al. (9), Page and Avon (16), and Ford et al. (18). Yet the addition of an HECD system increases its sensitivity to MeBr over that of an ECD system. HECD also increases method ruggedness by reducing potential interference from endogenous sample effect. Although MeBr recovery is somewhat lower than desired, the method offers rapid, efficient screening of assorted nut samples and products suspected of MeBr contamination. It is also adaptable to ordinary laboratory equipment and does not emit MeBr fumes into the laboratory atmosphere.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Analysis of Fatty and Nonfat Foods for Chlorophenoxy Alkyl Acids and Pentachlorophenol

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A multiresidue method has been developed to analyze low-level residues of chlorophenoxy alkyl acids and pentachlorophenol (PCP) in fatty and nonfat foods. The acidified sample is extracted, cleaned up using gel permeation chromatography, methylated by ion-pair alkylation with tetrabutylammonium hydroxide and methyl iodide, cleaned up using Florisil, and determined by gas chromatography using electron capture and/or electrolytic conductivity detectors. Recoveries ranged from 53 to 75% for 2,4-D at 200 ppb, 61 to 93% for 2,4,5-T at 80 ppb, and 76 to 84% for PCP at 20 ppb fortified in a variety of food items. Also, 31 other herbicides were evaluated through this procedure.

The U.S. Food and Drug Administration (FDA) initiated the Total Diet Study (TDS) (1, 2) in May 1961. TDS monitors the levels of various contaminants, radionuclides, selected nutrients, toxic elements, industrial chemicals, and pesticides in table-ready foods and in the diets of specific age/sex groups. The study provides baseline information that is used to identify trends in the food supply and in diets over time. This information is also used to identify potential public health problems.

Since initiation of TDS in May 1961, the collection sites, foods collected, analytes, and analytical methodologies of TDS have changed significantly. The collection sites are rotated among major population centers in the United States. The number of foods collected has grown from 82 in 1961 to 234 in 1991. Initially, food items were composited for analysis, but beginning in 1982, all food items have been analyzed individually. The food items in TDS are examined using one or more multiresidue methods, developed over the years and described in the FDA *Pesticide Analytical Manual*, Vol. I (PAM I) (3), compiled and issued by FDA.

The method for the analysis of chlorophenoxy alkyl acids (CPAs) and pentachlorophenol (PCP) residues in TDS has been in development since 1982 and recently has been included in PAM I as sec. 221.2. As analytical methods are developed, new analytes are added to the analytical scheme; the number of herbicide analytes has increased from 11 in 1961 to over 28 in 1991. Most of the common CPAs and PCP can be determined by using this methodology. This paper describes the current methodology used for analyzing 234 food items of TDS for CPAs and PCP residues. Recoveries of selected CPAs and PCP through this procedure are presented.

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Mention of trade names and suppliers is for information only and does not imply endorsement by the U.S. Food and Drug Administration.

Experimental

Principle

The sample is acidified with sulfuric acid, and residues are extracted by use of various techniques depending upon the nature of the sample (4). The sample extract is cleaned up by gel permeation chromatography (GPC) using a Bio-Beads SX-3 column and a 50% methylene chloride–hexane eluting solvent (5). The concentrated extract is then methylated by ion-pair alkylation with tetrabutylammonium hydroxide (TBAH) and methyl iodide (6). The methylated extract is then further cleaned up by Florisil column chromatography (7). Determination is by gas chromatography (GC) with electron capture and/or electrolytic conductivity detectors.

Apparatus

(a) *Blender.*—High speed; Waring, Lourdes, Omni-Mixer, or equivalent. Explosion-proof model recommended. Quart container is suitable for routine use.

(b) *Centrifuge.*—Explosion proof.

(c) *Centrifuge bottles.*—500 mL glass-stoppered.

(d) *Chromatographic column.*—300 × 10 mm id, with Teflon stopcock and coarse-porosity fritted disk; TS 24/40 joint at top of column (Kontes Glass Co., Vineland, NJ, Cat. No. K-422450), or equivalent.

(e) *Cylinders, graduated.*—10 mL glass-stoppered; 25 mL, 100 mL, 250 mL.

(f) *Siphon tube.*—For removing solvent layer.

(g) *Kuderna-Danish concentrators.*—500 or 250 mL, with Snyder column and receiving flask (Kontes Glass Co., Cat. No. 570001-0500, 570001-0250, 503000-0121, and 621400-0525), or equivalent.

(h) *Separatory funnels.*—250 mL, 1 L, 2 L.

(i) *Automated gel permeation chromatograph.*—Model 1001, fitted with a Kontes column (500 × 25 mm) containing 35 g Bio-Beads SX-3 200 × 25 mm (Analytical Biochemistry Laboratories, Inc., Columbia, MO), or equivalent. Permits preprogrammed cleanup of up to 22 samples without supervision.

(j) *Manual gel permeation chromatograph.*—Prepare using the following: *(1) Sample introduction valve.*—Model SU-8031 (Chromatrix, Inc., Berkeley, CA), or equivalent; *(2) Pump.*—Milton Roy instrument minipump 1000-psig capacity (Milton-Roy Co., Riviera Beach, FL), or equivalent; *(3) Sample loading loop.*—A 5.0 mL calibrated loop consisting of 1/16 in. Teflon tubing coiled in cylindrical form; *(4) Pulse dampener.*—Made from ca 6 ft of 1/8 in. copper tubing coiled and closed at one end, installed with a "Tee" in the line between pump and sample introduction valve; and *(5) GPC column.*—500 × 25 mm id with organic solvent plunger kit (Analytical Biochemistry Laboratories, Inc.), or equivalent.

(k) *Syringe.*—10 mL with Luer-Lok tip, fitted with a Millipore Swinny stainless adaptor (Cat. No. IEAXX 3001200) with Millipore 5.0 μm LS-type filter material (Cat. No. LSW 01300, Millipore Corp., Bedford, MA).

(l) *Microliter syringes.*—25, 50, or 100 μL Hamilton syringes (Hamilton Co., Reno, NV), or equivalent, for adding reagents.

Reagents

Some reagents are used only for specific sample types, as indicated in method discussion.

(a) *Sulfuric acid.*—10%, reagent grade.

(b) *Boiling chips.*—Carborundum, 20-mesh, or other suitable boiling chips.

(c) *Florisil.*—PR grade, 60–100 mesh (Floridin Co., Berkeley Springs, WV).

(d) *Sodium sulfate.*—Anhydrous, granular, reagent grade.

(e) *Sodium chloride.*—Reagent grade, prepared as a saturated solution in water.

(f) *Sodium or potassium oxalate.*—Reagent grade.

(g) *Solvents.*—Acetonitrile, ethyl ether (ethanol stabilized), hexane, methanol, methylene chloride (cyclohexene stabilized), acetone, and petroleum ether, pesticide grade (Burdick and Jackson, Muskegon, MI).

(h) *Gel beads.*—Bio-Beads SX-3 resin (200–400 mesh, Bio-Rad Laboratories, Richmond, CA) and/or Envirobeads SX-3 Select pretested (Analytical Biochemistry Laboratories, Inc.).

(i) *Tetrabutylammonium hydroxide.*—Titrant, 1.0M in methanol (Fisher Scientific Co., Springfield, NJ).

(j) *Methyl iodide.*—Certified grade (Fisher Scientific Co.).

(k) *Standard reference materials.*—Dissolve sufficient quantity of chlorophenoxy acid herbicides and pentachlorophenol standards in acetone to prepare a 1 mg/mL stock solution. Dilute subsequent standards with acetone. Dissolve sufficient quantity of methyl esters of chlorophenoxy acids and pentachlorophenyl methyl ether standards in 10% acetone–isooctane (v/v) to prepare a 1 mg/mL stock solution. Dilute subsequent standards with isooctane. All standards were obtained from the Pesticide and Industrial Chemicals Repository, U.S. Environmental Protection Agency, Research Triangle Park, NC.

Systems Suitability

GPC Column Preparation and Validation.—Either automated or manual GPC equipment can be used in validating GPC columns. Weigh 35 g Bio-Beads SX-3 into a 400 mL beaker. Add 150 mL methylene chloride–hexane (50 + 50, v/v). Stir the beads with a glass or steel rod until all beads have swelled and no clumps are present. Hold the column in an upright position with the plunger tightened 125 mm from the midpoint of the column. Pour the slurry into a 500 × 25 mm column with the aid of a powder funnel. Add the slurry to the column continuously until all beads have been added. The beads should not be completely settled during the addition. Place the other plunger in the column after the beads have settled and the liquid has drained off. Compress each plunger an equal distance from its respective end until a bed length of ca 200 mm is achieved. Connect the column to the GPC solvent delivery system, and pump solvent from the bottom to the top of the column until all the air is expelled. Adjust the flow rate of the system to 5 mL/min and check the column pressure. Adjust the operating pressure for the column to 8–11 psig by moving the plunger(s). Allow the GPC system to equilibrate by pumping solvent through it for several minutes, and readjust the flow rate to 5 mL/min.

The goal in GPC cleanup is to separate the lipid material from the analytes; the lipid fraction is discarded. New GPC columns must be validated before use to ensure that sample coextractives and pesticides are adequately separated. The following validation procedure confirms the suitability of the GPC column. Validated columns can be used repeatedly for sample cleanup. The validation procedure includes (a) elution of a butterfat solution, (b) elution of a solution of organochlorine and organophosphorus pesticides, (c) elution of a butterfat solution fortified with those pesticides, and (d) elution of CPAs and PCP solution.

(a) *Elution of fat.*—Melt butter and filter through a fluted filter paper into a suitable container. Weigh 5 g warm filtered butter (do not include water layer) into a 25 mL glass-stoppered graduated cylinder, and dilute to 25 mL with 50% methylene chloride–hexane. Mix until fat is dissolved (0.2 g fat/mL). Load 5 mL fat solution onto GPC column and elute with 50% methylene chloride–hexane. Collect the column effluent in tared beakers in 10 mL aliquots until a total of 100 mL is collected. Evaporate the solvent, cool and weigh each beaker, and calculate the amount of fat collected in each 10 mL aliquot. (For manual GPC, collect 10 mL aliquots in graduated cylinders and transfer to tared beakers for evaporation and calculation of fat content.) Visual scrutiny of the column during the validation procedure can be helpful in assessing the condition of the column. The yellow fat band should enter the column in a relatively narrow band that widens as it transverses the column. The band for an inadequate GPC column normally tails or streaks through the column. If the column is adequate, a minimum of 95% of the fat will elute in the first 60 mL collected. In an inadequate column, more than 5% of the fat will elute in

the 60–100 mL fraction. The column should be repacked with the existing beads. Before the column is repacked, the column plunger assembly should be inspected to make sure that the Teflon screen holding the beads is not partially plugged. Partial blockage can cause sample tailing. A different lot of beads should be used to pack the column if the repacked column also fails the validation procedure.

(**b**) *Elution of pesticides.*—Organochlorine and organophosphorus pesticides are used in the validation procedure to avoid the extensive methylation required for the CPAs and PCP. Prepare a mixed standard containing 1.2 μg/mL ethion, 0.4 μg/mL diazinon, 0.4 μg/mL heptachlor epoxide, 0.2 μg/mL dicloran, and 0.6 μg/mL cieldrin in 50% methylene chloride–hexane. Load 5 mL mixed standard onto the GPC column and elute with 50% methylene chloride–hexane. Collect 10 mL aliquots until a total of 160 mL is collected. Transfer each 10 mL aliquot to a 250 mL Kuderna-Danish concentrator fitted with a graduated collection flask, and add 50 mL hexane and 2–3 boiling chips. Concentrate to 10 mL. The recoveries for organochlorine and organophosphorus pesticides are determined using the GC conditions found in Chapter 3 of PAM I. The GPC column is suitable for use if diazinon and ethion elute in the 50–70 mL range, and dicloran starts to elute in the 90–100 mL fraction. Determine the volume to be discarded (typically 0–60 mL) and collected (typically 60–160 mL) by examining the fat and mixed standard elution profiles. Record the discard and collection volumes for subsequent steps in the validation procedure.

(**c**) *Elution of pesticides from fat.*—Weigh 2 g warm, filtered butterfat into a tared 10 mL glass-stoppered graduated cylinder, add 5 mL mixed pesticide standard solution, adjust the volume to 10 mL with 50% methylene chloride–hexane, and mix until the fat is dissolved. Load 5 mL fortified fat onto the GPC column, and elute with 160 mL 50% methylene chloride–hexane. Collect the appropriate volume of eluant as determined above, transfer to a Kuderna-Danish concentrator fitted with a 10 mL receiving flask, add 2–3 boiling chips, and concentrate to 5 mL. Determine the recoveries for the organochlorine and organophosphorus pesticides using the conditions found in Chapter 3 of PAM I. The pesticide fraction for a suitable GPC column will contain a minimum of 80% of the diazinon, parathion, and ethion and a minimum of 95% of the organochlorine pesticides.

(**d**) *Elution of herbicides.*—Prepare a mixed standard solution of 0.1 μg/mL 2,4,5-T and 0.05 μg/mL pentachlorophenol in 50% methylene chloride–hexane. Load 5 mL of this solution onto the GPC column and elute with 160 mL 50% methylene chloride–hexane. Collect the appropriate volume of eluant as determined above, transfer to a Kuderna-Danish concentrator fitted with a graduated receiving flask, add 2–3 boiling chips, and concentrate to ca 3 mL. Cool solution, add 50 mL acetone and fresh boiling chips, and reconcentrate to 3 mL. Cool solution and methylate as below. Clean up the methylated solution on Florisil and concentrate to 5 mL. Examine by EC/GC as described. Gel column is acceptable if at least 80% of the 2,4,5-T and pentachlorophenol are recovered.

Methylation Validation.—Standard solutions of CPAs and PCP have been found susceptible to degradation. To determine completeness of methylation, compare authentic standard solutions of chlorophenoxy acid methyl esters and pentachlorophenyl methyl ether to the methylated chlorophenoxy acids and pentachlorophenol. If the methylated standard values do not compare well to the authentic standards, i.e., they are consistently less than 40% or greater than 120% of authentic standards, prepare a new mixed standard solution of the acids and pentachlorophenol. Methylate the freshly prepared mixed standard and the old mixed standard, and clean up both by Florisil chromatography. Determine the degree of methylation in both old and new standards by comparison to authentic standards. If recoveries are acceptable in the new standards, the problem can be attributed to degradation of the old standard. If unusual recoveries persist, then the methylation step is at fault.

Florisil Column Preparation and Validation.—Each new batch of purchased Florisil must be validated before use. By using PAM I, sec. 121.3, calculate the lauric acid value. The amount of Florisil to use in the 300 × 10 mm id chromatographic column is calculated by dividing 110 by the lauric acid value and multiplying by 4. Prepare the Florisil column by adding the calculated amount of Florisil to the column, tapping the column gently to settle the absorbent. Top the column with ca 2 cm Na_2SO_4. Prewash the column with 15 mL hexane, but do not allow the column to go to dryness. Add 5 mL mixed standard containing 2,4,5-T methyl ester (0.1 μg/mL), pentachlorophenyl methyl ether (0.05 μg/mL), and picloram methyl ester (0.2 μg/mL). Elute the column with 35 mL of 20% methylene chloride–hexane (Eluate 1). Change receivers and elute the column with 60 mL 50% methylene chloride–0.35% acetonitrile–49.65% hexane (Eluate 2). Add 100 mL ethyl ether (Eluate 3), and collect 10 mL aliquots. Concentrate eluates to 5 mL, examine by EC/GC and calculate recoveries. The pentachlorophenyl methyl ether should be in Eluate 1 and the 2,4,5-T methyl ester in Eluate 2. Using the 10 mL aliquots of Eluate 3 collected, determine and record the volume needed to elute the picloram methyl ester from the column. Florisil that does not conform to this elution profile should be discarded.

Method

Sample Extraction

(**a**) *Dairy products.*—Grind cheese and other solid samples before analysis. Weigh 100 g sample into a 500 mL glass-stoppered centrifuge bottle. Add 100 mL methanol, 10 mL 10% H_2SO_4, and ca 2 g sodium or potassium oxalate, and mix. Add 50 mL ethyl ether and shake vigorously 1 min; then add 50 mL petroleum ether and shake vigorously 1 min. Centrifuge ca 5 min at 1500 rpm. Proceed to liquid-liquid partitioning.

(**b**) *Animal tissue.*—Weigh 50 g sample into a 1 L blender cup. Add 50 mL distilled water, 100 mL methanol, 10 mL 10% H_2SO_4, and 2 g sodium or potassium oxalate to the blender cup. Blend 3 min at high speed, and transfer to a 500 mL glass-stoppered centrifuge bottle with the aid of a powder funnel. Rinse blender and funnel with 50 mL ethyl ether and combine with sample extract. Shake vigorously 1 min; then add 50 mL petroleum ether and shake vigorously 1 min. Centrifuge ca 5 min at 1500 rpm. Proceed to liquid-liquid partitioning.

(c) *Fats and shortenings.*—Weigh 25 g sample into a 500 mL glass-stoppered centrifuge bottle. Add 50 mL distilled water, 100 mL methanol, 10 mL 10% H_2SO_4, and ca 2 g sodium or potassium oxalate, and mix. Add 50 mL ethyl ether and shake vigorously 1 min; then add 50 mL petroleum ether and shake vigorously 1 min. Centrifuge 5 min at 1500 rpm. Proceed as in *Dairy products* after initial centrifuging. Sample is ready for liquid-liquid partitioning.

For vegetable oils, weigh 5 g pure vegetable oil into a 25 mL volumetric flask. Dilute to volume with 50% methylene chloride–hexane (fat concentration 0.2 g/mL) and proceed directly to GPC cleanup.

(d) *Grains and cereal products.*—Weigh 50 g sample into a blender cup. Add 10 mL 10% H_2SO_4 and 340 mL 30% water–methanol. Blend 2 min at high speed and pour into a 500 mL centrifuge bottle with the aid of a powder funnel. Centrifuge 5 min at 1500 rpm, and pour the liquid layer through a funnel containing a glass wool plug into a 250 mL graduated cylinder. Save 250 mL, pour the extract into a 2 L separator, and add 100 mL methylene chloride. Shake the separator for 30 s, and then add 30 mL saturated NaCl solution, 10 mL 10% H_2SO_4, and 650 mL water. Shake 30 s and allow the emulsion to settle. Transfer methylene chloride layer to a 100 mL graduated cylinder and record volume.

(**Note**: If emulsion does not break, treat as for *Legume vegetables.*)

Transfer measured volume of methylene chloride to a Kudema-Danish concentrator fitted with a 10 mL receiving flask. Add boiling chips and concentrate the extract on a steam bath to 5 mL. Dilute to 10 mL with hexane and mix. Clean up extract by GPC. Equivalent weight of sample cleaned up by GPC is:

$$50\text{ g} \times [250/(340 + 10)] \times (\text{mL } CH_2Cl_2 \text{ recovered}/100\text{ mL}) \times (\text{mL loaded onto GPC}/10\text{ mL})$$

(e) *Fruits, vegetables other than legumes, and beverages.*—Weigh 100 g sample into a blender cup. Add 10 mL 10% H_2SO_4 and 250 mL methylene chloride. Blend 2 min at high speed and pour into a 500 mL centrifuge bottle with the aid of a powder funnel. Centrifuge 5 min at 1500 rpm. Siphon off and discard the top water layer.

Carefully decant methylene chloride (leave the cake in the centrifuge bottle) through a funnel containing a glass wool plug into a 250 mL separator. Let stand at least 30 min to ensure complete separation of any remaining water. Transfer methylene chloride to a 250 mL graduated cylinder and record volume. Transfer the measured volume of methylene chloride to a Kudema-Danish concentrator fitted with a 10 mL receiving flask. Add boiling chips and concentrate the extract to 5 mL on a steam bath. Dilute sample extract to 10 mL with hexane and mix. Clean up extract by GPC. Equivalent weight of sample cleaned up by GPC is:

$$100\text{ g} \times (\text{mL } CH_2Cl_2 \text{ recovered}/250\text{ mL}) \times (\text{mL loaded onto GPC}/10\text{ mL})$$

(f) *Sugars and high-sugar processed foods.*—Weigh 50 g sample into a blender cup. Add 10 mL 10% H_2SO_4, 100 mL water, and 200 mL methanol. Blend 2 min at high speed. Pour mixture through a powder funnel containing a glass wool plug into a 2 L separator.

Add 250 mL methylene chloride and shake 30 s. Add 700 mL distilled water, 10 mL 10% H_2SO_4, and 35 mL saturated NaCl solution. Shake separator 1 min and allow emulsion to settle. Drain remaining emulsion and methylene chloride into a 500 mL centrifuge bottle and centrifuge 5 min at 1500 rpm. Siphon off and discard the water layer. Pour methylene chloride layer through a funnel containing a glass wool plug into a 250 mL separator. Let stand at least 30 min to ensure complete separation of any remaining water. Transfer methylene chloride layer to a 250 mL graduated cylinder and record volume.

Transfer measured volume of methylene chloride to a Kudema-Danish concentrator fitted with a 10 mL receiving flask. Add boiling chips and concentrate extract on a steam bath to ca 5 mL. Dilute sample to 10 mL with hexane and mix. Clean up extract by GPC. Equivalent weight of sample cleaned up by GPC is:

$$50\text{ g} \times (\text{mL } CH_2Cl_2 \text{ recovered}/250\text{ mL}) \times (\text{mL loaded onto GPC}/10\text{ mL})$$

(g) *Water.*—Weigh 500 g sample and transfer to a 1 L separator. Add 10 mL 10% H_2SO_4 and 60 mL methylene chloride. Shake vigorously 1 min. Let layers separate, and drain methylene chloride layer into a Kudema-Danish concentrator fitted with a 10 mL receiving flask. Extract sample twice more with 60 mL methylene chloride each time. Combine all extracts. Add boiling chips and evaporate to near dryness on a steam bath. Add 50 mL acetone and evaporate to 3 mL.

No GPC cleanup is necessary. Proceed to methylation. Entire solution (therefore, entire weight of original sample) is methylated.

(h) *Legume vegetables.*—Weigh 100 g sample into a 1 L blender cup. Add 10 mL 10% H_2SO_4 and 200 mL methanol. Blend 2 min at high speed and pour into a 500 mL centrifuge bottle with the aid of a powder funnel. Centrifuge 5 min at 1500 rpm and carefully pour the top layer through a funnel containing a glass wool plug into a 250 mL graduated cylinder. Save 250 mL. Pour the 250 mL extract into a 2 L separator and add 100 mL methylene chloride. Shake separator 30 s, and then add 30 mL saturated NaCl solution, 10 mL 10% H_2SO_4, and 650 mL water. Shake 30 s and allow emulsion to settle 30 min. Transfer methylene chloride layer to a 100 mL graduated cylinder and record volume. (**Note**: If emulsion does not break, drain emulsion into a 500 mL centrifuge bottle and centrifuge 5 min at 1500 rpm. Siphon off and discard the water layer. Pour methylene chloride through a funnel containing a glass wool plug into a 250 mL separator. Let stand at least 30 min to ensure complete separation of any remaining water. Transfer methylene chloride layer to a 100 mL graduated cylinder and record volume.)

Transfer the measured volume of methylene chloride to a Kudema-Danish concentrator fitted with a 10 mL receiving

Table 1. Recoveries of 2,4-D from a variety of food items

Food group	Spiking level, ppm	No. of samples	Av. rec., %	CV, %
Nonfat fruits/ veg./beverages	0.1	140	77.8	26.2
Dairy	0.2	51	66.1	33.8
Animal tissue	0.2	104	54.6	41.1
Veg./legumes	0.1	25	78.4	23.8
Grains	0.1–0.2[a]	23	62.4	25.3
Sugar products	0.1–0.2[a]	10	72.1	18.8
Fats/shortenings	0.2	11	81.7	22.8
Egg products	0.2	3	14.3	8.5
Liver	0.2	2	14.0	10.0
Water	0.01	2	62.5	37.3

[a] Different fortification levels are calculated together.

flask. Add boiling chips and concentrate extract on a steam bath to ca 5 mL. Dilute to 10 mL with hexane and mix. Clean up extract by GPC. Equivalent weight of sample cleaned up by GPC is:

$$100\ g \times 250/[200 + 10 + (100\ g \times \%water)] \times (mL\ CH_2Cl_2\ recovered/100\ mL) \times (mL\ loaded\ on\ GPC/10\ mL).$$

Liquid-Liquid Partitioning

Transfer organic phase, with a siphon tube, to a 1 L separatory funnel containing 500–600 mL water, 30 mL saturated NaCl solution, and 10 mL 10% H_2SO_4. Reextract the original sample aqueous phase twice, shaking vigorously 1 min with a separate 50 mL ethyl ether–petroleum ether (1 + 1) portion. Centrifuge and transfer organic phase to the separatory funnel after each extraction. Mix combined organic phase and aqueous phase cautiously to prevent emulsion formation. Drain and discard the aqueous phase. Gently rewash organic phase twice with 100 mL water, 10 mL 10% H_2SO_4, and 30 mL saturated NaCl solution; discard the aqueous phase each time. (If emulsions form, add an additional 5 mL saturated NaCl solution to the wash). After the final wash is discarded, transfer the organic phase to a 250 mL separatory funnel. Let stand at least 30 min. Drain and discard any aqueous phase and emulsion that separates. By using petroleum ether, quantitatively transfer the organic phase to a Kuderna-Danish concentrator fitted with a 125 mL receiving flask, add boiling chips, and evaporate solvent. Cool, add 50 mL methylene chloride to the sample extract, and mix. Add boiling chips and evaporate on a steam bath until the level in the receiving flask does not change but methylene chloride remains in the Snyder column traps. Allow to cool. Use the approximate fat content of the sample, as listed in Table 202 of PAM I, to determine what dilution is required to achieve a concentration of no more than 0.2 g fat/mL (the optimum fat concentration for GPC cleanup is 0.15 g/mL). Quantitatively transfer the sample extract from the 125 mL receiving flask to a glass-stoppered graduated cylinder, and dilute to volume using 50% methylene chloride–hexane. Transfer an aliquot of the solution to a tared vessel for drying and weighing to determine the fat concentration. If necessary, adjust the remaining solution volume so that the solution contains no more than 0.2 g fat/mL. Clean up extract by GPC. Use the following formula to calculate the equivalent weight of whole sample cleaned up by GPC:

$$(sample\ weight \times mL\ loaded\ onto\ GPC)/final\ extract\ volume$$

Table 2. Recoveries of 2,4,5-T from a variety of food items

Food group	Spiking level, ppm	No. of samples	Av. rec., %	CV, %
Nonfat fruits/ veg./beverages	0.04	174	83.3	24.7
Dairy	0.08	65	77.2	31.1
Animal tissue	0.08	116	63.2	40.2
Veg./legumes	0.04	30	82.4	28.3
Grains	0.04–0.08[a]	29	74.5	27.0
Sugar products	0.04–0.08[a]	12	76.6	20.5
Fats/shortenings	0.08	21	69.1	33.4
Egg Products	0.08	3	14.3	8.7
Liver	0.08	2	13.0	0.0
Water	0.004	1	63.0	—
	0.008	1	83.0	—

[a] Different fortification levels are calculated together.

GPC Cleanup

Use a GPC column prepared as described in *GPC Column Preparation and Validation*. Centrifuge cloudy solutions before loading them onto the GPC system. Use a 5 μm pore size Millipore filter, with a Swinny adapter, and a 10 mL syringe with a Luer-Lok tip to fill the GPC sample loading loops. Load sample extract onto the GPC column in 5 mL loops. Approximately 1 g fat can be loaded in each loop. Use the original sample weight, aliquots taken during extraction, volume of final sample solution, and loading loop size to calculate the amount loaded. If the required limit of quantitation is lower than can be achieved with these sample size restrictions, load additional aliquots of the sample extract in separate 5 mL loops and combine concentrated eluates. Elute column with 160 mL 50% methylene chloride–hexane, and, using the elution profile determined in *GPC Column Preparation and Validation*, collect the appropriate volume in a beaker. Quantitatively transfer the eluate to a Kuderna-Danish concentrator fitted with a 10 mL receiving flask, using acetone to rinse the collection beaker. Add 2–3 boiling chips and concentrate to ca 3 mL. Cool, add 50 mL acetone and fresh boiling chips, and reconcentrate to ca 1 mL. Use a micro-Snyder column to obtain the final volume if necessary. The sample extract is ready for methylation.

Table 3. Recoveries of PCP from a variety of food items

Food group	Spiking level, ppm	No. of samples	Av. rec., %	CV, %
Nonfat fruits/ veg./beverages	0.01–0.02[a]	178	68.1	20.9
Dairy	0.02–0.04[a]	67	77.4	20.4
Animal tissue	0.02–0.04[a]	117	76.7	24.4
Veg./legumes	0.01–0.02[a]	30	62.2	29.4
Grains	0.01–0.02[a]	29	65.3	26.0
Sugar products	0.02	11	64.6	33.1
Fats/shortenings	0.02–0.04[a]	22	80.6	36.1
Egg products	0.02–0.04[a]	4	78.3	16.7
Liver	0.02–0.04[a]	3	69.3	30.8
Water	0.001	1	61.0	—
	0.003	1	67.0	—

[a] Different fortification levels are calculated together.

Methylation Procedure

Use a well-ventilated hood and protective gloves when adding reagents for methylation. With each batch of samples, also methylate an aliquot of the same mixed standard solution of CPAs and PCP used to fortify the recovery test samples above. Dilute sample extracts to 3 mL with acetone. Add 80 µL 1.0M TBAH in methanol and 40 µL methyl iodide. Immediately stopper the tube and mix. Place the stoppered tube in a 40°C water bath for 1.5 h with the water level of the bath above the fluid level in the tube. Remove the tube from the water bath, attach to a 250 mL Kuderna-Danish concentrator, and add 50 mL hexane and boiling chips. Evaporate to ca 1 mL (avoid dryness). Dilute to an appropriate volume with hexane, add 2 mL distilled water, and shake stoppered tube. Discard water and clean up methylated extracts using a Florisil column.

Florisil Cleanup

Prepare the column as described in *Florisil Column Preparation and Validation.* Place a Kuderna-Danish concentrator fitted with an appropriate receiving flask under the column. Quantitatively transfer the methylated sample extract to the column. Rinse the flask with hexane and add to the column; the sample extract and rinse volumes together should not exceed 15 mL. Elute the column with 35 mL Eluate 1. Change receiver and elute column with 60 mL Eluate 2. Change receiver and elute column with the appropriate volume of Eluate 3 as determined in *Florisil Column Preparation and Validation.* Add boiling chips to each Kuderna-Danish concentrator and concentrate eluates on a steam bath. Add 50 mL hexane to the Kuderna-Danish concentrator containing Eluate 2 and reconcentrate to remove final traces of acetonitrile. Add 50 mL hexane to the Kuderna-Danish concentrator containing Eluate 3 and reconcentrate.

Determination

Inject an appropriate amount of methylated extract onto the GC system for quantitation of residues. (Equivalent milligrams of sample injected depends on the limit of quantitation required.) The chromatograph should be equipped with a nonpolar column, such as OV-101, and a ^{63}Ni electron capture detector (sec. 311.4, PAM Vol I). Calculate quantities of CPAs and PCP by comparison to standard reference materials. Use standard solutions made from reference materials of chlorophenoxy acid methyl esters and pentachlorophenyl methyl ether. Report residues in terms of the methylated products unless directed otherwise. The methylated extract is also amenable to GC determination using other detectors. Certain residues are recovered through the extraction, cleanup, and methylation steps of this method but can be determined only when examined by GC with a detector other than the electron capture detector (e.g., electrolytic conductivity detector).

Results and Discussion

The herbicide procedure above has been developed to analyze 234 fatty and nonfat food items for CPAs, PCP, and other herbicide residues. Following the analytical scheme described under *Method*, the food items were separated into 8 general groups: dairy foods (32 items); animal tissues (66 items); fats and shortenings (10 items); grain and cereal products (18 items); fruits, vegetables other than legumes, and beverages (85 items); sugars and high-sugar processed foods (8 items); water (1 item); and legume vegetables (14 items). Each food item is assigned to a particular general group depending on its percent fat, percent moisture, sample matrix, and sample weight required for analysis. A specific extraction step is used for each general group, and all extracts are cleaned up with GPC. The herbicides are determined after the GPC eluates are methylated and cleaned up with a Florisil minicolumn.

This procedure has been used in the Total Diet Study since 1986 to analyze 3978 food items for herbicide residues. Food items fortified with 2,4-D, 2,4,5-T, and PCP were carried through the procedure with each series of samples. All but 5 of the 234 items have been fortified at least once. The overall recoveries for the fortified 2,4-D, 2,4,5-T, and PCP, as shown in Tables 1–3, are acceptable. The average recoveries for 2,4-D, 2,4,5-T, and PCP at different fortification levels, as shown in Tables 1–3, were calculated together because the individual statistics for each level were very similar. The recoveries for 2,4-D, 2,4,5-T, and PCP fortified in water were comparable to the recoveries obtained in the other food groups and are presented in Tables 1–3.

Table 1 shows the average recoveries obtained for 2,4-D fortified at 100 and 200 ppb in 7 general food groups. As indicated, the recoveries for 2,4-D are acceptable but tend to be slightly lower than the recoveries for 2,4,5-T and PCP, which are fortified at lower levels. Table 2 shows the average recoveries for 2,4,5-T fortified at 40 and 80 ppb in 7 general food groups. The recoveries were acceptable and generally higher in comparison to those for 2,4-D. The overall average recoveries for the general animal tissue food group fortified with 2,4-D and 2,4,5-T were lower than the recoveries for the other general food groups. Liver and eggs, included in the animal tissue group, contributed to the lower recoveries and higher coefficients of variation (CVs) for 2,4-

Table 4. Herbicides evaluated by procedure for CPAs and PCP

Chemical	Rec., fatty foods[a]	Rec., nonfat foods[a]	Notes[b]
Alloxydim-sodium	NR	NR	Does not methylate
Benazolin[c]	P (28–32%)	C	Elutes in ethyl ether
Bifenox	C	C	Parent is methyl ether
Bromofenoxim[c]	P (57–86%)	C	—
Bromoxynil[c]	P (50–68%)	C	—
Chloramben	P (40–43%)	P (49–59%)	—
4-Chlorophenoxyacetic acid[c]	P (32–69%)	C	—
Chloroxuron	NR	NR	Does not methylate
Cloprop[c]	P (50–66%)	C	—
Dalapon[c]	NR	NR	Does not methylate
2,4-DB	C	C	—
Dicamba	P (71–76%)	C	—
Dichlorprop[c]	C	C	—
Dinoseb	NR	NR	Does not methylate
Disul-sodium	C	P (52–56%)	Soluble only in acidified acetone: does not methylate, but parent (Na salt) elutes in ethyl ether
Dodine	NR	NR	Does not methylate
DNOC[c]	P (45–50%)	C	N detector required
Fenac[c]	C	C	—
Fluroxypyr[c]	P (27–33%)	C	Two peaks from methylation; elutes partially in ethyl ether
Ioxynil[c]	C	C	—
MCPA	C	C	—
MCPB[c]	C	C	—
Mecoprop[c]	C	C	—
Picloram	P (6–10%)	C	Elutes in ethyl ether
Silvex	C	C	—
2,4,5-TB	C	C	—
2,3,6-TBA	C	C	—
TCA	NR	NR	Methyl ester elutes in GC solvent front
Triadimenol	NR	NR	Ether does not elute from Florisil
Triclopyr[c]	C	C	—
2,3,5-Triiodobenzoic acid[c]	V (66–86%)	V (79–138%)	—

[a] C = complete, recoveries >80%; P = partial, recoveries <80% with actual range; V = variable, actual recoveries; and NR = not recovered.
[b] Ester/ether elutes from Florisil in Eluate 2 unless otherwise noted.
[c] No standard reference material available for the ester/ether; recoveries calculated against the methylated acid/phenol according to the method.

D and 2,4,5-T because the recoveries for these items ranged from 13 to 18%, as shown in Tables 1 and 2. Adequate recoveries cannot be obtained for 2,4-D and 2,4,5-T fortified in these items. The cause of these low recoveries is unknown at this time. The low recoveries appear to be confined to the CPAs because reasonable recoveries can be obtained for the same items fortified with PCP. The average recoveries for PCP fortified at 20 and 40 ppb were acceptable, as shown in Table 3. Other herbicides were evaluated using the same procedure as used for CPAs and PCP; the results are shown in Table 4. The herbicides were fortified in duplicate milk and tomato samples and analyzed. Specific observations for several herbicides are noted in Table 4. These notes help explain inconsistences found during the evaluation. The method has been successfully applied to a variety of fatty and nonfat food items collected under the FDA's Total Diet Study.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Analysis of Polychlorinated Biphenyls in Aqueous Samples Using C_{18} Glass Column Extraction

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A method for polychlorinated biphenyl (PCB) trace extraction from contaminated water at the part-per-trillion levels is described. This procedure involves quantitative adsorption of PCBs on C_{18} glass microcolumns. PCBs are retained on the surface and subsequently eluted with *n*-hexane (5 mL) before gas chromatography. Recovery of water fortified with PCBs was 83.6–108.5%. Compared to liquid-liquid extraction methods, the C_{18} glass microcolumns give comparable results, lessen solvent costs, and are less time-consuming.

Because of the chemically stable and nonflammable nature of polychlorinated biphenyls (PCBs), together with their high boiling point, low solubility, and nonconductive nature, PCBs are nearly ideal for many industrial applications, including capacitors and transformers (1). Unfortunately, these same properties cause PCBs to persist in the environment and to bioconcentrate; therefore, PCPs are potential hazards to biota.

Aquatic ecosystems have been contaminated by direct dumping of PCB and waste fluids containing PCBs (2). Because of their insoluble character, PCBs are usually quickly bound in some organic entity, such as river and lake sediments, algae, and protozoa. The concentration of PCBs in water is very small and can only be measured by sensitive techniques (3).

Multipesticide residue methods are effective in recovering PCBs. The 2 methods most often used are based on either extraction with an organic solvent (4–7) or adsorption on a filter containing activated carbon (8, 9). These methods, however, are rather time-consuming because of the long extraction time from both water and carbon. Other analytical procedures involve adsorption on XAD-2 (10), polyurethane foam (11), a mixture of *n*-undecane and Carbowax 4000 monostearate on Chromosorb W (12), or silica gel (13) followed by selective desorption with the appropriate eluents. The use of reversed-phase partitioning to analyze organic pollutants incorporates octadecylsilica (C_{18}) reversed-phase partitioning for gas chromatographic (GC) analysis of aqueous samples (14, 15). C_{18} extraction is economical and requires fewer organic solvents and operational steps. However, commercial C_{18} cartridges are hindered by interfering peaks on GC with electron capture detection (ECD). Potential interferences originate from different components of the commercial cartridges, such as the propylene cartridge and polyethylene frit, when an electron capture unit is used for detection (16, 17). The use of glass cartridges (18) or disk technology (19) could eliminate the interferences caused by plastic cartridges.

The purpose of the present study was to evaluate the use of a C_{18} glass microcolumn for solid-phase trace extraction of PCBs, taking advantage of the excellent extraction capacity of C_{18} bonded to porous silica for organic compounds and avoiding the interferences caused by commercial cartridges (18).

This report examines how the recovery is affected by the nature and volume of the eluting solvent, sample size, flow rate, pH, and presence of a surfactant in the sample.

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Experimental

Apparatus and Reagents

(a) *Reference materials.*—Nine biphenyls (2-PCB; 2,2′-PCB; 2,4-PCB; 4,4′-PCB; 2,4,5-PCB; 3,3′,4,4′-PCB; 2,2′,4,5,5′-PCB; 2,2′,4,4′,5,5′-PCB; and decachlorobiphenyl) were chosen as model compounds. Analytical standards were purchased from Riedel de Haen, Seelze, Germany. Commercially available PCB mixtures, Aroclor 1016, 1242, 1248, 1254, and 1260, were purchased from Supelco, Inc., Bellefonte, PA 16823.

(b) *Solvents.*—Dichloromethane, ethyl acetate, ethyl ether, *n*-hexane, petroleum ether (b.p. 40–60), and methanol; pesticide grade (J.T. Baker Inc., Phillipsburg, NJ 08865). Solvents were shown to be free from interfering residues by GC/ECD following 200-fold concentration.

(c) *Buffers.*—Solutions of pH 2–9 were prepared (20).

(d) *Tensioactives.*—Sodium lauryl sulfate, Triton 100-X, and Cetrimide; analytical grade.

(e) *Preparative C_{18}.*—55–105 μm (Waters Chromatography, Div. of Millipore, Milford, MA 01757).

(f) *GC system.*—Model 2000 C gas chromatograph (Konik Instruments Inc., Westport, CT 06880) equipped with ^{63}Ni electron capture detector and 2 fused silica columns, 1 primary column, 30 m × 0.25 mm id cross-linked with BP-5–5% phenylmethylsiloxane–0.25 μm bonded-phase (Supelco), and

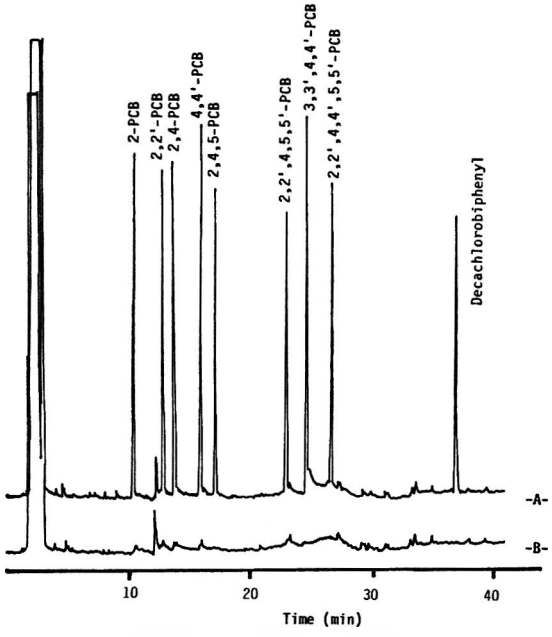

Figure 1. Chromatograms of (A) fortified water sample containing 9 PCBs and (B) nonfortified water sample extracted on C_{18} bonded-phase sorbent in a glass microcolumn: volume injected, 1 μL. Amounts were as follows: 2-PCB, 2500 pg; 2,2′-PCB, 1500 pg; 2,4-PCB, 250 pg; 4,4′-PCB, 1500 pg; 2,4,5-PCB, 150 pg; 3,3′,4,4′-PCB, 150 pg; 2,2′,4,5,5′-PCB, 100 pg; 2,2′,4,4′,5,5′-PCB, 50 pg; and decachlorobiphenyl, 30 pg.

the other used as the confirmation column, 30 m × 0.24 mm id cross-linked with DB-17–50% phenylmethylsiloxane–0.25 μm bonded-phase (J & W Scientific, Folsom, CA 95630).

Microcolumn Preparation

Place 0.5 g C_{18} into glass column (9 × 100 mm id) with coarse frit (n°3), and cover with plug of glass silanized glass-wool. Activate column before use by passage of 10 mL methanol followed by 10 mL distilled water.

Analytical Procedure

Pass 1 L sample through column at 33 mL/min (vacuum pressure = 2.7 Pa). After entire sample has passed through column, dry solid phase by aspirating ambient air through column. Elute PCBs with 5 mL hexane. Before GC/ECD, concentrate extracts to 200 μL under gentle stream of nitrogen.

GC Analysis

Inject 1 μL sample into GC system by splitless mode. Temperature program: initial, 50°C, hold 0.8 min; ramp to 140°C at 30°C/min, hold 2 min; program to 280°C at 5°C/min, hold 4 min. Injector temperature: 285°C. Detector temperature: 300°C. Makeup gas was argon–methane (95 + 5) at 60 mL/min. Carrier gas was helium at 1.5 mL/min.

Results and Discussion

A typical chromatogram of the 9 biphenyls extracted from a water sample and a chromatogram of a blank are shown in Figure 1. The BP-5 capillary column provides excellent resolution at the picogram level for the 9 PCBs, with a run time of approximately 45 min. All water sample results were confirmed on a DB-17 column.

First, we examined the ability of various solvents to elute PCBs from a C_{18} glass microcolumn. Table 1 shows the recovery of PCBs from 1 L spiked samples of distilled water. The best results were obtained with hexane.

We also studied the effect of eluting solvent volume (from 1 to 10 mL) from C_{18} glass microcolumns with 500 mg C_{18} packing in a small glass column. At least 5 mL eluting solvent was needed. Table 2 shows the results obtained. Recoveries with elution volumes of more than 5 mL were almost identical, but they inevitably involved longer concentration times.

Table 3 shows the effect of sample size. Tests performed to determine whether sample volume affected recovery showed that recovery values were the same over a 0.1–10 L range of water. Extraction and GC analysis of 1 L samples can be completed in about 1 h.

Recoveries listed in Table 1 were obtained by GC of a 1 μL aliquot of hexane eluate concentrated to 200 μL. The 200 μL

Table 1. Effect of various elution solvents (5 mL) on analyte recovery from C_{18} bonded-phase column

Compound	Concn in fortified water, ng/L	Rec., %[a]		
		n-Hexane $\bar{X}$ ± RSD	Light petroleum ether $\bar{X}$ ± RSD	Ethyl acetate $\bar{X}$ ± RSD
2-PCB	500	92 ± 8	80 ± 9	81 ± 10
2,2′-PCB	300	94 ± 7	76 ± 7	76 ± 8
2,4-PCB	50	100 ± 8	79 ± 7	74 ± 8
4,4′-PCB	300	92 ± 6	73 ± 8	67 ± 10
2,4,5,-PCB	30	108 ± 5	75 ± 7	80 ± 7
3,3′,4,4′-PCB	30	91 ± 9	73 ± 6	69 ± 7
2,2′,4,5,5′-PCB	16	85 ± 7	67 ± 7	72 ± 10
2,2′,4,4′,5,5′-PCB	12	86 ± 7	84 ± 8	77 ± 9
Decachlorobiphenyl	6	83 ± 8	93 ± 7	81 ± 8

[a] $n = 5$; values are expressed as $\bar{X}$ ± RSD (mean ± relative standard deviation).

Table 2. Effect of various volumes of *n*-hexane on analyte recovery from C_{18} bonded-phase column

	Rec., %[a]				
Compound	1 mL	2 mL	3 mL	5 mL	10 mL
2-PCB	45 ± 10	60 ± 11	72 ± 9	92 ± 8	94 ± 5
2,2′-PCB	55 ± 12	69 ± 14	79 ± 15	94 ± 7	93 ± 7
2,4-PCB	49 ± 15	63 ± 19	76 ± 12	100 ± 8	100 ± 4
4,4′-PCB	40 ± 15	52 ± 15	63 ± 9	92 ± 6	93 ± 5
2,4,5,-PCB	45 ± 14	75 ± 13	89 ± 10	108 ± 5	104 ± 5
3,3′,4,4′-PCB	51 ± 18	53 ± 20	60 ± 13	91 ± 9	93 ± 7
2,2′,4,5,5′-PCB	30 ± 13	50 ± 15	69 ± 8	85 ± 7	89 ± 8
2,2′,4,4′,5,5′-PCB	32 ± 12	60 ± 16	69 ± 12	86 ± 7	89 ± 6
Decachlorobiphenyl	24 ± 13	64 ± 20	70 ± 17	83 ± 8	84 ± 9

[a] $\bar{X}$ ± RSD (n = 5).

Table 3. Effect of sample size on analyte recovery from C_{18} bonded-phase column using 5 mL *n*-hexane as elution solvent

	Rec., %[a]					
Compound	Sample size, 0.1 L	Concn, µg/L	Sample size, 1 L	Concn, µg/L	Sample size, 10 L	Concn, µg/L
2-PCB	93 ± 7	5.00	91 ± 8	0.50	92 ± 6	0.05
2,2′-PCB	87 ± 5	3.00	94 ± 7	0.30	91 ± 6	0.03
2,4-PCB	94 ± 6	0.50	99 ± 8	0.05	97 ± 8	5.0×10^{-3}
4,4′-PCB	92 ± 8	3.00	92 ± 6	0.30	87 ± 8	0.03
2,4,5,-PCB	101 ± 9	0.30	108 ± 5	0.03	105 ± 7	3.0×10^{-3}
3,3′,4,4′-PCB	89 ± 8	0.30	91 ± 9	0.03	96 ± 9	3.0×10^{-3}
2,2′,4,5,5′-PCB	82 ± 7	0.16	85 ± 7	0.02	83 ± 8	2.0×10^{-3}
2,2′,4,4′,5,5′-PCB	84 ± 9	0.12	86 ± 8	0.01	86 ± 8	1.0×10^{-3}
Decachlorobiphenyl	81 ± 8	0.06	83 ± 7	6.0×10^{-3}	80 ± 5	6.0×10^{-4}

[a] $\bar{X}$ ± RSD (n = 5).

Table 4. Effect of various flow rates on recovery of 9 PCBs

	Rec., %[a]				
Compound	3.5	10	30	60	85
2-PCB	90 ± 7	91 ± 9	94 ± 7	94 ± 8	92 ± 8
2,4-PCB	96 ± 7	96 ± 8	94 ± 7	93 ± 7	94 ± 7
2,4′-PCB	100 ± 8	101 ± 7	98 ± 9	100 ± 9	96 ± 7
4,4′,-PCB	93 ± 8	87 ± 10	92 ± 9	96 ± 8	85 ± 6
2,4,5-PCB	95 ± 9	100 ± 7	108 ± 6	106 ± 5	103 ± 5
3,3′,4,4′-PCB	93 ± 6	91 ± 5	90 ± 7	91 ± 4	92 ± 8
2,2′,4,5,5′-PCB	77 ± 7	82 ± 8	78 ± 6	85 ± 7	79 ± 5
2,2′,4,4′,5,5′-PCB	86 ± 7	81 ± 5	84 ± 7	88 ± 7	83 ± 5
Decachlorobiphenyl	83 ± 4	80 ± 8	89 ± 8	85 ± 7	82 ± 6

[a] $\bar{X}$ ± RSD (n = 5).

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Table 5. Effect of various pHs on recovery of 9 PCBs

Compound	Rec., %[a]						
	pH 2	pH 3.4	pH 4.8	pH 6	pH 7	pH 8	pH 9
2-PCB	94 ± 7	93 ± 8	97 ± 7	94 ± 6	91 ± 8	93 ± 9	95 ± 8
2,2′-PCB	88 ± 9	91 ± 9	93 ± 8	89 ± 7	91 ± 9	92 ± 7	89 ± 6
2,4-PCB	97 ± 10	99 ± 8	101 ± 9	99 ± 9	98 ± 7	103 ± 9	100 ± 9
4,4′-PCB	93 ± 10	92 ± 7	90 ± 8	94 ± 6	87 ± 5	91 ± 9	93 ± 9
2,4,5,-PCB	108 ± 9	100 ± 9	105 ± 9	103 ± 7	100 ± 9	99 ± 7	107 ± 2
3,3′,4,4′-PCB	89 ± 8	91 ± 7	93 ± 7	88 ± 6	93 ± 9	88 ± 7	94 ± 8
2,2′,4,5,5′-PCB	88 ± 8	85 ± 5	87 ± 6	89 ± 5	82 ± 8	84 ± 8	83 ± 7
2,2′,4,4′,5,5′-PCB	85 ± 9	86 ± 8	86 ± 8	87 ± 7	84 ± 5	84 ± 9	82 ± 7
Decachlorobiphenyl	83 ± 9	82 ± 7	81 ± 8	81 ± 9	79 ± 9	80 ± 6	83 ± 9

[a] $\bar{X}$ ± RSD (n = 5).

of eluate from 1 L water corresponds to a 5000:1 concentration factor, which allows detection limits of 53 and 0.6 ng/L (for 2-PCB and decachlorobiphenyl, respectively). The detection limit was calculated from diluted samples that produced a chromatographic peak with a height 3 times the standard deviation of the baseline noise (21).

The effect of the sample flow rate through the C_{18} column was investigated at 3.5, 10, 30, and 85 mL/min. Table 4 shows that recovery measurements of the resulting eluents did not differ significantly at these flow rates.

We also tested the possible effect of pH and surfactants on the percent recovery. Recovery was the same for pH between 2 and 9 (see Table 5). Table 6 shows that the presence of a surfactant in the sample increases solubility of the biphenyls in the sample and results in lower pesticide recovery.

Our results were similar to those from other investigators (14, 15) who demonstrated that a glass microcolumn does not modify the effectiveness of commercial cartridges but does reduce interfering peaks.

Figure 2 shows that results obtained with fortified tap water were similar to those obtained with classic organic solvent extraction methods such as those proposed by Rodier (4) and the American Public Health Association (5). Comparisons in all cases indicate that PCBs can be extracted from water with 500 mg C_{18} bonded to porous silica in a glass microcolumn.

Finally, we investigated recovery of the 9 compounds from various types of water samples. Table 7 shows that sea, distilled, and tap water gave similar recoveries. The biggest problem encountered was restricted water flow caused by suspended sediments; therefore, a prefiltration step was added so that 1 L water volumes could be processed. This step was needed to perform the analysis; however, a substantial portion of hydrophobic compounds like PCBs may reside on particulates in water (22, 23). The prefiltration step could diminish recoveries of these compounds.

Thirty-four samples taken from natural waters in the Valencia Community (a predominantly industrial area), which flow directly into the Mediterranean Sea, were analyzed for the presence of Aroclors. Samples were collected in June and September, 1990. Table 8 shows results. Aroclors 1016, 1242, and 1254 were identified in the natural water samples analyzed. GC peak retention times from standard mixtures were used to identify the contaminant Aroclor, and peak area was used to quantitate each individual peak. The detection limit estimated for Aroclors was 10–100 ng/L, depending on the mixture detected. Aroclor levels were low (84–313 ng/L), but they were of a sufficient order of magnitude to prompt us to do a corrected quantitation. Figure 3 shows the chromatograms corresponding to the waste water sample collected from the Belcaire River, which was contaminated with an Aroclor that we identified as Aroclor 1242.

Table 6. Effect of different surfactants (20 mg/L) in sample

Compound	Rec., %[a]			
	Distilled water	Anionic sodium lauryl sulfate	Nonionic Triton X-100	Cationic Cetrimide
2-PCB	92 ± 8	52 ± 11	41 ± 10	49 ± 9
2,2′-PCB	94 ± 7	55 ± 8	59 ± 9	53 ± 6
2,4-PCB	100 ± 8	61 ± 8	63 ± 6	58 ± 8
4,4′-PCB	92 ± 6	56 ± 10	52 ± 9	55 ± 11
2,4,5,-PCB	108 ± 5	60 ± 9	63 ± 10	64 ± 9
3,3′,4,4′-PCB	91 ± 9	58 ± 10	49 ± 9	54 ± 10
2,2′,4,5,5′-PCB	85 ± 7	49 ± 8	43 ± 8	49 ± 10
2,2′,4,4′,5,5′-PCB	86 ± 7	52 ± 7	50 ± 9	53 ± 9
Decachlorobiphenyl	83 ± 8	62 ± 8	67 ± 10	63 ± 6

[a] $\bar{X}$ ± RSD (n = 5).

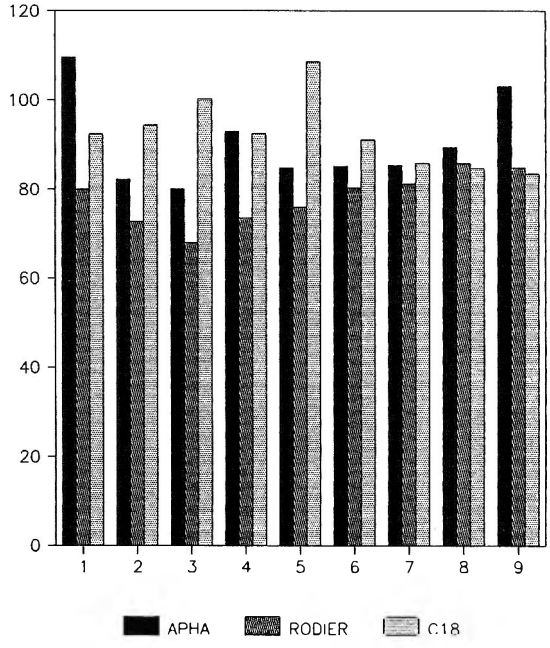

Figure 2. Comparison of C_{18} bonded porous silica glass microcolumn method and the Rodier and American Public Health Association methods for PCBs in waters. The fortified levels are the same as in Table 1, and each data is the mean of 5 determinations.

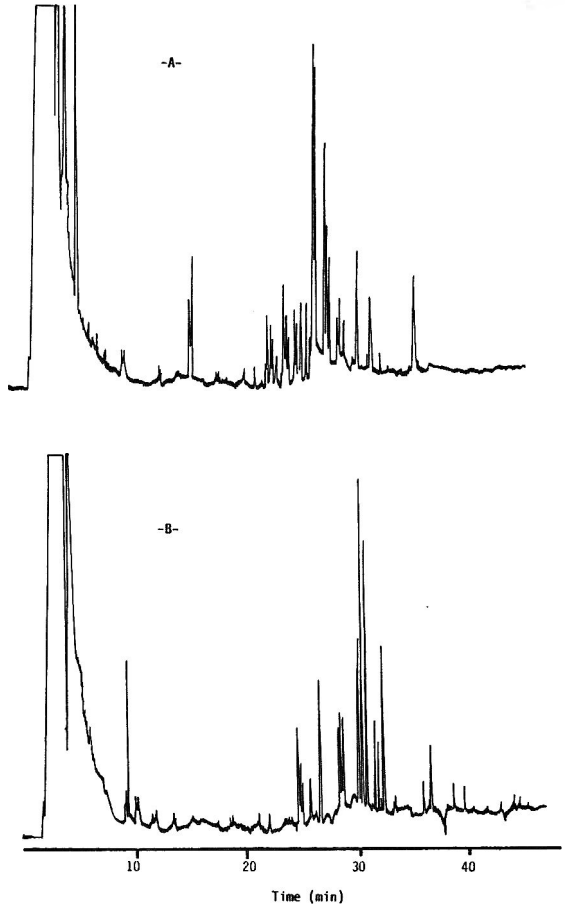

Figure 3. Gas chromatography of a Belcaire River water sample contaminated with Aroclor 1242 using (A) BP-5 column and (B) DB-17 column.

Comparison of our results with those recently obtained by other authors in the same area shows similar Aroclor levels (24).

In conclusion, the C_{18} glass microcolumn extraction method presented here is very fast and allows quantitative recoveries of PCBs. Because a low volume (5 mL) of stripping eluent is used, a high preconcentration ratio is reached (1:200). The glass microcolumn allows a single operator to perform a large number of extractions in a time period.

Acknowledgments

The authors thank CICYT (ALI-88-471) and the Ministry of Science and Education for financial support of this project and of the researchers, respectively.

Table 7. Recovery of PCBs from various 1 L water samples

Compound	Rec., %[a]		
	Distilled	Lake	Sea
2-PCB	92 ± 8	92 ± 8	94 ± 9
2,2′-PCB	94 ± 7	95 ± 6	103 ± 8
2,4-PCB	100 ± 8	96 ± 8	101 ± 10
4,4′-PCB	92 ± 6	90 ± 7	95 ± 5
2,4,5,-PCB	108 ± 5	99 ± 5	101 ± 5
3,3′,4,4′-PCB	91 ± 9	88 ± 6	99 ± 8
2,2′,4,5,5′-PCB	85 ± 7	80 ± 7	81 ± 10
2,2′,4,4′,5,5′-PCB	86 ± 7	95 ± 9	77 ± 7
Decachlorobiphenyl	83 ± 8	77 ± 7	57 ± 8

[a] $\bar{X}$ ± RSD (n = 5).

Table 8. PCBs in natural waters

Sampling place	Aroclor	PCB, ng/L water
Canyoles River (Xàtiva)	1016	129
Meeting Albaida and Clarià Rivers (Montaverner)	1242	84
Serpis River, gate Beniarres drain (Alcocer de Planes)	1242	193
Serpis River, Cotes Baixes polygon (Alcoi)	1254	313
Belcaire River (Moncofar)	1242	212

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Liquid Chromatographic Screening Method for Fluorescent Derivatives of Chlorophenoxy Acid Herbicides in Water

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A liquid chromatographic method for screening 9 chlorophenoxy acids (2,4-D; 2,4-DP; 2,4-DB; MCPA; MCPP; MCPB; 2,4,5-T; 2,4,5-TP; and 2,4,5-TB) and their ethyl esters in ground- and tap water is presented. The water samples are acidified and subjected to either liquid-liquid or solid-phase extraction. The extracts obtained are saponified in 0.03N NaOH–50% methanol (1 + 1) and the acidic compounds are re-extracted with ethyl acetate–*n*-hexane (8 + 2) after acidification and derivatized with 9-anthryldiazomethane. Derivatized compounds are analyzed using reversed-phase column chromatography with fluorescence detection (excitation, 365 nm; emission, 412 nm). Recoveries of analytes from 20 mL water samples were greater than 90%, and the average coefficient of variation was within 5.0% at 0.5 ppb for both extraction methods. These methods are simple and useful for the determination of small amounts of chlorophenoxy herbicides. Solid-phase extraction is suitable for screening a large number of samples simultaneously, and liquid extraction for separate determination of the acids and ethyl esters of the herbicides was improved by introducing a saponification step.

In Japan, ground- and tap water pollution by herbicides, pesticides, and fungicides used at golf courses has become an object of recent public concern. Chlorophenoxy acids are the most common group of herbicides used for controlling broadleaf weeds. Commercially available products of the chlorophenoxy acids are their ethyl esters, sodium and potassium salts, and dimethylamine salts.

Chlorophenoxy acids in water samples are extracted by either liquid-liquid extraction with organic solvents under acidic conditions (1, 2), or solid-phase extraction (SPE) with C_{18} (3), Carbopack B (4), or anion exchange (5) columns.

Determination of the phenoxy acids has been performed by gas chromatography (GC) (1, 2, 5, 6) and liquid chromatography (LC) (3, 4, 7–12). For GC analysis, derivatization by esterification or silylation is necessary to enhance the volatility of the compounds and to improve sensitivity and selectivity of detection.

Chlorophenoxy acids are separated by reversed-phase column on liquid chromatography, and peaks are detected mainly by UV monitoring at 280 or 230 nm (3, 4, 7–10). Di Corcia et al. (4) used SPE cartridges for the pretreatment of the water samples, and Hamann and Kettrup (8) introduced the column switching method. These 2 methods were effective in improving sensitivity and selectivity of the compounds by UV detection.

Although these methods are useful for determination of chlorophenoxy acids, they have not been applied to their ethyl ester forms.

A previous study (13) described the screening method for the acid forms of 2,4-D, MCPA, MCPP, and MCPB in groundwater samples by reversed-phase LC using precolumn derivatization with 9-anthryldiazomethane (ADAM). In the present study, we report an application of the method with modifications to some other chlorophenoxy acids and their ethyl esters in ground- and tap water.

Experimental

Reagents and Materials

(a) *Solvents.*—Acetonitrile (LC grade), dry acetone, ethanol, methanol, benzene, *n*-hexane, dichloromethane, and ethyl acetate (pesticide grade, Wako, Osaka, Japan). Benzene is a carcinogen.

(b) *SPE cartridges and vacuum manifold.*—C_{18}-SPE cartridges containing 100 mg C_{18} bonded silica in 1 mL polypropylene cartridge and Vac Elute system with 25 mL polypropylene reservoir (Analytichem International Inc., Harbor City, CA).

(c) *Reaction vials.*—3 mL reaction vials with Teflon-lined screw caps (GL Science, Tokyo, Japan).

(d) *Derivatization reagent.*—0.025% ADAM (Funakoshi, Tokyo) (14) in dry acetone, kept at −20°C.

(e) *Chlorophenoxy acids and their ethyl esters.*—2,4-D and MCPP (GL Science, Tokyo, Japan); MCPA, 2,4-DP, and 2,4,5-T (Tokyo Kasei Kogyo, Tokyo, Japan); and 2,4,5-TP (Sigma, St. Louis, MO). 2,4-DB and 2,4,5-TB were synthesized by reacting γ-butyrolactone, 2,4-dichlorophenoxide, and 2,4,5-trichlorophenoxide (the latter compounds were converted from

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2,4-dichlorophenol and 2,4,5-trichlorophenol, respectively) with NaOH at 160°C for 60 min (15).

The synthesized 2,4-DB and 2,4,5-TB were purified by liquid-liquid partition and recrystallized from benzene, and their structures were confirmed by ^1H NMR and mass spectra. The purity of each chlorophenoxy acid standard was higher than 95%. Each ethyl ester of the 9 herbicides was synthesized by the method of Fast et al. (16).

(**f**) *Standard solutions.*—(*1*) *Stock standard solution of the phenoxy acids*: 100 mg of each phenoxy acid was dissolved in acetone, diluted to 100 mL (1000 ppm), and stored at 4°C. (*2*) *Working standard solution of the phenoxy acids*: Stock standard solution was diluted with acetone to 0.5, 1.0, 2.5, 5.0, 7.5, and 10 ppm. (*3*) *Stock and working standard solution of ethyl esters of 9 chlorophenoxy acids*: Solutions were prepared in the same way as for phenoxy acids.

Apparatus

(**a**) *LC system.*—Pump, Model 880-PU (Jasco, Tokyo, Japan); fluorescence detector, Model 820-FP at excitation 365 nm, emission 412 nm (Jasco); injector, Model 7125 with 20 µL loop (Rheodyne Inc., Cotati, CA); integrator, Model C-R3A (Shimadzu, Kyoto, Japan); analytical column, Model TSK-gel ODS 120T, 250 × 4.6 mm id (Tosoh, Tokyo, Japan); guard column, Model Tsk-gel ODS 120T, 15 × 3.2 mm id (Tosoh); column temperature, 25°C.

(**b**) *Mobile phase.*—Acetonitrile–water (3 + 1) at 1 mL/min flow rate.

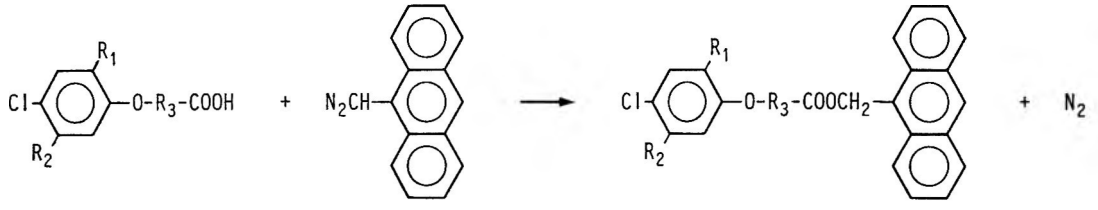
Sample Acidification

Each water sample (20 mL) was dispensed into a 30 mL glass-stoppered test tube and acidified with 150 µL 35% HCl. When free available chlorine was present in the sample, it was removed by the addition of Na_2SO_3 before acidification. Free available chlorine in water samples was determined by chlorine comparator using *o*-tolidine (Shibata Kagaku, Tokyo, Japan) according to the method described previously (17).

Herbicide Extraction

(**a**) *Liquid extraction.*—Phenoxy acids and their ethyl esters were extracted from the aqueous sample first with 3 mL benzene and then with 4 mL ethyl acetate–*n*-hexane (8 + 2) by vigorous shaking for 1 min. The organic phases were combined by transferring with Pasteur pipet into a 10 mL glass-stoppered test tube. Phases were then concentrated to <0.1 mL with rotary evaporator under reduced pressure by heating in a water bath at 40°C.

(**b**) *C_{18}-SPE cartridge.*—The C_{18}-SPE cartridges were prewashed with 3 mL each of dichloromethane, methanol, and 0.1N HCl, sequentially. Water samples were passed through the C_{18}-SPE cartridge at 4 mL/min. Phenoxy acids and ethyl esters were eluted with 0.5 mL methanol, followed by 3 mL dichloromethane–methanol (8 + 2) at 1 mL/min, and they were collected in 10 mL glass-stoppered test tubes. The solution was concentrated by evaporation to <0.1 mL by heating in a water bath at 40°C under a stream of nitrogen.



	R_1	R_2	R_3
MCPA	$-CH_3$	-H	$-CH_2-$
MCPP	$-CH_3$	-H	$-CH(CH_3)-$
MCPB	$-CH_3$	-H	$-CH_2-CH_2-CH_2-$
2,4-D	-Cl	-H	$-CH_2-$
2,4-DP	-Cl	-H	$-CH(CH_3)-$
2,4-DB	-Cl	-H	$-CH_2-CH_2-CH_2-$
2,4,5-T	-Cl	-Cl	$-CH_2-$
2,4,5-TP	-Cl	-Cl	$-CH(CH_3)-$
2,4,5-TB	-Cl	-Cl	$-CH_2-CH_2-CH_2-$

Figure 1. Reaction of chlorophenoxy acid herbicides with 9-anthryldiazomethane (ADAM).

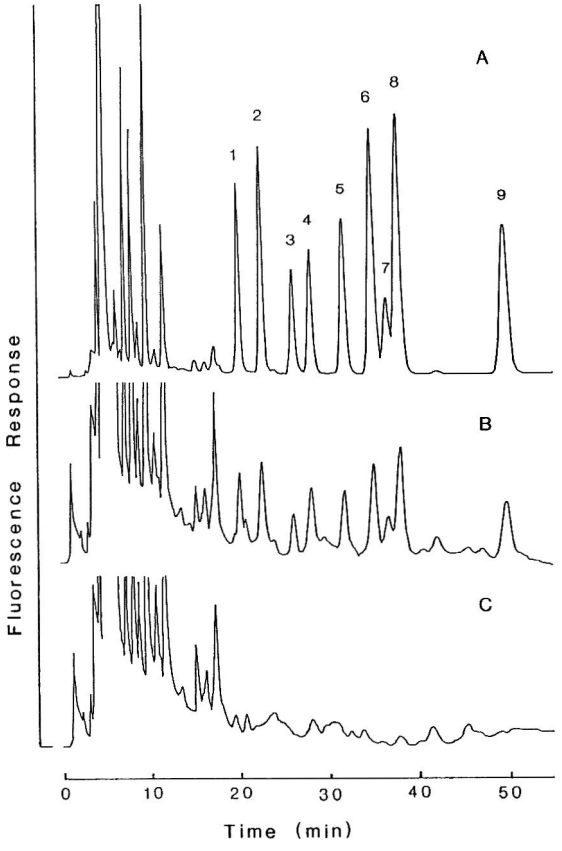


Figure 2. Liquid chromatograms of ADAM-derivatized phenoxy acids. (A) Working standard solution: 1 = 2,4-D; 2 = MCPA; 3 = 2,4,5-T; 4 = 2,4-DP; 5 = MCPP; 6 = 2,4-DB; 7 = 2,4,5-TP; 8 = MCPB; 9 = 2,4,5-TB (20 ng each per injection). (B) Groundwater sample spiked with 0.5 μg/L phenoxy acids. (C) Blank groundwater sample.

Saponification

The concentrated sample was hydrolyzed 15 min with 1 mL 0.03N NaOH–50% methanol (1 + 1) at 70°C.

Re-extraction

After cooling samples, 4 mL 0.1N HCl was added to the test tubes, and the phenoxy acids were extracted first with 2 mL and then with 1 mL ethyl acetate–*n*-hexane (8 + 2) by vigorous shaking for 1 min. Extracts were collected in a 3 mL reaction vial and evaporated to dryness under a stream of nitrogen.

Derivatization of Phenoxy Acids

Phenoxy acids were derivatized by adding 50 μL 0.025% ADAM solution in acetone and allowing mixture to stand 30 min at room temperature in the dark. A 10 μL aliquot of the resulting solution was injected on the LC system. For working standard solution of phenoxy acids, 20 μL of each standard solution was dispensed to 3 mL reaction vials, evaporated to dryness under a stream of nitrogen, and then derivatized as described above.

Results and Discussion

Chromatography

Chlorophenoxy acids were derivatized with ADAM (Figure 1) under mild conditions (13). The yield of derivative forms for each phenoxy acid was reproducible, with an average coefficient of variation (CV) of less than 2%. Figure 2A shows a typical chromatogram of the ADAM-derivatized phenoxy acids from the working standard solution. Under these chromatographic conditions, the resolution factor (Rs) of 2,4-DB and 2,4,5-TP and that of 2,4,5-TP and MCPB were 1.00 and 0.75, respectively. The latter Rs decreased with the rise in column temperature. For instance, these peaks were not separated at 40°C. The column should be kept at 20–25°C to detect 2,4,5-TP and MCPB as distinct peaks. The lowest detection limit of the herbicides was approximately 500 pg/injection (S/N = 3), except for 2,4,5-TP, for which the limit was 1 ng/injection. The calibration curves, plots of the amount of each phenoxy acid versus peak height of the fluorescence response, showed excellent linearity from 1 to 40 ng/injection (r = 0.998–0.999) in the range examined.

Recovery from Groundwater Samples

Groundwater samples were spiked with either 9 chlorophenoxy acids or their ethyl esters at 0.5 or 10.0 ppb levels. Recoveries of these compounds with liquid or SPE extraction at pH 1.1–1.2 are presented in Table 1.

With liquid extraction, recoveries were more than 90% at 2 different levels examined. Average CVs at 0.5 ppb were 5.1% for the acids and 5.3% for the ethyl esters. In the previous paper (13), the acid forms of 2,4-D, MCPA, MCPP, and MCPB were extracted twice with ethyl acetate–*n*-hexane (8 + 2). Extraction conditions were suitable for the 9 chlorophenoxy acids, but the recoveries of the ethyl esters of 2,4-DB, MCPB, and 2,4,5-TB were lower than 50% (data not shown). The low recoveries were improved by using benzene as the first extraction solvent before ethyl acetate–*n*-hexane (8 + 2) in the present study. For liquid extraction, the 9 chlorophenoxy acids were readily determined without saponification. By introducing a saponification step, ethyl esters, in addition to acid forms, are estimated together as phenoxy acids. Therefore, the amount of ethyl esters of the 9 herbicides can be determined by subtracting acid concentration from total phenoxy acids. Compounds spiked at 0.5 ppb were detected as distinct peaks on the chromatogram (Figure 2B) relative to the background groundwater (Figure 2C).

The SPE technique with 1 g C_{18} bonded silica and methanol as an elution solvent gave good extraction efficiency for chlorophenoxy acids in acidified water samples (3, 4). We examined the previously untried technique using C_{18}-SPE cartridges for the ethyl esters of the 9 herbicides. The ethyl esters of the herbicides in water were retained on the cartridge at both acidic and neutral pH (data not shown). To extract both acids and ethyl esters of the herbicides simultaneously, water samples were acidified before applying to the cartridge, and the elution was performed with dichloromethane–methanol (8 + 2). Recoveries of herbicides from groundwater were more than

Table 1. Recovery (%) of phenoxy acids and their ethyl esters from groundwater[a]

Form	Phenoxy acid	Rec. after liquid extraction at spike level		Rec. after C_{18}-SPE extraction at spike level	
		0.5, ppb	10.0, ppb	0.5, ppb	10.0, ppb
Acid	2,4-D	93 ± 5	98 ± 5	96 ± 5	96 ± 2
	MCPA	99 ± 6	95 ± 4	98 ± 6	99 ± 3
	2,4,5-T	100 ± 6	100 ± 4	94 ± 5	98 ± 2
	2,4-DP	98 ± 5	96 ± 6	103 ± 5	98 ± 3
	MCPP	104 ± 5	92 ± 3	94 ± 5	94 ± 4
	2,4-DB	100 ± 5	99 ± 4	95 ± 4	98 ± 2
	2,4,5-TP	93 ± 5	100 ± 3	94 ± 4	96 ± 4
	MCPB	96 ± 2	94 ± 3	99 ± 2	95 ± 1
	2,4,5-TB	93 ± 6	99 ± 3	99 ± 5	98 ± 1
Ester	2,4-D	93 ± 6	91 ± 3	95 ± 2	97 ± 4
	MCPA	96 ± 4	98 ± 2	97 ± 5	98 ± 4
	2,4,5-T	92 ± 3	92 ± 4	92 ± 3	99 ± 4
	2,4-DP	102 ± 4	94 ± 3	105 ± 4	96 ± 2
	MCPP	94 ± 6	95 ± 5	91 ± 4	92 ± 4
	2,4-DB	100 ± 5	92 ± 3	98 ± 5	95 ± 1
	2,4,5-TP	97 ± 6	93 ± 2	96 ± 5	99 ± 2
	MCPB	100 ± 6	94 ± 4	96 ± 4	96 ± 3
	2,4,5-TB	94 ± 6	94 ± 2	97 ± 3	94 ± 3

[a] Each value is the average of 5 samples ± SD.

90%. When spiked at 5 ppb, average CVs were 4.6% for acids and 4.0% for ethyl esters (Table 1).

Unfortunately, the phenoxy acids that eluted from the SPE cartridges could not be directly derivatized with ADAM. HCl contained in the water sample was trapped in the column bed and eluted with the elution solvent from the cartridge. The residual HCl, which was not completely removed by the evaporation step, is likely to decompose ADAM; therefore, the reaction of the chlorophenoxy acids with ADAM does not occur. The saponification of the eluate from the cartridge leads to the determination of the sum of acids and esters. When only the acid forms must be determined, a step to remove HCl is required. However, the SPE method is simple and effective for the simultaneous determination of total acids and ester forms in a large number of samples.

Recovery from Tap Water Samples

Tap water samples were shown to contain 0.3–0.4 ppm free available chlorine, as measured by the *o*-tolidine method. The forms of free available chlorine, HOCl and OCl^-, which are present in the ratio of about 60:40 (18) at pH 7.2–7.4, are derived from Cl_2 in chlorinated water. We examined the effect of free available chlorine in the samples on the recovery of the 9 herbicides (Table 2). Recoveries of the acids and the ethyl esters of MCPA, MCPP, and MCPB were poor, either by liquid or C_{18}-SPE extraction, when they were not treated with Na_2SO_3 for dechlorination; however, recoveries of the other compounds were excellent without dechlorination. If the free available chlorine was not removed, peaks were observed with retention times at 31.3, 45.0, and 55.4 min on the chromatograms of MCPA, MCPP, and MCPB, respectively, in addition to small peaks at the usual retention times. This would imply that the 3 compounds were structurally modified. When the tap water samples were spiked with the 9 phenoxy acids or their ethyl esters at levels of 5 ppb and were kept for 15 h at room temperature before dechlorination with Na_2SO_3, more than 90% of each compound was recovered (data not shown). Recoveries of MCPA, MCPP, and MCPB spiked at concentrations as low as 0.5 ppb were improved by dechlorination before sample acidification with HCl (Table 2).

These results suggested that the poor recoveries were not caused by free available chlorine, although free available chlorine is known to react with organic compounds and modify their structure at neutral pH (19). Chlorine (Cl_2) may have been formed when free available chlorine was mixed with HCl, which was used for sample acidification. Chlorine, but not free available chlorine, might be responsible for the structural changes of phenoxy acids such as MCPA, MCPP, and MCPB by the reaction with the methyl group at the R_2 position of the benzene ring (Figure 1) during extraction and/or concentration steps.

Interferences and Detection Limits

Most acidic compounds in the water samples were extracted by the procedure described in the method. Compounds containing carboxyl groups were confirmed to take part in the reaction with ADAM. Short fatty acids, including *n*-butyric, *n*-capric, and *n*-caprylic acids, were separated as ADAM derivatives from the 9 phenoxy acids under the same chromatographic conditions; retention times were 14.2, 24.4, and 46.3 min, respectively. When the organic acids such as glutaric, malonic, fumaric, tartaric, and succinic acid were spiked at the concentrations of 5 ppb and subjected to either liquid or C_{18}-SPE extraction, no peaks were observed on the chromatogram.

Table 2. Recovery (%) of phenoxy acids and their ethyl esters from tap water processed with chlorine

Form	Phenoxy acid	Without dechlorination[a]		With dechlorination[b]	
		Liquid extraction	C_{18}-SPE	Liquid extraction	C_{18}-SPE
Acid	2,4-D	92–94	98–100	97 ± 4	100 ± 6
	MCPA	83–91	48–71	102 ± 3	96 ± 4
	2,4,5-T	93–98	96–100	98 ± 6	98 ± 6
	2,4-DP	95–98	92–95	99 ± 4	101 ± 4
	MCPP	70–83	29–56	95 ± 4	98 ± 4
	2,4-DB	94–97	92–94	98 ± 4	96 ± 3
	2,4,5-TP	95–98	98–100	93 ± 3	93 ± 3
	MCPB	<5	<5	97 ± 4	95 ± 4
	2,4,5-TB	94–96	94–96	92 ± 4	97 ± 5
Ester	2,4-D	92–96	93–95	99 ± 4	98 ± 3
	MCPA	82–91	64–91	95 ± 4	97 ± 3
	2,4,5-T	96–100	96–100	93 ± 4	97 ± 3
	2,4-DP	91–96	93–97	94 ± 4	95 ± 2
	MCPP	56–84	55–87	97 ± 4	95 ± 2
	2,4-DB	95–100	94–100	99 ± 3	95 ± 3
	2,4,5-TP	96–98	97–98	95 ± 5	97 ± 8
	MCPB	<5	<5	97 ± 5	94 ± 3
	2,4,5-TB	98–99	93–95	97 ± 7	95 ± 8

[a] Water samples contained 0.3–0.4 ppm free available chlorine. Concentrations of acids and esters were both 5 ppb. Each datum is recovery range of 3 samples.

[b] Before addition of HCl, dechlorination was performed by the addition of 50 μL 1% Na_2SO_3. Concentrations of acids and ethyl esters were 0.5 ppb. Each value is the average of 5 samples ± SD.

The compounds examined may be present in the water that was contaminated by bacteria and algae. In this method, the effects of these compounds on the determination of the 9 chlorophenoxy acids are negligible.

The detection limits of the herbicides from water samples were about 1 ng/injection (S/N = 5) under the conditions described above, except for 2,4,5-TP, for which the limit was 2 ng/injection. The limits of detection for the phenoxy acids were 0.2–0.4 ppb in a 20 mL water sample. The U.S. Environmental Protection Agency sets the maximum contaminant levels for 2,4-D and 2,4,5-TP in drinking water at 70 and 50 ppb, respectively (20); the World Health Organization sets the level for 2,4-D at 100 ppb (21). The method described in this paper can be applied to estimate a very low level of chlorophenoxy herbicides in drinking water samples.

Acknowledgment

Tropotox, a herbicide of MCPB used in this study, was a generous gift from Nissan Kagaku Kogyo Co., Ltd, Tokyo, Japan.

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SPICES AND OTHER CONDIMENTS

Stable Carbon Isotope Ratio Method for Detection of Corn-Derived Acetic Acid in Apple Cider Vinegar: Collaborative Study

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The stable carbon isotope ratio analysis method for determining the presence of corn-derived acetic acid in cider vinegar has been collaboratively studied. The method is based upon natural differences in the $^{13}C/^{12}C$ ratio; pure cider vinegars yield $\delta^{13}C$ results near –25 ‰, while corn vinegar yields results near –10 ‰. Samples are combusted at 500°C in sealed glass tubes over CuO. The purified CO_2 is analyzed by isotope ratio mass spectrometry relative to the Pee Dee belemnite limestone (PDB) standard. The precision of this method was observed to be similar to that of other isotope ratio methods that have been collaboratively studied: s_R ranged from 0.6 to 1.2, while s_r ranged from 0.1 to 1.1. The results indicate that as little as 10% added corn vinegar may be detected in cider vinegar. On the basis of published data of the natural variability of cider vinegar $^{13}C/^{12}C$ ratios, it is recommended that samples yielding results more positive than –22.0 ‰ be classified as not pure cider vinegar using this method. This method has been adopted first action by AOAC International.

The price difference between vinegar from apple cider stock and vinegar from grain alcohol stock makes it financially advantageous to extend cider vinegar with grain-based vinegar. A method that will detect the addition of corn-based vinegar to cider vinegar (1) was recently presented. It was shown that this type of adulteration has been a substantial market problem for cider vinegar. The method is based on the natural differences in the $^{13}C/^{12}C$ ratio of carbon in the products. Pure cider vinegars were shown to have a $\delta^{13}C$ range of –24.1 to –27.2 ‰ (δ mean = –26.0 ‰), while corn vinegar gave values near or more positive than –10.0 ‰.

Stable carbon isotope ratio analysis (SIRA) methods for detection of food adulteration have been adopted by AOAC International for apple juice, **981.09**; orange juice, **982.21**; honey, **978.17**; and maple syrup, **984.23** (2). This report presents a collaborative study of SIRA for the detection of corn-derived acetic acid in apple cider vinegar.

Submitted for publication August 29, 1991.
This report was presented at the 100th AOAC International Meeting, Scottsdale, AZ, September 15–18, 1986.
The recommendation was approved by the General Referee, Committee Statistician, Committee Safety Advisor, and the Committee on Foods II and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1993) *J. AOAC Int.* **76**, January/February issue.

Collaborative Study

Six samples were prepared for distribution to the collaborators: 2 samples (A, B) of cider vinegar, 1 sample (C) of distilled white corn vinegar, and 3 mixtures of these samples in the proportions 10%C–90%B, 25%C–75%A, and 50%C–50%A. All samples were of 5% acidity calculated as acetic acid.

The collaborators were instructed to practice the method on commercial vinegar samples until comfortable, and then analyze each of the 6 samples once only, reporting all results.

992.08 Corn-Derived Acetic Acid in Apple Cider Vinegar—Carbon Ratio Mass Spectrometric Method

First Action 1992

(Applicable to detection of 25–90% corn-derived vinegar in apple cider vinegar)

Method Performance

s_r = 0.11–1.12; s_R = 0.64–1.18;

RSD_r = –0.49 to –4.54%; RSD_R = –2.78 to –4.98%

A. Principle

Sample is burned completely to CO_2 and H_2O; CO_2 is purified and $^{13}C/^{12}C$ ratio is measured in isotope ratio mass spectrometer. Differences in $^{13}C/^{12}C$ values for cider vinegar [$\delta^{13}C$ range: –24.1 to –27.2 ‰; δ mean: –26.0 ‰ (per mil)] and corn-derived vinegar ($\delta^{13}C$ mean: ca –10 ‰) provide measure of corn-derived vinegar in cider vinegar.

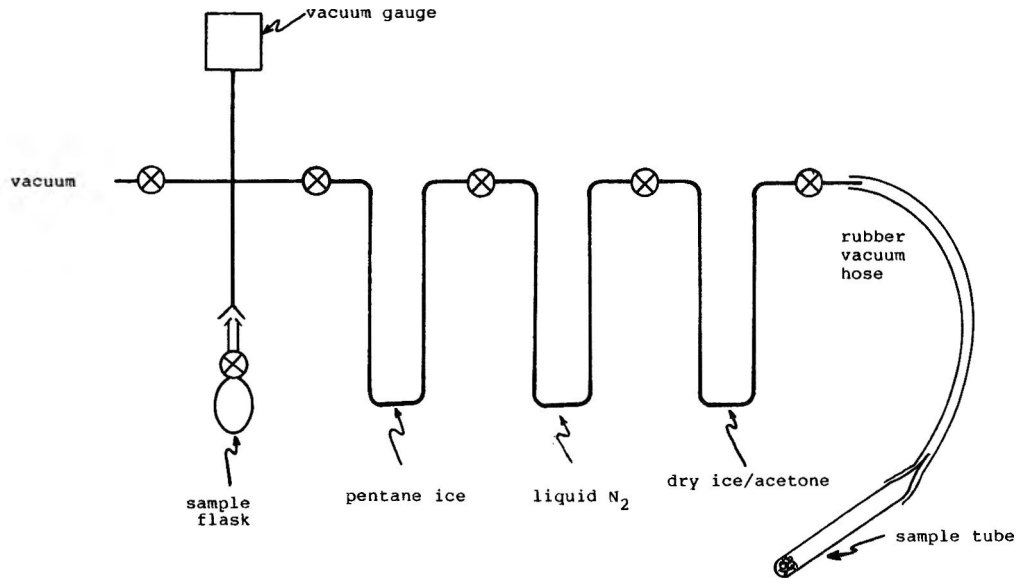

Figure 992.08A. CO_2 purification system.

B. Apparatus

(a) *Capillary pipets.*—25 μL, disposable. Heat pipets in oven at 500°C overnight before use.

(b) *Combustion tubes.*—Borosilicate glass, 10 mm od × 22 cm, sealed at one end. Load tubes with 2 g CuO wire and heat in oven at 500°C overnight before use.

(c) *Oven.*—Muffle or ashing oven capable of maintaining 500–550°C, large enough to hold sealed combustion tubes.

(d) *Purification system.*—Glass manifold with vacuum pump and gauge, sample flask and rubber hose for connection of combustion tubes (see Figure **992.08A**).

(e) *Mass spectrometer.*—Designed or modified for isotope ratio measurement and capable of accuracy of 0.01% of abundance at mass 45 (Micromass 602, 602D, 903, or 602C, Kearns Group, 58 Buckingham Dr, Stamford, CT 06902, or equivalent).

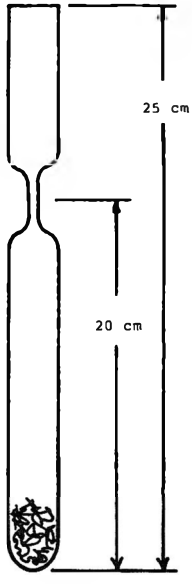

Figure 992.08B. Detail of constriction of combustion tube.

C. Preparation of Sample

Using laboratory torch, make constriction 3 cm from end of combustion tube by heating and drawing out glass tubing (see Figure **992.08B**). Fill 25 μL pipet with vinegar. Freeze vinegar by immersing pipet in liquid N_2 for 10 s. Insert frozen pipet into bottom of combustion tube, and attach tube to rubber hose of purification line. Immerse lower half of combustion tube into flask containing dry ice–acetone mixture. After 1 min, evacuate combustion tube. When pressure falls below 200 μm, quickly replace dry ice–acetone bath with liquid N_2. Seal combustion tube at constriction with torch, leaving short, thin nub at end of tube.

Heat sealed combustion tube in oven at 500°C for ≥1 h. (*Caution*: Considerable pressure is generated in combustion tubes at 500°C. Provide adequate protective shielding.) Let oven cool to room temperature and remove combustion tube.

Score flame sealed nub with steel file, and insert nub end of tube into connecting hose of purification system. Evacuate connecting hose, and open combustion tube by bending hose until scored nub breaks off. Pass combustion products through traps of dry ice–acetone and liquid N_2, evacuating any noncondensible gases. Warm liquid N_2 traps, and pass liberated CO_2 through pentane ice trap to recondense in sample flask cooled with liquid N_2.

D. Determination

See **984.23E**.

E. Calculations

See **984.23F**. Report results as $\delta^{13}C$ ‰. Sample with $\delta^{13}C$ value more positive than −22.0 ‰ relative to Pee Dee belemnite limestone (PDB) is considered not pure cider vinegar.

Ref.: *JAOAC* **75**, July/August issue (1992)

Table 1. Collaborative results for determination of $\delta^{13}C$ values for cider vinegar, corn vinegar, and mixed cider and corn vinegar samples (‰)[a]

	Collaborators					
Sample	1	2	3	4	5	6
1	−10.9	−25.0	−23.8	−22.2	−25.3	−19.0
2	−15.8[b]	−25.6	−24.6	−23.4	−25.4	−20.9
3	−10.5	−24.6	−23.6	−22.1	−26.5	−23.5[b,c]
4	−12.0	−24.8	−23.3	−22.1	−22.6	−19.4
5	−11.1	−23.5	−24.0	−22.6	−25.5	−19.7
6	−10.9	−23.0	−22.8	−21.4	−24.2	−18.0
x	−11.1	−24.4	−23.7	−22.3	−24.9	−19.4
s	0.6	1.1	0.6	0.7	1.3	1.2

[a] Samples: 1, white distilled vinegar, C; 2, pure cider vinegar, A; 3, 90% B–10% C mix; 4, 75% A–25% C mix; 5, pure cider vinegar, B; 6, 50% A–50% C mix.
[b] Outlier by Dixon's test, not used in statistical calculations.
[c] Collaborator reported sample gave anomalously high gas yield; sample probably contaminated.

Table 2. Performance parameters for collaborative study of detection of corn-derived acetic acid in apple cider vinegar

Sample pair	Mean, ‰	s_r	s_R	RSD_r, ‰	RSD_R, ‰
1,6	−15.2	0.46	0.76	−3.02	−4.98
3,4	−23.0	0.11	0.64	−0.49	−2.78
2,5	−24.7	1.12	1.18	−4.54	−4.78

Results and Discussion

Results obtained from the 6 collaborators are tabulated in Table 1, and the statistical summary of the results is presented in Table 2. The reference standards used by the collaborators are listed in Table 3. The means of the results for the 2 pure cider vinegars fell within the reported natural $\delta^{13}C$ range (−24.1 to −27.2, δ mean = −26.0); the means of the results on samples consisting of 100, 50, 25, and 10% corn vinegar fell outside of the reported natural range. It, thus, appears that SIRA is a sensitive test for determining the presence of corn vinegar in cider vinegar.

After exclusion of 2 outliers (Dixon's test), the standard deviation(s) for each sample was calculated. The magnitude of these standard deviations (0.6–1.2) is similar to that obtained in previous collaborative studies of SIRA of other materials. The statistical parameters s_R and s_r for the method were estimated by analysis of results of paired Samples 1 and 6, Samples 2 and 5, and Samples 3 and 4 (3). The s_R values were 0.76 (Samples 1 and 6), 1.18 (Samples 2 and 5), and 0.64 (Samples 3 and 4). The s_r values were 0.46 (Samples 1 and 6), 1.12 (Samples 2 and 5), and 0.11 (Samples 3 and 4). In the author's laboratory, the repeatability (s_r) was in the range of 0.1–0.2 for nonblind duplicates run on different days.

A ranking test (Table 3) and 2-sample chart (Figure 1) indicate that interlaboratory bias makes a significant contribution to s_R (3). In particular, results from Collaborators 2 and 6 were significantly different from the other collaborators.

Collaborator 6 used a combustion method slightly different from the specified protocol. This difference in sample preparation may explain the relatively positive bias of those results. Results from Collaborator 2 showed a slight negative bias, particularly for the more positive valued samples. This might be caused by minor contamination in sample preparation. Because the level of carbon in the 25 µL sample is quite small, it would not require a large contamination to affect the results substantially. The most likely contaminants (solvents, dust, finger oils, dry ice CO_2) generally have relatively negative $\delta^{13}C$ values; thus, such contamination will affect more positive valued samples disproportionately, as observed with Collaborator 2. The instruction to heat the sample tubes and pipets overnight at 500°C is designed to reduce the likelihood of this type of contamination.

The author proposes a $\delta^{13}C$ value of −22.0 ‰ as a criterion for classifying a sample of cider vinegar. Samples with a value more positive than −22.0 ‰ will be classified as adulterated with a very high degree of confidence. The value selected is 4 standard deviations from the mean of the distribution of pure cider vinegars; this value correlates to approximately 25% corn vinegar as the limit for reliable detection of grain-based vinegar, absent other confirming evidence. In conjunction with other analytical data, or when evaluating mean values of mul-

Table 3. Ranking test, working standards, and remarks of collaborators

Coll.	Ranking test	Working standard (assumed value relative to PDB)		Remarks
1	21.5	UQ-2 Marble	(+1.91)	—
2	9	Tank CO_2	(not stated)	—
3	20.5	Tank CO_2	(−10.299)	No pentane trap on purification line
4	24.5	Marble	(0.0)	—
5	17	Tank CO_2	(−48.31)	37 µL sample[a]
6	33.5	Paris $CaCO_3$	(+2.35)	Recirculation of sample over CoO

[a] Collaborator 5 reported that 25 µL sample yielded too little CO_2 for analysis and that 37 µL yielded sufficient sample without failure of the combustion tubes due to excess pressure.

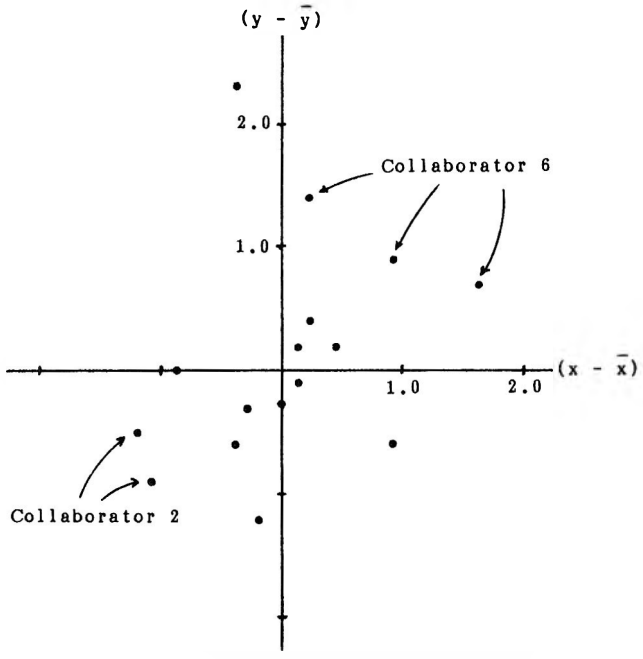

Figure 1. Two sample chart for pairing of Samples 1 and 6, Samples 2 and 5, and Samples 3 and 4.

tiple analyses, classifying criteria closer to the mean value of pure cider vinegar may be appropriate. Criteria closer to the natural mean may also be appropriate for some quality control purposes.

Recommendation

The Associate Referee recommends that the SIRA method for vinegar be adopted first action and, in addition, that the $\delta^{13}C$ value of –22.0 ‰ be established as a criterion of proof of addition of grain-based vinegar to cider vinegar.

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J. Hayes, K. Baker, and K. Freeman, Indiana University, Bloomington, IN

C. Sullivan and R. Olsen, Krueger Enterprises, Inc., Cambridge, MA

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S. Fabris, University of Waterloo, Waterloo, ON, Canada

W. Stichler, GSF-Institute fur Radiohydometrie, Neuherberg, Germany

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TOBACCO

Equivalency of Gas Chromatographic Conditions in Determination of Nicotine in Environmental Tobacco Smoke: Minicollaborative Study

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Alternative gas chromatographic (GC) conditions were collaboratively studied for equivalency with conditions specified in the official AOAC method 990.01 for nicotine in environmental tobacco smoke (ETS). Test conditions under evaluation were the use of a 30 m × 0.32 mm id fused silica capillary column with split injection as compared to the method-specified 30 m × 0.53 mm id fused silica capillary column with direct or on-column injection. The 6 samples analyzed in duplicate by 7 laboratories included 3 spiked samples (concentration ranging from 0.18 to 1.8 μg nicotine per sample) and 3 ETS samples (concentration in air ranging from 4 to 36 μg nicotine/cu m). Four laboratories used the AOAC approved GC conditions and 3 used the test conditions. Data analysis detected no difference in results ($P = 0.72$) obtained using either the 0.32 mm or 0.53 mm id column (and associated conditions). Average repeatability (RSD_r) and reproducibility (RSD_R) relative standard deviations for the 0.53 mm id column were 5.2 and 8.3%, respectively, for spiked nicotine samples, and 5.3 and 8.5%, respectively, for ETS samples. Average repeatability (RSD_r) and reproducibility (RSD_R) relative standard deviations for the 0.32 mm id column were 4.1 and 4.7%, respectively, for spiked nicotine samples, and 3.5 and 6.3%, respectively, for ETS samples. The method incorporating the use of the 0.32 mm id column and associated conditions is recommended for adoption as official first action.

Submitted for publication May 23, 1991.
This report was presented at the 104th AOAC International Annual Meeting, September 10–13, 1990, New Orleans, LA.
The recommendation was approved by the General Referee, Committee Statistician, Committee Safety Advisor, and the Committee on Feeds, Fertilizers and Related Materials and was adopted by the Official Methods Board of the Association. See "Changes in Official Methods of Analysis" (1992) *J. AOAC Int.* **75**, 223.

The assessment of public exposure to environmental tobacco smoke (ETS) is an important area of research as a result of reports by the U.S. Department of Health and Human Services (1) and the U.S. National Research Council (2) alleging that ETS exposure represents a health risk. Nicotine is the most widely used chemical marker of ETS exposure in indoor air quality surveys, although a firm quantitative relationship between concentrations of nicotine and other analytes present in ETS is tenuous at best (3, 4). Although not unique to tobacco (5, 6), nicotine should be present in indoor air specifically as a result of tobacco smoking.

Over the past decade or so, there has been an increasing trend in routine gas chromatographic analyses away from packed columns toward open-tubular (or capillary) columns. The reasons for this change are numerous and any detailed discussion is beyond the present scope, but briefly, they center around significant improvements in speed, efficiency, inertness, and (in most cases) sensitivity imparted to the analytical determination. For many laboratories, this evolution toward use of small-diameter capillary columns (0.20–0.32 mm id) to realize the greatest improvements has taken an intermediary path through large-diameter capillary columns (0.53–0.75 mm id) due mostly to limitations in existing gas chromatographs designed for packed-column use but that can be altered for use with the large diameter columns by minimal modification. It was primarily for this reason that the official AOAC method **990.01** (7) was originally implemented using a 0.53 mm id column. As GC instrumentation improves and is replaced, so too should the methods to take full advantage of benefits available from existing capillary column technology. Although a change in capillary column diameter from 0.53 to 0.32 mm accompanied by a change in pertinent instrument parameters would normally be expected only to improve results, it is considered a change in methodology and, therefore, requires further evaluation before official action can be sanctioned.

Collaborative Study

A minicollaborative study was designed to test the equivalency of a 30 m × 0.32 mm id capillary column with split injec-

tion to the method-specified (**990.01**) 30 m × 0.53 mm id column with direct or on-column injection. Seven collaborating laboratories analyzed 6 samples in duplicate, including 3 spiked samples and 3 ETS samples. Spiked samples (concentration ranging from 0.18 to 1.8 μg nicotine per sample) were generated by adding 3–4 μL quantities (Hamilton 10 μL syringe; accuracy and precision, ±1%) of methanolic solutions of known nicotine concentration to the primary section of XAD-4 resin in the sample tubes. True ETS was generated and sampled in a controlled-environment test chamber by delivering sidestream plus exhaled mainstream smoke (human smoking) from 1R4F Kentucky reference cigarettes. Nominal flow rate for each sample tube was 1 L/min and sampling duration was 2 h. Operating characteristics of the chamber were identical to those used previously (8).

All samples were prepared in sufficient quantity to provide each laboratory with blind duplicates at each concentration. None of the personnel assisting in the operation of the environmental chamber was involved in sample analysis so the requirement that all samples be analyzed blind was preserved. Analysts were asked to report the quantities of nicotine determined in micrograms per tube. The conversion of micrograms per tube to micrograms per cubic meter for ETS samples was made by the Associate Referee, using the volumetric flow rate and sample duration data recorded during sample collection. With the exception of the alternative GC column and conditions under evaluation, the remainder of the procedure used was as specified in AOAC method **990.01**.

991.50 Nicotine in Environmental Tobacco Smoke Gas Chromatographic Method—Alternative Conditions

First Action 1991

(Applicable to determination of airborne vapor-phase nicotine at concentrations dependent on air flow rate and sampling duration, e.g., 0.1–2500 μg/cu m for 2 h sampling at 1 L/min.)

Method Performance:

(pooled data for 0.53 and 0.32 mm id columns):

$s_r = 0.024$; $s_R = 0.035$; $RSD_r = 4.9\%$; $RSD_R = 6.8\%$

A. Principle

See **990.01A**.

B. Apparatus

(**a**) *Pump.—See* **990.01B(a)**.

(**b**) *Sorbent sampling tubes.—See* **990.01B(b)**. Note: Tubes with glass wool spacers replace tubes with urethane foam spacers as standard item (Cat. No. 226-30-11-04, SKC Inc., Eighty Four, PA 15330, or equivalent).

(**c**) *Gas chromatograph.—See* **990.01B(c)**.

(**d**) *Column.*—(*1*) 30 m × 0.53 mm id fused silica capillary, coated with 1.5 μm film of 5% phenyl methylpolysiloxane (e.g., DB-5, Cat. No. 125-5032, J & W Scientific, Inc., Folsom, CA 95630, or equivalent); or (*2*) 30 m × 0.32 mm id fused silica capillary, coated with 1.0 μm film of 5% phenyl methylpolysiloxane (e.g., DB-5, Cat. No. 125-5033, J & W Scientific, Inc., or equivalent).

(**e**) *GC conditions.*—(*1*) Helium carrier gas flow ca 15 mL/min (12 psig); direct or on-column injection 1–2 μL, or (*2*) helium carrier gas flow ca 2 mL/min (15 psig); split injection (split ratio ca 5–10:1) 2 μL. Oven temperature 150° programmed at 5°/min to 180° (run time ca 6 min); injector and detector temperatures 225 and 300°, respectively. Suitable detector conditions: helium makeup gas 15 mL/min; air 75 mL/min; hydrogen 3 mL/min; "bead current" sufficient to give signal-to-noise ratio >50 for nicotine in 0.1 μg/mL calibration standard. Approximate retention times are 1.9 min for quinoline and 2.6 min for nicotine under conditions (*1*), and 3.3 min for quinoline and 4.2 min for nicotine under conditions (*2*).

(**f**) *Sample containers.—See* **990.01B(f)**.

C. Reagents

See **990.01C**.

D. Preparation of Standard Solutions

See **990.01D**.

E. Collection and Preparation of Samples

See **990.01E**.

F. Determination

See **990.01F**.

G. Determination of Desorption Efficiency

See **990.01G**.

H. Calculations

See **990.01H**.

I. Potential Interference

Typical field-sampling results in 0–2 μg nicotine collected on XAD-4 resin, with mass of quinoline collected on resin below detection limits (ETS contains quinoline at ca 1% of nicotine concentration). This level of quinoline poses no interference with 5 μg quinoline internal standard. If nicotine collected is >10 μg, modify method by (*1*) decreasing sampling flow rate and/or duration, (*2*) extracting XAD-4 resin in larger solvent volume and adding specified aliquot of internal standard solution to only 1 mL of extract, (*3*) using increased amount of quinoline internal standard, or (*4*) using an alternate internal standard such as *N*-ethylnornicotine (9).

Ref.: JAOAC **72**, 1002 (1989). JAOAC **75**, July/August issue (1992).

CAS-54-11-5 (nicotine)

CAS-5979-92-0 (*N*-ethylnornicotine)

Results and Discussion

Table 1 presents the collaborative study results for nicotine determination in blind duplicate samples. Levels 1, 2, and 3 (values converted to μg nicotine per cu m air sampled) corre-

Table 1. Collaborative results on GC determination of nicotine in blind duplicate samples

		Nicotine, μg/cu m air sampled[a]			Nicotine, μg/tube[b]		
		Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Coll.	GC analysis[c]	(1 puff)	(4 puff)	(9 puff)	(0.179)	(0.538)	(1.793)
1	1	3.04[d]	13.25	—[e]	0.178	0.517[d]	1.769
		5.37[d]	14.22	—	0.233	0.604[d]	1.727
2	1	3.57	14.03	35.95	0.160	0.539	1.764
		4.11	13.06	36.01	0.171	0.534	1.693
3	2	4.41	11.63	—	0.177	0.539	1.745
		4.37	11.29	—	0.181	0.540	1.754
4	2	4.10	13.56	35.92	0.172	0.536	1.815
		4.29	13.66	33.39	0.168	0.534	1.792
5	2	4.33	14.19	37.67	0.154	0.569[f]	1.876
		4.43	13.42	34.53	0.188	0.556[f]	1.731
6	1	3.14	14.38	—	0.182	0.541	1.820
		3.44	11.74	—	0.157	0.529	1.829
7	1	3.64	13.46	31.89	0.155	0.479[f]	1.715
		3.65	12.74	32.46	0.154	0.486[f]	1.670
		Summary statistics: outliers included					
Mean		3.99	13.19	34.73	0.174	0.536	1.764
s_r		0.65	0.85	1.44	0.019	0.024	0.047
RSD_r, %		16.3	6.4	4.1	10.9	4.5	2.7
Repeatability value		1.82	2.38	4.03	0.053	0.067	0.132
s_R		0.65	1.01	2.09	0.021	0.031	0.058
RSD_R, %		16.3	7.7	6.0	12.1	5.8	3.3
Reproducibility value		1.82	2.83	5.85	0.059	0.087	0.162
		Summary statistics: outliers excluded					
Mean		3.96	13.19	34.73	0.174	0.532	1.764
s_r		0.19	0.85	1.44	0.019	0.006	0.047
RSD_r, %		4.8	6.4	4.1	10.9	1.1	2.7
Repeatability value		0.53	2.38	4.03	0.053	0.017	0.132
s_R		0.46	1.01	2.09	0.021	0.027	0.058
RSD_R, %		11.6	7.7	6.0	12.1	5.1	3.3
Reproducibility value		1.29	2.83	5.85	0.059	0.076	0.162

[a] ETS nicotine collected in environmental chamber; sampling time 2 h. Number of puffs of 1R4F cigarette in parentheses.
[b] Solutions of known nicotine concentration spiked on XAD-4 tubes; concn, μg/tube, in parentheses.
[c] Refers to GC column and conditions as outlined in method section B (Apparatus), subsections (d) and (e).
[d] Inhomogeneity of variance indicated by Cochran test; value excluded.
[e] — Indicates invalid datum.
[f] Extreme laboratory average indicated by Grubbs paired-value test; value not excluded (see text).

spond to the 3 concentrations of ETS nicotine generated and sampled in the environmental chamber for 1, 4, and 9 puffs, respectively, of the 1R4F cigarette. Levels 4, 5, and 6 (values reported as μg nicotine per tube) correspond to the 3 concentrations of nicotine spiked on XAD-4 tubes; the "true" values were 0.179, 0.538, and 1.793 μg/tube, respectively. Mean values, repeatability and reproducibilty standard deviations, and the corresponding relative standard deviations (RSD) are also shown both before and after outlier removal.

Initial screening of the data resulted in excluding certain values as invalid. These are indicated by the blank entries in Table 1 for Level 3. In all cases, these data were excluded for failure to follow the method; i.e., the range of instrument calibration standards was not extended to encompass the range of nicotine found in samples. Statistical analysis of all valid data was conducted as recommended by AOAC (10) for detection of outliers according to the Cochran test for inhomogeneity of variances and the Grubbs test (single and paired-value) for extreme laboratory averages.

Values reported by Collaborator 1 for Levels 1 and 5 were identified as outliers based on the Cochran test and were removed. The Grubbs paired-value test indicated that Collabora-

Table 2. Comparison of method performance parameters by GC analysis type (outliers excluded as indicated in Table 1)

	Nicotine, µg/cu m air sampled						Nicotine, µg/tube					
	Level 1		Level 2		Level 3		Level 4		Level 5		Level 6	
GC analysis[a]	1	2	1	2	1	2	1	2	1	2	1	2
Mean	3.59	4.32	13.36	12.96	34.08	35.38	0.174	0.173	0.518	0.546	1.748	1.786
s_r	0.25	0.09	1.08	0.35	0.29	2.02	0.022	0.014	0.006	0.005	0.033	0.060
RSD_r, %	7.0	2.1	8.1	2.7	0.9	5.7	12.6	8.1	1.2	0.9	1.9	3.4
Repeatability value	0.70	0.25	3.02	0.98	0.81	5.66	0.062	0.039	0.017	0.014	0.092	0.168
s_R	0.33	0.13	1.08	1.32	2.78	2.02	0.027	0.014	0.031	0.015	0.061	0.060
RSD_R, %	9.2	3.0	8.1	10.2	8.2	5.7	15.5	8.1	6.0	2.7	3.5	3.4
Reproducibility value	0.92	0.36	3.02	3.70	7.78	5.66	0.076	0.039	0.087	0.042	0.171	0.060

[a] Refers to GC column and conditions as outlined in method section B (Apparatus), subsections (d) and (e).

tors 5 and 7 were probable outliers for Level 5; however, these values were not removed. The decision to retain the values reported by Collaborator 5 was made based on the unusually small variation noted for Level 5 (RSD_r = 1.1% after removal of Cochran outlier laboratory) and the fact that the average laboratory value reported by Collaborator 5 deviated only 5% from the "true" value. Without the indication of the paired Grubbs test, there is no basis for excluding the data reported by Collaborator 7, and thus, they were retained also.

Along with the blind duplicate pair at each concentration, each collaborator also received 6 blank XAD-4 tubes, which had been opened, labeled, and capped in an identical manner as the samples. The reported nicotine values for the primary and backup sections of XAD-4 resin in the blanks were used to correct all sample values as stated in the method. Desorption efficiency was determined to be 100% by 5 of the 7 collaborators. However, Collaborators 2 and 7 determined nicotine recovery to be less than 100%, and desorption efficiencies were calculated and applied as stated in the method.

Mean reported values for Levels 4, 5, and 6 are in excellent agreement with theoretical values, with the average bias being less than 2% (both including and excluding outliers). The problems encountered previously with the spiking procedure using 7 µL (8) were avoided by using only 3 or 4 µL of spiking solution. In experiments preliminary to this collaborative study, significant bias was noted for a spiking solution volume of 1 µL and also for volumes more than 5 µL. Therefore, it is recommended that any spiking onto XAD-4 resin inside the tubes be done with volumes between 2 and 5 µL, inclusive.

Comparison of repeatability and reproducibilty RSD values (RSD_r and RSD_R) reveals that virtually all of the variation noted among laboratories (reproducibility) is accounted for by the precision within individual laboratories (repeatabilty). In addition, analysis of variance of data in Table 1 shows that there is not sufficient evidence to indicate a difference among laboratories and also no evidence of a significant laboratory × sample interaction (P = 0.22 and 0.14, respectively). Additional data analysis (analysis of variance) was conducted to detect a difference between the 0.32 mm and 0.53 mm id columns (and associated GC conditions) and none was found (P = 0.72).

Presented in Table 2 are method performance parameters calculated for each GC method. From these data, there is no apparent trend in repeatability, reproducibility, or bias between the 2 methods studied.

These same columns and conditions were compared previously in a ruggedness test in the Associate Referee's laboratory, and no effect on results was detected (8). Also, performance parameters calculated here are slightly better than those published previously (8). As a result, it can be concluded that the method under study (GC method 2) yielded results equivalent to those of AOAC method **990.01** (7).

Using the same 0.32 mm id column recommended here, the GC parameters can be modified further (lower flow rate, splitless injection) to determine other vapor-phase components of ETS that are trapped by the XAD-4 resin. Although not studied here, one such example is the simultaneous determination of nicotine and 3-vinylpyridine (11, 12). Resulting from combustion of nicotine, 3-vinylpyridine appears to be an additional tracer of ETS in indoor air (11–13).

Recommendation

The data indicate no evidence for a difference in method performance due to capillary column dimensions and associated instrument conditions. It is recommended that this method for determining nicotine in environmental tobacco smoke, describing use of either a 0.53 mm id capillary column (with direct or on-column injection) or a 0.32 mm id capillary column (with split injection), be adopted official first action as a replacement to the existing method, **990.01**.

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VITAMINS AND OTHER NUTRIENTS

Comparison of Three Methods for Calculating Protein Content in Developing Apple Flower Buds[1]

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Three methods (Kjeldahl, sulfuric acid–hydrogen peroxide, and summation of amino acid content) for determining and calculating the protein content of apple flower buds were compared. Quantitation of protein content based on summation of amino acids appears to be the most accurate method. A new nitrogen:protein conversion factor (5.51) was calculated based on total amino acid analysis. This new conversion factor could replace the conventional 6.25 factor for estimating total protein content of apple flower bud by the Kjeldahl method. However, Kjeldahl is not an accurate method for estimating protein content in apple flower bud tissue, regardless of the conversion factor, and probably would not be a good method for estimation of protein in other plant species.

Research on cold hardiness of fruit trees can be divided into 2 general areas: studies of the processes that control the ability of the plant to survive freezing temperatures (mechanism of cold acclimation) and studies on the mechanism of freezing resistance and injury (1). Much of the research on cold acclimation and freezing resistance has centered on changes in proteins and amino acids, nucleic acid, cell wall properties, and carbohydrates (2–4). In most studies, an increase in cold hardiness was accompanied by a positive or negative correlation with amino acids and protein (4).

Quantitative measurements of the protein content of plant tissues present many difficulties, partly due to the presence of large quantities of other complex plant constituents but mainly due to the relatively small amounts of protein present in buds (2). The most commonly used procedures for total protein determination include the Folin phenol method (5), the Biuret method (6), the 280/260 nm ultraviolet (UV) absorption method or the improved 224–236 nm isoabsorbance method (7), the 280/205 nm absorption procedure of Scopes (8), the relatively new Coomassie Blue dye binding method of Bradford (9), and the classical Kjeldahl nitrogen procedure (10).

The Folin phenol, Bradford, and UV absorption methods are not well-suited for measuring total proteins in plant tissue, because complete protein extraction and solubilization are required. Also, the sensitivity of these methods is affected by the presence of numerous interfering compounds, especially phenolics and tannins (11–13). The Kjeldahl nitrogen method (10), in which total nitrogen is multiplied by a conversion factor of 6.25, is the procedure most frequently used by investigators to measure protein because of its simplicity. Heidelbaugh et al. (14) found that the best estimate of the protein content of a biological sample is the summation of the amino acid nitrogen content. They recommended that, whenever accurate data on the protein content of individual tissues are required, conversion factors based on the actual amino acid nitrogen content should be used. The method of protein quantitation, presented by Horstmann (15) and Peterson (16), is based upon knowledge of the amino acid composition of a protein or protein mixture.

The purpose of this study was to compare the following 3 methods for determining the protein content of typical apple flower bud tissues for future studies on apple flower buds, which often are subjected to spring freeze damage in Eastern Canada (17, 18): (*1*) determination of total protein by summation of the amino acid content of apple flower buds as described by Horstmann (15), (*2*) multiplication of Kjeldahl nitrogen by 6.25 and a new conversion factor calculated from the amino acid analyses, and (*3*) measurement of the nitrogen content of apple flower buds by the sulfuric acid–hydrogen peroxide ashing procedure of Thomas et al. (19) and multiplication by 6.25 and the new conversion factor. The second and third procedures are commonly used methods for total protein determination because of their simplicity; the first method is not as simple as the others but is more accurate.

Materials and Methods

Materials

Type W-3 cation-exchange spherical resin, sized to 9.0 ±0.5 μm, was obtained from Beckman Instruments Inc., Palo

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Alto, CA; type DC-6A, 11.0 ±1.0 μm spherical resin, was purchased from Dionex Corp., Sunnyvale, CA. L-Tryptophan, D-glycosamine hydrochloride, D-galactosamine hydrochloride, and 4-hydroxyproline were obtained from Calbiochem-Behring Corp., La Jolla, CA. 3-Nitro-L-tyrosine was purchased from Aldrich Chemical Co., Milwaukee, WI. The standard amino acid calibration mixture, Piercesolve (ethylene glycol monomethyl ether), ninhydrin, and stannous chloride hydrate were purchased from Pierce Chemical Co., Rockford, IL. Sodium citrate dihydrate (crystal) was obtained from Allied Fisher Scientific, Fairlawn, NJ.

Collection of Plant Material and Sample Preparation

Flower buds were collected from six 30-year-old apple trees (*Malus domestica* Borkh. cv. McIntosh/M7) grown at MacDonald College of McGill University, Ste.-Anne-de-Bellevue, Quebec. Forty buds were harvested from each tree at bud break (March 29, 1985), and each bud was weighed immediately after harvest. All the buds from each tree replicate (ca 2.68 g) were combined, frozen in liquid nitrogen (−170°C), freeze-dried, pulverized in a Thomas-Wiley mill equipped with a 64 mm stainless steel hopper and stationary blades (Arthur H. Thomas Co., Philadelphia, PA), and then stored at −20°C in air-tight containers until needed.

Extraction of Apple Flower Bud

To remove all traces of soluble amino acids and other compounds from apple flower bud tissues, 2–3 g samples of the pulverized tissues were extracted with a mixture of 0.1M HCl in 75% ethyl alcohol. Samples were suspended in 200 mL extraction solvent and homogenized for 3 min in a VirTis Model 45 homogenizer (VirTis, Gardiner, NY), speed set at 30/100; homogenates were centrifuged 30 min at 50 000 × *g* (SS-34 Sorvall rotor) and 2°C. Supernatants were removed and dried under vacuum (Buchi, Rotavapor, Switzerland) at 45°C. The pellet was suspended in the same extraction solvent, and the extraction procedure was repeated twice. The final pellets were suspended in 20 volumes of acetone, and the suspension was again centrifuged as before. The pellets from the final centrifugation were dried at 50°C overnight and then placed under vacuum to remove the last remnants of solvent. The dried pellets were finally ground to pass through a 40 mm screen and stored at −20°C until needed.

Amino Acid Analysis

Amino acid analyses were performed on a semiautomated amino acid analyzer (Beckman Spinco Model 120C) modified for accelerated chromatography to accommodate both the 0.6 and 0.9 cm diameter columns with adjustable column fittings, as described previously (20). Amino acids and proteins of apple flower buds were determined on 50 mg samples of lyophilized powders. Samples were hydrolyzed in 18 × 150 mm Pyrex test tubes under vacuum (below 10 μm Hg) with 15.0 mL triple-glass-distilled, constant-boiling HCl (6.0M) at 110°C in duplicate for each of 4 times (24, 48, 72, and 96 h, respectively).

The data reported for serine, threonine, and tyrosine represent the average of values extrapolated to zero time of hydrolysis. Addition of phenol (10–15 μL) to the hydrolysates usually prevented chlorination of tyrosine. The values for valine, isoleucine, leucine, and phenylalanine are averages of data from 48, 72, and 96 h hydrolysis. All others are reported as the average values from 24, 48, 72, and 96 h hydrolysis.

The 4-hydroxyproline was determined separately from concentrated hydrolysate (equivalent to 0.1 mg protein/analysis) by the procedure of Zarkadas et al. (20). Recoveries of 4-hydroxyproline were calculated relative to alanine. Methionine and cyst(e)ine were determined separately (50 mg) by the performic acid procedure of Moore (13). The recoveries of cystine plus cysteine as cysteic acid and of methionine as the methionine *S*,*S*-dioxide were calculated relative to both the yields obtained by the performic acid treatment of standard solutions of these amino acids and to alanine and leucine present in the samples. Tryptophan in apple flower bud samples (50 mg) was also determined separately after alkaline hydrolysis by a rapid method (20), with 3-nitro-L-tyrosine as the internal standard.

Total Protein Determination

Protein in the hydrolysates was quantitated as described by Horstmann (15). According to this method, a mean residue weight (WE in μg/nmol) was calculated for the amino acids constituting the proteins in the tissue by the following equation:

$$WE = \sum_{i=1}^{19} (a_i \times b_i) \qquad (1)$$

where a_i is the mole fraction of a specific amino acid, i, found in the analyzed aliquot, and b_i is the molecular weight of amino acid residue, i. A conversion factor, F, which is the apparent average residue molecular weight (in mg/nmol) of the apple flower bud protein mixture, increased in proportion to the missing tryptophan and cyst(e)ine residues from acid hydrolysates, was used to determine the protein mass in each hydrolysate sample analyzed as follows:

$$F = WE/[1 - (a_{Trp} + a_{Cys})] \qquad (2)$$

where a_{Trp} and a_{Cys} are the respective mole fractions of the specific amino acids tryptophan and cyst(e)ine per mole of total amino acid composition. Similarly, the F′ factor was also calculated from Equation 2 but in the absence of tryptophan, cyst(e)ine, proline, and 4-hydroxyproline. Both F and F′ calculated values are constants characteristic of apple flower buds and can be used in all subsequent quantitations of this tissue by standard procedures described by Horstmann (15) and Peterson (16).

The protein concentration (P) of each hydrolysate was then calculated from the following expression:

$$P = F \sum_{i=1}^{17} X_i \qquad (3)$$

where X_i = nanomoles of amino acid, i, found in the analyzed aliquot.

Table 1. Amino acid composition and protein content of apple flower bud tissues harvested at bud break (March 29, 1985)

Amino acid (AA)	g AA/kg total protein, mean ± SEM[a]	CV[a]	Mean mole fraction, % (a_i)[b]	Mean residue weight, μg ($a_i \times b_i$)[b]
Aspartic acid	108.76 ± 1.07	2.42	10.36	0.011919
Threonine	43.13 ± 0.77	4.36	4.68	0.004727
Serine	41.31 ± 1.51	8.96	5.20	0.004526
Glutamic acid	124.62 ± 1.40	2.75	10.59	0.013659
Proline	46.35 ± 0.64	3.40	5.58	0.005413
Glycine	52.35 ± 0.50	2.35	10.06	0.005731
Alanine	50.09 ± 0.46	2.25	7.73	0.005487
Cyst(e)ine	11.04 ± 0.25	5.56	1.17	0.001210
Valine	57.41 ± 0.84	3.58	6.35	0.006290
Methionine	27.32 ± 0.67	6.02	2.28	0.002992
Isoleucine	51.83 ± 0.59	2.77	5.02	0.005674
Leucine	80.89 ± 0.90	2.74	7.84	0.008857
Tyrosine	50.84 ± 1.24	5.98	3.42	0.005570
Phenylalanine	51.69 ± 0.43	2.02	3.85	0.005662
Histidine	75.91 ± 1.38	4.45	6.49	0.008314
Arginine	65.92 ± 0.98	3.65	4.63	0.007129
Tryptophan	5.35 ± 0.13	5.82	0.32	0.000585
4-Hydroxyproline	29.95 ± 0.37	3.82	2.32	0.003043
Total AA recovered	994.84 ± 0.41	0.11	100.00	—
Ammonia	27.42 ± 0.60	5.15	—	—
Total AA nitrogen, g/kg total protein[c]	181.60 ± 0.80	1.15	—	—
Mean residue wt. (WE), μg (defined by Eq. 1)[d]	—	—	—	0.109685 ± 0.00007
Conversion factor, μg (defined by Eq. 2)[d], F	—	—	—	0.111343 ± 0.00006
Conversion factor, μg (defined by Eq. 2)[d], F′	—	—	—	0.121056 ± 0.00016
Total protein, g/kg dry weight (calcd from Eq. 3)[d]	60.29 ± 0.59	2.37	—	—

[a] Mean values and standard error of the means (SEM) for 3 replicates and 24 determinations; CV, coefficient of variation.
[b] a_i is the mole fraction of a specific amino acid, i, found in the analyzed sample; b_i is the molecular weight of amino acid residue, i; and Eq. 1 gives the mean weight equivalent (WE) of AA in that protein mixture.
[c] Calculated as described previously (2, 14).
[d] The total protein, WE, and F and F′ constants were calculated using Eq. 1–3.

Total Nitrogen Determination (Kjeldahl)

The total nitrogen content of the freeze-dried samples of apple flower buds was determined by the Kjeldahl method (10, modified version, sec. **2.058**). A 0.5 g sample was digested by the Kjeltec digestion system (Model 20, Tecator AB, Hoganas, Sweden) with selenium as the catalyst. Total N was determined by distilling the ammonia from the Kjeldahl digests into 4% boric acid and titrating with standard acid, using the Kjeltec distilling and titration unit (Model 1002).

New Protein Conversion Factor

The new factor for converting Kjeldahl nitrogen into protein content was calculated by the following analytical convention:

$$\text{Protein conversion factor} = 100 \text{ g total nitrogen}/\%\text{ amino acid nitrogen (\%N)}$$

where %N = 18.16 calculated from the amino acid data in Table 1.

Sulfuric Acid–Hydrogen Peroxide

Samples were digested by the sulfuric acid–hydrogen peroxide wet ashing procedure of Thomas et al. (19), and total nitrogen in these digests was determined as ammonia by the indophenol blue method using the automated Technicon AAII analyzer (Technicon Instruments Co., Tarrytown, NY).

Protein Nitrogen Determination

Total nitrogen based on summation of the amino acid nitrogen was calculated as described previously (2, 14).

Results and Discussion

The 3 methods used gave estimates of nitrogen that differed by as much as 42.9% (Table 2). Similar differences between methods have been reported previously by Heidelbaugh et al. (14) and Benedict (11). The sulfuric acid–hydrogen peroxide ashing procedure, a colorimetric method, gave higher estimates of nitrogen than the other 2 methods, probably because of interference from other substances. Nitrogen determinations by the Kjeldahl and the sulfuric acid–hydrogen peroxide ashing procedures were significantly greater than that from the sum of amino acid nitrogen because of incorporation of many nonprotein nitrogenous compounds, e.g., alkaloids, amides, ammonium salts, nitrogenous glucosides, porphyrins, fats, hor-

Table 2. Comparison of 3 methods for total nitrogen and protein content of apple flower buds harvested at bud break (g/100 g dry matter)[a]

Component	H_2SO_4–H_2O_2 ashing (19), mean ± SEM[b]	CV	Kjeldahl (10), mean ± SEM[b]	CV	Sum of amino acid N (14), mean ± SEM[c]	CV
Nitrogen	1.917 ± 0.107	1.51	1.654 ± 0.045	4.75	1.095 ± 0.015	2.37
Total protein calcd by:						
Sum of amino acids	—	—	—	—	6.029 ± 0.058	2.37
N × 6.25	11.979 ± 0.104	1.51	10.337 ± 0.284	4.75	6.800 ± 0.032	2.37
N × 5.51	10.555 ± 0.129	1.51	09.108 ± 0.250	4.75	6.031 ± 0.084	2.37

[a] Mean ± SEM within a row for each component are significantly diffferent at the 99% confidence level by ANOVA and least significant difference test.
[b] Mean values and standard error of the means (SEM) for 6 replicates and 30 determinations; CV, coefficient of variation.
[c] Mean values and standard error of the means (SEM) for 6 replicates and 24 determinations.

mones, and nucleic acids (Table 2). To correct for this variance, a new factor (5.51) for the conversion of Kjeldahl nitrogen to protein content was calculated on the basis of the summation of the amino acid nitrogen content of this plant tissue (Table 1).

Although these results are in accord with the factors reported by Benedict (11) for almonds (5.2), peanuts and Brazil nuts (5.5), tree nuts and coconuts (5.3), etc., the data of Table 2 show that even with the use of the new factor, the protein content calculated for apple flower buds was higher by the Kjeldahl procedure than by amino acid analyses. The values in Table 1 show deviations of less than 3.0% from the average values obtained among 3 replicates within the same treatment. The least variability in amino acid content was found when the results were expressed as amino acids (g)/anhydrous, fat, and ash-free tissue protein (kg), because the influence of all nonprotein constituents of this tissue was eliminated. The average weight equivalent (WE, μg/nmol) and conversion factors, F and F′ (g/nmol), obtained are listed in Table 1 and can be used in all subsequent quantitations of protein in this tissue.

During acid hydrolysis of proteins in 6M HCl, tryptophan is destroyed, cystine is converted to cysteine, and asparagine and glutamine are converted, respectively, to aspartic and glutamic acids. These considerations were taken into account in calculating the conversion factor, F, which is the apparent average residue molecular weight of this protein mixture increased in proportion to the missing tryptophan and cyst(e)ine residues (Equation 2). Similarly, the F′ factor is also calculated from Equation 2, but in the absence of tryptophan, cyst(e)ine, proline, and 4-hydroxyproline. Apple flower buds harvested at bud break contained 6.03% total protein on a dry weight basis (Table 1). This value can be calculated by summing amino acids or by multiplying total protein nitrogen by 5.51.

Although the Kjeldahl method is recognized as accurate and precise for nitrogen determination, its use for protein assay in plant materials has been questioned because of the conversion factor (11). Protein quantitation by the Kjeldahl method makes the assumption that all plant proteins have a mean nitrogen content of 16%. We found that the nitrogen content of apple flower buds is 18.16%. The calculated conversion factor of 5.51 should be substituted for 6.25 in estimating total protein content of apple flower buds; however, this conversion factor will still give an erroneously high protein content when used with Kjeldhal total nitrogen analysis. The Kjeldahl method is not accurate for estimating protein content in apple flower bud tissue, regardless of the conversion factor, and probably will not be a good method for protein determination for other plant materials.

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TECHNICAL COMMUNICATIONS

In Vitro Effect of Multiple Antibiotic/Antimicrobial Residues on the Selection for Resistance in Bacteria

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A method using a gram-positive and a gram-negative organism was used to investigate the selection for resistant populations after exposure to residue levels of 7 antibiotics and 1 antimicrobial. The organisms were exposed to individual compounds and combinations of 3 compounds for 14 days. The changes in minimum inhibitory concentration (MIC) of a panel of 8 antibiotics and 1 antimicrobial were used as the measure of resistance development/selection. For *Staphylococcus aureus* ATCC 9144, exposure to residue levels of oxytetracycline, tylosin, penicillin, and virginiamycin resulted in an increased MIC of the compound itself; most individual residues did not result in increased cross-resistance. With combinations of residues, 13 of 45 determinations resulted in significant increases in MIC. *Enterobacter cloacae* B520, which was much less sensitive to 4 of 9 markers, showed MIC increases only for tylosin and the combination of neomycin–sulfamethazine–oxytetracycline. The results indicate an interaction among residue levels of antibiotics in selection for resistance.

Foods of animal origin contain antibiotic/antimicrobial residues, including multiple residues. The *Domestic Residue Data Book* of the Food Safety and Inspection Service, U.S. Department of Agriculture, for 1988 and 1989 indicates that violative residues occur in products of animal origin at an incidence that usually is less than 5% (1). However, neither the residues present at less than the violative level nor the incidence of multiple residues have been completely tabulated. Whether a product contains more than 1 residue is difficult to ascertain from the published information. Consumers who eat a normal, diverse diet should expect to be exposed to multiple residues on a continuing basis.

Although Brady et al. (2) developed some data on the significance of individual antibiotic/antimicrobial residues in the development/selection for resistance, essentially no data are available on the effect of multiple residues on the development/selection for resistance in bacteria. Previous studies (2) reflect only the effects of individual antibiotics/antimicrobials upon 2 representatives of the Enterobacteriaceae. The potential for multiple residues to select for resistant bacterial populations has not been adequately evaluated.

The present study uses *Staphylococcus aureus* ATCC 9144, an organism that is extremely sensitive to low levels of antibiotics in general, and the β-lactams specifically, in addition to the *Enterobacter cloacae* used in previous studies. If resistance is likely to develop in bacterial strains, these organisms have the potential to exhibit that effect. Representatives of the Enterbacteriaceae might have a more limited ability because of a higher degree of native resistance to antibiotics and may not be as good an indicator organism for measuring the potential of resistance development.

The antibiotics used in these studies are representative of some of the major antibiotic families used in animal agriculture.

The supplementation levels of the compounds used were the maximum residue levels allowed in that meat product in which the residue would be expected to occur (3).

The purpose of this report is to evaluate the potential effects of combinations of antibiotics and antimicrobials to select for resistant populations in both gram-positive and gram-negative microorganisms.

Experimental

Buffers

All buffer salts were dissolved and diluted to 1 L with water and then stored at room temperature according to method **957.23** (4).

(a) *Phosphate buffer, pH 4.5.*—13.6 g monopotassium phosphate.

(b) *Phosphate buffer, pH 6.0.*—8.0 g monopotassium phosphate and 2.0 g dipotassium phosphate.

(c) *Phosphate buffer, pH 8.0.*—16.73 g dipotassium phosphate and 0.523 g monopotassium phosphate.

Antibiotic Standard Solutions for Supplementation

(a) *Neomycin, 100 μg/mL.*—Dissolve 10 mg neomycin sulfate in 100 mL pH 8 buffer. Dilute to 10 μg/mL on day of use.

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Table 1. Concentrations of antibiotics/antimicrobial used for MIC determinations with 2 organisms

Antibiotic/antimicrobial	Concentration of standard, µg or units/mL	Range in well, µg or units/mL
	S. aureus	
Neomycin	100	50–0.025
Oxytetracycline	100	50–0.025
Gentamicin	100	50–0.025
Sulfamethazine	800	400–0.20
Tylosin	100	50–0.025
Penicillin	5	2.5–0.00125
Streptomycin	100	50–0.025
Virginiamycin	100	50–0.025
Chloramphenicol	100	50–0.025
	E. cloacae	
Neomycin	100	50–0.025
Oxytetracycline	100	50–0.025
Gentamicin	100	50–0.025
Sulfamethazine	1000	500–0.25
Tylosin	800	400–0.20
Penicillin	800	400–0.20
Streptomycin	100	50–0.025
Virginiamycin	800	400–0.20
Chloramphenicol	100	50–0.025

(**b**) *Gentamicin, 100 µg/mL.*—Dissolve 10 mg gentamicin in 100 mL pH 8 buffer. Dilute to 10 µg/mL on day of use.

(**c**) *Penicillin, 100 units/mL.*—Dissolve penicillin in 10 mL acetone–pH 6 buffer (equal parts), and dilute with pH 6 buffer to 100 mL. Dilute to 10 units/mL. Prepare fresh daily.

(**d**) *Oxytetracycline, 100 µg/mL.*—Dissolve 10 mg oxytetracycline hydrochloride in 10 mL 0.1N HCl, and dilute with pH 4.5 buffer to 100 mL. Dilute to 10 µg/mL on day of use.

(**e**) *Sulfamethazine, 100 µg/mL.*—Dissolve 10 mg sulfamethazine in 10 mL 0.1N HCl, and dilute with water to 100 mL. Dilute to 10 µg/mL on day of use.

(**f**) *Tylosin, 100 µg/mL.*—Dissolve 10 mg tylosin tartrate in 5 mL methanol, and dilute with pH 8 buffer to 100 mL. Dilute to 10 µg/mL on day of use.

(**g**) *Streptomycin, 100 µg/mL.*—Dissolve 10 mg streptomycin sulfate in 100 mL pH 8 buffer. Dilute to 10 µg/mL on day of use.

(**h**) *Virginiamycin, 100 µg/mL.*—Dissolve 10 mg virginiamycin in 10 mL ethanol, and dilute with pH 8 buffer to 100 mL. Dilute to 10 µg/mL on day of use.

Antibiotic Standard Solutions for Minimum Inhibitory Concentration Determination

(**a**) *Neomycin, oxytetracycline, gentamicin, and streptomycin.*— Prepare 100 µg/mL solutions as described.

(**b**) *Sulfamethazine.*—Prepare 1000 µg/mL solution as described.

(**c**) *Tylosin.*—Prepare 100 and 800 µg/mL solutions as described.

(**d**) *Penicillin.*—Prepare 5 and 800 µg/mL solutions as described.

(**e**) *Virginiamycin.*—Prepare 100 and 800 µg/mL solutions as described.

(**f**) *Chloramphenicol.*—Prepare 100 µg/mL solution by dissolving 10 mg chloramphenicol in 10 mL acetone and diluting with pH 6 buffer to 100 mL.

Each antibiotic solution in both sets of standards was filter-sterilized with a 5 mL syringe and Acrodisk (Gelman Sciences, Ann Arbor, MI) and then dispensed aseptically.

Media

(**a**) *Maintenance medium.*—Tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI) was used for maintenance of organisms.

(**b**) *Inoculation broth.*—Tryptic soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD) was used for selection studies.

Microorganisms

Two microorganisms were used: *S. aureus* ATCC 9144, and a strain of *E. cloacae*, B520, isolated from the human intestinal tract and identified by the Enterotube system (Roche Diagnostic Systems, Nutley, NJ). Baseline minimum inhibitory concentration (MIC) values for 9 antibiotics were determined for both organisms.

MIC Determination

Inoculate flask of TSB with *E. cloacae* B520 or *S. aureus* ATCC 9144, and incubate 18 h with shaking at 37°C. Dilute 18 h culture and inoculate TSB flask so that final concentration is ca 10^3 organisms/mL. For each antibiotic MIC determination, dispense 125 µL seeded TSB into 12 wells of 1 row of 96-well disposable, sterile, flat-bottomed well plate (Corning Glass Works, Corning, NY). Add 125 µL antibiotic solution to first well of each row. (Concentrations of each antibiotic used with each organism are shown in Table 1.) Mix contents of first well by drawing up liquid and expelling 4 times, using micropipet fitted with sterile tip. Withdraw 125 µL from first well and expel into second well, mixing contents as before. Continue dilutions across row, using fresh sterile tip for each transfer. Each MIC determination for 1 antibiotic and 1 organism requires 1 row of wells. Incubate well plates 18 h at 37°C, and measure turbidity at 620 nm with microtiter plate reader (Easy Reader ATC, SLT Labinstruments).

Selection for Resistance

On Day 0 of the study, tubes containing 1 mL sterile TSB were supplemented aseptically with individual antibiotics/antimicrobials or combinations at concentrations shown in Table 2. Tubes were inoculated from the appropriate 18 h culture and incubated 18 h with shaking at 37°C.

Each day for 14 days, tubes of 1 mL TSB were aseptically supplemented with antibiotics/antimicrobials and inoculated with previous day's growth. Growth from Day 14 tubes was

streaked onto TSA slants, incubated 18 h at 37°C, and stored at 4°C until used for MIC determination.

Results and Discussion

The rationale for using in vitro studies with bacteria is that the intestinal populations of many common animals have high levels of resistance, and preventing contamination of animals with resistant bacteria is extremely difficult. In vitro bacterial studies offer a system that can be protected from contamination and can provide many generations of exposed organisms. Because the purpose of the study is selection for resistant bacterial populations, pure cultures of sensitive strains are the ideal test systems.

E. cloacae B520 was used as one of the indicator organisms because it had been used in previous studies. *S. aureus* ATCC 9144 was chosen because it is a gram-positive organism and is sensitive to a large number of antibiotics. Neither organism was selected for its ability to develop resistance when exposed to antibiotics.

The antibiotics chosen were common representatives of several of the antibiotic families used in animal agriculture. The concentrations used were tolerance levels established for residues in muscle tissue (3). Combinations of 3 residues were chosen as a possible maximum number of drugs that might be found in the general diet or in specific products. Obviously, other combinations and permutations of residues are possible; the ones chosen were designed to give some reasonable insight into the problem.

The procedure for evaluating the tendency of compounds to select for resistance at residue tolerance levels was the MIC determination (5). This assay uses a halving dilution sequence, i.e., addition of the antibiotic to the first well of seeded broth reduces the antibiotic concentration by half. This dilution series is continued as many times as is indicated. The greatest accuracy in determining the MIC is achieved at the lower concentrations. A difference in the MIC of only 1 well (a doubling or halving) between determinations is not considered significant; significance requires determinations that differ by more than 1 well. The results should be interpreted by this commonly used approach (5).

Table 3 shows the results of exposing *S. aureus* ATCC 9144 to residue levels of individual antibiotics/antimicrobials and combinations of antibiotics/antimicrobials. The combination of neomycin–sulfamethazine–oxytetracycline caused a rise in the MIC for neomycin. The remainder of the compounds did not cause an MIC change for neomycin of more than 1 well. Neomycin alone raised the MIC of the organism to oxytetracycline; the combinations of neomycin–sulfamethazine–oxytetracycline and penicillin–sulfamethazine–oxytetracycline raised the MIC 4-fold to oxytetracycline. Oxytetracycline alone doubled the MIC to itself, but this 1-well difference was not considered significant. However, in light of the effect of combinations, any slight synergy of the other compounds could increase the MIC.

None of the individual compounds or combinations increased the MIC to gentamicin. Sulfamethazine doubled the MIC to itself, which was not considered significant. Some synergy was apparent among sulfamethazine and the other compounds in some mixtures, which resulted in a large rise in the MIC to sulfamethazine. Tylosin raised the MIC to itself approximately 8-fold, as did the combination penicillin–sulfamethazine–oxytetracycline. Exposure to penicillin increased the MIC to penicillin 5-fold; the MIC to penicillin was increased 4- to 16-fold by the combinations of neomycin–sulfamethazine–oxytetracycline, streptomycin–tylosin–oxytetracycline, and sulfamethazine–virginiamycin–penicillin.

Table 2. Concentrations of antibiotics/antimicrobial used for supplementation

Antibiotic/antimicrobial or combination	Concentration(s), µg or units/mL
Neomycin	0.25
Gentamicin	0.10
Penicillin	0.05[a]
Oxytetracycline	0.10
Sulfamethazine	0.10
Tylosin	0.20
Streptomycin	0.50
Virginiamycin	0.10
Neomycin–sulfamethazine–OTC	0.25–0.10–0.10
Gentamicin–sulfamethazine–tylosin	0.10–0.10–0.20
Penicillin–OTC–sulfamethazine	0.05–0.10–0.10[a]
OTC–tylosin–streptomycin	0.10–0.20–0.50
Sulfamethazine–virginiamycin–penicillin	0.10–0.10–0.05[a]

[a] For *S. aureus* only, penicillin concentration was 0.025 units/mL.

None of the individual compounds or combinations increased the resistance to streptomycin or chloramphenicol. Exposure to virginiamycin increased the MIC to virginiamycin 4-fold; exposure to tylosin increased the MIC to virginiamycin 8-fold. Exposure to the combinations of penicillin–sulfamethazine–oxytetracycline and sulfamethazine–virginiamycin–penicillin increased the MIC to virginiamycin 8-fold. The other combinations doubled the MIC to virginiamycin; this is not considered significant except for the observation of a trend.

The results indicate an additive effect or synergy among compounds in increasing the MICs to *S. aureus*. Of the 45 MIC determinations for the combinations, 13 showed an increase in MIC greater than 2-fold and 6 showed a 2-fold increase. For 19 of the possible 45 determinations to show such a trend strongly suggests an additive/synergistic effect.

The picture is far less clear for the results obtained with *E. cloacae* B 520 as the test strain. Examination of Table 4 indicates that this member of the Enterobacteriaceae was grossly insensitive to 4 of the 9 markers. None of the compounds or combinations raised the MIC to neomycin, gentamicin, or streptomycin. It is not surprising that these 3 aminoglycosides respond in a similar fashion. Tylosin raised the MIC to oxytetracycline 4-fold. The combination of neomycin–sulfamethazine–oxytetracycline increased the MIC to chloramphenicol 4-fold.

Table 3. MIC values (μg or units/mL) of *S. aureus* ATCC 9144 after exposure to antibiotics/antimicrobial at residue levels

Antibiotic/antimicrobial[a]	Neo	Otc	Gen	Smz	Tyl	Pen	Stp	Vir	Cmp
Expected MIC	0.8	0.1	0.4	25	0.8	0.02	6.3	0.2	3.2
Control (av.)	0.7	0.1	0.4	25	0.8	0.02	5.5	0.25	2.4
Neo	1.6	0.8[b]	0.2	25	0.8	0.02	1.6	0.2	3.2
Gen	0.8	0.1	0.4	25	0.8	0.02	6.3	0.2	3.2
Pen	0.8	0.1	0.4	200[b]	0.8	0.1[b]	12.5	0.4	3.2
Otc	0.8	0.4[b]	0.4	25	0.8	0.025	12.5	0.2	3.2
Smz	1.6	0.2	0.4	50	0.8	0.02	6.3	0.2	3.2
Tyl	0.8	0.2	0.4	25	6.3[b]	0.02	12.5	1.6[b]	3.2
Stp	0.4	0.4[b]	0.4	25	0.8	0.02	6.3	0.2	3.2
Vir	0.8	0.1	0.4	12.5	1.6	0.02	6.3	0.8[b]	3.2
Neo–Smz–Otc	3.2[b]	0.8[b]	0.4	100[b]	0.4	0.08[b]	3.2	0.4	3.2
Gen–Smz–Tyl	0.8	0.1	0.4	25	1.6	0.02	3.2	0.4	3.2
Pen–Smz–Otc	0.8	0.8[b]	0.4	100[b]	6.3[b]	0.04	6.3	0.8[b]	3.2
Stp–Tyl–Otc	1.6	0.4[b]	0.4	400[b]	0.8	0.32[b]	12.5	0.4	3.2
Smz–Vir–Pen	0.8	0.2	0.4	400[b]	1.6	0.16[b]	12.5	0.8[b]	3.2
95% confidence limit upper range (n = 5)	1.0	0.1	0.4	25	0.8	0.04	10.0	0.4	3.9

[a] Neo = neomycin; Otc = oxytetracycline; Gen = gentamicin; Smz = sulfamethazine; Tyl = tylosin; Pen = penicillin; Stp = streptomycin; Vir = virginiamycin; Cmp = chloramphenicol.
[b] Considered significant.

Statistical treatment was not used to determine the significance of a rise in the MIC. Examination of Tables 3 and 4 shows the upper level of the 95% confidence limits of the controls. It is quite apparent that statistical treatment would cause a skewing of the results and indicate that many more compounds and combinations caused a statistically significant rise in the MIC. This would be a misuse of logic, experimental design, and statistics.

The data developed with *S. aureus* as the test strain suggest that combinations of antibiotics at residue levels can cause increases in the MIC. The tester strain of *E. cloacae* did not prove to be as versatile, because 4 of the 9 marker drugs had MICs that were high to begin with. Nevertheless, there are suggestions of an interaction among residues that should be considered potentially important in the selection for resistant populations of bacteria.

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Table 4. MIC values (μg or units/mL) of *E. cloacae* B 520 after exposure to antibiotics/antimicrobial at residue levels

Antibiotic/antimicrobial[a]	Neo	Otc	Gen	Smz	Tyl	Pen	Stp	Vir	Cmp
Expected MIC	1.6	1.6	0.8	500	400	100	3.2	400	3.2
Control (av.)	1.9	1.4	0.8	500	400	105	3.8	400	3.8
Neo	1.6	1.6	0.8	500	400	200	3.2	400	6.3
Gen	1.6	1.6	0.8	500	400	62.5	3.2	400	6.3
Pen	3.2	1.6	1.6	500	400	100	6.3	400	3.2
Otc	3.2	1.6	1.6	500	400	100	6.3	400	6.3
Smz	3.2	1.6	0.8	500	400	100	6.3	400	3.2
Tyl	3.2	6.3[b]	0.8	500	400	100	6.3	400	6.3
Stp	3.2	0.8	1.6	500	400	100	6.3	400	3.2
Vir	1.6	0.8	0.8	500	400	100	6.3	400	6.3
Neo–Smz–Otc	3.2	3.2	0.8	500	400	125	3.2	400	12.5[b]
Gen–Smz–Tyl	1.6	1.6	0.8	500	400	62.5	3.2	400	6.3
Pen–Smz–Otc	3.2	1.6	1.6	500	400	100	6.3	400	6.3
Stp–Tyl–Otc	3.2	1.6	0.8	500	400	100	6.3	400	6.3
Smz–Vir–Pen	3.2	1.6	0.8	500	400	100	6.3	400	6.3
95% confidence limit upper range (n = 5)	2.8	1.8	0.8	500	400	118.9	4.5	400	4.5

[a] Neo = neomycin; Otc = oxytetracycline; Gen = gentamicin; Smz = sulfamethazine; Tyl = tylosin; Pen = penicillin; Stp = streptomycin; Vir = virginiamycin; Cmp = chloramphenicol.
[b] Considered significant.

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TECHNICAL COMMUNICATIONS

Training Modules To Develop Analytical Proficiency for Pharmaceutical Chemists

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As a part of a training program for new personnel in pharmaceutical analysis and for visiting scientists from other government laboratories, the Division of Drug Analysis (DDA) developed training modules to raise the job performance of incoming college students, technical aides, and newly hired chemists to an acceptable level. The modules include Liquid Chromatography, Gas Chromatography, Dissolution Testing, Thin-Layer Chromatography, Ultraviolet-Visible Spectrophotometry, Infrared Spectrophotometry, and Data Collection and Recording (as related to preparing worksheets in accordance with the requirements of the U.S. Food and Drug Administration). Each module is designed to be delivered in minimal time. By using a combination of commercial audiovisual materials and a training concept developed at DDA, the targeted group can be trained with minimal supervision. This approach does not require experienced analysts to be placed as full-time, one-on-one trainers; thus, the cost of the training is cut. The training scheme could be extended to other applications in industry, academia, or government by targeting training modules to their characteristic operation. These modules are also used as refresher courses for experienced analysts who have not practiced these techniques recently. The modular concept provides an excellent way to train employees quickly.

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Colleges and universities do not have the time or resources to educate their students in the array of techniques required to perform the specific pharmaceutical analyses required by the Division of Drug Analysis (DDA) of the U.S. Food and Drug Administration (FDA). To address these needs, DDA established internal training programs to ensure that our analysts have proper instruction in the analytical techniques and methods that are common to our laboratory. In the past, training programs were developed and conducted whenever new analysts were hired. Assignment of trainers depended on who was most readily available and on our current work loads. Each trainer had to spend much time collecting the materials and covering them on a one-to-one basis with the students. Such programs were very expensive, because our experienced analysts were selected to perform this training. In addition, the program suffered because there was no syllabus or structure to ensure that all incoming analysts covered the same materials adequately.

Analysts at the DDA usually follow the compendium procedures listed in *United States Pharmacopeia* (USP) (1). These directions are written for the experienced analyst; the untrained analyst finds the procedures difficult to follow. For the untrained analyst to reach the expected efficiency, some additional training is needed. This need prompted the development of the training modules.

Information Gathering

The first task was to find the prior level of training or instruction for the targeted groups. Comprehensive interviews were conducted with employees representing most of the targeted group with the aim of establishing the level and type of training needed.

All employees interviewed had majored in chemistry or physical science and had some background in analytical chem-

istry. Many had taken a course in instrumental analysis and were familiar with analytical terminology. Most of the student employees were majoring in physical science or pharmaceutical chemistry and had training in the instrumental methods. College students do not have enough time to become experts in any analytical technique. The instrumental analysis courses vary, but generally they are 1-semester courses that include an introduction to many different instrumental techniques and some short illustrative laboratory experiments. The course contents depend upon the facilities and the staff of the various colleges. Many of the laboratory experiments are performed on instruments set up by the laboratory assistant so that the student does not have the opportunity to become familiar with any of the instruments. The interviews established that a training level equivalent to a 3rd-year college course was needed.

Development of Training Modules

Each training module was supplemented with a commercial audiovisual presentation in conjunction with hands-on training covering the techniques that DDA chemists normally use. Savant (Sloan Audio Visuals for Analysis and Training, PO Box 3670, Fullerton, CA 92634) has developed a series of audiovisual training courses that cover many of the analytical methods and give the basic theory and principles of operation. The Savant courses are available either as slides with audiotape cassettes or as videotapes. The tape–slide versions proved to be the most useful, because the presentation could be stopped at any point to allow discussion. These courses were prepared by recognized experts in their fields. Because the Savant series did not cover dissolution, the DDA developed a tape–slide course for this test.

Because pharmaceutical chemists use USP methods in most assay work, the analysis of drug formulations found in USP XXII formed the target goal of the training. All training modules consisted of the following sources of materials: Savant tape–slide course, simplified instrument operating instructions taken from manufacturers' instrument manuals, and a method of analysis selected from an accepted official source (1, 2).

The next step was to select training samples of dosage forms of the drugs for each training module. Drug formulations were selected to give the trainees experience in handling and preparing solutions required for analysis of different types of dosage forms, i.e., tablets, syrups, injections, pure powders, etc. The samples were selected to highlight the analytical techniques involved and to fulfill specific aims that were deemed important. Included in the training was preparation of FDA worksheets to record the laboratory data, which might later be used in a court of law. Good laboratory and analytical practices were included in the preparation of samples and handling of all chemicals. A draft of each module was prepared and circulated to senior chemists and supervisors for their comments and recommendations. Their comments were included in the final draft of the module.

The training modules are designed to be applicable to the instruments in use in any laboratory, not a particular brand of instrument. Each module is supplemented by additional information about specific equipment to make it easier for the trainees to use the instruments.

Preliminary Testing of Modules

The training modules were tested in the laboratory by part-time student employees to see if the modules were written clearly and were at the correct instructional level. Three students at the 3rd-year college level were assigned to undertake each training module. Although the students worked as a team, each student was required to perform the analysis and submit results. The team worked with a trainer who was available at all times to answer questions. Questions on the theory or the operation of the instrument were answered with special care. Each student noted any unclear portions of the training and was debriefed to obtain his or her recommendations on the training module and analysis. The trainers discussed the results of the analysis with the trainees and evaluated their performance. Their recommendations were incorporated into a revised version of the module. Each training module was evaluated similarly. The final draft of each module was reproduced after these comments and further evaluated by a professional technical journal editor.

Final Testing of Modules

The final draft of each instrument module was further tested by 3 visiting foreign chemists from the Ministry of Commerce, Saudi Arabia, to ensure that the training modules were not ambiguous and were consistent with their needs. This allowed the opportunity to clarify any misunderstandings because of different interpretations of language. Words with only 1 meaning were chosen.

The trainer met with the visiting scientists to introduce the planned program. After the introduction, the trainees began each module by studying the Savant tape–slide presentation on the analytical technique. Sufficient time was allowed for individual study and for making notes before discussion with the trainer. The theory was clarified as needed. The trainees worked as a team, with each member preparing samples and performing the instrumental analysis.

As the trainees began to operate the instrument, they became familiar with all aspects, including its setup and the performance of suitability tests. The trainer was available for consultation or for explanation of the instrument. Whenever possible, the training was performed on a manual instrument rather than on a computer-controlled one. Use of the manual instrument allowed a better understanding of the instrument and the method, and no time was lost in learning specific keystrokes of computer operation. After the objectives specified in the training module were completed, the training was extended, time permitting, to an automated system.

After becoming familiar with the instrument operation and establishing instrument suitability, the trainees were ready to begin the analysis of the selected sample. Most of the training methods of analysis were based on the method described in

USP. USP does not include methods that use all of the instrumental techniques that DDA felt were important; therefore, we used the well-tested procedures in *Official Methods of Analysis* of the AOAC (2).

The trainees consulted with the trainer when clarification was needed or when questions arose. After the analysis, the trainees were debriefed and the data were compared. If the results were not acceptable, discussion with the trainees usually uncovered the problem, and calculations were corrected or the analysis was repeated. This format was used for all the training modules. The trainees' recommendations were incorporated into the final draft of the training module.

Determinations of the accuracy and precision for each analysis were included. The trainee was able to observe firsthand how to follow USP procedures. Many such procedures give only a shortened version of the necessary calculation, which makes it difficult for the untrained analyst to follow the steps required to arrive at the final solution. A logical stepwise equation was developed that was applicable to most analyses.

Training Modules

Liquid Chromatography

The liquid chromatography (LC) training module calls for reversed-phase columns, because they are most commonly used in drug analysis, and includes care of the columns and troubleshooting. Tableted aspirin was selected as the drug sample to be analyzed (3). The analysis consisted of an assay for aspirin and the measurement of a possible hydrolysis product (salicylic acid). The method allows the determination of the major component at a high concentration and the determination of a possible breakdown product present at very low concentration. Samples are prepared according to USP procedure for measuring both components. Often, no salicylic acid will be present. USP requires a minimum resolution requirement between aspirin and salicylic acid. Careful attention to the instrument setup, column, mobile phase, sample preparation, and proper integration was required to obtain reliable results.

Gas Chromatography

Few USP monographs use gas chromatography (GC) for assay of the active ingredient of a drug formulation. Many former USP methods have been replaced by methods that use LC. The greatest use of GC in USP is the analysis for residual solvents or alcohols. Testosterone cypionate injection in oil was selected as the formulation for the test (4). After the analysis of testosterone cypionate, the training could be extended to the determination of alcohols in cough syrups, which is recommended.

Thin-Layer Chromatography

Thin-layer chromatography (TLC) is used extensively to identify the main component and to establish the presence of impurities in drug formulations. This training module is based on the use of silica gel-coated plates (normal-phase chromatography).

Sulindac powder (USP Raw Material) was chosen as the drug for TLC training (5). This drug may contain several impurities that can be separated and detected. Impurity standards must be available to mark the R_f of the impurities.

Two methods for estimating the concentrations of impurities are used. In one method, a quantitatively diluted solution of the bulk drug is used as a reference to estimate the concentration of the impurities in the bulk drug; this technique is more economical for training purposes than the use of expensive reference standards. The other method requires the use of impurity standards to identify and calculate directly the concentration of the impurity.

Ultraviolet-Visible Spectrophotometry

The ultraviolet-visible absorbance spectra of drugs have been used extensively for quantitative determinations. Currently, UV is the detector of choice in LC and dissolution. USP has reduced the number of monographs based on nonstability-indicating UV absorbance while dramatically increasing the number of LC monographs on stability-indicating UV absorbance. Most drugs have a chromophoric group or groups that give excellent absorbance spectra in such solvents as water or methanol. A sample of chlorzoxazone tablets was selected for this analysis (6). The first analysis was performed at a single wavelength according to USP instructions. The training was modified to scan the solutions over the entire UV-vis spectral range.

After the trainees gained experience with USP and modified procedures, some training was given on the use of diode-array instrumentation as a UV-vis spectrophotometer and as an LC detector. The trainees had the opportunity to experience different characteristics of the UV instruments, potential interferences, and calculations. The application of Beer's Law was demonstrated.

Infrared Spectrophotometry

This training module included qualitative (identification) and quantitative portions. Infrared (IR) spectrophotometry is a technique used routinely by USP for identification. USP identity tests usually require diluting the drug with potassium bromide, making a solid pellet, and comparing the spectrum with that of the standard. IR spectophotometry can also be used quantitatively under certain conditions.

The AOAC assay for meprobamate tablets (7) offered a suitable system for quantitative analysis, because the drug's carbonyl group absorbs in a wavelength region free from solvent interference. We wanted to demonstrate that IR spectrophotometry can be used quantitatively under certain conditions, i.e., if the drug can be prepared in solutions having known concentrations in a suitable solvent. A standard grating spectrometer was used so that the student could learn basic principles and manually calibrate the instrument.

The trainees were shown how to extract the active drug from the tablet matrix and prepare potassium bromide pellets for

identification purposes. Fourier transform IR spectrophotometry was also used to examine the identification pellet. The student compared the spectra obtained with the diffraction grating and Fourier transform IR instruments.

Dissolution Testing

All oral dosage forms must allow the drug to be delivered to the proper system in the body. For each dosage form, USP and FDA have requirements that specify a certain minimum fraction to be dissolved at a specified time. The dissolution training experiment was performed on a common 6-spindle drive unit using 1 L kettles to hold the dissolution medium. Both USP Apparatus 1 (basket) and Apparatus 2 (paddle) were used.

The training covered the setup of the instrument, including proper alignment of all parts. Standard USP calibrators were used to test the instrument suitability before measurements were made on the sample. Aspirin tablets were used as the drug sample (3). This drug had been used earlier for the LC training module; therefore, 2 modules could use the same sample.

Discussion

The order of training for each instrument is not critical; however, we recommend that the initial module dealing with the preparation of samples and standard solutions and a discussion on writing the worksheet be covered before dealing with actual pharmaceutical formulations. That should be followed by the UV training, because UV spectrophotometry is used in dissolution methods and as a detector in LC procedures. Training in the LC technique required the greatest time; therefore, at least 2 weeks should be allowed for LC training. After the analysis of the drug formulation outlined in the training module, another drug sample might be analyzed to reinforce the training if time permits. After successfully completing the training methods, the analyst should be able to perform other drug analyses as specified in USP. No effort was made to include method development in the training, but approaches to development of methods and validation were discussed with the trainees as opportunities arose.

Videotaping the demonstrations of recently installed instruments by factory representatives is a technique the DDA has found useful to supplement training or to review instrument operation. These videotapes are used to orient inexperienced analysts or to refresh experienced analysts in the correct operation of that particular instrument. We have found that, by having individuals view the videotape and then review the operation manuals, our training time for correct operation of the instruments is substantially reduced. Also, individuals may review these presentations at their convenience, without feeling pressured by the presence of a trainer or other analysts.

We are currently examining computer software simulations (Software 1B, JCE Software, Dept of Chemistry, University of Wisconsin, 1101 University Ave, Madison, WI 53706) that have the capacity to solve problems similar to a method development problem, and we plan to add them to our training modules. Computerized simulations could be added for an advanced training program.

The aspirin tablet monograph was chosen for the LC training for several reasons. Aspirin hydrolyzes to salicylic acid; thus, the trainees should realize that salicylic acid must be determined first. Yet many do not make this association. The trainees are made aware of the importance of sample preparation and potential solvent-related problems. Different wavelengths are used for detection of aspirin and salicylic acid; this shows the power of a variable-wavelength detector vs a fixed-wavelength detector. USP aspirin tablet monograph, as written, seems complicated to the novice. The trainees must organize the manipulations and do so expeditiously to achieve success with the salicylic acid determination. The resolution value specified by USP may require the trainees to look at several columns before choosing one that meets the requirement. This process makes the trainees aware of the characteristics of good and bad columns. Gradient elution and diode-array detectors were discussed.

Testosterone cypionate injection in oil was chosen not only because it is one of the few remaining packed-column GC assays remaining in USP but also because the oil base poses a sampling problem to the trainees. The trainees found that the formulation is best sampled by the use of a TC (to contain) pipet instead of a TD (to deliver) pipet, and they quickly discovered why an internal standard is needed when making manual injections. In addition, the trainees determined the alcohol content of a cough syrup formulation by the procedure outlined in USP. Some time was allocated to discuss the use of capillary columns.

The TLC training includes the use of 2 methods to estimate impurity levels in drug samples. The USP monograph was initially followed. This procedure uses normal-phase chromatography with a silica gel plate. The trainees estimate the concentrations of the impurities by spotting a series of dilutions of the standard with a maximum concentration of 1.0% of the main spot. The plate is developed and the impurity spots are examined. The sum of the intensities of the impurity spots should fall below the intensity of the standard spot corresponding to 1.0%, the USP limit. The trainees are asked to repeat the experiment, except that they are given secondary impurity standards to weigh, dilute, and spot, along with the sample. They are then asked to identify and quantitate impurity spots found in the sample. The results found by this direct quantitative procedure are compared with the estimated results obtained with the USP procedure. The trainees can readily see that the absorptivity of sulindac is different from that of the impurities identified so that different results are obtained with the 2 techniques.

Before the introduction of LC, almost every sample received for analysis was first tested by TLC to determine identity and to check for impurities. TLC can be used for quantitative analysis, and USP uses the technique for analysis in certain steroid monographs.

The UV-vis spectroscopy training module was included to show the trainees how the absorption spectrum of a molecule changes with wavelength. The USP monograph calls for making the determination at a single wavelength. After the trainees

finished the assay, they were asked to obtain the entire UV-vis spectrum in the range of the sample and standard (200–800 nm) and to repeat the calculation at the wavelength of maximum absorbance if it was different from the wavelength specified in the USP procedure. This training not only demonstrated how an assay can be accomplished but also enlightened the trainees as to how variable-wavelength and diode-array detectors can be better used for LC analysis and identification. Discussion topics included sources of error from formulation excipients, other potential problems, the effect on absorbance of changing wavelength, and calculations.

Meprobamate in tablet form is a classic example for illustrating an IR analysis. The IR training consisted of quantitative and qualitative portions. The AOAC method was modified because pentaerythritol tetranitrate was not an ingredient of the tablet. Meprobamate has a strong carbonyl band in the range of 5.0–6.5 μm that can be used for the quantitative determination. In USP, IR analysis is mainly used for identification. Occasionally, however, IR spectrophotometry may be the easiest or the only approach to obtaining quantitative results. We wanted the trainees to be aware of this capability.

The trainees performed the quantitative experiment in the transmission mode of the instrument, and then converted transmission to absorbance before quantitative calculations were made in accordance with Beer's Law.

The trainees were made aware of problems associated with quantitative and qualitative IR techniques. Limited discussion about identifying the absorption bands in the IR spectrum were held.

The dissolution training with aspirin tablets highlighted some important points. One observation is that the paddle technique usually gives lower results. The wavelength specified in the USP method is 265 nm, which is the isosbestic point for aspirin and salicylic acid. Questions posed about why this wavelength is used were answered. The advantages and disadvantages of the paddle and the basket techniques were discussed in detail, especially in applying the test to nonofficial formulations.

A financial savings was realized by using this modular method of training instead of the one-on-one system. On-the-job training required a fully trained analyst at a salary range of $20–25 per hour for a period of at least 3 months, and the need for several trainers caused scheduling problems. The modular method required 1 trainer for approximately 1 day per week, and this person could train 3 persons at a time.

All trainees received identical training, which permitted evaluation of the individual performances. Any deficiencies were corrected before they were allowed to perform an unsupervised analysis. The trainees were considered capable of independent analysis on any of the instruments after completion of the training modules in 12 weeks.

The trainees concluded that they were adequately trained and could perform analyses described in USP. They were assigned an analysis on a drug sample that was not a part of the training to make sure that they had been adequately trained. A more efficient and economical training program is realized by the modular method.

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TECHNICAL COMMUNICATIONS

Liquid Chromatographic Determination of Ivermectin in Bovine Milk: Interlaboratory Study

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A laboratory trial was completed for an analytical method that can quantitate the marker residue of ivermectin, 22,23-dihydroavermectin B_{1a}, in bovine milk at 1 ng/mL. Currently, ivermectin is not approved for use in lactating dairy cows. In this method, the ivermectin residues are isolated from the milk matrix by a series of liquid–liquid extractions. A fluorescent derivative of the marker compound is prepared and then quantified by liquid chromatography with fluorescence detection. The interlaboratory study was successfully completed by using dosed milk and milk fortified with marker residue at 1, 2, and 4 ng/mL. The average recoveries by the 3 participating laboratories were 87, 59, and 95% at 1 ng/mL; 90, 61, and 96% at 2 ng/mL; and 90, 73, and 99% at 4 ng/mL. The concentrations of the marker residue in the dosed milk were 4.3, 3.7, and 4.7 ng/mL; coefficients of variation were 4.0, 24.8, and 5.9%, respectively.

Ivermectin (Figure 1) is an antiparasitic drug (1, 2) that is not currently approved for use in lactating dairy cows. Ivermectin consists of 2 homologues, 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and 22,23-dihydroavermectin B_{1b} (H_2B_{1b}). The drug consists of not less than 80% H_2B_{1a} and not more than 20% H_2B_{1b} (3). Tolan et al. (4) developed a determinative method for avermectins in plasma based on liquid chromatography (LC) with fluorescence detection. Tway et al. (3) modified the reaction conditions for the formation of the fluorescent derivative and developed a method for the detection of ivermectin residues in cattle and sheep tissues in which the H_2B_{1a} homologue was used as the marker residue. This method was later adapted by Schenck et al. for the determination of ivermectin in milk (5).

The method for the determination of ivermectin in bovine milk was successfully validated in the following 3 laboratories of the U.S. Food and Drug Administration (FDA) in 1988–1990: (*A*) Division of Veterinary Medical Research (DVMR), Beltsville, MD, (*B*) Denver District Office, Denver, CO, and (*C*) Minneapolis District Office, Minneapolis, MN. (It should be noted that according to the terminology used by the DVMR, the complete method would also contain a confirmatory step.)

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METHOD

Principle

Ivermectin is isolated from milk by adding ammonium hydroxide and ethanol to 10 mL milk and extracting with ethyl acetate and isooctane. The ethyl acetate–isooctane extract is evaporated to an oil, and the oil is dissolved in hexane and partitioned with acetonitrile. The acetonitrile is placed in a silylated test tube, partitioned with hexane to remove residual oil, and evaporated to dryness. The residue is derivatized with a solution of 1-methylimidazole and acetic anhydride in *N,N*-dimethylformamide (DMF). The derivatized sample is dissolved in chloroform and loaded onto a silica solid-phase extraction (SPE) tube. The tube is rinsed with additional chloroform, and the eluates are evaporated to dryness. The residue is taken up in methanol, and the H_2B_{1a} derivative is detected and quantified by LC with fluorescent detection.

Apparatus

(**a**) *Centrifuge.*—IEC Model HN-SII (Damon/IEC Division, Needham Heights, MA) equipped with rotor(s) for handling 50 and 15 mL centrifuge cups, or equivalent.

(**b**) *Oil bath.*—95–100°C.

(**c**) *Centrifuge tubes.*—Glass, 15 and 50 mL, with polyethylene stoppers; silylated glass, 15 mL, with glass stoppers; polypropylene, 50 mL, screw-capped.

(**d**) *SPE columns.*—3.0 mL silica, Supelco Supelclean LC-Si (Supelco, Bellefonte, PA), or equivalent; with vacuum manifold, Supelco 5-7030M, or equivalent.

(**e**) *Ultrasonic bath.*—500 W output (see *Results and Discussion*).

(**f**) *Chromatographic system.*—Kratos FS 970 LC fluorometer (Kratos Analytical, Ramsey, NJ). Operating conditions: excitation wavelength 364 nm; 7-54 excitation filter; 418 nm emission filter; time constant set to 6; range set to 1.0 or 0.5 μA. Analytical column: Econosil C_{18}, 5 μm, 4.6 × 250 mm (Alltech Associates, Inc., Deerfield, IL) or Zorbax ODS 4.6 × 150 mm (DuPont, Wilmington, DE); guard column: Brownlee OD-GU, or equivalent (Applied Biosystems, Inc. San Jose, CA), ambient temperature to 30°C; 50 μL injection.

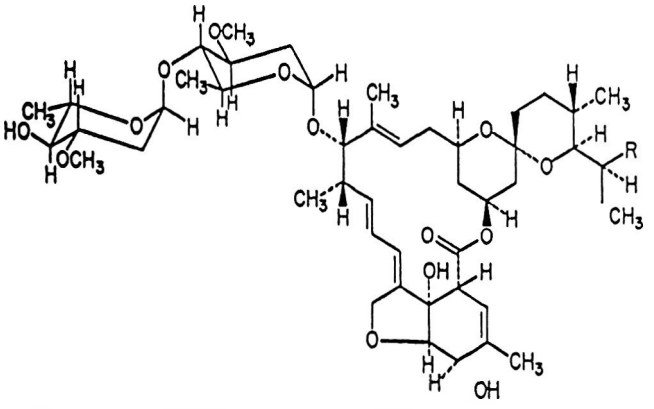

Figure 1. The structure of ivermectin: R = C_2H_5 for H_2B_{1a}, and R = CH_3 for H_2B_{1b}.

Pump: capable of isocratic flow at 1.8–2.0 mL/min (see *Results and Discussion*).

(g) *Water bath.*—Temperature controlled to 50–80°C.

(h) *Dispensers.*—0–10 mL, Repipet, or equivalent (Labindustries, Berkeley, CA).

(i) *Tape.*—1/2 in., Ace Scientific No. 132956, or equivalent (Ace Scientific, East Brunswick, NJ).

(j) *Mixer.*—Vortex, or equivalent (Baxter, McGaw Park, IL).

Reagents

(a) *Solvents.*—LC grade acetonitrile, chloroform, ethyl acetate, hexane, isooctane, methanol, and water. Reagent grade acetic anhydride, ammonium hydroxide, and DMF. Technical grade methylene chloride, acetone, and toluene; 95% USP grade ethanol; and 99% 1-methylimidazole.

(b) *LC mobile phase.*—Water–methanol (95 + 5), premixed (see *Results and Discussion*).

(c) *Silylation reagent.*—Sylon-CT, Supelco, or equivalent (Supelco).

(d) *Derivatizing reagent.*—Mix 0.9 mL DMF, 0.3 mL acetic anhydride, and 0.2 mL 1-methylimidazole, in that order, immediately before use.

Ivermectin Standard Solution

(Ivermectin standard solutions are stable in methanol up to 1 year at −20°C.)

(a) *Analytical standard.*—1.38% H_2B_{1a} in propylene glycol (available from Merck Chemical Division, Merck & Co. Inc., Rahway, NJ 07065).

(b) *Stock standard solution.*—Weigh 0.36 g H_2B_{1a} analytical standard into 50 mL volumetric flask, and dilute to volume with methanol (100 µg/mL).

(c) *Working standard solution.*—Dilute 0.5 mL stock standard H_2B_{1a} solution to volume with methanol in 100 mL volumetric flask. Use this 500 ng/mL solution to prepare fortified milk samples and working standards to generate standard curve. Prepare working standards by using 10, 20, 40, and 80 µL 500 ng/mL solution as described under *Derivatization of Ivermectin.* The working standards are equivalent to 0.5, 1.0, 2.0, and 4.0 ng H_2B_{1a}/mL in milk, respectively.

Glassware Cleaning and Silylation Methods

(a) *Preliminary glassware treatment.*—Soak all glassware overnight in 2 µg/mL H_2B_{1a} in methanol (see *Results and Discussion*).

(b) *Glassware cleaning.*—Wash with detergent, then chromic acid, followed by thorough washing with distilled water. (Trace amounts of acid, alkali, and detergent can lead to loss of ivermectin.)

(c) *Silylated tube preparation.*—Fill 15 mL centrifuge tube with silylation reagent and let stand 20 min. Immediately rinse with toluene followed by methanol. Soak in additional methanol 20 min, rinse with acetone, and dry. Repeat silylation method every 2 months.

(d) *Silylated tube cleaning.*—Soak tube in methylene chloride and then in detergent several hours each. Rinse several times each with hot tap water and then distilled water, and rinse with acetone. Clean freshly silylated tube twice by this method before first use. Do not machine-wash.

Sample Treatment

(a) *Storage.*—Store milk samples in 50 mL polypropylene centrifuge tubes at 0°C or below. (Concentration of H_2B_{1a} in dosed milk did not decrease after 1 year in storage.)

(b) *Preparation of fortified sample.*—Add appropriate amount of working standard to milk and vortex mix before beginning analysis.

(c) *Sample extraction.*—Mix 10 mL milk with 2.5 mL concentrated ammonium hydroxide in 50 mL centrifuge tube. Add 10 mL ethanol, 10 mL ethyl acetate, and 10 mL isooctane sequentially, shaking mixture 30, 30, and 20 s after each respective addition. Centrifuge 4 min to separate liquid phases; transfer upper layer to clean 50 mL centrifuge tube with Pasteur pipet. Take care not to transfer any of the lower layer. Add additional 10 mL ethyl acetate and 10 mL isooctane to first tube, shaking mixture as above; repeat centrifugation. Combine top layers in second tube, and evaporate solvents at 70°C under nitrogen. Dissolve remaining oil in 3 mL hexane by briefly vortexing and sonicating sample. Add 4 mL acetonitrile to tube. Shake tube 1 min, centrifuge 4 min to separate liquid phases, and transfer lower acetonitrile layer to silylated test tube. Repeat acetonitrile extraction. Shake combined acetonitrile extracts 1 min with 1 mL hexane, centrifuge 4 min, and discard hexane. Take care to remove entire hexane layer. Evaporate acetonitrile to dryness under nitrogen at 70°C.

Derivatization of Ivermectin

(Less than 0.05 mL residue should remain for each sample before derivatization. Excess residue, methanol, and atmospheric moisture can lead to poor derivatization reaction. Excess residue in sample is caused by incomplete removal of isooctane and hexane layers during sample preparation.)

For preparation of standards, add amounts of 500 ng/mL working standard in methanol as given above to silylated centrifuge tubes and evaporate to dryness. Using 1 mL measuring

Table 1. Percent recovery of H_2B_{1a} from fortified and control bovine milk

Added, ng/mL	Laboratory A	Laboratory B	Laboratory C
0	ND[a]	ND	ND[b]
0	0.17[c]	ND	ND
0	ND	ND	ND
0	0.09[c]	ND	ND
0	ND	ND	ND
1	78	62	94
1	84	47	101
1	94	55	99
1	103	73	98
1	77	58	85
2	93	67	96
2	84	67	99
2	88	59	96
2	94	50	99
2	93	NS[d]	92
4	86	73	99
4	89	68	101
4	85	80	99
4	99	72	96
4	92	NS	99

[a] ND = Not detected.
[b] The analyst at Laboratory C noticed a slight response at the retention time of H_2B_{1a} in all 5 control samples.
[c] Concentration measured in control sample (ng/mL).
[d] NS = No sample.

pipet, add 0.1 mL freshly prepared derivatizing reagent to samples and standards. Stopper tubes, vortex briefly, and centrifuge for a few seconds at slow speed. Place tubes in 95°C oil bath for minimum of 1 h. Solution will turn a tar-black color. Cool tubes until they are warm to touch, add 1 mL chloroform to each tube, and vortex. Elute chloroform on silica SPE tube prewashed with 4 mL chloroform. Wash each centrifuge tube with three 1 mL portions of chloroform and elute each wash through SPE tube. Wash SPE tube with additional 2 mL chloroform, collecting all eluates. Evaporate chloroform to dryness at 60°C under nitrogen; dissolve residue in 0.5 mL methanol for analysis.

Table 2. Determination of H_2B_{1a} (ng/mL) in dosed milk samples

Laboratory A	Laboratory B	Laboratory C
4.37	4.84	4.81
4.12	4.01	4.69
4.29	3.67	4.99
4.58	3.38	4.25
4.24	2.36	4.78

Generation of Standard Curve and Calculation of H_2B_{1a} Concentration in Milk

Calculate slope, a, and intercept, b, of standard curve by linear regression analysis of standards based on peak height of H_2B_{1a} derivative peak, using $y = ax + b$, where y = peak height and x = concentration of H_2B_{1a} standard in ng/mL. Use peak height of H_2B_{1a} measured for milk sample to calculate concentration injected as follows:

$$H_2B_{1a}, \text{ng/mL (injected)} = (\text{peak height} - b)/a$$

Calculate concentration of H_2B_{1a} in milk sample as follows:

$$H_2B_{1a}, \text{ng/mL (sample)} = H_2B_{1a}, \text{ng/mL (injected)}/20$$

Results and Discussion

Each participating laboratory analyzed 5 control milk samples, 5 fortified milk samples at each of 3 levels (1.0, 2.0, and 4.0 ng/mL), and 5 dosed milk samples. Table 1 gives the results of the analysis of the control and fortified samples at the 3 participating laboratories. The recoveries averaged 87, 59, and 95% at 1 ng/mL; 90, 61, and 96% at 2 ng/mL; and 90, 73, and 99% at 4 ng/mL in Laboratories A, B, and C, respectively. The coefficients of variation (CVs) were 11.3, 16.6, and 6.5% at 1 ng/mL; 5.0, 13.4, and 3.1% at 2 ng/mL; and 6.5, 7.1, and 1.9% at 4 ng/mL, respectively.

Table 2 contains the results of the analysis of dosed milk. Chromatograms of dosed and control milk samples are shown in Figure 2. The dosed milk contained an average of 4.3, 3.7, and 4.7 ng H_2B_{1a}/mL as determined by Laboratories A, B, and C, respectively. The respective CVs were 4.0, 24.8, and 5.9%. In the analysis of the dosed milk sample provided by the developer of the method, the average concentration was 4.1 ng H_2B_{1a}/mL with a CV of 4.4%. The samples were reassayed after a year in storage at –20°C and found to have no significant change in H_2B_{1a} concentration (unpublished data, Frank Schenck, FDA, Baltimore District Office, Baltimore, MD).

The interlaboratory CVs were 22.7, 19.4, and 12.9% for the samples fortified at 1, 2, and 4 ng/mL, respectively. The interlaboratory CV for the dosed milk samples was 16.2%. The interlaboratory CVs are within the range expected for a method designed to measure residues at the low nanogram/milliliter level (6).

Laboratory B initially reported recoveries of 39, 18, 29, 56, and 34% at 1 ng/mL. Closer examination of the data revealed that the standard curve used to calculate the recoveries at this level was not linear, r = 0.980. Laboratory B had used 5 working standards instead of 4 working standards as specified above to generate the standard curve. It was noted that the 2 high standards, equivalent to 3.75 and 5 ng/mL, clearly generated less signal per ng of H_2B_{1a} than did the 3 low standards. By omitting the 2 highest of the 5 standards in calculating the standard curve, a linear curve was generated, r = 0.999. This curve was used to calculate the recovery data given in Table 1. Laboratory B analyzed only 4 samples at the 2 and 4 ng/mL levels because of problems with the derivatization reaction.

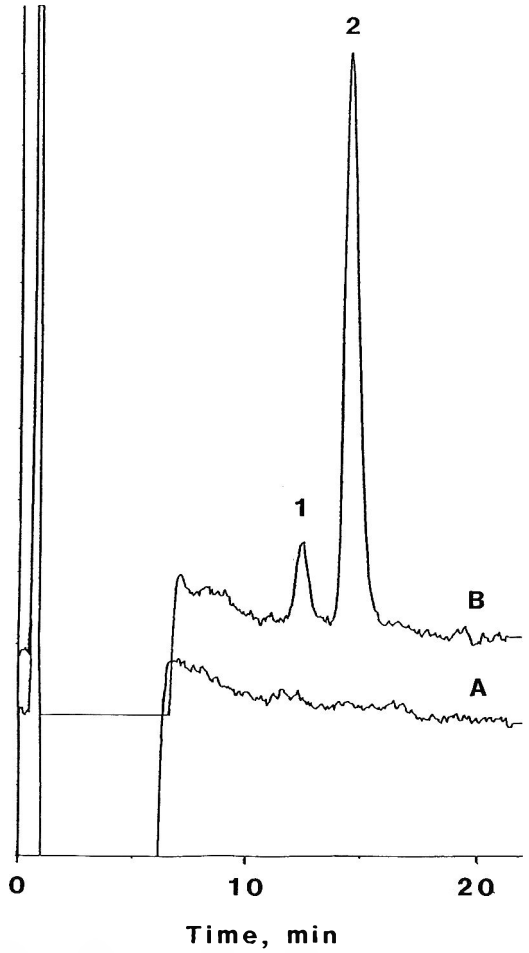

Figure 2. Chromatogram of (A) control milk and (B) ivermectin-dosed milk containing approximately 4 ng H_2B_{1a}/mL. Peak 2 in chromatogram B is the H_2B_{1a} fluorescent derivative used for quantitation; Peak 1 is the fluorescent derivative of H_2B_{1b}. The zero base line between 1 and 6 min in each chromatogram is caused by the detector automatically turning off at 1 min because of excessive signal. The detector was manually reset at 6 min.

At the retention time of H_2B_{1a}, the control milk contained no interferences greater than those allowed under the method guidelines of the FDA Center for Veterinary Medicine, namely, 10% of the value obtained from the determination of a standard at the target level. For H_2B_{1a}, the target level was 2 ng/mL. The chemist in Laboratory C noted a minor peak at the retention time of ivermectin in all the control milk samples, but the peaks were not large enough to quantify. Two of the 5 control milk samples analyzed in Laboratory A also showed a minor peak at the retention time of ivermectin. The largest of these peaks had a height of less than 10% of that obtained with an H_2B_{1a} standard of 2.0 ng/mL, the target level. This sample was the only one of the 15 control samples that had a significant interference at the retention time of H_2B_{1a}.

The chemist in Laboratory B had problems with an incomplete reaction when preparing the fluorescent derivative of H_2B_{1a}. It was subsequently determined that the problem was probably caused by the presence of excessive residue after the sample extraction. This excess, together with the loss of 2 samples as noted above, may partly explain the lower recoveries observed at this laboratory.

The method recommends the use of a 500 W ultrasonic bath. Laboratory A did not have this equipment available and used a 185 W ultrasonic bath instead. No problems were encountered; however, the method states that, in the past, the use of ultrasonic baths of lower power had proved to be unacceptable. Care must be taken to ensure that the recovery of H_2B_{1a} is reproducible if a low-power ultrasonic bath is used.

It is recommended in the method that glassware be soaked in 2 μg/mL H_2B_{1a} solution before use. In the past, the use of untreated glassware was found to lead to the loss of H_2B_{1a}. Probably, H_2B_{1a} binds irreversibly to active sites on the glassware. However, in this trial, the analyst in Laboratory C did not pretreat the glassware and obtained acceptable results.

Several other observations about the method were noted by the analysts. The use of the proportioning capabilities of an LC pump to mix the solvents led to unacceptable variations in the retention time of H_2B_{1a}. One analyst noted a problem with column overload after a relatively few injections. This problem was not seen in the other 2 laboratories.

This method is time-consuming and must be carefully followed to ensure good results. Even small amounts of residue from improperly prepared samples can lead to low recoveries because of incomplete formation of the fluorescent derivative. However, when used properly, the method can quantify very low levels (1 ng/mL) of ivermectin in bovine milk.

Acknowledgments

The author thanks the following for their contributions: Frank Schenck, FDA, Baltimore District Office, Baltimore, MD, for analysis and preparation of the dosed milk samples used in the trial; H.F. Righter, Animal Nutrition and Biology Branch, FDA, Beltsville, MD, who generated the dosed milk used in this study; Roger Burkepile, Denver District Office, FDA, Denver, CO, and Richard Thompson, Minneapolis District Office, FDA, Minneapolis, MN, for participating in this trial.

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TECHNICAL COMMUNICATIONS

Rapid Analysis for Ivermectin Residue in Liver and Muscle Tissue by Liquid Chromatography

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A rapid procedure has been developed for the isolation and determination of ivermectin in liver and muscle tissue. The analyte is extracted from the target tissue with isooctane and isolated by coagulation of extraneous matrix residue at reduced temperature. A fluorescent derivative is prepared and analyzed by liquid chromatography. Recoveries of ovine, bovine, and porcine liver and muscle tissues fortified within a 0–60 ppb range averaged 75%, with a 12.7% coefficient of variation.

The use of the antiparasitic animal drug ivermectin is monitored by the Food Safety and Inspection Service, an agency of the U.S. Department of Agriculture (1). The agency tests 4000–5000 domestic and imported animals annually for the presence of the drug. The method of testing was provided by the manufacturer as part of a New Animal Drug Application (NADA). The method is reliable, but is also labor-intensive because it incorporates 6 partitioning steps (2).

The method described here addresses the need for a faster screening test to process the large number of samples generated by the monitoring program. It is a modification of the manufacturer's NADA method. The partitioning steps have been replaced by a freezing technique. The reduction of manipulations has increased productivity 300% without affecting reliability.

Experimental

Apparatus

(a) *Laboratory blender and control.*—Cat. No. 3392-C10 and 3392-C40 (Thomas Scientific, Swedesboro, NJ 08065-0099), or equivalent.

(b) *Meat grinder.*—Model No. 4612 (Hobart Corp., Troy, OH 45373), or equivalent.

(c) *Polypropylene tube, 50 mL.*—No. 222-3937-G80 (Evergreen Scientific, Los Angeles, CA 90058), or equivalent.

(d) *Mechanical shaker and box carrier.*—Eberbach 610 shaker and 6040 box carrier, Cat. No. 8287-C30 and 8287-J20 (Thomas Scientific), or equivalent.

(e) *Centrifuge.*—Sorvall T 6000B (DuPont Co., Newtown, CT 06470), or equivalent.

(f) *Pipet aid.*—Cat. No. 4-00-110 (Drummond Scientific Co., Broomall, PA 19008), or equivalent.

(g) *Glass centrifuge tubes, 15 mL.*—Pyrex No. 8084 (Corning Glassworks, Corning, NY 14831), or equivalent.

(h) *Plug Tite caps.*—Cat. No. 127-0019-200 (Elkay Laboratory, Shrewsbury, MA 01545), or equivalent.

(i) *N-EVAP.*—Model No. 112 (Organomation Association, Inc., Berlin, MA 01503), or equivalent.

(j) *Ultrasonic bath.*—Model B5200R1 (Branson Ultrasonics Corp., Danbury, CT 06810-1961), or equivalent.

(k) *High-temperature oil bath.*—Model 160 high-temperature bath (Fisher Scientific, Fair Lawn, NJ 07410), or equivalent.

(l) *Solid-phase extraction (SPE) cartridge.*—Silica gel, No. 51900 (Waters Chromatography Div., Milford, MA 01757), or equivalent.

(m) *Autosampler vial.*—Cat. No. 54-45VC3 (Rainin Instrument Co., Woburn, MA 01801-4628), or equivalent.

(n) *Liquid chromatograph.*—M-6000A pump, Model 710B Wisp autosampler, Model CX4 column heater, Model 420A fluorescence detector (Waters Associates Inc.), or equivalent.

(o) *Precolumn.*—Brownlee Labs Spheri-5 RP-18, 30 × 4.6 mm, 5 μm (Applied Biosystems, Inc., Foster City, CA 94404), or equivalent.

(p) *Analytical column.*—Zorbax ODS 150 × 4.6 mm id (DuPont Co., Wilmington, DE 19898), or equivalent.

Reagents

(a) *Solvents.*—Acetonitrile, isooctane, acetone, chloroform; LC grade (Burdick and Jackson, Muskegon, MI 49442), or equivalent.

(b) *Water.*—Distilled.

(c) *Acetone–water extraction solution (1 + 1, v/v).*

(d) *Ivermectin standard.*—Cat. No. L$640,471-076P004 (Merck, Sharpe and Dohme Inc., Rahway, NJ 07065). (**Note**: The homologue ivermectin is shipped as a glycerol formulation.)

(e) *Ivermectin stock solution (125 μg/mL).*—Weigh 0.23 g glycerol formal (1.38%, w/w dihydro B_{1a}) solution, and dilute to 25 mL with methanol.

(f) *Ivermectin intermediate stock solution (25 μg/mL).*—Dilute 2 mL ivermectin stock solution to 10 mL with methanol.

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(g) *Ivermectin spiking solution (0.5 μg/mL).*—Dilute 2 mL ivermectin intermediate stock solution to 100 mL with methanol. (**Note**: 5 μL ivermectin spiking solution represents 1 ng/g.)

(h) *Cylon CT.*—Cat. No. 3-3065 (Supelco Inc., Bellefonte, PA 16823-0048), or equivalent.

(i) *Acetic anhydride.*—Cat. No. 2420-500 (Mallinckrodt Inc., Paris, KY 40361), or equivalent.

(j) *N,N-Dimethylformamide.*—Cat. No. D119-500 (Fisher Scientific), or equivalent.

(k) *1-Methylimidazole.*—Cat. No. M5, 083-4 (Aldrich Chemical Co., Inc., Milwaukee, WI 53233), or equivalent.

(l) *Derivatizing reagent.*—Mix 1-methylimidazole, acetic anhydride, and *N,N*-dimethylformamide (2 + 3 + 9, v/v) in a clean, dry 15 mL glass centrifuge tube with vortex mixer. Mix before use and discard unused portion.

(m) *Mobile phase.*—Methanol–distilled water (97 + 3, v/v).

Determination

Under isocratic conditions, equilibrate system by using mobile phase at flow rate of 1.8 mL/min. Set column heater at 30°C. Injection volume is 100 μL. Detector attenuation is 2; excitation wavelength is 360 nm; and emission wavelength is 425 nm. Recorder speed is 5 mm/min; recorder range is 50 mV; and run time is 20 min. Calculate concentration of ivermectin in each sample using the formula:

$$A/B \times D = C$$

where A is representational concentration of external standard in ng/g, B is peak height of external standard in mm, D is sample peak height in mm, and C is sample concentration in ng/g.

Sample Extraction

All tissue must be fresh or frozen. Discard rancid or putrid tissue. Liver tissue is blended to uniform consistency. Muscle tissue is ground to uniform consistency. Weigh 2.5 g prepared tissue into 50 mL polypropylene tube. Add an appropriate amount of ivermectin spiking solution to test samples used as recoveries. Add 15 mL acetone–water solution and 15 mL isooctane. Cap tube and shake by hand to disperse tissue. Shake 10 min by using mechanical shaker. Centrifuge 10 min at 2000 rpm. Transfer top isooctane solvent layer to clean, 15 mL glass centrifuge tube by using pipet aid and disposable pipet. Evaporate isooctane to ca 0.5 mL with an N-EVAP (60°C).

Re-extract sample and acetone–water solution 2 more times with 15 mL portions of isooctane as previously described. Combine all 3 extracts. Evaporate third and final 15 mL portion of isooctane completely.

Add 4 mL preheated acetonitrile (60°C) to residue in 15 mL glass centrifuge tube. Stopper tube and vortex mix. Return tube to N-EVAP bath for 5 min. Vortex mix tube to dissolve matrix residue and sonicate 2 min. Place tube in freezer (0°C) for 1 h.

Remove tube from freezer and centrifuge 5 min at 1500 rpm. Decant acetonitrile into clean, 15 mL glass centrifuge tube and discard sample residue. Completely evaporate acetonitrile by using an N-EVAP (60°C).

Prepare external standard by pipetting an appropriate amount of ivermectin spiking standard into clean, 15 mL glass centrifuge tube and evaporate methanol by using an N-EVAP (60°C). Add 150 μL derivatizing reagent to each tube, cap, vortex, and sonicate. Centrifuge tubes 1 min at 1500 rpm. Place tubes, submerged 2–3 cm, into high-temperature oil bath (96°C) for 1.5 h.

Remove tubes from oil bath and wipe off excess oil. Add 1 mL chloroform to each tube. Prewet silica SPE cartridge with 3–4 mL chloroform. Transfer contents of tube onto SPE cartridge, rinse tube with 3 mL chloroform onto SPE cartridge. Pass 8 mL chloroform through SPE cartridge after initial 4 mL has passed. Retain all chloroform eluates in clean, 15 mL glass centrifuge tube. Completely evaporate chloroform and take to final volume with 1 mL methanol. Cap, vortex, and sonicate tube. Place tubes in freezer (0°C) for 30 min. Remove cold tubes and centrifuge 5 min at 1500 rpm. Transfer to autosampler vials for liquid chromatographic (LC) determination.

Results and Discussion

This analysis is intended as a rapid screening method. Analyte isolation was simplified to increase speed. Liver samples, in particular, and muscle samples, to a lesser degree, produce a late-eluting artifact peak. The derivatization step produces early eluting artifact peaks in both tissues. Both types of interference can affect quantitation. However, there are critical control points that can maximize analytical performance.

It is very important to transfer only the isooctane layer during the tissue extraction step. To exclude the lower layer, transfer only 13–14 mL from each of the three 15 mL isooctane extraction phases. This is the most effective way to avoid late-eluting artifact peaks.

The elution rate for the SPE step is also important. The cartridges contain only 0.8 g silica gel and are sensitive to elution rate. A fast elution rate produces dirty samples; a slow elution rate leaves analyte retained on the column. The optimum rate is 3 mL/min.

The condition of the 15 mL glass centrifuge tubes affects performance. Silanizing all glass tubes before use makes them easier to clean and prevents adsorption of ivermectin by the reactive glass surface.

The derivatizing reagent is sensitive to alkaline and acidic residues; therefore, a rigorous cleaning regimen for the glass tubes should be used (3). Remove all sample residue by hand; do not machine wash tubes. Soak 2 h in methylene chloride and 2 h in detergent solution. Rinse with hot tap water, distilled water, acetone, and methanol before drying.

The late-eluting artifact peak previously mentioned has a retention time of about 60 min. The run time for each injection is 20 min. This run time causes the artifact peak to coelute with an injection front. These parameters can be changed if they do not work. Flush the LC system 40 min with 100% methanol after use to remove all late-eluting peaks.

This method is now being used by FSIS instead of the Tway method (2). The validation study done to establish the depend-

ability of this method used the same standards of performance as the NADA method it replaced [recovery range, 60–100%; coefficient of variation (CV), <20%]. The protocol for the study used liver and muscle tissue from bovine, ovine, and porcine species for a total of 60 tests per analyst. Tissues were spiked within a 0–60 ppb range. Individual analytical results for the 4 participating analysts were as follows: $\bar{x}$ = 75%, CV = 8.3%; $\bar{x}$ = 77%, CV = 13.7%; $\bar{x}$ = 74%, CV=13.3%; and $\bar{x}$ = 76%, CV=14.7%.

Acknowledgments

The author thanks the following laboratory members: Suzanne Moon, Beverly Barnett, and Teresa Spratlin for intralaboratory validation of the method, and Ralph Bakowski, Richard Dalrymple, and Sher Ali for enlightening discussions and suggestions.

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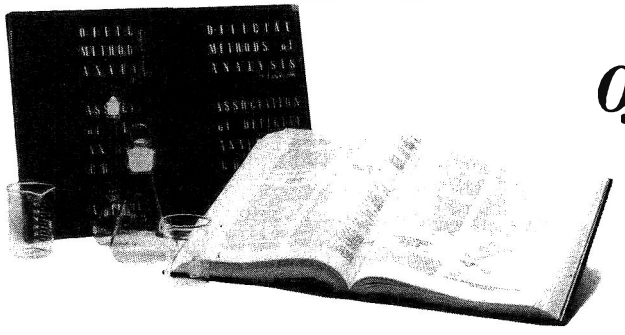

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REGULATORY ANALYTICAL METHODS

Introduction

Regulatory agencies approve and promulgate methods of analysis that regulate commodities in their markets. In many instances, these internally approved methods are not widely disseminated outside the agencies. In order to give a method a wider audience, an agency may submit it for publication in the *Journal of AOAC International*. This "Regulatory Analytical Methods" section in the Journal will provide select methods submitted by an agency. Final decisions to publish a method rests solely with the Editor-in-Chief of the Journal.

The methods published under this section have received approval by a national, state/provincial, or international regulatory agency or body and are used to regulate commodities that fall within the scope of the Journal. Each regulatory analytical method published contains its own introduction that explains the origin, nature, and approval that the particular method has undergone.

Having been approved by the regulatory agency submitting the method, the methods published under this section do not undergo the peer review accorded AOAC Journal articles before being published. These methods are ***not*** AOAC Official Methods of Analysis, since they have not been subjected to the full AOAC collaborative study process.

The intent of publishing these regulatory methods in the Journal is to give them a wider distribution and provide them with a publication reference. It is hoped that this section will be used by regulatory bodies of the world to disseminate their methods to scientists everywhere. They are not, however, meant to supplant the AOAC collaborative study process, and AOAC encourages all regulatory bodies and associated industries to support that process.

Animal Drugs

JOHN R. MARKUS and JOHN O'RANGERS
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The U.S. Food and Drug Administration (FDA) is required by the general safety provisions of sections 409, 512, and 706 of the Federal Food, Drug and Cosmetic Act to determine if each food additive, new animal drug, or color additive proposed for use in food producing animals is safe. The pertinent regulations implementing the statutory provisions are found in 21 CFR Parts 70 and 500.

The sponsor of an application for use of an animal drug is required as part of the approval to submit scientific data to demonstrate that the use of the drug is safe for the animals proposed and any edible food to be used for consumption. To demonstrate the safety of the drug, the sponsor must submit acceptable analytical methods. These methods must be capable of determining and confirming the amount and presence of the animal drug or its metabolites in a variety of matrixes.

FDA typically requires analytical methods for finished pharmaceutical and medicated feed dosage forms and for residues of the drug in edible animal tissues. The sponsor must present data or information that demonstrates the method can perform what it purports according to sound analytical principles. In addition, FDA performs a trial of the method according to strict procedures to assure the method is acceptable. The U.S. Drug Administration laboratories also participate with FDA in testing tissue residue methods.

The types of methods described above, because they are used for regulatory purposes by FDA in its compliance programs, are available for release to the general public. These methods are releasable under the Freedom of Information Act. 21 CFR 514.11(e)(6) specifically permits the public disclosure of assay methods after approval of the animal drug has been published in the *Federal Register*.

Because of the volume and types of feeds containing approved animal drugs and used for food-producing animals, the FDA Center for Veterinary Medicine (CVM) has made it a policy to make available to the public methods for animal drugs in Type A Medicated Articles (premixes) and the corresponding Type B and C Medicated Feeds and tissue residues. CVM is the unit responsible for evaluating the effectiveness and safety of animal drugs.

Before 1973, the methods were incorporated into a manual called the *Food Additives Analytical Manual*. This manual, which is no longer available, included methods for direct and indirect food additives and animal drugs. In 1985, CVM issued a new manual called *Animal Drug Analytical Manual*. This manual, often referred to as ADAM, contains only methods for animal drugs. The 1985 edition contained only 8 methods. The manual is published and distributed by AOAC International. Information for the manual is furnished by CVM.

Since 1985, there have been no updates to ADAM. CVM, in cooperation with AOAC International, is now introducing a new procedure for making methods more readily available. It is the intention of CVM in conjunction with AOAC International to first publish the methods in the *Journal of AOAC International* and then incorporate the same in a later update to ADAM.

Ivermectin

The following methods are approved regulatory methods to determine and confirm residues of ivermectin in cattle, swine, and sheep.

REGULATORY ANALYTICAL METHODS

Method I. Liquid Chromatography/Fluorescence Determination of Ivermectin in Animal Tissue and Plasma

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Avermectins, which are isolated from the mycelia of *Streptomyces avermitilis*, are a broad family of compounds that are potent parasiticides at very low dosage levels. Ivermectin is a mixture of 2 homologs, not less than 80% 22,23-dihydroavermectin B_{1a} (dihydro B_{1a} or H_2B_{1a}) and not more than 20% 23,23-dihydroavermectin B_{1b} (dihydro B_{1b} or H_2B_{1b}). Radiometabolism studies conducted at the Merck & Co., Inc., laboratories have demonstrated that H_2B_{1b} is generally metabolized more rapidly than H_2B_{1a}. In addition, these studies concluded that H_2B_{1a} is not rapidly metabolized and, therefore, is the major residue found at the proposed withdrawal times. 22,23-Dihydroavermectin B_{1a} is the marker substance for ivermectin. Radioactive depletion studies indicated liver as the logical choice for target tissue.

Regulation Information

Ivermectin is approved in the dosage forms and for use in animals listed in Table 1.

Tolerances: 21CFR556.344

Marker Residue: 22,23-Dihydroavermectin B_{1a}

Cattle and reindeer.—Target tissue, liver for both species; tolerance, 15 ppb in target tissue for marker residue (15 ppb in liver corresponds to a concentration for total residues of 50 ppb ivermectin in liver); safe concentrations for total residues of ivermectin in uncooked, edible tissues of both species are as follows: muscle, 25 ppb; liver, 50 ppb; kidney, 75 ppb; and fat, 100 ppb.

Swine.—Target tissue, liver; tolerance, 20 ppb in target tissue for marker residue (20 ppb in liver corresponds to a concentration for total residues of 75 ppb ivermectin in liver); safe concentrations for total residues of ivermectin in uncooked, edible tissues of swine are as follows: muscle, 25 ppb; liver, 75 ppb; kidney, 100 ppb; and fat, 100 ppb.

Received January 14, 1991. Accepted December 4, 1991.
[1] Current address: 1709 Burning Tree Dr., Vienna, VA 22182.

Sheep.—Target tissue, liver; tolerance, 30 ppb in target tissue for marker residue (30 ppb in liver corresponds to a concentration for total residues of 125 ppb ivermectin in liver); safe concentrations for total residues of ivermectin in uncooked, edible tissues of sheep are as follows: muscle, 25 ppb; liver, kidney, and fat, 125 ppb.

Method I. Liquid Chromatography/Fluorescence Determination of Ivermectin in Animal Tissue and Plasma

Scope

Ivermectin residues are determined in liver, muscle, kidney, fat, and plasma from cattle, sheep, and swine (1).

Principle

The tissue sample is homogenized with acetone–water (1 + 1, v/v). The dihydro B_{1a} is extracted from the homogenized tissue with isooctane. Following removal of the isooctane, methanol is added, and the sample is chilled overnight to congeal the fat. Methanol is separated from the fat and evaporated. Solvent–solvent distributions from hexane into acetonitrile and then into hexane from acetonitrile–water are performed. Solvent is removed, and fluorescence is produced by heating at 95°C with 1-methylimidazole reagent. After chloroform is added, the reaction mixture is washed through a silica gel Sep-Pak cartridge, the solvent is removed, and liquid chromatographic (LC) analysis using fluorescence detection is performed on the residue dissolved in methanol. The fluorescent derivative is produced by the reaction shown in Figure 1. Figure 2 is a flow diagram of the tissue residue assay procedure.

The following is a possible assay schedule that allows an analyst to complete 11 samples and 1 recovery analysis in 2 working days. *Day 1.*—Grind 11 samples plus 1 control fortified with an appropriate level of standard. Complete isooctane extractions, evaporations, and dissolution in methanol. Place in freezer to cool. (Point A in Figure 2.) *Day 2.*—Do the acetonitrile, hexane, and water extractions and evaporations. Make derivative, wash through Sep-Pak cartridge, and dissolve for LC. (Point C in Figure 2.) *Day 3.*—Same as Day 1. Also, run LC

Table 1. Approved Ivermectin dosage forms and animal species

Regulation (21CFR)	Dosage form	Animal
520.1192	Paste	Horses, cattle
520.1193	Tablets and chewable cubes	Dogs
520.1194	Drench	Sheep
520.1195	Liquid (oral)	Horses
522.1192	Injection	Horses, cattle, reindeer, swine
522.1193 (combination with clorsulon)	Injection	Cattle

for samples from Day 2 and calculate results. If a fortified sample is not included, the schedule allows analysis of 6 samples/day.

Limit of Reliable Measurement

The determinative method has been validated by Merck Sharp & Dohme with recovery studies of dihydroavermectin B_{1a} in liver, kidney, muscle, and fat of cattle and sheep spiked at 9.7–97 ppb. U.S. Food and Drug Administration (FDA) validation studies were performed at the 15 ppb level in fortified control cattle liver and the 30 ppb level in sheep liver. The limit of detection of the method is 1–2 ppb.

Apparatus

(All references to commercial apparatus and chemicals in this section are for descriptive purposes only and do not constitute endorsement or recommendation of a product by FDA and the U.S. Government; equivalent products may be substituted.)

(**Note**: Glassware should be completely free of all acidic and alkaline residues.)

(**a**) *Balance.*—Analytical, capable of weighing 1 mg accurately.

(**b**) *Balance.*—Capable of weighing 5 g accurately into an approximately 60 g cup.

(**c**) *Water bath.*—With variable temperature range from 40 to 80°C.

(**d**) *Oil bath.*—95–100°C.

(**e**) *Centrifuge.*—IEC Model HN-S-II, with 6-place rotor IEC No. 958 and 15 and 50 mL cups.

(**f**) *Centrifuge tubes.*—Glass, 15 and 50 mL, with polyethylene stoppers.

(**g**) *Centrifuge tubes.*—50 mL polypropylene (Corning No. 25331) for storing standard solutions.

(**h**) *Centrifuge tubes.*—15 mL, silylated approximately once every 2 months, to be used only for the derivatization reaction. Select tubes with stoppers that fit tightly. Fill each tube to the top with Sylon-CT. Let stand 20 min. Immediately and quickly rinse thoroughly, first with toluene and then with methanol. Fill with methanol. Let stand 20 min. Discard methanol. Rinse thoroughly with acetone and dry. Clean tubes by hand by first soaking in methylene chloride immediately after use and then in detergent for at least 3 h, followed by thorough rinsing with hot water, distilled water, and acetone before thorough drying. Variations in the washing regimen are not recommended, because poor results can occur when the above standard washing method is not followed.

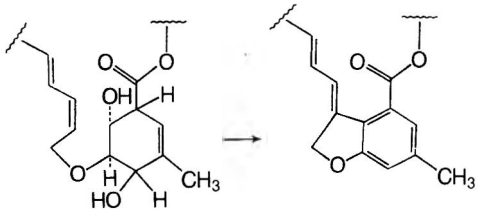

Figure 1. Reaction for production of fluorescent derivative.

(**i**) *Dispensing pipettors.*—10, 15, and 20 mL.

(**j**) *Gloves.*—Disposable PVC (Fisher).

(**k**) *Freezer.*—Capable of reaching temperatures of –20°C.

(**l**) *Graduated cylinder.*—25 mL.

(**m**) *Pipets.*—Disposable.

(**n**) *Pipets.*—Graduated, 1.0 and 2.0 mL.

(**o**) *Parafilm.*—American Can Co.

(**p**) *Pipets.*—Volumetric, 0.5, 1, 2, 3, 4, and 5 mL.

(**q**) *Reciprocating shaker.*—Variable speed (Eberbach, J.T. Baker Catalog No. 8278-E30).

(**r**) *Sep-Pak silica cartridges.*—Waters Associates No. 51900.

(**s**) *Omnimixer.*—With 50 mL stainless steel cups and corresponding blades (Sorvall Model 17105).

(**t**) *Spatula.*—Stainless steel.

(**u**) *Syringes.*—50 and 250 µL and 5 mL.

(**v**) *Tape.*—0.5 in. (Ace Scientific No. 13 2956).

(**w**) *Ultrasonic bath.*—Sonogen automatic cleaner (Bronson Model 520).

(**x**) *Vortex mixer.*

(**y**) *Liquid chromatograph.*—Consisting of a Beckman-Altex Model 110A pump, Valco sample valve with syringe loading sample loop or Waters Wisp autosampler, a Kratos-Schoeffel Instruments Model FS950 or FS970 fluorescence detector, and a 1 mV recorder.

(**z**) *LC column.*—15 cm × 4.6 mm id, Zorbax ODS-C_{18} (E.I. duPont de Nemours & Co.).

(**aa**) *Guard column.*—5 µm, 4.6 mm id, C_{18} standard Brownlee Labs guard column (Spheri-5 RP-18 OD-GU, obtained from Raman Instrument Co.). Place guard column before analytical column. Replace monthly or when pressure reaches 2000 psi.

Reagents and Solutions

(**a**) *Acetic anhydride.*—Reagent grade (J.T. Baker).

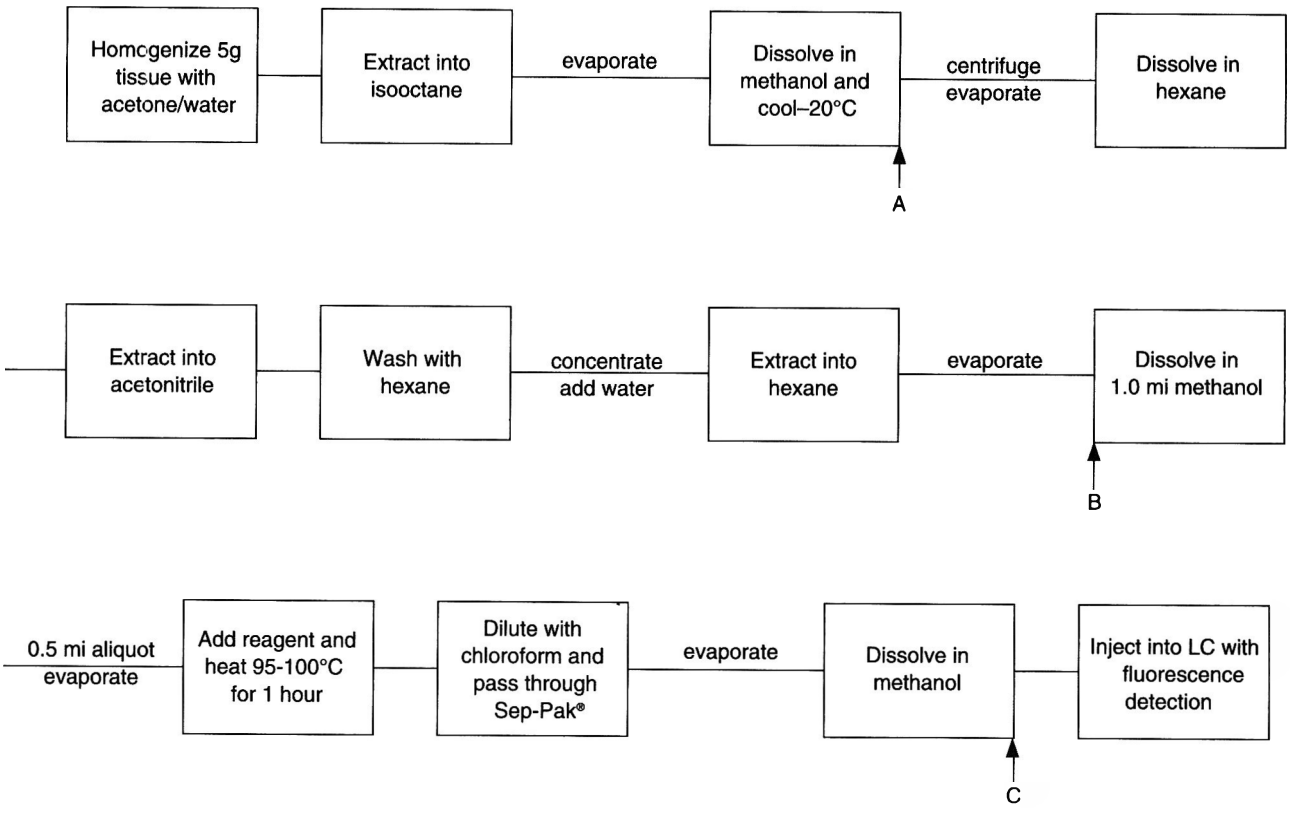

Figure 2. Flow diagram of ivermectin tissue assay.

(**b**) *Acetone.*—Nanograde (Mallinckrodt).

(**c**) *Acetonitrile.*—LC grade (Fisher) or nanograde (Mallinckrodt).

(**d**) *Chloroform, ethyl acetate.*—Distilled-in-glass (Burdick and Jackson Laboratories). (**Caution**: Chloroform is a carcinogenic agent; use reagent carefully.)

(**e**) *Dimethylformamide (DMF).*—Reagent grade (J.T. Baker).

(**f**) *Hexane, methanol.*—Distilled-in-glass (Burdick and Jackson Laboratories), or nanograde (Mallinckrodt).

(**g**) *Methylene chloride.*—Any grade available.

(**h**) *1-Methylimidazole.*—99% (Aldrich).

(**i**) *1-Methylimidazole derivating agent.*—Mix 0.2 mL 1-methylimidazole with 0.3 mL acetic anhydride and 0.9 mL DMF. Prepare just before use.

(**j**) *Nitrogen.*—Extra-dry compressed gas (Matheson).

(**k**) *Sodium chloride.*—Reagent grade (J.T. Baker).

(**l**) *Isooctane.*—Nanograde (Mallinckrodt).

(**m**) *Water.*—Double-distilled. Redistill distilled, deionized water in an all-glass apparatus.

(**n**) *Acetone–water (1 + 1, v/v).*—Mix equal volumes of nanograde acetone and double-distilled water.

(**o**) *Methanol–water.*—Dilute 100 mL double-distilled and filtered water to 2 L with methanol. De-aerate 10 min by slowly bubbling nitrogen through the mixture.

(**p**) *Sylon-CT.*—Supelco.

(**q**) *Analytical standards for tissue and plasma.*—For the most sensitive scale, add accurate aliquots of 50, 100, 150, 200, and 250 µL of a 500 ng/mL standard solution of dihydro B_{1a} in methanol to silylated 15 mL centrifuge tubes. Evaporate liquid in tubes to dryness at 60°C with a nitrogen purge. After reaction and Sep-Pak treatment, etc., redissolve samples in 1 mL methanol to give 25, 50, 75, 100, and 125 ng/mL standard solution equivalents for use on the 0.2 scale. For samples expected to contain higher amounts of dihydro B_{1a}, use larger amounts of standard and less sensitive fluorescence scales. Generally, unknown samples in the part-per-million range are diluted before the LC step to fit the 25–125 ng/mL scale.

Starting with solutions of ca 0.045% (w/w) dihydro B_{1a} in propylene glycol, prepare stock solutions of ca 10 µg/mL by weighing accurately ca 1 g solution into a 50 mL volumetric flask. Dilute to mark with methanol and thoroughly mix. Store this stock solution and all dilutions of it in polypropylene tubes at –20°C (Note 1, *Notes*).

Analysis

Analysts attempting the ivermectin residue analysis for the first time are advised to conduct a trial run and prepare a standard curve before attempting the complete method.

Safety precautions.—The procedure described is not intrinsically difficult or dangerous to accomplish. Nevertheless, because ivermectin is a known weak teratogen, analysts should wear protective gloves at all times while performing the method. Solvent transfers are best made in a well-ventilated hood. When homogenizing liver samples, a proper shield

should be in place. All homogenizations of nonaqueous solvents should always be performed surrounded by an ice-water bath.

Isolation procedures for liver, muscle, and kidney.—Accurately weigh 5.0 g tissue into 50 mL Sorvall homogenizer cup. Add 15 mL acetone–water (1 + 1, v/v) into cup. Homogenize 2 min. Pour sample carefully and as completely as possible into 50 mL centrifuge tube. Wash off cup and blades with small portions of isooctane until a total of 15 mL isooctane is used (see Note 2, *Notes*). Collect washing in centrifuge tube.

Stopper tube. Shake well for 1 min. Centrifuge 10 min. Transfer upper (isooctane) layer to second 50 mL centrifuge tube with disposable pipet. Completely avoid lower layer. Break up plug by using vortex mixer and/or shaking. Add 15 mL fresh isooctane, and repeat extraction combining isooctane layers. Evaporate combined isooctane layers down to a small volume using 80°C bath and nitrogen purge. Repeat extractions with 2 more 15 mL isooctane portions, adding isooctane in each case to tube that had previous 2 isooctane layers. Again, evaporate down as far as possible by using 80°C bath and purge. Add 6 mL methanol and dissolve or resuspend residue completely with ultrasonic bath and/or vortex mixer. Place samples in refrigerator until thoroughly cooled. At this point, sample is best left overnight in refrigerator. Sample can be stored up to 4 days.

Centrifuge cold 50 mL tube for 5 min. Decant off clear supernatant to fresh 15 mL centrifuge tube. Wash residue in 50 mL tube with 1 mL methanol by using vortex mixer. Centrifuge. Decant off into same 15 mL tube. If solids are decanted off in either step, recentrifuge 15 mL tube and decant off into new 15 mL tube to get clear solution. For muscle only, do a further 2 mL extraction of sample, and add to combined methanol solutions.

Evaporate all methanol by using 60°C bath and nitrogen purge. Add 3 mL hexane to tube previously containing methanol by using ultrasound to remove all material from walls of centrifuge tube. Add 4 mL acetonitrile and repeat ultrasonic treatment briefly. Shake thoroughly. Centrifuge 5 min. Transfer lower (acetonitrile) layer to fresh 15 mL tube. Re-extract hexane layer with second 4 mL portion of acetonitrile. Combine acetonitrile layers. Extract combined acetonitrile layers with 1 mL hexane. Centrifuge to clear layers. Transfer upper (hexane) layer to waste by disposable pipet or by aspiration. Proceed to *Extraction of ivermectin from samples.*

Fat samples.—Accurately weigh 5.0 g tissue into 50 mL Sorvall homogenizer cup. Add 20 mL acetone–water and 10 mL isooctane to cup. Homogenize 2 min. Pour sample into 50 mL centrifuge tube. Take care not to lose sample. Use spatula to help effect transfer. Wash off cup and blades with small portions of isooctane until an additional 10 mL isooctane is used. Collect in centrifuge tube. Add 1 g solid sodium chloride to tube. Stopper tube. Shake well 1 min. Centrifuge 10 min. Transfer upper (isooctane) layer to second 50 mL centrifuge tube with disposable pipet. Completely avoid lower layer. Break up plug by using vortex mixer and/or shaking. Add 15 mL fresh isooctane and repeat extraction by combining isooctane layers. Evaporate combined isooctane layers down to ca 5 mL by using an 80°C bath and nitrogen purge. Repeat extractions with 2 more 15 mL portions of isooctane, adding isooctane in each case to tube that had previous 2 isooctane layers. Again, evaporate as far as possible by using 80°C bath and purge.

Add 2 mL hexane and 5 mL acetonitrile to hot and molten fat, and continue below, or freeze overnight in freezer at −20°C.

For samples frozen overnight, remelt in 80°C bath, and add hexane and acetonitrile the next day. Shake still-warm mixture thoroughly for ca 1 min. Centrifuge immediately. Cool. Use disposable pipet to transfer upper (acetonitrile) layer into a fresh 15 mL centrifuge tube. For horse and swine samples, the last drop of acetonitrile normally forms a bubble, which should be transferred as completely as possible by pulling at its center with the pipet. Sheep and cattle samples can be cooled briefly in ice water and the acetonitrile layer decanted off. Repeat melting and extraction with 5 mL acetonitrile and transfer into same 15 mL tube. Add 2 mL hexane to 15 mL tube. Shake thoroughly. Centrifuge. Transfer upper (hexane) layer to waste with disposable pipet or by aspiration. Proceed to *Extraction of ivermectin from samples.*

Extraction of ivermectin from samples.—Evaporate acetonitrile solution to ≤1.0 mL using ca 60°C bath and nitrogen flush. If acetonitrile is <1 mL, make up to 1 mL with fresh acetonitrile. Use ultrasound to get homogeneous mix, if necessary. Add 4 mL distilled water (2 mL for kidney samples) and 5 mL hexane. Shake ca 1 min. Centrifuge 5 min. Transfer upper (hexane) layer to fresh 15 mL centrifuge tube by using disposable pipet. Avoid lower layer. Repeat hexane extraction with 5 mL and then 4 mL, combining all 3 hexane extracts. Evaporate solution to dryness (or as near dryness as possible) by using 40°C bath and nitrogen purge. To obtain dryness, more heat may have to be applied to end of evaporation. Water bath may go up to ca 80°C at this point. Dissolve residue in exactly 1.0 mL methanol by using vortex mixer and ultrasonic bath. Mix thoroughly. Centrifuge 5 min.

Pipet exactly 5.0 mL supernatant into bottom of silylated 15 mL centrifuge tube. Evaporate off methanol carefully by using 60°C bath and nitrogen purge. Avoid spattering. Add 0.1 mL freshly made acetic anhydride–methylimidazole–DMF mixture to each tube and series of standard tubes by using a 1.0 mL measuring pipet. Stopper. Vortex mix briefly. Centrifuge 3 s. Tape stoppers in place, and put all samples and standards in well-stirred 95°C oil bath. Bottom of tubes should be ca 1 in. below oil surface. After 1 h, remove tubes. Wash oil off with acetone wash bottle. Cool briefly. (After cooling, samples can be stored in freezer at −20°C, if necessary.)

Add ca 1 mL chloroform to each tube and vortex mix. Wash Sep-Pak cartridge with 3–4 mL chloroform by using syringe to force liquid through. Add sample by disposable pipet to syringe, and force sample through cartridge. Wash centrifuge tube 3 times with 1 mL each of chloroform into syringe and through cartridge. Elute cartridge with further 8–9 mL chloroform (3, 3, and 2 or 3 mL). Collect all chloroform eluant, and leave Sep-Pak cartridge in 15 mL centrifuge tube. Evaporate chloroform with a 60°C water bath and nitrogen purge. Dry residue completely. Proceed to LC determination.

Table 2. LC operating conditions

Parameter	FS 950 detector	FS 970 detector
Excitation lamp	FSA 110, std 365 nm	Deuterium SFA 101
Standard Kratos flow cells	FSA 210	FSA 980
Excitation filter	FSA 403, 365 nm band filters	Corning 7–54 entrance filter with monochrometer set at 364 nm
Emission filter	418 nm	418 nm
Sensitivity	0.2 scale, 5.5 sensitivity (Lo background setting)	0.05 scale, 5.5 sensitivity (Hi position)
Time constant	about 6	about 6
Recorder	1 mV	1 mV

Plasma.—Pipet 5.0 mL plasma and 7.5 mL acetone into 50 mL centrifuge tube. Shake briefly. Let stand 15 min. Add by pipet 7.5 mL distilled water and 15 mL ethyl acetate. Shake moderately 1 min by hand. Shaking should be thorough enough to allow extraction but not violent enough to cause emulsions. (If less than 5 mL plasma is used, add 7.5 mL acetone, and, after the 15 min wait, add enough distilled water to make plasma plus water volume total 12.5 mL.)

Centrifuge at least 10 min. Transfer upper (ethyl acetate) layer to second 50 mL tube as thoroughly as possible by using a disposable pipet. Absolutely do not transfer any of lower phase. Thoroughly break up plug in bottom of first tube with wide end of disposable pipet. Repeat ethyl acetate extraction. Combine ethyl acetate layers.

Evaporate ethyl acetate completely with nitrogen purge and 75°C water bath. Pipet exactly 1.0 mL methanol into dry tube. Dissolve residue thoroughly by using an ultrasonic bath. (Methanol must contact all of the tube. Great care is needed to assure that everything is dissolved.)

Pour methanol solution into 15 mL centrifuge tube. Centrifuge at least 5 min. Pipet exactly 0.5 mL clear solution into bottom of silylated 15 mL centrifuge tube. Evaporate completely to dryness with nitrogen purge and 60°C water bath.

Add 0.1 mL imidazole reagent to samples and standards by using 1 mL graduated pipet. Stopper tubes. Vortex mix to dissolve residue. Centrifuge briefly. Tape stoppers. Place centrifuges (sample and standards together) in well-stirred 90°C oil bath for 1 h. Remove tubes and rinse off oil with acetone. Residue should be black. (If residue is not black, use remaining sample in 15 mL centrifuge tube to repeat derivatization with next set of standards.)

Add ca 1 mL chloroform to residue in each tube. Vortex mix thoroughly. Wash silica gel Sep-Pak cartridge with 3–4 mL chloroform by using a syringe to force liquid through. Add chloroform sample to syringe with disposable pipet, and force liquid through cartridge. Wash centrifuge tube 3 times with ca 1 mL each of chloroform; add chloroform sample to syringe, using the same disposable pipet used in the previous step, and force liquid through cartridge. Elute cartridge with a further 8–9 mL chloroform (3, 3, and 2 or 3 mL). Collect all chloroform eluent, and leave Sep-Pak cartridge in 15 mL centrifuge tube. Evaporate chloroform with 70°C bath and nitrogen purge. Residue must be completely dry. Proceed to LC determination.

Determinative Procedures

LC conditions.—Mobile phase, methanol–water (95 + 5, v/v); flow rate, 1.8 mL/min (usual pressure, 1000 psi; 500–2500 psi acceptable); column temperature, 30°C. See Table 2 for detector parameters.

The appropriate retention time of the dihydro B_{1a} derivative under these conditions is 14 min. A 10 ppb standard should give a peak height of ca 20% full scale deflection under these conditions.

Analysis of samples for tissues and fat.—Pipet 0.5 mL methanol (or other suitable quantity, V_1 in following equation) into tube. Use vortex mixer and ultrasound to completely dissolve residue. (Samples can be stored at this point in freezer at −20°C, if necessary.) Centrifuge briefly.

Inject 50 µL supernatant of each sample and standard into LC system. Measure peak heights at retention time of dihydro B_{1a} derivative as indicated by standards.

Plot standard graph of peak height vs ng/mL standard. Curve should be linear and should go through origin.

Read ng/mL for each unknown from graph and calculate concentration as follows:

$$\text{ppb} = \text{ng/mL} \times V_1/V_2 \times D/G = V_1/V_2 \times \text{ng/mL} \times D/5$$

where V_1 = milliliter solvent used to dissolve sample, V_2 = milliliter sample taken to make derivative (see *Extraction of ivermectin from samples*), D = dilution of sample at end of assay, or 1 if no dilution is made, and G = grams of sample taken, 5.

Analysis of samples for plasma.—Pipet exactly 0.5 mL methanol (or other larger volumes for high assay plasmas, V_1 in following equation) into tube. Use vortex mixer and ultrasound to completely dissolve residue. Centrifuge. Inject 50 µL supernatant from each sample and each standard, in turn, into LC system. Measure peak heights of dihydro B_{1a} derivative at retention time indicated by standards.

Plot standard graph of peak height vs ng/mL standard. Curve should be linear and should go through origin.

Read ng/mL for each unknown from graph and calculate concentration as follows:

$$\text{ng/mL for sample} = (\text{ng/mL})u \times V_1/V_2 \times D/V_3 = (\text{ng/mL})u \times x\ V_1/V_2 \times D/5$$

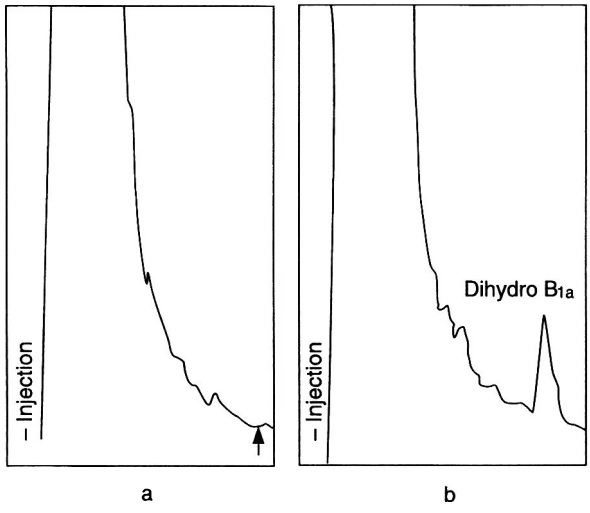

Figure 3. Typical chromatograms: (a) control cattle liver sample; (b) cattle liver containing 8 ppb dihydroavermectin B_{1a} for drawing base line for H_2B_{1a} peak.

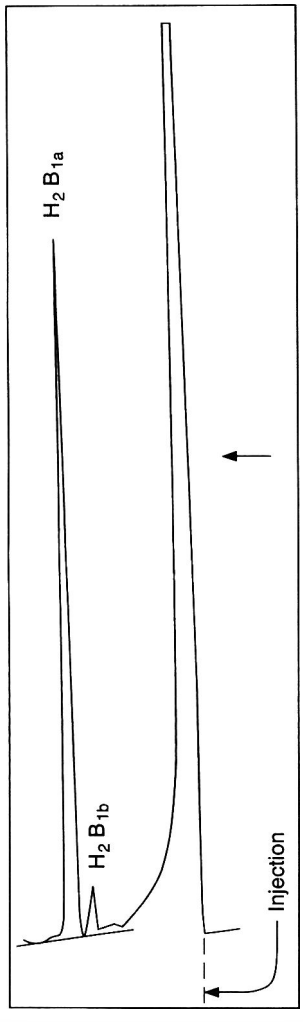

Figure 4. LC separation of dihydroavermectins B_{1a} and B_{1b}.

where (ng/mL)u = ng/mL read from standard curve, V_1 = mL solvent in which sample is dissolved, V_2 = mL aliquot taken in, see *Analysis, Plasma*, paragraph 2), D = dilution of sample at end of assay before LC, or 1 if no dilution is made, and V_3 = mL of sample taken, 5.

Typical chromatograms and details of LC analysis.—Figure 3 shows typical chromatograms obtained in the analysis. The method has a lower limit of reliable measurement of ca 10 ppb. At this level, a peak of ca 5 cm is generally observed, and reproducible quantitation can be readily achieved. The limit of detection is 1–2 ppb because discernible peaks are observed at these levels. At levels below 10 ppb, assay results are not reproducible enough to be totally quantifiable.

Figure 4 illustrates that the separations of H_2B_{1a} from H_2B_{1b}, under the specific LC conditions, are adequate to allow determination of both compounds, if desired. However, the usual practice is to determine only H_2B_{1a}. Also shown in Figure 4 is the method for drawing the base line for the H_2B_{1a} peak. For quantitation, peak height is measured from the base line to the apex of the curve.

Figure 5 shows chromatograms of spiked beef liver (top) and control liver (bottom) that were run for an extended period of time. In both chromatograms, the base line is essentially flat after elution of the solvent/junk peak and the ivermectin (top) peak for a period of 2 h after injection. Just beyond this period, peaks are seen in both chromatograms that can cause chromatographic problems unless they are carefully accounted for.

Additional samples can be run during the 2 h postinjection of the first liver sample with no interference from peaks due to this first sample. About 6 samples can be analyzed starting with the first liver sample with no interferences at the 3/h rate. Beyond 2 h, a different pattern emerges. Figure 6 demonstrates what can happen if the interference is ignored. Pictured are a set of H_2B_{1a} standards. Overlaid are 2 interference peak traces taken from Figure 5. The overlay at A2 would cause very little if any interference for the analyses on which it is overlaid. On the other hand, overlay A1 would obviously cause considerable interference. Therefore, to perform LC beyond the initial 2 h period requires careful planning. There are several ways to handle the potential interference. The LC peaks vary slightly in elution time from day to day, with slight variations in the mobile phase composition and with the aging of column and precolumn. A wait of more than 2 h between 2 consecutive LC assays could be necessary for the interferences to clear. This wait is usually unrealistic because of the very long assay times resulting.

When an automatic sampler like the WISP is used, a better solution involves running standards and samples for 2 h beyond the first liver injection, temporarily stopping injections but not the flow or recorder until the first interferences are visible. Then, the subsequent injections are fitted to the interferences. For example, to get chromatography like A2, start the automatic injector early enough so that the next injection occurs exactly at the top of the highest interference peak. Because the interferences are at constant times for a particular day, running at 20 min intervals will keep the peaks at a harmless pace for the next 6 samples. After the next 6 samples, the process is

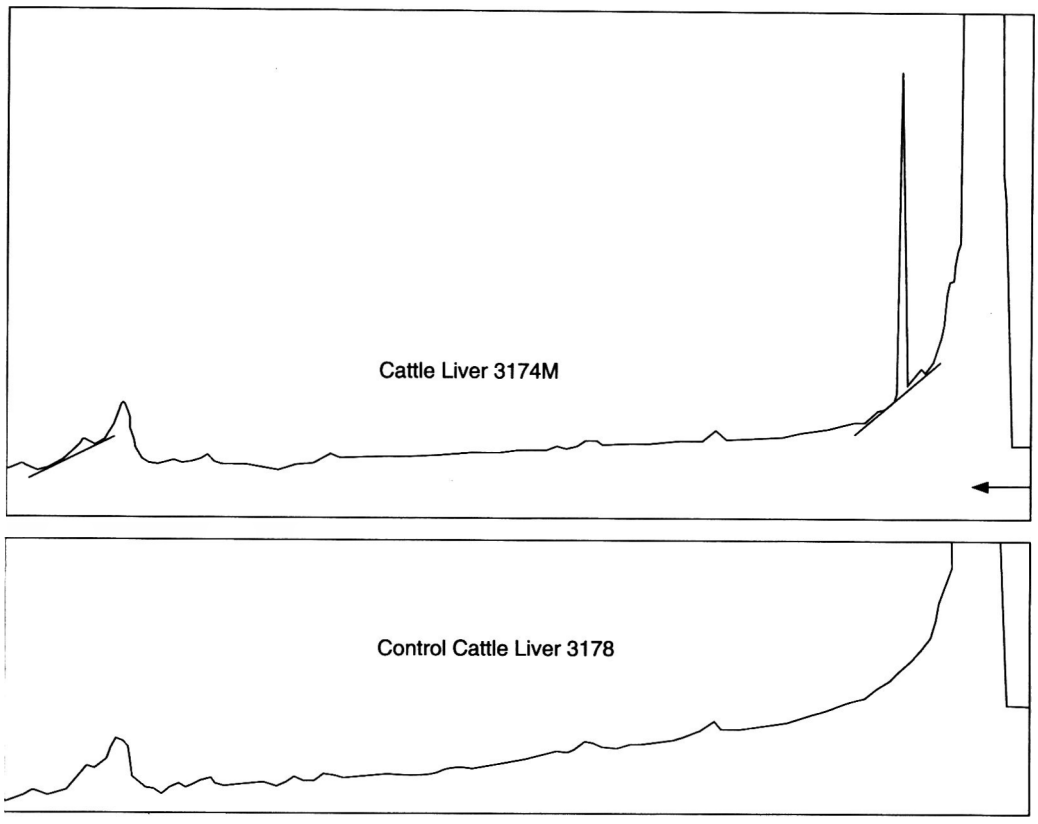

Figure 5. Chromatograms for liver and control liver samples.

repeated, or better still, the WISP is programmed to delay on the basis of the first 6-sample set. Obviously, similar things can be done with manual injection, or other solutions to the problem can be devised. Experience with Dupont 15 cm column indicates that interfering peaks always approximate 2 h, so that only a slight adjustment should be necessary. Also, the peaks presented are the worst case encountered; the problem normally would not be as acute.

Recovery procedures.—Analyze nonmedicated control tissue or plasma samples fortified with dihydroavermectin B_{1a} to determine recovery levels. Prepare spiked samples by adding small amounts of a methanol solution of H_2B_{1a} directly into the homogenization vessel on top of the tissue.

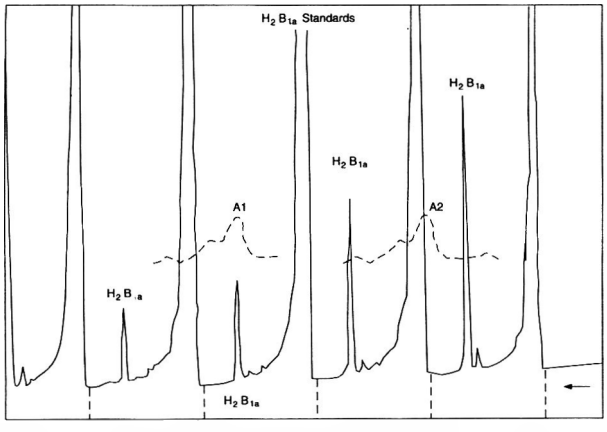

Figure 6. Illustration of possible interference with H_2B_{1a} analysis because of peaks eluting from earlier samples.

Confirmatory procedures.—Confirm the presence of ivermectin by the modified LC procedure described in Method II.

Reliability of the Method

Validation.—*(1) Cattle and sheep liver.* A validation study was conducted under the sponsor's new animal drug applications (NADA) 128–909 (for cattle) and 131–392 (for sheep). See Tables 3 and 4 for summary of results of the validation trial.

Nonmedicated cattle tissues were spiked with dihydroavermectin B_{1a} at levels of 9.7–97 ppb and immediately carried through the assay procedure in the Merck Sharp & Dohme research laboratories. The recoveries, expressed as the average ±1 standard deviation, were 84 ±5% for muscle, 81 ±8% for liver, 84 ±10% for kidney, and 82 ±9% for fat. The overall recovery for all fortified tissues was 83%. Similar recovery studies were run on sheep tissues. The recoveries averaged 83 ±10% for muscle, 79 ±6% for liver, 85 ±12% for kidney, and 81 ±7% for fat, with an overall recovery of 82% for all tissues. The assay worked equally well for all 4 tissues in both animal species. Similar results were also obtained with swine and horse recovery studies (2).

Data generated during the FDA trial of the determinative procedure are tabulated in Tables 3 and 4. Overall recoveries from control cattle liver fortified at the 15 ppb level averaged 72%, with an interlaboratory coefficient of variation (CV) of

Table 3. FDA/USDA validation data for recovery of Ivermectin from cattle liver (ppb)

	FDA		USDA, FSIS				
	Beltsville		Beltsville		Athens		Summary
Controls	0.0	0.0	0.0	0.0	—	—	—
	0.0	0.0	0.0	0.0	—	—	—
	0.0	—	0.0	—	—	—	—
			Fortified				
7.5 ppb	4.9	5.3	6.9	6.2	6.5	6.9	S = 0.687
	4.8	5.3	6.3	5.8	6.2	5.7	N = 15
	5.2	—	5.2	—	6.1	—	—
$\bar{X}$	5.1		6.1		6.3		5.8 (82%)[a]
CV, %	4.5		10.3		7.5		11.8
15 ppb	9.0	10.3	11.3	12.0	11.4	11.1	S = 0.972
	9.8	9.8	12.0	12.0	9.7	11.2	N = 15
	10.1	—	11.7	—	10.6	—	—
$\bar{X}$	9.8		11.8		10.9		10.8 (72%)[a]
CV, %	5.1		2.8		7		9.0
30 ppb	21.1	23.0	25.5	19.5	21.6	25.0	S = 2.471
	22.3	21.9	24.8	24.5	21.8	16.2	N = 15
	20.5	—	24.3	—	23.9	—	—
$\bar{X}$	21.8		23.7		21.7		22.4 (74.6%)[a]
CV, %	4.4		10.1		15.5		11.0
Dosed[b]	11.0	11.0	19.7	19.6	9.9	11.3	S = 4.355
14 and 21 day	11.8	13.0	14.7	19.1	12.4	9.0	N = 16
Tissue mixed	11.3	—	18.7	—	11.7	10.8	—
$\bar{X}$	11.7		18.4		10.9		12.3 (82%)[a]
CV, %	6.7		11.4		11.4		35.4

[a] Percent relative to sponsor's data.
[b] Sponsor's data, NADA 128–409, 15,15,16,15,16,14,16,14,13 ppb; $\bar{X}$, 15 ±1 ppb.

9%. No individual laboratory averaged less than 65% recovery. No laboratory had an intralaboratory CV greater than 7% for these 15 ppb fortified samples. The overall average value obtained for the dosed cattle liver was 12.3 ppb, with a rather large interlaboratory CV of 35%. The intralaboratory CV did not exceed 11.4% in any laboratory. One laboratory averaged an 18 ppb recovery from these dosed tissues, one 11.7 ppb, and the third, 10.9 ppb. The drug sponsor's values for these dosed samples varied from 13 to 16 ppb, with an average of 15 ppb.

Recoveries from control sheep liver fortified at the 30 ppb level ranged from 63 to 89%, with an overall average of 75%. The interlaboratory CV was 9.9%. Recoveries from the dosed sheep liver tissues ranged from 25.2 to 32.4 ppb and averaged 29.1 ppb, with a 7.7% interlaboratory CV. For samples of these dosed tissues, the drug sponsor's values ranged from 26 to 34 ppb, with an average of 29 ppb.

On the basis of these results, the proposed method was recommended as an acceptable regulatory method by FDA for the determination of ivermectin marker, H_2B_{1a}, in cattle liver at the 15 ppb level and above and in sheep liver at the 30 ppb level and above.

(2) *Swine liver.*—A validation study was conducted under the sponsor's NADA (135–008) submission for use of ivermectin in swine. See Table 5 for summary of results of the validation trial.

Values reported by the Denver District Office (DEN-DO) laboratory for the spiked control samples produced CVs that always exceeded the CV criteria of 20%. However, after reviewing the individual assays, it was noticed that with each succeeding sample assay the recovery values increased. This may be indicative of the glassware becoming "conditioned" to ivermectin. This observation was further supported because the dosed tissue assays, which were performed last, produced results that gave a CV of only 4.2%.

As reported by all 3 laboratories, no interferences were encountered in the determinative procedure.

The percent recoveries for the fortified tissues fell between the 60 and 120% range, which is the Center for Veterinary Medicine (CVM) permitted range for a method that determines residues below 0.1 ppm. Averages and ranges of recoveries from fortified tissue determined by 3 laboratories are as follows: CVM, Beltsville laboratory, 94% and 90–99%; Balti-

Table 4. FDA/USDA validation data for recovery of Ivermectin from sheep liver (ppb)

	FDA		USDA, FSIS				
	Beltsville		Beltsville		Athens		Summary
Controls	0.0	0.0	0.0	0.0	—	—	—
	0.0	0.0	0.0	0.0	—	—	—
	0.0	—	0.0	—	—	—	—
			Fortified				
15 ppb	9	11	11.0	10.9	10.1	13.1	S = 1.301
	13	10	10.3	11.0	8.6	11.1	N = 16
	12	—	11.7	—	12.8	11.5	—
$\bar{X}$	11.0		11.0		11.2		11.1 (74%)[a]
CV, %	14.4		4.5		14.9		11.7
30 ppb	21	25	22.9	21.5	20.0	24.9	S = 2.233
	22	23	22.5	20.5	18.8	25.4	N = 16
	20	—	22.3	—	24.0	26.8	—
$\bar{X}$	22.2		21.9		22.4		22.5 (75%)[a]
CV, %	8.7		4.3		14.2		9.9
60 ppb	47	57	41.3	34.0	42.4	44.3	S = 7.422
	55	50	40.0	39.0	39.5	54.4	N = 16
	56	—	39.0	—	47.3	55.1	—
$\bar{X}$	53.0		38.7		47.2		46.4 (77.3%)[a]
CV, %	8.1		7.2		13.6		16.0
Dosed[b]	30.7	29.8	25.5	27.4	27.0	30.0	S = 2.251
	26.9	29.3	29.2	31.9	32.4	29.4	N = 15
	29.4	—	25.2	—	31.9	—	—
$\bar{X}$	29.2		27.8		30.1		29.1 (100.3%)[a]
CV, %	4.8		10.0		7.2		7.7

[a] Percent relative to sponsor's data.
[b] Sponsor's data, NADA 131–392, 34, 30, 32, 28, 29, 26, 30, 26, 30, 26 ppb; $\bar{X}$, 29 ±3 ppb

more District Office laboratory, 84% and 74–90%; and DENDO, 84% and 61–101%.

Collaborative studies.—No formal AOAC collaborative study of the method has been performed.

Notes

Note 1.—A concentrated propylene glycol standard solution is prepared by weight and its concentration verified by conventional LC techniques. Standards for the LC verification must still be weighed. Concomitant purity and moisture difficulties can be overcome by careful handling of ivermectin. The glycol solution is best protected from moisture and should be stored at –20°C and allowed to come to room temperature before opening to weigh sample. Dilutions of standards should be made with methanol Dilute solutions of ivermectin are unstable to even trace amounts of acid, alkali, and detergents and also to air and light. Even in the absence of all of these, ivermectin is often lost on glassware and/or plasticware. The difficulties involving air and light are largely avoided by using alcoholic solvents. Those involving acids and bases are handled by sufficient cleaning of all vessels used in the analysis. Other glassware losses are avoided by closely following the described procedures. When stored at –20°C, the most dilute methanolic standard solution has been shown to be completely stable for at least 1 year.

Note 2.—The following is an alternate extraction procedure. Accurately weigh 5.0 g liver into 50 mL centrifuge tube. Add 15 mL acetone–water (1 + 1, v/v) by using a dispensing pipetter. Homogenize with Polytron homogenizer set at 5 or 6 until complete homogenization occurs (15–20 s, or less). Pour isooctane into first 50 mL tube. Clean generator between uses with isooctane, acetone–water (1 + 1, v/v), water, and acetone.

Discussion

Complete separations.—In general, the derivatization reaction will not proceed in the presence of even small amounts of alcohol and/or water. Additionally, too high a residue from tissue will seriously retard the reaction. When adding the derivatizing reagent to the tube, a small amount (probably on the order of 0.05 mL, or less) of waxy looking solid should be present. Care must be taken that all solvent is removed in the drying step and that condensation of water vapors from the bath does not take place in the tube. The tubes are best removed

Table 5. Summary validation data for recovery of ivermectin from swine liver (ppb)[a]

Laboratory	Sample	Found, ppb	Rec., %	Confirmed
CVM	Control	0	—	ND
BALT–DO	Control	0	—	ND
DEN–DO	Control	0	—	ND
CVM	10 ppb spiked	8.2	82.3 (CV = 6.8)	N/A
BALT—DO	10 ppb spiked	9.2	92.2 (CV = 5.2)	N/A
DEN—DO	10 ppb spiked	7.3	72.7 (CV = 25.2)	N/A
CVM	20 ppb spiked	18.2	93.7 (CV = 3.4)	Yes
BALT—DO	20 ppb spiked	16.9	84.4 (CV = 8.6)	Yes
DEN—DO	20 ppb spiked	17.0	84.8 (CV = 22.4)	Yes
CVM	40 ppb spiked	32.3	80.2 (CV = 6.7)	N/A
BALT—DO	40 ppb spiked	42.2	105.6 (CV = 9.1)	N/A
DEN—DO	40 ppb spiked	37.3	93.3 (CV = 24.5)	N/A
CVM	Dosed[b]	15.0 (CV = 6.7%)	—	Yes
BALT—DO	Dosed	21.6 (CV = 6.3%)	—	Yes
DEN—DO	Dosed	16.9 (CV = 4.2%)	—	Yes

[a] Data developed under NADA 135-008; values reflect 4–5 determinations.
[b] Value reported by the sponsor was 20 ppb.

from the bath while some nitrogen flow is maintained. Also, the flow of nitrogen should be increased markedly toward the end of the drying period.

In addition to holding the amount of residue going into the derivatization to a minimum, the separations involving collection of the upper isooctane and hexane phases must be cleanly made. Absolutely no bottom phase should be transferred. Transfer of the bottom acetonitrile phase need not be made quite as selectively.

In most every case, successful separations and, therefore, successful derivatization can be determined by the color of the tube contents after the heating period. The 0.1 mL reagent should be at least dark brown in color. Most observers would call the color black.

Dissolution after evaporation to dryness.—Quantitative redissolution of trace amounts of ivermectin from glassware is difficult. The best procedure involves vortex mixing to initiate redissolving the residue followed by use of an ultrasound device with considerable power.

Silylated reaction tubes.—Silylated tubes are not absolutely necessary for the derivatization reaction, but they are strongly recommended for routine use because they contribute a great deal to the reproducibility of results. The silylated tubes must be carefully and thoroughly hand-washed before and between uses. They should be resilylated about once every 2 months. Machine washed tubes may require resilylating after each use.

Derivatization timing.—The peak yield of the derivative is not quite achieved in 1 h at 95°C. The yield is more or less constant in the range of 1–4 h. A 1 h reaction time was selected to shorten the total procedure time and to lessen potential interferences from protracted heating. For example, lengthening reaction time definitely broadens the "junk" peak at the injection point of the chromatogram.

Derivatization procedure.—Stoppers should be kept in place during the 95°C heating by use of No. 13 2956 tape (Ace Scientific). This tape is removed from the tube after reaction more easily than scotch tape. It also does a good job of holding the stopper in place. Oil baths are more reliable and give more precise data. All tubes containing standards and samples should be heated together for the same time period and at the same temperature.

Glassware and plasticware.—Unexplained losses of ivermectin on glassware surfaces are minimized by routinely using the same glassware. Glassware that is relatively easy to clean by hand should be used. After use in the assay, pipets should be rinsed well with methanol and acetone, and immediately dried in a 60°C oven. All glassware must be spotless. Scratched ware should be discarded or not used during this analysis scheme. Centrifuge tubes and similar glassware should be cleaned by hand and then cleaned with chromic acid followed by a very thorough rinsing in a central glass washing unit. All glassware should be visually inspected before use. New glassware should be soaked overnight in ca 2 µg/mL methanolic H_2B_{1a} before the usual thorough cleaning. This preconditioning of glassware seems to lead to more reproducible assay values.

Acceptable assay stopping places.—In general, standard solutions and all assay solutions kept overnight or longer should be stored at –20°C. Only storage in methanol during the assay has proven to be acceptable. Any stopping place other than those specified in the procedure write-up should be suspect, particularly a dry storage and/or storage in hexane. In general, if other stopping places are needed, evaporating to dryness and taking up in methanol is suggested. The next day, methanol should be removed by nitrogen evaporation and the procedure continued.

Automatic sampling for LC.—Only the Waters WISP autosampler has been routinely used for the ivermectin assay. Use of WISP requires small capacity inserts for the sampling vials because of the low-volume sample size. The self-sealing type

of caps for these inserts has caused erratic peak heights, apparently because of pressure problems in the vials. Only the PTFE single-use septums are recommended for this purpose.

References

(1) Method was submitted by the applicant, Merck Sharp & Dohme Research Laboratories, Merck & Co., Inc., of Rahway, NJ, in conjunction with the requirements for approval of their new animal drug applications: NADA 128-409, for use in cattle; 131–392, for use in sheep; and 135–008, for use in swine.

(2) Tway, P.C., Wood, J.S., & Downing, G.V. (1981) *J. Agric. Food Chem.* **29**, 1059

REGULATORY ANALYTICAL METHODS

Method II. Liquid Chromatography/Fluorescence Confirmatory Assay of Ivermectin in Cattle, Sheep, and Swine Liver Tissues

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Avermectins, which are isolated from the mycelia of *Streptomyces avermitilis*, are a broad family of compounds that are potent parasiticides at very low dosage levels. Ivermectin is a mixture of 2 homologs, not less than 80% 22,23-dihydroavermectin B_{1a} (dihydro B_{1a} or H_2B_{1a}) and not more than 20% 23,23-dihydroavermectin B_{1b} (dihydro B_{1b} or H_2B_{1b}). Radiometabolism studies conducted at the Merck & Co., Inc., laboratories have demonstrated that H_2B_{1b} is generally metabolized more rapidly than H_2B_{1a}. In addition, these studies concluded that H_2B_{1a} is not rapidly metabolized and, therefore, is the major residue found at the proposed withdrawal times. 22,23-Dihydroavermectin B_{1a} is the marker substance for ivermectin. Radioactive depletion studies indicated liver as the logical choice for target tissue.

Regulation Information

Ivermectin is approved in the dosage forms and for use in animals listed in Table 1.

Tolerances: 21CFR556.344

Marker Residue: 22,23-Dihydroavermectin B_{1a}

Cattle and reindeer.—Target tissue, liver for both species; tolerance, 15 ppb in target tissue for marker residue (15 ppb in liver corresponds to a concentration for total residues of 50 ppb ivermectin in liver); safe concentrations for total residues of ivermectin in uncooked, edible tissues of both species are as follows: muscle, 25 ppb; liver, 50 ppb; kidney, 75 ppb; and fat, 100 ppb.

Swine.—Target tissue, liver; tolerance, 20 ppb in target tissue for marker residue (20 ppb in liver corresponds to a concentration for total residues of 75 ppb ivermectin in liver); safe concentrations for total residues of ivermectin in uncooked, edible tissues of swine are as follows: muscle, 25 ppb; liver, 75 ppb; kidney, 100 ppb; and fat, 100 ppb.

Sheep.—Target tissue, liver; tolerance, 30 ppb in target tissue for marker residue (30 ppb in liver corresponds to a concentration for total residues of 125 ppb ivermectin in liver); safe concentrations for total residues of ivermectin in uncooked, edible tissues of sheep are as follows: muscle, 25 ppb; liver, kidney, and fat, 125 ppb.

Method II. Liquid Chromatography/Fluorescence Confirmatory Assay of Ivermectin in Cattle, Sheep, and Swine Liver Tissues.

Scope

Ivermectin residues are confirmed in cattle, sheep, and swine liver tissue (1).

Principle

Samples are homogenized in acetone–water (1 + 1, v/v), and the dihydro B_{1a} is extracted with isooctane. The solvent is evaporated, methanol is added, and the solution is chilled overnight to congeal fat. The methanol is separated from the fat and evaporated. The residue is partitioned first between hexane and

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Table 1. Approved Ivermectin dosage forms and animal species

Regulation (21CFR)	Dosage form	Animal
520.1192	Paste	Horses, cattle
520.1193	Tablets and chewable cubes	Dogs
520.1194	Drench	Sheep
520.1195	Liquid (oral)	Horses
522.1192	Injection	Horses, cattle, reindeer, swine
522.1193 (combination with clorsulon)	Injection	Cattle

acetonitrile and then between acetonitrile, water, and hexane. The hexane extracts are evaporated. Up to this point, the procedure is identical to determinative Method I.

The residue is dissolved in methanol. Two portions are prepared, one labeled A (aglycone) and another labeled M (monosaccharide). The contents of Tube A are hydrolyzed with 1% H_2SO_4 in methanol, and those of Tube M are hydrolyzed with 1% H_2SO_4 in isopropanol. Methanol is added to Tubes A and M, and the contents are mixed. Methylene chloride–hexane–isobutyl alcohol (20 + 30 + 2, v/v) is added, and the contents are mixed. Distilled water is added, the contents are mixed, and the upper phase is collected. A third aliquot of the methanol solution is added to a tube labeled I (H_2B_{1a}). This tube and the collected upper phase are evaporated and derivatized with imidazole.

Methanol is added and the solution is mixed. Isobutyl alcohol–hexane (1 + 99, v/v) is added, and the solution is mixed. Water is added and mixed; the tube is centrifuged. The upper phase is collected. The solvent is evaporated, methanol is added, and the tube is centrifuged; and D–I, D–A, and D–H_2B_{1a} are confirmed by liquid chromatography (LC) on a Zorbax C_{18} column with fluorescence detection (Figure 1). Confirmation results when peaks are observed at appropriate levels for H_2B_{1a} itself and the monosaccharide and aglycone derivatives.

Limit of Reliable Measurement

An FDA study showed that the method confirms H_2B_{1a} in cattle liver at the 15 ppb level and above and in sheep liver at the 30 ppb level and above.

Apparatus

(All references to commercial apparatus and chemicals in this section are for descriptive purposes only and do not constitute endorsement or recommendation of a product by FDA and the U.S. Government; equivalent products may be substituted.)

The following equipment is required in addition to that listed in *Apparatus* of Method I.

(a) *Graduated cylinder.*—50 mL, glass and stoppered.

(b) *Pipets.*—Graduated, 5.0 and 10.0 mL.

Reagents and Solutions

The following reagents are required in addition to those listed in *Reagents and Solutions* of Method I.

(a) *Isobutyl alcohol, isopropanol, methylene chloride.*—Distilled-in-glass (Burdick and Jackson Labs).

(b) *Methylene chloride–hexane–isobutyl alcohol.*—Fill a 500 mL stoppered graduated cylinder to 200 mL mark with methylene chloride. Add hexane to 500 mL mark and then 20 mL isobutyl alcohol. Mix.

(c) *Sulfuric acid–isopropanol (1 + 99, v/v).*—Pipet 0.5 mL concentrated sulfuric acid carefully into ca 40 mL isopropanol in 500 mL volumetric flask. Mix. Dilute to mark with isopropanol. Thoroughly mix. Make fresh just before use.

(d) *Sulfuric acid–methanol (1 + 99, v/v).*—Pipet 0.5 mL concentrated sulfuric acid carefully into ca 40 mL methanol in 50 mL volumetric flask. Mix. Dilute to mark with methanol. Thoroughly mix. Make fresh just before use.

(e) *Standard solutions.*—Dilute a standard ivermectin solution in methanol with methanol until each 2 mL of solution contains 5 times the number of nanograms of ivermectin as the level (in ppb) in the meat to be detected. Example:

$$C + 5 \times L$$

where C = concentration of the diluted standard in ng/2 mL, L = level analyst desires to determine in liver in ppb, and 5 = number of grams of liver sample taken.

Analysis

Isolation procedures.—Process samples through evaporation to dryness in the 40–80°C bath, as described in Method I, *Analysis, Extraction of ivermectin from samples.*

Dissolve residue in exactly 2 mL methanol by using vortex mixer and ultrasonic bath. Mix thoroughly. Centrifuge 5 min. Pipet exactly 0.5 mL supernate into each of 2 clean, silylated 15 mL tubes. Evaporate completely to dryness with nitrogen in 70°C bath. Dried samples should appear like a small drop of oil; be sure all solvent is completely removed. Store remaining 1 mL in freezer. Add 0.1 mL sulfuric acid–methanol (1 + 99, v/v) to one of the 2 samples (A sample) and 0.1 mL sulfuric acid–isopropanol (1 + 99, v/v) to the second (M sample). Vortex mix samples 10 s. Thoroughly mix by ultrasound. Repeat vortex mixing. Let stand 16–18 h (overnight) at room temperature. To all A samples and A standards at one time, add 0.9 mL methanol by using 5 mL measuring pipet. Add methylene chloride–hexane–isobutyl alcohol to 7 mL mark and mix. Add 4.0 mL distilled water by using 10 mL measuring pipet. Shake 1 min. Centrifuge 5 min. Transfer mixed solvent (upper) phase by disposable pipet to fresh, silylated 15 mL tube. Transfer as much upper phase as possible but absolutely no lower phase.

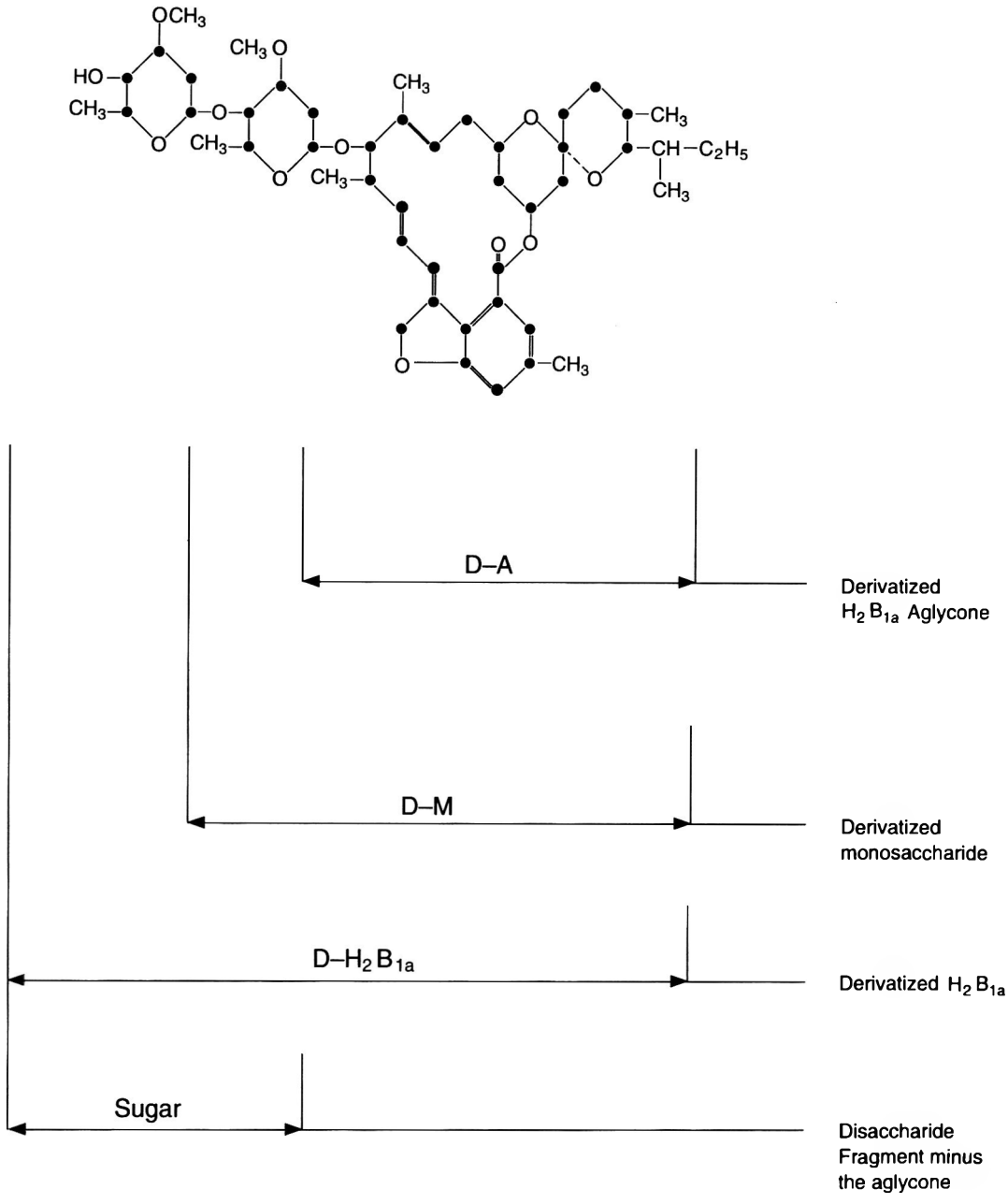

Figure 1. Confirmatory fragments for H_2B_{1a}.

Repeat extraction of lower phase with second 6 mL portion of mixed solvent Combine extracts in silylated tube.

Do the same extractions with M samples, except with moderate shaking. Centrifuge 10 min or more (to avoid emulsions).

Into third 15 mL silylated tube, pipet another 0.5 mL methanol solution of sample. This is the sample labeled I. Evaporate M and A extracts to dryness. Remove all solvent. Guard against absorption of moisture. Use 40°C bath temperature for M and A samples and up to 70°C bath temperature for I sample. Add 0.1 mL derivatizing reagent to all M, A, and I dry tubes and standards. Vortex mix 10 s. Tape stoppers in place. Centrifuge briefly. Place tubes in 95–100°C oil bath for 1 h, all at the same time. Cool to room temperature. Add 0.9 mL methanol by using 5 mL measuring pipet. Mix. Add isobutyl alcohol–hexane (1 + 99, v/v) to 7 mL mark. Mix. Add 4.0 mL water from measuring pipet. Shake 1 min. Centrifuge 5 min. Transfer upper phase as completely as possible to fresh, silylated 15 mL tube. Transfer no lower phase. Repeat extraction with second 6 mL portion of isobutyl alcohol–hexane. Combine extracts. Evaporate solution completely to dryness with nitrogen flush and 40°C bath. At end of evaporation, allow temperature to rise to 60–70°C.

Determinative Procedures

LC operating conditions.—Column, 15 cm × 4.6 mm id, Zorbax ODS–C_{18}; mobile phase, water–methanol (5 + 95,

Table 2. FDA confirmatory data for ivermectin in cattle and sheep liver

	Cattle						Sheep					
	A		M		I		A		M		I	
Sample	ppb	%RM[a]	ppb	%RM	ppb	%RM	ppb	%RM	ppb	%RM	ppb	%RM
Controls	0	0	16.8	112	0	0	0	0	3.6	12	0	0
A, M + I	0	0	1.3	8	0	0	0	0	15.3	51	0	0
Absent in sheep and cattle	0	0	1.3	8	0	0	0	0	4.9	17	0	0
	0	0	0.0	0	0	0	0	0	3.6	12	0	0
	0	0	0.0	0	0	0	0	0	5.3	18	0	0
Fortified	—	—	—	—	—	—	38.2	127	29.0	130	30.0	100
	11.1	73	20.1	134	15.4	103	43.6	145	52.5	175	30.0	100
Control	10.7	71	21.4	142	15.8	106	17.6	59	20.6	69	26.5	88
Cattle-15 ppb	15.9	107	17.8	119	15.0	100	24.7	82	41.6	138	27.0	90
Sheep-30 ppb	13.7	91	18.5	123	15.3	102	19.0	63	36.6	122	24.3	81
Fortified (control cattle-30 ppb)	23.3	78	33.3	110	20.5	68	—	—	—	—	—	—
Dosed	17.3	115	32.8	218	15.0	100	30.0	100	52.5	175	25.9	86
Cattle-15 ppb	13.2	88	14.1	94	15.0	100	55.0	181	52.5	175	24.5	82
	12.5	83	14.6	97	15.4	102	28.9	96	46.6	155	30.3	101
Sheep-30 ppb	15.0	100	12.8	86	13.7	91	27.1	40	49.9	166	29.4	98
	15.0	100	12.8	86	11.6	78	26.0	88	49.9	166	30.3	101

[a] %RM = marker recovery.

v/v); mobile phase flow rate, 1.8 mL/min (usual pressure, 1000 psi; 500–2500 psi acceptable); column temperature, 30°C.

FS950 detector conditions.—Excitation lamp, FSA 110, standard 365 nm; standard Kratos flow cells, FSA 210; excitation filter, FSA 403, 365 nm band filters; emission filter, 418 nm; sensitivity range, 0.2 A or higher; time constant, ca 6.

The approximate retention time of the dihydro B_{1a} derivative under these conditions is 14 min.

Analysis of samples.—Dissolve residues from *Analysis, Isolation procedures* in exactly 0.5 mL methanol by using vortex mixer and ultrasound. Process M samples first and inject into LC system before handling A or I samples. After extraction, protect M samples from light as much as possible. If A and I samples are to be delayed significantly, store in freezer at –20°C before adding methanol. Centrifuge and inject 50 µL clear phase (M samples) into LC system. Inject 1 standard of a type, unknowns, and finally the second standard, in that order. Do the same with A and I samples.

Examine unknown sample chromatograms for presence of A, M, and I peaks at the same elution time as the standard peaks. Compare unknown A to standard A, etc. Average 2 standard values for each type (A, M, and I). Calculate each unknown as a percentage of that standard average. A value of 60% or more of the theoretical amount of all 3 (A, M, and I) is proof that this particular level of ivermectin is present in meat (liver).

Procedure to follow for suspected interferences.—If 1 of the 3 assay peaks for H_2B_{1a}, the monosaccharide, or the aglycone is out of line with the others, particularly if the out-of-line assay is on the high side, use the following procedure. Prepare solvent systems containing 7.5% water and 10% water. Rerun chromatography at 40–45°C using 7.5% water solvent and at 70–75°C using the 10% water solvent. For each set of conditions, chromatograph a standard of the offending derivative first. Standard peak should appear at roughly the same retention time as the time for the 30°C/5% water conditions. If it does not, adjust mobile phase until it does. Then, run unknown on LC system. Compare LC results. At either or both temperatures, improved separation should result. Select temperature at which maximum separation occurs. Adjust water content upward until separation of known peak from interfering peak is adequate to allow confirmation. Calculate results and report confirmation or lack of confirmation as usual.

Recovery procedures.—Recovery is determined by analysis of fortified samples and comparison of experimental and theoretical concentrations of ivermectin.

Confirmatory procedures.—No additional confirmatory procedures have been provided.

Reliability of the Method

Validation.—For cattle and sheep liver, recoveries from samples spiked with dihydroavermectin B_{1a} and the aglycone were 60–110%, the normal range for the assay method (Method I).

Monosaccharide recovery values tended to be higher, probably because of a light stability problem for this compound or by the presence of tissue components leading to higher recoveries than for standard samples. In addition, 18 different blank and/or zero assay samples were run. All were readily determined to have no ivermectin present (2).

In the FDA validation study of the confirmatory method, all participating laboratories reported having problems. Table 2 was prepared from data in the FDA Beltsville report. The FDA

Beltsville analysts reported that chromatograms of sheep liver extracts showed large amounts of interferences at the retention volume (RV) of derivatized monosaccharide (D-M). One control cattle liver showed large amounts of interferences at the D-M RV. Two others showed interferences of 8% of the D-M response for 15 ppb H_2B_{1a}. The last 2 cattle livers showed no interferences at the D-M RV. The values in excess of 100% for D-M in fortified control cattle liver may result from interferences. FDA Beltsville analysts concluded that the confirmatory procedure should not be accepted until the problems were resolved, and the procedure was successfully validated in the U.S. Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS), and FDA laboratories. Analysts at USDA Beltsville FSIS also reported interferences at the D-M retention volume in both control sheep and beef livers. Analysts at USDA Athens FSIS reported the D-M interferences in sheep liver but not in cattle liver.

The confirmatory procedure, as originally proposed, involved the derivatives of 2 different fragments of the H_2B_{1a} homolog, D-A and D-M, plus D-H_2B_{1a}, which had been cleaned up by solvent-solvent partitioning rather than by silica gel Sep-Pak chromatography, as in the determinative procedure. However, this difference in the cleanup of D-H_2B_{1a} did not necessarily enhance the specificity of the cleanup procedure. Although the proposed procedure did not perform as predicted during the method trial, enough of the data was salvaged to satisfy the minimum requirements for confirmatory procedures. The data in Table 2 indicate that there are no obvious interferences at the retention volume of D-A or D-H_2B_{1a} in either cattle liver or sheep liver. Four of the 5 beef controls had D-M interferences of less than 8% of that expected for D-M levels equivalent to 15 ppb H_2B_{1a}. Neither USDA Beltsville FSIS nor USDA Athens FSIS reported interferences at the retention volume of D-A or D-H_2B_{1a} from either cattle or sheep liver. USDA Athens FSIS reported no interferences at the retention volume of D-M in cattle liver.

A confirmatory procedure for ivermectin residues in liver that is based on the presence of D-M, D-A, and D-I, each at a minimum level of 60% of that theoretically possible, would have excellent specificity. However, a confirmatory procedure for ivermectin residues in liver that is based upon the presence of D-A and D-I, each at a level of at least 60% of that theoretically possible, meets the current minimum requirements for specificity.

For validation of residues in swine liver, attempts to use the same MeOH–H_2O ratio in the confirmatory procedure that was used in the determinative procedure resulted in the same interference that was observed in the validation study for residues in sheep tissues. This interference occurred at the same retention volume (or time) as the monosaccharide derivative. However, this problem was resolved using the alternate LC condition suggested by the sponsor.

The conditions used were as follows: column temperature, 40°C; MeOH–H_2O ratio, 92.5 + 7.5.

Collaborative studies.—No formal AOAC collaborative study of the method has been performed.

References

(1) Method was submitted by the applicant, Merck Sharp & Dohme Research Laboratories, Merck & Co., Inc., of Rahway, NJ, in conjunction with the requirements for approval of their new animal drug applications: NADA 128-409, for use in cattle; 131–392, for use in sheep; and 135–008, for use in swine.

(2) Tway, P.C., Wood, J.S., & Downing, G.V. (1981) *J. Agric. Food Chem.* **29**, 1059

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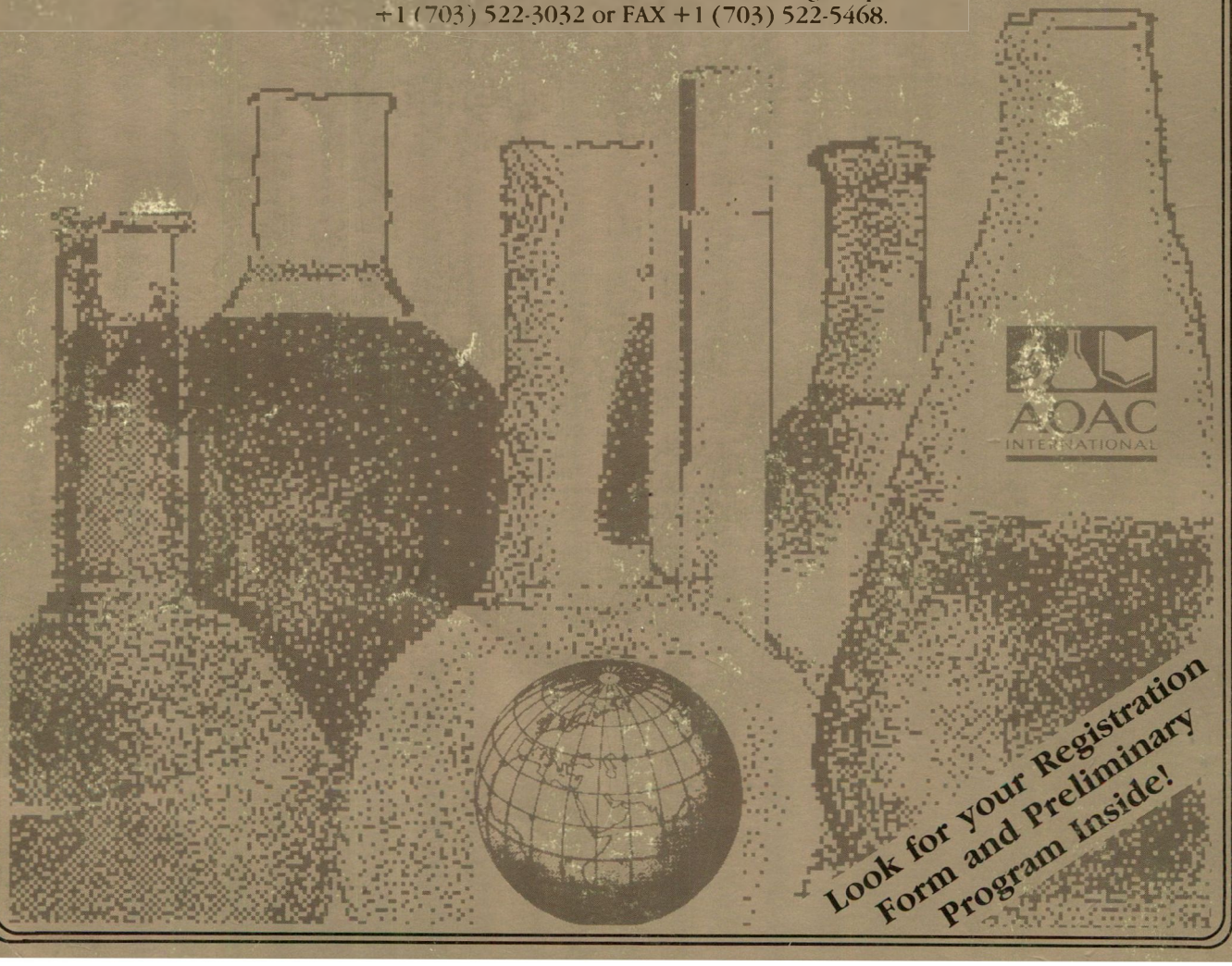
Open Forum, Short Courses, Lab Equipment Exposition, Modern Lab Workshop

For more information, contact the AOAC Meetings Department:
+1 (703) 522-3032 or FAX +1 (703) 522-5468.



Look for your Registration Form and Preliminary Program Inside!