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AOAC INTERNATIONAL

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JOURNAL OF AOAC INTERNATIONAL

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, 481 North Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA. **Subscriptions** for Vol. 81 (1998) are: Members, $181.00 in North America, $232.00 outside North America; Nonmembers, $249.00 in North America, $300.00 outside North America. Periodicals postage paid at Gaithersburg, MD, 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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The Journal of *AOAC INTERNATIONAL* publishes articles that present, within the fields of interest of the Association: unpublished original research; new methods; further studies of previously published methods; background work leading to development of methods; compilations of authentic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.

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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*. For describing collaborative studies, contact AOAC Technical Services for guidelines.

1. Write in clear, grammatical English.

2. To Managing Editor, AOAC INTERNATIONAL, 481 N Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA, submit typewritten original plus 3 photocopies (1 side only, white bond, 8½ × 11 in. [21½ × 28 cm] of complete manuscript in order as follows—*1.* Title page; *2.* Abstract; *3.* Text (introduction, method or experimental, results and/or discussion, acknowledgments, references); *4.* Figure captions; *5.* Footnotes; *6.* Tables with captions, one per page; *7.* Figures.

3. Suggest in a cover 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.

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1. **Title page** (separate sheet, **DOUBLE SPACED**): title of article, authors' names (full first, middle initial if any, full last), authors' addresses including mail codes.

2. **Abstract** (separate sheet, **DOUBLE SPACED**): 200 words. Provide specific information, not generalized statements.

3. **Text** (consecutive sheets, **DOUBLE SPACED**): *Introduction*. Include information on why work was done, previous work done, use of compound, or process being studied.

Method or Experimental. Consult recent issue of *Journal* for proper format. Separate special reagents/apparatus from details of procedure and list in sections with appropriate headings; 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. 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. Van Vunakis & 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 Book Co., New York, NY

OFFICIAL METHODS REFERENCE

(1) *Official Methods of Analysis* (1995) 16th Ed., AOAC INTERNATIONAL, 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 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. Lines, letters, numbers, and symbols must be sharp and unbroken. 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 μ. 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").

For Your Information

Meetings

April 20–22, 1999: AOAC Southeast-USA Section, Atlanta, GA, USA. Contact: Pat Beckett (becketp@doacs.state.fl.us), Florida Department of Agriculture, Chemical Residue Laboratory, 3125 Conner Blvd, Tallahassee, Fl 32399-1650, USA, +1-850-488-9670, fax +1-850-922-9110

August 1–5, 1999: AOAC Latin American and Caribbean Section, Santiago, Chile. Contact: Nuri Gras (ngras@gopher.cchen.cl), fax +562-3646277, or Patricia Bravo (corthon@ibm.net), fax +562-2439299

September 26–30, 1999: The 113th AOAC INTERNATIONAL Annual Meeting and Exposition, Houston, TX, USA. Contact: Meetings and Education Department (meetings@aoac.org), AOAC INTERNATIONAL, 481 N. Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA, +1-301-924-7077, fax +1-301-924-7089

September 10–14, 2000: The 114th AOAC INTERNATIONAL Annual Meeting and Exposition, Philadelphia, PA, USA. Contact: Meetings and Education Department (meetings@aoac.org), AOAC INTERNATIONAL, 481 N. Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA, +1-301-924-7077, fax +1-301-924-7089

Pohland Is Incoming AOAC President

Albert E. Pohland, U.S. Food and Drug Administration, was named 1998–1999 President of AOAC INTERNATIONAL at the 112th AOAC INTERNATIONAL Annual Meeting and Exposition held in Montréal, Canada, September 13–17, 1998.

Pohland has a long history of service to AOAC. He served as an Associate Referee on Confirmative Methods from 1969 to 1973 and as a member of the Foods Methods Committee from 1988 to 1991. Over the years, he has been a member of the Committee on Meetings, Symposia, and Educational Programs (1981–1987), the Constitution (now Bylaws) Committee (1987–1990), and has been a member of the Joint Mycotoxin Committee since 1979. He currently is a section editor of the *Journal of AOAC INTERNATIONAL*, a member of the Liaison Committee, and an AOAC liaison officer to the International Union of Pure and Applied Chemistry (IUPAC). He was elected to the Board of Directors in 1990 and became President-Elect in 1997. AOAC recognized his contributions to the Association in 1995 by naming him a Fellow.

A Ph.D. graduate of Colorado State University, Pohland spent 2 years as a post-doctoral fellow at the University of Nebraska before joining FDA in 1965. At FDA, Pohland's work focused on the natural toxins, particularly mycotoxins. As Program Manager for Natural Toxins, he guided the development of research programs not only for mycotoxins but also for two new areas: seafood toxins and plant toxins. His efforts are documented in an extensive record of nearly 90 publications and have resulted in many awards, the most prestigious of which was the FDA's Award of Merit for "foresight, outstanding leadership, and extraordinary cooperation in studies of natural toxicants in foods."

Pohland's research interests and activities have not been restricted to the natural toxicants, however. In 1971, for example, he was awarded the FDA's Award of Merit for his work on the highly toxic, chlorinated dibenzo-*p*-dioxins. In 1991, he was a member of the L-tryptophan Research Group, which received the FDA Group Recognition Award for "outstanding performance during the L-tryptophan contamination crisis."

Pohland's interest in the analytical sciences, particularly as they are applied to natural toxins in foods, has taken him all over the world and resulted in his serving since 1980 on the Joint U.S.–Japan Natural Resources Program, Panel on Toxic Microorganisms, and since

Paul Beljaars (left) passes the gavel to Al Pohland.

1979 on the IUPAC Commission on Food Chemistry, which he chaired from 1983–1994. He has had a continuing interest in helping to disseminate knowledge about the naturally occurring toxicants and the analytical sciences and, consequently, has served as lecturer, adjunct professor, and professor at various institutions while working for the FDA, including the U.S. Department of Agriculture Graduate School, American University, and the University of Maryland. Most recently, he joined the Joint Institute for Food Safety and Applied Nutrition (JIFSAN), a collaborative effort between the University of Maryland and the FDA. The Institute serves as the vessel for scientific contributions to national food safety programs and international food standards. In this new capacity, Pohland will be director of the new FAO/WHO Collaborating Center for Mycotoxins, and as such will be active in the development of joint research projects with international participants.

AOAC INTERNATIONAL looks forward to Pohland's term as President of the Association.

AOAC Board Slate Elected; Proposition and Methods Approved

The election of a slate of officers and directors, a proposition to increase membership dues, and 12 "final action" *Official Methods*℠ were among the items re-

cently approved by vote of the AOAC membership.

The following Board of Directors slate, which the Nominating Committee proposed, was elected: **President-Elect, George H. Boone**, U.S. Food and Drug Administration, Brooklyn, New York, USA; **Secretary-Treasurer, Michael H. Brodsky**, Ontario Ministry of Health, Toronto, Ontario, Canada; **Director, Maria Ines Santoro**, University of São Paulo, São Paulo, Brazil; **Director, Jonathan W. DeVries**, General Mills, Inc., Minneapolis, Minnesota, USA. Both Santoro and DeVries were elected to 3-year terms.

Albert E. Pohland will serve as 1998–1999 Board **President** and **Paul R. Beljaars** will serve as **Immediate Past President**. Also serving existing Board terms as Directors are: **Claire A. Franklin**, Pest Management Regulatory Agency, Ottawa, Ontario, Canada; **Thomas L. Jensen**, Nebraska Department of Agriculture, Lincoln, Nebraska, USA; **James A. Ault**, Ricerca, Inc., Painesville, Ohio, USA; and **Jon E. McNeal**, U.S. Department of Agriculture, Washington, DC, USA.

The ballot included a section for "final action" member adoption of AOAC® *Official Methods*℠. There were 12 methods recommended for final action and, by a majority of the votes cast, all were approved.

The membership also approved a proposition on the ballot to increase membership dues from the current level of US$75.00 to US$85.00, which will help offset part of the difference between direct membership expenses and dues income while AOAC pursues alternative revenue sources to support member benefits.

Beljaars Praises AOAC for "Eliminating Borders"

AOAC INTERNATIONAL is "eliminating borders for better science," President Paul Beljaars declared in his opening address at the 112th AOAC INTERNATIONAL Annual Meeting and Exposition, September 13–17, 1998, at the Queen Elizabeth Hotel in Montréal, Quebec, Canada.

As evidence, the first AOAC president from outside North America cited the annual meeting's location—the first outside the United States—and a meeting last May of the Official Methods Board in Bilthoven, The Netherlands. He offered greetings in several languages, including French and his native Dutch.

Listing accomplishments this past year, Beljaars hailed progress in the peer-verified methods program. "Numerous methods are being submitted, reviewed, approved, and—for the first time—published," he said, citing nine peer-verified methods published in the July/August issue of the *Journal of AOAC INTERNATIONAL*.

Beljaars praised *Inside Laboratory Management* magazine as "a vital resource for members," citing articles on HACCP, accreditation, LIMS, and laboratory automation. He also called attention to the new Technical Division for Laboratory Management, noting that TDLM membership has grown to more than 350 individuals.

"One of the group's most significant actions to date is acceptance within its organizational structure of the ad hoc Food Laboratory Accreditation Group. Under TDLM, this group will be known as ALACC—the Analytical Laboratory Accreditation Criteria Committee—and will work to present AOAC-recommended criteria to accrediting bodies," he said.

Beljaars said AOAC is "thrilled to announce the kickoff of the new proficiency testing program, with invaluable assistance form the Proficiency Testing Advisory Group and the technical divisions!" He forecast expansion "to be one of the most extensive programs available, covering food, agricultural, and environmental matrixes."

Other accomplishments cited by the outgoing AOAC president included:

- Formation of a formal liaison relationship with the governing council of the food chemistry division of the Federation of European Chemical Societies;
- Correspondence with the Federation of German Agricultural Control and Research Institutes;
- Development of a closer relationship with the Pan American Health Organization;
- Appointment of AOAC as technical advisor to the Inter-American Network of Food Analytical Laboratories; and
- Formation of the Southern California Subsection of the AOAC Pacific Southwest Section.

Noting that the AOAC sections program is enjoying impressive successes internationally, Beljaars reported that the Europe Section had arranged travel to the Montréal meeting for Petras Serapinas of the Institute of Theoretical Physics and Astronomy in Lithuania. The AOAC president also reported approval of the charter for the Japan Section and invited its first president, Hiroshi Kurata, to join him on the stage.

Looking toward 1999, Beljaars offered the following predictions: AOAC will continue to offer member benefits and services, including a better computer database and a Website redesign. The AOAC awards program will be reviewed to reward important areas of success and encourage young scientists and volunteers. Technical staff and volunteers will seek ways to make the collaborative study process faster, more fluid, and more user-friendly. AOAC will provide more electronic publications on CD-ROM and the Web, as well as more books in languages such as Spanish and Japanese.

Beljaars urged AOAC members to create an organization that "(*1*) is truly global, with sections and/or offices in every populated continent, offering products and services in many lan-

guages; (2) maximizes use of on-line technologies that allow us to communicate more information faster; but (3) remains in touch personally with scientists that make up the analytical community."

The outgoing president expressed hope that the annual meeting would bring "people, ideas, and energy from around the world together in one place, to personally experience the successes we all share—past, present, and future!"

1998 AOAC Awards

Presented September 14, 1998, at the 112th AOAC INTERNATIONAL Annual Meeting and Exposition, The Queen Elizabeth Hotel, Montréal, Quebec, Canada

1998 Harvey W. Wiley Award

Viorica Lopez-Avila of the Midwest Research Institute in Mountain View, California, USA, is the 1998 recipient of AOAC's most prestigious award, the Harvey H. Wiley Award. This award is presented each year to a scientist who has produced significant advances in analytical methodology in an area of interest to AOAC.

Lopez-Avila is a researcher whose hallmark traits are common to many Wiley Awardees: enthusiasm for a great diversity of analytical fields and an intellect that allows her to seek out innovative methods. Primarily an environmental analyst specializing in extraction and sample preparation, Lopez-Avila is nationally recognized for pioneering the use of microwave-oven technology to simplify complex extractions.

These microwave-assisted methods work in much the same way as everyday microwave ovens, using microwave radiation to heat water or other solvents capable of absorbing microwave energy. But instead of cooking vegetables, the goal is to superheat a solvent to dramatically speed up extraction processes.

One use for microwave heating is with a standard reflux procedure called the Soxhlet extraction, which calls for boiling a solvent and condensing it so that it percolates through the matrix. As conventionally performed, this process takes many hours, but, according to Lopez-Avila, by using the microwave the time to complete the process is reduced to 15–20 minutes.

The process works with a variety of solvents, including methylene chloride–acetone and hexane–acetone mixtures, methyl *tertiary*–butyl ether, or toluene–methanol mixes. It is particularly valuable with aqueous buffers, because samples extracted from such buffers are immediately ready for immunoassay testing typically performed in aqueous solutions. Not surprisingly, Lopez-Avila is a leader in combining immunoassay methodologies with microwave extractions.

Lopez-Avila is always on the lookout for ways to apply old and new technologies in innovative ways. One example is a process called membrane introduction mass spectrometry (MIMS), new for environmental chemistry. It uses a special capillary tube that inserts into the ion source of a mass spectrometer. The tube is composed of a silicon membrane that prevents the diffusion of water molecules but not organic pollutants. This allows the organics to be immediately introduced into the ion source of the mass spectrometer, without the need for conventional, time-consuming extraction techniques (which require purging the organics from the water, trapping them on an absorbent material, thermally desorbing them from the trap, and separating them by gas chromatography). A 45-minute technique can now be completed in about 5 minutes.

Lopez-Avila is editing a compendium of field analytical methods, including the new MIMS technology. She believes that much of the future of environmental analysis will be in field technology. People in the field—who do the remediation work—cannot make decisions if they have to wait 3 or 4 weeks for results and they cannot wait to extract a sample for 24 hours; they need to extract it in 10 minutes and analyze it in another 10. Technologies that can be used on site are needed. Lopez-Avila is committed to the development of methods that are more environmentally friendly. One of the advantages of microwave-assisted and supercritical fluid extraction techniques is that the use of organic solvents can be minimized or eliminated.

The Harvey W. Wiley Award is AOAC INTERNATIONAL's highest scientific honor. Named for one of the Association's founders—a scientist who in 1906 was instrumental in establishing the U.S. Pure Food, Drug, Cosmetic, and Meat Inspection laws—it has been awarded annually since 1957. It carries a cash prize of $5,000 as well as the opportunity to address the Wiley Award Symposium at the Association's Annual Meeting. Nominations for this award are accepted on an ongoing basis and are not restricted to members of AOAC INTERNATIONAL.

Fellow of AOAC INTERNATIONAL

Henry B. Chin, National Food Processors Association, Dublin, California, USA, as current member of Official Methods Board; current Chair of the Methods Committee on Residues and Related Topics; Peer Reviewer for *J. AOAC Int.*, and service as an Associate Referee.

Mark R. Coleman, Eli Lilly & Company, Greenfield, Indiana, USA, as current member of the Methods Committee on Feeds, Fertilizers, and Related Agricultural Topics; President of AOAC Central Section; Organizer of forum on Methods for Antibiotics and Drugs in Feeds; and former Associate Referee of the Year.

Nancy Thiex, South Dakota State University, Brookings, South Dakota, USA, as current member of the Editorial Board; General Referee for Feeds; service as an Associate Referee of multiple

methods; and former Collaborative Study of the Year Award winner.

David F. Tomkins, Monsanto Company, Muscatine, Iowa, USA, as member of Official Methods Board; current member of the Methods Committee on Pesticide and Disinfectant Formulations; service as General Referee and Associate Referee for Pesticide and Disinfectant Formulations; and former Associate Referee of the Year.

Mary W. Trucksess, U.S. Food and Drug Administration, Washington, DC, USA, as member of *Inside Laboratory Management* Advisory Board; Chair of Joint AOAC/AOCS/AACC/IUPAC/IDF Mycotoxin Committee; peer reviewer for the *J. AOAC Int.*, and former General Referee of the Year.

Máire C. Walsh, State Laboratory, Dublin, Ireland, as current member of Safety Committee; Committee on Feeds, Fertilizers, and Related Agricultural Topics; Quality Assurance Committee; and AOAC Europe Selection Committee; member of Task Force on Integration of AOAC Method Programs; and former Safety Advisor of the Year Award winner.

Lou C. Zygmunt, Arlington Heights, Illinois, USA, as Associate Referee on Sugars in Cereals since 1980; work on five collaborative studies; and former Associate Referee of the Year.

The Fellow of AOAC INTERNATIONAL Award is presented in recognition of 10 or more years of meritorious service to the Association. Any member who has given such service is eligible for nomination.

General Referee of the Year Award

David Firestone, U.S. Food and Drug Administration, Washington, DC, USA. Firestone was chosen for his research and analytical support in the area of fats and oils analysis. He has served AOAC and its members as the General Referee for Fats and Oils for 35 years.

The General Referee of the Year is awarded by the AOAC INTERNATIONAL Official Methods Board in recognition of outstanding volunteer commitment and leadership in methods development.

Collaborative Study of the Year Award

"Synthetic Pyrethroids in Agricultural Products, Multi-Residue Gas Chromatographic Method" under the Methods Committee on Residues and Related Topics. Associate Referee **Guo-Fang Pang**, Import and Export Commodity Inspection Bureau, Qinhuangdao, People's Republic of China. The study was noteworthy for its level of international participation with nine laboratories from the People's Republic of China, two laboratories from the United States, and laboratories from Taiwan, Italy, Slovakia, and Greece.

The Collaborative Study of the Year is awarded by the AOAC INTERNATIONAL Official Methods Board in recognition of the collaborative study that sets a standard of excellence in development, design, execution, and analysis.

Methods Committee Advisor of the Year Award

Jung-Keun Lee, U.S. Food and Drug Administration, Washington, DC, USA. Lee was selected for his outstanding service as Statistics Advisor to the Methods Committee on Pesticide and Disinfectant Formulations and his assistance as an AOAC representative to the Eurachem/CITAC Working Group, reviewing the "Uncertainty in Analytical Measurement" guideline.

The Committee Advisor of the Year Award recognizes outstanding service by a Committee Statistician, Safety, or other Advisor in designing suitable methods and studies, fit for their purpose.

Associate Referee of the Year Award

David M. Barbano and **Joanna Lynch**, Associate Referees for nitrogen and fat in dairy products under the Methods Committee on Commodity Foods and Commodity Products, Cornell University, Ithaca, New York, USA

Denise Hughes, Associate Referee for *Salmonella*, *E. coli*, and *Listeria* in foods under the Methods Committee on Microbiology and Extraneous Materials, TECRA Diagnostics, Roseville, New South Wales, Australia

Jeff Nielsen, Associate Referee for Neomycin under the Methods Committee on Feeds, Fertilizers, and Related Agricultural Topics, Pennfield Animal Health, Omaha, Nebraska, USA

Guo-Fang Pang, Associate Referee for Synthetic Pyrethroids under the Methods Committee on Residues and Related Topics, Qinhuangdao Import and Export Commodity Inspection Bureau, Qinhuangdao, People's Republic of China

John Scussel, Associate Referee for Alcohol in Distilled Spirits by NIRT under the Methods Committee on Additives, Beverages, and Food Process-Related Analytes, Castleton Beverage Corporation, Jacksonville, Florida, USA

Olof Theander, Associate Referee for Total Dietary Fiber by Colorimetric (Uppsala) Method under the Methods Committee on Food Nutrition, Swedish University of Agricultural Science, Uppsala, Sweden

The Associate Referee Awards—up to one per Methods Committee—are selected by the Methods Committee to recognize volunteer commitment to the development and validation of analytical methods.

Harvey W. Wiley Scholarship Award

Sharel Menezes, San Jose State University, San Jose, California, USA

Sharel Menezes, a graduate student at San Jose University, USA, received the Harvey W. Wiley Scholarship. She earned her BS degree in biochemistry and chemistry at Sophia College in Bombay, India, in 1993. At graduation, she received the best student award from both the Rotary Club of Bombay and Sophia College. Menezes worked as a

research assistant at the Indian School of the Kuwait University in the Department of Botany and Microbiology from 1993 to 1995, and in the Department of Biochemistry from 1995 to 1996.

Menezes initiated her MS degree at San Jose State University in the fall of 1997. In her research, Menezes focuses on capillary electrophoretic methods for the separation of optical isomers. Under the guidance of Joseph Pesek, she will use etched capillaries to bind chiral selectors to the surface and then test them with electrochromatography.

The U.S.$1,000 Harvey W. Wiley Scholarship is awarded to upper division undergraduate or graduate students to encourage and assist study in the analytical sciences. A college chosen by the current year's Harvey W. Wiley Award recipient makes the scholarship selection based on criteria established by the Association.

Staff Service Award

Ronald R. Christensen, Executive Director and General Counsel, 10 years of service to AOAC INTERNATIONAL

Christensen earned his B.S. degree in Forest Science, his M.S. in Forest Administration, and his Juris Doctorate in General Law—all from the University of Minnesota. Early in his adult life Christensen served 8 years in the U.S. Navy as a submariner in the Polaris/Poseidon Fleet Ballistic Missile Submarine Program where he specialized in satellite navigation systems. Prior to joining AOAC in 1988, Christensen served from 1979 through late 1987 as Director of Science and Education and then Executive Vice President of the Society of American Foresters in Bethesda, Maryland, USA.

As Executive Director and General Counsel of AOAC INTERNATIONAL, Christensen is responsible for the day-to-day activities of a staff of 31 headquarter employees and one European representative. He also serves as ex officio Secretary/Treasurer of the AOAC Research Institute.

Robert Rathbone, Managing Editor, 10 years of service to AOAC INTERNATIONAL

Rathbone holds a master's degree from Iowa State University's School of Agriculture, and began his professional career in publications work by covering research on campus for Iowa State's Cooperative Extension Service. Having spent 5 years living and working in the publication departments of agricultural research farms in Nigeria and Zaire, he also brings some technical expertise to AOAC, especially regarding how to interpret scientific information to various audiences.

Rathbone has been editor of *Inside Laboratory Management* since its inception in early 1997. He is also managing editor of the *Journal of AOAC INTERNATIONAL*. He is currently busy working on the development of other new publications for AOAC on a variety of topics.

Official Methods Board Actions

Collaborative Study Manuscripts Approved

All approved manuscripts were reviewed during the September 1998 meeting of Official Methods Board and adopted as First Action Official Methods℠.

Methods Adopted First Action

998.10, Determination of the Efficacy of Preservation of Non-Eye Area Water Miscible Cosmetic and Toiletry Formulations—Contact Neal Machtiger, Colgate-Palmolive Co., Microbiology and Environmental Science, 909 River Rd, PO Box 1343, Piscataway, NJ 08855-1343, USA

998.11, Rapid Test for Screening Nitrates in Forages Using Test Strip—Contact Anant Jain, University of Georgia, College of Veterinary Medicine, Athens Veterinary Diagnostic Laboratory, Athens, GA 30602-7382, USA

Paul Beljaars celebrates the Grand Opening at the AOAC INTERNATIONAL annual meeting with fellow Board members. Pictured (l to r) are: Kenneth W. Boyer, George H. Boone, Jon E. McNeal, Michael H. Brodsky, Paul R. Beljaars, Maria Ines Santoro, James A. Ault, Albert E. Pohland, and Raymond M. Matulis.

Journal Information

The Association

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

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

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

European Representatives. For information about AOAC INTERNATIONAL and its publications, persons outside the United States may also contact the following: Margreet Lauwaars, PO Box 153, 6720 AD Bennekom, The Netherlands, telephone +31-318-418725.

The Journal

The *Journal of AOAC INTERNATIONAL* (ISSN 1060-3271) is published bimonthly by AOAC INTERNATIONAL, 481 N Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA. Each volume (one calendar year) will contain about 1400 pages. The scope of the *Journal* encompasses the development and validation of analytical procedures pertaining to the physical and biological sciences related to foods, drugs, agriculture, and the environment. Emphases is on research and development of precise, accurate, and sensitive methods for the analysis of foods, food additives and supplements, contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment.

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

Manuscripts. Prepare manuscripts double spaced throughout and carefully revise before submission; 4 copies should be submitted to Managing Editor, AOAC INTERNATIONAL, 481 N Frederick Ave, Suite 500, Gaithersburg, MD 20877, USA. AOAC INTERNATIONAL reserves the right to refuse manuscripts produced on dot matrix printers of less than letter quality.

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Books in Brief

Microbiological Analysis of Food and Water: Guidelines for Quality Assurance. Edited by N.F. Lightfoot and E.A. Maier. Published by Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK, 1998. Price: $210.00. 266 pp. ISBN 0-444-82911-3.

This book represents the outcome of work carried out by European microbiology specialists, under the auspices of the Community Bureau of Reference, to develop procedures for quality assurance and quality systems in microbiological laboratories. The importance of this work was reinforced by the implementation of national accreditation systems and their mutual recognition with the European Union following the resolution (90/C1/01 of December 21, 1989) of the European Council on the "global approach to conformity assessment." The accreditation systems are based on European Standard EN 45001, which lists several requirements to be fulfilled by laboratories. Several procedures (e.g., statistical control, use of reference materials, validation of methods, proficiency testing, etc.) were still not used by microbiologists and needed to be translated into microbiological terms or adapted to the particular situation of microbiological measurements or testing. *Microbiological Analysis of Food and Water: Guidelines for Quality Assurance* has been written with the express objective of using simple but accurate wording, to be accessible to all microbiology laboratory staff. To facilitate reading, the more specialized items, in particular some statistical treatments, have been added as an annex to the book. All QA and QC tools mentioned within these guidelines have been developed and applied by the authors in their own laboratories.

GC/MS: A Practical User's Guide. By Marvin McMaster and Christopher McMaster. Published by John Wiley & Sons, Inc., 605 Third Ave, New York, NY 10158-0012, USA, 1998. Price: $59.95. 167 pp. ISBN 0-471-24826-6.

Though gas chromatography/mass spectrometry (GC/MS) is one of the most effective and popular methods of separating, identifying, and quantifying compounds in complex mixtures, there have been no comprehensive handbooks to date that clearly explain the setup and maintenance of a functional GC/MS system. Now, Marvin and Christopher McMaster have created the hands-on resource that researchers and students need to get their own systems up and running quickly. Covering everything from necessary components to tuning, troubleshooting, and processing data, it allows even those with little prior knowledge of GC/MS to perform their own analyses and gather the data they require. *GC/MS: A Practical User's Guide* contains full coverage of vital equipment, including the function, costs, and advantages of both desktop and floor-standing systems; a walkthrough of a basic GC/MS analysis and an examination of key methods of structural data interpretation; extensive information on GC/MS system optimization; an exploration of the various research and environmental uses of GC/MS systems; and an extended section on liquid chromatography/mass spectrometry to enhance comprehension of the gas method. This book is for organic, analytical, clinical, environmental, and forensic chemists in all types of laboratories—and for students in all of these specialities.

Handbook of HPLC. Edited by Elena Katz, Roy Eksteen, Peter Schoenmakers, and Neil Miller. Published by Mar-

cel Dekker, Inc., 270 Madison Ave, New York, NY 10016, USA, 1998. Price: $225.00. 989 pp. ISBN 0-8247-9444-3.

The *Handbook of HPLC* discusses the principles, techniques, and instrumentation involving HPLC within a detailed, comprehensive framework. It delineates HPLC usage in separation, purification, and detection processes across a wide variety of disciplines from industry to applied research. Intended as an on-the-job guide for experienced practitioners as well as an introduction to the subject for novices, the *Handbook of HPLC* assesses the limitations of reversed-phase HPLC, ion-exchange, size-exclusion HPLC, and other separation modes and shows how to select techniques that procure desirable results. It also details the practical operation of pumps, detectors, injection devices, and data handling systems, and explores applications in areas such as pharmaceuticals, biotechnology, environmental monitoring, art conservation, nutrition, food processing, and more. With over 4,100 references, equations, drawings, and photographs, the *Handbook of HPLC* is for analytical, food, pharmaceutical, environmental, organic, physical, and bioanalytical chemists; molecular biologists; chromatographers; biotechnologists; pharmacologists, and graduate-level students in these disciplines.

Quality in the Food Analysis Laboratory. By Roger Wood, Anders Nilsson, and Harriet Wallin. Published by the Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Rd, Cambridge CB4 4WF, UK, 1998. Price: L52.50. 314 pp. ISBN 0-85404-566-X.

'Fit for purpose' is a phrase familiar to all users of analytical data, who need to be assured that data provided by laboratories are both appropriate and of the required quality. *Quality in the Food Analysis Laboratory* surveys the procedures that a food analysis laboratory must consider to meet such requirements. The need to introduce quality assurance, the different quality models available and the legislative requirements are considered. Specific aspects of laboratory practice and particular areas of accreditation, which may cause problems for analytical laboratories, are also discussed. Covering for the first time those areas of direct importance to food analysis laboratories, this book will serve as an aid to those laboratories when introducing new measures and justifying those chosen.

Analytical Instrumentation Handbook, Second Edition. Edited by Galen Wood Ewing. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, USA, 1998. Price: $250.00. 1453 pp. ISBN 0-8247-9455-9.

This book is intended primarily to act as a guide for analytical chemists who may be called to decide what approach to pursue in solving specific problems. No person can be expected to be expert in more than a few of the techniques available in a modern analytical laboratory. A particular method may be adopted simply because it is more familiar to the operator (or perhaps less expensive) than other alternative methods, some of which might be better adapted to the problem at hand. Rather than choose a method arbitrarily, the chemist is urged to consult this handbook for an overview of other possible techniques. Once a technique has been selected, the handbook will present pertinent information about options within each domain, including the pros and cons of different kinds of instrumentation. The handbook will also be of value as a library reference for all persons interested in this basic area of chemical science, from college and university students through the technician and research levels, to industrial management.

European Pharmacopoeia Third Edition: Supplement 1999. Published by the Council of Europe, F-67075 Strasbourg, France, telephone +33/3-88-41-25-60, fax +33/3-88-41-27-89, e-mail PressUnit@coe.fr, Internet www.coe.fr.

The 1999 Supplement of the third edition of the European Pharmacopoeia adds 105 new European standards, or monographs, and 124 revised monographs that incorporate the latest scientific advances. They either harmonize standards that existed in several national pharmacopoeias or cover new substances or new technologies such as gene amplification used for biologicals to detect viral contaminants or unusual pathogenic organisms. This third edition is the main tool for the standardization of medicines in Europe and in many non European countries and has been designed to meet the specific needs of authorities in charge of licensing medicines for human or veterinary use; public or private quality control services; and manufacturers of raw materials, excipients, and pharmaceuticals. It covers approximately 1,450 harmonized European standards on subjects such as synthetic molecule, biologicals, human and veterinary vaccines, and herbal preparations. It also contains 250 general methods of analysis and 1,000 reagents and a specific chapter on dosage forms such as eye preparations, tablets, and transdermal patches.

New Products

NuGenesis™ by Mantra Software

NuGenesis™ is an application-independent software data management system. It interprets data from the wide variety of applications used in the laboratory, integrates it, and manages it within a single software structure. NuGenesis allows users to unify scientific knowledge into a common database and allows them access to this information from anywhere in the corporation. "Print-to-database" archiving technology permits scientists to save data, reports, graphics, and other information within centralized databases for later use. NuGenesis is available in a fully scalable architecture, extendable from PC workstations to client/server networks running in either Windows 95 or WindowsNT environments. The client/server version is available for both Access and Oracle platforms.

Contact: Dave Levy, Mantra Software Corp., 1900 West Park Dr, Westborough, MA 01581, telephone + 1-508-616-9876.

Fujifilm Presents Neutron-Detecting Imaging Plate

The task of capturing and analyzing data from neutron crystallography, neutron radiology, and monitoring has now become much easier and more accurate. Jointly developed through collaborative research with the Japan Atomic Energy Research Institute, the BAS-ND-IP is the only imaging plate for research using neutrons. Fujifilm Neutron Imaging Plates (ND IP) are composed of a layer of gadolinium oxide and photo-stimulable phosphor sandwiched between a flexible substrate and a protective coating. Images are recorded when neutrons stimulate the gadolinium oxide, initiating secondary radiation stored in the photo-stimulable phosphors. BAS-ND Imaging Plates are linear over five orders of magnitude, providing quantitative accuracy with high resolution never before attainable with neutrons. Irradiation time using BAS-ND-IP can be reduced to $1/10$ to $1/100$ of traditional X-ray film.

Contact: Vanessa Hartin, Science Systems Group of Fuji Medical Systems USA, Inc., Stamford, CT, telephone + 1-800-431-1850 x2649.

High Precision Pumps by Bellco Glass

Bellco announces the release of two new volumetric, high precision pumps for solution handling applications. The Accu™ multiple metering pumps allow for simultaneous feed scheduling of multiple bioreactor and for multisolvent dispensing and batch formulations. The ChemTec™ pumps provide programmable, automated bioreactor feed either by volume or weight. In contrast to volumetric metering pumps, the ChemTec mass flow delivery of nutrients is not affected by temperature, viscosity changes, or pump tube wear.

Contact: Eddie Leonelli, Bellco Glass, Inc., 340 Edrudo Rd, Vineland, NJ 08360, telephone + 1-800-257-7043.

Misonix Tray Horn Provides Sonication to All Wells in Microtiter Plates

The new Misonix 431-T Tray Horn allows even sonication to all 384- and 96-well microtiter plates. The ultrasonic sonication accelerates reactions for high throughput screening and dramatically reduces processing times. With the 431-T Tray Horn, sonication is performed uniformly. The need to introduce a probe into each sample well is eliminated, avoiding sample loss and the possibility of cross contamination. Applications for the Tray Horn include high throughput screening, ELISAs, drug screening, genomics, cell disruption, DNA or RNA probe labeling, and hybridations.

Contact: Kim Brecher, Misonix, Inc., 1938 New Highway, Farmingdale, NY 11735, telephone + 1-800-645-9846.

Revolution™ Threaded Glassware

Harvard Apparatus announces a full line of new glassware products using their Revolution™ Threaded technology. Threaded Teflon valves, caps, and adapters eliminate the breakage and "freeze-ups" that occur with traditional glassware. Threaded caps provide large openings, making your glassware more versatile. Together with adapters, they make air sensitive chemistry, chromatographic techniques, and freeze drying possible.

Contact: Harvard Apparatus, Inc., 84 October Hill Rd, Holliston, MA 01746-1371, telephone + 1-800-272-2775.

Automated Dissolution HPLC System

Shimadzu Scientific and Logan Instruments have introduced a new, automated Tablet Dissolution HPLC Analysis System. The fully integrated system provides a fast, easy setup and PC control of all dissolution and HPLC parameters. Automated, simultaneous sampling and sample transfer from all six vessels to the HPLC ensures complete sample integrity with no cross contamination. The system enables unattended operation from tablet drop to report review. The system also offers comprehensive report generation plus LIMS and network connectivity. For regulatory compliance, the system enables five report options, including raw data, raw data summary, percent dissolved, amount dissolved, and graphic dissolution profile. Multipoint and single point tests can be performed and HPLC reports can be created for individual and batch runs.

Contact: Mary Madden, Shimadzu Scientific Instruments, Inc., 7102 Riverwood Dr, Columbia, MD 21046, telephone 1-800-477-1227.

UV MAX Microplate by Whatman

Whatman announces the first optically clear, flat bottom microplate for *in situ* UV or visible spectroscopic analysis of well contents, enabling scientists to further automate the spectroscopic analyses of samples isolated in the wells of the microplate. The 96-well UV MAX microplates are molded from specially formulated polystyrene that is transparent to UV light down to 260 nm, and they have the same size footprints as standard 96-well microplates.

Contact: Sharman V. Pate, Whatman, Inc., 9 Bridewell Pl, Clifton, NJ 07014, telephone + 1-888-942-8626.

New Quantitative Test for Deoxynivalenol

Vicam has introduced a new fluorescence test product for deoxynivalenol (DON). The DONtest TAG™ implements new technology to measure DON with greater speed, precision, and accuracy than previous technologies. It offers users the benefit of running a variety of samples in either single or multiple batches.

Contact: Jennifer Smith, Vicam Science Technology, 313 Pleasant St, Watertown, MA 02172, telephone + 1-800-338-4381.

New Glassware Washer Offers Dual Spindle Rack Capability

The new Heinicke 1100 Series Undercounter Laboratory Glassware Washer offers dual spindle capability for narrow-neck glassware, microprocessor control, and 82°C/180°F cycle temperature setting for high-level disinfection. It is equipped with three preset wash cycles and three preset cycle temperature settings and includes deionized rinsing and convection drying cycles as standard. A new 1/3 hp pump provides enhanced flow rate and improves cleaning performance.

Contact: Hotpack, 10940 Dutton Rd, Philadelphia, PA 19154, telephone + 1-800-523-3608.

New Simulated Distillation Software for EZChrom *Elite* Chromatography Data Systems

SimDist 200 by Envantage Analytical Software is a 32-bit software application that performs simulated distillation using ASTM D2887, D3710, and D5307 methods. Its streamlined design uses tabbed sections for direct access to all analysis settings, and includes complete reporting with automatic export to Microsoft Excel, text, or CSV files. The software is designed to work with its EZChrom *Elite* chromatography systems.

Contact: Lisa Fay, Scientific Software, Inc., 6612 Owens Dr, Pleasanton, CA 94588, telephone + 1-925-416-9000.

New Milk Urea Nitrogen Testing Instrument for Real Time Data Capture

Bentley Instruments announced the ChemSpec 150, a milk urea nitrogen (MUN) testing device, that operates as a stand-alone unit, allowing real time data capture though serial port, printer, floppy, or zip drive. It is capable of testing 150 milk samples per hour, at a cost of $0.05 per sample, and realizes five times greater accuracy than the infrared method while offering greater ease of calibration and use.

Contact: David W. Dorlé, Dorlé Communications, Inc., 5209 Silver Maple Circle, Minnetonka, MN 55343, telephone + 1-612-935-3644.

X-Y Micropositioner Features Stainless Steel Construction

The Supper Stainless Steel X-Y Micro Slide features all stainless steel construction, including a permanently lubricated drive screw for smooth operation and measures only 1 1/8 square inches by 5/16 inches thick. This compact device provides 0.125 inches travel in both axes and can be equipped with an optional "Z" attachment. The Micro Slide incorporates multiple tapped holes for mounting in any orientation and can accommodate a variety of optics, mirrors, photodiodes, infrared sensors, and other devices.

Contact: Donald E. Goodwin, Charles Supper Co., Inc., 15 Tech Circle, Natick, MA 01760, telephone + 1-508-655-4610.

Fast Pulse Preamplifier-Discriminator

Advanced Research Instruments Corp. announces Version 7 of the F-100T amplifier-discriminator. This new unit amplifies and processes pulses from a photomultiplier tube, or electron multiplier and replaces the traditional preamplifier, linear amplifier, and lower level discriminator. It is compatible with virtually any rate meter or counter/timer. Hand-sized, it is easily placed next to a detector to minimize noise and maximize useful sensitivity. Input sensitivity is 100 µV–200 mV, for TTL output pulses, and the maximum pulse repetition rate for equally spaced pulses is 50 MHz. The F-100T applications are photon, electron, and ion counting; fast PMT (photo multiplier tube) pulse processing; electron multiplier preamplifier-discriminator; SIMS (secondary ion mass spectroscopy); ISS (ion scattering spectroscopy); and X-ray.

Contact: Advanced Research Instruments Corp., 2434 30th St, Boulder, CO 80301, telephone + 1-303-449-2288.

Sulfur Selective Detection for GC Analysis

The GC–SCD from Antek Instruments offers analysts a powerful new sulfur selective detection system. Using patented oxidative/reductive pyro-

lysis technology, this system allows low parts per billion detection of all sulfur compounds. Equimolarity enables analysts to quantitate all sulfur present—whether identified or unknown. The SCD is equipped with a large 14 in. dual-zone furnace that provides complete sample combustion.
Contact: Becky Wreyford, Antek Instruments, Inc., 300 Bammel Westfield Rd, Houston, TX 77090-3533, telephone + 1-800-365-2143.

New AC/DC Specialty Gas Monitors

CEA Instruments announces its new TG–KA series of portable toxic gas detectors. These compact, direct reading instruments with digital displays use patented gas membrane galvanic sensors available for formaldehyde, ozone, phosgene, hydrogen fluoride, phosphine, and many others. The sensors are unaffected by normal interfering gases and can detect as little as 0.01 ppm. Adjustable audible and visual alarms can be set as low as 0.1 ppm. The TG–KA weighs less than 1 lb, and comes with battery charger, AC power supply, carrying case, recorder output, and other accessories. The unit will operate for 30 h between charges, or continuously on AC power.
Contact: Steven R. Adelman, CEA Instruments, Inc., 16 Chestnut St, Emerson, NJ 07630, telephone + 1-201-967-5660.

New Stainless Steel Quick Disconnect Couplings

The SST Series quick disconnect couplings from Colder Products Co. features a flow path of 316 stainless steel. The SST Series was designed for use with analytical instruments, printing, pharmaceutical, semiconductor, chemical handling, and food/beverage industries. SST couplings feature a thumb latch for easy one-handed disconnects; an automatic shutoff; and an audible "click" to verify a secure, leak-free connection. They are available in both $\frac{1}{8}$ inch and $\frac{1}{4}$ in. nominal flow capacity, and they operate in working pressures from vacuum to 150 psi, and in temperatures from –40° to 200°F continuous, or 400°F intermittent.
Contact: Phil Allen, Colder Products Co., 1001 Westgate Dr, St. Paul, MN 55114, telephone + 1-800-444-2474.

Special Support-Service Bundles for In-House Technical Support Groups

Hewlett-Packard Co. announced a special support-service bundle for in-house technical support groups that perform their own maintenance and repair on HP chemical analysis instruments. The bundle allows laboratory managers to integrate HP resources with in-house technical support. It includes parts and unlimited telephone assistance to isolate and resolve hardware problems; preferred response time for on-site assistance; access to HP service notes; and eligibility for HP's instrument repair training.
Contact: Hewlett-Packard Co., (102) Chemical Analysis Group, 3010 PO Box 954, Santa Clarita, CA 91380-9971.

Automated Fluoride Analysis

MagicChem 650/Fluoride by Labtronics, Inc. automates standard fluoride analysis. The system consists of a robotic XYZ sampler, dilutor, dosing pump, ion meter, flow-through electrode, and software. It automatically measures correct sample volume, adds reagent, transfers prepared samples, and runs regular standards and QC samples. Off-scale samples can be automatically diluted and rerun. Other chemistry tests can be added to the systems and performed simultaneously. The MagicChem software included with the system provides advanced graphic capabilities, password protection, audit trail, extensive reporting functions, and comprehensive LIMS interfacing.
Contact: Susan Riekels, Labtronics, Inc., 95 Crimea St, Guelph, Ontario, N1H 2Y5, Canada, telephone + 1-519-767-1061.

New Reagents Provide Safer KF Moisture Determinations

A new line of Karl Fischer reagents designed to minimize exposure to methanol and other hazardous chemicals is now available from Riedel-de Haën Laboratory Chemicals. HYDRANAL-E reagents use ethanol instead of methanol as the solvent and are free of halogenated hydrocarbons. These reagents also offer improved solubility for long chained hydrocarbons, less pronounced side reactions when titrating ketones, and are more suitable for solid materials. HYDRANAL-E reagents are available for one-component and two-component volumetric reagent systems.
Contact: Don Hobbs, Riedel-de Haën Laboratory Chemicals, PO Box 14508, St. Louis, MO 63178, telephone + 1-800-521-8956.

Whatman VectaSpin Tube Filters Enhanced with Ultrafiltration and Microfiltration Media

The VectaSpin 3 Microcentrifuge and VectaSpin 20 Centrifuge tube filters can be used to prepare HPLC samples, environmental samples, and biomolecular samples. They permit scientists to combine ultrafiltration or microfiltration operation with centrifugation into one step. The VectaSpin 3 is rated at 5000 g maximum, and the VectaSpin 20 is rated at 2075 g maximum. The filtered samples can be stored in the tubes at temperatures between –70° and 50°C.
Contact: Sharman V. Pate, Whatman, Inc., 9 Bridewell Pl, Clifton, NJ 07014, telephone + 1-888-942-8626.

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SPECIAL REPORT

Structure–Retention Relationships and Physicochemical Characterization of Solutes in Thin-Layer Chromatography

TIBOR CSERHÁTI and ESTHER FORGÁCS
Hungarian Academy of Sciences, Chemical Research Center, Institute of Chemistry, PO Box 17, 1525 Budapest, Hungary

This review enumerates and critically evaluates experimental methods and calculation procedures used to elucidate the molecular basis of thin-layer chromatography (TLC) separations in both adsorption and reversed-phase modes. It addresses the quantitative relationship between molecular structure and retention and the determination of the solute physicochemical parameters by TLC.

Thin-layer chromatography (TLC) is a convenient and rapid analytical method for separation and semi-quantitative determination of a wide variety of organic and inorganic compounds present in low quantities in complicated matrixes (1, 2). In the past decade, the number of practical applications (3) and theoretical studies of the relationships between molecular structure and retention behavior have increased considerably (4). Retention of solutes in TLC strongly depends on the chemical structure of the solutes (5, 6) and on their physicochemical parameters (7). It has been proved many times that the quantitative relationship between molecular structure and retention behavior can bee used successfully to predict retention of various solutes (8, 9). Because molecular parameters define retention, the corresponding physicochemical parameters of solutes can be calculated easily by measuring their retention in appropriate TLC systems (10).

This review considers the latest achievements in quantitative structure–retention relationships and in determination of molecular characteristics by TLC.

Quantitative Structure–Retention Relationships in TLC

Many efforts have been devoted to elucidate quantitative relationships between molecular structure and chromatographic retention (11). A wide variety of measured or calculated physicochemical parameters have been tested as descriptors of retention behavior of individual solutes or solute series (12). Topological indexes (13) have been used extensively to describe the relationship between retention behavior and molecular structure in TLC (14, 15).

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Significant multilinear correlations were found between retention behavior of 16 barbituric acid derivatives in 2 adsorption TLC systems (system A: chloroform–acetone, 9 + 1; system B: 2-propanol–chloroform–25% ammonia, 9 + 9 + 2) and the following connectivity indexes (8):

$$R_{F(\text{System A})} = -0.410 + 0.052 \times {}^{0}X^{v} + 0.128 \times {}^{4}X_{pc} \quad (1)$$

$$n = 16;\ r = 0.892;\ s = 0.055;\ F = 25.31$$

$$R_{F(\text{System B})} = -0.812 + 0.056 \times {}^{0}X^{v} + 6.447 \times {}^{4}X_{c}^{v} \quad (2)$$

$$n = 16;\ r = 0.940;\ s = 0.044;\ F = 30.15$$

where X is the connectivity index, n is the number of solutes, r is the coefficient of correlation the square of which is related to the ratio of variance explained by the independent variables, s is the standard deviation of the dependent variable, F is a calculated statistical parameter characterizing the fitness of measured data to the equation, R_F is the distance of the spot of solute from the start divided by the distance of the eluent front from the start, ${}^{0}X^{v}$ is the indicator of the number of carbon atoms and the size of the molecule, ${}^{4}X_{pc}$ is the indicator of the number and length of adjacent substituent pairs, and ${}^{4}X_{c}^{v}$ is the indicator of the number of quaternary carbons and the presence of non-σ adjacent electrons. The results emphasize the marked impact of molecular size and of the number and length of substituents on the retention behavior of barbituric acid derivatives in adsorption TLC.

Similar results were obtained for the retention behavior of 23 phenol derivatives in 2 adsorption TLC systems (system A: benzene–dioxane–acetic acid, 90 + 25 + 4; system B: benzene–methanol–acetic acid, 45 + 8 + 4) (16):

$$R_{M(\text{System A})} = -0.706 + 7.214 \times {}^{3}X_{c}^{v} + 1.144 \times {}^{4}X_{pc} - 4.975 \times {}^{4}X_{pc}^{v} \quad (3)$$

$$n = 23;\ r = 0.918;\ s = 0.134;\ F = 34.07$$

$$R_{M(\text{System B})} = -0.488 + 5.736 \times {}^{3}X_{c}^{v} + 0.892 \times {}^{4}X_{pc} - 4.271 \times {}^{4}X_{pc}^{v} \quad (4)$$

$$n = 23;\ r = 0.892;\ s = 0.117;\ F = 24.67$$

where R_M is $\log(1/R_f - 1)$. This method can be used successfully to predict retention behavior of phenol derivatives in adsorption TLC (16). Furthermore, topological indexes have been used to describe the retention behavior of phenol (17) and fatty acid isomers (18).

Other solute descriptors such as R_2 (excess molar refraction), π_2^H (the dipolarity/polarizability), $\Sigma\alpha_2^H$ and $\Sigma\beta_2^0$ (the overall or effective hydrogen-bond acidity and basicity, respectively), and V_x (the McGowan characteristic volume) have been used to assess the linear correlation between R_M values of nonhomologous series of compounds extrapolated to water (R_{Mw}; 19) and the solute descriptors mentioned above (20):

$$R_{Mw} = 0.259 + 0.239 \times R_2 - 0.662 \times \pi_2^H - 0.666 \times \Sigma\alpha_2^H - 3.006 \times \Sigma\beta_2^0 + 3.603 \times V_x \quad (5)$$

$$n = 76;\ s = 0.206;\ F = 635$$

where the statistical parameters are the same as for equations 1 and 2. The results of equation 5 prove that these decsriptors are suitable for prediction of the retention behavior of solutes in reversed-phase TLC (RP-TLC).

Retention parameters of 38 nonionic surfactants on an alumina layer coated with paraffin oil were determined with methanol–water mixtures as the mobile phase, and the relationship between molecular structure and retention behavior was elucidated by canonical correlation analysis (21). The character of the surfactant's hydrophobic moiety exerts a significant impact on retention whereas the number of polar ethylene oxide groups has negligible effect.

The study of quantitative relationships between molecular characteristics and retention behavior of solutes is a relatively new research field in TLC and RP-TLC. Because research groups use different sets of physicochemical parameters and molecular descriptors, the suitability of these parameters and descriptors for prediction of retention behavior cannot be compared. We strongly hope that these methods will continue to contribute to rational optimization of TLC separations, thereby increasing the efficiency of control of TLC methods and the number of research laboratories using the methods.

Determination of Physicochemical Parameters of Solutes by TLC

Lipophilicity is the molecular parameter most frequently used in quantitative structure–activity relationship studies (22, 23). This parameter governs penetration of bioactive compounds through hydrophobic cell membranes, uptake by target organs or organisms, etc. Lipophilicity can be determined by the traditional partition method between water and *n*-octanol (24). However, this method is time-consuming, and the compound has to be very pure because impurities influence the partition of the original bioactive compound, resulting in a biased lipophilicity value. In the search for more rapid methods, various chromatographic techniques, such as RP-TLC (25), reversed-phase liquid chromatography (RP-LC; 26), micellar electrokinetic chromatography (27), and gas–liquid chromatography (28), have been explored for determination of molecular lipophilicity.

Determination of lipophilicity by chromatography offers many advantages: It is rapid and relatively simple, it does not need pure solutes because impurities are separated during the process, and a very small amount of compound is needed. Many RP-TLC systems used to determine molecular lipophilicity generally use a silica support. The hydrophobic ligand can be bound to the surface of the silica support by adsorption or by covalent bonding. Paraffin (29) or silicone oils (30) dissolved in an appropriate solvent (*n*-hexane, diethyl ether, chloroform, etc.) have been used to impregnate the silica. The concentration of the oils is generally 5 vol %, but lower (1 vol %) and higher (15 vol %) concentrations also have been reported (31). The oils are bound by adsorptive forces to the polar adsorption centers on the surface of silica support and do not move during the separation process. Silica supports with covalently bonded hydrophobic ligands—silanized silica (32) and octyl- (33) or octadecyl-bonded silica (34)—also have been successfully applied for determination of lipophilicity. Other inorganic and organic supports such as alumina and cellulose have not been used frequently for studies of solute lipophilicity (35).

Because most compounds show negligible mobility in water, the solvent strength of the mobile phase has to be increased by adding an organic modifier miscible with water. Methanol, acetone, and acetonitrile are the most frequently used organic modifiers (36). 2-Propanol, dioxane, and tetrahydrofuran also have been used in RP-TLC (37). To increase the reliability of the lipophilicity determination, R_M values [$R_M = \log(1/R_f - 1)$] characterizing lipophilicity in RP-TLC have been extrapolated to zero concentration of organic modifier (38). The suitability of various RP-TLC methods for the exact determination of the lipophilicity of bioactive compounds has been vigorously discussed and results have been compared with those obtained by RP-LC by using $\log k'$ (capacity factor) values as indicator of lipophilicity (39). Results are somewhat contradictory. Good correlations were found for xanthine and adenosine derivatives (40, 41):

$$\log k' = (0.921 \pm 0.037) + (0.763 \pm 0.056) \times R_M \quad (6)$$

$$n = 34;\ r = 0.922;\ s = 0.173;\ F = 182.0;\ P < 0.005$$

However, for nonhomologous series of commercial pesticides, lipophilicity values determined by RP-TLC and RP-LC were slightly different (42).

Lipophilicity values determined by RP-TLC have been correlated successfully with biological activity for various compounds such as 1-[3-(aroylo)]- and 1-[3-(aryloxy)]-propyl aminothiazoles (43), substituted ethylenediamines and ethanolamines (44), phenylcarbamic esters (45), β-blocker benzoxasine derivatives (46), *N*-indol-3-yl(acetyl)amino acids (47), quaternary ammonium bolaamphiphiles (48), N_1-aryl-substituted quinolone antibacterials (49), and benzimidazole and benztriazole derivatives (50). Exact knowledge of the quantitative correlations between physicochemical parameters and biological activity may promote rational design of new pharmaceuticals

and pesticides and better understanding of the biochemical and biophysical bases of biological activity.

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AGRICULTURAL MATERIALS

Determination of Maleic Hydrazide in Pesticide Formulations by Capillary Electrophoresis

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A capillary electrophoresis method was developed to quantitate the growth regulator maleic hydrazide (MH) in pesticide formulations. Liquid formulations were dissolved in water, injected into a capillary electrophoresis instrument, and monitored at 220 nm. The running buffer was 10 mM dibasic sodium phosphate at pH 9.0. Total analysis time was 10 min. Four liquid formulations with a guarantee of 30.2 or 30.3% potassium salt of MH were analyzed. Within-day coefficients of variation (CVs) ranged from 3.0 to 7.7%, and between-day CVs ranged from 5.5 to 10.0%. Purity of each MH peak was checked by using a photodiode array detector in the spectrum mode. No interferences were observed.

Maleic hydrazide (MH) is a plant growth regulator that is absorbed by the leaves and roots and inhibits cell division (1). It is an isomer of uracil, a pyrimidine base in RNA and may become incorporated into RNA (2). MH also exhibits some herbicidal activity. It is registered for use on tobacco, potatoes, onions, nonbearing citrus fruit, turf, utility and highway rights-of-way, airports, industrial land, lawns, recreational areas, ornamental or shade trees, and ornamental plants (3). The formulation types registered by the U.S. Environmental Protection Agency include single-active-ingredient products and a multiple-active-ingredient product. For single-active-ingredient products, MH can be sold as an emulsifiable concentrate (8%), soluble concentrate/liquid (21.6–33.3%), and soluble concentrate/solid (80%). The formulation registered for multiple ingredients is an emulsifiable concentrate (11.1% plus one other active ingredient; 3).

Numerous liquid (4–7) and gas chromatographic (8–10) methods exist that measure MH in various matrixes such as tobacco, potato tubers, onion, and garlic. However, none of these methods have been used for formulations. Methods for analyzing MH in formulations involve either titration or liquid chromatography (LC; 1). Both procedures were developed by the MH manufacturer Uniroyal.

Capillary electrophoresis (CE) is beginning to find its niche in pesticide analysis. In CE, a fused-silica capillary is filled with an aqueous running buffer and an electric field is applied to the capillary. Separation is achieved by migration of charged particles in the running buffer. Cations migrate to the cathode and anions migrate to the anode under the influence of an electroosmotic flow. CE offers many advantages over conventional chromatographic techniques, including decreased use of organic solvents, use of small sample volume, and increased efficiency and resolution. The major disadvantage of CE is decreased sensitivity due to online detection, but this is being corrected by use of capillaries with longer path lengths.

This paper describes a CE method for analysis of the most prevalent MH formulation, MH potassium salt at 30.2 or 30.0%.

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METHOD

Apparatus and Reagents

(**a**) *CE system.*—Hewlett-Packard 3D CE system (Avondale, PA) equipped with a photodiode array detector and an extended light path capillary. Prior to injection, flush the capillary for 1 min with 0.1M NaOH followed by 2 min flush with running buffer. Operating conditions: injection, hydrodynamically for 1 s at 5 mbar; amperage, 40 A; wavelength, 220 nm; capillary temperature, 20°C; temperature of carousel, 25°C.

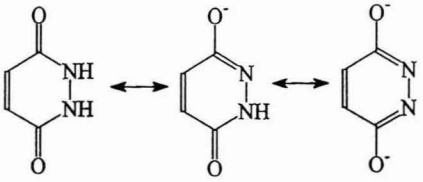
(**b**) *Capillary column.*—Internal diameter, 75 μm; bubble factor, 2.7; total length, 48.5 cm; effective length, 40 cm (Hewlett Packard).

(**c**) *Running buffer.*—10 mM dibasic sodium phosphate (Sigma, St. Louis, MO), prepared daily and filtered prior to use through a 0.45 μm filter disk (Millipore Co., Bedford, MA).

(**d**) *Working standard.*—Accurately weigh ca 20 mg MH reference standard (99% pure; Riedel-de-Haen, Seelze, Germany) into a 25 mL volumetric flask and bring to volume with LC grade methanol (EM Science, Gibbstown, NJ). Dilute the stock 1/1600 in water.

Sample Preparation

MH formulations were liquid containing 30.2 or 30.3% MH potassium salt, all from Drexel Chemical Co. (Memphis, TN), Uniroyal Chemical Co. (Middlebury, CT), and Platte Chemical Co. (Greeley, CO). The pH of the formulation should be between 8.5 and 9.5 to avoid ionization of the second enol moiety in the molecule (Figure 1). Accurately weigh amount of technical liquid formulation containing ca 110 mg MH into a 100 mL volumetric flask and dilute to volume with LC grade



Maleic Hydrazide Free Acid | Maleic Hydrazide Mono-salt pH 8.5-9.5 | Maleic Hydrazide Di-salt pH 10.5 -11.5

Figure 1. Ionization of maleic hydrazide.

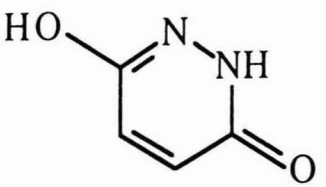


Figure 2. Structure of maleic hydrazide.

water. Make a 1/2000 dilution, filter a portion with a 0.2 μm syringe filter (Gelman, Inc., Ann Arbor, MI), and inject into the CE system.

Determination

Inject standard twice, followed by 2 injections of sample. Finally, inject standard twice. Use peak height to calculate content:

$$\text{Compound, \%} = (R/R') \times (W'/W) \times 500 \times \text{purity of standard} \times 1.34$$

where R and R' = average peak heights of sample and standard, respectively; W' = weight of standard in stock solution (mg); W = weight of sample (mg); 500 = dilution factor; and 1.34 = conversion factor for potassium salt.

Results and Discussion

Because of the chemical structure of MH (Figure 2), it is possible to use UV detection. Furthermore, MH is water soluble. These 2 properties make it an ideal candidate for CE determination. An electropherogram of MH is shown in Figure 3. Baseline separation was accomplished and MH migrated in

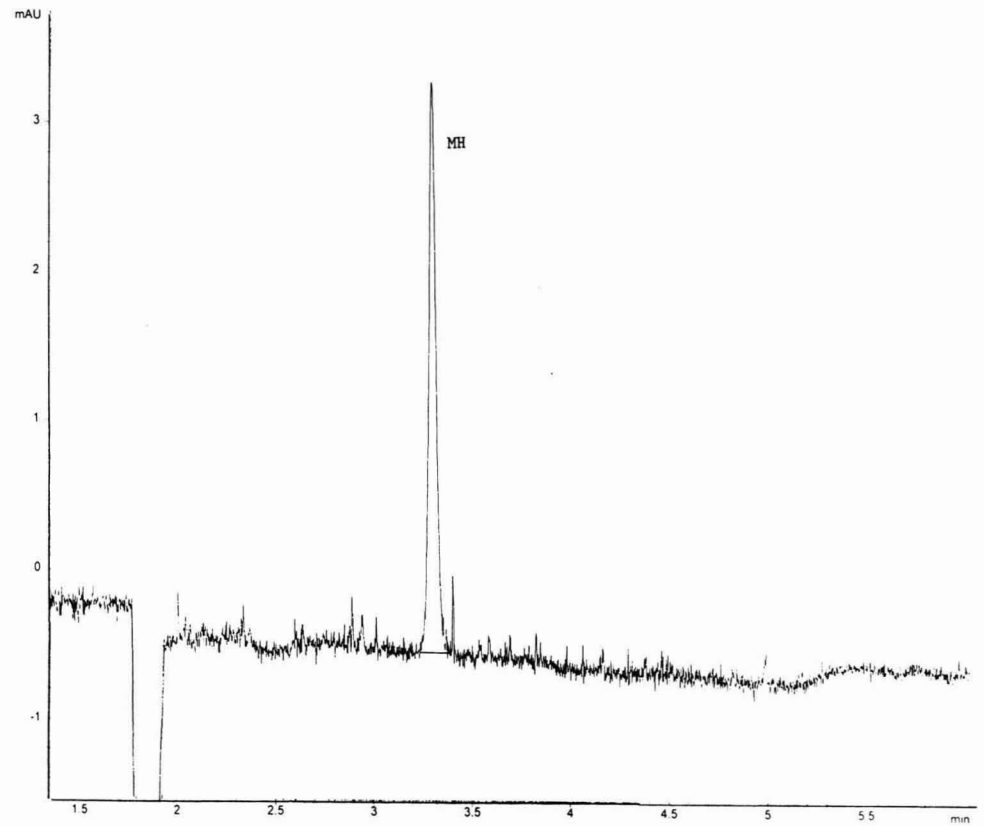


Figure 3. CE electropherogram of MH formulation.

3.5 min. Thus, the total analysis time for each sample, including conditioning, is 10 min.

Using a photodiode array detector, a UV spectrum from 200 to 340 nm was taken for each MH peak. Purity checks indicated that there were no coeluting compounds present. MH can be monitored at either 220 or 313 nm, but at 220 nm the extinction coefficient is much greater and thus a larger peak height is obtained. Peak height was used for all calculations. Although peak height is preferred over peak area if migration time fluctuates, migration time does not vary in this method, and thus, use of peak area should also be fine.

Both within-day and between-day reproducibility studies were performed on 4 formulations. All formulations were guaranteed for 30.2 to 30.3% MH potassium salt. Two were from Uniroyal, one was from Platte Chemical, and the last was from Drexel Chemical. For within-day variations, each formulation was analyzed 10 times in 1 day. For between-day variations, each formulation was analyzed 10 times on 5 different days. Results are presented in Table 1. Within-day coefficients of variation (CVs) ranged from 3.0 to 7.7%. Between-day CVs varied from 5.5 to 10%. Linearity for MH was shown to be from 0.31 to 5 μg/mL. Pearson's correlation for the standard curve was 0.99. The detection limit was 0.25 μg/mL.

The method is very reliable. Because of the short preparation step and fast CE migration, it is very cost and time effective. Because organic solvents are not a hazard, the need for waste disposal is eliminated.

Table 1. CE determination of MH in pesticide formulations

Formulation	Guarantee, %	MH found, % (CV, %)	
		Within-day[a]	Between-day[b]
A	30.3	30.6 (6.5)	29.7 (5.5)
B	30.2	29.9 (7.7)	29.6 (7.6)
C	30.3	31.8 (3.5)	31.1 (6.8)
D	30.3	30.5 (3.0)	31.0 (10.0)

[a] Each value is an average of 10 determinations in 1 day.
[b] Each value is an average of 10 determinations per day performed over 5 days.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Multiresidue Liquid Chromatographic Method for Determining Residues of Mono- and Dibasic Penicillins in Bovine Muscle Tissues

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A liquid chromatographic method with UV detection at 325 nm was developed for simultaneous determination of amoxicillin, ampicillin, penicillin G, and cloxacillin residues in bovine muscle tissue as their mercaptide derivatives. The penicillins are extracted from bovine tissues with 0.1M phosphate buffer (pH 8.5), cleaned up on a t-C_{18} Sep-Pak cartridge, and eluted with 2 mL acetonitrile. After the acetonitrile in the eluate is evaporated to dryness, the residue is dissolved in 200 μL (40 + 60, v/v) acetonitrile–phosphate buffer (pH 6.5) and derivatized with acetic anhydride and mercuric chloride in the presence of 1,2,4-triazole at 65°C for 30 min. Gradient analysis on a Spherisorb 5 μm ODS(2) (octadecyl silane) analytical column using a binary mobile phase consisting of acetonitrile and 0.10M phosphate buffer (pH 6.5) in the presence of 0.0157M sodium thiosulfate at 1 mL/min permits determination of each intact penicillin in bovine muscle tissue at ≥10 ppb with recoveries ≥72%. This laboratory method provides detection sensitivities equivalent to those of rapid tests used for screening β-lactam drug residues in bovine tissue samples for regulatory enforcement.

β-Lactam veterinary drugs besides penicillin G (PENG), such as amoxicillin (AMOX), ampicillin (AMPI), cloxacillin (CLOX), ceftiofur (CEFT), and cephapirin (CEPH), are used extensively in food animal production. Except for PENG, there are no suitable, sensitive analytical methods available for routinely monitoring residues of the other β-lactams in food animals. Therefore, a sensitive, multiresidue chemical method for simultaneous determination of β-lactams including PENG would be more practical to a regulatory laboratory than individual methods developed for analysis of each β-lactam.

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Several multiresidue methods based on UV detection have been developed for determining either only monobasic (1–3) or only amphoteric penicillins. Very few methods are available for simultaneous determination of both (4–6). Palmer and Bywater (4) and Zomer et al. (5) recently used liquid chromatography (LC) with bioautographic detection to determine AMOX, AMPI, PENG, oxacillin, CLOX, and dicloxacillin in animal tissues and body fluids at concentrations down to 10 ng/g. Moats and Harik-Khan (6) also reported a multiresidue LC method for these same β-lactams in milk.

Even though these multiresidue methods have the sensitivity required for regulatory analyses, the extraction and analytical procedures are lengthy and impractical for routine analyses. However, with a mass spectrometer as a specific detector, it is possible to simultaneously analyze amphoteric and monobasic β-lactams at trace concentrations suitable for regulatory monitoring and surveillance. For example, Parker et al. (7) recently determined AMOX, sulbenicillin, PENG, carbenicillin, piperacillin, CLOX, and dicloxacillin in human plasma by LC-electrospray ionization-mass spectrometry (LC-ESI-MS) at concentrations down to 25 ng/mL. Most recently, Tyczkowska et al. (8) determined simultaneously picogram levels of AMOX, AMPI, PENG, CLOX, CEPH, and CEFT in milk by LC-ESI-MS. However, the same procedure using a UV detector in place of the mass spectrometer could detect only about 100 ng of the same analytes/mL in milk. Even though the method seems simple, the detection limits are well above the maximum residue limits (MRLs) defined for these antibiotics in milk. Thus, the LC-UV method is unsuitable for regulatory analyses.

We report here a simple sample preparation procedure that features a precolumn derivatization reaction previously described by Bundgaard (9) and applied to analysis of human serum and urine by Haginaka and Wakai (10) for simultaneous determination of AMOX, AMPI, PENG, and CLOX (i.e., both amphoteric and monobasic penicillins: *see* Figure 1 for chemical structures) in bovine tissues, each at a concentration of ≥10 ppb, with penicillin V (PENV) as internal standard.

METHOD

Materials and Reagents

(a) *Acetonitrile.*—LC grade (Canlab Division, Baxter Diagnostics Corp., Mississauga, ON, Canada).

(b) *Water.*—Obtained from a Barnstead RO/Nanopure ultrafiltration unit.

(c) *Sodium penicillin G, potassium penicillin V, sodium cloxacillin, and amoxicillin reagents.*—Sigma Chemical Co., St. Louis, MO.

(d) *Sodium ampicillin.*—Obtained from Aldrich, Milwaukee, WI.

(e) *Derivatizing reagent.*—2M 1,2,4-triazole containing 10^{-3}M mercuric chloride. Weigh 34.45 g 1,2,4-triazole (Aldrich) into 400 mL beaker, add 150 mL water, and stir with a magnetic bar to dissolve. Add 25 mL 0.01M $HgCl_2$, mix, and adjust pH to 9.0 ± 0.5 with 5M NaOH. Transfer quantitatively into a 250 mL volumetric flask and dilute to volume with water. *Note*: Mercuric chloride is highly toxic. Avoid contact. Consult Material Safety Data Sheets for its handling and disposal.

(f) *Mobile phase phosphate buffer.*—0.1M containing 0.0157M thiosulfate (pH 6.5). Weigh 4.696 g dibasic anhydrous sodium phosphate, 8.969 g monobasic sodium phosphate (monohydrate), and 2.482 g anhydrous sodium thiosulfate. Dissolve in 800 mL water and dilute to volume in a 1 L volumetric flask.

(g) *Extraction solution.*—0.1M NaH_2PO_4 (pH 8.5). Weigh 13.9 g monobasic sodium phosphate and dissolve in ca 900 mL water. Adjust pH with saturated NaOH to ca 8 and then to 8.5 with 5M NaOH. Transfer quantitatively into a 1 L volumetric flask and dilute to volume with water.

(h) *Acetic anhydride (0.2M).*—Pipet 1.9 mL anhydrous acetic anhydride (Mallinckrodt, Point-Claire, PQ, Canada) into ca 80 mL LC grade acetonitrile and mix to dissolve. Transfer quantitatively into a 100 mL volumetric flask and dilute to volume with acetonitrile.

(i) *Borate buffer (0.1M, pH 9).*—Dissolve 1.262 g boric acid (98%, Aldrich) and 8.3 mL 1M NaOH in ca 100 mL water. Transfer quantitatively into 200 mL volumetric flask and dilute to volume with water.

(j) *Sulfuric acid (0.17M).*—Measure 9.4 mL concentrated sulfuric acid (18M) and slowly add to ca 500 mL water in a 1 L volumetric flask. Gently mix and make up to volume with water.

(k) *Aqueous sodium chloride (2%).*—Dissolve 20 g analytical reagent grade NaCl in 880 mL water.

Apparatus

(a) *Solid-phase extraction (SPE) cartridges.*—t-C_{18} Sep-Pak Vac (3 cc, 500 mg, 17% carbon loading, end-capped; Waters Chromatography Ltd., Mississauga, ON, Canada).

(b) *Vortex mixer.*—Variable speed mixer (Canlab Division, Baxter Diagnostics Corp.).

(c) *SPE vacuum manifold.*—Speed-Mate, 30 vacuum manifold (Applied Separations, Bethlehem, PA).

(d) *Solvent evaporator.*—Zymark Turbo LV (Zymark Ltd., Mississauga, ON, Canada).

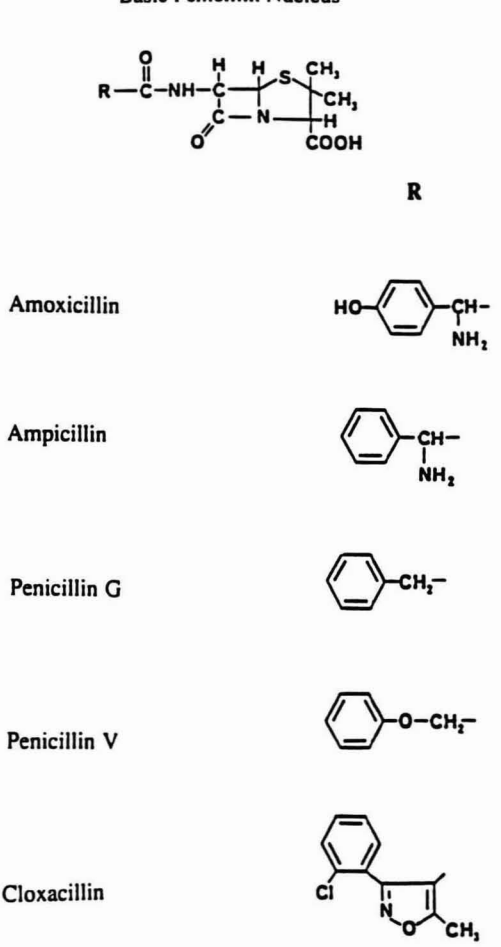

Figure 1. Chemical structures of β-lactams included in this study.

(e) *MSE Coolspin 2 centrifuge with fixed-angle rotors.*—Fisons, Sussex, UK.

(f) *LC-UV equipment.*—Waters Multisolvent Delivery System consisting of a 610 fluid unit, a 610 valve unit and a 600E system controller, a 994 photodiode array detector (PDA), a 5200 printer/plotter, and an ABB SE-120 analog strip chart recorder. A Waters Millilab Workstation with a 50 µL fixed injection loop (Rheodyne) served as the automated injection unit. The components in the tissue extracts were separated on a 250 × 4.6 mm, 5 µm Spherisorb ODS(2) (octadecyl silane) stainless steel column with 12% carbon loading and end-capped (Phenomenex, Torrance, CA). The column was preceded by a Spherisorb C_{18} guard cartridge.

Preparation of Standard Solutions

(a) *Stock standard solutions of AMOX, AMPI, PENG, and CLOX (100 µg active drug/mL).*—Weigh 0.0107 g sodium am-

Table 1. Mobile phase composition for gradient analysis of β-lactam antibiotic residues

Run time, min	Buffer[a] composition, %	Acetonitrile composition, %	Gradient curve
0	82	18	—
8	75	25	6
25	65	35	6
30	0	100	11
33	82	18	11
60	82	18	11

[a] Buffer: 0.10M phosphate buffer (pH 6.5) containing 0.0157M sodium thiosulfate.

picillin, 0.0107 g sodium penicillin G, and 0.0111 g sodium cloxacillin into individual 100 mL calibrated volumetric flasks and make up to volume with water. Weigh 0.0120 g AMOX into a 100 mL calibrated volumetric flask and add ca 80 mL water. Ultrasonicate sample for ca 5 min to dissolve and make up to volume with water.

Note: Store AMPI stock solutions at a concentration of 10 μg/mL at –20°C in 4 mL glass vials. However, if possible, store standards at –76°C or lower.

(**b**) *Stock standard solution of PENV as internal standard (100 μg active drug/mL).*—Weigh 0.0111 g potassium penicillin V and dissolve with water in 100 mL calibrated volumetric flask.

(**c**) *Mixed standard working solution, 2 μg/mL.*—Prepare a solution containing AMOX, AMPI, PENG, and CLOX by serial dilution of stock solutions. This mixed standard may be stored in 4 mL glass vials at –76°C.

(**d**) *PENV working standard solution, 20 μg/mL.*—Prepare from stock standard solution.

Sample Preparation

Accurately weigh 2 g homogenized blank (drug-free) beef muscle tissue into each of five 50 mL polypropylene centrifuge tubes. Accurately weigh 2 g homogenized test samples into 50 mL polypropylene centrifuge tubes. Fortify blank tissue samples with 10, 20, 50, 100, and 200 μL of a 2 μg/mL mixed working standard to prepare tissue calibration standards containing 10, 20, 50, 100, and 200 ng each of AMOX, AMPI, PENG and CLOX, per gram, respectively. Add 25 μL of a 20 μg/mL PENV standard solution to each test and standard sample to provide a constant amount, 250 ng/g, as internal standard. Add 7 mL 0.1M NaH_2PO_4 (pH 8.5) to each sample, and agitate on a Vortex mixer at high speed for 2 min. Add 2 mL 0.17M H_2SO_4, agitate on a Vortex mixer at high speed for 20 s, and centrifuge at 2500 × *g* for 10 min. Decant supernatant into 15 mL polypropylene centrifuge tube. Repeat extraction with 5 mL 0.1M NaH_2PO_4 (pH 8.5) and centrifuge at 2500 × *g* for 10 min. Combine supernatants from 2 extractions in a 15 mL polypropylene centrifuge tube. Add 100 μL 5M NaOH, mix, and centrifuge at 1000 × *g* for 10 min. To avoid analyte losses and clogging of C_{18} cartridges, the pH of the extract at this point should be between 8.3 and 8.5. More NaOH should be added if the pH of the extract is lower than 8.3. However, if the pH is greater than 8.5, the extract must be discarded, the extraction repeated on a freshly weighed tissue sample, and a smaller volume of NaOH added.

Cleanup of Tissue Extracts on t-C_{18} Sep-Pak Cartridges

Condition a t-C_{18} Sep-Pak (3 mL vacuum-type SPE cartridge, Waters Chromatography) with 20 mL methanol followed by 20 mL water, 10 mL 2% NaCl, and 10 mL 0.1M NaH_2PO_4 (pH 8.5) buffer. To maintain anticipated recoveries of analytes from the SPE cartridge, do not substitute other SPE cartridges for this cleanup step. Load extract onto t-C_{18} cartridge at ca 3 mL/min. Rinse centrifuge tube with 10 mL 0.1M NaH_2PO_4 buffer (pH 8.5) and load the rinse onto the t-C_{18} cartridge. Continue evacuating cartridge for another 2 min. Elute penicillins slowly (ca 1 mL/min) with 2 mL acetonitrile. Evaporate eluate to dryness at 50°C (Zymark TurboVap LV evaporator) with prepurified nitrogen. (With nitrogen tank pressure regulator set at 58 psi and inlet pressure of 18 psi, solvent evaporation takes only 6–8 min.) Add 200 μL (40 + 60, v/v) acetonitrile–0.1M phosphate buffer (pH 6.5) to the residue and leave mixture in 50°C water bath for 5 min. Remove sample from the water bath and agitate on a Vortex mixer at high speed for 20 s.

Table 2. Intra-assay precision of method for determining β-lactam residues in bovine muscle tissue[a]

Fortification level, ng/g	Mean response ratio ± SD (CV, %) measured for AMOX	AMPI	PENG	CLOX
10.0	0.060 ± 0.01 (16.6)	0.050 ± 0.004 (8.0)	0.103 ± 0.006 (5.8)	0.090 ± 0.004 (4.4)
20.0	0.150 ± 0.016 (1.7)	0.103 ± 0.005 (4.9)	0.233 ± 0.010 (4.3)	0.158 ± 0.015 (9.5)
50.0	0.371 ± 0.022 (5.9)	0.205 ± 0.013 (6.3)	0.508 ± 0.033 (6.5)	0.345 ± 0.013 (3.8)
100.0	0.791 ± 0.036 (4.6)	0.428 ± 0.022 (5.1)	1.125 ± 0.050 (4.4)	0.720 ± 0.054 (7.5)
200.0	1.732 ± 0.130 (7.5)	0.885 ± 0.066 (7.5)	2.225 ± 0.189 (8.5)	1.475 ± 0.150 (10.2)

[a] Four replicate analyses ($N = 4$) were conducted for each analyte at each of the 5 calibration points. Response ratio is defined as the ratio of the detector response (peak height) of the identified β-lactam to that of the internal standard, PENV. Linear calibration curves with correlation coefficients of 0.9994, 0.9993, 0.9996, and 0.9996 were obtained for the determination of AMOX, AMPI, PENG, and CLOX, respectively, from bovine muscle tissue.

Table 3. Interassay precision and accuracy[a] of method for determining β-lactams added to control tissues at 10, 35, and 150 ng/g

β-Lactam	Concn added, ng/g	Concn found on indicated day, ng/g				Mean ± SD (CV, %)	Accuracy, %
		1	2	3	4		
AMOX	10	12.2	12.2	13.3	—[b]	12.6 ± 0.6 (4.8)	126
	35	34.0	39.0	41.1	40.8	39.0 ± 3.3 (8.5)	111
	150	153	141	154	147	149 ± 6 (4.0)	99
AMPI	10	9.7	10.8	11.5	—	10.7 ± 0.9 (8.4)	107
	35	39.0	36.1	36.0	40.7	38.0 ± 2.3 (6.1)	109
	150	152	148	145	144	147 ± 4 (2.7)	98
PENG	10	10.9	10.0	10.0	—	10.3 ± 0.5 (4.9)	103
	35	39.6	38.2	39.0	40.2	39.3 ± 0.9 (2.3)	112
	150	158	150	152	150	153 ± 4 (2.6)	102
CLOX	10	12.3	12.3	11.5	—	12.0 ± 0.5 (4.2)	120
	35	38.2	41.7	44.4	40.3	41.2 ± 2.6 (6.3)	118
	150	156	143	144	155	150 ± 7 (4.7)	100

[a] Accuracy is defined as the ratio of the amount found by experiment to the actual amount added.
[b] —, samples were analyzed on 3 consecutive days.

Precolumn Derivatization of Extracted Penicillins

Add 20 µL 0.2M acetic anhydride solution to sample, agitate on a Vortex mixer at high speed for 20 s, and add 300 µL pH 9 borate buffer (0.1M). Agitate sample again on a Vortex mixer at high speed for 20 s. Add 500 µL derivatizing reagent, mix, and allow to react in a 65°C water bath or dry bath for 30 min. Filter derivatized penicillins through a 0.45 µm Acrodisc polyvinyledene difloride (PVDF) filter. The sample is now ready for LC-UV analysis.

LC Analysis

The gradient flow conditions shown in Table 1 were used for LC analysis of penicillins on the Waters LC system described.

Recovery and Validation Studies of Analytical Method with Fortified Tissue Samples

Intra-assay precision of the method was determined by replicate analyses ($N = 4$) of blank (drug-free) muscle tissue fortified with all 4 β-lactams at various concentrations (Table 2). Interassay precision (Table 3) was determined by analyses on 3 or 4 consecutive days of blank muscle tissue blind-fortified with each of the 4 analytes at 10, 35, and 150 ng/g. Method accuracy was evaluated by interpolation from the calibration curve to calculate amounts of each analyte in each blind-fortified sample. To determine recoveries of β-lactams added to blank muscle tissues, UV detector responses for each of the analytes in fortified samples taken through the procedure were

Table 4. Recovery of β-lactams from fortified blank (drug-free) bovine muscle tissues

β-Lactam	Concn added, ng/g	N[a]	Recovery, %	RSD, %[b]	Mean recovery, %
AMOX	20	4	64	7	72 ± 6
	50	3	77	3	
	100	4	76	7	
	200	4	71	3	
AMPI	20	4	90	12	81 ± 7
	50	3	76	7	
	100	4	83	3	
	200	4	75	2	
PENG	20	4	90	3	85 ± 6
	50	3	79	1	
	100	4	89	1	
	200	4	81	2	
CLOX	20	4	72	2	76 ± 4
	50	3	76	2	
	100	4	81	8	
	200	4	73	3	

[a] N = number of replicates.
[b] RSD = relative standard deviation.

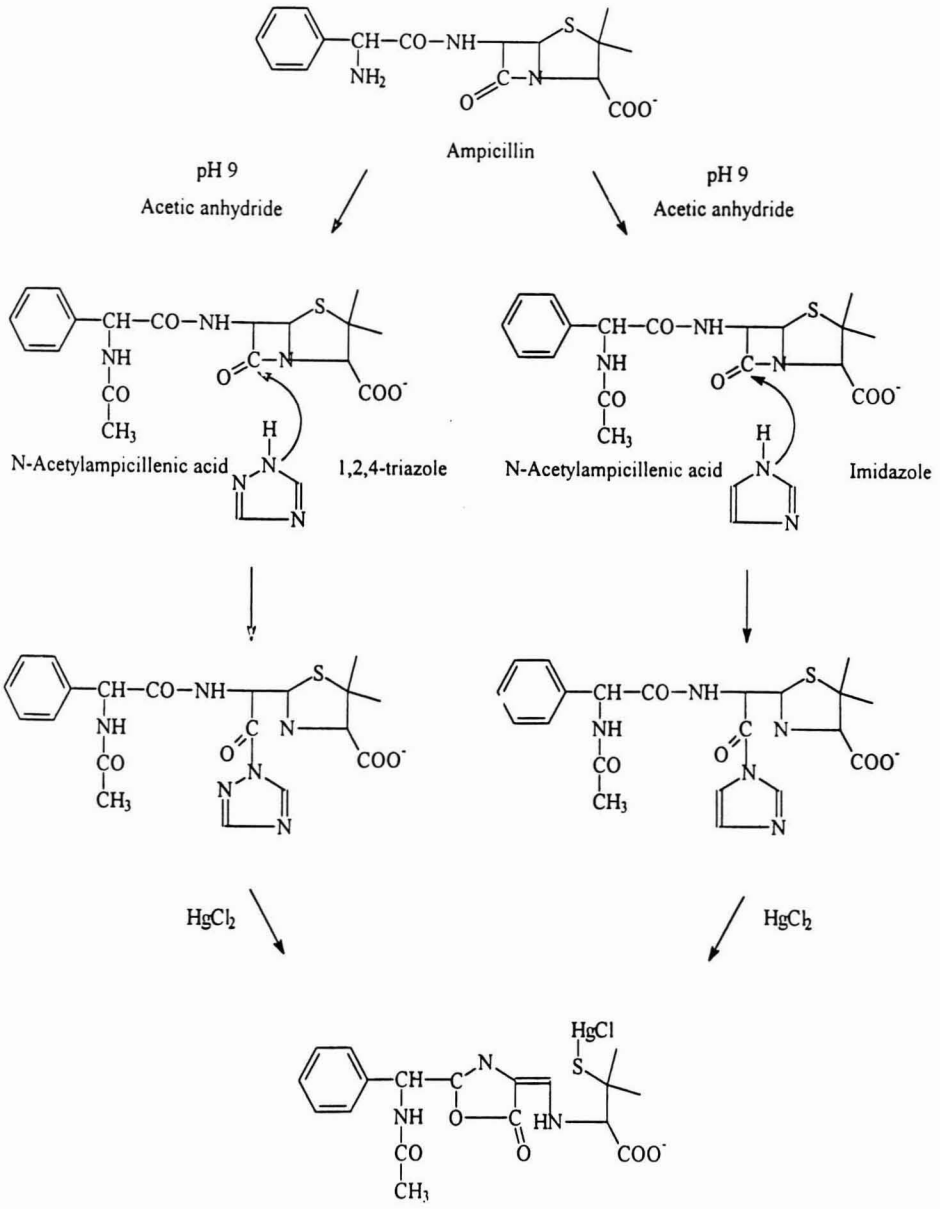

Figure 2. Mechanism of imidazole- or 1,2,4-triazole-catalyzed formation of mercuric mercaptide derivatives of amphoteric penicillins (AMPI or AMOX) after intial N-acetylation (9).

compared with those obtained from equivalent external standards dissolved in 40% acetonitrile–60% phosphate buffer (pH 6.5) and directly derivatized (Table 4).

Results and Discussion

Figure 1 shows the chemical structures of the penicillins included in this study. AMPI and AMOX are dibasic (amphoteric), while CLOX, PENG, and PENV are monobasic. These chemical differences present formidable problems to analysts trying to develop sensitive analytical methods for simultaneous determination of these penicillins in animal tissues at the parts-per-billion (ppb) levels suitable for regulatory enforcement. The success of the analytical procedure described in this paper hinges on the judicious choice of reaction conditions that favor formation of stable products by both dibasic and monobasic penicillins with mercuric chloride with the same degree of specificity.

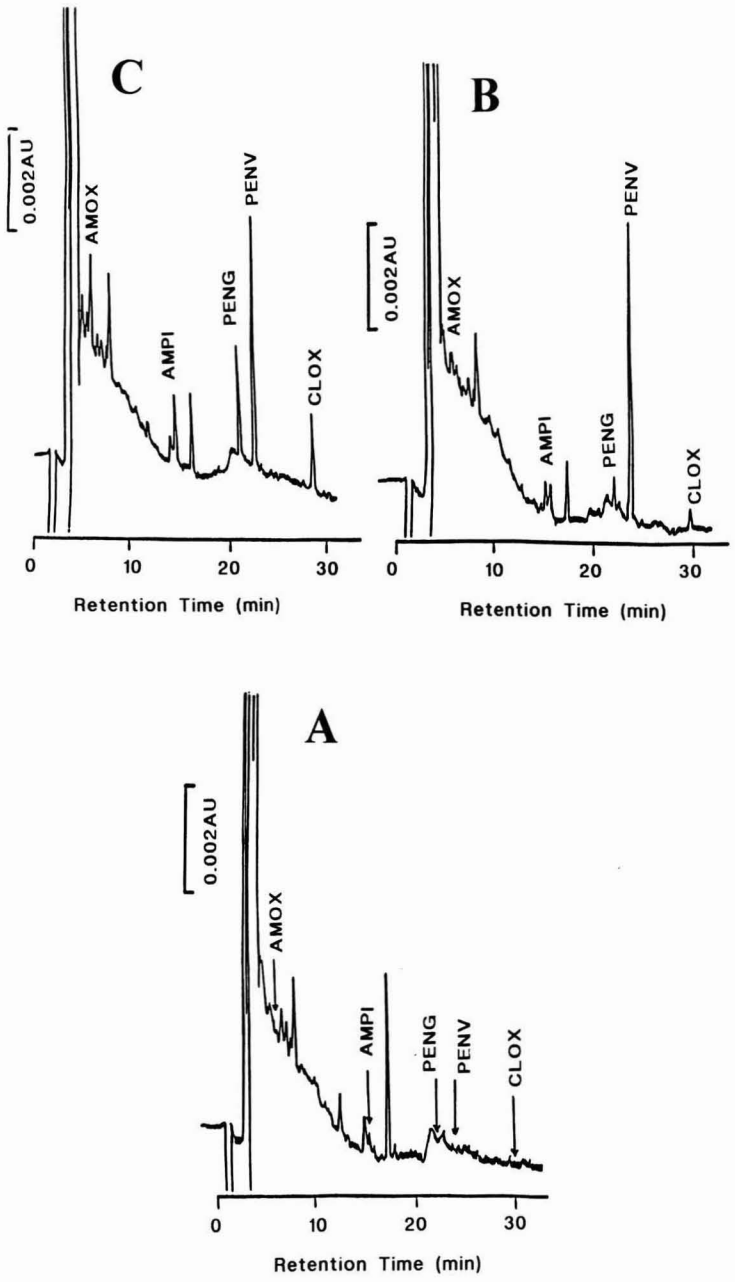

Figure 3. Typical gradient elution chromatogram of an extract of blank bovine muscle tissue (A), and blank bovine muscle tissue fortified with 10 ng/g (B) and 50 ng/g (C) of each of AMOX, AMPI, PENG, and CLOX and the internal standard, PENV at 250 ng/g.

When an aqueous solution of AMPI, an amphoteric penicillin, is treated with 1,2,4-triazole or imidazole, AMPI rearranges at least partly to form the corresponding penicillenic acid, which exhibits a maximum UV absorbance at 322 nm. In the presence of mercuric chloride, an unstable product with an absorption maximum at 311 nm is formed. Under the same conditions, however, monobasic penicillins react to form very stable products with absorption maxima at 325 nm. This unstable product is believed to be α-aminobenzylpenicillenic acid, a compound in which both the thiol and the side-chain amino groups have competed for the available mercuric chloride. If, however, the side-chain amino group in the amphoteric penicil-

Table 5. Maximum residue limits (MRL) defined for β-lactam antibiotics in bovine tissues included in this study (ng/g)

β-Lactam	Codex MRL	Canadian MRL[a]	U.S. MRL[a]	EU MRL[b]
AMOX	—[c]	TND[d]	10	50
AMPI	—	10	10	50
PENG	50	50	50	50
CLOX	—	—	10	300

[a] Reference 11.
[b] EU = European Union; reference 12.
[c] — = not defined by Canada or Codex.
[d] TND = tolerance not defined.

Table 6. Evaluation of validated operating parameters

	Level of fortification (concn found), ng/g			
Sample No.[a]	AMOX	AMPI	PENG	CLOX
138	88 (90)	88 (90)	88 (83)	88 (84)
139[b]	15 (21)	15 (—)	15 (13)	15 (13)
140	75 (86)	75 (73)	75 (73)	75 (60)
141	88 (86)	88 (85)	88 (90)	88 (92)
143	15 (17)	15 (16)	15 (14)	15 (14)
142	75 (83)	75 (80)	75 (76)	75 (77)

[a] Samples were fortified by quality assurance chemist and analyzed according to the described method.
[b] A detectable amount of AMPI was observed but could not be quantitated.

lin (AMPI or AMOX) is initially acetylated to form α-acetamidobenzylpenicillin, then the resulting product will react like any of the monobasic penicillins with mercuric chloride in the presence of 1,2,4-triazole or imidazole to form α-acetamidobenzylpenicillenic acid mercuric mercaptide, a stable product. This product exhibits an absorption maximum at 325 nm and a molar absorbance comparable with those of other monobasic penicillins. It is this reaction, whose mechanism (Figure 2) was first explained by Bundgaard (9), that was used in this method to permit simultaneous analyses of both monobasic and dibasic penicillins. The acetylation reaction occurs instantaneously at room temperature and does not, therefore, introduce any delays in the total reaction time for the penicillins.

The precolumn derivatization reaction of the penicillins with mercuric chloride provides optimal chromatographic parameters when conducted in an acetonitrile–buffer (40 + 60, v/v) medium. When the composition of acetonitrile in the reaction medium is increased, the resulting mixture shows decreased analyte sensitivity, usually accompanied by broad tailing and split peaks. Acetonitrile (100%) significantly suppresses the derivatization reaction between β-lactams and mercuric chloride. Thus, even though it is the most suitable solvent for eluting β-lactams from the t-C_{18} cartridge, it must be evaporated prior to chemical derivatization. Petz (13) has discussed extensively the problems associated with evaporating solutions containing penicillins to dryness using rotary evaporation and advised against that practice. However, we did not find such problems when we evaporated the acetonitrile–penicillin extracts to dryness at 50°C with the Zymark LV sample evaporator. This, we believe, is due to the different mechanism by which sample evaporation is effected by the Zymark Sample evaporator (centrifugal versus surface). Use of the Zymark evaporator, therefore, permitted us to reproducibly and rapidly remove the solvent without any deleterious effects. Therefore, we strongly recommend that this apparatus be used exclusively for this stage of the evaporation.

Figure 3A shows a typical chromatogram of a blank (drug-free) muscle tissue extract. Figures 3B and 3C show chromatograms of extracts from blank muscle tissues fortified at 10 and 50 ng/g, respectively, with AMOX, AMPI, PENG, CLOX, and the internal standard, PENV (250 ng/g), detected as their mercaptide derivatives at 325 nm. The chromatograms demonstrate that the β-lactams are separated from one another and from other endogenous and exogenous components in the tissue extracts. Additionally, Figure 3B demonstrates that these analytes can be detected at the MRLs (11, 12) defined in the United States, Canada, and the European Union for regulatory enforcement of these veterinary drugs in bovine muscle tissue (Table 5).

In selecting a suitable analytical column for chromatographic resolution of the β-lactams, we found that the Spherisorb 5 μm ODS(2) column gives the maximum detector response among the columns we tested. This column provides 15 500 theoretical plates for benzophenone in the LC column test mixture when operated with a 75% acetonitrile–water mobile phase at 1 mL/min.

A maximum ion-pair (sodium thiosulfate) concentration of 0.0157M and a buffer concentration of 0.10M were optimal for chromatographic analyses of the β-lactams. Even though sensitivities of monobasic penicillins are not significantly affected by buffer concentration, a reasonably high molarity of phosphate buffer in the mobile phase must be maintained to obtain suitable detector responses for amphoteric penicillins. However, because phosphate buffer concentrations <0.10M caused mixing problems in the pumps and occassionally precipitated in the LC transfer lines in the presence of high percentage of organic modifier, we limited the concentration of phosphate buffer in the mobile phase to 0.10M.

Within-day (Table 2) and between-day (Table 3) precisions (coefficient of variation, CV) of the method were less than 10% for concentrations ranging from 10 to 200 ng/g muscle tissue. Linear calibration curves with correlation coefficients >0.999 were obtained for each β-lactam (Table 2). Concentrations of each of the 4 β-lactams added to blank muscle tissue (at or above the MRLs defined in Table 5) can be determined with accuracies ranging from 99 to 120% (Table 3). Mean recoveries of AMOX, AMPI, PENG, and CLOX from fortified bovine muscle tissue were 72 ± 6, 81 ± 7, 85 ± 6, and 76 ± 4%, respectively (Table 4). Recovery of each β-lactam antibiotic added to blank bovine muscle tissue was calculated by comparing the

UV detector response (peak height) measured for an analyte of interest in the sample that has been subjected to the procedure with that of an equivalent external standard of β-lactam dissolved in 40% acetonitrile–60% 0.1M phosphate buffer (pH 6.5) directly derivatized as described previously. It is very important, however, to use control tissue fortified with various concentrations of the drugs of interest for generating calibration curves for quantitative analysis, because the β-lactams are unstable in the dissolving solution for extended periods (1–2 h) in the absence of a tissue matrix.

The method incorporates PENV as an internal standard. In addition to correcting for losses in sample preparation, the internal standard provides predictable response ratios for each of the β-lactams, which may be used to monitor the validity of the analytical measurements. For example, at a fortification of 100 ng/g of tissue, response ratios of about 0.8, 0.4, 1, and 0.7 are expected for AMOX, AMPI, PENG, and CLOX, respectively. Sample preparation for LC analyses required about 2.5 h for a set of 12 samples, including 4 calibration standards.

The method was used to determine the concentrations of β-lactams in 2 sets of fortified bovine tissue (samples 138–140 and 141–143) prepared by a quality assurance chemist from drug-free tissues. One set, samples 138–140, was analyzed immediately. The second set, samples 141–143, was stored at –76°C and analyzed a week later. Results are shown in Table 6. Except for sample 139, in which a trace amount of AMPI was detected but could not be quantitated, the concentrations of all the other analytes added to the other samples were correctly detected and quantitated.

Conclusions

The simple and accurate LC-UV method developed permits simultaneous detection and analysis of β-lactam antibiotic residues, be they monobasic or amphoteric, at the MRLs defined for bovine muscle tissue. This has been made possible by use of precolumn derivatization reaction conditions favorable for the formation of the mercaptide complex of each intact β-lactam, regardless of whether it is dibasic or monobasic. The method will permit regulatory laboratories that previously could not afford an electrospray ionization mass spectrometer, which is required by the only suitable method currently available for simultaneous analyses of these β-lactams, to determine residual concentrations of β-lactams commonly used in food animal production with simple, easily available, and affordable laboratory instrumentation.

Acknowledgment

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DRUGS, COSMETICS, FORENSIC SCIENCES

Solid-Phase Extraction Cleanup and Liquid Chromatography with Ultraviolet Detection of Ephedrine Alkaloids in Herbal Products

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A solid-phase extraction (SPE) cleanup and a liquid chromatographic (LC) method with UV detection is presented for analysis of up to 7 ephedrine alkaloids in herbal products. Alkaloids from herbal products are extracted with acidified buffer, isolated on a propylsulfonic acid SPE column, eluted with a high-ionic-strength buffer, and separated by LC with detection at 255 nm. LC separation is performed by isocratic elution on a YMC phenyl column with 0.1M sodium acetate–acetic acid (pH = 4.8) containing triethyl-amine and 2% acetonitrile. Ephedrine alkaloids are completely separated in 15 min. Average recovery of 5 common alkaloids from 3 spiked matrixes is 90%, with an average relative standard deviation (RSD) of 4.4% for alkaloid spikes between 0.5 and 16 mg/g. Average quantitation of ephedrine and pseudoephedrine from 6 herbal products is 97% of declared label claims, and average quantitation of synephrine from an herbal dietary product is 85% of label claim (RSD, 3.2%). Recoveries of synephrine, norephedrine, ephedrine, pseudoephedrine, *N*-methylephedrine, and *N*-methylpseudoephedrine spiked in 4 herbal products averaged 95%. Results of ruggedness testing and of a second laboratory validation of the procedure are also presented.

Ephedrine alkaloids are derivatives of 2-amino-1-phenyl-1-propanol where the amino group is free, methylated, or dimethylated. The 3 pairs of diastereomeric alkaloids include norephedrine (NOR), norpseudoephedrine (NPE), ephedrine (EPH), pseudoephedrine (PSE), methylephedrine (MEP), and methylpseudoephedrine (MPE). These alkaloids are sometimes found in dietary supplements that promote weight loss, body building, and increased energy (1, 2). The main sources of ephedrine alkaloids are raw botanicals and extracts from plants of the genus *Ephedra*. About 100 000 kg *Ephedra* powder and extracts were imported into the United States in 1993 alone (2). The principal ephedrine alkaloids in *ma huang*, a traditional Chinese medicine derived from the dried stem of the plants, are EPH and PSE, with EPH comprising up to 80% of the total alkaloid content (2). Typical *ma huang* is approximately 1% ephedrine alkaloids by weight; however, concentrated extracts frequently contain 4–8% ephedrine alkaloids (2).

Ephedrine alkaloids are chemical stimulants and can affect the cardiovascular and nervous systems of humans. Misuse of these alkaloids is common (2, 3), and since 1993, the U.S. Food and Drug Administration (FDA) has reported more than 800 instances of illnesses and injuries associated with use of products containing or suspected to contain ephedrine alkaloids (1, 2). As a result of these reports, FDA proposed a rule to limit the amount of ephedrine alkaloids in dietary supplements to 8 mg per serving up to 24 mg per day and to limit intake of ephedrine-containing products to 7 days (1, 4).

A quick, reliable, and powerful analytical method is needed to separate and detect these ephedrine alkaloids in herbal matrixes at the levels of concern. However, analysis of herbal products containing ephedrine alkaloids is complicated because of the potential occurrence of up to 7 similar alkaloids, the wide range in concentrations possible, the presence of matrix contaminants, the need to analyze large numbers of samples, and the occurrence of many different matrixes. Several potential multiresidue procedures exist. Liu et al. (5–7) and Flurer (8) used capillary electrophoresis (CE) with UV detection to analyze Chinese herbal products. Betz et al. (9), LeBelle et al. (10), Chiu et al. (11), and Yamasaki et al. (12) analyzed herbal products by gas chromatography (GC) with various detectors. Jian-Sheng et al. (13), Sagara et al. (14), and Price et al. (15) used liquid chromatography (LC) with UV detection to analyze ephedrine alkaloids in plant tissue.

These methods involved very little cleanup. Alkaloids usually were extracted and injected directly into the instrument of choice. Although lack of a cleanup procedure was adequate for CE methods, LC and GC methods suffered from either short column life or from the necessity of derivatization.

Several authors have used cyano, reversed-phase, and mixed-phase, solid-phase extraction (SPE) for cleanup of urine and plasma samples containing ephedrine alkaloids (16–27).

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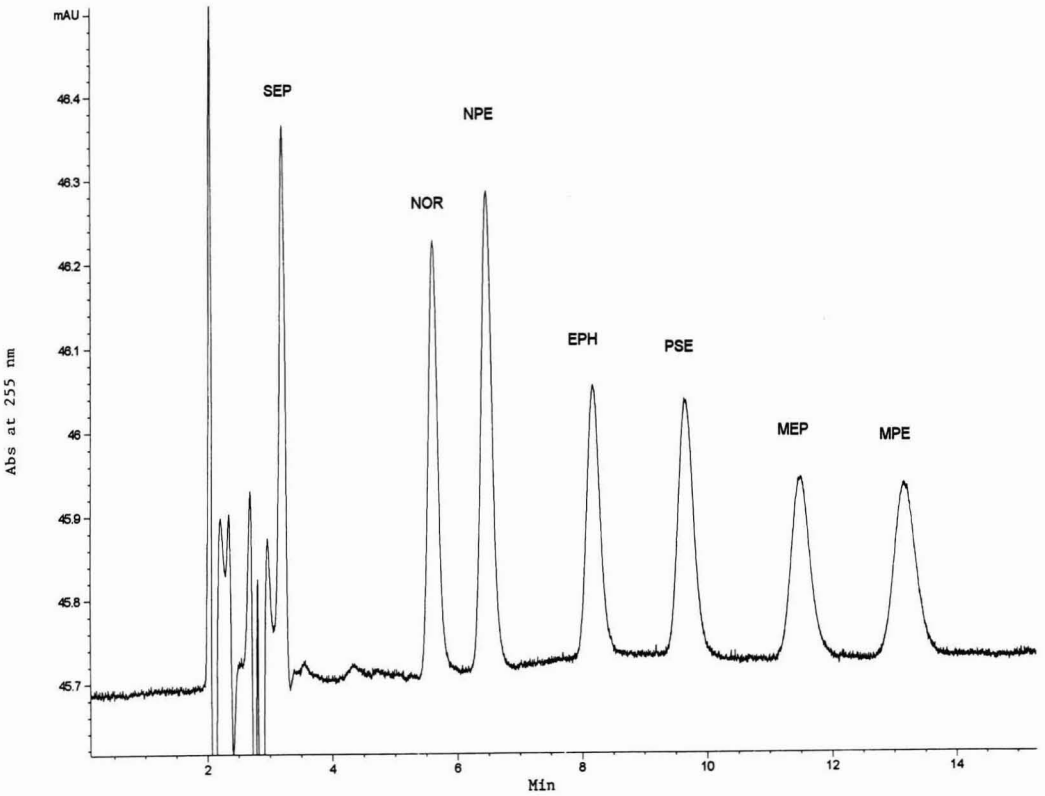

Figure 1. Representative chromatogram of a standard solution containing SEP at 10 μg/mL and NOR, NPE, EPH, PSE, MEP, and MPE, each at 20 μg/mL.

For our preliminary cleanup, separation, and detection method (27), we used a propylsulfonic acid (PRS) SPE column to clean up herbal products containing ephedrine alkaloids. However, ruggedness testing revealed that the method suffered from a wandering internal standard, interfering peaks, plasticizers coming from the SPE column during the organic wash, and low recoveries of late-eluting alkaloids.

On the basis of available equipment, the need for a rugged method, and the desire to keep the procedure simple, we reinvestigated that method (27) and developed a modified procedure using acidic extraction, SPE cation-exchange cleanup, LC separation using an isocratic mobile phase and a YMC phenyl column, and UV detection at 255 nm.

METHOD

Apparatus

(**a**) *LC systems.*—(*1*) *Laboratory 1.*—Beckman 110B pumps (Fullerton, CA), isocratic flow at 0.80 mL/min, Beckman 420 controller, Rheodyne 7725 injector (20 μL) (Rohnert Park, CA), Beckman 163 UV detector at 255 nm. (2) *Laboratory 2.*—Hewlett-Packard 1040 LC (Palo Alto, CA) with a diode array detector at 255 nm.

(**b**) *pH Meter.*—Orion Model 601A (Cambridge, MA), calibrated at pH 4.00 and 7.00.

(**c**) *Column.*—(*1*) *Analytical.*—3.0 × 250 mm, S-5 μm, 120A, Phenyl, Cat. No. PH12S052503WT, YMC, Inc. (Wilmington, NC). (2) *Guard.*—30 × 4.6 mm, 5 μm, Ultremex Phenyl, Cat. No. 03A-0052-E0, Phenomenex (Torrance, CA).

(**d**) *SPE.*—500 mg, 6 mL PRS, Cat. No. 540-0050-C, Isolute (Mid-Glamorgan, UK); Varian Vac-Elute SPE manifold (Palo Alto, CA).

(**e**) *Pipettes.*—Calibrated 100–1000 μL Eppendorf (Brinkmann Instruments, Westbury, NY); 4 and 25 mL class A volumetric; 10 mL Mohr.

(**f**) *Filter paper.*—Whatman No. 1 qualitative, 110 mm filter papers, Cat. No. 1001-110 (Clifton, NJ).

Reagents and Solutions

(**a**) *Standards.*—(–)-(1*R*,2*S*)-norephedrine, (–)-norpseudoephedrine, (–)-(1*R*,2*S*)-ephedrine, (+)-(1*S*,2*S*)-pseudoephedrine, (–)-(1*R*,2*S*)-*N*-methylephedrine, (+)-(1*S*,2*S*)-*N*-methylpseudoephedrine, and synephrine, all with purity ≥98%, Aldrich Chemical Co. (Milwaukee, WI). HCl salts can be substituted as long as they are appropriately converted to the free

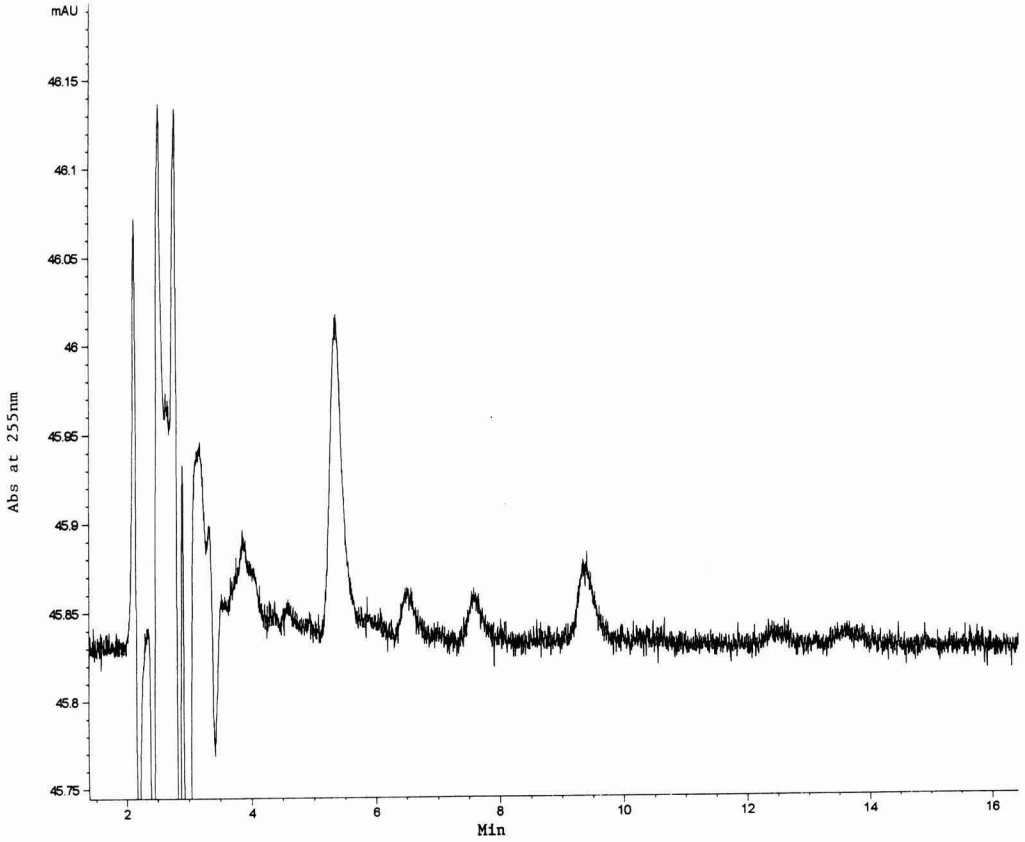

Figure 2. Chromatogram of matrix C blank (1.0 g).

bases. (Norpseudoephedrine is no longer available from Sigma and Aldrich).

(**b**) *Chemicals.*—Anhydrous sodium acetate, Mallinckrodt, AR grade (Paris, KY), Cat. No. 7372, or equivalent; acetonitrile, Baker Analyzed LC Grade, Cat. No. 9255-03 (J.T. Baker, Phillipsburg, NJ), or equivalent; methanol, J.T. Baker, LC grade, Cat. No. 9093-33, or equivalent; glacial acetic acid, Baker Analyzed, Cat. No. 9508-01, or equivalent; 99% triethylamine (TEA), Sigma (St. Louis, MO), Cat. No. T-0886, or equivalent. *Note*: If TEA is yellow, then distill.

(**c**) *Mobile phase (MP).*—Dissolve 16.4 g sodium acetate in 1.94 L LC grade water and add 16 mL acetic acid, 6.0 mL TEA, and 40 mL acetonitrile; pH should be 4.8. Filter through 0.45 μm filter and store at 4°C when not in use.

(**d**) *Diluted mobile phase (DMP).*—Dilute 100 mL MP to 500 mL with LC grade water. Store at 4°C when not in use.

(**e**) *Elution buffer (EB).*—Dissolve 16.4 g sodium acetate in 970 mL LC grade water and add 8.0 mL acetic acid, 3.0 mL TEA, and 20 mL acetonitrile; pH should be 4.8. Store at 4°C.

(**f**) *Standard solutions.*—Prepare individual stock standards of SEP, NOR, NPE, EPH, PSE, MEP, and MPE at 4.0 mg/mL each in DMP. Prepare a combination stock standard of these alkaloids at 4.0 mg/mL each in DMP. Sonication is necessary for dissolution. Prepare working standards by diluting stock standard with DMP. All standards are stable for >1 month if stored at 4°C. MEP and MPE solids are hygroscopic and must be protected from moisture.

Calibration Curve

(**a**) *Retention times.*—Prepare individual 20 μg/mL standards of each ephedrine alkaloid in DMP from the 4.0 mg/mL stock standards. Inject each into the LC system and determine retention times.

(**b**) *Calibration curve.*—Prepare 5 combination working standards in DMP with ephedrine concentrations ranging from 4.0 to 150 μg/mL. Each standard will contain all of the ephedrine alkaloids. Inject each of the 5 combination standards into the LC system and obtain retention times and peak areas. Prepare calibration curves for each ephedrine alkaloid by plotting peak area vs the alkaloid concentration (μg/mL). Correlation coefficients (r^2) should all be ≥0.999. Resolution of all adjacent ephedrine peaks should be >2; the tailing factors for all peaks should be <1.4; and the maximum retention time should be <15 min for flow rates greater than 0.7 mL/min.

Table 1. Recoveries[a] of ephedrine alkaloids from spiked matrixes

Spike, mg/g	Matrix[b]	Recovery, % (RSD, %)				
		NOR	EPH	PSE	MEP	MPE
0.48	A	98.3 (4.2)	99.7 (6.9)	101 (5.8)	91.0 (8.8)	88.6 (8.0)
2.0	A	88.3 (5.3)	102 (2.1)	89.4 (5.3)	88.5 (4.7)	87.8 (5.0)
4.0	A	82.1 (3.8)	93.5 (2.5)	90.4 (2.4)	85.7 (3.7)	82.2 (2.9)
8.0	A	97.8 (2.5)	99.3 (2.8)	88.3 (2.0)	95.0 (3.6)	91.7 (4.3)
4.0	B	90.2 (3.6)	96.8 (6.8)	94.8 (1.9)	89.8 (2.0)	87.2 (2.5)
8.0	B	85.5 (4.4)	96.4 (4.6)	98.0 (3.3)	82.6 (6.1)	90.2 (7.2)
2.0	C	84.9 (3.7)	83.1 (2.9)	84.1 (2.7)	81.4 (6.1)	71.5 (2.6)
4.0	C	88.3 (1.4)	89.1 (1.8)	87.8 (4.9)	79.3 (6.2)	72.3 (7.4)
8.0	C	81.7 (6.6)	88.3 (2.0)	89.0 (11)	80.0 (3.3)	80.0 (7.2)
16	C	94.6 (4.7)	93.7 (3.0)	96.1 (2.7)	88.7 (1.0)	83.4 (2.6)
2.0	C[c]	88.7 (5.4)	95.5 (3.7)	98.6 (7.5)	86.4 (4.0)	79.9 (5.5)
4.0	C[c]	91.4 (5.8)	99.6 (4.7)	91.7 (7.1)	90.0 (7.0)	82.0 (3.2)
8.0	C[c]	97.1 (5.8)	102 (3.2)	94.0 (2.4)	94.7 (4.6)	89.3 (5.0)

[a] Results are based on analyses of 5 separate samples.
[b] Matrix consisting of green tea, kola nut, and black tea at ratios of 2:1:0 (A), 1:2:0 (B), and 1:1:1 (C).
[c] Samples were analyzed in laboratory 2 by a different analyst using different sets of reagents and a different LC system.

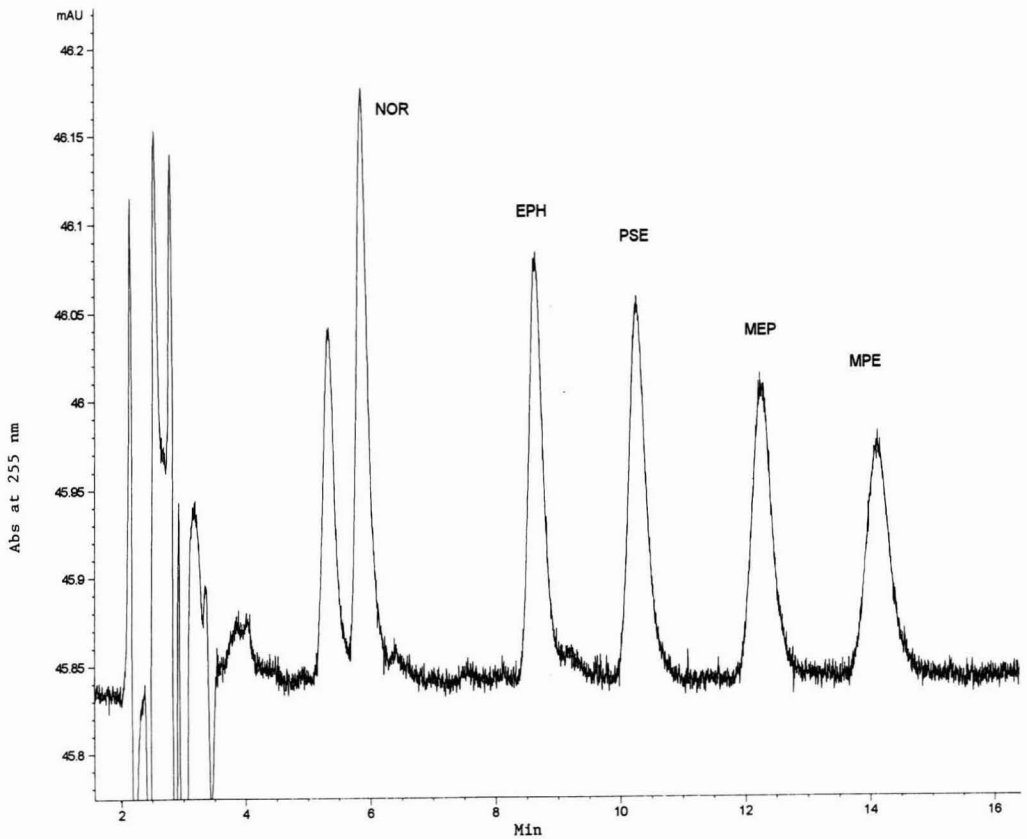

Figure 3. Representative chromatogram of matrix C (1.0 g) spiked with NOR, EPH, PSE, MEP, and MPE, each at 2.0 mg/g.

Sample Procedure

(a) *Step 1.*—Accurately weigh 0.5 g herbal product into a 50 mL Erlenmeyer flask. If desired, a wet spike of the 4.0 mg/mL combination standard can be added at this time.

(b) *Step 2.*—Add a magnetic stir bar and 25.0 mL DMP. Cover flask with Parafilm and stir for 20 min at room temperature on a magnetic stir plate.

(c) *Step 3.*—Gravity filter extract through 11 cm Whatman qualitative filter in a 60° glass funnel, and catch several milliliters of extract in a dry 125 mL Erlenmeyer flask. Use 1.00 mL filtered extract in step 5 if alkaloid content is ≥8 mg/g, and use 2.00 mL if alkaloid content is <8 mg/g.

(d) *Step 4.*—Prepare 500 mg, 6 mL PRS SPE column by washing with 2 to 5 mL each of methanol, followed by water, and followed by DMP. Discard all washes. Do not let column dry. Use vacuum to pull washes through column.

(e) *Step 5.*—Pipet either 1.00 or 2.00 mL filtered extract (step 3) onto top of prepared SPE column and allow extract to soak into column. A mild vacuum may be needed to start the flow. Do not allow column to dry.

(f) *Step 6.*—Wash column with 4.0 mL DMP followed by 5 to 6 mL methanol. With vacuum, pull air through column for 1 to 2 min. Discard all washes.

(g) *Step 7.*—Place 10 mL volumetric flask under column to collect alkaloids. Add 4.0 mL EB to column. Allow 4 mL EB to sink into column, apply vacuum, and elute at 0.5 to 1 mL/min. Pull air through for 1 to 2 min. Bring collected eluate up to 10.0 mL with water.

(h) *Step 8.*—Inject 20 µL of 10 mL extract from step 7 into LC system and obtain retention times and peak areas.

(i) *Step 9.*—Inject appropriate working standard containing ephedrine alkaloids 3 times during analysis. Obtain retention times and average peak areas for each alkaloid. Calculate concentration of each alkaloid in sample.

Results and Discussion

This rapid and reliable procedure for separating and detecting several ephedrine alkaloids in herbal matrixes resulted from ruggedness testing of an earlier procedure we developed (27). This earlier procedure involved use of 4 items that caused problems: a (1 + 1) ethyl acetate–acetone wash, phentermine as internal standard, Phenomenex phenyl column, and 0.15M sodium acetate EB. The ethyl acetate–acetone wash extracted plasticizers from the SPE column, resulting in 2–4 interfering peaks in the chromatogram. Switching to a methanol wash resulted in no detectable plasticizers and cleaner chromatograms. Use of phentermine as an internal standard lengthened and complicated the procedure because of its rapidly changing retention times when used with the Phenomenex column: Resolution of phentermine and PSE peaks varied from 1.2 to 2.1 within 20 injections. Elimination of the internal standard and use of external calibration curves resulted in a reliable and more rapid procedure. Interfering peaks from herbal products were sometimes observed with the Phenomenex column. Switching to the YMC phenyl column eliminated this problem by moving interfering peaks away from the NOR and EPH peaks. A small peak sometimes interfering with <0.5 mg/g PSE was observed. Finally, low recoveries of the late-eluting MEP and MPE alkaloids were traced to retention on the SPE column when EB was 0.15M sodium acetate. Use of 0.20M sodium acetate corrected this.

Standards containing SEP, NOR, EPH, PSE, MEP, and MPE were run through the method, and several variables were examined. Varying the DMP wash volume between 3 and 6 mL and the methanol wash volume between 4 and 8 mL had less than a 5% effect on recoveries on 5 alkaloids. However, up to 10% of early-eluting SEP was washed off with volumes of DMP > 4 mL. The volume of the 0.2M sodium acetate EB was also investigated: A plot of alkaloid recoveries versus EB volumes levels off at 3.5 mL; 4.0 mL is adequate to recover >90% of NOR, EPH, and PSE and >80% of SEP, MEP, and MPE

Table 2. Quantitation[a] of EPH and PSE in various herbal products

Herbal product[b]	Laboratory	Label claim, mg EPH/g	Recovery, mg/g		Recovery, % of label claim[c]	RSD, %
			EPH	PSE		
Product 1, finished product	1	15	14.8	0.4	98.5	4.7
Product 1, finished product	2	15	15.2	<0.5	101	5.2
Product 2, raw product	1	60	58.9	<0.4	98.2	6.3
Product 3, raw product[d]	1	60	56.9	—[e]	94.8	15
Product 3, raw product	2	60	59.1	2.8	98.5	5.6
Product 4, finished product	1	20	20.8	6.10	104	6.2
Product 5, finished product	1	25	20.9	<0.4	83.7	4.5
Product 6, finished product	1	10.6[f]	7.78	2.54	97.4	1.5
Product 6, finished product	2	10.6[f]	7.93	2.53	98.7	5.0

[a] Results are based on analyses of 5 separate samples.
[b] Finished products were on-shelf products; raw products were *ma huang* extracts.
[c] Recoveries were based on label claims for EPH concentration, except those for product 6, which were based on label claims for total ephedrine alkaloid concentration.
[d] PSE peaks were present but not integrated.
[e] —, PSE analysis was not performed due to poor baseline from a noisy deuterium lamp.
[f] Unit of label claim is mg alkaloids/g.

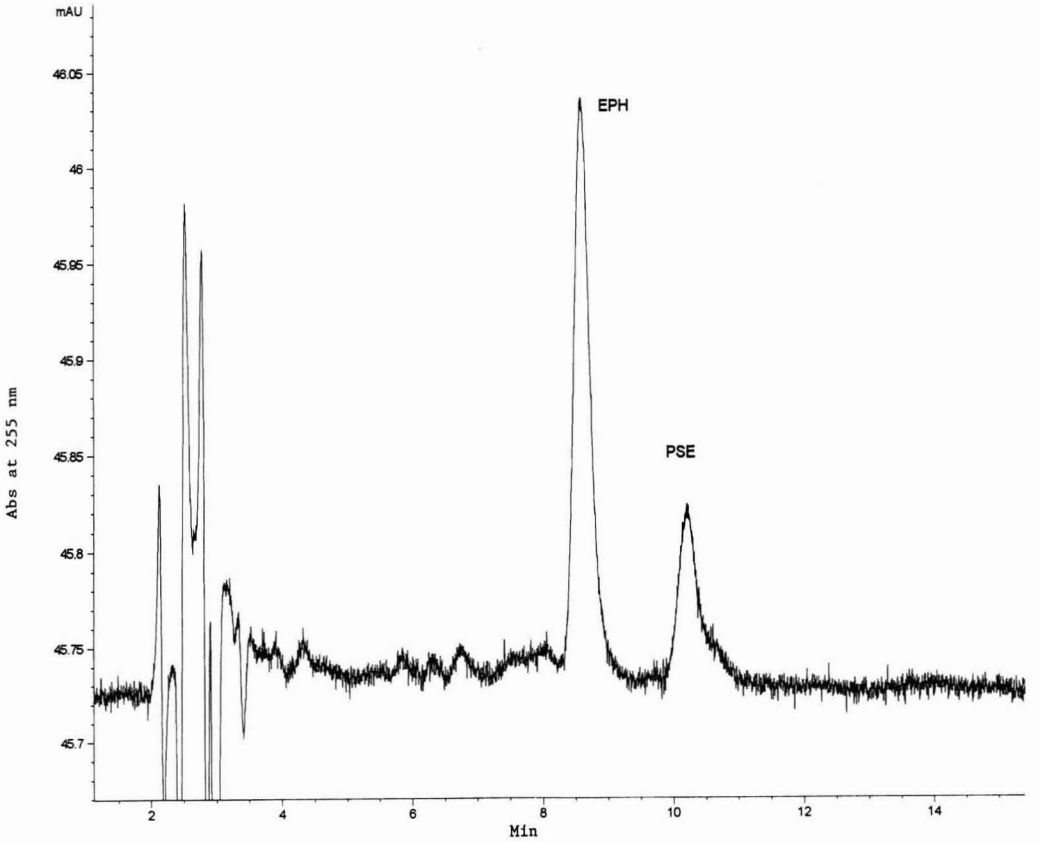

Figure 4. Chromatogram of herbal product (0.62 g) containing 8.8 mg EPH/g and 3.2 mg PSE/g.

(step 7). Flow rates through the SPE column can vary between 0.5 and 2 mL/min with no change in results. Four lots of Isolute SPE columns and 3 YMC analytical columns gave identical results. However, different brands of both PRS SPE and phenyl analytical columns require changes in the procedure in order to obtain good recoveries. Changes in column temperatures (15° to 40°C), flow rates (0.5 to 1.2 mL/min), LC pump brands (Beckman, Hewlett-Packard, and Perkin Elmer), detector brands (Beckman, Hewlett-Packard, and Perkin Elmer), and acetonitrile concentration (1.0 to 4.0%) did not significantly influence results. Retention times typically varied less than ± 0.4% during a full day of manual injections; however, variations of up to 5% were experienced from one day to another, and these changes were traced to changes in the actual flow rate from bubbles in the pump heads. Over a 6-month period, retention times and column pressure on the YMC column did not change. Freshly prepared or 3-month-old stock standards and freshly prepared or 2-week-old working standards gave similar results when stored at 4°C; no protection from light was needed. The 500 mg SPE columns could retain up to 4 mg total ephedrine alkaloid standard with less than 10% breakthrough. However, when herbal matrix was used, then maximum alkaloid load on the SPE column was 1 mg. Finally, both recoveries and precision (relative standard deviation, RSD) were within a few percent among several analysts. Both experienced analytical chemists and junior-level chemistry students quickly mastered the method with standards and actual samples. NPE was not used because it is not readily available at present.

Synephrine (SEP) can be isolated and detected with this procedure, even though it elutes close to the solvent front. It is a biologically active ephedrine-like compound (28) that is found in *Citrus aurantium*. SEP recently has been found in herbal diet products, and it is apparently used in place of ephedrine. Analysis of a commercial dietary product containing 13 mg SEP/g and advertised as "ephedrine free" gave an average recovery of 85% based on the label claim (RSD for 5 runs was 3.2%). Recoveries of spikes adding 4 mg SEP/g averaged 102%.

Calibration curves for NOR, EPH, PSE, MEP, and MPE were linear for each alkaloid at concentrations between 4 and 300 μg/mL. Peak areas produced better linear relationships than peak heights. Slopes ranged from 0.14 area units/ppm for MEP to 0.31 area units/ppm for PSE. Y intercepts ranged from 0.051 for MPE to 0.27 for EPH. Correlation coefficients (r^2) were all greater than 0.9999. A typical chromatogram of a standard containing 10–20 μg/g each of SEP, NOR, NPE, EPH, PSE, MEP, and MPE is shown in Figure 1.

Three herbal matrix blanks were used to test the new procedure. The matrixes were prepared from commercially available green tea, kola nut, and black tea because these 3 are common herbal dilutants. Matrix A was a 2 + 1 mixture of green tea and kola nut; matrix B was a 1 + 2 mixture of green tea and kola nut; and matrix C was a 1 + 1 + 1 mixture of green tea, black tea, and kola nut. Blanks containing 0.5 or 1.0 g of each of these matrixes produced no interfering peaks that caused >5% error at an ephedrine level of 2 mg/g. Matrix C gave the most complicated blank and was used for the study by the second laboratory (Figure 2). These 3 matrixes were spiked with 3–4 levels of NOR, EPH, PSE, MEP, and MPE and then analyzed. Spike levels corresponded to individual ephedrine concentrations between 0.5 and 16 mg/g. Average recovery (Table 1) based on 325 determinations of 5 alkaloids was 89.9%, and average RSD was 4.4%. Average recoveries of MEP and MPE (87.2 and 83.5%) could be increased to >90% by increasing the volume of EB from 4 to 5 mL. Average recoveries of SEP (not shown in Table 1) and NOR (85.0 and 89.9%) could be increased a few percent by decreasing the volume of DMP during SPE wash from 6 to 4 mL. Figure 3 shows a chromatogram of matrix C spiked with 5 ephedrine alkaloids each at 2 mg/g.

Six commercial products containing ephedrine alkaloids and with quantitative label claims were analyzed. These products contained various amounts of EPH and PSE along with caffeine, *ma huang* extract, green tea, black tea, St. John's wort extract, guaifenesin, and kola nut. Average recovery based on label claims and on 45 runs of 6 commercial products was 97.2% of label claim, and average RSD was 6.0% (Table 2). Figure 4 shows a chromatogram of a typical herbal dietary product with a label claim of 10.6 mg total ephedrine alkaloids (EPH and PSE)/g. Four herbal products were also spiked with an additional 2 mg of each of the ephedrine alkaloids per 0.5 g and run through the procedure in triplicate. Recoveries of spiked SEP, NOR, EPH, PSE, MEP, and MPE varied from 76 to 112%; average recovery was 95%.

A second analyst in a second laboratory analyzed the experimental matrixes and herbal products, using different sets of standards, different reagents, and different equipment. Average recovery of 2, 4, and 8 mg/g spikes of matrix C was 92.1%, and average RSD was 5.0%. By contrast, laboratory 1 obtained an average recovery of 85%, with an RSD of 4.4%. Average recovery from 3 commercial herbal products by laboratory 2 was 99.4% of label declaration, with an average RSD of 5.3%. By contrast, analysis by laboratory 1 gave an average of 96.9% of label declaration, with an RSD of 7.0% (Table 2).

The procedure is rapid, is consistent from laboratory to laboratory, produces good recoveries, yields acceptable RSD values, and produces very little hazardous waste. Recoveries from spiked herbal matrixes averaged 90%, and analysis of commercial herbal products yielded results that were 97% of label claims. Recoveries of SEP, NOR, EPH, PSE, MEP, and MPE spiked in commercial products averaged 95%. Two laboratories and 3 analysts obtained recoveries and RSD values that were within a few percent of each other. Few modifications are required for samples containing 2–20 mg ephedrine alkaloids/g, alkaloid concentrations as low as 0.5 mg/g can be detected, and analysis time is short: A complete analysis of 5 samples can be performed in less than 5 h. We are continuing this work by developing a GC/MS confirmation based on double derivatization of the ephedrine alkaloids, as suggested by Clouette (17).

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Simultaneous Determination of Naphazoline Hydrochloride and Chlorpheniramine Maleate by Derivative Spectrophotometry and by Densitometry

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Two methods were developed for simultaneous determination of naphazoline hydrochloride and chlorpheniramine maleate in bulk drug and dosage forms (eye and nose drops). Derivative spectrophotometry was used to eliminate band overlapping during analysis of such 2-component mixtures. Naphazoline hydrochloride was determined by recording first-derivative (D_1) absorbance curves: at 295.5 nm, naphazoline hydrochloride exhibits a maximum D_1 absorption whereas chlorpheniramine maleate has zero D_1 absorption. Chlorpheniramine maleate was determined by scanning the second-derivative (D_2) absorption spectra: at 261.7 nm chlorpheniramine maleate exhibits a D_2 maximum absorption whereas naphazoline hydrochloride has negligible D_2 absorption. Linear relations between concentration and absorbance were obtained for the concentration range 20–100 g/mL for both bulk drugs. Mean recoveries were 99.96 ± 0.97 and 99.92 ± 0.62%, for naphazoline hydrochloride and chlorpheniramine maleate, respectively. The second method involves quantitative densitometric evaluation of thin-layer chromatograms of ethanolic solutions of the drug mixtures. Separation was performed on silica gel plates (20 × 20 cm) with acetone–25% ammonium hydroxide (90 + 10, v/v) as mobile phase. R_f values were 0.68 and 0.86 for naphazoline hydrochloride and chlorpheniramine maleate, respectively. Densitometric evaluation was done by recording peak areas at 280 nm for naphazoline hydrochloride and at 262 nm for chlorpheniramine maleate. Linear relationships between concentration and peak area were obtained for the concentration ranges 2–10 g/spot and 4–10 g/spot for bulk naphazoline hydrochloride and chlorpheniramine maleate, respectively. Mean recoveries were 99.99 ± 0.75 and 99.67 ± 0.73%, respectively. The presence of the preservative cetrimide did not interfere with determination of the 2 drugs or with accuracy and precision of the 2 proposed methods.

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Naphazoline hydrochloride, [2-(1-naphthyl methyl)-2-imidazoline hydrochloride], is a sympathomimetic agent having marked α-adrenergic activity. It has rapid and prolonged vasoconstrictor action in reducing swelling and congestion and is used to treat rhinitis and sinusitis (1).

Chlorpheniramine maleate, [3-(4-chlorophenyl)-3-(2-pyridyl) propyl dimethylamine] hydrogen maleate, has histamine H_1-receptor antagonistic action and is used to treat anaphylactic reaction, rhinitis, and allergic conjunctivitis. It is also used to treat nausea and vomiting, cough, motion sickness, sedation, and hypnosis (1).

Naphazoline hydrochloride and chlorpheniramine maleate are listed in the European Pharmacopoeia (1), the United States Pharmacopoeia (USP; 2), and the British Pharmacopoeia (3).

Spectrophotometric (4, 5), fluorometric (6), liquid chromatographic (LC; 7), and gas chromatographic (GC; 8, 9) methods have been reported for determination of naphazoline hydrochloride, either alone or after separation from other drugs. Chlorpheniramine maleate has been determined by spectrophotometric (10–12), LC (13), GC (14), thin-layer chromatographic (TLC; 15, 16), polarographic (17), fluorometric (18), nuclear magnetic resonance spectrometric (19), complexometric (20–22), and potentiometric (23) methods either alone or after separation from other drugs. Simultaneous determination of the 2 drugs in eye and nose drops and in spray solutions is difficult (24), requiring a preliminary extraction step that is tedious and time consuming (personal communication, Kahira Pharmaceutical and Chemical Industries, Cairo, Egypt). For example, eye and nose drops solutions are made alkaline and then extracted with ether. The extract is acidified, and the 2 drugs are determined in acidic aqueous solution by measuring the absorbance at 280 and 262 nm for naphazoline hydrochloride and chlorpheniramine maleate, respectively, and calculating concentrations by solving 2 equations simultaneously. Simple and rapid methods for simultaneous analysis of the 2 drugs without prior extraction will facilitate routine quality control. This paper describes spectrophotometric and densitometric methods for simultaneous determination of the 2 compounds in raw material and dosage forms.

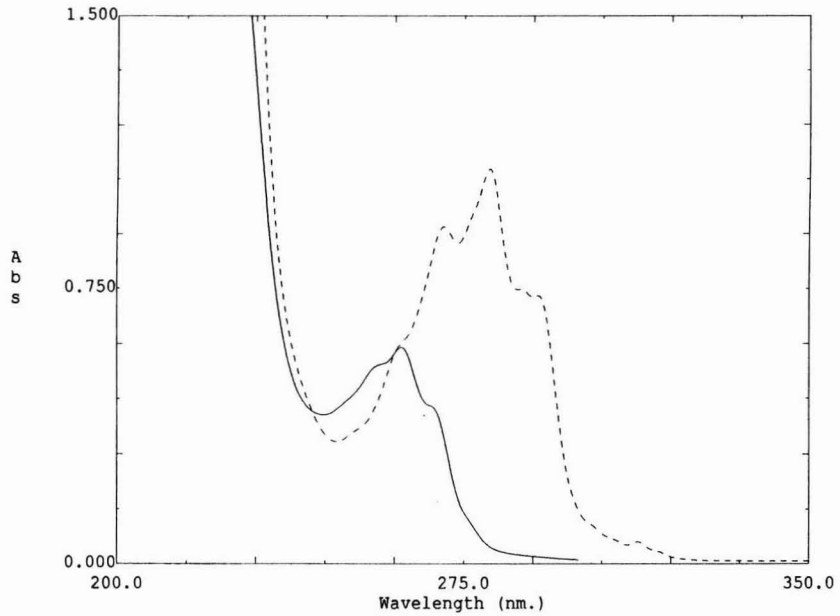

Figure 1a. Zero-order spectra (D_0) of 40 μg/mL naphazoline hydrochloride (– – –) and chlorpheniramine maleate (———).

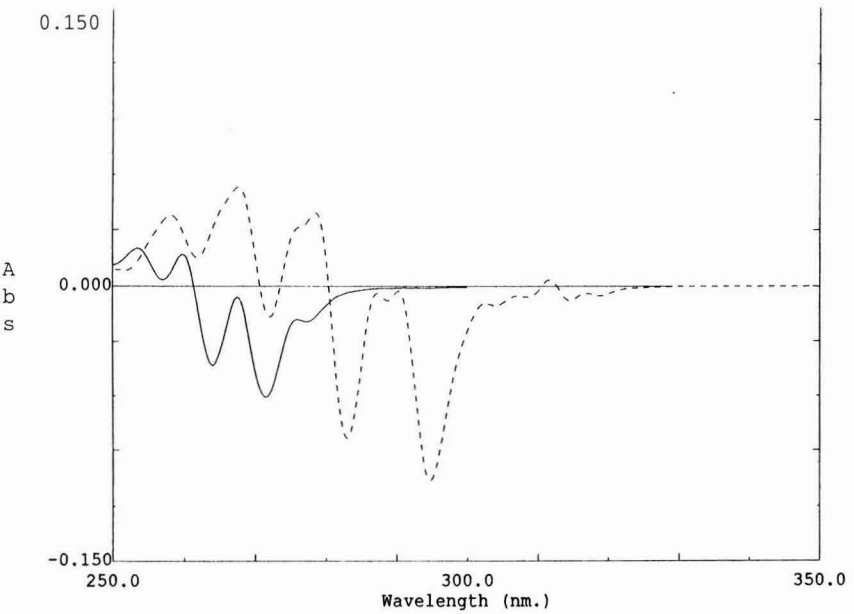

Figure 1b. First-derivative spectra (D_1) of 40 μg/mL naphazoline hydrochloride (– – –) and chlorpheniramine maleate (———).

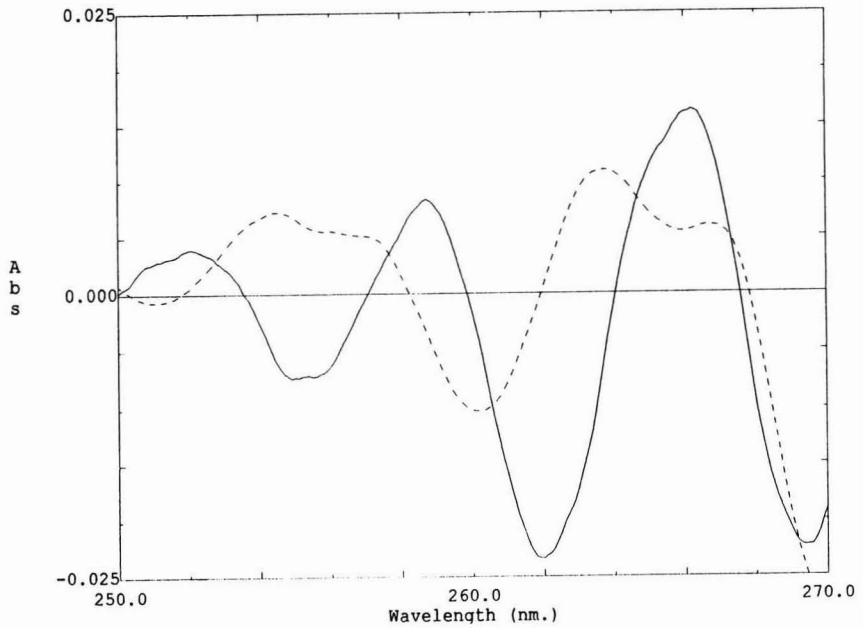

Figure 1c. Second-derivative spectra (D_2) of 40 μg/mL naphazoline hydrochloride (– – –) and chlorpheniramine maleate (———).

Experimental

Apparatus

(**a**) *UV/visible spectrophotometer.*—Shimadzu 1601 PC attached to IBM computer with UVPC personal spectroscopy, S/N UH3-0293, and Hewlett-Packard printer for Windows 6.1 Desk Jet 600 series version, software version 3.7.

(**b**) *Densitometer.*—Dual-wavelength Shimadzu flying CS-9000 with video display and high-speed, high-quality, parallel-head printer/plotter.

(**c**) *TLC and high-performance TLC (HPTLC) plates.*—Precoated with Silica gel GF, 0.25 mm thickness (E. Merck, Darmstadt, Germany).

(**d**) *Micropipet.*—10 μL (Desaga, Heidelberg, Germany).

(**e**) *Tank.*—20 × 21 × 9 cm (Desaga).

Reagents

(**a**) *Naphazoline hydrochloride.*—Kindly provided by Kahira Pharmaceutical and Chemical Industries, Cairo, Egypt. Purity, 100.02 ± 0.4% determined by USP method (2).

(**b**) *Chlorpheniramine maleate.*—Kindly supplied by Kahira. Purity, 99.95 ± 0.6% determined by USP method.

(**c**) *Cetrimide.*—Kindly provided by Kahira.

(**d**) *Prisoline eye and nasal drops.*—Kahira, batch No. 760218, obtained from retail market in Cairo. Each 100 mL is labeled to contain 50 mg each of naphazoline hydrochloride and chlorpheniramine maleate and 2 mg cetrimide.

(**e**) *Chemicals and solvents.*—All chemicals were analytical grade, and all solvents were spectroscopic grade: ethanol (BDH, London, UK), acetone (El Nasr Pharmaceutical Chemical Com-

Table 1. Determination of naphazoline hydrochloride and chlorpheniramine maleate in mixtures by derivative spectrophotometry

Concentration in bulk powder, μg/mL		Recovery, % (n = 3)	
Naphazoline hydrochloride	Chlorpheniramine maleate	Naphazoline hydrochloride (D_1)	Chlorpheniramine maleate (D_2)
20	80	98.75	100.55
30	70	99.06	99.05
40	60	100.02	100.17
50	50	99.92	99.82
60	40	100.01	98.99
70	30	101.14	100.20
80	20	100.81	100.64
Mean ± RSD, %		99.96 ± 0.97	99.92 ± 0.62

Table 2. Determination of naphazoline hydrochloride and chlorpheniramine maleate in laboratory-prepared eye and nose drops

Concentration in laboratory-prepared nose and eye drops, mg/100 mL			Recovery, % ($n = 3$)			
			Derivative spectrophotometry		Densitometry	
Cetrimide	Naphazoline hydrochloride	Chlorpheniramine maleate	Naphazoline hydrochloride	Chlorpheniramine maleate	Naphazoline hydrochloride	Chlorpheniramine maleate
1	25	25	99.59	99.24	99.99	100.05
2	50	50	101.05	100.10	100.70	99.90
3	75	75	100.15	98.95	100.01	98.92
Mean ± RSD			100.26 ± 0.60	99.43 ± 0.33	100.23 ± 0.33	99.62 ± 0.50

pany, Cairo, Egypt), 25% ammonium hydroxide (immediately determined before use; Prolabo, Paris, France), distilled water (freshly prepared).

Standard Stock Solutions

Accurately weigh 50 mg each of naphazoline hydrochloride, chlorpheniramine maleate, and cetrimide and dissolve in a small amount of distilled water or ethanol in 50 mL volumetric flask. Make up to volume with the same solvent to obtain the following concentrations: naphazoline hydrochloride, 1 mg/1 mL water and 1 mg/1 mL ethanol; chlorpheniramine maleate, 1 mg/1 mL water and 1 mg/1 mL ethanol; and cetrimide, 1 mg/mL water.

Derivative Spectrophotometric Method

(**a**) *Construction of calibration curves.*—Transfer accurately measured portions equivalent to 1–5 mg naphazoline hydrochloride from standard stock aqueous solution (1 mg/mL) into a series of 50 mL volumetric flasks. Fill to the mark with distilled water. Record the first-derivative (D_1) curve of each solution at 4 nm interval against distilled water as a blank at 250–350 nm. Measure D_1 absorbance at 295.5 nm (maximum absorption) and plot amplitude vs concentration. Repeat the procedure for chlorpheniramine maleate. Scan the second-derivative (D_2) curve at 250–270 nm at 4 nm interval, measure D_2 absorbance of each solution at 261.7 nm (maximum absorption), and construct calibration curve.

(**b**) *Assay of laboratory-prepared eye and nasal drops.*—Dissolve 25 mg each accurately weighed naphazoline hydrochloride and chlorpheniramine maleate and 1 mg accurately weighed cetrimide in distilled water in 100 mL volumetric flask. Dilute to volume with distilled water. Prepare separate drops containing 50 and 75 mg each accurately weighed naphazoline hydrochloride and chlorpheniramine maleate and 2 and 3 mg accurately weighed cetrimide. Pipet 5 mL prepared drops solution into 50 mL calibrated flask and dilute to mark with distilled water. Record derivative spectrum curves D_1 and D_2 for each solution and measure D_1 and D_2 absorbances at

Table 3. Comparison of proposed methods with reported method for simultaneous determination of naphazoline hydrochloride and chlorpheniramine maleate in Prisoline[a] eye and nasal drops

	Derivative method		Densitometric method		Reported method[b]
Preparation	Found ± RSD, %	Recovery by standard addition ± RSD, %[c]	Found ± RSD, %	Recovery by standard addition ± RSD, %[c]	Found ± RSD, %
			Naphazoline hydrochloride		
Drug bulk powder	99.96 ± 0.97	99.47 ± 0.69	99.99 ± 0.75	100.1 ± 0.24	100.50 ± 0.59
	$t = 0.66$		t = 0.74		
	$F = 1.04$		$F = 1.70$		
Prisoline	100.08 ± 0.16		100.32 ± 0.28		100.10 ± 0.78
			Chlorpheniramine maleate		
Drug bulk powder	99.92 ± 0.62	99.82 ± 0.19	99.67 ± 0.73	100.42 ± 0.47	99.50 ± 0.58
	$t = 0.38$		$t = 0.27$		
	$F = 3.25$		$F = 2.0$		
Prisoline	97.84 ± 0.24		97.6 ± 0.23		98.01 ± 0.28

[a] Label claims that each 100 mL contains naphazoline hydrochloride, 50 mg; chlorpheniramine maleate, 50 mg; and cetrimide, 2 mg (batch No. 760218).
[b] Personal communication, Kahira Pharmaceutical and Chemical Industries, Cairo, Egypt.
[c] Average of 5 analyses.

Table 4. Determination of naphazoline hydrochloride and chlorpheniramine maleate in mixtures by densitometry

Amount of bulk powder applied on HPTLC plates, μg/spot		Recovery, % (n = 3)	
Naphazoline hydrochloride	Chlorpheniramine maleate	Naphazoline hydrochloride	Chlorpheniramine maleate
2	8	98.70	100.50
3	7	99.92	99.03
4	6	100.02	100.60
5	5	101.05	99.30
6	4	100.32	98.91
Mean ± RSD		99.99 ± 0.75	99.67 ± 0.73

previously chosen wavelengths. Calculate concentration of each drug.

(**c**) *Assay of pharmaceutical formulations.*—Pipet 5 mL eye and nasal drops solution of the dosage form and proceed as described above for laboratory-prepared eye and nasal drops starting from "into 50 mL calibrated...."

Densitometric Method

(**a**) *Construction of calibration curves.*—Transfer a volume of stock alcoholic standard solution of each drug equivalent to 1–5 mg in separate 5 mL volumetric flasks and dilute to volume with absolute ethyl alcohol. Using a 10 μL micropipet, apply 10 μL of each prepared solution to a precoated TLC or HPTLC aluminum sheet (20 × 20 cm). Space spots 2 cm (TLC) or 1 cm (HPTLC) apart and 1.5 cm from the bottom edge of the plate. Place plate in chromatographic tank (with filter paper) previously saturated for 1 h with developing mobile phase, acetone–25% ammonium hydroxide (90 + 10, v/v). Develop plate by ascending chromatography to a distance of 14–16 cm (TLC) or 7–8 cm (HPTLC), dry at room temperature, detect spots under UV lamp, and determine spots densitometrically (in flying-spot mode) at 280 nm for naphazoline hydrochloride and 262 nm for chlorpheniramine maleate. Construct calibration curves by plotting area under the peak vs concentration of drug.

(**b**) *Assay of laboratory-prepared eye and nasal drops.*—Prepare eye and nose drops as described in *Derivative Spectrophotometric Method.* With a pipet, transfer a suitable accurately measured volume (10 mL) of eye and nasal drops solutions to a flat-bottom dish and place the dish in a vacuum desiccator over sulfuric acid. Apply vacuum until sample is almost dry, dissolve dish contents in the least amount of ethanol, and quantitatively transfer into a 5 mL volumetric flask. Complete to volume with ethanol to bring up a range of concentrations (0.5–1.5 mg/mL) from each of the 2 drugs. Proceed as described under *Construction of calibration curves*, starting from "Using a 10 μL micropipet...." For every determination, apply both standard and sample solutions on the same TLC or HPTLC plate. Calculate concentrations of naphazoline hydrochloride and chlorpheniramine maleate from regression equations or by comparing with standard solutions.

(**c**) *Assay of pharmaceutical formulations.*—Proceed as described for *Assay of laboratory-prepared eye and nose drops* starting from "With a pipet, transfer a suitable accurately measured volume...."

Results and Discussion

The presence of both naphazoline hydrochloride and chlorpheniramine maleate in ophthalmic and nasal preparations creates problems in their determination during quality control assays. The purpose of this work was to develop methods for simultaneous determination of naphazoline hydrochloride and chlorpheniramine maleate in dosage forms without prior extraction. Methods using spectrophotometry and densitometry are described.

Zero-order absorption spectra (D_0) of naphazoline hydrochloride and chlorpheniramine maleate show significant band overlap at the wavelengths of maximum absorption of naphazoline hydrochloride (280 nm) and chlorpheniramine maleate (262 nm; Figure 1a). However, first-derivative absorption spectra (D_1) show that naphazoline hydrochloride has a typical trough at about 295.5 nm while chlorpheniramine maleate shows zero or negligible absorption at that wavelength (Figure 1b). Second-derivative absorption spectra (D_2) show that chlorpheniramine maleate has maximum absorption at 261.7 nm while naphazoline hydrochloride shows negligible absorption (D_2) at that wavelength (Figure 1c). Thus, D_1 and D_2 spectrophotometry is suggested for determination of both drugs simultaneously.

For quantitative application, linear relationships have been found between amplitude heights in D_1 and D_2 spectra of naphazoline hydrochloride and chlorpheniramine maleate, respectively, and their respective concentrations in the range 20–80 μL/mL for bulk materials. Corresponding mean recoveries are 99.96 ± 0.97 and 99.92 ± 0.62%, respectively.

Calibration curves were represented by the following regression equations:

$$Y_1 = 0.0074 + 0.002365\ C$$

(r = 0.9997) for naphazoline hydrochloride

$$Y_2 = 0.00025 + 0.005688\ C$$

(r = 0.9998) for chlorpheniramine maleate

where Y_1 is D_1 absorbance at 295.5 nm, Y_2 is D_2 absorbance at 261.7 nm, C is concentration in μg/mL, and r is the correlation coefficient. These equations were used for direct evaluation of the 2 drugs. Mixtures containing different ratios of the 2 drugs and laboratory-prepared drops were analyzed. Results are pre-

sented in Tables 1 and 2. They showed no interference from cetrimide used as preservative in both drops.

Results of recovery experiments using the standard addition technique are presented in Table 3.

A densitometric technique is also suggested for simultaneous determination of these drugs based on difference in R_f values. Naphazoline hydrochloride and chlorpheniramine maleate were separated on TLC and HPTLC silica gel plates, whereas cetrimide remained at the base. Cetrimide has no UV absorption. The R_f values of naphazoline hydrochloride and chlorpheniramine maleate were 0.68 and 0.86, respectively, with acetone–25% ammonium hydroxide (90 + 10, v/v) as mobile phase. The 2 separated drug spots can be determined densitometrically on the same plate by scanning quantitatively at 280 and 262 nm for naphazoline hydrochloride and chlorpheniramine maleate, respectively.

Substances were spotted at equivalent locations on precoated TLC and HPTLC plates (20 × 20 cm), which were developed with the same solvent system. On TLC plates, spots were applied 2 cm apart, for a maximum of 9 spots per plate. Optimum resolution was reached at 14–16 cm migration of solvent. Spot diameters rarely exceeded 8 mm, and separation time was about 45 min. By comparison, on HPTLC plates, spots were spaced at 1 cm apart, allowing 18 spots per plate. Optimum resolution was reached at 7–8 cm migration of solvent. Spot diameters exceeded 4 mm only in very rare cases, and separation time was only 20–25 min. Thus HPTLC has advantages over TLC in routine analysis with respect to cost per plate.

For HPTLC, linear correlations were obtained between areas under the peak and concentrations in the ranges 2–10 μg and 4–10 μg per spot for naphazoline hydrochloride and chlorpheniramine maleate, respectively. Mean recoveries were 99.99 ± 0.75 and 99.67 ± 0.73%, respectively. Calibration curves were represented by the following regression equations:

$$Y = 0.0118 + 0.0521\ C$$

(r = 0.9986) for naphazoline hydrochloride

$$Y = -0.0282 + 0.0282\ C$$

(r = 0.9988) for chlorpheniramine maleate

where Y is area under the peak, C is concentration in μg, and r is correlation coefficient. These equations were used for direct evaluation of the 2 drugs.

Mixtures of the 2 drugs in different ratios and laboratory-prepared eye and nasal drops were analyzed. Results (Tables 4 and 2) show the applicability of the method and the absence of interference from the cetrimide preservative.

Compared with the official and reported methods for analysis of the 2 drugs, HPTLC is relatively inexpensive, capable of rapidly producing quantitative results with accuracy and precision comparable with those of official and reported methods. Advantages of HPTLC include simultaneous determination of mixtures of the 2 drugs in many samples under the same conditions without prior extraction or cleanup. A large number of samples and reference standards can be chromatographed simultaneously and then quantitatively evaluated on a single plate. Thus separation of complex mixtures and reliability of identification of individual substances are enhanced enormously. Chromatographic separation is elegantly coupled with quantitative determination of spots directly on the plate. No problems are caused by UV-absorbing mobile phase systems because densitometric evaluation is done after the mobile phase has been removed by evaporation. No time- and material-consuming prior extractions are necessary.

Precision

Precision was evaluated by performing 5 analyses of each sample. Relative standard deviations were 0.97 and 0.62% with derivative spectrophotometry and 0.75 and 0.73% with densitometry for naphazoline hydrochloride and chlorpheniramine maleate, respectively. Results obtained were compared statistically with those obtained by applying the reported method (personal communication, Kahira Pharmaceutical and Chemical Industries; Table 3).

Method Validation

Validity and reproducibility of the proposed methods were assessed further by applying the standard addition method. Known amounts of standard naphazoline hydrochloride and chlorpheniramine maleate were added to a fixed amount of sample solution at 3 concentration levels, and recoveries were calculated. Results (Table 3) show that both methods are precise and reproducible. There was no interference from cetrimide or contaminants.

Conclusion

The 2 proposed methods were successfully applied for simultaneous determination of naphazoline hydrochloride and chlorpheniramine maleate in bulk drug and dosage forms without preliminary extraction or interference from cetrimide. Calculated t and F values were less than the theoretical values (Table 3), indicating that there is no difference between the 2 methods with respect to precision and accuracy. In addition, both are simple, rapid, and less costly than other methods. They are therefore more suitable for quality control and routine analysis than other methods.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Identification of β-Lactam Antibiotics in Tissue Samples Containing Unknown Microbial Inhibitors

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Antibiotic residues in animal tissues can be detected by various screening tests based on microbial inhibition. In the 7-plate assay used by the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS), penicillinase is incorporated into all but one plate to distinguish β-lactam antibiotics from other types. However, β-lactams such as cloxacillin and the cephalosporins are resistant to degradation by penicillinase. They may not be identified as β-lactams by this procedure, and thus, they may be identified as unidentified microbial inhibitors (UMIs). However, these penicillinase-resistant compounds can be degraded by other β-lactamases. The present study describes an improved screening protocol to identify β-lactam antibiotics classified as UMIs. A multiresidue liquid chromatographic procedure based on a method for determining β-lactams in milk was also used to identify and quantitate residues. The 2 methods were tested with 24 tissue FSIS samples classified as containing UMIs. Of these, 3 contained penicillin G, including one at a violative level, and 5 contained a metabolite of ceftiofur. The others were negative for β-lactam antibiotics.

Antibiotic residues in animal tissues can be detected by various screening tests based on microbial inhibition (1–7), as well as rapid milk-screening tests (8–11). However, identification of specific antibiotics is more difficult than detection. And analysis may be complicated by the presence of natural microbial inhibitors in some tissues (12–15).

In the 7-plate assay (7) of the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS), penicillinase is incorporated in all but one plate to distinguish β-lactam antibiotics from other types. However, β-lactams such as cloxacillin and the cephalosporins are resistant to degradation by penicillinase (16–18). Thus, they may not be identified as β-lactams by this procedure and are classified as unknown microbial inhibitors (UMIs). However, these penicillinase-resistant compounds can be degraded by other β-lactamases.

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Chromatographic methods have been described for determining penicillins in tissues from several species (19–30), but none is suitable for determining cephalosporins or their metabolites in tissues. The 2 cephalosporins approved for use in food-producing animals in the United States are ceftiofur and cephapirin. Both are converted to metabolites in animal tissues, ceftiofur to desfuroylceftiofurcysteine (DFCC; 31), and cephapirin to the desacetyl form (W. Moats, S. Buckley, unpublished data).

Ceftiofur metabolites can be identified tentatively by rapid screening assays such as the *Bacillus stearothermophilus* diffusion assay (Delvotest) and enzyme immunochemical tests (LacTek-BL and LacTek-Cef) before and after hydrolysis with Penase and lactamase II. Portions of aqueous extracts of tissues are treated separately with these enzymes, and results are compared with those of untreated samples and positive controls. Bioactive ceftiofur metabolites are present if extracts retain inhibitory activity after Penase treatment, but lose the activity after lactamase II treatment and if they are positive to the LacTek-Cef test but negative to the LacTek-BL assay. However, the presence of nonbioactive ceftiofur metabolites is suspected if the extract shows no microbial inhibitory activity and tests are negative by LacTek-BL but positive by LacTek-CEF. If inhibitory activities are shown after Penase and lactamase II treatments, the presence of antibiotics other than β-lactams is suspected. The efficacy of using Penase and lactamase II to tentatively identify ceftiofur metabolites was evaluated by analyzing tissues containing unidentified antibiotic residues.

The present study describes an improved screening protocol (32) for identifying β-lactam antibiotics classified as UMIs. A multiresidue liquid chromatographic (LC) procedure based on a method for determining β-lactams in milk (33, 34) was also used to identify and quantitate residues. The 2 approaches were tested with 30 samples from FSIS, 24 of which contained UMIs and 5 of which were identified as containing β-lactams.

Experimental

Equipment

(a) *Stomacher.*—Seward Model 80 (Tekmar-Dohrman, Cincinnati, OH).

(**b**) *High-speed microcentrifuge.*—Tomy (Peninsula Laboratories, Belmont, CA).

(**c**) *Photometer.*—For 12 × 75 mm tubes (Idexx Laboratories, Westbrook, ME).

(**d**) *Multiblock heater.*—Lab-Line, Melrose Park, CA.

(**e**) *Incubator.*—VWR Model 1525 (VWR Scientific, Philadelphia, PA).

(**f**) *Extraction and digestion tubes.*—Polypropylene tubes with caps, 12 × 72 mm (5 mL) and 16 × 92 mm (10 mL); conical tubes, 17 × 110 mm (12 mL) and 25 × 107 mm (30 mL).

Reagents

(**a**) *Extraction and dilution buffer.*—73 mM phosphate buffer (pH 6) containing 8.71 g KH_2PO_4 and 2.04 g $K_2HPO_4 \cdot 3H_2O$ diluted in 1 L. This is equivalent to 1% KH_2PO_4 adjusted to pH 6 with 1M NaOH (7).

(**b**) *β-Lactams.*—Cephapirin sodium salt and penicillin G sodium salt were obtained from Sigma Chemical Co., St. Louis, MO. DFCC was a gift from Pharmacia and Upjohn Co., Kalamazoo, MI.

(**c**) *Stock solutions of β-lactams.*—Stock A contained 1 mg β-lactams/mL in deionized water. Stock B (10 μg/mL) was prepared by diluting 0.1 mL Stock A with 9.9 mL phosphate buffer. Stock C (1 μg/mL or 1 ppm) was prepared by taking 0.5 mL Stock B and diluting with 4.5 mL phosphate buffer.

(**d**) *Lactamase enzymes.*—Lactamase II (E.C.3.5.2.6; molecular weight, 23 000) produced from *Bacillus cereus* 569/H (K_m, 1100 mM; specific activity, 2000 Units (U)/mg vs cephalosporin C), Cat. No. 19143, ICN Pharmaceuticals (Costa Mesa, CA). A stock solution of 1000 U/mL (Stock 1) was prepared in phosphate buffer. Aliquots in 100 μL portions were prepared and stored in a –80°C freezer. A working dilution of 100 U/mL (Stock 2) was prepared by diluting Stock 1 1:10 with phosphate buffer (pH 6). Bacto Penase (E.C.3.5.2.8) containing 20 000 LU (Levy units)/mL/min and equivalent to 10 million International Units (IU)/mL was purchased from Difco, Inc., Detroit, MI.

(**e**) *Screening assays.*—LacTek-BL for β-lactams and LacTek-CEF for ceftiofur (Idexx Laboratories). Delvotest P-mini (Gist-Brocades, Delft, The Netherlands) was purchased from Eastern Crown, Inc., Vernon, NY.

Chemicals and Reagents for LC Analysis

(**a**) *Acetonitrile.*—EM Omnisolv (Gibbstown, NJ) or equivalent.

(**b**) *Tetraethylammonium chloride; 1-decanesulfonic acid, sodium salt, 98%; dodecyl sulfate, sodium salt, 98%.*—Aldrich Chemical Co., Milwaukee, WI.

(**c**) *Antibiotic standards.*—Ceftiofur and DFCC were gifts from Pharmacia and Upjohn Co. Other antibiotics were purchased from Sigma.

(**d**) *KH_2PO_4, H_3PO_4, and Na_2HPO_4.*—Reagent grade, from several sources.

(**e**) *β-Lactamase.*—β-Lactamase-Liq (Charm Sciences, Malden, MA). The dry powder was reconstituted in water according to manufacturer's instructions. The reconstituted enzyme was dispensed in 0.1 mL portions into small vials and stored frozen until needed.

Glassware and Equipment for LC Analysis

All glassware were cleaned in special detergent (Micro, International Products, Trenton, NJ, or equivalent) at ca 60°C for 30 min (a longer period may etch glassware), rinsed in deionized water, rinsed 5 min or longer in dilute acid bath (ca 0.01M HCl or H_2SO_4), and rinsed again with deionized water.

(**a**) *Graduated cylinders.*—25 and 50 mL.

(**b**) *Conical graduated centrifuge tubes.*—15 mL, calibrated to 1 and 4 mL.

(**c**) *Glass-stoppered side-arm flasks.*—250 mL.

(**d**) *Conical flasks.*—125 mL.

(**e**) *Blender.*—Waring type, base with 100 or 300 mL stainless steel jars with covers.

(**f**) *Vortex evaporator.*—Buchler Instrument Co., Ft. Lee, NJ.

(**g**) *Thermostated hot plate.*—With shallow tray.

(**h**) *Plastic-coated lead rings.*—I^2R Corp., Cheltenham, PA; used to weigh down flasks during evaporation.

Apparatus for LC Cleanup

(**a**) *Pump.*—Varian (Sugarland, TX) Model 9012.

(**b**) *Autosampler.*—Waters (Milford, MA) WISP 712 with a 2000 μL loop.

(**c**) *Fraction collector.*—Isco (Lincoln, NE) Foxy.

(**d**) *Data system.*—Waters 990 diode array detector.

(**e**) *Column.*—Supelcosil LC-18, 4.6 × 150 mm, 5 μm particle size (Supelco, Bellefonte, PA).

Apparatus for LC Analysis

(**a**) *Pump.*—Varian Model 9012.

(**b**) *Autosampler.*—Varian Model 9090.

(**c**) *Detector.*—Waters 481 UV/visible detector.

(**d**) *Data system.*—Varian Model 654.

Procedures

(**a**) *Sample extraction.*—Thirty incurred samples of beef tissue (Table 1) were obtained from FSIS Midwestern Laboratories. Twenty-four samples contained UMIs, and 5 contained β-lactams, as tested with the 7-plate assay (7). Five gram samples were weighed, cut into small pieces (ca 5 mm^2), and transferred quantitatively into Tekmar stomacher bags (9 × 16 cm). Extraction buffer was added at a ratio of 4 mL/g. Mixtures were blended in a stomacher for 60 s and then allowed to stand for 1 h at 4°C. Extracts were transferred to polypropylene tubes and centrifuged at 12 000 rpm (11 850 × *g*) for 10 min. (If a high-speed centrifuge is not available, samples may be centrifuged at 3000 × *g* for 15 min.) Supernatants or clarified extracts were analyzed as described below.

(**b**) *Enzyme hydrolysis.*—To 2 mL clarified extracts, 20 μL Penase concentrate was added to give 400 LU/2 mL extract. To another 2 mL extract, lactamase II (20 μL Stock 2) was added to give 2 U/mL extract. Enzyme-treated extracts were hydrolyzed for 45 min at 37°C. After hydrolysis, samples were kept at 4°C or in an ice bath to inhibit further enzymatic hydrolysis

Table 1. Analysis of FSIS samples

Sample No. (type[a])	FSIS screen[b]	New screen	Delvotest P on LC fractions					LC analysis, ppm
			A[c]	B	C	D	E	
1 (L)	UMI	Ceftiofur	–	+	±	–	–	0.68 DFCC
2 (L)	UMI	—	–	–	–	–	–	—
3 (L)	UMI	Pen G < 10 ppb	–	–	–	+	–	0.004 Pen G
4 (L)	UMI	Ceftiofur	–	+	±	–	–	1.53 DFCC
5 (L)	UMI	—	–	–	–	–	–	—
6 (L)	UMI	Pen G trace	–	+	±	–	–	No DFCC
7 (L)	UMI	—	–	–	–	–	–	—
8 (L)	UMI	Ceftiofur, not bioactive	–	–	–	–	–	—
9 (K)	UMI	Ceftiofur	–	+	–	–	+	0.37 DFCC
10 (L)	UMI	—	–	–	–	–	–	—
11 (L)	UMI	Ceftiofur, not bioactive	–	–	–	–	–	—
12 (L)	UMI	—	–	–	–	–	–	—
13 (L)	UMI	—	–	–	–	–	–	—
14 (L)	UMI	—	–	–	–	–	–	—
15 (K)	UMI	—	–	–	–	–	–	—
16 (L)	UMI	Pen G ca 10 ppb	–	–	–	+	–	0.064 Pen G
17 (L)	UMI	—	–	–	–	–	+	No cloxacillin
18 (L)	UMI	Pen G < 10 ppb	–	–	–	+	–	0.010 Pen G
19 (K)	UMI	Ceftiofur	–	+	±	–	–	0.69 DFCC
20 (K)	Negative	—	–	–	–	–	–	—
21 (K)	UMI	Ceftiofur + UMI	–	+	+	–	+	0.31 DFCC
22 (L)	UMI	—	–	–	–	–	–	—
23 (L)	UMI	—	–	–	–	–	–	—
24 (K)	UMI	Pen G < 10 ppb	–	–	–	–	–	—
25 (L)	UMI	Pen G > 10 ppb	–	–	–	+	–	0.021 Pen G
26 (L)	β-Lactam	Pen G > 10 ppb	–	–	–	+	–	0.28 Pen G
27 (L)	β-Lactam	Pen G, ceftiofur, not bioactive	–	–	–	+	–	0.18 Pen G
28 (M)	β-Lactam	Pen G > 10 ppb + UMI	–	–	–	+	–	0.058 Pen G
29 (K)	β-Lactam	Pen G > 10 ppb	–	–	–	+	–	0.18 Pen G
30 (K)	β-Lactam	Pen G > 10 ppb	–	–	–	+	+	2.92 Pen G

[a] K = kidney; L = liver; M = muscle.
[b] *Micrococcus luteus* plate assay (7).
[c] A, amoxicillin; B, desfuroylceftiofurcysteine; C, ampicillin; D, penicillin G; E, cloxacillin.

at room temperature. Treated, negative control, and positive control samples were assayed by Delvotest, LacTek-BL, and LacTek-CEF. Bovine kidney extract was used for negative control. To evaluate use of screening tests with tissue extracts, 2 mL portions of clarified kidney extract were spiked with 10 ppb penicillin G (20 μL of 1 μg/mL solution) or 100 ppb cephapirin (20 μL of 10 μg/mL) for use as positive reference standards. Control and unknown samples were tested for β-lactams with Delvotest, LacTek-BL, and LacTek-CEF before and after digestion with Penase and lactamase II. Samples retaining inhibitory activity after Penase treatment but losing the activity after lactamase II hydrolysis were suspected to contain bioactive ceftiofur metabolites. These samples were analyzed further by LC to determine the presence of ceftiofur metabolites.

(c) *Rapid screening tests.*—The protocols described by the manufacturers of the commercial screening tests were followed. Samples were analyzed within 20 min or stored at 4°C or in an ice bath for later analysis. Hydrolyzed samples were kept in a freezer (–20°C) for storage overnight or a few days. (*1*) *Delvotest.*—A multiblock heater with precise temperature control was used. Sample tube holders of the heating block were filled about one-third full with water and equilibrated at 65°C. Hydrolysates (0.1 mL) were transferred to vials, and a nutrient tablet was added to each vial. Sample and nutrient tablet were gently mixed and incubated for 2.5 h at 65°C in the block heater. (*2*) *LacTek-BL and LacTek-CEF.*—Portions (0.25 mL) of the hydrolysate were transferred to antibody-coated tubes. Tracer reagent (enzyme-labeled β-lactam) was added, and the mixture was incubated for 3 min at room temperature. Tube contents were discarded, and tubes were washed 3 times with LacTek wash buffer. Color developer was added, and the mixture was incubated for another 3 min at room temperature. Stopping reagent was added, and absorbance was measured at 405 nm with the Idexx photometer.

(d) *Extraction and deproteinization, procedure 1.*—Tissue was cut into small pieces, and 5 g was transferred to a 100–

300 mL blender jar. Then 5 mL water, 2 mL 0.1M tetraethylammonium chloride (Et_4NCl) (for liver and kidney tissues, use 1 mL 0.2M Et_4NCl and 1 mL 0.005M KH_2PO_4), and 40 mL acetonitrile were added. The mixture was blended for 1 min at one-half full power as measured by a variable-resistance transformer (final volume = 50 mL). After the mixture was allowed to stand 10 min, the supernatant was decanted through a small plug of glass wool in the stem of a funnel, and 40 mL filtrate (20 mL for liver and kidney) was collected, equivalent to 4 g tissue (2 g for liver and kidney). The filtrate was transferred to a 250 mL side-arm flask, and then 2 mL 0.01M pH 6 buffer, 5 mL water, and 5 mL *tert*-butyl alcohol (to suppress foaming) were added. The flasks were weighed down with plastic-coated lead rings and connected to a water pump vacuum. After the contents had stopped boiling, the flasks were placed in a shallow (1–2 cm) water bath heated to 40°–50°C. If foaming persisted, more *tert*-butyl alcohol was added, always with an equal volume of water. The contents were evaporated to 1–2 mL (not to dryness), rinsed into graduated tubes with several small portions of water to a final volume of 4 mL, and filtered through a 25 mm 0.45 µm polyvinylidene fluoride (PVDF) syringe filter into a 4 mL autosampler vial.

(**e**) *Extraction and deproteinization, procedure 2 (β-lactamase).*—Tissue was cut into small pieces, and 15 g was weighed into a 100–300 mL blender jar and blended with 45 mL water for 1 min at one-half full power. Ten milliliters homogenate was measured into a 125 mL conical flask and mixed with 2 mL 0.1M Et_4NCl. Then 40 mL acetonitrile was added slowly with continual stirring (final volume = 50 mL). After the mixture was allowed to stand 10 min, the supernatant was decanted through a plug of glass wool in the stem of a funnel, and 40 mL filtrate (equivalent to 2 g tissue) was collected. The filtrate was transferred to a 250 mL glass-stoppered side-arm flask, and 2 mL 0.01M pH 6 buffer (5 + 1, KH_2PO_4–Na_2HPO_4) and 5 mL each *tert*-butyl alcohol and water were added. The contents were evaporated and filtered as described for procedure 1.

For the β-lactamase treatment, before beginning the extraction procedure, 0.1 mL reconstituted β-lactamase was added to 10 mL tissue homogenate, and the mixture was incubated 1 h at room temperature.

(**f**) *LC fractionation.*—Two milliliters sample extract was loaded onto the LC column with a flow of 100% 0.01M KH_2PO_4. After 3 min, an acetonitrile gradient was started that gave 60% acetonitrile in 40 min. The column was returned to starting conditions at 41 min, and another sample could be loaded at 55 min. Standards were run initially to determine retention times. The fraction collector was set to collect 1.5–2.0 time windows centered on the retention time of the analytes. For penicillin G and cloxacillin fractions, 0.2 mL 0.01M Na_2HPO_4 was added to the tubes prior to fraction collection. Elution of β-lactam standards is shown in Figure 1. Fractions of 1.5–2.0 mL corresponding to each compound of interest were collected. For the present study, these compounds were amoxicillin, desfuroylceftiofurcysteine and desacetylcephapirin, ampicillin, penicillin G, and cloxacillin.

(**g**) *Analysis of fractions.*—Fractions were evaporated to <1 mL under reduced pressure in the Vortex evaporator, and volumes were adjusted to 1 mL with water. They were then tested for antimicrobial activity with Delvotest P-mini as described for milk (other milk-screening tests may be satisfactory). Fractions testing positive were analyzed by LC. Prior to LC analysis, 0.2 mL of a solution containing 0.01M KH_2PO_4, 0.01M H_3PO_4, and 0.01M sodium decanesulfonate was added to amoxicillin, ampicillin, DFCC, and cephapirin fractions. LC conditions for analysis were different from those used for cleanup.

LC analysis conditions for each fraction were as follows: (*1*) *Amoxicillin.*—Derivatize by the method of Ang and Luo (35). (*2*) *DFCC.*—0.015M H_3PO_4, 0.0075M sodium dodecylsulfate–acetonitrile (60 + 40), Supelcosil LC-18 column. (*3*) *Ampicillin.*—0.01M H_3PO_4, 0.005M KH_2PO_4, 0.005M sodium dodecylsulfate–acetonitrile (65 + 35), Supelcosil LC-18 column. (*4*) *Penicillin G.*—0.0133M KH_2PO_4, 0.0067M H_3PO_4–acetonitrile (68 + 32), Supelcosil LC-18-DB or Inertsil ODS-2 column. (*5*) *Cloxacillin.*—0.008M KH_2PO_4, 0.002M H_3PO_4–acetonitrile (62 + 38), Supelcosil LC-18-DB column. Residues were confirmed by repeating LC analysis with a tissue homogenate treated with β-lactamase (β-Lactamase-Liq, Charm Sciences).

Results and Discussion

Thirty tissue samples—1 control and 29 that tested positive for microbial inhibitors by the *Micrococcus luteus* plate assay—were obtained from FSIS field laboratories. Five of the 29 samples were classified by FSIS laboratories as positive for β-lactams on the basis of their degradation by penicillinase. The other 24 had antimicrobial activities that could not be identified by the FSIS 7-plate assay (7) and were classified as UMIs. The 30 samples—21 liver samples, 8 kidney samples, and 1 muscle sample—were analyzed by the new screening test and by LC. Results are summarized in Table 1.

The new screening protocol was based on a previous procedure (32). It includes a rapid test for antimicrobial activity (Delvotest P-mini) and 2 immunoassay tests, LacTek-BL for β-lactams in general and LacTek-Cef for ceftiofur, and classifies β-lactams by relative rates of degradation by penicillinase and by β-lactamase II.

The screening test gave positive results for penicillinase-sensitive penicillins in 5 samples already identified as containing β-lactams. It also indicated low levels of penicillins in 6 other samples. Seven samples tested positive for ceftiofur, including 2 that were positive by LacTek-Cef but showed no antimicrobial activity. These results suggested that protein-bound ceftiofur metabolites might be present. One ceftiofur-positive sample and one β-lactam-positive sample also contained antimicrobials other than β-lactams.

The multiresidue LC analysis of β-lactams was based on a previous procedure for β-lactam residues in milk (33, 34). Cleanup was by LC fractionation. Because residues of penicillin G in tissue homogenates, especially liver and kidney, degrade rapidly (36), resulting in low and erratic recoveries, tissues were extracted by blending directly with acetonitrile.

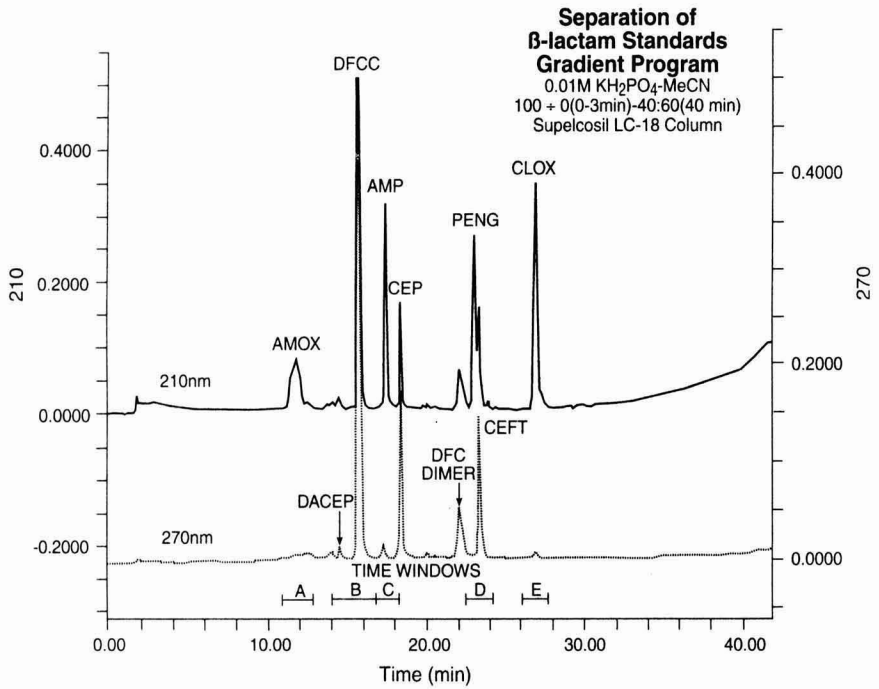

Figure 1. Gradient elution of standards: AMOX = amoxicillin; DACEP = desacetylcephapirin; DFC dimer = desfuroylceftiofur dimer; DFCC = desfuroylceftiofurcysteine; CEP = cephapirin; AMP = ampicillin; PENG = penicillin G; CEFT = ceftiofur; CLOX = cloxacillin. Time windows collected are shown.

Residues were confirmed by repeating the analysis after a water homogenate of the tissue had been treated with β-lactamase. In the β-lactamase procedure, stability of residues in water homogenate is not of concern because the intention is to degrade the residues. If a suspect peak disappears or diminishes, the presence of a β-lactam is confirmed. Procedures for penicillin G (36) and DFCC (37) are described in more detail elsewhere.

LC analysis of each fraction would have been extremely tedious. Thus, fractions were first screened for antimicrobial activity after acetonitrile was removed under reduced pressure. The Delvotest P-mini used as described for milk testing was satisfactory for this purpose. LC fractions that tested negative contained no antibiotic, and further analysis was not necessary. Fractions from the β-lactamase-treated replicate corresponding to those testing positive from the untreated extract could also be tested for antimicrobial activity. Loss of antimicrobial activity indicated presence of a β-lactam. However, if another antimicrobial was present in the fraction, antimicrobial activity remained after β-lactamase treatment even if a β-lactam was also present. When samples contained high levels (>1 ppm) of penicillin G (for example, sample 30), the cloxacillin fractions frequently tested positive but no cloxacillin was ever found, suggesting that penicillin G tailed slightly. LC confirmation of fractions testing positive was therefore essential, because the fractions did not always contain the expected antibiotic.

None of the amoxicillin fractions tested positive by the screening test. Derivatization would have been necessary to determine amoxicillin. The method of Ang and Luo (35) was simple and worked well with fractions prepared from sample extracts. A few ampicillin and cloxacillin fractions tested positive in the screening test but were all found negative by LC analysis. Thus, penicillin G and the ceftiofur metabolite DFCC were the only β-lactams found in the UMI tissues. This report is the first of chromatographic identification of ceftiofur metabolites in samples from commercial sources. DFCC serves as a marker residue for ceftiofur metabolites, which are mostly bound to proteins (31). Confirmation by analysis of a replicate after treatment with β-lactamase provided a simple and effective confirmatory test. Any residual background was subtracted, thus improving quantitation.

In general, the LC results agreed well with the new double-enzyme screening test. The presence of DFCC, the principle free metabolite of ceftiofur, was confirmed in samples testing positive for ceftiofur and showing antimicrobial activity. The 5 samples testing positive for β-lactams by the FSIS screen contained violative levels of penicillin G. Four of 6 other samples testing positive for penicillin G by the new screen were found to contain detectable levels of penicillin G by LC analysis, one at violative levels.

The improved screening protocol was slightly more sensitive than LC analysis and detected ceftiofur metabolites, which are not bioactive. Otherwise, the 2 methods agreed well. Samples that were negative by the screening procedure did not require further examination by LC, saving considerable time. The

multiresidue LC procedure can be used to determine any β-lactam antibiotic by collecting suitable time windows, and it can be used with most other antibiotics.

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DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Apramycin in Swine Kidney Tissue by Liquid Chromatography with Fluorescence Detection

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A liquid chromatographic method for determining apramycin in swine kidney tissue is described. Apramycin is extracted from tissue with basic methanol and purified by ion-pair extraction. By using an automated derivatization and injection procedure, the purified extract is derivatized with *o*-phthaldehyde, separated on a C_{18} column, and detected with a fluorescence detector. For fortified kidney samples, between-run coefficients of variation ranged from 4.8 to 7.1% at 1.00 ppm and from 9.6 to 14.3% at 0.50 ppm. Recoveries ranged from 76 to 86%. Standard curves were linear over the range 10–100 ng/mL.

Apramycin, also called nebramycin factor 2, is an aminoglycoside antibiotic (Figure 1) produced by *Streptomyces tenebrarius*. Commercially available oral and parenteral preparations are used to control gram-negative infections in swine (1) and calves (2) and to treat and control colibacillosis in poultry (3, 4). Unpublished radiolabeled residue and metabolism studies at our facility have determined that, in swine, kidney is the edible tissue in which residues accumulate at the highest levels and deplete at the slowest rate. Apramycin is the marker residue comprising greater than 80% of radiolabeled residue. As with other aminoglycosides, the high concentrations of apramycin in kidney may be due to its binding to renal tubular epithelial cell luminal membranes. The European Community (EC) has set maximum residue limits (MRLs) for apramycin in swine at 1 ppm in muscle, fat, liver, and skin and at 5 ppm in kidney.

An unpublished apramycin microbiological bioautographic method with a limit of detection of 0.1 ppm has been developed at our facility. However, a more specific liquid chromatographic (LC) method was requested by the EC for monitoring purposes. Two LC methods with pulsed amperometric detection have been published. One method can separate and quantitate apramycin in standard solutions from 1 mg to 10 ng on column (5) but has not been extended to biological matrixes. The other method detects both apramycin and tobramycin in blood serum at 0.6 ppm but no statistical data regarding method reproducibility were reported (6).

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This paper describes a sensitive and selective method for determining apramycin developed prior to the setting of MRLs for swine tissue by the EC. This method was later expanded by the Veterinary Laboratories Agency in the United Kingdom to other edible tissue and has been validated with a limit of quantitation of 0.5 ppm in muscle, fat, liver, and skin and 2.5 ppm in kidney (7). The data presented here are from the preliminary validation done prior to setting of MRLs.

Experimental

Apparatus

(**a**) *LC system.*—Model 600E multisolvent delivery system and a 715 WISP injection system (Waters Associates, Milford, MA), Model 980 programmable fluorescence detector (Applied Biosystems, Foster City, CA) with excitation wavelength set at 230 nm and a 389 nm emission cutoff filter installed. Pump flow rate was 1.0 mL/min.

(**b**) *Mass spectrometer.*—Sciex Model API 1 (Thornhill, Ontario, Canada) equipped with an ion spray interface operated at a source potential of 5.0 kV. Nebulizing and curtain gases were nitrogen at ca 1 and 1.2 L/min, respectively.

(**c**) *LC column.*—Nova-Pak C_{18}, 3.9 × 300 mm × 5 μm (Waters).

(**d**) *Sonicator.*—Sonifer cell disruptor 350 with 1/4 in. microtip (Branson, Danbury, CT).

(**e**) *Rotator.*—Roto Torque rotator (Cole-Parmer, Niles, IL).

(**f**) *Heating block.*—Reacti-Therm heating module (Pierce, Rockford, IL).

Reagents

(**a**) *Water.*—LC grade.

(**b**) *Solvents.*—Distilled in glass, LC grade.

(**c**) *Di-(2-ethylhexyl) phosphate (DEHP).*—Sigma, St. Louis, MO.

(**d**) *Hydrochloric acid.*—Reagent grade.

(**e**) *1-Octanesulfonic acid sodium salt.*—LC grade.

(**f**) *Acetic acid.*—ACS grade.

(**g**) *Sodium phosphate monobasic monohydrate.*—ACS grade.

(**h**) *Anhydrous sodium phosphate dibasic.*—ACS grade.

(**i**) *Fluoraldehyde.*—*o*-Phthaldehyde (OPA) derivatizing agent (Pierce, Rockford, IL). Stored at 4°C. New reagent was used every 2 months.

(**j**) *Sodium chloride.*—ACS grade.

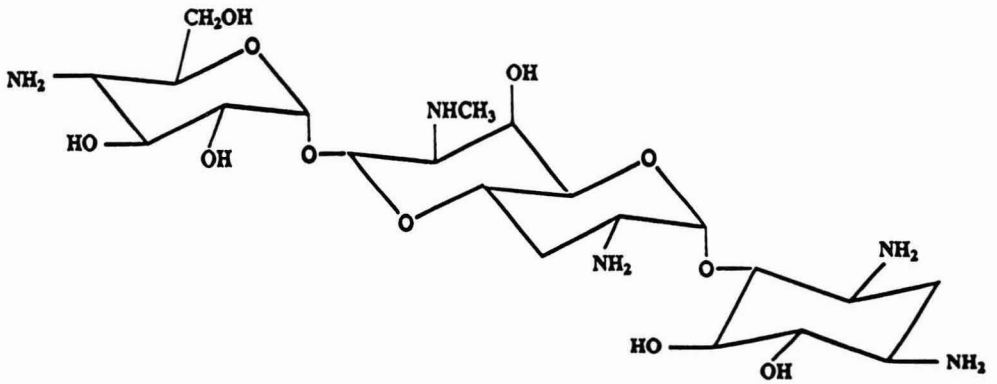

Figure 1. Structure of apramycin.

(**k**) *Sodium hydroxide.*—ACS grade.

(**l**) *Ammonium hydroxide concentrated.*—ACS grade.

(**m**) *Phosphate buffer solution.*—Solution was prepared by dissolving 13.8 g sodium phosphate monobasic and 14.2 g anhydrous sodium phosphate dibasic in water in a 1 L volumetric flask. Octanesulfonic acid (0.5 g) and NaCl (0.3 g) were added, and the solution was diluted to volume with water.

(**n**) *LC mobile phase.*—Acetonitrile–water–acetic acid (40 + 60 + 2) with 0.005M octanesulfonic acid.

(**o**) *Mobile phase for LC/ion spray mass spectrometry (LC/SP-MS).*—Acetonitrile–water–acetic acid (40 + 60 + 2) with 0.005M ammonium acetate.

(**p**) *Dilution solution.*—Solution was prepared by combining 300 mL 0.25N HCl and 100 mL 0.75N NaOH.

(**q**) *Reference standard.*—Apramycin (Eli Lilly, Indianapolis, IN).

(**r**) *Stock standard.*—1.00 mg/mL standard stock solution was prepared by adding 50.0 mg apramycin reference standard to 50 mL volumetric flask and diluting to volume with water.

(**s**) *Spiking solution.*—Stock solution was diluted 1:100 with water to give a 10 mg/mL solution.

(**t**) *Diluted standards.*—Standards of 100, 50, 25, and 10 ng/mL were prepared by diluting stock solution to these concentrations in control swine tissue extract.

Spiked Kidney Tissues

Swine kidney tissue was ground to a homogenous consistency in a tissue grinder and stored frozen at –20°C until use. Thawed 10 g portions of tissue were spiked with various amounts of spiking solution. For 1.0 and 0.5 ppm samples, 1.00 and 0.50 mL spiking solutions were added respectively, and the tissue was mixed thoroughly with a spatula.

Tissue Extraction

Ground kidney tissue (10 g) was weighed into a 50 mL polypropylene centrifuge tube. Concentrated ammonium hydroxide (10 mL) was added, the tube was allowed to sit at room temperature for 10 min, and then 25 mL methanol was added. The tube was sonicated for 30 s with a Sonifer cell disruptor set at 4 and centrifuged for 20 min at 1300 × *g*. Tube contents were decanted into a 100 mL volumetric flask and another 25 mL methanol was added to the polypropylene tube. The tube was sonicated for 1 min at a setting of 4 and centrifuged for 20 min at 1300 × *g*. Tube contents again were decanted into the 100 mL volumetric flask, and flask contents were diluted to volume with methanol.

A 2.5 mL portion (equivalent to 250 mg tissue) was taken from the volumetric flask and dispensed into a test tube. Tube contents were evaporated to dryness at 60°C under a stream of air, and then 3 mL phosphate buffer solution and 2 mL ethyl acetate containing 1% DEHP were added. The tube was capped and rotated for 15 min on a rotator set at low with indicator on 10. (This setting gives ca 20 rotations/min.) The tube was removed from the rotator and centrifuged at 1300 × *g* for 10 min. The top 1.5 mL of the organic layer was removed and combined with 1.5 mL 0.25N aqueous HCl in another test tube. The tube was rotated for 7 min on a setting of low with the indicator on 10. The tube was removed from the rotator and heated at 60°C for 10 min in a heating block. The tube was removed from the heating block and 0.5 mL 0.75N NaOH was added. The tube was allowed to sit for 5 min, and then the organic layer was aspirated.

A 2.0 mL volume of toluene was added to the tube. The tube was rotated for 5 min on a setting of low with the indicator on 10 and then centrifuged at 1300 × *g* for 5 min. The top toluene layer was aspirated, and 22.5 µL of the aqueous layer was transferred to a limited-volume insert injection vial (Waters). Finally, 22.5 µL dilution solution was added.

Derivatization and LC Determination

(**a**) *Preparation of WISP 715 autoinjector.*—A 4 mL vial filled with OPA derivatizing agent was placed in vial position 1. The autotransfer functions were set as follows: Auto Transfer, On; Transfer Before, Vial; Purge After Transfer, Off; Dispense Needle Depth, 4; Vial Transfer, 1; Transfer Volume, 90 µL; Mix Cycles, 4; Mix Volume, 135 µL; Delay Time, 35 min; Injection Volume, 90 µL.

For each sample injected, 90 µL fluoraldehyde was taken from vial 1 and mixed with 45 µL sample 4 times. The sample and derivatization reagent then were allowed a reaction time of

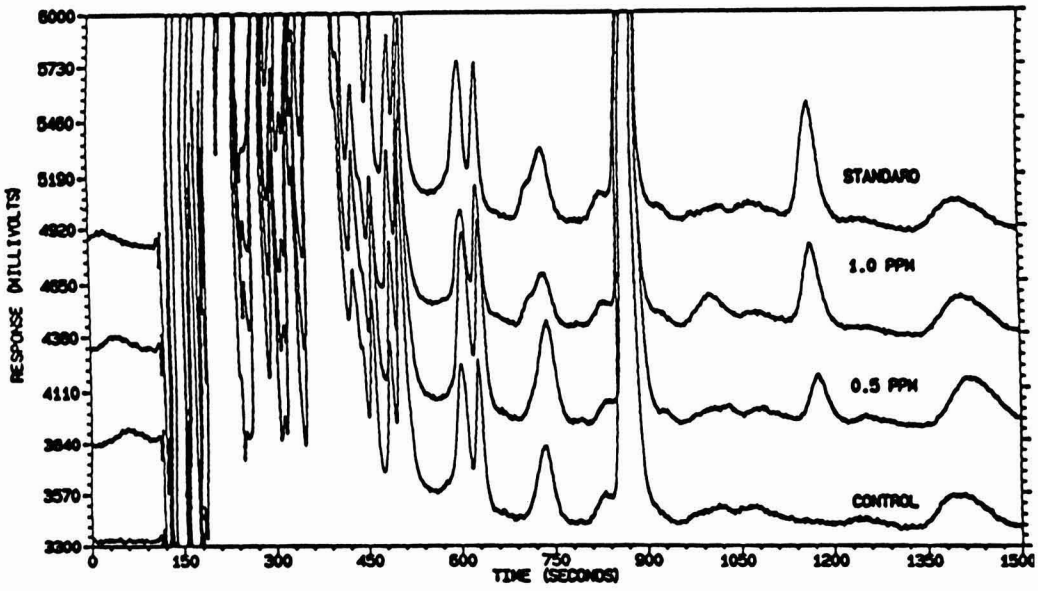

Figure 2. Chromatograms of a 100 ng/mL standard, kidney samples fortified at 1.00 and 0.50 ppm, and a control kidney sample.

35 min prior to injection to the LC system. After the first injection, the next sample was being derivatized while the first sample was eluting from the LC system.

Apramycin concentration in tissue was calculated as follows:

$$\text{Apramycin, ppm} = A \times n/(f \times c) \times V \times 1\ \mu\text{g}/1000\ \text{ng}$$

where A = apramycin concentration (ng/mL) determined from regression line, n = volume of neutralized acid (mL), f = fraction of ethyl acetate/DEHP phase extracted into acid, c = amount of methanolic extract evaporated to dryness (mL), and V = volume of methanol in extract equal to 1 g tissue (mL/g). For validation of the method for kidney tissue, n = 2.0 mL, f = 1.5 mL/2.0 mL, c = 2.5 mL, and V = 10 mL/g.

Results and Discussion

Figure 2 shows chromatograms of a control, kidney tissues fortified at 1.0 and 0.5 ppm, and a 100 ng/mL standard prepared in control extract. For fortified kidney tissue (Table 1), within-assay coefficients of variation (CVs) ranged from 4.8 to 7.1% at 1.0 ppm and from 9.6 to 14.3% at 0.50 ppm. Assay recovery was consistently over 80%, except for day 2, for which recovery from tissue fortified at 0.50 ppm was 76.4%. A 4-point standard curve ranging from 10 to 100 ng/mL was linear over this range. Correlation coefficients averaged 0.999, and the intercept was close to zero.

Aminoglycosides are difficult to extract from tissue because they bind to anionic tissue constituents (8). Initially, attempts were made to extract apramycin from fortified kidney tissue by homogenization with methanol or acetonitrile in various proportions with water. Recoveries from these extractions were less than 5%. Trichloroacetic acid precipitation was also tried but resulted in incomplete recovery. Gilbert and Kohlhepp (9) reported a 37% recovery for the aminoglycoside gentamicin when extracted from animal tissue by trichloroacetic acid precipitation. However, recovery improved to 96% with NaOH digestion. For this method, we used digestion with concentrated ammonium hydroxide and methanol and sonic disruption to obtain 95% recovery of apramycin.

Apramycin has numerous amino and hydroxyl groups (Figure 1), making it highly polar and difficult to isolate from matrix components after base extraction. Apramycin will not move from the aqueous phase into organic immiscible solvents at any pH. Therefore cleanup by liquid–liquid extraction could not be used. Reversed-phase solid-phase extraction columns do not retain apramycin completely on the column and thus could not be used to separate apramycin from other polar matrix components. Cation-exchange solid phase columns with either a sulfonylpropyl or propylbenzenesulfonyl functional group

Table 1. Recovery of apramycin from spiked swine kidney tissue on 3 separate days

Day	Spike level, ppm	n	Mean recovery, %	CV, %
1	1.0	4	81.5	5.6
	0.5	3	84.4	11.7
2	1.0	4	85.2	7.1
	0.5	4	76.4	9.6
3	1.0	4	80.3	4.8
	0.5	4	86.3	14.3

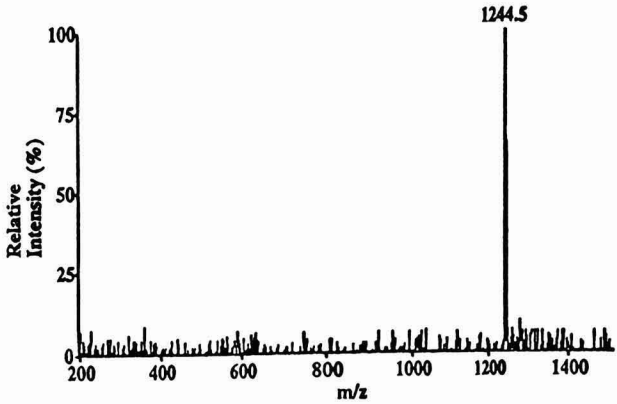

Figure 3. Mass spectrum of OPA–apramycin derivative.

gave inconsistent recoveries of apramycin because of their sensitivity to matrix interferences. A 90% recovery was achieved by ion-pair partitioning of apramycin into ethyl acetate. The ion-pair was broken by adding aqueous HCl, and apramycin was quantitatively recovered in the aqueous phase. Evaporation of larger amount of methanolic tissue extracts prior to ion-pair partioning resulted in poorer recoveries, presumably because tissue constituents interfered with ion-pair formation. Therefore, ion-pair extraction was limited to methanolic tissue extracts equivalent to 250 mg.

Apramycin has no chromophore for absorption in the UV or visible region. Therefore pre- or postcolumn derivatization is

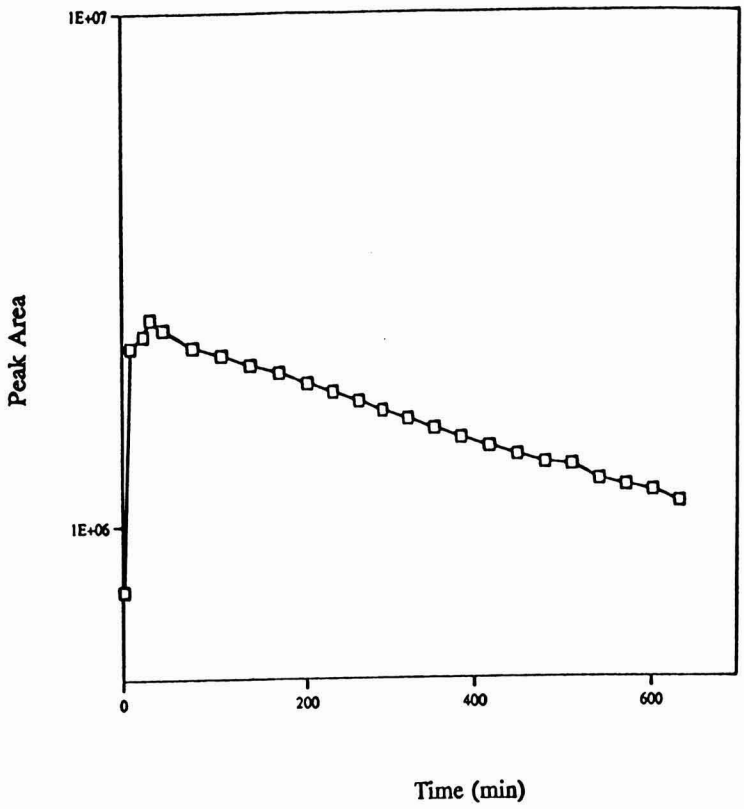

Figure 4. Formation and decay of OPA–apramycin derivative.

necessary for UV or fluorescence detection. Precolumn OPA derivatization allowed formation of the OPA–apramycin derivative, which provided sufficient sensitivity to quantitate apramycin in swine kidney at 0.5 ppm. Precolumn OPA derivatization had the added advantage of decreasing analyte polarity, thereby increasing retention and reducing tailing of apramycin on the C_{18} column. Typically, OPA fluorescence derivatives are detected at an excitation wavelength of 340 nm. However, an excitation wavelength of 230 nm almost doubled the sensitivity of the OPA–apramycin derivative. Higher sensitivities for OPA–amino acids have been reported previously at this lower excitation range (10).

Any of the 4 primary amines in apramycin could react with OPA to form fluorescence derivatives. If all 4 primary amines reacted with OPA, the mass of the OPA–apramycin derivative would be 1243.5. LC/ISP-MS was used to determine the mass of the derivative formed in this method. To make the mobile phase compatible with the mass spectrometer, ammonium acetate was substituted for octanesulfonic acid. The substitution caused only a slight shift in retention time.

One hundred twenty-five microliters of a 333 μg/mL standard of apramycin with OPA (1:2) was injected into the LC/ISP-MS system. Figure 3 shows the mass spectrum of the derivative peak. A peak at *m/z* 1244.5 is the $[M + H]^+$ ion for the derivative. Thus, the derivative monitored in this method is apramycin that has reacted with OPA on all 4 primary amines.

The formation and decay of the OPA–apramycin derivative were measured by making repeated injections of 10 μg/mL standards of apramycin and plotting peak area versus time. The OPA-apramycin derivative had a maximum peak area 33 min after addition of OPA and declined from this maximum with a half life of 9.13 h (Figure 4). Other primary amines such as amino acids have been reported to react with OPA almost immediately (9). The time delay in the formation of the OPA–apramycin derivative might be due to steric hindrance slowing the rate of the reaction of OPA with the 4 primary amines. Lai and Sheehan (11) reported that precolumn derivatization of tobramycin, an aminoglycoside antibiotic that has 5 primary amines, resulted in formation of 2 derivatives: an early eluting peak at 4 min and a later eluting peak at 10 min. The peak area ratio of the 2 peaks varied with time with the later eluting peak reaching a maximum at 30 min. The OPA–apramycin peak behaved similarly to the later eluting peak in the tobramycin assay, requiring approximately 30 min to reach maximum peak height. Other partial OPA–apramycin derivatives might also be forming in this method and could be obscured by the solvent front.

The OPA–apramycin derivative reached a maximum after approximately 0.5 h and declined thereafter. Therefore, to achieve maximum sensitivity an automated precolumn derivatization procedure using the WISP 715 was devised. Fluoraldehyde was withdrawn from vial position 1, mixed with the sample, allowed to react for 35 min, and then injected onto the LC column. After injection of the first sample, the subsequent sample was simultaneously being derivatized while the previous sample was eluting from the column. This automated procedure ensured that all samples reacted with OPA for the same amount of time prior to injection.

Acknowledgment

We thank Doug Kiehl for determing the mass spectrum of the apramycin derivative.

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FOOD BIOLOGICAL CONTAMINANTS

Performance Tested Method Certification of BAX™® for Screening/*Salmonella*: A Case Study

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This report details the independent laboratory study of the BAX™® for Screening/*Salmonella* assay to complete AOAC Performance Tested Method certification. The performance of the BAX system was compared with those of BAM culture methods on food samples inoculated with *Salmonella*. This study validated product claims. Performance Tested Method status was granted for the screening assay.

Performance Tested Method certification is a program administered by AOAC Research Institute (AOAC RI) to validate performance claims of commercial test kits. Data supporting test kit performance claims, product literature, labels, manufacturing specifications, and quality assurance/quality control procedures were supplied by the kit manufacturer. After initial review by AOAC RI, 2 independent reviewers evaluated the submitted information and designed a laboratory study to be conducted at an independent facility. This report details the independent laboratory study of the BAX for Screening/*Salmonella* assay (Qualicon, Inc., Wilmington, DE) at Campden & Chorleywood Food Research Association, Chipping Campden, Gloucestershire, UK. Although harmonization of AOAC method validation programs will lead to minor changes in the Performance Tested Method certification process, the independent laboratory study phase described here will remain substantially unchanged.

The importance of *Salmonella* spp. as a leading cause of food-borne bacterial disease continues to be emphasized by national epidemiological reports (1–3). The low infective dose of *Salmonella* requires that the presence of a single organism be detected. Additional difficulty in detection of *Salmonella* in food stems from the fact that organisms may be injured and/or may be outnumbered by closely related competitor organisms (4). The need for a rapid, reliable, and extremely sensitive detection method for *Salmonella* is vitally important to the food industry, particularly for products with limited shelf life. BAX for Screening/*Salmonella* is a new assay that easily and definitively detects *Salmonella* in food 18–20 h after sampling, thereby shortening the time to result for product release.

BAX for Screening/*Salmonella* uses polymerase chain reaction (PCR) technology for rapid and highly specific amplification of a conserved region in the *Salmonella* genome that is then detected by agarose gel electrophoresis. The process requires a standard overnight enrichment of food samples and includes use of tableted reagents (primers, *Taq* DNA polymerase, deoxynucleotides, and excipients). The test procedure includes use of control reactions that allow any food-mediated PCR inhibition (5) to be detected.

PCR is a complex enzymatic reaction capable of amplifying very low levels of target DNA to detectable levels. Theoretically, a single copy of target DNA can be amplified to over 10^{10} copies in a 35-cycle reaction. However, small variations in the sample will affect amplification efficiency, and sampling statistics cannot guarantee that a single copy will always get into the reaction tube (6). Therefore, BAX for Screening/*Salmonella* protocols have been optimized to ensure that at least 10 copies of target *Salmonella* DNA get into the reaction tube from a sample with 10^4 colony-forming units (cfu)/mL after primary enrichment.

METHODS

Part 1. Comparative Recovery: BAX System versus BAM Culture Method

(**a**) *Inoculation of foods and sample preenrichment.*—*Salmonella* serotypes, 2 from groups B–I and 3 from groups J–Z, were obtained from the Campden & Chorleywood Food Research Association. *S. typhimurium* (CRA3510), *S. waycross* (CRA1961), *S. champaign* (CRA1327), and *S. driffield* (CRA1430) were cultured in 10 mL nutrient broth (NB; Oxoid, Unipath, Hampshire, UK) at 37°C for 18–24 h. A freeze-dried culture of *S. virchow* (CRA1424) was prepared by the UK National Collection of Type Cultures method (7) for use as the inoculum for dried milk.

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1. *Homogenize 25 g sample of food in LB. Incubate 24 h at 35°C.*
2. *Add 1 mL of enrichment broth to 9 mL of BHI. Incubate 3 h at 37°C.*
3. *Add 5 μL of enrichment into 200 μL of lysis reagent.*
4. *Incubate 20 min at 37°C, then 10 min at 95°C.*
5. *Add 50 μL of lysate to sample and positive control tablets.*
6. *Amplify DNA in thermal cycler.*
7. *Add dye and load 15 μL of each reaction on pre-cast, pre-stained gel.*
8. *Run electrophoresis for 30 min at 180 volts.*
9. *Photograph gel.*
10. *Analyze results. (Detail)*

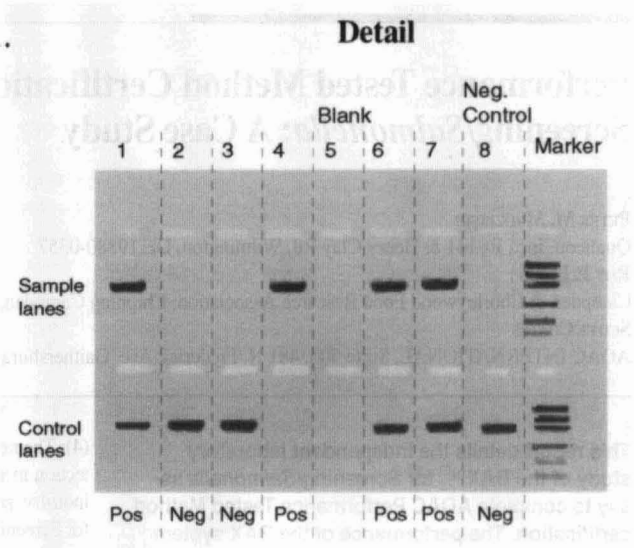

Figure 1. Assay steps and gel photograph for BAX™ for Screening/*Salmonella*.

Cultures were diluted in maximum recovery diluent (MRD; Oxoid) and added to the foods at an inoculum level of ca 1–5 cfu/25 g. Each food was inoculated with a single serotype. Inoculated meat samples were frozen at –20°C for 24 h. Inoculum levels were determined by the *Bacteriological Analytical Manual* (BAM), most probable number (MPN) method (8), and by serial 10-fold dilutions and pour plates on nutrient agar (NA; Oxoid). After 24 h incubation at 37°C, all visible colonies on the plates were counted. Ten replicate samples (25 g) of inoculated foods (dried milk, raw ground beef, raw chicken livers, raw ground pork, and pasteurized milk) and 3 replicates (25 g) of uninoculated foods were prepared by homogenization in lactose broth (LB), and were incubated at 35°C for 24 h.

(**b**) *Culture method for Salmonella detection based on BAM method (9).*—Preenriched samples were subcultured into 10 mL selenite cystine broth (Oxoid) and 10 mL tetrathionate broth (Oxoid) with brilliant green. Broths were incubated at 35°C for 18–24 h. After incubation, a loopful of each broth was streaked onto xylose lysine desoxycholate agar (Oxoid), Hektoen enteric agar (Oxoid), and bismuth sulfite (BS) agar (Oxoid). Plates were incubated at 35°C for 24 h. BS plates were reincubated for a further 24 h. Two colonies that were characteristic of *Salmonella* were selected from each plate for confirmation. In the absence of typical colonies, up to 2 atypical colonies per agar were chosen. Colonies were inoculated into triple sugar iron agar (Oxoid) and lysine iron agar (Oxoid), and isolates showing typical reactions after incubation at 35°C for 24 h were further confirmed by testing for agglutination with *Salmonella* poly-O and poly-H agglutination antiserum (Mast Diagnostics, Bootle, Merseyside, UK) and by API 20E (bioMerieux, Basingstoke, UK).

(**c**) *BAX for Screening/Salmonella.*—Preenriched food samples (1 mL) were subcultured into 9 mL brain heart infusion broth (CM225; Oxoid; Figure 1) and incubated at 37°C for 3 h. A portion (5 μL) of the resulting culture was added to 200 μL lysis buffer containing protease enzyme (Qualicon, Inc., Wilmington, DE). After incubation, first at 37°C for 20 min and then at 95°C for 10 min, 50 μL of the lysed samples was transferred to a PCR sample tube (Qualicon, Inc.). These tubes contained in tablet form all reagents (*Taq* polymerase, deoxynucleotides, and selective primers) necessary for a PCR reaction (Figure 2). In addition, 50 μL lysed sample was transferred to a control reaction tube. The tube contains all previously described reagents, as well as target nucleic acid. Amplification in this tube indicates that there was no PCR inhibition introduced with the sample and that proper reaction conditions were maintained. Samples were then subjected to thermal cycling (Perkin Elmer 9600 thermocycler, Branchburg, NJ) via a 2-temperature PCR protocol (hold period 94°C/2 min; 35 cycles of 94°C/15 s and 72°C/3 min; hold period 72°C/7 min, then 4°C). Amplified product was then detected by agarose gel electrophoresis at 180 V for 30 min with 2.0% Seakem Gold Reliant agarose gels, prestained with ethidium bromide (FMC, Rockland, ME). Gels were photographed under UV illumination with a FOTO/Phoresis UV documentation system (Fotodyne, Inc., Heartland, WI). A positive result (*Salmonella*-specific band) is indicated by a fluorescent band at the 725-base-pair level (Figure 1). This molecular weight corresponds to the third band of 6 that arise in a line on the gel that is loaded with a Low DNA Mass Ladder (Life Tech-

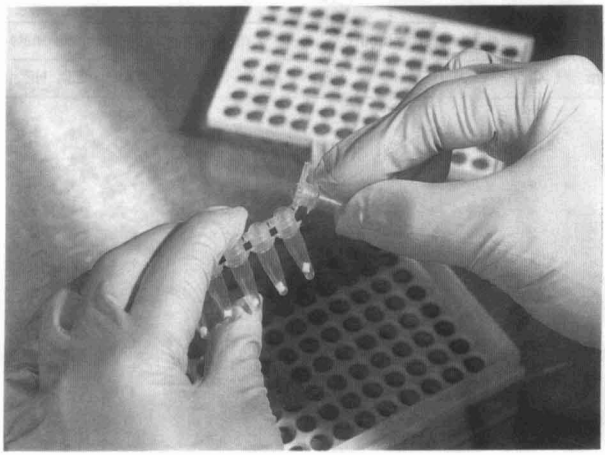

Figure 2. Tableted reagents and primers used with BAX for Screening/*Salmonella* assay. All materials required for PCR are supplied in the tube shown.

nologies, Gaithersburg, MD). Visualization of all 6 bands demonstrates the adequacy of the detection system. A positive control reaction is indicated by a band in the control lane of the gel (Figure 1).

Part 2. Effect of Non-Salmonella Flora

S. enteritidis (CRA1002), *S. typhimurium* (CRA1008), *Pseudomonas fluorescens* (CRA8298), *P. aeruginosa* (CRA8299), *Acinetobacter calcoaceticus* (CRA1564 and CRA7438), and *Moraxella* species (CRA1500 and CRA1504) were cultured overnight in NB. Non-*Salmonella* cultures were combined in equal portions to produce a cocktail of microflora. These organisms were chosen because they are commonly found in the raw meat samples tested here (10). *Salmonella* and non-*Salmonella* cultures were diluted in MRD, and appropriate dilutions were inoculated into samples (25 g) of raw chicken wings and raw pork sausages. A second group of food samples was temperature-abused (25°C for 24 h) to increase the levels of natural background flora. Intended *Salmonella* inoculum levels were 1–15 cfu/g, and inoculum levels and background flora levels were confirmed by plate counts on NA.

Inoculated samples were enriched in LB by incubation at 35°C for 24 h. Samples were analyzed for *Salmonella* by the culture method previously described. Five replicate portions of enrichment culture were analyzed for *Salmonella* by the BAX system as previously described.

Results

Part 1. Comparative Recovery: BAX System versus BAM Culture Method

Table 1 lists the foods tested, inoculation levels (plate count and MPN) and BAX system and BAM results. The dried milk was run twice because the first sample had a high inoculation of 49 cfu/25 g. The second dried skim milk sample was inocu-

Table 1. Results of inoculated food samples: BAX system versus BAM method[a]

		Selenite cystine broth			Tetrathionate broth			
Food	BAX system	XLD	HE	BS	XLD	HE	BS	BAM confirmed
Pasteurized milk	–	–	–	–	–	–	–	N/A
Plate count	–	–	–	+	–	–	–	–
(2.5 cfu/25 g)	+	+[b]	–	+	+[c]	–	+	+
	+	–	–	+	+	–	–	+
MPN	+	–	–	–	+[c]	–	–	+
(0.5 cfu/25 g)	–	–	–	–	–	–	–	N/A
	–	–	–	–	–	–	–	N/A
	–	–	–	–	–	–	–	N/A
	–	–	–	–	–	–	–	N/A
	–	–	–	–	–	–	–	N/A
Uninoculated	–	–	–	–	–	–	–	N/A
Uninoculated	–	–	–	–	–	–	–	N/A
Uninoculated	–	–	–	–	–	–	–	N/A

Table 1. (*continued*)

Food	BAX system	Selenite cystine broth			Tetrathionate broth			BAM confirmed
		XLD	HE	BS	XLD	HE	BS	
Chicken liver	+	+	+	−	+	+	+	+
Plate count	+	+	+	+	+	+	+	+
(27.5 cfu/25 g)	+	+	+	+	+	+	+	+
	+	+	+	−	+	+	+	+
MPN	+	+	+	+	+	+	+	+
(8 cfu/25 g)	+	+	+	−	+	+	+	+
	−	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
	+	+	−	+	+	+	+	+
Uninoculated	+	−	−	−	+	+	+	+
Uninoculated	+	+	+	−	+	+	−	+
Uninoculated	−	−	−	−	−	−	−	N/A
Dried skim milk	+	+	+	+	+	+	+	+
Plate count	+	+	+	+	+	+	+	+
(49 cfu/25 g)	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
MPN	+	+	+	+	+	+	+	+
(115 cfu/25 g)	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
	+	+	+	−	+	+	+	+
Uninoculated	−	−	−	−	−	−	−	N/A
Uninoculated	−	−	−	−	−	−	−	N/A
Uninoculated	−	−	−	−	−	−	−	N/A
Dried skim milk	+	+	+	+	+	+	+	+
Plate count	+	+	+	+	+	+	+	+
(4.7 cfu/25 g)	+	+	+	−	+	+	+	+
	+	+	+	+	+	+	+	+
MPN	+	+	+	+	+	+	+	+
(2.3 cfu/25 g)	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
Uninoculated	−	−	−	−	−	−	−	N/A
Uninoculated	−	−	−	−	−	−	−	N/A
Uninoculated	−	−	−	−	−	−	−	N/A
Ground beef	+	+	+	−	+	+	−	−
Plate count	+	+	+	−	+	+	+	+
(6.5 cfu/25 g)	+	+	+	−	+	+	+	−
	+	+	+	−	+	+	+	+
MPN	+	+	+	+	+	+	+	+
(60 cfu/25 g)	+	+	+	−	+	+	−	+
	+	+	+	−	+	+	−	+
	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+
	+	+	+	−	+	+	−	+
Uninoculated	−	+	+	−	+	+	−	−
Uninoculated	−	+	−	−	+	−	−	−
Uninoculated	+	+	+	−	+	+	−	−

Table 1. (*continued*)

Food	BAX system	Selenite cystine broth			Tetrathionate broth			BAM confirmed
		XLD	HE	BS	XLD	HE	BS	
Ground pork	+	+	+	–	+	+	+	+
Plate count	+	+	+	–	+	+	–	+
(13 cfu/25 g)	+	+	+	–	+	+	–	+
	+	+	+	+	+	+	+	+
MPN	+	+	+	+	+	+	+	+
(6 cfu/25 g)	+	+	+	+	+	+	+	+
	+	+	+	–	+	+	+	+
	+	+	+	–	+	+	–	+
	+	+	+	–	+	+	–	+
	+	–	–	–	+	+	+	+
Uninoculated	–	–	–	–	–	–	–	N/A
Uninoculated	–	–	–	–	–	–	–	N/A
Uninoculated	–	–	–	–	–	–	–	N/A

[a] XLD = xylose lysine desoxycholate agar; HE = Hektoen enteric agar; BS = bismuth sulfite agar; N/A = only positive results are confirmed.
[b] Atypical colony.
[c] Only 1 colony visible.

lated in the target range (4.7 cfu/25 g). A total of 78 samples were tested, representing 10 inoculated and 3 uninoculated control samples for each of the 6 food types. Plate count data and MPN data (9) were statistically equivalent except those for ground beef samples. BAX for Screening/*Salmonella* recovered inoculated samples as well as or better than did the BAM method at all inoculation levels from 2.5 to 49 cfu/25 g.

Of the 78 samples tested (Table 2), the BAX system and BAM were in agreement on 74 occasions (52 positive and 22 negative for *Salmonella*). On 3 occasions involving ground beef samples, the BAX system gave positive results that were not confirmed by BAM. Two samples were inoculated with 6.5 cfu/25 g *Salmonella*, and one was the uninoculated control. Only once did the BAX system fail to detect *Salmonella* in a sample that was BAM-positive. This was on a chicken liver sample inoculated with 27.5 cfu/25 g *Salmonella*.

Table 2. Summary of comparative recovery: BAX system versus culture method[a]

BAX	Culture +	Culture –	Total
BAX system +	52	3	55
BAX system –	1	22	23
Total	53	25	78

[a] Of 25 culture-negative samples, 3 were BAX system-positive: 2 of these were inoculated and 1 was uninoculated. Assuming that the 1 uninoculated sample is a false positive, the assay yields a false-positive rate of 4.00%. This is not significantly different from the claim of 3% or less ($\chi^2 = 0.09$, p = not significant). Of 53 confirmed culture-positive samples, 1 was BAX system-negative for a false-negative rate of 1.89%. This supports the claim of 2% or less. False positive = BAX system-positive but culture-negative/total culture-negative (1/25). False negative = BAX system-negative but culture-positive/total culture-positive (1/53). Sensitivity = $1 - f_n$. Specificity = $1 - f_p$. (f_n is the false-negative rate; f_p is the false-positive rate.)

Part 2. Effect of Non-Salmonella Flora

Tables 3–6 list foods tested, inoculation levels of *Salmonella* and non-*Salmonella*, and BAX system and BAM results. Five BAX system assays and 1 BAM assay were run for each sample. Low inoculation levels for non-*Salmonella* microflora were less than 3×10^4 cfu/g. High inoculation levels reached nearly 10^7 cfu/g.

In total, 160 BAX assays were run on 32 independent samples inoculated with *Salmonella* (Table 7). All 32 samples were confirmed positive by the BAM culture method. Of the 160 BAX system assays, 156 were positive for *Salmonella*. The 4 BAX system-negative results were all from the same raw pork sausage sample. Seven uninoculated control samples were confirmed positive by the BAM culture method. All 7 were also positive on all 5 replicates by the BAX system. Of 9 control samples that were negative by BAM, 2 were negative on all 5 replicates with the BAX system, 6 were positive on 1 of 5 BAX system replicates, and 1 was positive on 4 BAX system replicates.

Discussion

This study was undertaken to validate performance claims of BAX for Screening/*Salmonella*. Earlier studies (11, 12) comparing BAX for Screening/*Salmonella* directly to culture methods were used to develop product claims of sensitivity, ≥98%; specificity, ≥97%; false-negative rate, ≤2%; false-positive rate, ≤3%; accuracy, 98%.

In part 1 of this study, 60 samples were inoculated with *Salmonella*. To test the effects of injury, inoculated meat samples were frozen before analysis and milk samples were inoculated from freeze-dried cultures. There were 3 instances of BAX system-positive and BAM-negative results. In 2 of these cases, *Salmonella* had been deliberately introduced to the sample. The

Table 3. Non-*Salmonella* microflora study using raw chicken inoculated with *S. typhimurium*[a]

Salmonella inoculum, cfu/g	Non-*Salmonella* inoculum, cfu/g	BAX system (n/5 + ve)	Selenite cystine broth			Tetrathionate broth			Confirmed
			XLD	HE	BS	XLD	HE	BS	
3	1.0×10^{6}[b]	5	+	+	−	+	+	+	+
9	1.0×10^{6}[b]	5	+	+	−	+	+	+	+
0	1.0×10^{6}[b]	5	+	+	−	+	+	+	+
3	1.0×10^{4}[b]	5	+	+	−	+	+	+	+
9	1.0×10^{4}[b]	5	+	+	−	+	+	+	+
0	1.0×10^{4}[b]	5	+	+	−	+	+	+	+
3	2.7×10^{6}[c]	5	+	+	−	+	+	+	+
9	2.7×10^{6}[c]	5	+	+	−	+	+	+	+
0	2.7×10^{6}[c]	0	+	+	−	+	−	−	−
29.7	4.9×10^{4}[c]	5	+	+	+	+	+	+	+
9.9	4.9×10^{4}[c]	5	+	+	+	+	+	+	+
0	4.9×10^{4}[c]	1	+	−	−	+	−	+	−

[a] XLD = xylose lysine desoxycholate agar; HE = Hektoen enteric agar; BS = bismuth sulfite agar.
[b] Inoculated non-*Salmonella* microflora.
[c] Natural background flora.

large number of suspect colonies found on plates originating from raw ground beef can often obscure typical colonies, making confirmation difficult. An independent study (12) has shown that the BAX system is often more sensitive in detecting *Salmonella* than culture methods in such situations. In this previous study, the BAX system was shown to detect *Salmonella* consistently at 10^4 cfu/g after enrichment and often at levels as low as 10^3 cfu/g. There was no cross-reactivity with the BAX system when tested on pure cultures of organisms normally found in raw meat samples. It is believed, therefore, that these were true positive samples. The third instance of BAX system-positive and BAM-negative results is also in question because of this argument. However, accepting this sample as a false-positive yields a false-positive rate of 4% (Table 2), which is not statistically different from the claimed false-positive rate of 3% ($\chi^2 = 0.09$, p = not significant).

Table 4. Non-*Salmonella* microflora study using raw chicken inoculated with *S. enteritidis*[a]

Salmonella inoculum, cfu/g	Non-*Salmonella* inoculum, cfu/g	BAX system (n/5 + ve)	Selenite cystine broth			Tetrathionate broth			Confirmed
			XLD	HE	BS	XLD	HE	BS	
5	1.0×10^{6}[b]	5	+	+	−	+	+	−	+
15	1.0×10^{6}[b]	5	+	+	−	+	+	+	+
0	1.0×10^{6}[b]	5	+	+	+	+	+	+	+
5	1.0×10^{4}[b]	5	+	+	−	+	+	−	+
15	1.0×10^{4}[b]	5	+	+	−	+	+	+	+
0	1.0×10^{4}[b]	1	+	+	+	+	+	−	−
5	2.7×10^{6}[c]	5	+	+	−	+	+	−	+
15	2.7×10^{6}[c]	5	+	+	−	+	+	−	+
0	2.7×10^{6}[c]	4	+	−	+	+	+	−	−
29.1	4.9×10^{4}[c]	5	+	+	+	+	+	+	+
9.7	4.9×10^{4}[c]	5	+	+	+	+	+	+	+
0	4.9×10^{4}[c]	1	+	−	−	+	−	+	−

[a] XLD = xylose lysine desoxycholate agar; HE = Hektoen enteric agar; BS = bismuth sulfite agar.
[b] Inoculated non-*Salmonella* microflora.
[c] Natural background flora.

Table 5. Non-*Salmonella* microflora study using raw pork sausages inoculated with *S. typhimurium*[a]

Salmonella inoculum, cfu/g	Non-*Salmonella* inoculum, cfu/g	BAX system (n/5 + ve)	Selenite cystine broth			Tetrathionate broth			Confirmed
			XLD	HE	BS	XLD	HE	BS	
6.9	1.1×10^{6}[b]	5	+	+	+	+	+	+	+
2.3	1.1×10^{6}[b]	5	+	+	+	+	+	+	+
0	1.1×10^{6}[b]	5	+	+	+	+	+	+	+
6.9	1.1×10^{4}[b]	5	+	+	+	+	+	+	+
2.3	1.1×10^{4}[b]	5	+	+	+	+	+	+	+
0	1.1×10^{4}[b]	5	+	+	+	+	+	+	+
6.9	3.1×10^{4}[c]	5	+	+	+	+	+	+	+
2.3	3.1×10^{4}[c]	5	+	+	+	+	+	+	+
0	3.1×10^{4}[c]	1	+	–	+	+	–	+	–
29.7	9.5×10^{6}[cd]	1	+	+	+	+	+	+	+
9.9	9.5×10^{6}[cd]	5	+	+	+	+	+	+	+
0	9.5×10^{6}[cd]	1	–	–	–	–	–	–	N/A[e]

[a] XLD = xylose lysine desoxycholate agar; HE = Hektoen enteric agar; BS = bismuth sulfite agar.
[b] Inoculated non-*Salmonella* microflora.
[c] Natural background flora.
[d] Temperature-abused.
[e] N/A = only positive results are confirmed.

In one instance, the BAX system failed to detect *Salmonella* in a sample that was positive by BAM. This yields a false-negative rate of 1.9%, which is consistent with the claim of <2%.

Part 2 of this study was designed to determine the effect of non-*Salmonella* microflora on the ability of BAX to detect low levels of *Salmonella*. In 4 of 160 assays (Table 7), the BAX system failed to detect *Salmonella* in samples confirmed by BAM. These 4 were not statistically independent, because they were all replicates of one sample. If, however, these samples are treated independently, the results of part 2

Table 6. Non-*Salmonella* microflora study using raw pork sausages inoculated with *S. enteritidis*[a]

Salmonella inoculum, cfu/g	Non-*Salmonella* inoculum, cfu/g	BAX system (n/5 + ve)	Selenite cystine broth			Tetrathionate broth			Confirmed
			XLD	HE	BS	XLD	HE	BS	
17.7	1.1×10^{6}[b]	5	+	+	+	+	+	+	+
5.9	1.1×10^{6}[b]	5	+	+	+	+	+	+	+
0	1.1×10^{6}[b]	5	+	+	+	+	+	+	+
17.7	1.1×10^{4}[b]	5	+	+	+	+	+	+	+
5.9	1.1×10^{4}[b]	5	+	+	+	+	+	+	+
0	1.1×10^{4}[b]	5	+	+	–	+	+	+	+
17.7	3.1×10^{4}[c]	5	+	+	+	+	+	+	+
5.9	3.1×10^{4}[c]	5	+	+	+	+	+	+	+
0	3.1×10^{4}[c]	0	–	–	–	–	–	–	N/A[d]
29.1	9.5×10^{6}[ce]	5	+	–	+	+	+	+	+
9.7	9.5×10^{6}[ce]	5	+	+	+	+	+	+	+
0	9.5×10^{6}[ce]	1	–	–	–	–	–	–	N/A

[a] XLD = xylose lysine desoxycholate agar; HE = Hektoen enteric agar; BS = bismuth sulfite agar.
[b] Inoculated competitors.
[c] Natural background flora.
[d] N/A = only positive results are confirmed.
[e] Temperature-abused.

Table 7. Summary of microflora study (inoculated samples): BAX system versus culture method[a]

Microflora study	BAX system –	BAX system +	Total
Without microflora	1	52	53
With microflora	4	156	160
Total	5	208	213

[a] $\chi^2 = 0.071$, $p = 0.79$, not significant. No inhibitory effect due to competing flora. The 4 BAX system-negative samples with competition were from a single meat sample and were not independent ($p = 3.7078 \times 10^{-5}$). However, they have been treated as independent for this analysis.

(156 positive and 4 negative on inoculated samples) are equivalent to the results of part 1 ($\chi^2 = 0.071$, p = not significant). There was no significant effect of increased microflora levels on the ability of the BAX system to detect *Salmonella*.

The number of BAX system and BAM confirmed positives on uninoculated samples indicates the presence of natural *Salmonella* contamination in part 2 of this study. Because of this and because multiple BAX system assays were run on each sample, the BAX system-positive results on BAM-negative samples are consistent with intermittent natural contamination.

On the basis of these results, AOAC RI awarded Performance Tested Method status to the BAX for Screening/*Salmonella* assay, the first PCR-based product to receive this status.

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FOOD CHEMICAL CONTAMINANTS

Identification of the Source of Reagent Variability in the Xanthydrol/Urea Method

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Contamination of food and food packaging material by rodent urine is evidence of insanitary conditions. Urea from rodent urine is used as a chemical indicator of contamination. The limit of detection of the xanthydrol/urea AOAC Method 959.14 by formation of dixanthylurea crystals is 4 μg urea isolated from urine on packaging material. Six different lots of xanthydrol from 5 different manufacturers were compared. Differences in urea detection sensitivity of the xanthydrol of up to 1000-fold were observed. Melting points showed further evidence of variability and impurities in xanthydrol lots. A liquid chromatographic method was developed to separate and identify the impurities. Confirmation of analytes was performed by gas chromatography/mass spectrometry.

Storage of food and food packaging materials under insanitary conditions can be demonstrated by the presence of rodent urine, as indicated by urea. Xanthydrol is used in AOAC Method **959.14**, Urine Stains on Foods and Containers: Xanthydrol Test for Urea for identification of urea by formation of dixanthylurea crystals (1). The lower limit of detection of the method is 4 μg urea from an extract concentrated on a glass slide (1). Variations have been observed in the sensitivity of the xanthydrol used in the test. Six different lots of xanthydrol from 5 different manufacturers were compared through limit of detection study, melting point determination, liquid chromatography (LC) with photodiode array detection, and gas chromatography/mass spectrometry (GC/MS).

METHOD

Apparatus

(**a**) *Olympus model BH2 polarizing light microscope using bright field and cross-polar illumination.*

(**b**) *Microprobe or fine-tip dissecting needle.*

(**c**) *Depression slide.*

(**d**) *Pipet.*—10 μL.

(**e**) *Capillary tubes.*

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(**f**) *Thomas melting point apparatus.*

(**g**) *0.45 μm nylon filters.*

(**h**) *LC system.*—Model 600E series multisolvent pump (Waters, Milford, MA), Model 7125 injector with 100 μL injection loop (Rheodyne, Cotati, CA), Model 38000 temperature control module (Waters), Model 996 photodiode array detector (Waters), and Millennium software data station version 2.1 (Waters). Column: J.T. Baker, Bakerbond, 5 μm, octadecyl, 4.6 × 250 nm (J.T. Baker, Inc., Phillipsburg, NJ). Operating conditions: 100 μL injection, 40°C column temperature; a 10 min isocratic run with LC mobile phase at 1 mL/min; photodiode array detection at 200–400 nm.

(**i**) *GC/MS system.*—Model 7673 automated liquid injector (Hewlet-Packard, Palo Alto, CA), Model 5890A gas chromatograph with a capillary direct interface to a Model 5970B mass selective detector (MSD; Hewlett-Packard). The GC/MS system was controlled with a Pascal Chemstation data system (Hewlett-Packard). Column: 30 m × 0.25 mm; Restek, RTx-20, (80% dimethyl, 20% diphenyl polysiloxene), fused silica capillary, 0.53 mm id × 0.5 μm film (Restek, Bellefonte, PA). Operating conditions: 2 μL split-splitless injection, split vent opened 1 min after injection. Temperatures: injector 290°C; interface, 290°C. Oven program: 100°C for 2 min, 10°C/min to 280°C, and hold 5 min. Total program time, 25 min. MSD operating conditions: electron ionization, scan mode, scan 40–450 *m/z* at 1.02 scans/s.

Reagents

ACS reagent grade unless otherwise noted.

(**a**) *Acetic acid solution.*—66% solution, dilute 100% glacial acetic acid 2:1 with water (J.T. Baker, 9507-01).

(**b**) *Urea solution.*—CAS [57-13-6], 1.0, 0.1, and 0.01 mg/mL aqueous urea solution, Spectrum U-1016.

(**c**) *Water.*—LC grade (Burdick & Jackson, Muskegon, WI; 365-4).

(**d**) *Acetonitrile.*—LC grade, CAS [75-05-8] (Burdick & Jackson, 015-4).

(**e**) *LC mobile phase.*—Prepare solvent A with 950 mL acetonitrile and 50 mL water and mix. Prepare solvent B with 950 mL water and 50 mL acetonitrile and mix. Mix 900 mL solvent A with 100 mL solvent B, filter through 0.45 μm membrane, and degas.

(**f**) *Xanthene.*—CAS [92-83-1] (Aldrich, X20-1).

Table 1. Xanthydrol lot information, melting points, and urea detection limits[a]

Label	Manufacturer, lot No.	(Date opened) Label purity, %	Melting point, °C	Urea detection limit, μg
Mnfr1	Aldrich, KM00615CM	(7/86) 98	117–176	0.2
Mnfr2	Sigma, 92H2628	(2/93) 99	121–123	2
Mnfr3A	Kodak, B13A	(5/84) 95	118–194	2
Mnfr3B	Kodak, E13A	(9/85) 95	118–190	0.2
Mnfr4	Spectrum, HG358	(11/92) 98	125–199	200
Mnfr5	Fluka, 33219 984	(8/86) 97	117–176	0.2

[a] These data are for specific lots analyzed and may not be representative of other lots produced by the manufacturer.

(**g**) *Xanthone.*—CAS [90-47-1] (Sigma, X 6125).

(**h**) *Xanthydrol.*—CAS [90-46-0], *see* Table 1. The xanthydrol was stored according to manufacturer's directions.

Limit of Detection

A limit of detection for urea was determined for each xanthydrol solid by adding 20 μL of a known urea solution, 10 μL acetic acid solution, and a small amount of xanthydrol solid to a well slide. A microprobe or a fine-tip dissection needle can be used to transfer a small amount of the xanthydrol solid to a depression slide and to carefully mix the components. The slide was examined at a magnification of 100× immediately and at 5 min intervals until dixanthylurea crystal formation was observed or 20 min had passed (1).

Melting Point Determination

Capillary tubes of each xanthydrol lot were packed in triplicate. The melting point of each was determined with a Thomas melting point apparatus. An average melting point range was obtained from the 3 melting point runs.

LC Analysis

A stock solution of 0.1 mg/mL in acetonitrile was prepared for each xanthydrol lot and for the xanthone and xanthene standards. The solutions were stored for no more than 1 week in the dark at 4°C until use. A portion was diluted 1:3 with acetonitrile and used immediately for further testing. Before injection, the diluted portions were filtered through a 0.45 μm nylon filter. The filtered solutions were stored in aluminum foil-covered vials to protect them from light prior to LC analysis. Each test injection was scanned from 200 to 400 nm with photodiode array detection. The peak area at a wavelength of 238 nm was used for measurement.

GC/MS Analysis

A solution of 1 mg/mL in acetonitrile was prepared for each xanthydrol lot and for the xanthone and xanthene standards. The solutions were immediately diluted 1:10 in acetonitrile and analyzed directly with an automated liquid injector by capillary

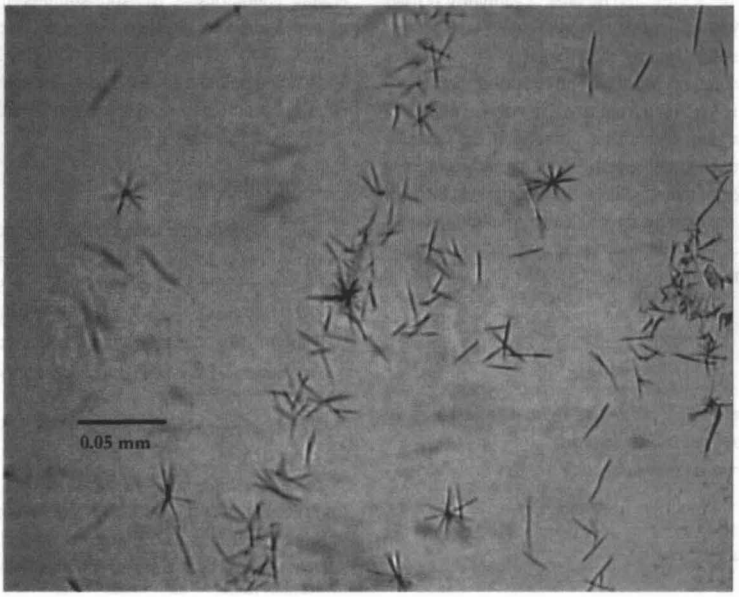

Figure 1a. The rosette crystal form of dixanthylurea at 100× magnification.

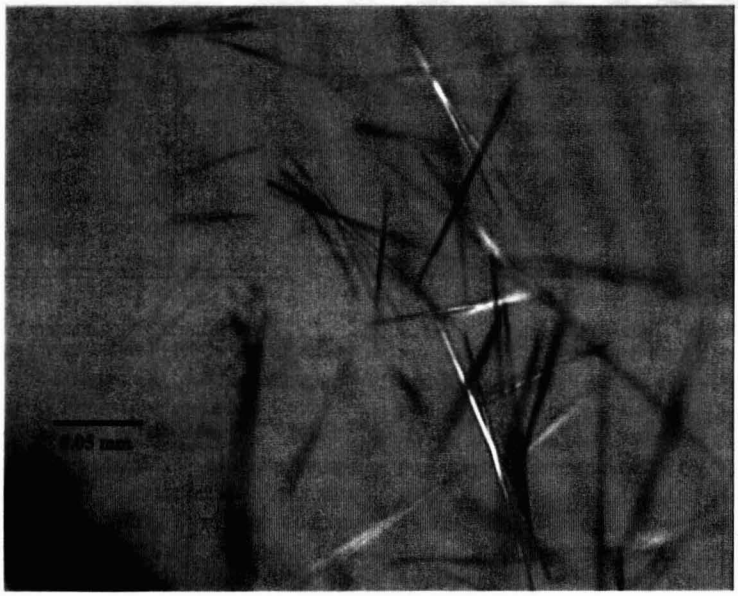

Figure 1b. The needle blade crystal form of dixanthylurea at 100× magnification.

GC with MSD. Total ion chromatograms and mass spectra for each injection were obtained.

Results and Discussion

The xanthydrol solids were stated to be 95–99+% pure and ranged from fine to coarse crystals, white to slightly yellowish in coloration depending on the lot and manufacturer. The xanthydrol detection limits varied from 0.2 to 200 μg urea (Table 1). Five of the 6 xanthydrol lots met AOAC Method **959.14** specification of 4 μg urea detection sensitivity (1).

Use of the depression slide and the microprobe and a study of the crystalline nature of the reagents were helpful in AOAC Method **959.14**. The depression slide or well slide is superior to the flat slide in use with the method (2). The test drop is prevented from drying out as quickly; thus time for crystal formation is increased. In addition, the well slide provides a better medium for observing the dixanthylurea crystals microscopically. Crystals grown under a microscope are likely to be smaller, but more perfect, than those prepared in greater quantities (3).

A great excess of xanthydrol reagent should be avoided. By use of the microprobe a small crystal of the xanthydrol can be introduced into the droplet. This small crystal will minimize the common occurrence of saturating the solution with the reagent in a zone about the crystal and consequently crystallizing the reagent itself (4). Only dixanthylurea crystals seen in the droplet should be considered positive because dried unreacted xanthydrol assumes needle-like crystalline forms similar to the blade form of dixanthylurea (2). The inexperienced analyst may mistake these dried xanthydrol crystals for the compound formed by the reagent with the urea present in the test drop. This source of error can be avoided by studying the crystal habit of all reagents involved in the reaction (4). A negative control slide can be run simultaneously with a test slide to further help the analyst differentiate a positive from a negative reaction.

Two forms of crystals in the reaction of xanthydrol with urea were observed in this study (Figures 1a and 1b). Intermediate forms of the reaction of xanthydrol with urea have been determined by nuclear magnetic resonance (NMR; 5). In an NMR study, Coxon and Fatiadi (5) revealed the presence of a monoxanthen-9-yl derivative, which upon treatment with xanthydrol and acetic acid yielded the dixanthylurea. Thus, monoxanthen-9-yl is a precursor of the dixanthylurea, and the reaction is indeed a 2-step process (5). This finding suggests that the 2 crystalline forms of the reaction of xanthydrol with urea are monoxanthyl-urea and dixanthylurea.

For low urea concentrations (2–200 μg), small rosette crystals with low birefringence were observed. Little spidery rosettes are characteristic of dixanthylurea crystals. Visualization of the almost translucent rosettes with their feathery blades can be enhanced by stopping down the the microscope field diaphragm to increase the contrast in the field of view. The field diaphragm controls the size of the illuminated field of view (6). Higher concentrations of urea (50–1000 μg) form needles, often in sheaves or in clusters, of much greater birefringence, in addition to the rosettes (7). The needle form of the crystal is most likely the monoxanthylurea as found by Coxon (5). Needles form at or near the edge of the droplet, while rosettes tend to float just below the surface (2). The rosette crystal form of dixanthylurea was noted in all xanthydrol lots at the limit of urea detection.

A narrow melting point range of 2°–3°C has been used traditionally as an indicator of compound purity (8). Only one

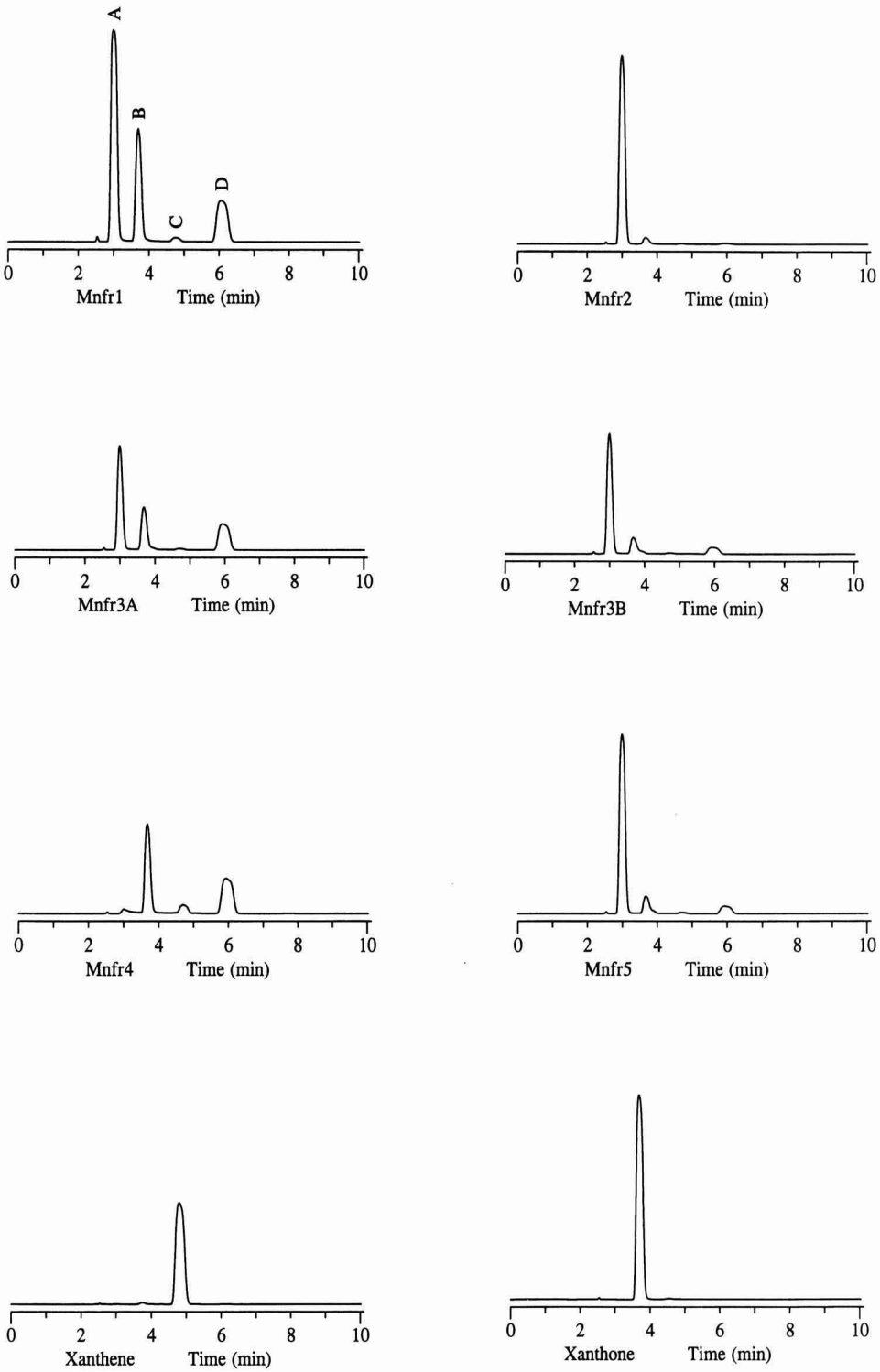

Figure 2. LC traces of xanthydrol lots from Mnfr1, Mnfr2, Mnfr3a, Mnfr3b, Mnfr4, and Mnfr5, and of xanthene and xanthone at 238 nm.

Table 2. GC/MS data

Compound	GC retention time, min	*m/z*	LC identification
Xanthene	15.4	181	C
9-Methoxyxanthene	16.3	212, 181	None
9-Ethoxyxanthene	16.7	226, 181	None
Xanthydrol	17.5	197, 181	A
Xanthone	18.3	196, 168	B

manufacturer, Mnfr2, reported a narrow melting point of 3°C (121°–123°C), which corresponds to the range reported for xanthydrol (Table 1; 9). The remaining 5 lots had melting point ranges greater than 60°C, which is well over the 3°C expected range for pure compounds.

An LC method was developed to separate the impurities in the xanthydrol compounds. Test portions of 100 µL were analyzed. UV visible spectra at 200–400 nm were taken of the xanthydrol and the xanthene and xanthone standards. A wavelength of 238 nm was chosen for LC analysis because it is an absorbance maxima for the primary compound of interest, xanthydrol; is within the solvent transmittance range; and is near a peak maxima of the xanthene and xanthone standards. All lots tested showed a mixture of compounds, indicating that the materials were not pure. This explains the observed wide range in melting point temperatures. LC revealed peaks with the following retention times: peak A, 3.00 min; peak B, 3.70 min; peak C, 4.73 min; and peak D, 5.97 min (Figure 2). Although xanthydrol from Mnfr2 had the fewest observable peaks on the LC trace, it did not have the sensitivity of several other lots tested (2 versus 0.2 µg urea; Table 1). For the most sensitive xanthydrol lots, peak A was the most prominent LC peak, with peaks B, C, and D present as small extraneous peaks. The least sensitive xanthydrol lot from Mnfr4 had a prominent peak B. The retention time and UV/Vis spectrum of peak B were the same as those of the xanthone standard. The retention time and UV/Vis spectrum of peak C were the same as those of the xanthene standard. Comparison of the UV/Vis data with published spectra shows that peaks A, B, and C correspond to xanthydrol, xanthone, and xanthene, respectively (9). Xanthone is a major reactant in the production of xanthydrol (10). Three of the 4 compounds in the xanthydrol solutions were identified by GC/MS.

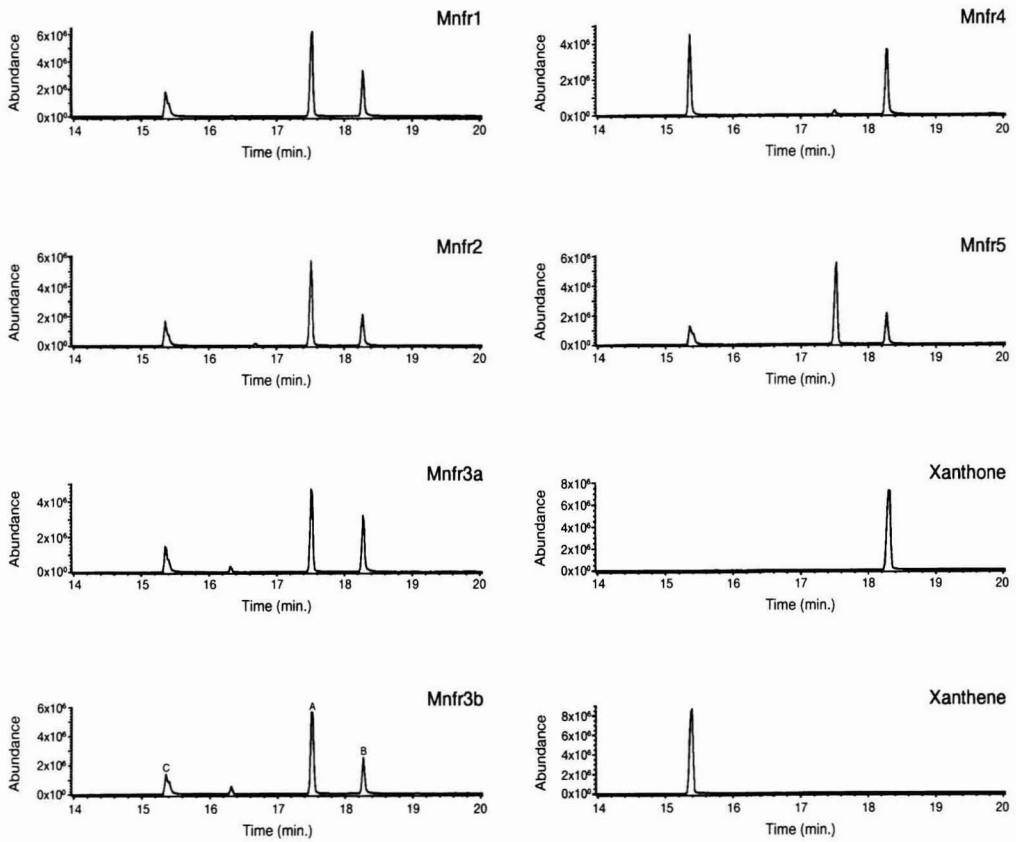

Figure 3. GC/MS total ion chromatograms of xanthydrol lots from Mnfr1, Mnfr2, Mnfr3a, Mnfr3b, Mnfr4, and Mnfr5, and of xanthene and xanthone.

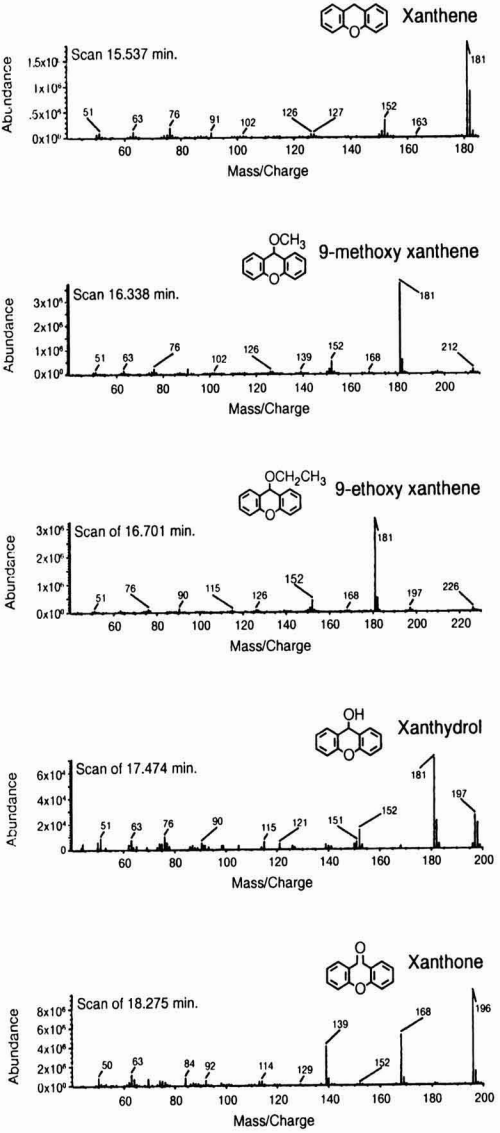

Figure 4. Mass spectra of xanthene, 9-methoxyxanthene, 9-ethoxyxanthene, xanthydrol, and xanthone.

GC/MS analysis of xanthydrol lots identified the compounds xanthene, xanthydrol, and xanthone. The mass fragmentation pattern of 9-methoxyxanthene was observed in lots from Mnfr1, Mnfr3a, and Mnfr3b. The mass fragmentation pattern of 9-ethoxyxanthene was observed in the lot from Mnfr2. Only trace amounts of these 2 compounds were noted in the analysis (Table 2 and Figures 3 and 4). Reference standards were not available for these 2 compounds. The 9-ethoxyxanthene peak was found only in xanthydrol from Mnfr2 and was not observed in LC analysis. 9-Ethoxyxanthene and 9-methoxyxanthene also have been identified by MS as cleavage products of the substituted xanthydrol *N*-xanthyl-L-valine (11). Xanthydrol lots from Mnfr1, Mnfr2, Mnfr3a, and Mnfr5 showed a doublet xanthene peak in the total ion chromatograms (TICs), which could be xanthene and the isomer 6H-dibenzo[b,d]pyran. This isomer has been reported to form in aqueous acetonitrile systems of xanthene (12). Xanthydrol from Mnfr4 gave a pure xanthene peak with no doublet. Xanthone was present in all 6 xanthydrol lots. Xanthene, xanthone, and a trace amount of xanthydrol were observed in the TIC of the lot from Mnfr4. In the remaining 5 lots tested, xanthydrol was the predominant compound found in the TICs (Figure 3).

Conclusions

For AOAC Method **959.14**, the well slide is superior to the flat slide. The microprobe allows the analyst to easily add a small crystal of xanthydrol reagent to the test solution. The 2 forms of crystals observed in the reaction of xanthydrol with urea were the rosette and the needle blade. Although some crystals were observed after the slide had dried, only those seen on the wet slide were considered positive because the dried xanthydrol crystals resemble the needle blade form.

Initial testing of the xanthydrol reagent's sensitivity to urea provided evidence that differences exist among lots and manufacturer of the reagent. A limit of urea detection, a melting point determination, LC, and GC/MS analysis confirmed these differences. LC analysis revealed 4 peaks. GC/MS analysis identified xanthydrol as peak A, xanthone as peak B, and xanthene as peak C. We were unable to identify peak D. Xanthone and xanthene were found in all lots tested. In lot Mnfr4, only a trace amount of xanthydrol was found by GC/MS. Xanthydrol was the predominant peak in all other lots tested. The xanthydrol from lot Mnfr4 was the only one tested that did not meet the minimum urea detection method specification of 4 μg.

Recommendation

It is recommended that the xanthydrol reagent initially be tested with 4 μg urea. The well slide and the microprobe dissecting needle are helpful for use with the method. At 4 μg, urea should be detected easily by AOAC Method **959.14**. If the xanthydrol does not have a reliable positive response with 4 μg urea, the LC method can be used to confirm the existence of reagent contaminants. The xanthydrol reagent must meet the minimum specification of 4 μg urea detection sensitivity for use in AOAC Method **959.14**. Proper storage of xanthydrol (protect from light, store with dessicant at <4°C) and initial testing of the reagent with 4 μg urea should ensure reliable results.

Acknowledgment

We thank Timothy McNeal of the U.S. Food and Drug Administration, Division of Product Manufacture and Use, for conducting the GC/MS analysis.

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FOOD CHEMICAL CONTAMINANTS

Variability Associated with Testing Shelled Corn for Fumonisin

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Variances associated with sampling, sample preparation, and analytical steps of a test procedure that measures fumonisin in shelled corn were estimated. The variance associated with each step of the test procedure increases with fumonisin concentration. Functional relationships between variance and fumonisin concentration were estimated by regression analysis. For each variance component, functional relationships were independent of fumonisin type (total, B1, B2, and B3 fumonisins). At 2 ppm, coefficients of variation associated with sampling (1.1 kg sample), sample preparation (Romer mill and 25 g subsample), and analysis are 16.6, 9.1, and 9.7%, respectively. The coefficient of variation associated with the total fumonisin test procedure was 45% and is about the same order of magnitude as that for measuring aflatoxin in shelled corn with a similar test procedure.

Fumonisins are mycotoxins produced by several fungi of the genus *Fusarium* (1). Fumonisin is found in various grains including shelled corn and is carcinogenic in laboratory animals such as rats (2). At present, there is no U.S. Food and Drug Administration action level for fumonisin in food or feed products produced in the United States (3).

The test procedure used to estimate the concentration of fumonisin in a bulk lot is similar to test procedures used to measure other mycotoxins, such as aflatoxin, in agricultural products. The test procedure consists of 3 steps. First, a random sample (test sample) is taken from the lot (sampling step). Second, the entire test sample is comminuted in a mill or grinder, and a random subsample is removed from the comminuted test sample. Grinding and subsampling are collectively called the sample preparation step. Third, the fumonisin in the subsample is extracted with solvent and quantitated (analytical step).

The variability associated with each of the 3 steps contributes to the total variability associated with the test procedure. The variability associated with the test procedure makes it difficult to estimate the true fumonisin concentration of a bulk lot with a high degree of confidence and consequently makes it difficult to accurately classify lots into categories such as that required by regulatory activity. If the variability of the overall fumonisin test procedure can be reduced, the lot concentration can be estimated with more confidence and lots can be classified more accurately.

Previous studies that measured the variability associated with test procedures used to measure aflatoxin in corn, peanuts, and cottonseed lead one to expect that the variability of each step of the test procedure would be different and that the cost associated with reducing the variability of each step would be different (4–6). It is important to design a fumonisin test procedure that will have the lowest variability that resources will allow. Therefore, the objectives of this study were to measure the variabilities of sampling, sample preparation, and analytical steps of the test procedure used to measure fumonisin in shelled corn and to show how to change the design of the test procedure to decrease variability and achieve more precise results.

Experimental

Sample Preparation

Twenty-four bulk lots of shelled corn harvested from 24 different fields in North Carolina were identified as having possible fumonisin contamination. A bulk sample of ca 45 kg (100 lbs) was taken from each of the 24 lots. Each bulk sample of shelled corn was riffle divided into thirty-two 1.1 kg test samples. Each test sample was comminuted in a Romer mill.

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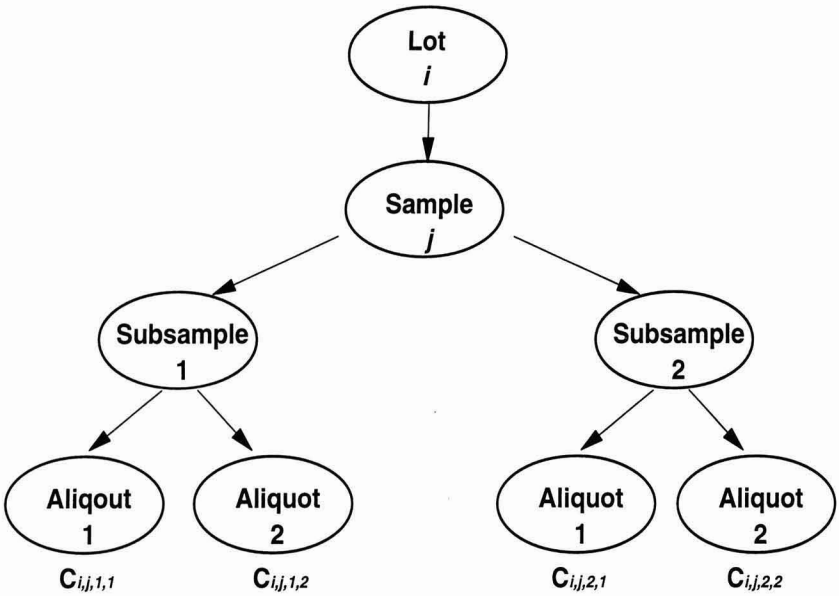

Figure 1. Schematic diagram of nested experimental design showing how the fumonisin test result $C_{i,j,k,l}$ was obtained. The identification for lot is *i*, where *i* = 1 to 10; for sample is *j*, where *j* = 1 to 10; for subsample is *k*, where *k* = 1 to 2; and for analysis is *l*, where *l* = 1 to 2.

The comminuted test samples were placed in plastic bags, each plastic bag was identified by lot number and sample number, and the bags were stored at 5°C until needed for several planned fumonisin studies.

Experimental Design

A nested design was used to determine the variability associated with each step of the fumonisin test procedure (Figure 1). Ten lots, with an expected wide range in fumonisin concentration, were chosen from the 24 lots by using fumonisin estimates for each of the 24 lots obtained from a previous study. For each of the 10 lots, 10 comminuted test samples were selected from the 32 test samples by arbitrarily taking every third sample. By using a riffle divider, two 25 g subsamples were taken from each of the 10 comminuted test samples. The fumonisin content of the 25 g subsamples was measured by using AOAC Official Method **995.15** (7). As specified by the analytical method, each 25 g subsample was blended with 50 mL methanol–water (3 + 1, v/v) for 3 min. Two portions were removed from the blended extract. The concentrations of B1, B2, and B3 fumonisins, in parts per million (ppm), were quantitated. Total fumonisin (sum of B1, B2, and B3) was also calculated for each portion. The nested design resulted in 400 analyses (10 lots × [10 samples/lot] × [2 subsamples/sample] × [2 aliquots/subsample]).

Variability Estimates

The error structure or sources of variability associated with the fumonisin test procedure are also illustrated in Figure 1. The total variance among fumonisin test results is composed of at least 3 variance components: sampling, sample preparation, and analysis (5). The variances in this study were estimated with a model in which an observed fumonisin test result, C, may be represented as follows:

$$C = \mu + S + SP + A \quad (1)$$

where μ is the true fumonisin concentration in the lot being tested, S is the random deviation of sample concentrations about the lot concentration with expected value 0 and variance δ^2_s, SP is the random deviation of subsample concentrations about the sample concentration with expected value 0 and variance δ^2_{sp}, and A is the random deviation of analytical assay results about the subsample concentration with expected value 0 and variance δ^2_a. By assuming independence among the random deviations in equation 1, the following variance relationship is obtained:

$$\delta^2_t = \delta^2_s + \delta^2_{sp} + \delta^2_a \quad (2)$$

where δ^2_t is the total variance associated with the fumonisin test procedure. With a Statistical Analysis System (SAS) nested analysis of variance procedure (8), each of the variance components in equation 2 was determined for each lot. Estimates of the true variance components and the true fumonisin concentration by experimental values are denoted by s^2 and C, respectively. Variances were determined for total, B1, B2, and B3 fumonisins.

Table 1. Fumonisin concentration and sampling, sample preparation, and analytical variances associated with measuring fumonisins in shelled corn

Fumonisin type	Lot ID	Fumonisin concentration (μg/g)	Total	Variance[a]		
				Sample	Sample preparation	Analytical
B1	5	0.6	0.0319	0.0150	0.0080	0.0089
B1	18	3.0	0.6806	0.5874	0.0572	0.0360
B1	16	4.3	0.9774	0.7102	0.2229	0.0443
B1	15	5.5	1.0022	0.7571	0.0572	0.1879
B1	23	6.5	2.4380	1.6142	0.6259	0.1979
B1	19	8.6	1.8009	1.1260	0.2752	0.3997
B1	2	11.6	3.0845	1.9999	0.6331	0.4515
B1	22	14.5	4.3885	2.4088	0.5325	1.4472
B1	14	14.7	2.5686	—[b]	2.1026	0.4661
B1	3	16.0	3.7095	2.1837	0.4854	1.0404
B2	5	0.2	0.0134	0.0014	0.0022	0.0098
B2	18	1.1	0.1003	0.0830	0.0098	0.0076
B2	16	1.3	0.0843	0.0358	0.0379	0.0106
B2	15	1.9	0.1382	0.0807	0.0210	0.0365
B2	23	2.0	0.3734	0.3017	0.0412	0.0305
B2	19	4.0	0.5881	0.4274	—	0.1607
B2	14	5.1	0.4742	0.2216	0.1661	0.0866
B2	2	5.4	1.2444	0.9021	0.2187	0.1236
B2	22	6.7	1.5876	1.0461	0.1651	0.3764
B2	3	7.6	1.2589	1.0141	0.0676	0.1772
B3	5	0.0	—	0.0001	—	—
B3	18	0.3	0.0062	0.0045	0.0002	0.0015
B3	16	0.5	0.0258	0.0052	0.0039	0.0167
B3	15	0.6	0.0126	0.0078	0.0015	0.0034
B3	23	0.7	0.0481	0.0330	0.0110	0.0042
B3	19	1.0	0.1137	0.0762	0.0240	0.0135
B3	2	1.4	0.0615	0.0367	0.0095	0.0153
B3	14	2.0	0.1290	0.0178	0.0804	0.0307
B3	22	2.1	0.1329	0.0618	0.0224	0.0487
B3	3	2.2	0.0892	0.0644	0.0111	0.0138
Total[c]	5	0.8	0.0892	0.0280	0.0265	0.0347
Total	18	4.4	1.3421	1.1658	0.1009	0.0754
Total	16	6.2	1.8246	1.1685	0.5330	0.1231
Total	15	8.0	0.1057	1.5223	0.1695	0.4139
Total	23	9.3	5.3131	3.8800	1.0168	0.4163
Total	19	13.7	4.3813	2.7495	0.3825	1.2494
Total	2	18.5	9.1663	6.4071	1.5971	1.1621
Total	14	21.8	5.6314	—	4.4938	1.1376
Total	22	23.3	12.9603	7.4069	1.5820	3.9714
Total	3	25.7	10.1210	6.7057	1.0758	2.3395

[a] Sample variance reflects 1.1 kg sample. Sample preparation variance reflects Romer mill and 25 g subsample. Analytical variance reflects AOAC Method **995.15** and LC.
[b] —, missing value.
[c] Total fumonisin = B1 + B2 + B3.

Results

Table 1 shows the total, sampling, sample preparation, and analytical variance estimates for each type of fumonisin (B1, B2, B3, and total) for each of the 10 lots. Because of experimental error, some variance estimates were calculated to be negative (not physically possible values) and were treated as missing values in Table 1 (negative values ignored and not used in any subsequent analysis). For each type of fumonisin, variance results are ordered by the fumonisin concentration, which varied from less than 1 to about 26 ppm. In general, each variance component increases with fumonisin concentration regardless of fumonisin type as has also been observed in aflatoxin studies for corn, peanuts, and cottonseed (4–6).

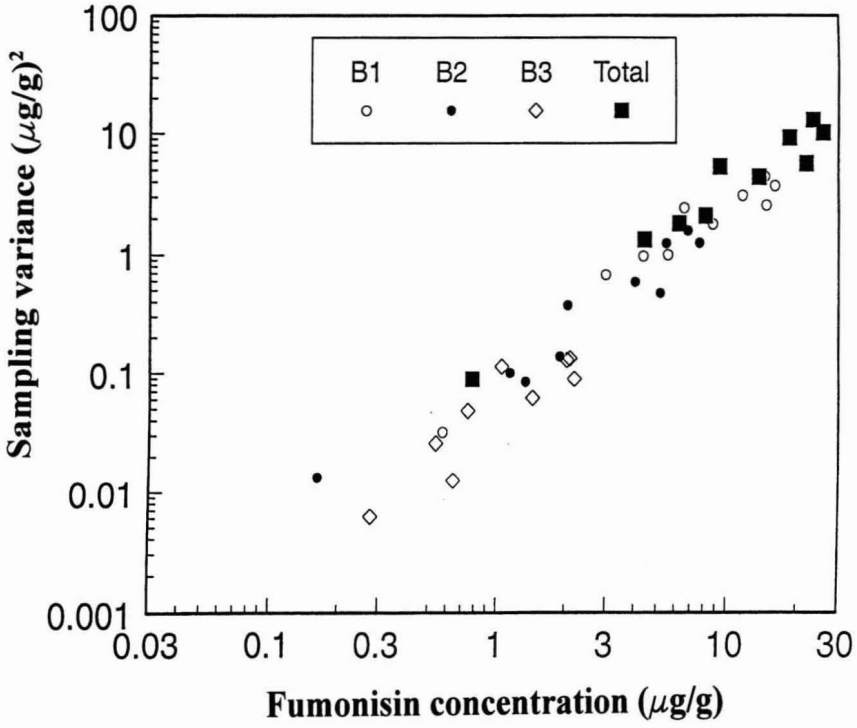

Figure 2. Sampling variance versus fumonisin concentration for 1.1 kg sample of shelled corn.

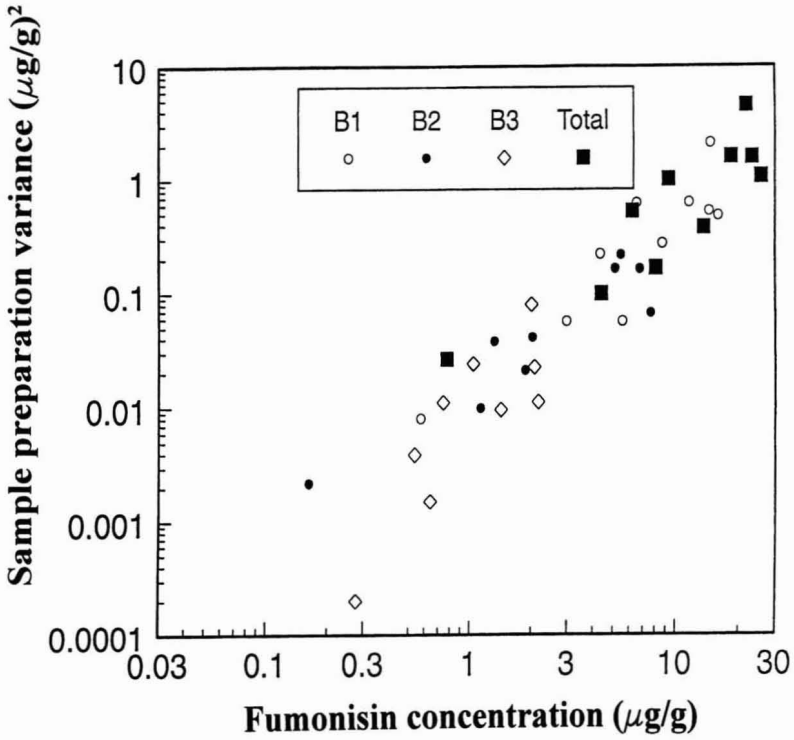

Figure 3. Sample preparation variance versus fumonisin concentration in 25 g comminuted subsample of shelled corn.

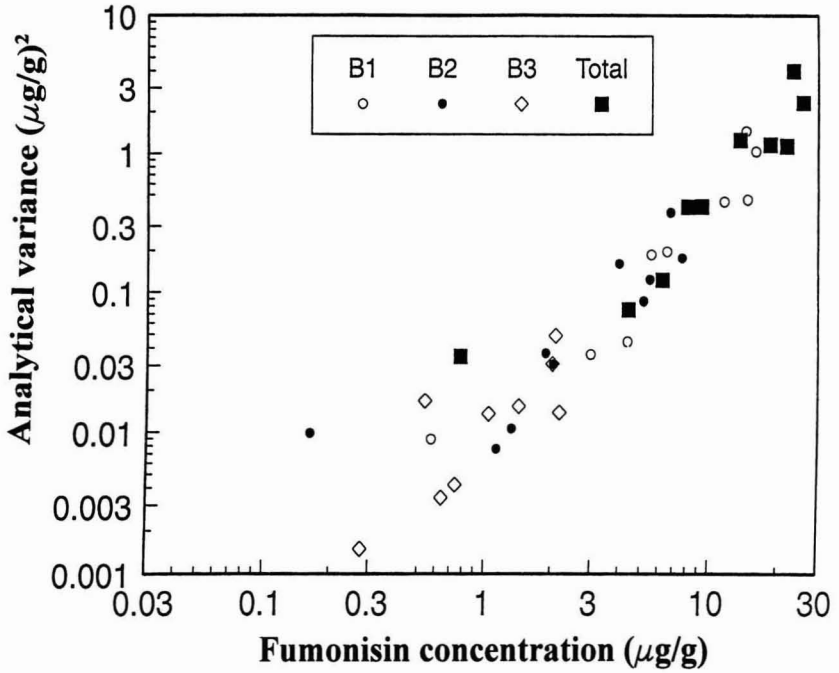

Figure 4. Analytical variance versus fumonisin concentration for shelled corn in 1 portion of extract analyzed by AOAC Official Method 995.15.

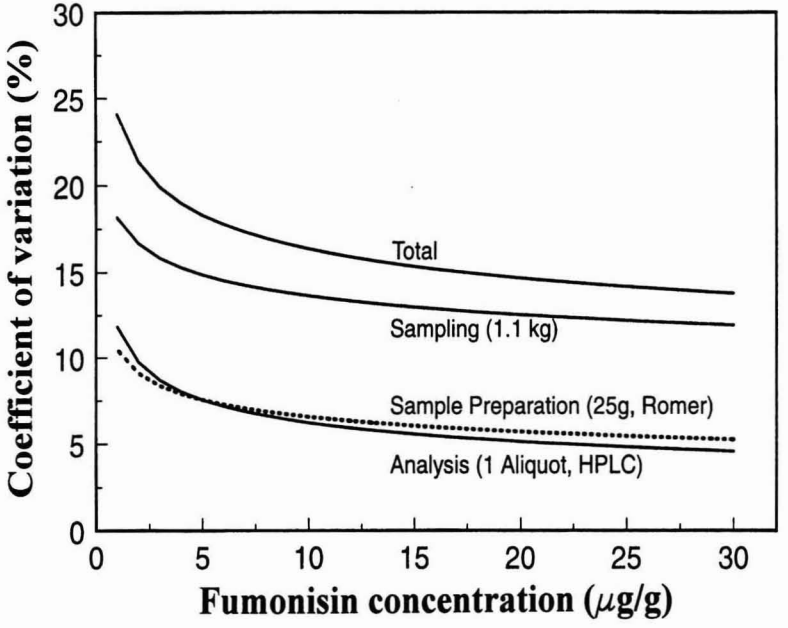

Figure 5. Coefficient of variation associated with each step of the fumonisin test procedure for shelled corn. Coefficients of variation are specific for 1.1 kg sample, Romer mill, 25 g subsample, LC, and 1 portion.

Figures 2–4 show that sampling, sample preparation, and analytical variances increase linearly with fumonisin concentration (either B1, B2, B3, and total) when plotted in full log plots. Two important observations can be made from the plots in Figures 2–4. First, the figures suggest that the relationship between variance, s^2, and fumonisin concentration, C, can be described by a power function:

$$s^2 = a \times C^b \quad (3)$$

where a and b are constants determined from regression analysis.

Second, for each variance component (Figures 2–4), the variance values, identified by type of fumonisin, appear to have about the same slope. If this is true, then the variances for each type of fumonisin can be pooled, and equation 3 for each variance component would be independent of fumonisin type. The general linear model (GLM) procedure in SAS was used to check the homogeneity of the slope values associated with fumonisin type for each variance component. The test indicated that the slopes were not significantly different at the 95% confidence level.

From regression analysis, the values of a and b in equation 3 that describe the sampling, sample preparation, and analytical variances as function of fumonisin concentration (independent of the type of fumonisin) were determined:

$$s^2_s = 0.033 \times C^{1.75} \quad (4)$$

$$s^2_{sp} = 0.011 \times C^{1.59} \quad (5)$$

$$s^2_a = 0.014 \times C^{1.44} \quad (6)$$

From the regression analysis, the coefficients of determination (R^2) for equations 4–6 are 0.946, 0.87, and 0.882, respectively. R^2, is the square of the correlation coefficient and $100R^2$ indicates the percentage of the sum of squares or variability that is accounted for by the regression equation.

The total variance associated with the fumonisin test procedure can be estimated by adding the sampling (equation 4), sample preparation (equation 5), and analytical (equation 6) variances, as shown in equation 2.

$$S^2_t = (0.033 \times C^{1.75}) + (0.011 \times C^{1.59}) + (0.014 \times C^{1.44}) \quad (7)$$

The total variance in equation 7 is specific for the test procedure used in this study (1.1 kg sample, Romer mill, 25 g subsample, 1 aliquot, and liquid chromatography [LC]).

As an example, the sampling, sample preparation, analytical, and total variances expected for the test procedure when fumonisin concentration is 2 ppm are 0.111, 0.033, 0.038, and 0.182, respectively. Here, sampling variance accounts for 61% of the total testing variability (s^2_s/s^2_t), sample preparation variance accounts for 18.2% of the total testing variability (s^2_{sp}/s^2_t), and analytical variance accounts for 20.8% of the total testing variability (s^2_a/s^2_t). As with other mycotoxins, sampling is the largest source of variation, especially for small sample sizes. For a normal distribution, a total variance of 0.182 indicates that repeated fumonisin test results will vary about the true lot concentration of 2 ppm by ± 0.85 ppm (2 standard deviations or 95% confidence limits).

The total testing variation can be reduced by reducing the variance associated with one or more of the steps of the testing procedure. Sampling variance can be reduced by increasing sample size, ns, or increasing the number of sampling units. Sample preparation variance can be reduced by increasing subsample size (assume use of the same mill), nss, or increasing the number of subsampling units. Analytical variance can be reduced by quantitating fumonisin in more than one portion from the blended extract, na (assume use of the same analytical method). The effects of ns, nss, and na on sampling, sample preparation, and analytical variances are shown in equations 8–10, respectively.

$$s^2_s = (1.1/ns) \times 0.033 \times C^{1.75} \quad (8)$$

$$s^2_{sp} = (25/nss) \times 0.011 \times C^{1.59} \quad (9)$$

$$s^2_a = (1/na) \times 0.014 \times C^{1.44} \quad (10)$$

where ns is in kg, nss is in g, and na is number of portions.

The total variance associated with a fumonisin test procedure for any sample size, any subsample size (Romer mill), and any number of aliquots (LC) can be determined by adding equations 8–10.

$$s^2_t = (0.0363/ns) \times C^{1.75} + (0.275/nss) \times C^{1.59} + (1/na) \times 0.014 \times C^{1.44} \quad (11)$$

Because a different cost is associated with reducing the variability of each step of the fumonisin test procedure, it is important to consider cost as well as the expected reduction in variability when designing a fumonisin test procedure.

It would be of interest to compare the variability of the test to measure fumonisin in shelled corn to the variability of the test to measure aflatoxin in shelled corn. It is difficult to compare the variabilities by using variance because aflatoxin concentration is usually reported in parts per billion (ng aflatoxin/g corn) and fumonisin is usually reported in ppm (μg fumonisin/g corn). However, the coefficient of variation (CV) can be used to compare the variabilities of the 2 mycotoxin test procedures, because CV is a relative measure of variation and is a dimensionless variable. The CV associated with each step of the fumonisin test procedure was computed from variance equations 4–6 over a range of fumonisin concentrations and is plotted as a continuous or smooth curve in Figure 5. For example, at a fumonisin concentration of 2 ppm, the CVs associated with sampling, sample preparation, and analysis are about 16.6, 9.1, and 9.7%, respectively. The CV for the fumonisin test procedure is about the same as that for testing shelled corn for aflatoxin (4, 9).

Summary and Discussion

The variability associated with the test procedure to measure fumonisin in shelled corn is similar to that associated with the test procedure to measure aflatoxin in shelled corn. For small sample sizes, sampling variance is the largest source of the total

testing variation. For example, when testing a bulk corn lot with a fumonisin concentration of 2 ppm, the CVs associated with sampling, sample preparation, and analysis are 16.6, 9.1, and 9.7%, respectively. The variances associated with each step of the test procedure increase with fumonisin concentration. Regression equations were developed to predict the variance as a function of fumonisin concentration for each step of the fumonisin test procedure. They were independent of fumonisin type (B1, B2, B3, or total).

From variance estimates, the effect of the test procedure on confidence limits with which fumonisin concentration is being estimated can be determined. Further studies are needed to determine the type of distribution (symmetrical, skewed, etc.) that will best describe the distribution of sample test results from a given lot so the performance of fumonisin sampling plans can be predicted more accurately.

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FOOD CHEMICAL CONTAMINANTS

Immunoaffinity Column as Cleanup Tool for a Direct Competitive Enzyme-Linked Immunosorbent Assay of Cyclopiazonic Acid in Corn, Peanuts, and Mixed Feed

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An immunoaffinity column (IAC) for cyclopiazonic acid (CPA) was prepared by coupling a CPA-specific monoclonal antibody to CNBr-activated sepharose 4B. A direct competitive enzyme-linked immunosorbent assay (dc-ELISA) was used to study the chromatographic behavior of a 0.2 mL gel column with a binding capacity of 4 μg CPA/column as well as to evaluate its efficacy as a cleanup tool for analysis of naturally occurring CPA. Sample extract either in buffer solution or in a solution containing up to 35% methanol could be loaded onto the column. After the column is washed with 5 mL deionized water and 5 mL 50% methanol, CPA could be quantitatively eluted with 2 mL 100% methanol. The column could be regenerated at least 10 times by washing with 10 mL equilibrating buffer and then storing in a cold room overnight before reuse. Recoveries of CPA added to corn, peanut, and mixed feed extracts in the range 10–200 ng/g were 88–105, 86–100, and 90–110%, respectively. Detection limits were 2.0, 4.4, and 4.7 ng/g for corn, mixed feed, and peanuts, respectively. Twenty-two peanut samples naturally contaminated with CPA were subjected to both IAC and solvent partition cleanup followed by dc-ELISA. Although a good correlation between data obtained from IAC-dc-ELISA and from SP-dc-ELISA ($r = 0.75$, $p < 0.0001$) was obtained, the slope of the linear regression was low (0.67), indicating loss during solvent partition cleanup. The overall data showed that the combination of IAC and dc-ELISA is an effective method for CPA analysis.

Cyclopiazonic acid (CPA) is a toxic secondary fungal metabolite produced primarily by aspergilli and penicillia. It was originally isolated from *Penicillium cyclopium* and *Aspergillus flavus* (1–3). The toxin causes degeneration and necrosis of the liver, lesions of the myocardium, and neurotoxic effects in many animal species through alteration of calcium homeostasis and cellular transduction (3–9). It has been found in a number of foods and feed (10–15). It can be transmitted into milk and eggs after animals ingest the toxin through contaminated feed (16). To minimize the potential risk to human and animal health, several methods, including spectrophotometry and thin-layer chromatography (11, 12, 14, 16), high-performance liquid chromatography (LC; 13, 15, 17–19), and enzyme immunoassays (20–24), have been established for analysis of CPA in a variety of samples.

Because CPA does not fluoresce and its UV absorption maximum (284 nm) is not specific, most chemical methods involve derivatization and extensive sample cleanup. Such methods are time consuming and not very sensitive. Some methods, such as those based on LC, also need expensive instrumentation. With immunoassays, on the other hand, sample matrix greatly interferes with assay sensitivity (24), as we found recently during analyses of CPA in agricultural commodities. Detection limits for CPA in corn, mixed feed, and peanuts in a direct competitive enzyme-linked immunosorbent assay (dc-ELISA) were estimated to be about 100, 300, and 600 ng/g (ppb), respectively (24).

To alleviate this problem, the present study was conducted. A monoclonal antibody (mAb) with high affinity to CPA was conjugated to Sepharose gel, which was then used to clean up the sample extract before dc-ELISA. Similar to results with other mycotoxins (25), our findings show that the CPA immunoaffinity column (IAC) effectively removes interfering materials from the matrix. Details of column preparation, chromatographic behavior, and conditions for regenerating the column, and protocols for application of this IAC in a dc-ELISA for corn, feed, and peanuts are presented in this paper.

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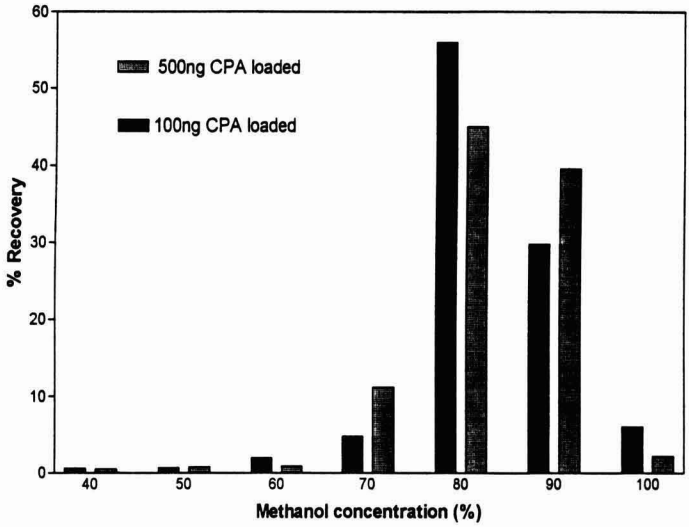

Figure 1. Sequential elution of CPA from IAC with different concentrations of methanol. CPA loaded was 100 ng (dark bars) or 500 ng (stripped bars). Columns were eluted sequentially with different concentrations of methanol. The amount of CPA in each fraction was determined by ELISA.

Experimental

Materials

CPA, Tween 20, sodium azide, Trizma hydrochloride, and pristane were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA, for blocking; cat. No. 3160-01) was obtained from Intergen Co. (Purchase, NY). Bakerbond ABx was from J.T. Baker (Phillipsburg, NJ). Virus-free Balb/c mice were purchased from Harlan Sprague-Dawley (Madison, WI). Microwell ELISA plates were obtained from Nunc Co. (No. 4-69914; Roskilde, Denmark). Horseradish peroxidase (HRP) was obtained from Boeringer Mannheim GmbH Biochemicals (Indianapolis, IN). CPA–BSA–HRP conjugate was prepared as previously described (24). All other chemicals and solvents were reagent grade or better.

Production and Purification of Anti-CPA Monoclonal Antibody

Specific mAb against CPA was generated in the ascites of Balb/c mice after injection of hybridoma cell line 5C8D1. Antibodies were purified first by precipitation with ammonium sulfate (final saturation, 33%) and then by ABx chromatography, as we described previously (24).

Preparation of CPA-Specific Affinity Column

(**a**) *Preparation of CPA-specific immunogel.*—CPA-specific immunogel was prepared by coupling CPA-specific antibody to CNBr-activated Sepharose 4B according to manufacturer's instructions (Pharmacia Fine Chemicals, Uppsala, Sweden). In a typical experiment, 5.2 g freeze-dried CNBr-activated Sepharose 4B was suspended in 20 mL 1 mM HCl and washed first with 1000 mL 1 mM HCl on a sintered glass funnel and then with 20 mL coupling buffer (CB, 0.1M $NaHCO_3$–0.5M NaCl, pH 8.3) twice. The swollen gel was immediately added to 25 mL CPA-specific antibody solution (220 mg lyophilized anti-CPA mAb powder containing 163 mg immunoglobulin G1 [IgG1]) that had been dialyzed overnight against 2 L CB. After mixing end-over-end on an orbital shaker (30 rpm) overnight in a cold room, excess IgG1 was removed by washing with several portions of CB. Absorbance of filtrates at 280 nm was measured. Unreacted sites on the gel were blocked by reacting with 25 mL 0.2M glycine (pH 8.0) at room temperature for 2 h. The product was washed alternatively with 5 cycles of buffers having high and low pH (CB and 0.1M sodium acetate buffer containing 0.5M NaCl, pH 4.0), followed by washing with phosphate buffered saline (PBS). The immunogel was stored in PBS plus 0.02% sodium azide. The final volume of the gel after settling was ca 17.5 mL.

(**b**) *Packing of CPA affinity column.*—The CPA-specific affinity column was prepared by filling a clean filter tube (Alltech Associates, Inc., Deerfield, IL) with 0.2 mL CPA immunogel in 0.57 mL slurry. It was held in place between 2 porous polyethylene frits (Alltech). The column was filled with PBS containing 0.02% sodium azide and stored at 4°C until use.

Determination of Column Capacity and Reusability

(**a**) *Determination of binding capacity of column.*—Column capacity for CPA was determined by passing 10 µg CPA in 10 mL PBS through an affinity column at 1 mL/min. To ensure maximum binding, the effluent was loaded to the column once more and washed with 30 mL PBS. Bound CPA was eluted with 2 mL 100% methanol at 0.5 mL/min. Eluates were air-dried, and the residue was reconstituted in 1 mL PBS. CPA in the reconstituted eluates were analyzed by dc-ELISA. The column was immediately regenerated by washing with 10 mL PBS.

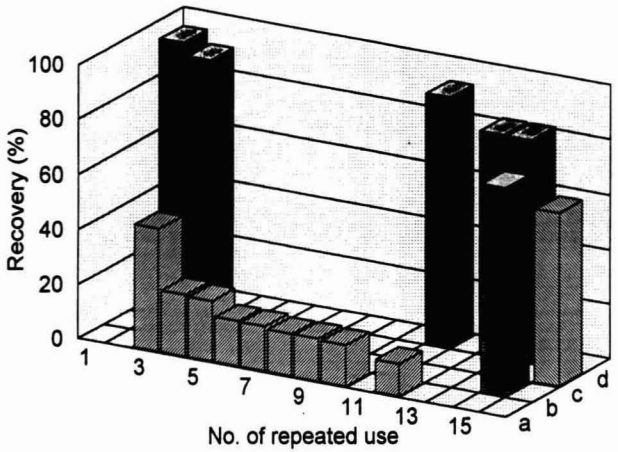

Figure 2. Change in binding capacity of IAC for CPA after repeated application of CPA standard solution, regeneration, and holding in equilibration buffer for various periods (a to d) after each use. CPA solution was loaded onto an IAC (initial capacity, 4 μg/column) and then washed with about 20 mL PBS. CPA was eluted from the column with 2 mL 100% methanol. The column was regenerated and kept in equilibrating buffer for <10 min (a), 5 h (b), 18 h (c), or >24 h (d) before application of next sample. Two milliliters CPA solution (0.5 μg/mL) was loaded each time. X axis represents the number of repeated use. Y axis represents relative percentage of CPA bound to column, with amount of CPA bound to the column the first time as 100% (column with no generation). Z axis represents withholding time before next time use. CPA (1 μg) was quantitatively bound onto the column in the first time of repeated use (bar in column 1 , row d). However, only 30% of CPA was bound when the column was used the third time and column had been subjected to regeneration and then kept in the equilibration buffer for <10 min (bar in column 3, row a). Binding of CPA onto the column was very low when the holding time was <10 min in subsequent application of the sample and regenerations (bars in columns 4–10, row a). Nevertheless, binding of CPA was still very high (11th application) when the same column was held >24 h after the 10th use and regeneration (bar in column 11 and row d).

(**b**) *Evaluation of conditions for elution of bound CPA from immunogel.*—To determine optimal conditions for eluting CPA from the immunogel, 100 or 500 ng CPA in 2 mL PBS was loaded on the column. After the column was washed with 10 mL PBS, it was eluted sequentially with 2 mL methanol at various concentrations. Eluates were air-dried and redissolved in 1 mL PBS; CPA in the reconstituted eluates was analyzed by dc-ELISA.

(**c**) *Column reusability.*—To determine column reusability, 2 mL CPA standard solution (0.5 μg/mL PBS) was loaded onto an IAC for CPA. After the column was washed, CPA was eluted. The column was regenerated with equilibrating buffer and allowed to stand for various periods, from <10 min to >24 h. The capacity of the regenerated column was then determined by reloading with standards. This process was repeated several times. CPA both in the PBS washing eluate and in the methanol eluate were determined by dc-ELISA. The distribution of CPA in the PBS washing eluate and in the methanol eluate was determined. Likewise, a series of experiments was conducted by loading extracts obtained from corn, peanuts, and mixed feeds contaminated with CPA.

Evaluation of IAC as a Cleanup Tool for dc-ELISA of CPA in Corn, Peanuts, and Mixed Feeds

(**a**) *Extraction and cleanup.*—CPA was extracted from samples with methanol—50 mM Tris-buffered saline (TBS, pH 8.0; 7 + 3, v/v), as described previously (24). Generally, 10 g sample was extracted with 50 mL solvent in a Waring blender at high speed for 1 min. The mixture was filtered through Whatman No. 1 filter paper, and a 5 mL portion (equivalent to 1.0 g sample) was withdrawn and diluted with an equal volume of deionized water. To prevent clogging of the column, turbid dilute mixtures were centrifuged or filtered first.

CPA affinity columns were mounted on a solid-phase extraction manifold (Supelco, Inc., Bellefonte, PA) with a 10 mL syringe barrel connected on top as a reservoir. To equilibrate the immunogel, the column was washed with 10 mL TBS at 2 mL/min. Diluted extracts (total CPA should not exceed 4 μg/column) were applied to the column at 1 mL/min. Then the column was washed with 5 mL water and 5 mL 50% methanol (aqueous solution). CPA was eluted with 2 mL methanol at 0.5 mL/min, air-dried, and reconstituted with 1 mL TBS, as described earlier. Depending on the CPA level in the samples, this solution was either directly subjected to dc-ELISA or diluted with TBS before the assay.

(**b**) *dc-ELISA.*—Protocols were essentially as previously described (24). Briefly, microwells of the ELISA plate were each coated with 120 μL purified anti-CPA mAb (1.5 μg/mL IgG1 diluted in PBS) by overnight incubation at 4°C. The plate was washed 4 times with 300 μL PBS containing 0.05% (v/v) Tween 20 (PBST). Then, 200 μL blocking solution [0.1%

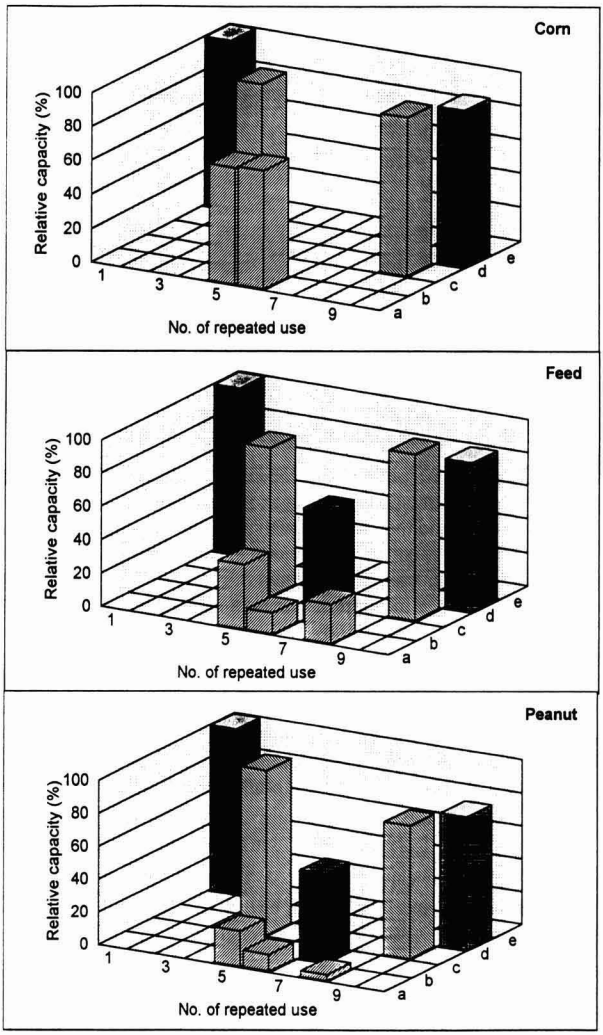

Figure 3. Change in binding capacity of IAC for CPA after repeated application of sample extracts naturally contaminated with CPA and holding in the equilibration buffer for various periods (a to d) after each use. Sample extract was loaded onto an IAC (capacity, 4 μg/column) and washed with 5 mL 50% of methanol; CPA was eluted with 2 mL 100% methanol. The column was regenerated and withheld for <5 min (a), 1 h (b), 14 h (c), and >24 h (d) before reloading again. Row e represents a new IAC (control). Five milliliters of sample extracts containing 151 ng (corn, 3A), 160 ng (peanuts, 3B), and 141 ng (mixed feed, 3C) CPA were loaded to the column each time. Y axis represents the relative column capacity (%), which is the amount of CPA bound to the column in a run divided by the amount CPA bound to a new column (or first time use with no regeneration) times 100. Z axis represents withholding time before use. Explanations for the data shown in this figure are similar to those for Figure 2.

(m/v) BSA in PBS (BSA–PBS)] was added to each well, and the plate was incubated at 37°C for 0.5 h. After the wells were again washed 4 times with PBST, 50 μL CPA standards or samples in 50 mM TBS plus 50 μL CPA–BSA–HRP conjugate (0.2 μg/mL BSA–PBS) were added. The conjugate was prepared according to the method of Yu and Chu (24). The plate was incubated at 37°C for 1 h and washed again, and 100 μL K-blue substrate solution (ELISA Technologies, Lexington, KY) was added. The reaction was terminated by adding 100 μL 1M HCl after incubation at room temperature in the dark for 15 min. Absorbance was determined in dual-wavelength mode at 450 and 650 nm in an automatic ELISA reader (Molecular Devices Co., Menlo Park, CA). Samples and standards were run in triplicate. Standard curves were generated from raw data by using a 4-parameter (sigmoidal) equation. Sample results were calculated from the curve.

Table 1. Recovery of CPA added to corn, peanuts, and mixed feed

	CPA detected								
	Corn			Peanuts			Mixed feed		
CPA added, ng/g	Detd, ng/g[a]	Rec, %[b]	CV, %	Detd, ng/g	Rec, %	CV, %	Detd, ng/g	Rec, %	CV, %
0	1.75	—[c]	5.2	3.74	—	8.5	3.83	—	5.0
1	2.93	118	14.1	4.87	113	5.6	5.38	155	15.0
10	11.1	93.5	13.5	13.7	99.6	16.4	14.8	110	4.9
20	19.4	88.3	1.8	22.0	91.3	2.7	21.8	89.9	1.4
50	54.4	105	16.8	46.6	85.7	14.6	52.7	97.7	3.5
100	106	104	4.5	101	97.3	4.8	111	107	8.0
200	208	103	9.6	204	100	9.3	209	103	11.8
Average	—	102	9.4	—	97.8	8.8	—	111	7.1

[a] Detd = mean CPA detected by dc-ELISA.
[b] Rec = mean recovery (%) after corresponding blank values had been subtracted.
[c] — = not applicable.

Results

Coupling Efficiency and Capacity of CPA Affinity Column

The efficiency of the coupling of the anti-CPA mAb to the CNBr-activated Sepharose 4B was measured by determining the difference between the amount of IgG1 added and the amount unconjugated after the immobilization reaction (26). The results showed that 98.2% of the added IgG1 (163 mg) was coupled to the 17.5 mL swelled CNBr-activated Sepharose 4B gel matrix. A 0.2 mL gel column contained about 1.8 mg IgG1. To determine column capacity, excess CPA was loaded onto the column repeatedly to ensure maximum binding and both bound and unbound CPA were determined (26). Results from 2 representative columns (0.2 mL each) showed that each column was capable of binding 4.2 μg CPA. Thus, each milliliter of gel could bind 21 μg CPA.

Evaluation of Conditions for Eluting Bound CPA from Immunogel

The effect of methanol concentrations on elution of bound CPA are shown in Figure 1. Bound CPA began to elute at a methanol concentration of 60%. Although bulk levels of CPA appeared in eluates with 80% methanol, 2 mL 80 or 90% methanol was insufficient to elute all the bound CPA from the column. However, 2 mL 100% methanol at 0.5 mL/min could quantitatively recover the CPA loaded to the column.

Change in Column Capacity after Repeated Use and Regeneration of Column

Two approaches were used to study the capacity of the column after repeated use. In one approach, 1 μg standard CPA was loaded to the column each time. After regenerating and holding for an appropriate period, the column was used again. Results are shown in Figure 2. Apparently the column was not well equilibrated when the column was held for less than 10 min after regeneration: A minimum holding time of 5 h is necessary. Best results were achieved by keeping the column in a cold room overnight or 24 h before reuse. Column capacity remained at >0.6 μg CPA/column (60%) after the column had been used 16 times.

In the second approach, extracts equivalent to 1 g corn, peanuts, or mixed feed naturally contaminated with CPA (5 mL containing 140–160 ng CPA) were loaded to columns with a capacity of 4 μg CPA/column. Results are shown in Figure 3. Recovery of CPA was low when regenerated columns were held for <5 min or 1 h. Good recoveries (92.3, 81.3, and 89.4% for corn, peanuts, and mixed feed, respectively) were obtained when columns were held >14 h after each use and reused 10 times.

Recovery of CPA from Spiked Corn, Peanuts, and Mixed Feed after IAC Cleanup

Extracts from CPA-free corn, peanuts, and mixed feed were spiked with different amounts of CPA, cleaned up by affinity chromatography, and analyzed by dc-ELISA. A small amount of CPA (1.75–3.83 ng/g) was present in nonspiked extracts (zero added) even after IAC cleanup (Table 1). Recoveries of added CPA (1–200 ng/g) after blanks (zero added) were subtracted, were 88–118% for corn, 86–113% for peanuts, and 90–155% for mixed feed. Mean coefficients of variation were 9.4, 8.8, and 7.1%, respectively. For CPA added at 10–200 ng/g, mean recoveries were 98.8, 94.8, and 102% for corn, peanuts and mixed feed, respectively.

Analysis of CPA in Naturally Contaminated Samples by dc-ELISA after IAC or Solvent Partition Cleanup

Twenty-two peanut samples naturally contaminated with CPA were used. Extracts prepared as described earlier were first cleaned up by IAC or by solvent partition (SP) according to the method of Lansden (17) and then analyzed by dc-ELISA. CPA concentrations in these samples ranged from 2.7 to 384 ng/g (Figure 4). Although there was good correlation between data obtained from SP-dc-ELISA and IAC-LC (correlation coefficient, $r = 0.75$, $p < 0.0001$), the regression slope was low (0.69), indicating that considerable loss occurred during SP cleanup.

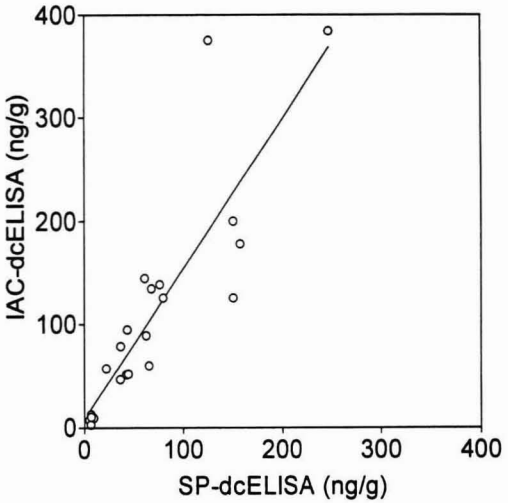

Figure 4. Correlation between CPA levels in peanuts determined by dc-ELISA after IAC cleanup versus after SP cleanup.

Discussion

With the advances of immunoassay techniques for low-molecular-weight naturally occurring compounds including mycotoxins and phycotoxins, immunoaffinity chromatography has become a very effective tool for concentrating and purifying compounds in a single step for subsequent analyses (25). This technology has gained wide application as a cleanup tool for a number of mycotoxins (25). In the present study, an IAC for CPA was prepared and its efficacy as a cleanup tool was evaluated. We found that the IAC could serve as an effective cleanup tool for CPA analyses. The IAC and the protocol developed in the present study provide the following advantages: high capacity binding of CPA, tolerance of high methanol concentration in samples (as high as 35% methanol could be loaded onto the column), effective removal of interferences by washing with 50% methanol, good analytical recovery, and reusability.

In CPA analysis, lack of an effective cleanup method greatly affects the sensitivity of many detection methods (11–23). Recently, we found that with most sensitive ELISAs the detection limits for CPA in complex matrixes such as corn, peanuts, and mixed feed were in the range 200–600 ng/g (24). Results from the present study indicate that the sensitivity of dc-ELISA improves considerably after IAC cleanup.

Spiking experiments showed that a small amount of CPA (1.75–3.83 ng/g) is present in blank samples. These blanks were analyzed for CPA by LC. Because the detection limit of CPA when analyzed by LC is about 10 ng/g, it is difficult to assess whether the amount found in blanks represents a true contamination or is due to interference with dc-ELISA. If these amounts were due to true contamination and were subtracted from the average, recoveries of CPA added to corn, peanuts, and mixed feed at 1–200 ng/g would be 102, 97.8, and 111%, respectively. With blank values plus 3 standard deviations as the basis for estimation (27), detection limits of IAC-dc-ELISA for CPA would be 2.0, 4.7, and 4.4 ng/g for corn, peanuts, and mixed feed, respectively. However, if those amounts were due to interference with no correction for the blanks, average recoveries of CPA spiked at 10–200 ng/g in corn, peanuts, and mixed feed would be 105, 109, and 115%, respectively. Thus, detection limits would be between 10–20 ng/g, an improvement of more than 20 times over dc-ELISA without cleanup, a method that we had recommended for screening (24). The method described in this paper is the most sensitive method for determining CPA in corn, peanuts, and mixed feed. Data from analyses of CPA in peanuts naturally contaminated with toxin further support this conclusion, because CPA concentrations in these samples were much lower than the detection limit of the previously established dc-ELISA (24). The enrichment and cleanup steps reported herein helped to solve the problem. The SP method was less effective because low recovery of CPA was apparent after this treatment.

A question often asked with use of IAC is the cost of preparing a column because columns generally require more antibodies than regular ELISAs, and, except in a few cases (25, 28, 29), each column can be used only once. In the present study, conditions for IAC reuse were optimized. Reusability could be affected by a number factors, including sample matrix (27), elution solvents (29), antibody properties, supporting matrix, storage conditions, and column capacity (25). Our study shows that the column's equilibration period after each use plays a key role. It is likely that once the antibodies in the IAC are conditioned with the eluting solvents through the dissociation process, kinetics for renaturation of the antibody to the conformation for binding is slow. In this regard, the time of the antibody/column exposure to the eluting solvent is also important and should be kept as short as possible, and regeneration of the column should be prompt. Conditions developed for reuse of IAC in the present study could be applied for other IAC systems to maximize IAC use.

Acknowledgments

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FOOD COMPOSITION AND ADDITIVES

Rapid Gas Chromatographic Method for Simultaneous Determination of Cholesterol and α-Tocopherol in Eggs

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A new method was developed for simultaneous determination of cholesterol and α-tocopherol in eggs. It involves rapid and simple sample preparation accomplished in one tube and chromatographic separation that does not require derivatization of analytes. Total analysis time per sample is 40 min. Labor, cost, and use of hazardous chemicals are minimized. To ensure selectivity, accuracy, and precision, critical analytical parameters were investigated. Overall recoveries were 98.8 and 99.2% for cholesterol and α-tocopherol, respectively. Linearity was acceptable for both analytes (r = 0.9964 for cholesterol and 0.9996 for α-tocopherol) in the fortification range examined. Precision data based on within-day and between-days variation gave overall relative standard deviations of 2.0% for cholesterol and 7.0% for α-tocopherol. The method was applied successfully for quantitation of cholesterol and α-tocopherol in eggs.

Cholesterol and α-tocopherol are among the nutritionally significant lipids in foods that increasingly require routine analysis. Cholesterol is a precursor of bile acids, steroid hormones, and vitamin D and is the principal steroid in foods of animal origin, occurring in both free and esterified form. Assessment of dietary cholesterol intake is of growing interest to consumers because cholesterol in serum has been implicated in atherosclerosis (1). α-Tocopherol is the prevalent congener of vitamin E. It is a natural antioxidant that extends the oxidative stability of dietary fat, showing also outstanding antioxidant activity in living cells. Accurate determination of these compounds is of great importance to the food industry and recently has been of special interest to poultry scientists who have placed considerable emphasis on altering, through changes in diet fed to chicken, the fatty acid and cholesterol composition of egg yolk (2).

The cholesterol and α-tocopherol contents of eggs are controversial. Data available from food composition tables and recent reports show wide variability, mainly because of differences in analytical methods used (3–7). Cholesterol and α-tocopherol usually are determined separately by methods including spectrophotometry (5, 6–9), liquid chromatography (LC; 5, 6, 10, 11), and gas chromatography (GC; 6, 12–19). When data for both analytes are required, it is possible to determine them in the same analysis because they are both found in the nonsaponifiable fraction and exhibit similar physicochemical properties.

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Simultaneous determination of cholesterol and α-tocopherol in eggs by LC has been reported (20). However, such a determination has not been achieved yet by GC. With its excellent precision, accuracy, and high degree of automation, GC has become the method of choice for cholesterol analysis, especially when many samples are to be analyzed (6, 21). The very few reports on simultaneous determination of cholesterol and α-tocopherol by GC either concern analysis of matrixes other than egg (22, 23) or are limited to issues relating to chromatographic behavior of analytes, which in many GC systems produce peaks with nearly identical retention times (10, 21).

Here, we describe a simple GC method for simultaneous determination of cholesterol and α-tocopherol in eggs. It involves rapid sample preparation accomplished in one tube and capillary GC analysis that does not require derivatization of analytes. To ensure selectivity, accuracy, and precision, critical analytical parameters were investigated.

METHOD

Apparatus

(**a**) *Capillary column GC system.*—Shimadzu Model GC-15A GC system equipped with Model AOC-17 autosampler, flame ionization detector, and Model Class-VP chromatography data system (Shimadzu Corp., Kyoto, Japan). Operating conditions: fused silica capillary column, 15 m × 0.32 mm id, coated with SPB-1 (Supelco, Inc., Bellefonte, PA) with film thickness of 1.0 μm; oven temperature, programmed from 250° to 275°C at 2°C/min and held there for 12 min; helium carrier gas, 2 mL/min; hydrogen, 30 mL/min; air, 300 mL/min; injection port temperature, 300°C; flame ionization detector temperature, 300°C; split ratio, 20:1; injection volume, 1 μL.

(**b**) *Sample preparation tubes.*—15 × 150 mm culture tubes with Teflon-lined screw cap suitable for sterilizing liquids (Corning, Inc., Corning, NY).

(**c**) *Water bath.*—Temperature regulated (±1°C; Model 3044, Kottermann, Hanigsen, Germany).

(**d**) *Vortex mixer.*—Model G-560E, Scientific Industries (Bohemia, NY).

Table 1. Influence of temperature, heating time, and KOH strength on efficiency of saponification of egg yolk samples, as represented by recovered cholesterol (mg cholesterol/100 g yolk)

Saponification time, min	0.5M KOH		2.0M KOH		Saturated KOH	
	60°C	80°C	60°C	80°C	60°C	80°C
5	946.0	986.2	995.8	1005.6	954.6	966.6
15	1144.2	1174.3	1135.2	1128.9	930.0	944.0
30	1175.0	1126.0	1054.8	1069.8	926.2	918.6
60	1154.8	1169.8	1035.6	1029.9	932.8	922.4
120	1164.2	1162.1	1018.0	1007.5	912.0	920.8

(**e**) *Centrifuge.*—IEC Model Centra-MP4, equipped with 6-position rotor with 15 mL carriers (Needman Heights, MA).

(**f**) *Solvent dispensers.*—5.0 mL (Model P5000), and 1.0 mL (Model P1000), precision pipettes (Gilson, Villiers-le-Bel, France) to conveniently dispense solvents.

(**g**) *Magnetic stirrer plate.*—With variable speed control (Fisher Scientific, Pittsburgh, PA).

(**h**) *Autosampler vials.*—Teflon-lined screw-cap vials with 1.5 mL capacity (Shimadzu).

Reagents

(**a**) *Hexane, methanol, and potassium hydroxide (KOH).*—Analytical grade (Merck, Darmstadt, Germany).

(**b**) *Cholesterol and α-tocopherol standard solutions.*—Using cholesterol and α-tocopherol (>99% purity) reference standards (Sigma Chemical Co., St. Louis, MO), prepare individual 2 mg/mL stock solutions in hexane. Prepare individual standard intermediate solutions by diluting portions of the stock solutions with hexane. Prepare mixed standard working solutions by transferring appropriate volumes from each standard intermediate solution into 10 mL flasks and diluting to volume with hexane to cover the range 6–54 μg/mL for each analyte (6.7, 13.3, 20.0, 33.3, and 53.3 μg/mL). Protect solutions from light, and keep them at –20°C when not in use. Prepare fresh standard intermediate solutions every month for cholesterol and each working day for α-tocopherol.

(**c**) *Methanolic KOH solution (0.5M).*—Prepare by dissolving, with stirring, 14 g KOH into methanol and diluting to 500 mL with methanol.

(**d**) *Pyrocatechol solution (200 mg/mL).*—Prepare by dissolving 1 g pyrocatechol (Sigma) in 5 mL methanol. Protect from light, and keep in a refrigerator when not in use. Prepare fresh every day.

Sample Preparation

Accurately weigh 0.2 g (±0.001) egg yolk into sample preparation tube. Add 100 μL pyrocatechol solution and 5 mL methanolic KOH solution, agitate immediately on Vortex mixer for 20 s, and cap tightly. Immerse lower half of tube in 80°C bath for 15 min; remove and agitate on Vortex mixer for 15 s every 5 min. Several tubes can be handled conveniently by placing them in a wire basket. Cool tube with tap water, remove cap, add 1 mL water and 5 mL hexane, and agitate vigorously on Vortex mixer for 1 min. Centrifuge 1 min at 2000 × *g*, transfer a portion of the upper phase to the autosampler vial, and close vial cap.

Chromatography, Preparation of Calibration Curve, and Calculations

Generate calibration curve by injecting 1 μL from each mixed standard working solution; plotting peak areas vs mass of analytes injected; and computing slope, intercept, and least-square fit of standard curves. Use calibration curve slopes and intercept data to compute mass of analytes in injected (1 μL) unknown sample extracts. For cholesterol determination, appropriately dilute sample extracts with hexane and reinject. Calculate concentration of cholesterol and α-tocopherol in unknown samples as follows:

$$\text{Analyte concentration, mg/100 g} = M \times V \times 2.5$$

where M is the mass (ng) of each analyte in injected sample extract (1 μL) according to corresponding calibration curve and V is the dilution factor, if any, applied.

Results and Discussion

Determination of cholesterol in eggs has been studied extensively. Among commonly used GC methods, some require extraction of total lipids, removal of solvents, hot saponification in alkaline media, extraction of nonsaponifiable material, repeated washes, concentration of extracts, and derivatization prior to analysis (13, 17). These steps are time consuming, as well as labor and material intensive. Of at least equal importance is the interlaboratory variability reportedly inherent in such highly manipulative treatments (24). Other more recent methods based on direct saponification of sample (15, 16, 18) have eliminated some of the steps. However, cholesterol determinations remain laborious and costly, requiring hazardous reagents, the procurement, recovery, and disposal of which are becoming increasingly expensive. Our method minimizes time, labor, and expendable materials, with sample preparation essentially completed in a single tube. To keep the procedure as simple and reliable as possible, all analytical steps were thoroughly investigated.

Table 2. Effect of vegetable fat on recovery of cholesterol and α-tocopherol[a]

Vegetable fat added, g/tube	Recovery of cholesterol, %	Recovery of α-tocopherol, %
0.050	99.2 ± 1.2	101.0 ± 1.6
0.100	96.8 ± 1.8	99.2 ± 2.6
0.150	100.4 ± 1.0	97.4 ± 2.8
0.200	99.6 ± 1.8	97.0 ± 1.2
0.250	98.2 ± 1.6	99.0 ± 2.8
0.300	91.6 ± 2.4	93.8 ± 1.8

[a] Values are means ± standard deviations; $n = 3$.

Direct-Saponification Step

Conditions for direct saponification of food samples are not well documented; various combinations of temperature, heating time, and KOH concentration are claimed to be suitable for converting esterified cholesterol to its free form (21). To establish optimum saponification conditions, 60 identical samples (0.2 g pooled egg yolks) were prepared and processed at various temperatures (60° or 80°C), heating times (5, 15, 30, 60, or 120 min), and strengths of methanolic KOH solution (saturated, 0.5M, or 2M). Two samples were processed for each set of conditions and then combined before hexane (10 mL) extraction and processing through the rest of the method.

When the methanolic KOH solution was 0.5M or 2.0M, cholesterol recovery increased as heating time increased from 5 to 15 min (Table 1). This result indicated that a 5 min heating time at either 60° or 80°C is not adequate for complete conversion of esterified cholesterol. Having attained its maximum value at 15 min, cholesterol concentration could remain essentially constant for heating times up to 120 min with 0.5M KOH. With 2.0M KOH, however, cholesterol recovery decreased gradually as heating time was increased. These results suggested that a 15 min saponification at 80°C with 0.5M KOH is sufficient for complete conversion of esterified cholesterol.

With saturated methanolic KOH, cholesterol recovery was very low no matter what temperature or heating time was applied (Table 1). Contrary to the findings in a recent report (18), these results unequivocally show that saponification with saturated methanolic KOH cannot proceed without loss of cholesterol content. Because auto oxidation of cholesterol has been observed in some rigorous hydrolysis schemes (9), the protective effect of antioxidants added before saponification was also evaluated. However, neither pyrogallol, pyrocatechol, nor butylated hydroxytoluene significantly reduced loss of cholesterol.

Unlike recovery of cholesterol, recovery of α-tocopherol was highly dependent on the level and type of added antioxi-

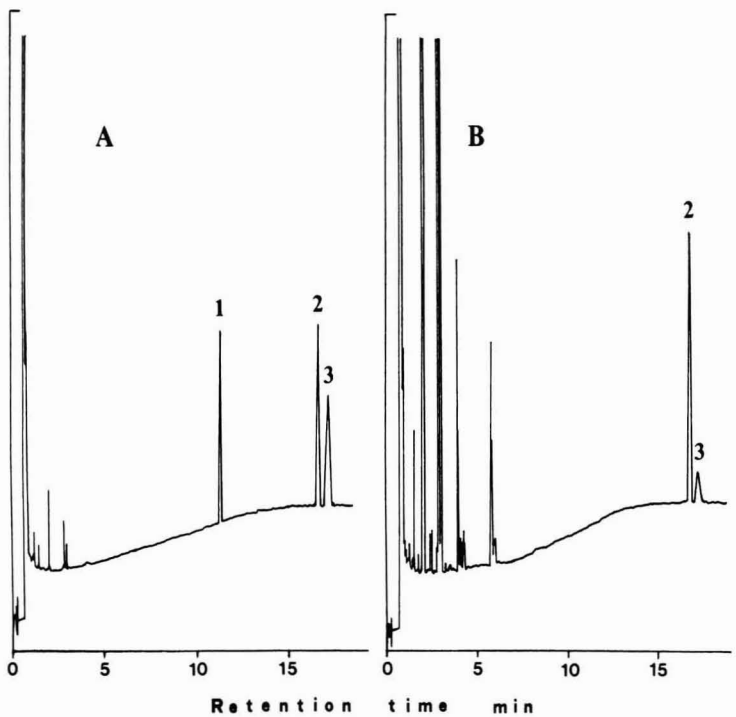

Figure 1. Typical chromatograms of (A) a standard solution of cholesterol and α-tocopherol containing 5a-cholestane as internal standard and (B) an egg yolk extract. Peaks: 1, 5a-cholestane; 2, cholesterol; 3, α-tocopherol.

Table 3. Recovery of cholesterol and α-tocopherol standards added to egg yolk

	Analyte added (n = 5), mg/100 g		Mean concn found, mg/100 g ± SD		Relative standard deviation, %	
Spiking level	Cholesterol	α-Tocopherol	Cholesterol	α-Tocopherol	Cholesterol	α-Tocopherol
0	0	0	1089.9 ± 19.0	7.3 ± 0.3	1.7	4.1
1	285.7	66.8	1356.0 ± 32.8	74.6 ± 1.1	2.4	1.5
2	571.4	133.6	1645.8 ± 23.9	139.4 ± 2.4	1.5	1.7
3	857.1	200.3	1934.0 ± 26.2	206.6 ± 2.8	1.4	1.3

dants. In the absence of antioxidants, α-tocopherol recovery after 15 min saponification at 80°C with 0.5M KOH was only 38%. Addition of 20 mg butylated hydroxytoluene increased recovery to 57%. But addition of 20 mg of either pyrocatechol or pyrogallol totally protected α-tocopherol from oxidation. Pyrocatechol seemed more suitable for use in this method than pyrogallol, because it did not darken as rapidly as pyrogallol did in the alkaline medium.

Extraction Step

When hexane or other nonpolar solvents are used for post-saponification extraction of cholesterol and α-tocopherol from foods and fats, addition of various amounts of water is, in most cases, a mandatory preliminary step (21). Our experiments to establish the amount of water needed for efficient extraction showed that when no water is added, extraction efficiency of hexane for both cholesterol and α-tocopherol is low, ranging from 30 to 40%. Addition of 0.5 mL water increases extraction efficiency to 85–93%. Further addition of 0.5 mL makes extraction quantitative, eliminating the need for a second extraction. Therefore, the minimum volume of water required for efficient extraction is 1 mL. These findings support most published procedures but disagree with a recent method (18) that claims quantitative extraction of cholesterol in the absence of water when saturated methanolic KOH is used. We checked this possibility, but the results did not differ significantly from those initially found.

Slover et al. (22) reported that fat in the saponification mixture also can affect the extraction efficiency. To investigate the matter, we prepared a series of 6 sample preparation tubes so that each one contained a fixed amount of both analytes but a variable amount of vegetable fat (0–0.3 g hydrogenated palm oil) free of either analyte. The content of each tube was saponified, extracted, and chromatographed as described, replicating each analysis 3 times. Results (Table 2) showed that extraction efficiency of hexane is not affected by fat, provided the amount in sample is ≤250 mg.

Chromatography

Although thin-film columns are generally preferred for analysis of high-molecular-mass, high-boiling-point compounds to minimize bleeding from the column, we achieved good results for cholesterol and α-tocopherol with a short thick-film column. As Figure 1 shows, both peaks are sharp without tailing. The thick film covering the active silanol groups on the surface of the fused silica seems to prevent adsorption of underivatized analytes, and thus, peak distortion does not occur. Some fatty acid methyl ester peaks are sizable, but they did not present any separation or contamination problem on the capillary column, eluting very early, just after the solvent front. On the other hand, plant sterols eluted long after α-tocopherol and were well-resolved from each other.

Because chromatographic results were acceptable, we did not consider derivatization of analytes prior to injection onto the GC system, in accordance with other workers (15, 16, 18, 25, 26). Trimethylsilylation of analytes might further improve peak shape, reduce retention time, and improve sensitivity. However, trimethylsilylation not only adds an extra step in the

Table 4. Precision of determination of cholesterol in egg yolk

Day	Concn of cholesterol found, mg/100 g	Mean value ± SD, mg/100 g	RSD, %
1	1073.4, 1044.1, 1098.0, 1069.0, 1096.3	1076.2 ± 19.8	1.8
2	1118.2, 1101.6, 1070.4, 1122.8, 1090.0	1100.6 ± 19.1	1.7
3	1087.0, 1068.2, 1114.6, 1098.0, 1101.6	1093.9 ± 15.6	1.4
	Overall mean	1090.2 ± 21.0	1.9
	Variance estimates		
	Source		RSD, %
	Between-days		2.6
	Within-day		1.9
	Overall		2.0

Table 5. Precision of determination of α-tocopherol in egg yolk

Day	Concn of α-tocopherol found, mg/100 g	Mean value ± SD, mg/100 g	RSD, %
1	7.5, 6.6, 7.0, 7.4, 6.4	7.0 ± 0.4	6.1
2	7.0, 7.2, 7.9, 7.5, 8.1	7.5 ± 0.4	5.5
3	7.0, 7.8, 7.2, 8.0, 6.8	7.4 ± 0.5	6.3
	Overall mean	7.3 ± 0.5	6.8
		Variance estimates	
	Source		RSD, %
	Between-days		8.7
	Within-day		6.7
	Overall		7.0

procedure but also could increase noise; lead to formation of artifacts; decrease recovery; result in poor linearity because of silicone deposits in the flame ionization detector; and raise safety concerns because many silylating agents are toxic, flammable, and corrosive.

Calibration

Both internal (15, 16, 18, 25) and external (10, 27, 28) standard calibration techniques have been proposed for analysis of cholesterol and α-tocopherol. Because delivery of sample volumes is quite precise with modern automatic sampling systems, the internal standard (IS) technique is most useful for assays that require extensive sample pretreatment including derivatization, where variable recoveries of the target analytes may occur. For an essentially manipulation-free and well-tested procedure such as the one we describe here, use of the IS technique may not be advantageous. On the contrary, it may actually increase precision error because of the frequent calibration needed for measuring 2 peak areas rather than one (29, 30).

To test the efficiency of external versus internal standard calibration in our system, we prepared 5 sample preparation tubes so that each one contained the same amount of 5a-cholestane (120 μg) as IS and a variable amount of cholesterol and α-tocopherol (33.5, 66.0, 100.0, 166.5, and 266.5 μg of each compound) in 5 mL hexane. Each mixture consisted of standards only, so they were analyzed by GC without saponification, with each analysis replicated 5 times. Cholesterol and α-tocopherol responses as either cholesterol/5a-cholestane and α-tocopherol/5a-cholestane peak area ratios or distinct cholesterol and α-tocopherol peak areas were plotted against amount of cholesterol or α-tocopherol. Regression analysis showed linear responses for both types of calibration and both analytes in the range examined: $y_{IC} = 0.003 + 0.0414x$, response factor = 1.001 ± 0.023, $r_{IC} = 0.99996$ for internal standard calibration of cholesterol; $y_{IT} = 0.001 + 0.0342x$, response factor = 0.838 ± 0.024, $r_{IT} = 0.99986$ for internal standard calibration α-tocopherol; $y_{EC} = 9.22 + 495.78x$, $r_{EC} = 0.99994$ for external standard calibration of cholesterol; and $y_{ET} = 0.50 + 409.63x$, $r_{ET} = 0.99992$ for external standard calibration of α-tocopherol, where y_{IC}, and y_{IT} represent peak area ratios, y_{EC} and y_{ET} represent peak areas, and x is the quantity (ng) of analyte injected. The excellent linearities suggested that both techniques are more than adequate for reliable quantitation of target analytes. We selected the external standard calibration technique, because it minimizes manipulations and analysis cost.

Table 6. Cholesterol and α-tocopherol in commercial eggs (mg/100 g yolk)

	Mean concn ± SD (n = 3)			Mean concn ± SD (n = 3)	
Sample No.	Cholesterol	α-Tocopherol	Sample No.	Cholesterol	α-Tocopherol
1	1120.3 ± 19.5	5.5 ± 0.4	11	1126.1 ± 21.0	7.0 ± 0.2
2	1096.0 ± 12.8	4.8 ± 0.3	12	1269.0 ± 25.4	6.8 ± 0.3
3	1077.0 ± 11.0	6.2 ± 0.4	13	1120.7 ± 26.3	<2
4	1298.2 ± 18.4	<2	14	1090.6 ± 15.8	2.8 ± 0.1
5	1164.2 ± 21.2	7.3 ± 0.3	15	1521.0 ± 21.1	3.0 ± 0.2
6	1196.7 ± 19.0	6.2 ± 0.4	16	1379.0 ± 10.1	26.4 ± 1.5
7	1084.8 ± 28.8	6.2 ± 0.3	17	1236.0 ± 13.6	112.8 ± 4.3
8	1095.8 ± 29.3	6.4 ± 0.4	18	1090.8 ± 18.2	5.2 ± 0.3
9	1110.0 ± 15.4	7.0 ± 0.4	19	1162.0 ± 20.4	4.8 ± 0.2
10	1089.8 ± 17.8	19.3 ± 1.0	20	1123.4 ± 17.6	5.0 ± 0.1

Accuracy

The standard addition procedure was used to study method accuracy. Fifteen of 20 samples from an egg yolk were spiked with standard cholesterol and α-tocopherol at 3 levels (5 samples at each level) from a methanolic solution containing both analytes. Least-squares and regression analyses of the data (Table 3) based solely on the 3-level spiking showed that the relationship between "added" (x) and "found" (y) for each analyte was adequately described by a linear regression: $y = 1067.3 + 1.011x$, r = 0.9931 for cholesterol; $y = 8.1 + 0.989x$, r = 0.9991 for α-tocopherol. The intercepts of these regression lines, which represent the values (mg/100 g) predicted for unspiked samples, were not significantly different from the arithmetic means of the unspiked samples (1067.3 versus 1089.9 for cholesterol; 8.1 versus 7.3 for α-tocopherol), suggesting the absence of interference in extracted samples. The absence of interference permitted evaluation of accuracy based on data from both spiked and unspiked samples. Least-squares and regression analyses of these data gave acceptable linearities: $y = 1083.1 + 0.988x$, r = 0.9964 for cholesterol; $y = 7.6 + 0.992x$, r = 0.9996 for α-tocopherol. Therefore, the slopes (0.988 and 0.992 for cholesterol and α-tocopherol, respectively) of these regression lines could be used as estimates of overall recovery (98.8% for cholesterol; 99.2% for α-tocopherol) for the proposed method.

Precision

Method precision was evaluated by assaying on each of 3 different days 5 egg yolk samples. To estimate overall precision, raw data were subjected to analysis of variance and expected mean squares for one-way classification-balanced design (31). Tables 4 and 5 show that the within-day precision was better than between-days precision for both analytes. Overall precisions were 2.0% for cholesterol and 7.0% for α-tocopherol in egg yolk.

Cholesterol and α-Tocopherol in Egg Samples

Results of analysis of eggs from various local markets (Table 6) demonstrate method applicability. Cholesterol concentrations in the egg yolks ranged from 1077 to 1521 mg/100 g. This variability cannot be attributed to analytical errors, because all values are means of triplicate analyses. Extensive studies on the modification of egg composition have shown that genetics (32), diet (33), and management (34) can influence cholesterol level in eggs. Nevertheless, cholesterol values found in this study are comparable with data obtained by other workers (5, 6, 15, 20), although higher than some recently reported results (18).

Unlike cholesterol levels, α-tocopherol levels show enormous variation. Although most samples contained α-tocopherol in the range 2.8–7.3 mg/100 g egg yolk, 2 samples did not contain α-tocopherol (limit of detection corresponded to 2 mg/100 g egg yolk for a peak-to-noise ratio of 3:1), and 3 samples contained 19.3, 26.4, and 112.8 mg/100 g egg yolk. Slover (35) reported an α-tocopherol level of 1.2 mg; McLaughlin et al. (36), 2.05 mg; and Syvaoja et al. (37), 5.5 mg/100 g egg yolk. Feeding vitamin E supplements to hens may be the reason for the higher α-tocopherol levels found in this study compared with earlier studies. Surai et al. (38) showed that vitamin E transfer from the diet to the egg yolk takes place very rapidly. An increase of vitamin E supplementation in the hen diet of up to 320 mg/kg feed results in eggs with a vitamin E level in the yolk of about 70 mg/100 g (39). Lower levels of vitamin E (about 40 mg/100 g) in egg yolk have been found after supplementation of the hen diet with α-tocopherol acetate at 100 mg/kg (40).

Most GC methods for determining cholesterol in eggs cannot discriminate between cholesterol and α-tocopherol, because the 2 compounds exhibit similar physicochemical properties. Therefore, faulty results may be obtained by these earlier methods when eggs contain the high levels of α-tocopherol found in this study or even higher.

Conclusions

The method has satisfactory analytical characteristics with respect to recovery, selectivity, and reproducibility. It is very rapid and simple, offering considerable savings in solvent, materials, sample manipulation, and analysis time. For analysis of 16 samples, sample preparation can be completed by a single analyst in about 1 h. The GC determinative procedure requires about 20 min for each sample but automation can extend analytical capacity. The method may be particularly suitable for laboratories where large throughput of compliance samples is obligatory.

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RESIDUES AND TRACE ELEMENTS

Gas Chromatographic Determination of Azoxystrobin, Fluazinam, Kresoxim-Methyl, Mepanipyrim, and Tetraconazole in Grapes, Must, and Wine

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Azoxystrobin, fluazinam, kresoxim-methyl, mepanipyrim, and tetraconazole were determined in grapes, must, and wine by a gas chromatographic method with nitrogen–phosphorus (NP) and mass spectrometric (MS) detectors. Pesticides were isolated from the matrixes by online microextraction with acetone–hexane (50 + 50, v/v). Because of the high selectivity of NP and MS detectors, no interferent peaks were present and no cleanup was necessary. Recoveries from fortified grapes, must, and wine ranged from 80 to 111%, with coefficients of variation ranging from 1 to 14%. Limits of determination were 0.05 mg/kg for kresoxim-methyl and 0.10 mg/kg for the other compounds.

New fungicides belonging to new chemical classes have been marketed in the 1990s to control the major pathogens of vine. These compounds belong to the chemical classes strobilurines, phenylpyrroles, anilinopyrimidines, and 2,6-dinitroanilines (1). A gas chromatographic (GC) method for determining some new fungicides used to control grey mold (*Botrytis cinerea*) in grapes, must, and wine was reported recently (2). No analytical method is reported for the new fungicides used to control downy mildew (*Plasmopora viticola*) and powdery mildew (*Uncinula necator*), such as the strobilurines azoxystrobin and kresoxim-methyl and the 2,6-dinitroaniline fluazinam. This paper describes a simple, rapid method to determine these fungicides in grapes, must, and wine by GC with nitrogen–phosphorus (NP) or mass spectrometric (MS) detection. The method also determines the anilinopyrimidine mepanipyrim and the latest generation triazole tetraconazole. An enzyme-linked immunosorbent assay (ELISA) has been reported for tetraconazole (3).

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METHOD

Apparatus

(**a**) *GC–NPD system.*—Fisons HRGC series Mega 2 gas chromatograph (Carlo Erba, Milan, Italy) equipped with an NP detector (NPD-80), a split-splitless injector, and an AS 800 autosampler (Carlo Erba) and connected to an HP 3396-A reporting integrator (Hewlett-Packard, Avondale, PA) was used. The capillary column was a WCOT fused-silica column CP-Sil 8 CB liquid phase (25 m × 0.25 mm id; film thickness, 0.12 μm; Chrompack Int., Middelburg, The Netherlands). The injector and detector were operated at 250° and 300°C respectively. The sample (2 μL) was injected in the splitless mode (60 s). The oven temperature was programmed as follows: 110°C for 1 min, raised to 260°C (10°C/min), and held for 5 min. Helium was the carrier gas at 120 kPa, and N_2 was the makeup gas at 80 kPa. The plasma of the detector was obtained with H_2 (60 kPa) and air (110 kPa), the current was 2.75 A, and the voltage was 3.5 V.

(**b**) *GC/MS system.*—GC HP-5890 (Hewlett-Packard, Palo Alto, CA) gas chromatograph equipped with GC/MS HP-5971 (Hewlett-Packard) and Durabond fused-silica column (30 m × 0.25 mm id; J&W Scientific, Folsom, CA), DB 5MS liquid phase (film thickness, 0.25 μm). The sample (2 μL) was injected in the splitless mode (60 s). The injector temperature was 250°C, and the oven temperature was programmed as follows: 110°C raised to 300°C (15°C/min) and held for 10 min. Helium was the carrier gas at 0.8 mL/min. Mass spectrometer operating conditions: electron ionization, 65 V; ion source, 180°C; dwell per ion, 100 ms; solvent delay, 8 min; selected monitoring (SIM), *m/z*: triphenylphosphate (internal standard) = 326, tetraconazole = 336, mepanipyrim = 222, kresoxim-methyl = 116/131/206, fluazinam = 371/387/417, and azoxystrobin = 344.

(**c**) *Rotatory shaker.*—GFL, Burgwedel, Germany.

Reagents

Active ingredient (AI) standards (purity, >99%) were provided by the manufacturer. Chemical Abstracts registry numbers are as follows: azoxystrobin, 131860-33-8; fluazinam, 79622-59-6; kresoxim-methyl, 143390-89-0; mepanipyrim, 110235-47-7; tetraconazole, 112281-77-3.

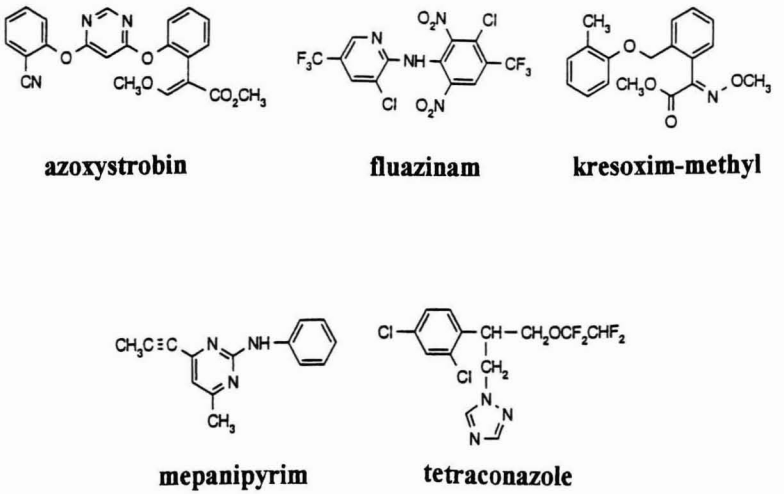

Figure 1. Structure of fungicides.

(**a**) *Acetone and hexane.*—Liquid chromatography and pesticide grade, respectively (Carlo Erba).

(**b**) *Sodium chloride, anhydrous sodium sulfate, and triphenylphosphate.*—Reagents for analysis (Carlo Erba and Janssen, Geel, Belgium).

(**c**) *Standard stock solutions (ca 500 mg/L).*—Prepared in methanol.

(**d**) *Internal standard solution.*—Add 0.1 mL triphenyl-phosphate solution (300 mg/L) to 1 L acetone–hexane (50 + 50, v/v) to obtain a final concentration of 0.03 μg/mL.

(**e**) *Spiking solution.*—Prepare intermediate solution containing all pesticides at 100 mg/L from standard stock solutions.

(**f**) *Working standard solutions (0.05, 0.50, 1.00, and 3.00 μg/mL).*—Prepare by evaporating to dryness portions of intermediate standard solutions under nitrogen stream and taking up the residue in the extract solution of acetone–hexane plus the internal standard from untreated (control) grapes, must, and wine.

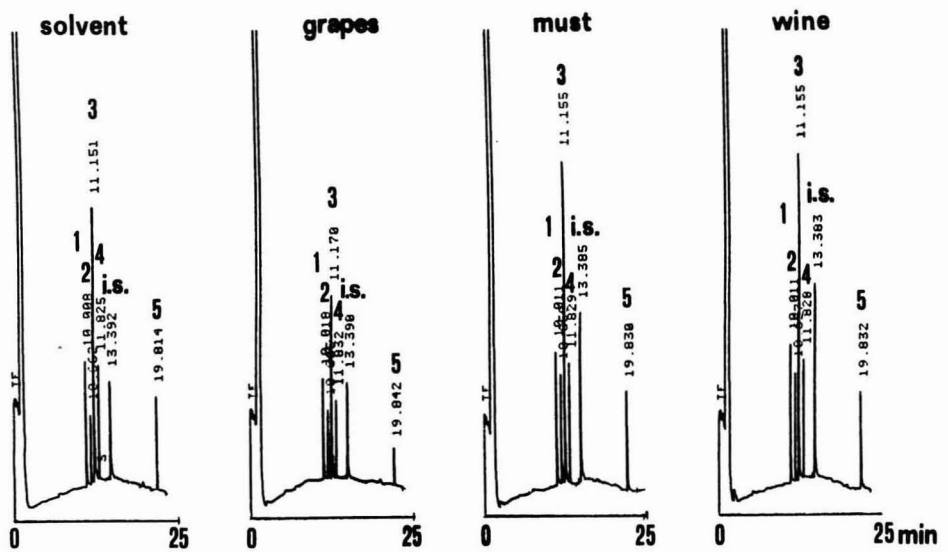

Figure 2. Comparison of GC–NPD chromatograms of pesticides in solvent and extracts of untreated grapes, must, and wine, in controls (c) and samples (s) fortified with fungicides at about 1.0 mg/kg. For GC conditions, *see* text. Peaks: tetraconazole (1), mepanipyrim (2), kresoxim-methyl (3), fluazinam (4), and azoxystrobin (5).

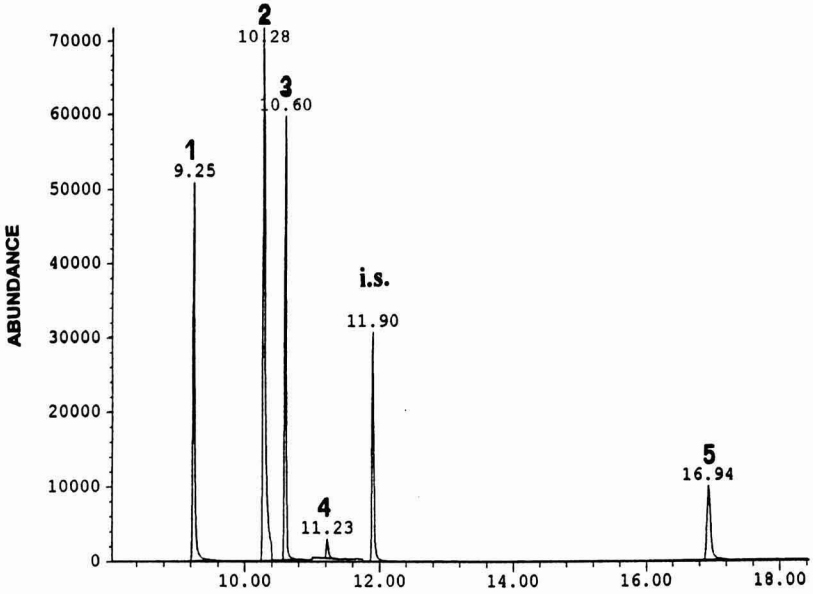

Figure 3. GC–SIM chromatograms of pesticides at about 1.0 mg/kg in extract of untreated grapes. For GC/MS conditions, *see* text. Peaks: tetraconazole (1), mepanipyrim (2), kresoxim–methyl (3), fluazinam (4), and azoxystrobin (5).

Sample Preparation

Use a blender to chop and homogenize grapes samples. Shake and/or stir must and wine.

Extraction

Weigh 10 g sample into 40 mL screw-capped tube; add 4 g NaCl and 10 mL acetone–hexane (50 + 50, v/v) containing internal standard. Homogenize resulting mixture for 30 min in a rotary shaker. Allow phases to separate, and pour organic layer into another tube containing 1 g anhydrous sodium sulfate. Inject sample without cleanup for GC–NPD determination. For GC/MS analysis, dry 1 mL organic extract under N_2 stream and dissolve in 100 µL acetone–hexane (50 + 50, v/v).

Recovery Assays

Untreated grapes, must, and wine samples were fortified with the fungicides and processed. Recovery assays were performed at 0.05–0.1 and 1.50 ppm. At each fortification level, 4 replicates were analyzed.

Results and Discussion

The 5 fungicides (Figure 1) were separated with DB 5 and CP-Sil 8 CB columns and eluted in the same sequence: tetraconazole, mepanipyrim, kresoxim-methyl, fluazinam, and azoxystrobin (Figures 2 and 3). The matrix effect on the peak response was evaluated. Working standard solutions at the same concentration (about 1 mg/kg) were prepared in the solvent (acetone–hexane, 50 + 50, v/v) and in the extract from untreated grapes, must, and wine. Peak responses were compared. No difference was found with the MS detector, but with the NP detector, responses were significantly different (Figure 2). Peaks corresponding to pesticides and internal standards increased to different degrees in must and wine. In grapes the internal standard peak was constant and the pesticide peaks de-

Table 1. Ratios of pesticide peak height to internal standard peak height in various matrixes[a]

Matrix	Azoxystrobin	Fluazinam	Kresoxim-methyl	Mepanipyrim	Tetraconazole
Solvent	100 ± 4	100 ± 5	100 ± 4	100 ± 3	100 ± 2
Grapes	43 ± 1	51 ± 3	60 ± 1	78 ± 3	55 ± 2
Must	51 ± 2	60 ± 3	68 ± 1	98 ± 5	62 ± 4
Wine	34 ± 1	67 ± 2	67 ± 2	99 ± 3	82 ± 3

[a] Values are percentages of the ratios in solvent; mean of triplicate analysis.

creased. Table 1 shows the ratios of peak height of pesticide to peak height of internal standard expressed as a percentage of the ratio for solvent. These data showed that pesticide calibration curves depend strongly on the matrix. Therefore, working standard solutions were prepared in the matrix extract of untreated grapes, must, and wine. Other have reported that the GC–NPD response of some pesticides are affected by the matrixes (4–6). Figure 4 shows the mass spectra of the fungicides. The major ions (*m/z*: azoxystrobin = 344, fluazinam = 371/387/417, kresoxim-methyl = 116/131/206, mepanipyrim = 222, tetraconazole = 336, triphenylphosphate (internal standard) = 326) were used to determine residues in the Selected Ion

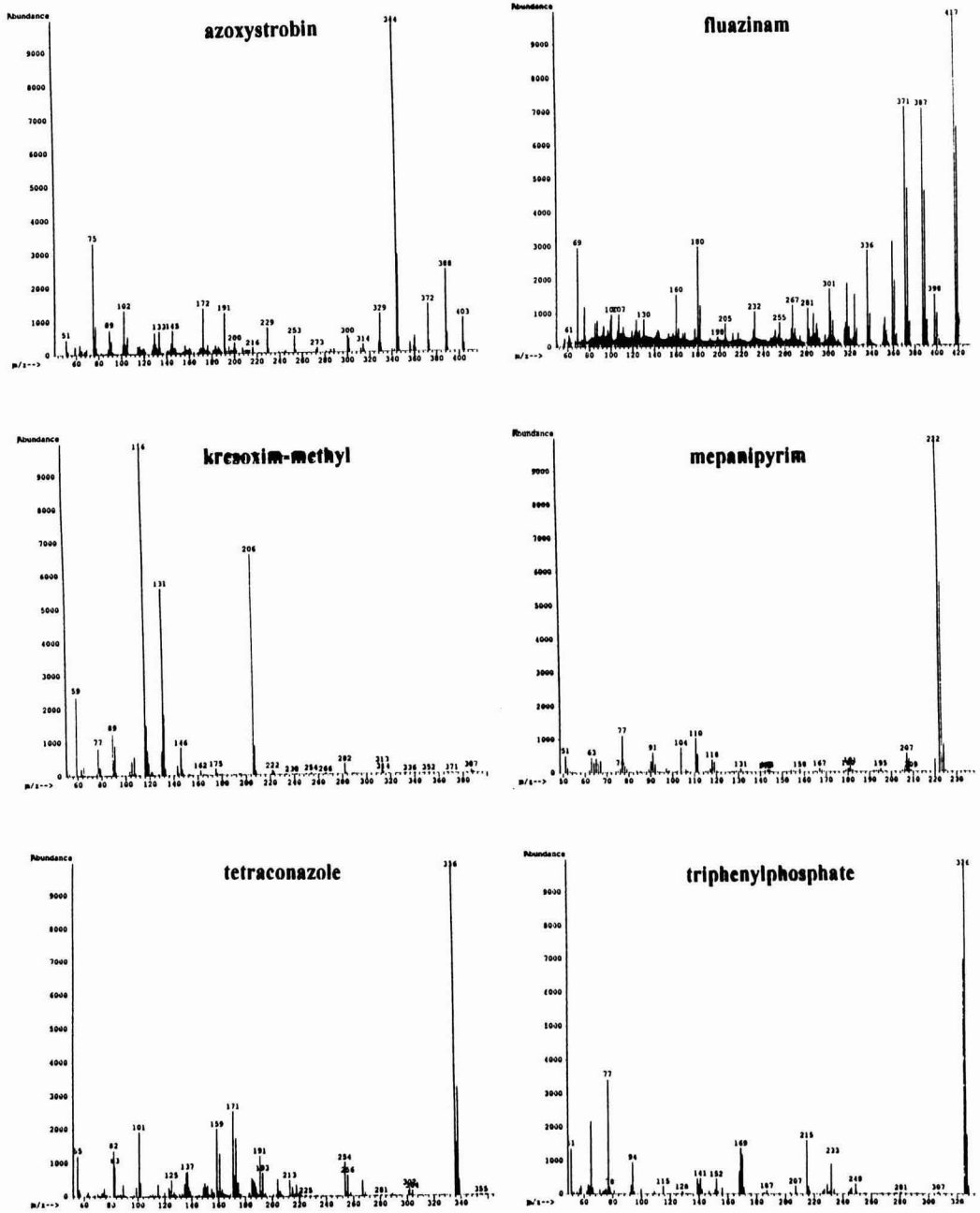

Figure 4. Mass spectra of fungicides.

Table 2. Recovery (% ± RSD) of fungicides from grapes, must, and wine samples

Fungicide	Fortification, mg/kg	Recovery		
		Grapes	Must	Wine
Azoxystrobin	1.50	98 ± 2	102 ± 4	90 ± 2
	0.10	85 ± 2	87 ± 1	81 ± 4
Fluazinam	1.50	103 ± 2	94 ± 11	102 ± 1
	0.10	111 ± 2	96 ± 1	80 ± 1
Kresoxim-methyl	1.50	102 ± 4	104 ± 3	95 ± 1
	0.05	107 ± 3	98 ± 2	85 ± 1
Mepanipyrim	1.50	100 ± 5	102 ± 10	93 ± 2
	0.10	109 ± 3	100 ± 1	96 ± 1
Tetraconazole	1.50	101 ± 4	104 ± 4	93 ± 1
	0.10	110 ± 2	95 ± 8	85 ± 14

Monitoring (SIM) mode. The method has a low sensitivity for fluazinam, which could not be determined at 0.1 mg/kg. Because the sensitivity of the MS detector was lower than that of the NP detector, extracts were concentrated by a factor of 10. Nevertheless, chromatograms of untreated samples were free from interfering peaks, and no cleanup was necessary.

Calibration curves for active ingredients were prepared by the internal standard method by plotting peak heights versus concentrations. Good linearity was achieved in the 0.05–3 mg/kg range, with correlation coefficients between 0.9987 and 0.9995 for both detectors.

Recovery

An online microextraction procedure according to Steinwandter (7) was used. With this method, extraction–partition was performed in one step. Thus, we gained speed and avoided losses due to transfers. Because of the high selectivity of the NP and MS detectors, no interfering peaks were present, and no cleanup was necessary. Because coextractive substances modify the peak height, quantitation of residues was performed by measuring samples fortified at 0.05–0.10 and 1.50 mg/kg against matrix-matched standards. Recovery and repeatability data are summarized in Table 2. Recoveries ranged from 80 to 111%. Accuracy was acceptable; coefficients of variation ranged from 1 to 14%. Under the above operating conditions the limits of determination (8) with GC–NPD were 0.05 mg/kg for kresoxim-methyl and 0.10 mg/kg for the other pesticides.

Conclusion

Extraction–partition was performed in one step. No cleanup was necessary, because the gas chromatograms of untreated samples were all free from interfering peaks. This allowed a simple and rapid determination of fungicides in grapes, must, and wine; an acceptable recovery and repeatability; and a sufficient limit of determination.

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RESIDUES AND TRACE ELEMENTS

Determination of Phosphine Residues in Whole Grains and Soybeans by Ion Chromatography via Conversion to Phosphate

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An ion chromatographic (IC) method was developed for determining phosphine (PH_3) in whole grains (barley, corn, oats, rice, rye, and wheat) and soybeans. The method converts phosphine to phosphate (i.e., orthophosphate) and isolates the phosphate by IC with eluent-suppressed conductivity detection. Recoveries of unbound phosphine by the method were similar to those obtained by an established colorimetric method for 7 different products fortified at 3 levels. Mean recoveries were low (i.e., 30–60%) and varied with product type and level of fortification. Recoveries of PH_3 from previously fumigated products fortified with aluminum phosphide ranged from 19.0% for barley fortified at 0.734 ppm to 88.3% for corn fortified at 1.691 ppm. Precision data from 3 products based on replicate analyses (n = 4 or 5) gave relative standard deviations of 1.78–4.66% for mean laboratory-fumigated PH_3 levels of 0.679–1.309 ppm. Estimated limits of detection (LOD) and quantitation (LOQ) for PH_3 were 0.010 μg/g (10 ppb) and 0.0275 μg/g (27.5 ppb) at signal-to-noise ratios (S/N) of 4:1 and 10:1, respectively. These values were also determined for a nonchemically suppressed IC system with LOD of 0.02 μg/g (20 ppb) and LOQ of 0.055 μg/g (55 ppb) at S/N of 4:1 and 10:1, respectively. Phosphate response was linear over the concentration range equivalent to 0.30–10.0 μg P/mL, with a mean correlation coefficient of 0.9988 based on replicate standard curves. The relationship of product composition to recovery from various products was also examined.

Phosphine (PH_3) is a highly toxic gaseous compound that has been used worldwide since the 1930s as a fumigant against insects (e.g., *Tribolium confusum* Duv.) in stored grains such as wheat, barley, oats, and rice. It is effective at air levels as low as 0.02 ppm over a 24 h period (1). Recently, use of phosphine has increased to replace halogenated fumigants (e.g., ethylene dibromide and methyl bromide). Typically, stored grain is exposed to the fumigant in the form of aluminum phosphide (AlP) in a gas-permeable container that allows slow hydrolysis by atmospheric moisture to phosphine gas as follows:

$$AlP + 3H_2O \rightarrow Al(OH)_3 + PH_3$$

Commercial preparations of aluminum phosphide in tablet or pellet form, for example, may contain the active ingredient, ammonium carbamate, and paraffin at levels of 55, 41, and 4%, respectively. Ammonium carbamate in combination with aluminum phosphide produces a nonflammable mixture of phosphine, ammonia, and carbon dioxide upon hydrolysis (2). Materials of this type are assayed by acid hydrolysis to phosphine. The gas is trapped in 1.5% mercuric chloride solution, and the HCl released is titrated with 0.1N NaOH (3). According to an early report regarding misuse of aluminum phosphide marketed as "Delicia-AIP," 12 persons became ill and one person died after fumigation of a grain elevator in a large city neighborhood (4). This report raised concern regarding application of aluminum phosphide as a fumigant (5). Similar tragedies have occurred more recently (6), including those caused by domestic misuse of zinc phosphide for rodent control (7). Therefore, consumers must know to take adequate precautions when using metal phosphides and to thoroughly aerate phosphine-treated products to reduce residues to negligible concentrations.

Grains adsorb PH_3 released by AlP while in storage (8). This surface uptake may be chemical or physical in nature and depends on temperature, product moisture content, particle size, and exposure time. The Food and Agriculture Organization (FAO), the World Health Organization (WHO), and the U.S. Environmental Protection Agency (EPA) have established a tolerance level of 0.1 ppm for total PH_3 (intact and derived from phosphide) in raw cereal grains and 0.01 ppm in certain foodstuffs including nuts (9, 10). It is important to have suitable analytical methodology for PH_3 residues that may be present on or in these products.

Early methodologies for detecting and estimating low levels of phosphine were based on gravimetry for phosphine in cereal grains (11) and on color reactions for phosphine in air samples (12, 13). Recent quantitative analytical procedures are based on colorimetry (14–17), titrimetry (18), and gas chromatography (GC; 19–22). Colorimetric and titrimetric methods are susceptible to interferences, time consuming, and manipulative. Although GC exhibits adequate sensitivity and specificity, it lacks retention control of the gaseous analyte at the low column tem-

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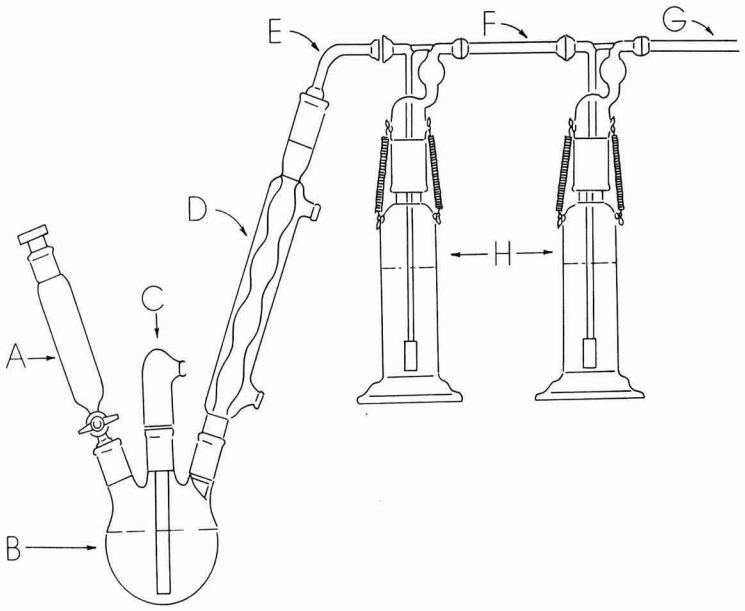

Figure 1. Phosphine generation apparatus: (A) addition funnel; (B) 3-neck reaction flask; (C) nitrogen inlet adapter; (D) Allihn condenser; (E) and (F) connector tubes; (G) outlet tube; (H) gas washing bottles.

peratures required (e.g., 30°–60°C). Preparation and use of reference PH_3 standards also hampers validation of this technique.

The procedure we propose uses modifications of existing methods (14, 15) for liberation of PH_3 from various matrixes and ion chromatography (IC) with suppressed conductivity for detection. Phosphine is generated by refluxing 100 g whole grain with 200 mL 10% H_2SO_4. The liberated phosphine gas is derived from either surface desorption of the fumigant or hydrolysis of residual aluminum phosphide (e.g., Phostoxin). The gas is transferred with the aid of nitrogen to 2 traps in series. Each trap contains 75 mL saturated bromine water, which oxidizes phosphine to phosphate ion:

$$2AlP + 3H_2SO_4 \rightarrow 2PH_3 + 2Al^{+3} + 3SO_4^{-2}$$

$$PH_3 + Br_2 + 4H_2O \rightarrow PO_4^{-3} + 2Br^- + 11H^+$$

Nonphosphine phosphorus residues (e.g., phosphite and phosphate) present in the acid media remain in the reflux mixture.

The trap liquids are combined, concentrated by boiling to about 5 mL, and then diluted prior to the determinative step. An aqueous solution of potassium dihydrogen phosphate (KH_2PO_4) is used as the reference standard.

The proposed IC procedure was compared with a colorimetric method (15) using the molybdenum blue reaction for phosphate anion. This reaction is based on complexation of the anion with molybdic acid followed by reduction with hydrazine sulfate (23).

IC has several advantages over spectrophotometry: Direct injection of concentrated sample extract into the chromatograph avoids the time-consuming and manipulative color development step, and phosphate anion retention is readily controlled by column selection, eluent composition (ionic strength and pH), and flow rate, providing greater specificity. Both IC and colorimetric methods are safer than GC because they do not require use of the toxic and difficult-to-handle phosphine gas reference material. The proposed procedure detects phosphine at the 10 ppb level via phosphate anion. The method is also more specific and easier to perform than the colorimetric method.

This study also considers the chemical and physical factors that may be associated with the low phosphine recoveries observed under acid reflux conditions. A brief comparative study of 2 IC systems—a chemically suppressed versus a nonchemically suppressed—was also conducted.

Experimental

Safety

Latex gloves were used in handling 10–20% HCl and 10% H_2SO_4 solutions. Manipulation of commercial aluminum phosphide pellets and bromine required use of both latex gloves and a fume hood.

Apparatus

(**a**) *Instrumentation.*—Dionex Model 4500i ion chromatograph with a pulsed electrochemical detector in the conductivity mode, a pneumatically actuated microinjection valve, and a Model SP-4400 integrator (Dionex Corp., Sunnyvale, CA). Injection volume, 50.0 µL; detector sensitivity range, 3–10 µS (microsiemens); column temperature, ambient.

Table 1. Recovery of phosphine derived from aluminum phosphide added to various whole grains and soybeans at 3 fortification levels

	Colorimetric detection			IC detection		
Product	PH_3 added, ppm	PH_3 recovered, ppm	Recovery, %	PH_3 added, ppm	PH_3 recovered, ppm	Recovery, %
White rice 1	0.212	0.121	57.1	0.179	0.101	56.4
White rice 2	1.290	0.724	56.1	1.195	0.650	54.4
White rice 3	2.744	1.840	67.1	2.465	1.660	67.3
Mean			60.1			59.4
Corn 1	0.200	0.082	41.0	0.159	0.061	38.4
Corn 2	1.246	0.546	43.8	0.920	0.378	41.1
Corn 3	2.357	1.234	52.4	2.095	1.087	51.9
Mean			45.7			43.8
Wheat 1	0.224	0.037	16.5	0.184	0.032	17.4
Wheat 2	1.214	0.271	22.3	1.099	0.250	22.7
Wheat 3	3.409	1.711	50.2	3.000	1.505	50.2
Mean			29.7			30.1
Oats 1	0.210	0.084	40.0	0.196	0.061	31.1
Oats 2	1.290	0.432	33.4	1.167	0.377	32.3
Oats 3	4.158	1.786	43.0	3.760	1.581	42.0
Mean			38.8			35.1
Rye 1	0.236	0.065	27.5	0.132	0.027	20.5
Rye 2	1.294	0.289	22.3	0.989	0.228	23.1
Rye 3	3.153	1.046	33.2	2.954	0.849	28.7
Mean			27.7			24.1
Barley 1	0.201	0.037	18.4	0.124	0.024	19.4
Barley 2	1.579	0.590	37.4	1.328	0.511	38.5
Barley 3	3.613	1.213	33.6	3.404	0.993	29.2
Mean			29.8			29.0
Soybeans 1	0.188	0.100	53.2	0.127	0.073	57.5
Soybeans 2	1.344	0.777	57.8	1.124	0.758	67.4
Soybeans 3	3.309	2.001	60.5	2.838	1.593	56.1
Mean			57.2			60.3

(**b**) *Columns and suppressor.*—Dionex AS4A, 15 μm particle size, 25 cm × 4 mm id analytical column; AG4A guard column and an anion micromembrane suppressor unit (AMMS-II). Eluent flow rate, 2.0 mL/min at ca 860 psi. Suppressor solution, 25 mM H_2SO_4; flow rate, 10 mL/min.

(**c**) *Eluent.*—1.5 mM Na_2CO_3–1.7 mM $NaHCO_3$ in deionized water, pH 10.1.

(**d**) *Sample pretreatment cartridge.*—On Guard-AG (Dionex No. 39637) or MaxiClean IC-Ag (Alltech Associates, Inc., Deerfield, IL) cartridges containing cation resin impregnated with Ag^+ for removal of excess bromide, preconditioned by washing with 15–20 mL deionized water.

(**e**) *Glassware cleaning protocol.*—Glassware (beakers, flasks, tubing, gas washing bottles, etc.) were soaked initially in 10–20% HCl for 2 h on a steam bath and then rinsed 6–8 times in deionized water. The condenser was rinsed with HCl solution and water. After each determination, beakers and flasks were soaked in dilute HCl while on the steambath and then thoroughly rinsed with water. Other glassware were rinsed with water only. Reagent blanks were run periodically to monitor apparatus cleanliness.

(**f**) *Phosphine generation apparatus.*—The phosphine gas generation assembly (Figure 1) consists of (A) 50 mL addition funnel with ℸ 24/40 joints, (B) 500 mL 3-neck round-bottom reaction flask with ℸ 24/40 joints and heating mantle, (C) nitrogen sweep inlet adapter with ℸ 24/40 joint and tube shortened to 85 mm, (D) Allihn condenser with a 200 mm jacket and ℸ 24/40 joints, (E) 240 mm connector tube with ʃ 18/9 spherical joint and ℸ 24/40 joint at opposite ends and a 60° bend at midpoint (custom made), (F) 160 mm connector tube with ʃ 18/9 ball and socket joint at opposite ends (custom made), (G) 75 mm straight outlet tube with ʃ 18/9 joint, and (H)

Table 2. Product composition[a] and phosphine recovery

Product	Moisture, %	Protein, %	Oil and fat, %	Carbohydrates, %	Crude fiber, %	Mineral, %	Recovery, %	
							Range	Mean
Wheat	13.4 (12.8)[b]	12.1	1.9	69.0	1.9	1.7	17.4–50.2	30.1
Barley	14.9 (9.3)	10.0	1.5	66.7	4.4	2.5	19.4–38.5	29.0
Corn	13.0 (11.3)	10.0	4.5	69.1	2.2	1.2	38.4–51.9	44.0
Oats	13.3 (9.9)	10.2	4.9	58.2	10.3	3.1	31.1–42.0	35.1
White rice	12.2 (14.0)	8.4	1.8	64.7	8.9	5.0	56.4–67.3	59.4
Rye	13.4 (11.5)	11.5	1.7	69.5	1.9	2.0	20.5–28.7	24.1
Soybeans[c]	10.0 (10.3)	34.1	17.7	33.5	4.9	4.7	57.5–67.4	60.3

[a] Data from reference 28.
[b] Values in parentheses are moisture contents determined by AOAC Method **925.10**.
[c] Data derived from reference 29.

125 mL gas washing bottles with $ 18/9 joints and adjustable-length dispersion tube [via polytetrafluoroethylene (PTFE) sieve] with a coarse fritted tip positioned ca 4 mm from the bottom. The assembly was secured with 4 size 18 metal pinch clamps at the $ 18/9 joints and 5 stainless steel spring wire clamps at the T 24/40 joints. All components were obtained from Kontes, Vineland, NJ. Assembled apparatus was maintained and operated in a well-ventilated hood with a 70 cm length of Tygon tubing directed to the rear of the hood from outlet G.

Reagents

(**a**) *Hydrochloric acid and bromine.*—AR grade, Mallinckrodt, Inc., Paris, KY.

(**b**) *Sulfuric acid.*—J.T. Baker, Instra-analyzed reagent, Phillipsburg, NJ.

(**c**) *Aluminum phosphide (Phostoxin).*—Degesch America, Inc., Weyers Cave, VA. Product in tablet form (3 g) containing 55% aluminum phosphide, 41% ammonium carbamate, and 4% paraffin.

(**d**) *Deionized water.*—Barnstead, Division of Sybron Corp., Boston, MA.

(**e**) *Antifoaming agent.*—Anti-Foam A concentrate No. A5633, Sigma Chemical Co., St. Louis, MO.

(**f**) *Potassium dihydrogen phosphate (KH_2PO_4).*—No. 60219, >99.5%, Fluka Chemical Corp., Ronkonkoma, NY.

(**g**) *Sodium carbonate and sodium bicarbonate.*—Liquid chromatography grade, Fisher Scientific Co., Itasca, IL.

Standard Solutions

A stock standard solution of KH_2PO_4 was prepared in deionized water at a concentration of 100 µg phosphorus/mL. Working standard solutions in deionized water were prepared weekly from stock standard solution at concentrations of 0.30–8.0 µg P/mL. A 50 µL volume of the appropriate working standard solution was injected into the ion chromatograph.

Nonsuppressed IC System

The system (24) consisted of a Waters 590 programmable pump, a Model 431 conductivity detector (Waters, Milford, MA), a Rheodyne Model 7125 valve (Rheodyne, Inc., Cotati, CA), and a HP-3396 Series II integrator (Hewlett-Packard Co., Avondale, PA). The system was passivated with 6N HNO_3 and washed thoroughly with deionized water prior to insertion of analytical and guard columns. Injection volume, 100 µL; detector sensitivity, 5 µS full scale; column temperature, ambient. A Waters IC-PAK A HR, 6 µm particle size, 75 × 4.6 mm analytical column was used with an IC-PAK anion Guard PAK insert in a precolumn module; eluent flow rate was 1.0 mL/min at ca 650 psi. Eluent preparation consisted of a borate/gluconate concentrate containing 16 g sodium gluconate, 18 g boric acid, and 25 g sodium tetraborate decahydrate dissolved in 500 mL deionized water in a 1 L volumetric flask; 250 mL glycerin was added, and the mixture was taken to volume and mixed. All chemicals were obtained from Fluka Chemical Corp. This concentrate can be stored in a refrigerator for 6 months. The working borate/gluconate eluent, pH 8.5, was prepared by adding 20 mL borate/gluconate concentrate, 20 mL *n*-butyl alcohol (Fluka) and 120 mL acetonitrile (Mallinckrodt) to a 1 L volumetric flask containing ca 500 mL deionized water. The mixture was diluted to volume with water, mixed, and passed through a 0.45 µm Nylon-66 membrane filter (AMF/CUNO, Inc., Meriden, CT). The observed conductivity was ca 270 µS. A stock standard solution at a concentration of 136.7 µg P/mL was prepared from KH_2PO_4 (0.854 g) in 100.0 mL deionized water. Working standard solutions in deionized water at concentrations of 0.30–2.80 µg P/mL were prepared.

Methods

(**a**) *Preparation of fortification standard.*—One 3 g Phostoxin tablet (Degesch America, Inc.) was ground to a powder with a mortar and pestle in a ventilated hood, with operator wearing latex gloves. The ground material (in 2 Petri dishes) was exposed to a current of air for ca 2 weeks to reduce the concentration of aluminum phosphide. To maintain stability, the partially depleted Phostoxin tablet material was stored at ambient temperature over silica gel in a polycarbonate desiccator.

(**b**) *Fortification of products.*—Partially depleted Phostoxin material (**a**) was diluted 1 + 10 with talc, mixed thor-

Table 3. Recovery of phosphine from whole grains and soybeans with laboratory-fumigated phosphine residues (calculations based on standard carried through procedure)

Product	Laboratory-fumigated phosphine, ppm	Phosphine added[a], ppm	Phosphine recovered, ppm	Recovery, %
Wheat 1	0.0018	0.552	0.135	24.5
Wheat 1	0.0956	1.062	0.493	46.4
Wheat 2	0.0632	1.275	0.501	39.3
Wheat 2	0.0632	2.046	1.108	41.1
Barley 1	0.0734	1.275	0.231	18.1
Barley 1	0.0734	2.406	0.809	33.6
Corn 1	1.691	1.371	1.255	91.5
Corn 1	1.691	2.288	1.835	80.2
Oats	1.391	1.104	0.288	26.1
White rice	1.053	0.553	0.404	73.1
Rye 1	0.178	1.136	0.215	18.9
Rye 2	0.389	0.553	0.209	37.8
Soybeans	1.602	1.062	0.828	78.0

[a] As diluted Phostoxin containing 0.2132% phosphine.

oughly, and stored over silica gel desiccant when not in use. This standard preparation was assayed by the proposed digestion oxidation step followed by colorimetry (15). Assays performed over a period of 42 weeks (n = 26) gave a mean concentration equivalent to 0.2132% PH_3, with a relative standard deviation (RSD) of 3.06%. Accurately weighed quantities of this diluted fortification standard were added to 100 g portions of each product in the reaction flask.

(**c**) *Preparation of phosphine-fumigated products.*—A 3 g Phostoxin tablet containing 55% aluminum phosphide was powdered and divided between 2 open 85 mm id Petri dishes, each placed in a separate polycarbonate desiccator having a volume of ca 10 L. An open 4 fl. oz. bottle containing a saturated solution of NaCl was placed in each desiccator for humidity control (75.3% relative humidity). Five 100 g portions of product (grains or soybeans) were weighed directly into 250 mL beakers or onto fluted filter paper (S & S No. 588, 185 cm) set in 250 mL beakers and placed in the desiccators. The product was exposed to liberated PH_3 gas for 1 week to obtain PH_3 levels of 1 ppm or greater. Lower PH_3 levels (ca 0.2 ppm or less) required up to 2 weeks exposure. On completion of the desired exposure time, the 100 g portions of product were transferred to 160 mL glass milk dilution bottles having a plastic screw cap (30 mm id) with Teflon liner and stored at –10°C until ready for analysis.

(**d**) *Assay procedure.*—One hundred grams product and 200 mL 10% H_2SO_4 were added to the reaction flask. One milliliter bromine (Br_2) and 75 mL deionized water were added to each of the 2 gas-washing bottles. All glass joints were sealed with water and clamped to prevent gas leakage. Nitrogen gas was swept through the apparatus at 95–100 mL/min for 30 min at ambient temperature. Then, heat was applied for 2 h to a maximum temperature of 95°C. For high-fat products such as soybeans, 2–3 drops antifoaming agent were added to eliminate excess foaming in the reaction flask. After heating, contents of the gas-washing bottles were transferred to a 600 mL beaker and combined with deionized-water rinses from the bottles and attached glass tubing. The aqueous mixture was concentrated to ca 5 mL on a hot plate in a fume hood and then cooled to ambient temperature. About 25 mL water was added, and the beaker contents were mixed. The pH of the product extract was adjusted to 3.0 ± 0.1 with 0.5M NH_4OH, quantitatively transferred to a 50.0 mL volumetric flask, and diluted to volume. A 1.5–2 mL portion of the diluted product extract was passed through a silver ion (Ag^+) cartridge attached to the loading syringe prior to injection of 50 µL into the ion chromatograph. The Ag^+ cartridge was washed with three 5 mL portions of water immediately after each injection to allow reuse 4–6 times. Excessive resistance by the cartridge material during subsequent injections indicated the necessity for replacement. Fifty microliters of the appropriate working standard solution having a chromatographic response approximating that of the sample solution was injected into the ion chromatograph. A 3–5 mL portion of the diluted product extract (based on fortification level) was removed for the molybdenum blue colorimetric procedure (15) in the comparative study (Table 1). Reagent and product blanks were included in all assay and recovery determinations. Standard curves were prepared to verify linearity of response for the 2 procedures.

(**e**) *Calculations.*—The amount of phosphine (µg/g or ppm) was calculated by direct comparison of sample peak area response obtained with that of the appropriate KH_2PO_4 working standard solution (as phosphorus) of similar magnitude as follows:

$$PH_3,\ \mu g/g\ (ppm) = \frac{R_u \times C \times DF \times 1.097}{R_s \times W}$$

where R_u and R_s = peak area responses of sample and standard solutions, respectively; C = concentration of working standard solution (µg P/mL); DF = dilution factor (mL); 1.097 = molecular weight conversion factor (PH_3/P); W = weight of sample portion (100 g).

Table 4. Recovery of phosphine from whole grains and soybeans with laboratory-fumigated phosphine residues (calculations based on known concentrations of standard)

Product	Laboratory-fumigated phosphine, ppm	Phosphine added[a], ppm	Phosphine recovered, ppm	Recovery, %
Wheat 1	0.0018	0.527	0.135	25.6
Wheat 1	0.0956	1.156	0.493	42.6
Wheat 2	0.0632	1.215	0.501	41.2
Wheat 2	0.0632	2.347	1.108	47.2
Barley 1	0.0734	1.215	0.231	19.0
Barley 1	0.0734	2.347	0.809	34.5
Corn 1	1.691	1.422	1.255	88.3
Corn 1	1.691	2.232	1.835	82.2
Oats	1.391	1.185	0.288	24.3
White rice	1.053	0.535	0.404	75.5
Rye 1	0.178	1.032	0.215	20.8
Rye 2	0.389	0.535	0.209	39.1
Soybeans	1.602	1.156	0.828	71.6

[a] As diluted Phostoxin containing 0.2132% phosphine.

Recoveries of phosphine were obtained by calculating the ratio of PH_3 recovered from the product fortified with diluted AlP material to that recovered from an equivalent amount of diluted AlP material carried through the procedure. Recoveries were also calculated from the known concentration of PH_3 in the diluted AlP material (0.2132% PH_3) added to the product. In addition, the recoveries from products with laboratory-fumigated PH_3 residues were obtained by subtracting the amount of PH_3 originally present from the total quantity of PH_3 found in a similar sample portion fortified with the diluted AlP preparation.

Results and Discussion

Phosphine Recovery: Colorimetric and IC Procedures

Comparative recovery data are shown in Table 1. For each of the 2 procedures, separate portions of product were fortified with diluted Phostoxin powder at 3 different levels. The added material was placed in direct contact with the product prior to introduction of the acid media. Quantities added ranged from 0.0124 to 0.4158 mg PH_3/100 g product (0.124–4.158 ppm PH_3). Recoveries in Table 1 were calculated by direct comparison of the response observed for an equal amount of PH_3 (as Phostoxin) carried through the procedure in the absence of product. Product blanks carried through the procedure prior to fortification indicated that all products were phosphine free.

The data show no significant differences in recoveries of phosphine when using either colorimetry or IC as the determinative step. Both procedures are based on the detection of phosphate ion, an oxidation product of phosphine. However, the colorimetric procedure provided slightly higher values for 5 of the 7 products, perhaps because of the less specific nature of the molybdenum blue reaction (25), making it subject to interferences from other ionic species that may be present in the concentrated extract. The colorimetric procedure (15) has a limit of detection (LOD) of 10 ppb phosphine and a limit of quantitation (LOQ) of about 25 ppb phosphine. Both are comparable with the limits of the proposed IC procedure. Although both procedures are equally sensitive, the IC approach requires less manipulation.

Mean recoveries with either detection step were low (<60%), ranging from 24.1% for rye to 60.3% for soybeans. Recoveries were consistent within the 3 fortification levels, with only corn and wheat exhibiting a gradual increase in recovery with an increase in the amount added. The increase shown for wheat agrees with results of Nowicki (20) for Phostoxin-fortified wheat but not with those of Scudamore and Goodship (22), both of whom analyzed laboratory-fumigated phosphine residues using acid reflux of the product followed by GC detection of phosphine. Scudamore and Goodship (22) fortified samples by introducing known amounts of phosphine gas into the reaction flask containing liberated phosphine. Their recoveries were consistently high, ranging from 87.0 to 95.6%. Bruce et al. (14) reported recoveries of 66.7–101.0% from wheat fortified with Phostoxin or phosphine in carbon disulfide and analyzed by colorimetry after acid reflux and oxidation with bromine water.

The inherent low recoveries obtained with the proposed procedure are related to substrate interaction in the acid medium. Similar findings have been reported for wheat at the 0.05–1.5 ppm range (20) and for sugar cane at the 0.01–1.0 ppm range under acid reflux conditions (26). Phosphine uptake by grains and cereal products has been studied under test chamber or simulated storage conditions (8, 18). The parameters examined include particle size, temperature, exposure time, and chemical composition. The terms chemisorption and physisorption have been used to describe the binding of phosphine to cereal substrate (8). Chemisorption refers to an irreversible, nonrecoverable, and substrate-specific sorption that increases with temperature and may involve the formation of covalent bonds between phosphine and substrate. An increase in temperature has been demonstrated to be a significant

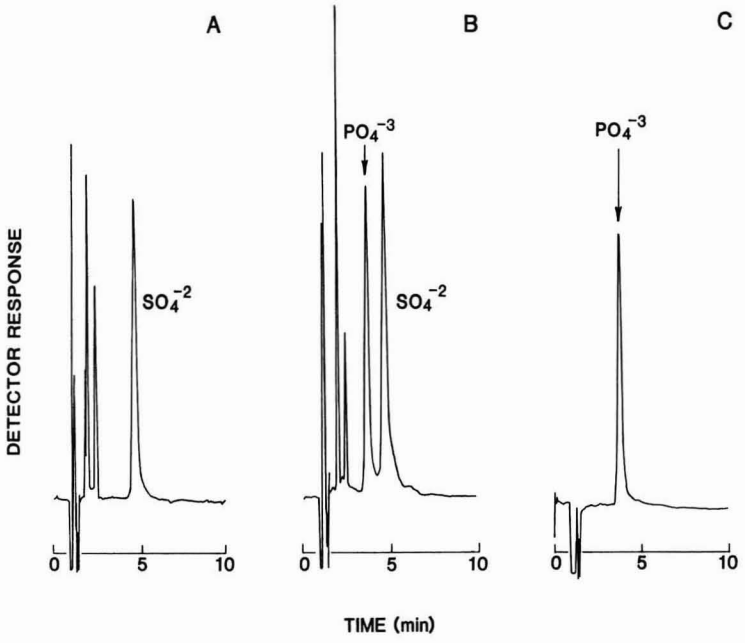

Figure 2. Ion chromatograms representing (A) extract from 100 g wheat blank; (B) extract from 100 g laboratory-fumigated wheat, 0.629 ppm P (0.690 ppm PH_3); and (C) phosphate standard, 1.000 μg P/mL. Conductivity range, 3 μS.

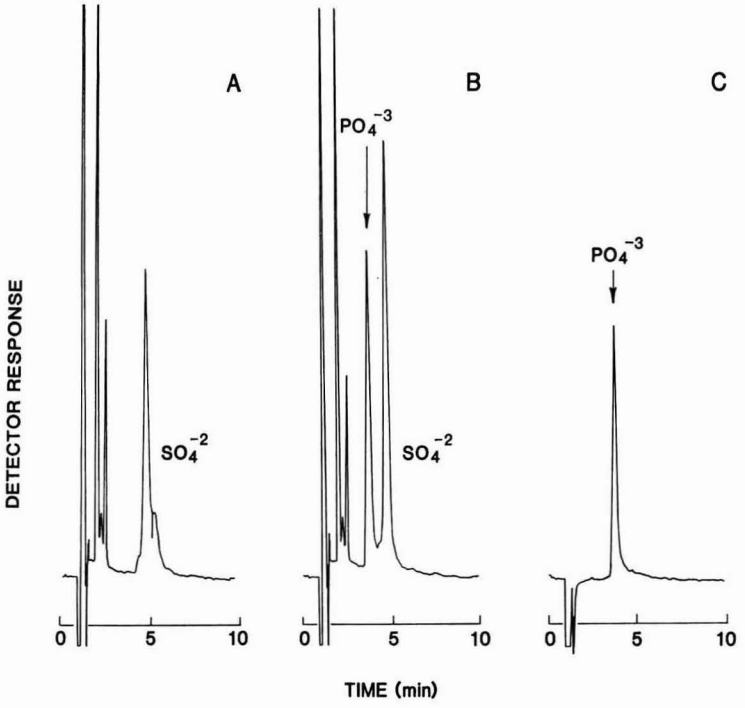

Figure 3. Ion chromatograms representing (A) extract from 100 g barley blank; (B) extract from 100 g laboratory-fumigated barley, 0.609 ppm P (0.668 ppm PH_3); and (C) phosphate standard, 1.000 μg P/mL. Conductivity range, 3 μS.

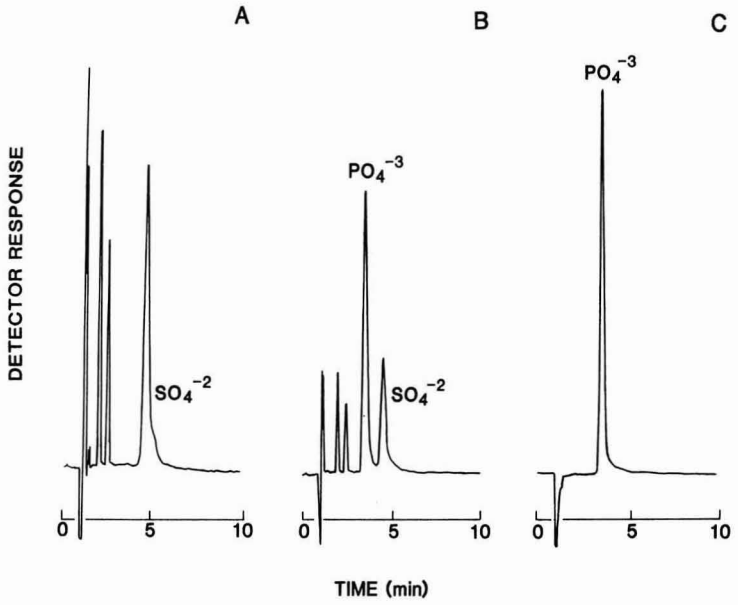

Figure 4. Ion chromatograms representing (A) extract from 100 g corn blank; (B) extract from 100 g laboratory-fumigated corn, 1.605 ppm P (1.761 ppm PH_3); and (C) phosphate standard, 4.000 μg P/mL. Conductivity range, 10 μS.

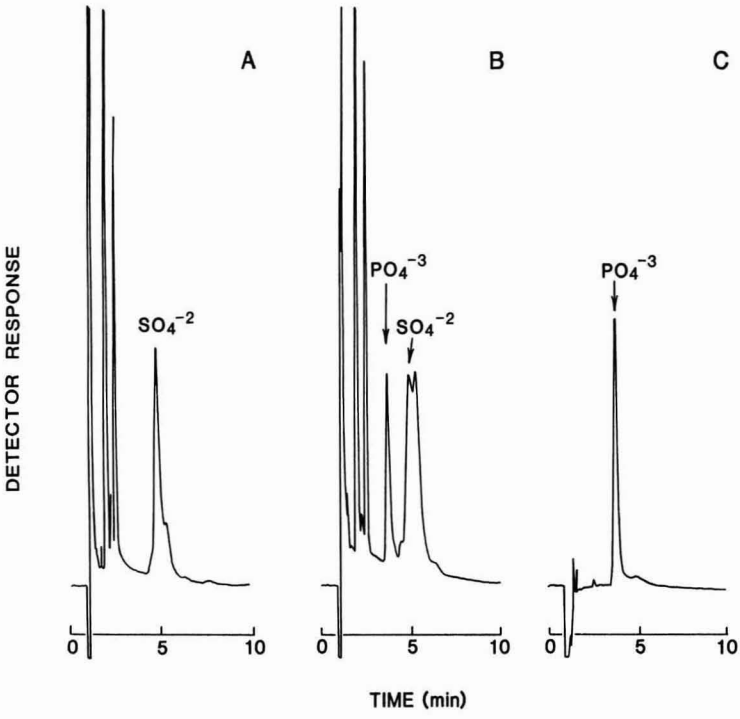

Figure 5. Ion chromatograms representing (A) extract from 100 g rye blank; (B) extract from 100 g laboratory-fumigated rye, 0.355 ppm P (0.389 ppm PH_3); and (C) phosphate standard 1.000 μg P/mL. Conductivity range, 3 μS.

Table 5. Precision data for whole grains with fortified and laboratory-fumigated phosphine PH_3 residues

Residue type	Product	Mean PH_3 fortification or fumigation level, ppm	RSD, %
Fortified	Wheat	0.679 ($n = 5$)	4.66
	White rice	0.791 ($n = 5$)	3.08
Fumigated	Corn	1.309 ($n = 5$)	4.45
	White rice	0.819 ($n = 4$)	1.78

factor for phosphine uptake (8). Conversion to other phosphorus-containing compounds has been demonstrated to explain the loss of phosphine after exposure. Experiments using wheat, flour, and whole insects (*Tribolium confusum* Duv.) exposed to radio-labeled phosphine derived from aluminum phosphide (^{32}P) have shown the presence of water-soluble phosphorus residues (phosphite, hypophosphite, and phosphate) isolated by paper chromatography (27). Physisorption is a temporary phenomenon that is non-substrate-specific and can be reversed by increased temperature, solvent treatment, or prolonged aeration. Both processes may be involved and related to the losses observed in this study.

Recovery versus Product Composition

Previous studies have shown that cereal products with high moisture, protein, and mineral contents take up higher levels of phosphine, presumably through chemisorption. Our study shows that chemical composition and the physical nature of the product are the main factors that affect recovery of phosphine generated from Phostoxin under acid reflux conditions. Table 2 provides comparative data for product composition and recoveries of phosphine from each product. Recoveries observed are due to matrix interaction and do not appear to be strongly influenced by any one component of composition. Moreover, certain composition factors may be interrelated. The possibility that the parent species, aluminum phosphide, may be interacting with the substrate cannot be excluded.

Most cereal grains have protein levels between 10 and 12%. Mean recoveries from cereal grains were generally low and less than 50%. An exception was white rice, which had the lowest protein content of 8.4% but gave the highest mean recovery, 59.4% (range, 56.4–67.3%). In a separate study, we observed 15% loss of phosphine fortified at the 0.15 ppm level in the presence of 0.76 g solubilized protein (gelatin). This suggests a phosphine–protein interaction under acid reflux conditions. The oil and fat contents of 4 of the 6 grains were less than 2%. Mean recoveries for these 4 grains were between 24 and 35%, except rice for which mean recovery was 59.4%. Both corn and oats with oil and fat levels of 4.5 and 4.9%, respectively, showed slightly higher recoveries of 35–45%. The carbohydrate contents of the grain products ranged from 58.2 to 69.5%. Mineral content has been suspected as the cause of low phosphine recovery under test chamber and storage conditions for certain cereal substrates (8). The mineral and fiber contents of the products in the current study may play a minor role in recovery. However, white rice, which had the highest mineral and fiber contents, also gave the highest recovery.

The legume (soybeans) included in the study was clearly an exception, giving results that are in contrast with those from cereal grains. Soybeans gave the highest mean recovery and range for phosphine. The chemical composition of soybean is different from those of cereal grains, being high in protein (34.1%) and oil and fat (17.7%), but low in carbohydrate (33.5%).

The physical nature of the bran or seed coat of a product also can affect phosphine recovery under acid reflux conditions. Highest mean recoveries and ranges were obtained for rice, corn, and soybeans. The smooth surface of these products may be more impervious to phosphine, thereby minimizing chemisorption. High recoveries from these products could be related to reduction in surface reaction sites.

Phosphine Recovery: Products with Laboratory-Fumigated PH_3 Residues

Samples representing each of the products were exposed to phosphine under controlled laboratory conditions to determine whether recoveries of phosphine introduced by fortification with Phostoxin are affected by the presence of fumigated phosphine residues on the various cereal grains and whether chemisorption is diminished. These laboratory-fumigated products were also used to evaluate method precision. Exposure times varied from 1 to 2 weeks. After exposure, the portions were stored at –10°C prior to analysis. The longer time required to achieve lower phosphine levels may be due to faster chemical sorption (non-recoverable PH_3) occurring initially with less physical uptake, in addition to other factors such as high moisture environment and dissipation of available PH_3 from the "closed system." Products were assayed "as is" and after fortification with diluted Phostoxin.

The data in Table 3 includes levels of laboratory-fumigated phosphine found after exposure and recoveries at various fortification levels. Equivalent quantities of diluted Phostoxin material added to the product were also carried through the procedure to determine recovery. Phosphine levels ranged from 0.0018 ppm for one wheat sample to 1.691 ppm for corn samples. Recoveries were lowest for barley (18.1%) and highest for corn (91.5%). Low recoveries were obtained for wheat, barley, oats, and rye and were similar to those obtained with phosphine-free products. By contrast, corn, rice, and soybeans yielded the highest recoveries with phosphine-free products and even higher values when residual phosphine is present. For example, recoveries for corn were 38.4–51.9% for phosphine-free products and 80.2–91.5% for similar samples with laboratory-fumigated residues. For these 3 products, irreversible

Table 6. Determination of phosphine in miscellaneous food products

Product No.	Product	Phosphine found, ppm[a]
1	Instant noodles A	4.06
2	Instant noodles B	0.68
3	Instant oatmeal	ND[b]
4	Oatmeal A	0.018
5	Oatmeal B	0.18
6	Cassava root	ND
7	Taro root	ND
8	Basmati rice	ND
9	Tea	ND

[a] Single determinations.
[b] ND = not detected.

chemisorption appears to be reduced by previous exposure to phosphine. Higher recoveries observed in both situations may be due partly to the more impervious nature of the product surface. The data in Table 4 represent levels of incurred phosphine but with fortification quantities based on diluted Phostoxin at a concentration equivalent to 0.2132% phosphine. Recoveries calculated by either technique (Tables 3 and 4) were similar and within ± 1–3%. Only one wheat sample and soybeans differed by approximately 6%.

Ion Chromatograms

Figures 2 through 5 are typical sets of ion chromatograms of product extract blank, product extract with laboratory-fumigated phosphine residue, and phosphate reference standard. Levels of laboratory-fumigated phosphine ranged from 0.389 ppm for rye to 1.761 ppm for corn. Retention times for phosphate anion varied from 3.5 to 3.8 min over a 2-month period for these samples. The phosphate anion peak was well resolved from early eluting anions and the later eluting sulfate anion. Sharp responses observed between 1.5 and 2.5 min are due to residual bromide and reagent impurities or oxidation by-products, including chloride and nitrate.

Optimization of Conditions

Various parameters were optimized during method development, notably those related to the acid reflux step used to liberate phosphine. The basic approach has been described previously (14). Accurately weighed portions of diluted Phostoxin (25–100 mg) and/or Phostoxin-fortified wheat were used for these comparative studies. The variables and conditions examined were (*1*) water condenser temperature, 10°–25°C; (*2*) reflux media, water versus 5–10% H_2SO_4; (*3*) reaction flask temperature, 25°–95°C; (*4*) reaction flask heating time, 1, 2, and 4 h; (*5*) reaction flask agitation, N_2 sweep versus magnetic stirring; (*6*) nitrogen sweep rate, 25–100 mL/min; (*7*) fortification media, free powder versus encapsulation; (*8*) number of bromine traps; (*9*) glass joint sealant, water versus silicone lubricant; (*10*) media and pH of extract (prior to IC), water versus eluent, pH 2–5; and (*11*) injection volume of extract, 25–100 µL.

Use of subambient condenser temperature and use of water as reaction medium were examined as a means of reducing or eliminating a sulfate response in the ion chromatograms. This peak was derived from SO_2 generation and carryover using aqueous sulfuric acid. When using 10% H_2SO_4, no difference in sulfate response was observed by lowering the condenser temperature. Although sulfate response was absent with water as the medium, a wheat sample fortified at 0.218 ppm PH_3 gave a recovery of 7.84% versus 36.7% with 10% H_2SO_4. Highest recoveries of phosphine were obtained with reaction flask at ambient temperature for 30 min followed by an increase to 95°C for 2 h in a 10% H_2SO_4 medium with a nitrogen sweep rate of 95–100 mL/min.

Fortification involved addition of diluted Phostoxin powder directly to reaction flask containing 100 g product prior to acidification with 200 mL 10% H_2SO_4. A brief comparative study was conducted regarding the Phostoxin addition sequence during a later stage of this work. For example, when Phostoxin (2.24 ppm PH_3) was added to wheat in the above manner, recovery was 35.7% (0.80 ppm PH_3). When Phostoxin at this same level was added to the flask first, followed by the product and aqueous sulfuric acid, recovery was 47.8% (1.07 ppm PH_3). On the basis of these results, it may have been preferable to fortify the product by the latter approach.

Use of gelatin capsules to contain the diluted Phostoxin to fortify 100 g wheat resulted in 37.7% reduction in recovery. This additional loss may have been due to phosphine reacting with the capsule's protein material. Use of a second bromine water trap increased recoveries by approximately 10%. The addition of a trap preceding the bromine water traps and containing either 3% H_2O_2 or 1M NaOH did not reduce response for sulfate ion. Use of silicone lubricant as a joint sealant for the generation apparatus produced a doublet in the phosphate response. Therefore, water was used for this purpose. Use of water also facilitated cleaning of glass components between determinations.

During the initial phase of this work, sample extracts were diluted with deionized H_2O prior to IC. The pH of these diluted extracts varied: rice, pH 2.3; rye, pH 1.8; barley, pH 2.0; wheat, pH 2.1; and, soybean, pH 2.0. In the absence of product matrix, the pH of the diluted extract was 3.0. The required nitrogen sweep rate of 95–100 mL/min increased the quantity of product-related volatile acids carried over, thus lowering extract pH. Extracts with pH values ranging from 2.5 to 2.0 reduced the phosphate anion response from 9 to 44%, respectively. The water diluent was briefly replaced with the eluent (1.5 mM Na_2CO_3–1.7 mM $NaHCO_3$). This medium provided a sample extract pH of about 3, which minimized suppression of the phosphate response. However, when eluent-diluted extract was passed through a Ag^+ cartridge to remove excess bromide, a precipitation reaction occurred that impaired flow. This blockage may have been due to formation of insoluble silver salts (i.e., silver carbonate). It was therefore necessary to revert to water as the diluent and to adjust the pH of the extract to 3.0 with 0.5M NH_4OH prior to bromide removal and IC. Without use of a Ag^+ cartridge, all anions in the diluted extract chromatogram are masked by the presence of bromide. The Ag^+ car-

tridges can be used repeatedly (4–6 times), provided they are flushed with three 5 mL volumes of deionized water between samples. When the cartridge resisted solvent flow because of partial obstruction, it was replaced.

Injection volumes of 25, 50, and 100 µL were compared with respect to linearity over a 30-fold range in concentration (0.1–3.0 µg P/mL, 5 points) and repeatability of peak response (peak width at 5% peak height) for the 5 phosphate standards. Correlation coefficient (r) values were 0.9999 for each volume, and relative standard deviation (RSD) values were 9.74, 2.96, and 10.43% for the 3 injection volumes, respectively. An injection volume of 50 µL was determined to be the most suitable.

Method Performance

Precision of the proposed procedure was assessed by replicate analysis of products fortified with diluted Phostoxin and of products with laboratory-fumigated phosphine residues (Table 5). Five 100 g portions of wheat and white rice were each fortified with identical weights of diluted Phostoxin. Mean levels of phosphine found were 0.679 ppm PH_3 (RSD = 4.66%) and 0.791 ppm PH_3 (RSD = 3.08%), respectively. Five 100 g portions of corn and four 100 g portions of white rice contained in fluted filter paper and placed in 250 mL beakers were exposed to phosphine under laboratory-controlled conditions. Mean levels of phosphine were 1.309 ppm PH_3 (RSD = 4.45%) for corn and 0.819 ppm PH_3 (RSD = 1.78%) for white rice. Analysis of replicate portions ($n = 5$) of wheat and rye exposed to phosphine contained in 250 mL beakers (without fluted filter paper) gave considerably higher RSD values (15–21%) due in part to the nonuniform exposure and the physical nature of these grains.

Replicate determinations ($n = 9$) of the diluted Phostoxin material (0.2132% PH_3) were performed over 3 months with sample portions varying from 25.1 to 110.1 mg. RSD for these analyses was 4.90%, indicating that there was no significant physical loss of phosphine during acid reflux and that this reference material was stable when stored at ambient temperature in a desiccator.

Estimated LOQ values (S/N, 10:1) for phosphine in 100 g product were 27.5 ppb for chemically suppressed conductivity IC system and 55 ppb for non-chemically suppressed system. Estimated LOD values (S/N, 4:1) were 10 ppb PH_3 for chemically suppressed IC system and 20 ppb PH_3 for non-chemically suppressed system. Both IC systems performed equally well and both were suitable for detection and quantitation of low levels of phosphate ion derived from phosphine. The chemically suppressed IC system provided slightly better chromatographic peak shape and resolution from extraneous responses and exhibited an overall sensitivity advantage of 2.

Phosphate standard response over the concentration range 0.3–8.0 µg P/mL (5 points) was linear, with r values varying from 0.9985 to 1.0000 ($n = 7$; mean, 0.9990). Response of phosphate ion derived from diluted Phostoxin carried through the procedure as a routine performance check displayed adequate linearity (r = 0.9982) over the concentration range 1.0–8.0 µg P/mL (3 points).

Applications

A recently analyzed sample of bulk wheat was found to contain 0.053 ppm PH_3. In addition to being used to monitor stored grain products and soybeans, the proposed procedure has been applied to other samples (Table 6). Instant noodles (products 1 and 2), analyzed by initially using a semiquantitative procedure based on acid hydrolysis and a Dräger indicator tube, were shown to contain 8 and 1.1 ppm PH_3, respectively (30). The time interval between analysis by this procedure and analysis by IC was approximately 3 months. Hence, loss of residue may have occurred during the interval. The presence of these residues could be due to inadequate aeration of the wheat ingredient. Products 3 and 6–9 had been stored in a warehouse near other products previously fumigated with phosphine. The 2 oatmeal samples (products 4 and 5) had been exposed to phosphine from a perforated pint jar containing Phostoxin material determined to have a concentration equivalent to 2.94% PH_3. The 4 finished food products with phosphine residues (Table 6) constitute violations of FAO/WHO and EPA tolerances of 0.01 ppm. By contrast, previous studies have shown that many consumer food products, if adequately packaged and aerated following exposure to phosphine under controlled conditions, have no measurable residue levels (<0.003 ppm; 31). Because of the increased worldwide use of phosphine as a fumigant for staple commodities, it is imperative that more attention be directed toward detection of phosphine and phosphide residues in these products and their potentially harmful affects to consumers. The IC method presented here would significantly aid in this task.

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RESIDUES AND TRACE ELEMENTS

Determination of Magnesium and Calcium in Foods by Atomic Absorption Spectrometry after Microwave Digestion: NMKL[1] Collaborative Study

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On the basis of results of the performed collaborative study, the 49th Annual General Meeting of the Nordic Committee on Food Analysis (NMKL) in The Faroe Islands, August 1995, approved this method to be printed and included in NMKL's collection of methods of analysis of foods. Eleven laboratories participated in an interlaboratory methods-performance (collaborative) study of a method for determining magnesium and calcium in foodstuffs by atomic absorption spectrometry (AAS) after wet microwave digestion. The study was preceded by a practice round of familiarization samples. The method was tested on 7 materials: 5 foods (apple, milk powder, minced fish, wheat bran, and chocolate cake) and 2 composite diets ranging in Mg content from 240 to 3900 mg/kg and in Ca content from 290 to 9300 mg/kg. The materials were presented to study participants as blind duplicates, and participants were asked to perform single determinations on each sample. Repeatability relative standard deviations (RSD_r) ranged from 1.9 to 4.9% for Mg and from 2.2 to 8.1% for Ca. Reproducibility relative standard deviations (RSD_R) ranged from 4.0 to 13% for Mg and from 5.9 to 23% for Ca. For Ca, lowest RSD_R values were found for samples with high concentrations of Ca (>3800 mg/kg sample) and with nitrate ion residues of <1.3% (w/v).

Magnesium and calcium are essential nutrients involved in bone and tissue formation. Analytical food laboratories need validated methods of analysis for determining these elements in foods in general. Validated methods for determining Mg and Ca may be found in the *Official Methods of Analysis* of AOAC INTERNATIONAL (1), but none seem to have been validated for a wide range of matrixes. For example, among the more modern methods using atomic absorption spectrometry, validated methods are available for drugs, cheese, and infant formulae but none for foods in general.

Flame atomic absorption spectrometry (FAAS) is still a much used analytical technique for determining elements in biological samples. Determinations are performed in sample solutions after digestion of the food material in acids. Microwave oven, wet digestion offers an alternative to traditional closed- and open-tube sample dissolution technique. Since the first description of the use of microwave radiation as an energy source in acid digestion (2), the technique has attracted considerable attention, and several successful methods have been described (3). However, digestion conditions must be established by taking into account individual analytical problems, that is, sample type, elements to be determined, and microwave system used.

The present method has been in use for several years at the Institute of Nutrition (Bergen, Norway). The method was selected by the Nordic Committee on Food Analysis (NMKL) to be evaluated in a collaborative study (repeatability and reproducibility) using judiciously chosen foodstuffs.

Prior to the collaborative study, the method was tested and optimized with respect to factors that could affect the trueness and reproducibility of the method, such as fat or carbohydrate content of sample to be digested, digestion programs for microwave ovens, concentration of lanthanum in the sample solution, ratio of acetylene and air in the flame, and concentration of nitric acid in the sample solutions to be measured by FAA.

Collaborative Study

A description of the method and an invitation to participate in the study were sent to 13 laboratories in Denmark, Finland, Norway, and Sweden. Eleven laboratories accepted and agreed

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[1] Nordic Committee on Food Analysis, Secretariat General, c/o National Veterinary Institute, Department of Food and Feed Hygiene, PO Box 8156, Dep. N-0033 Oslo, Norway.

to follow the method procedure and the time schedule of the study. The design, conduct, and interpretation of the study followed guidelines recommended by AOAC (4) and NMKL (5). Participating laboratories were from 4 countries and represent the food industry, commercial laboratories, universities, and government laboratories.

Study Materials

Each participant received 7 test materials: wheat bran, milk powder, and minced fish (materials obtained from the National Food Agency, Copenhagen, Denmark), 2 composite diet materials (diet D and diet F; obtained from the Swedish National Food Administration, Uppsala, Sweden), dried apple, and chocolate cake (produced at the Institute of Nutrition, Bergen, Norway). The composite diets were mixtures of different proportions of a number of foodstuffs, for example, meat, liver, potatoes, milk, and flour. These 2 diets were originally produced as reference materials for determination of metals for use in an interlaboratory study of a method for determination of lead, cadmium, zinc, copper, iron, chromium, and nickel (6). The expected values of Mg and Ca in the materials used in the study were obtained by analyzing 10 replicates per material (Table 1).

The homogeneity of the test materials was investigated by estimating within-container and between-container variations of the 7 study materials. The homogeneity test was performed by taking 5 subsamples, of 5 g, from each food sample. Two replicates of 0.25 g (according to the method) from each subsample were digested. In total, 10 replicates of each food sample were analyzed for Mg and Ca. Results were analyzed by 2-way analysis of variance (ANOVA) at the 5% level. Values of $p < 0.05$ were obtained for all test materials, indicating that test samples were homogeneous.

Protocol

Before the full collaborative study, participants were given the opportunity to become familiar with the method in a pretrial test. Test materials included 2 certified reference materials: wheat flour and oyster tissue, both purchased from the U.S. National Institute of Standards and Technology (Gaithersburg, MD). The oyster tissue material contained certified concentrations of Mg and Ca in the range 1000–2000 mg/kg. The wheat flour material contained between 100 and 500 mg/kg of the 2 elements. Nine laboratories reported results for the certified reference materials. All results for Mg concentration were acceptable and fell within the certified range. For Ca, only the results for the oyster tissue material having a high Ca concentration were acceptable. Several laboratories reported very low Ca results for the wheat flour material with a lower Ca concentration. The reason for these low results was most probably due to interference from nitrate ions in less diluted sample solutions. This fact was taken into account when elaborating the final text for the full collaborative study.

The 7 test materials of the collaborative study were all dry and packed in small plastic containers. They were presented to participants as blind duplicates, that is, as 14 randomly coded materials, but the fact that blind duplicates were included in the set of materials was not disclosed to the participants. Participants were asked to perform single determinations of the Mg and Ca concentrations of the materials according to the method described below and to report results in mg/kg on a dry weight basis. Participants were also asked to give information on the microwave oven used and the temperature program used, as well as to report the absorbance values obtained for the working standard solutions. All test samples were dried and had a moisture of 2 to 10%. The participants were asked to perform dry matter determinations on the test materials and report their values on dry weight basis.

Table 1. Types of materials included in the study and their expected magnesium and calcium concentrations (average of 10 independent decompsitions per sample)

Material	Expected values, mg/kg (dry weight)	
	Mg	Ca
Wheat bran	3900 ± 156	900 ± 30
Simulated diet (D)	636 ± 12	520 ± 10
Simulated diet (F)	600 ± 6	290 ± 15
Milk powder, freeze dried	820 ± 11	9300 ± 200
Minced fish, freeze dried	739 ± 13	4000 ± 200
Apple, dried	240 ± 7	300 ± 25
Chocolate cake, dried	270 ± 5	850 ± 40

METHOD

Field of Application

The method is applicable to quantitative determination of Mg and Ca in various types of foodstuffs, with the exception of oils, fats, and extremely fatty products. The method has been tested primarily on dry products but may, under certain conditions, be used for fresh samples. High residual concentrations of nitric acid in solutions to be measured by AAS using a flame of air and acetylene interfere with the determination of Ca.

Principle

Concentrated nitric acid and hydrogen peroxide are added to the weighed sample. The sample is digested in a microwave oven. Any commercially available microwave oven for laboratory use may be used. Lanthanum(III) oxide is added to standards and sample solutions to prevent interference from phosphate ions. The concentrations of Mg and Ca are determined by FAAS. The concentrations of the elements are calculated from standard curves.

Chemicals and Reagents

(**a**) *Concentrated nitric acid (HNO_3).*—65% Suprapur.

(**b**) *Nitric acid, 0.65% (w/v).*—Dilute 10 mL concentrated nitric acid (**a**) to 1000 mL with water.

(**c**) *Hydrogen peroxide (H_2O_2).*—30%, analytical quality.

(**d**) *Deionized and possibly filtered water.*—Specific resistance, >18 MΩ/cm.

(**e**) *Lanthanum(III) oxide (La_2O_3).*—For AAS.

(**f**) *Lanthanum solution, 5% (w/v).*—Weigh 14.66 g lanthanum(III) oxide (**e**) into 250 mL beaker, moisten with 10 mL

water, and cautiously add 62.5 mL concentrated HCl (**g**). Transfer to 250 mL volumetric flask and dilute to the mark with water. The solution will keep for 1 month.

(**g**) *Concentrated HCl.*—37%, analytical quality.

(**h**) *Magnesium standard.*—1000 mg/L in for example 2.5% (v/v) nitric acid (commercial standard solution).

(**i**) *Magnesium standard solution, 10 mg/L.*—Dilute 1 mL Mg standard (**h**) to 100 mL in a volumetric flask with 0.65% nitric acid (**b**). The solution will keep for 1 month.

(**j**) *Calcium standard.*—1000 mg/L in for example 2.5% (v/v) nitric acid (commercial standard solution).

(**k**) *Calcium standard solution, 100 mg/L.*—Dilute 10 mL Ca standard (**j**) to 100 mL in a volumetric flask with 0.65% nitric acid (**b**). The solution will keep for 1 month.

Apparatus, Equipment, and Gases

(**a**) *Analytical balance.*

(**b**) *Microwave oven.*—CEM microwave sample preparation system MDS-81D (maximum initial effect, 750 W) with capping station, rotational table, and 120 mL digestion containers capable of withstanding a pressure of 830 kPa.

(**c**) *Dispenser.*

(**d**) *Glassware.*—25, 50, 100, and 250 mL volumetric flasks; 250 mL beakers; 10 and 100 mL measuring cylinders.

(**e**) *Polyethylene tubes.*—10 mL.

(**f**) *Polyethylene tubes with screw caps.*—15 and 50 mL.

(**g**) *Automatic pipets with tips.*

(**h**) *Atomic absorption spectrometer.*

(**i**) *Computer calculation program.*—AA WinLab, Instrument Control Software (Perkin-Elmer Corporation).

(**j**) *Acetylene.*—Welding quality.

(**k**) *Air.*

Procedure

(**a**) *Preparation of test sample.*—The procedure for determining dry matter content involves freeze-drying and thermal drying at 105°C for 12 h until constant weight.

Weigh into the digestion container an amount of homogeneous sample corresponding to 0.2–0.25 g dry material. Each digestion series must contain 2 reagent blanks, that is, acids only without sample materials. If possible, include certified reference materials containing Mg and Ca in amounts corresponding to those found in samples to reveal systematic or random errors.

Note: The following 2 sections should be regarded as examples. Digestion programs and amounts of acids will vary with different digestion systems.

(**b**) *Digestion.*—Add 4 mL concentrated nitric acid (**a**) to each container. Seal the containers in the capping station. Place the carousel with the digestion containers in the microwave oven and start the program: S1, 250 W, 1.00 min; S2, 0 W, 1.00 min; S3, 250 W, 5.00 min; S4, 400 W, 5.00 min; and S5, 650 W, 5.00 min.

Open the cooled containers and add 0.5 mL H_2O_2 (**c**). Recap the containers, return them to the oven and start the next program: S1, 650 W, 1.00 min, and S2, 0 W, 20.00 min.

Note: In this example, H_2O_2 is added in a separate step, but the peroxide may also be added together with the nitric acid without affecting the accuracy of the determination.

Open the cooled containers and rinse down with water any condensed water in the cap and on the walls. Quantitatively transfer the sample solution to a 25 mL volumetric flask and dilute to the mark with water. Then transfer the sample solution to a polyethylene tube (**f**).

(**c**) *Dilution.*—Take out a suitable volume, add 5% La solution (**f**), and dilute this volume with 0.65% (w/v) nitric acid (**b**) so that the final concentration of Mg and/or Ca will be within the linear range of measurement of the metals. In this example, the following ranges are selected for the standard curves: for Mg, 0.05–0.4 mg/L, and for Ca, 0.5–4.0 mg/L. The lowest point may be lower if required by the concentrations of the sample solutions. Add 5% La solution (**f**) to a final concentration of La of 1% (e.g., 2 mL 5% La solution is diluted to 10 mL).

(**d**) *Preparation of working standard solutions.*—Prepare working standard solutions for Mg of 0.05, 0.1, 0.2, and 0.4 mg/L from the Mg standard solution (**i**): Add 0.25, 0.5, 1.0, and 2.0 mL to separate 50 mL volumetric flasks, add 10 mL 5% La solution (**f**), and dilute to the mark with 0.65% (w/v) nitric acid (**b**). Prepare fresh solutions daily.

Prepare working standard solutions for Ca of 0.5, 1.0, 2.0, and 4.0 mg/L from the Ca standard solution (**k**): Add 0.25, 0.5, 1.0, and 2.0 mL to separate 50 mL volumetric flasks, add 10 mL 5% La solution (**f**), and dilute to the mark with 0.65% (w/v) nitric acid (**b**). Prepare fresh solutions daily.

Note: Check that the residual concentration of nitric acid in the sample solution has no effect on the determination of Ca. Any interference can be eliminated in the following alternative ways: (*1*) Prepare a standard curve containing the same residual concentration of nitric acid as the sample solution, or (*2*) use the standard addition method. Prepare a zero solution for the standard curves by measuring 2 mL 5% La solution (**f**) into a 10 mL volumetric flask and dilute to the mark with 0.65% (w/v) nitric acid (**b**).

(**e**) *Determination.*—Connect the Mg and/or the Ca lamp and switch on the AAS instrument. Retrieve the stored program for Mg and/or Ca and adjust to the correct wavelength and slit. Allow the instrument to warm up for ca 30 min. Readjust the position of the lamp and the wavelength by using setup to maximum energy. Light the flame and adjust the instrument to zero against water. Measure the sample series (including the reagent blank), the working standard solutions, and the zero solution. Measure the standard and blanks first, last, and after every 15 sample solutions.

(**f**) *Calculations.*—Calculate standard curves for Mg and Ca by simple linear regression (method of least squares) on the basis of the data obtained from AAS measurements. Use the standard curves to calculate the amounts of the elements in reagent blanks and sample solutions as follows:

$$m_{bl} = A_{bl} \times k \times V_0 \qquad (1)$$

where m_{bl} = amount of element in the reagent blank solution V_0 (μg), A_{bl} = absorbance of the reagent blank solution (milliabsorbance units), k = slope of the standard curve (μg/mL per

Table 3. Results of the collaborative study for determination of Ca in foods

Sample	Ca, mg/kg, determined by indicated laboratory										
	1	2	3	4	5	6	7	8	9	10	11
Wheat bran	909	941	532	764	951	625	909	658	952	832	969
	913	970	476	741	1023	727	918	686	973	839	813
Simulated diet (D)	513	508	312	482	532	310	512	307	491	474	621
	522	539	270	451	511	351	524	303	528	452	572
Simulated diet (F)	254	275	207	291	278	176[a]	275	196	258	214	250[a]
	276	270	176	305	280	261[a]	262	210	249	212	423[a]
Milk powder, freeze dried	9460	9610	9450	8580	9340	9500	10100	10500	9460	9270	10500
	9280	9140	8560	8250	9090	9490	9070	10100	9440	9310	10300
Minced fish, freeze dried	4050	4250	3590	3440	3710	3850	3760	4010	3790	3880	4140
	3750	4200	3500	3660	3620	3800	3640	3970	3770	3890	4080
Apple, dried	334	256	154	273	260	152	229	201	254	274	244
	271	263	140	263	311	141	231	210	255	259	256
Chocolate cake, dried	983	1020	660	700	887	777	817	610	848	829	981
	881	1190	532	665	933	773	936	617	921	839	1050

[a] Outliers indicated by the Cochran test at $p < 0.01$.

milliabsorbance units), and V_0 = total volume of the reagent blank solution (mL).

$$m_{pr} = A_{pr} \times k \times V_1 \times f \quad (2)$$

where m_{pr} = amount of element in sample solution V_1 (μg), A_{pr} = absorbance of the sample solution (milliabsorbance units), k = slope of the standard curve (μg/mL per milliabsorbance units), V_1 = total volume of sample solution (mL), and f = dilution factor (further dilution of the sample).

Calculate the concentration of Mg and Ca in the sample from

$$c = (m_{pr} - m_{bl})/w \quad (3)$$

where c = concentration in sample (μg/g) and w = weighed amount of sample (g).

Results and Discussion

One of the critical factors studied was the effect of residual nitric acid on the Ca signal in FAAS. Standard curves of Ca constructed with 8% (v/v) or 5.2% (w/v) nitric acid showed an approximately 30% decrease in the slope of the curve compared with the slope of a curve constructed with 1% (v/v) nitric acid. A similar effect of nitric acid could be seen when a standard addition procedure was used. With our instrument settings, the suppressive effect of nitric acid started at a concentration of 2% (v/v). Users of the method are advised to apply a standard addition procedure for each dilution of the sample solution.

Results from the pretrial test for Mg and Ca in oyster tissue from 9 laboratories were 1180 ± 79 mg Mg/kg [1 SD (standard deviation)]; certified value, 1180 ± 170 mg Mg/kg (95% uncertainty); and 1964 ± 220 mg Ca/kg (1 SD); certified value, 1960 ± 190 mg Ca/kg (95% uncertainty). Results for Mg and Ca in wheat flour from 9 laboratories were 390 ± 20 mg Mg/kg (1 SD); certified value, 400 ± 20 mg Mg/kg (95% uncertainty); and 152 ± 28 mg Ca/kg (1 SD); certified value, 191 ± 4 mg Ca/kg (95% uncertainty).

Eleven laboratories reported results in the collaborative study (Tables 2 and 3). Results were statistically evaluated according to the IUPAC 1987 Protocol for the design, conduct, and interpretation of collaborative analytical studies (7). Results were tested for presence of outliers by using the Cochran and the Grubb's tests. Outliers were not included in the final estimation of method performance parameters. The following extreme (outlying) results were indicated by the Cochran test (extreme replicate results) at the $p < 0.01$ level (Tables 2 and 3): laboratory 11 results for Mg in apple and for Ca in diet material F, and laboratory 6 results for Ca in diet F.

The entire set of data indicated only one single extreme value as a result of the Grubb's test: laboratory 7 results for Mg in milk powder. Thus the set of data obtained in this study satisfies the requirement of the IUPAC 1987 protocol: The number of outliers in a collaborative study must not exceed a maximum of 2 out of 9 laboratories.

Tables 4 and 5 present estimated performance characteristics of the method.

Table 2. Results of the collaborative study for determination of Mg in foods

Sample	Mg, mg/kg, determined by indicated laboratory										
	1	2	3	4	5	6	7	8	9	10	11
Wheat bran	4230	3820	4050	4230	3610	4410	5290	4530	3750	3960	3630
	3780	3770	4120	3810	3800	4060	5570	4560	4030	4200	3370
Simulated diet (D)	660	746	642	679	624	626	686	623	642	715	649
	633	723	605	665	644	664	781	622	644	711	641
Simulated diet (F)	623	678	625	563	580	626	710	568	595	674	614
	584	681	585	635	602	605	635	574	563	662	603
Milk powder, freeze dried	822	908	881	851	848	845	1030[a]	839	808	953	862
	865	899	881	843	852	807	860	1020[a]	849	822	932
Minced fish, freeze dried	729	776	693	733	716	763	855	734	709	802	719
	738	837	710	722	675	740	857	740	727	789	721
Apple, dried	245	258	243	268	235	246	255	253	247	262	277[b]
	234	262	241	259	235	251	255	266	235	262	231[b]
Chocolate cake, dried	267	291	272	294	268	284	295	289	270	292	236
	286	296	264	288	277	290	300	290	274	292	283

[a] Outliers indicated by the Grubb's test at $p < 0.01$.
[b] Outliers indicated by the Cochran test at $p < 0.01$.

Table 4. Statistical analysis of collaborative study data for Mg

Sample	n^a	Mean	s_r	RSD_r, %	r^b	s_R	RSD_R, %	R^c	HORRAT
Wheat bran	11	4120	190	4.7	540	530	13	1490	2.8
Simulated diet (D)	11	665	25	3.8	70	46	6.9	129	1.2
Simulated diet (F)	11	618	27	4.4	76	43	6.9	120	1.2
Milk powder, freeze dried	10	859	18	2.1	51	39	4.6	111	0.8
Minced fish, freeze dried	11	749	18	2.4	50	51	6.8	144	1.2
Apple, dried	10	251	5.3	2.1	14.8	11.3	4.5	31.6	0.73
Chocolate cake, dried	10	284	5.7	2.0	15.9	11.3	4.0	31.8	0.58

[a] Number of laboratories remaining after elimination of outliers.
[b] $r = 2.8 \times s_r$.
[c] $R = 2.8 \times s_R$.

Table 5. Statistical analysis of collaborative study data for Ca

Sample	n^a	Mean	s_r	RSD_r, %	r^b	s_R	RSD_R, %	R^c	HORRAT
Wheat bran	11	824	47.8	5.5	128	157	19	439	3.3
Simulated diet (D)	11	458	22.5	4.7	61	104	23	290	3.6
Simulated diet (F)	9	254	11.5	4.3	30	38	15	107	2.1
Milk powder, freeze dried	11	9450	245	3.6	940	600	6.3	1670	1.5
Minced fish, freeze dried	11	3830	86.2	2.4	253	227	5.9	635	1.3
Apple, dried	11	238	19.2	7.8	52	53	22	149	3.1
Chocolate cake, dried	11	839	62.8	7.3	172	166	20	465	3.4

[a] Number of laboratories remaining after elimination of outliers.
[b] $r = 2.8 \times s_r$.
[c] $R = 2.8 \times s_R$.

For determination of Mg, the relative standard deviation for repeatability (RSD_r) of the method was estimated to be between 2.0 and 4.7%. The relative standard deviation for reproducibility (RSD_R) was estimated to be between 4.0 and 13%. For determination of Ca, RSD_r was estimated to be between 2.4 and 7.8%, and RSD_R was estimated to be between 5.9 and 22%.

RSD_R values were compared with those obtained from a large number of interlaboratory method performance studies involving a wide range of analytes, matrixes, and measurement techniques. Horwitz et al. (8) found that the RSD_R value generally can be predicted from a general equation, the so-called Horwitz equation:

$$RSD_R = 2^{(1-0.5 \log C)}$$

where C is the concentration as a decimal fraction. According to Horwitz, the ratio between observed RSD_R values and the RSD_R values predicted by this equation, designated HORRAT, can be regarded as an indication of the acceptability of a method with respect to its precision. The HORRAT values of this method are presented in the last columns of Tables 4 and 5. According to Horwitz et al. (8), a series of ratios close to or consistently smaller than 1.0 indicates acceptable precision of methods. Correspondingly, HORRAT values consistently near or greater than 2 probably indicate an unacceptable method with respect to precision. The same approach has been adopted by IUPAC (9).

Table 4 shows that the present method has an acceptable precision for Mg determination. HORRAT values are below 2.0 for all test materials except wheat bran. This material had a high concentration of Mg, 4 g/kg. The required large dilution may have given rise to the poor agreement in results between laboratories.

Table 5 shows that for Ca determination, acceptable HORRAT values were obtained only for materials with the highest Ca concentrations: milk powder and minced fish. HORRAT values were above 2.0 for other test materials. The most probable reason for this is failure to correct for interference from the concentration of nitric acid in less diluted sample solutions.

Collaborators' Comments

Laboratory 1 reported that the sample solutions were calibrated against both 1% nitric acid and solutions having a nitric acid concentration corresponding to that in the sample solutions. The Ca concentrations of the sample solution were analyzed by both FAAS and ICP (inductively coupled plasma). Results from FAAS and ICP agreed in the case of milk powder and fish. In the case of other test materials, results from FAAS and ICP agreed if the FAAS system was calibrated against a standard curve constructed from standards containing the same nitric acid level as the sample solutions.

Laboratory 5 indicated that results from the determination of Ca varied on a day-to-day basis.

Conclusions

The results of the collaborative study of this AAS method for determining Mg and Ca indicate that the method is suitable for determinations of Mg in foods in the concentration range 250–1000 mg/kg dry matter and Ca in foods containing concentrations of Ca above about 4000 mg/kg dry matter. In the case of Ca, method precision could be improved by correcting for the suspected interference of nitrate ions when measurements are made with less diluted sample solutions.

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RESIDUES AND TRACE ELEMENTS

Determination of 17β-Estradiol and Its Metabolites in Sewage Effluent by Solid-Phase Extraction and Gas Chromatography/Mass Spectrometry

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The paper describes a simple and quantitative method for monitoring non-conjugated 17β-estradiol (E_2) and its metabolites estrone (E_1) and estriol (E_3) as environmental contaminants in municipal sewage effluents. Estrogens were preconcentrated and cleaned up by solid-phase extraction using a reversed-phase C_{18} cartridge. They were derivatized with pentafluoropropionic acid anhydride, and the products were analyzed by gas chromatography/mass spectrometry. Recoveries from spiked distilled water and sewage were better than 87% at fortification levels of 100 and 20 ng/L. For a 1 L sewage sample and a concentration factor of 5000, detection limits were 5 ng/L for E_1 and E_2 and 10 ng/L for E_3. In a brief survey of Canadian wastewater, these estrogens were detected in many raw sewage and effluent samples at concentrations ranging from 6 to 109 ng/L for E_1, from <5 to 15 ng/L for E_2, and from <10 to 250 ng/L for E_3.

Many synthetic chemicals can mimic the function of the naturally occurring estrogen, 17β-estradiol (E_2), causing disruption of the endocrine system (1). These chemicals have been hypothetically linked to increased incidence of certain estrogen-dependent cancers in humans, falling sperm counts in men, feminization of fish, and abnormal reproductive organs of wildlife (2–4). Many of these endocrine disruptors are environmental contaminants, with examples including organochlorine insecticides such as DDT as well as its metabolites and derivatives, kepone, some hydroxylated polychlorinated biphenyls, chlorinated dioxins, alkylphenols such as bisphenol A, 4-nonylphenol, and 4-*tert*-octylphenol (5–7). However, data developed from MCF-7 (a human breast cancer cell) bioassay (8) or rainbow trout in vitro hepatocyte bioassay (9, 10) indicate that these xenobiotic compounds are 4 to 6 orders of magnitude weaker than E_2 in relative estrogen potency.

E_2 is the principal hormone that regulates cell proliferation and is responsible for the development and maintenance of the female reproductive system and sex characteristics. In humans, E_2 is metabolized to estrone (E_1) and estriol (E_3) in both conjugated and nonconjugated forms. These steroids are commonly found in human excreta and in urine, serum, and amniotic fluid of pregnant women (11). The synthetic estrogen 17α-ethynylestradiol (EE_2) is the major ingredient of many oral contraceptives that have been used since 1960. Structures of these steroids are shown in Figure 1.

Naturally occurring estrogens enter the environment through excretions of humans, domestic and farm animals, and wildlife. Although the concentrations of these steroids in sewage and environmental samples are very low (at nanogram-per-liter levels), it has been demonstrated that nonconjugated E_2 and EE_2 at concentrations as low as 1 ng/L can induce synthesis of vitellogenin (VG) in male fish (12). VG is a protein precursor of the egg yolk and is normally produced in large quantities by mature female fish. The observation of elevated levels of VG in male fish collected in rivers downstream of sewage treatment plants has led to the belief that estrogenic compounds are present in sewage (13). Although conclusive evidence is still unfolding, E_2 and EE_2 are suspected to be major contributors of estrogenic activities in sewage.

Very few analytical methods for determinating E_2 in environmental samples have been published. Previously, a radioimmunoassay technique was developed for determining E_2 and testosterone in sewage (14). Identification of estrogenic compounds in sewage effluent by gas chromatography/mass spectrometry (GC/MS) was also reported in a British study (15) that involved solid-phase extraction (SPE) of a 20 L sample, liquid chromatographic fractionation, and ion trap GC/MS analysis of underivatized E_1, E_2, and EE_2. To date, no data regarding occurrence of estrogens in the Canadian aquatic environment are available. In this work, we present a simple SPE and GC/MS method for determining free E_2 and its metabolites E_1 and E_3 in sewage effluent. The present study is a continuation of our current research in new analytical methods for detecting other estrogenic compounds such as nonylphenol (16), nonylphenol ethoxylates (17), and carboxylates (18) in sewage effluent and sludge samples.

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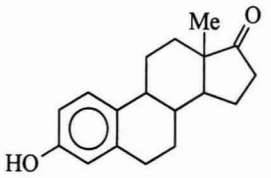
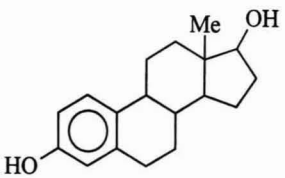
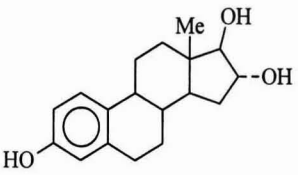


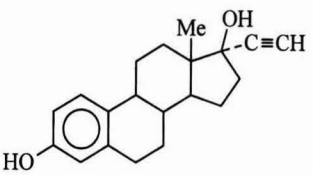
Estrone (E_1)



17ß-Estradiol (E_2)



Estriol (E_3)



17α-ethynylestradiol (EE_2)

Figure 1. Chemical structures of estrogens.

Experimental

Reagents and Chemicals

(**a**) *Solvents.*—Distilled-in-glass grade organic solvents (Burdick & Jackson, Muskegon, WI) were used without further purification.

(**b**) *Estrogens and anhydride.*—E_1, E_2, E_3, EE_2, and pentafluoropropionic acid anhydride (PFPA) were obtained from Sigma-Aldrich-Supelco Canada Ltd. (Oakville, ON, Canada). Stock solutions of each estrogen (1000 µg/mL) were prepared in acetone. Mixtures of the estrogens (100, 10, and 1 µg/mL) for sample fortification and standard preparation were also prepared in acetone. All stock solutions and mixtures were stored in screw-capped centrifuge tubes at –20°C in the dark.

Extraction Apparatus

A home-made system consisting of a 12-port SPE vacuum manifold (Supelco Visiprep DL 5-7044), a 20 L stainless steel tank, and a vacuum pump was used. A 6 mL, reversed-phase SPE cartridge containing 1 g C_{18} sorbent (ENVI-18, Supelco, 5-5706) was used to extract a sewage sample.

Collection of Sewage Samples

Grab or 24 h composite influent, primary, and final effluent samples from various municipal sewage treatment plants were collected. Samples extracted within 48 h after collection were kept at 4°C and no preservative was added. Other samples were preserved with formaldehyde (1%, v/v) and kept at 4°C in the dark until extraction.

Caution: Because of the presence of bacteria, viruses, and parasites that may pose a health hazard to workers, protective clothing, gloves, mask, and safety goggles with full side shields must be used when handling sewage samples as a safety precaution. It is also recommended that workers have proper immunization against diphtheria, tetanus, polio, and hepatitis A. For details, consult your local health authorities.

Extraction of Sewage Samples

Prior to extraction, each sewage sample (1 L) was vacuum filtered through a 47 mm Whatman GF/C filter with a pore size of 1.2 µm installed in an all-glass funnel support assembly (Kontes Ultra-Ware, Vineland, NJ; KT-953825-0000). A filter aid such as Celite 545 was used to minimize plugging of the filter. For recovery experiments, a portion of the free estrogens (e.g., 100 µL of the 1 µg/mL mixture in acetone) was spiked to 1 L distilled water or effluent. The sample was stirred for 15 min prior to SPE.

In preparation for extraction, each C_{18} tube was conditioned with 5 mL acetone, 5 mL methanol, and 10 mL water on an SPE manifold per manufacturer's instruction. The filtered sample was then applied to the extraction tube via a siphon tube and an adaptor (Supelco, 5-7275). An average flow rate of ca 10 mL/min was maintained by adjusting the vacuum to ca –15 in. Hg. As a safety precaution, the vacuum should not exceed –20 in. Hg. When extraction was complete, the tube was dried under vacuum for 5 min. A 5 mL portion of acetone–water (1 + 4, v/v), in 2 equal fractions, was used to rinse the tube, and the washes were discarded. Estrogens were removed from the C_{18} tube by eluting with 10 mL acetone. Because the

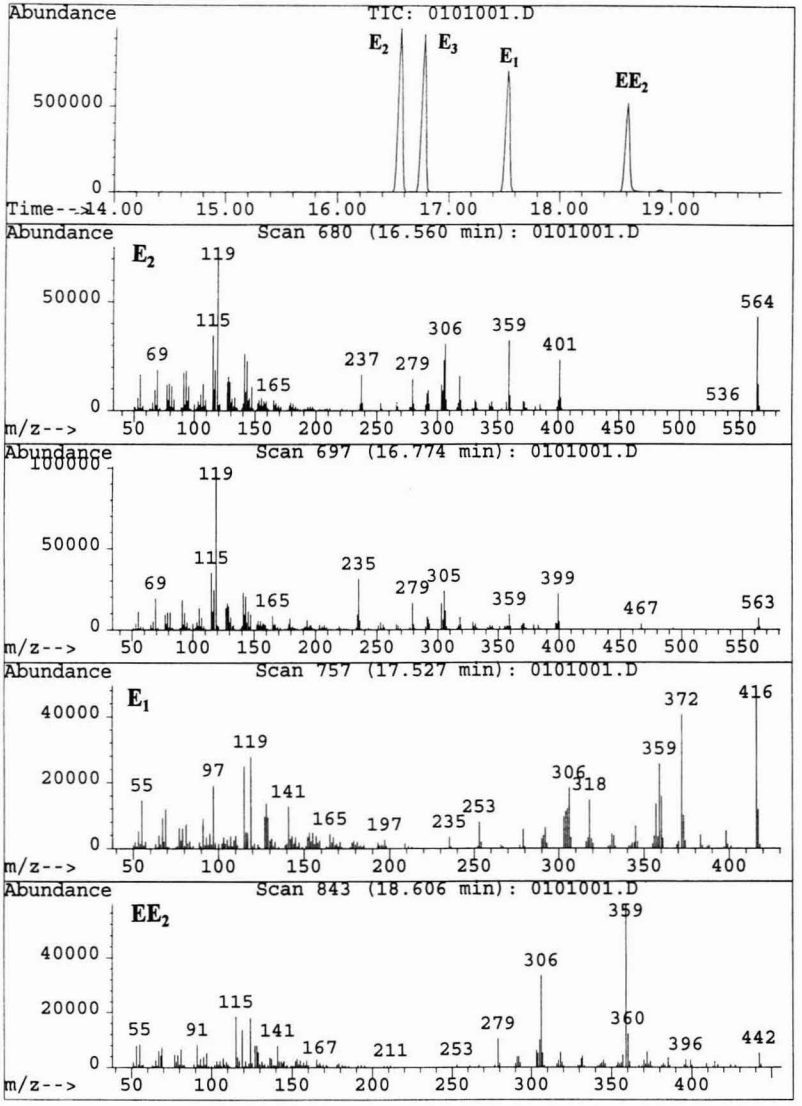


Figure 2. Total ion current chromatogram and mass spectra of PFP derivatives of E_2, E_3, E_1, and EE_2. Concentration, 10 ng/μL for each component.

acetone extract contained a small amount of water, it was gently evaporated to ca 100 μL with nitrogen on a water bath at 40°C. Estrogens were then back-extracted into four 1 mL portions of ethyl acetate, and the extract was dried by passing through a disposable pipet filled with 5 cm anhydrous sodium sulfate. All sewage samples (extracted and unextracted) were discarded down the toilet.

Derivatization of Estrogens

After the ethyl acetate extract was evaporated to 100 μL in a centrifuge tube, the sample was reacted with 50 μL PFPA at ambient temperature (22°C) for 20 min. At the end of reaction, 2 mL petroleum ether (boiling point, 30°–60°C) and 3 mL 1% K_2CO_3 were added and the products were partitioned into the organic layer by agitation on a Vortex mixer for 1 min. After phase separation (centrifugation was required for most sewage samples to break the emulsion), the upper layer was removed and passed through a disposable pipet filled with anhydrous sodium sulfate. The extraction was repeated twice, and the combined petroleum ether fraction was evaporated just to dryness. The residue was redissolved in 200 μL isooctane for GC/MS analysis. For a calibration standard, a mixture of these estrogens (e.g., 50 ng each) was derivatized and worked up as described above.

Table 1. Mass number and relative abundance of molecular and major ions of PFP derivatives of estrogens

PFP derivative	M^+, *m/z* (relative abundance, %)	Major ions, *m/z* (relative abundance, %)
E_1	416 (100)	372 (82), 359 (52), 306 (37), 119 (56)
E_2	564 (57)	401 (31), 359 (43), 306 (41), 119 (100)
E_3	726 (5)	563 (7), 399 (22), 359 (9), 119 (100)
EE_2	442 (9)	359 (100), 306 (57), 279 (18), 119 (24)

Analysis of Estrogen Derivatives by GC/MS

A Hewlett-Packard 5890 Series II GC equipped with a 5972 mass-selective detector and a 30 m × 0.25 mm id × 0.25 μm film thickness HP-5-MS column was used for quantitation of estrogens. GC conditions were as follows: injection port, 250°C; interface, 280°C; initial oven temperature, 70°C with a 1 min hold; programming rates, 30°C/min (from 70° to 180°C); and 5°C/min (from 180° to 290°C). Carrier gas (helium) linear velocity was held constant at 36.9 cm/s. Splitless injections (1 μL) were made by a HP7673 autosampler with a splitless time of 1 min. The electron energy and electron multiplier voltage were 70 eV and 400 V above autotune value, respectively. The detector was tuned each day by using perfluorotributylamine (PFTBA) and the standard spectra autotune program. Full-scan mass spectral data were collected from *m/z* 50 to 600. For quantitation of estrogens in a sample extract, selected ion monitoring (SIM) was used. Response factors for the estrogens were generated by injecting mixtures of their pentafluoropropionyl (PFP) derivatives at 2 levels (1000 and 200 pg/μL). The following quantitation and confirmation ions, respectively, were used in SIM work: *m/z* 372 and 416 (E_1), *m/z* 564 and 401 (E_2), *m/z* 563 and 399 (E_3), and *m/z* 359 and 306 (EE_2). Using the external standard method, we calculated the concentration of each estrogen in a sample by multiplying its area (for the quantitation ion) by the average response factor generated from the above 2 standards and dividing by an appropriate concentration factor (i.e., 5000 in this case).

Results and Discussion

Extraction of Estrogens from Sewage

Estrogens and other steroidal hormones in plasma and urine of a small sample size (i.e., a few milliliters) have been extracted successfully by solvents such as ethyl acetate (19), as well as a mixture of diethyl ether and dichloromethane (20). Extraction of estrogens and estrogen conjugates in late-pregnancy fluids has been reported by using a graphitized carbon black cartridge (11). Our extraction procedure was based on a previously published SPE method for estrogen and testosterone in sewage, using a C_{18} column (14). For convenience and consistency with our previous SPE procedures, suspended particulates in the sewage sample were removed by vacuum filtration through a layer of Celite and a glass fiber filter of 1.2 μm pore size. However, this process should be performed only immediately before the preconcentration step. Otherwise, reduction in flow rates or even plugging of the adsorption media may still occur if the filtered sample is stored for an extended period.

Prior to elution of estrogens, the C_{18} cartridge was washed with 5 mL acetone–water (1 + 4, v/v) and the washing was discarded. This cleanup step was very useful because it removed coextractives that were much more polar than the estrogens. Its effectiveness was best illustrated in the extraction of some sewage samples with known inputs from the textile industry: After the dark-colored dyes were removed by this cleanup step, the fraction containing the estrogens was nearly colorless.

In our experience, a small amount of water (100 to 200 μL) always remains on the C_{18} cartridge even if the cartridge is dried under vacuum for 30 to 60 min. The water, which is removed alongside the estrogen in the acetone elution step, must be removed before chemical derivatization. Rather than use a prolonged nitrogen evaporation step that causes loss of analytes to eliminate residual water, we stopped evaporation when the volume was about 100 μL, or when the solvent was almost entirely water. Then we back-extracted the estrogens into ethyl acetate, which was subsequently evaporated to about 100 μL for derivatization. A small volume of ethyl acetate was necessary to keep the sewage extract in solution, and because ethyl acetate is miscible with PFPA, its presence made derivatization efficient.

Derivatization and GC/MS Analysis

Several derivatization procedures have been published for GC determination of estrogens. For example, formation of trimethylsilyl (TMS; 19, 21, 22) and *tert*-butyldimethylsilyl derivatives of E_1, E_2, and E_3 (22), dimethylethylsilyl derivatives of EE_2 and other steroids used in oral contraceptive formulations (22), and PFP derivatives of testosterone acetate (20) have been demonstrated. In the beginning of this study, we evaluated several estrogen derivatives for ease of formation, stability of products, and chromatographic and mass spectral properties. In addition to TMS and PFP derivatives, we also studied acetyl and heptafluorobutyryl (HFB) derivatives. We selected PFP derivatives for this work because their formation produced very few side products and they were stable for weeks at –20°C. This stability made it possible to save sample extracts for further analysis. In addition, PFP derivatives were formed in the shortest time under the least vigorous conditions, requiring only 20 min of reaction at room temperature. Although we did not determine the absolute yield of PFP derivatives, we assumed the reaction to be complete and quantitative because longer reaction times (e.g., 60 and 120 min) or higher reaction temperatures (e.g., 50° and 65°C) did not improve yield. Pentafluoroacylation of estrogens also was reproducible. For 6 replicate reactions with 50 ng of each estrogen, coefficients of variation for the yields of the products ranged from 3.8% (for E_2) to 6.6% (for E_3).

As illustrated by the chromatogram shown in Figure 2, all PFP derivatives were baseline resolved and their order of elu-

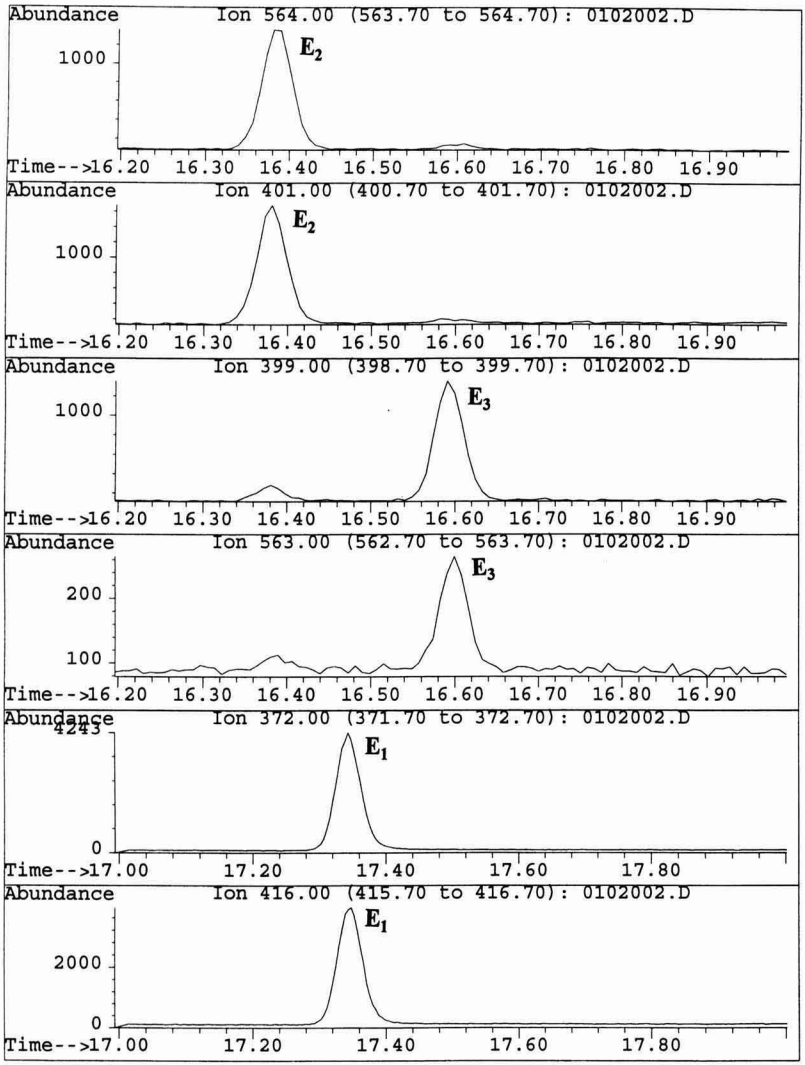


Figure 3. Extracted ion chromatograms for quantitation and confirmation ions of E_2, E_3, and E_1 from a 200 pg/μL standard.

tion is E_2, E_3, E_1, and EE_2. Tailing of the EE_2 derivative occurred, and its response to the mass-selective detector decreased as contaminants accumulated in the splitless liner. We think this derivative, which has one active OH group, became adsorbed to the polar coextractives, such as surfactants, that are common in sewage samples. The EE_2 response can be easily restored by replacing the liner and cutting a few centimeters of the capillary column in the injector end. By contrast, responses of the PFP derivatives of E_1, E_2, and E_3 were not greatly affected by cleanliness of the liner.

All hydroxyl groups in naturally occurring, endogenous estrogens—3-OH of E_1, 3- and 17-OH of E_2, and 3-, 16-, and 17-OH of E_3—reacted readily with PFPA to yield the respective mono-, di-, and tri-PFP derivatives, as evidenced by molecular ions at *m/z* 416, 564, and 726 (Table 1). The molecular ion (M^+) for the E_3 derivative was observed on a Hewlett-Packard MS Engine because its molecular weight is beyond the upper mass range of the mass-selective detector. Under the reaction conditions used, no partially derivatized E_2 and E_3 (i.e., a monosubstituted E_2 and either a mono- or disubstituted E_3) were observed in the chromatogram. The absence of such intermediates enabled straightforward quantitation of the estrogens. By contrast, a monosubstituted derivative of the synthetic estrogen EE_2 was formed (M^+ at *m/z* 442). Because mestranol, the 3-methyl ether derivative of EE_2, did not react with PFPA under such conditions, we think that the hydroxyl group at position 17 was protected from being derivatized by the presence of the ethynyl group at the same location.

Table 2. Accuracy and precision of recovery data of estrogens from fortified water and sewage samples ($n = 6$)

	Mean recovery ± standard deviation, %			
	From distilled water at indicated spiking level (ng/L)		From sewage at indicated spiking level (ng/L)	
Estrogen	100	20	100[a]	20[b]
E_1	101 ± 3	107 ± 5	96 ± 4	102 ± 8
E_2	95 ± 4	91 ± 5	105 ± 5	98 ± 9
E_3	93 ± 5	98 ± 6	101 ± 8	127 ± 13
EE_2	89 ± 6	102 ± 8	88 ± 8	87 ± 11

[a] A composite sewage final effluent (mixture of 3 samples from different treatment plants) was spiked. As all sewage final effluents are naturally contaminated with detectable amounts of E_1 and E_3, the results for these 2 estrogens in the spiking experiments have to be corrected (subtracted) for the blanks.

[b] A composite sewage final effluent that had been extracted previously by this procedure was spiked. Results were not corrected because the concentrations of estrogens in blanks were below detection limits.

All PFP derivatives displayed ions at *m/z* 69 (CF_3^+) and 119 ($C_2F_5^+$), which are characteristic of the PFP group (Figure 2). Molecular ions for the E_1 and E_2 derivatives were either the base peak or very strong. Those for E_3 and EE_2 were relatively weak. All the estrogens gave a prominent peak at *m/z* 359. This ion is likely due to loss of carbons at positions 15, 16, and 17 of the 5-member ring (ring D), their substituents, and a hydrogen from the molecular ion of each derivative. With the E_2 derivative for example, the ion at *m/z* 359 or $(M - 205)^+$ arises from loss of C_3H_5O, C_2F_5CO, and H (presumably from the carbon at position 14) from M^+. For EE_2, the ion corresponding to $(M - 83)^+$ arises from loss of C_3H_5O, the ethynyl group, and a hydrogen from M^+. The fragmentation pattern of EE_2 also provided direct evidence for the fact that the hydroxyl group at position 17 of this synthetic steroid was not derivatized.

The mass numbers and relative abundance of the major ions for the PFP derivatives are listed in Table 1. Their mass spectra are shown in Figure 2. Extracted ion chromatograms of the quantitation and confirmation ions derived from a calibration standard are depicted in Figure 3.

Method Performance

The accuracy and precision of this procedure was determined by analysis of distilled water and sewage effluent samples fortified with the estrogens at environmentally relevant levels (Table 2). At spiking levels of 100 and 20 ng/L, method precision was very good, with standard deviations of less than 10% for most samples. Recoveries of estrogens, ranging from 87 to 107% for all except one case, were close to quantitative.

Table 3. Levels of E_1, E_2, and E_3 in sewage influent, primary effluent, and final effluent[a]

				Estrogen found, mean ± standard deviation, ng/L		
Location	Sewage type	Sampling date	No. of replicates	E_1	E_2	E_3
Burlington	Primary	12/16/97	3	26 ± 1	7 ± 0.6	128 ± 10
Burlington	Final	12/16/97	3	6 ± 0.5	<5	18 ± 1
Burlington	Primary	1/20/98	3	53 ± 4	14 ± 2	220 ± 20
Burlington	Final	1/20/98	3	8 ± 1	<5	33 ± 1
Dundas	Primary	1/13/98	2	70, 68[d]	9, 8[d]	243, 203[d]
Dundas	Final	1/13/98	2	8, 10[d]	<5, <5	<10, <10
Edmonton	Primary, preserved[b]	8/20/97	3	109 ± 5	<5	209 ± 14
Edmonton	Final, preserved[b]	8/20/97	3	72 ± 2	<5	<10
Guelph	Influent, preserved[b]	12/18/97	2	58, 75[d]	<5	164, 158[d]
Guelph	Final, preserved[b]	12/18/97	2	18, 16[d]	<5	37, 30[d]
Guelph	Influent	1/22/98	3	41 ± 4	15 ± 2	250 ± 32
Guelph	Final	1/22/98	3	14 ± 1	<5	30 ± 2
Montreal, north	Influent[c]	11/17/97	3	28 ± 6	6 ± 1	72 ± 6
Montreal, south	Influent[c]	11/17/97	3	15 ± 2	7 ± 0.7	53 ± 3
Montreal	Primary[c]	11/17/97	3	19 ± 3	6 ± 0.7	68 ± 7

[a] All samples were grab and unpreserved except where noted.

[b] With formaldehyde (1%, v/v), 4°C in the dark.

[c] 24 h composite sample.

[d] For $n = 2$, individual results are given.

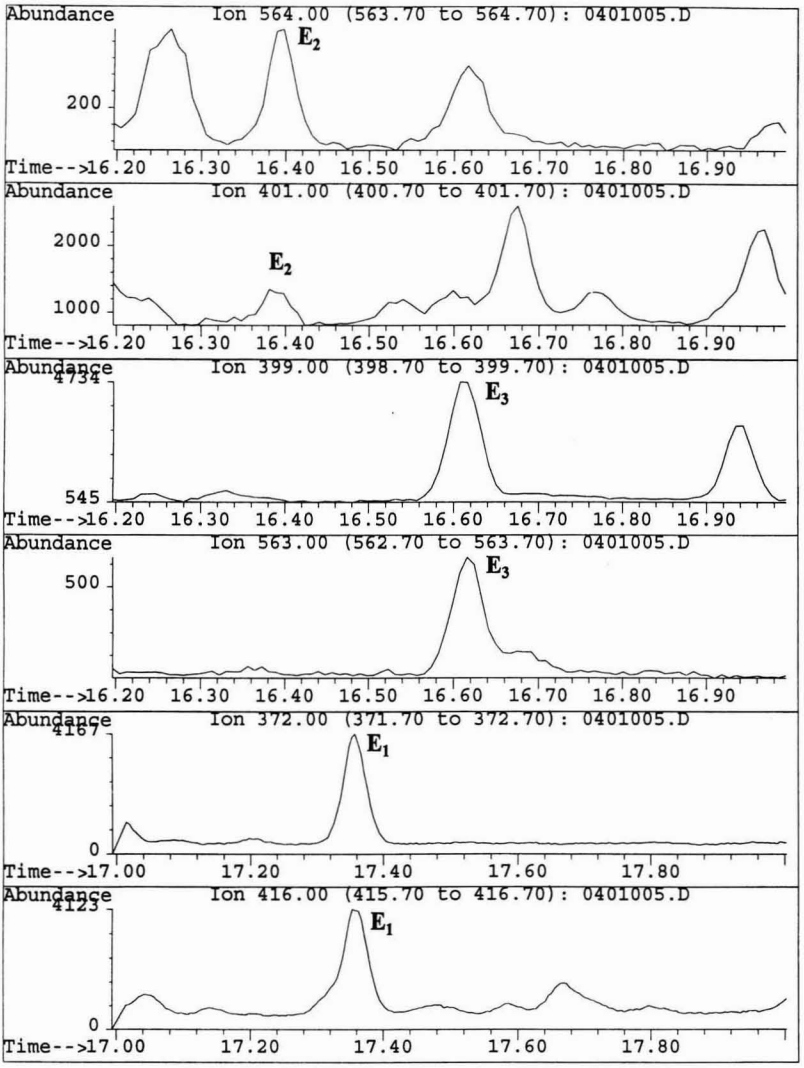


Figure 4. Extracted ion chromatograms of a derivatized, primary sewage effluent extract indicating the presence of E_2, E_3, and E_1 in the sample.

With a concentration factor of 5000 (1 L sample extracted and a final volume of 200 μL), detection limits (based on a signal-to-noise ratio of 10:1) for these estrogens in sewage samples were estimated to be 5 ng/L for E_2, E_1, and EE_2 and 10 ng/L for E_3. Detection limits could be reduced by a factor of 2 to 3 for distilled water samples where interference is minimal.

Application

This method has been used to determine steroidal estrogens in the sewage treatment plants of a few Canadian cities. E_1, E_2, and E_3 were found in all sewage influent and primary effluents that were extracted within 48 h after collection. In these samples, concentrations of E_1 and E_3 were relatively high, varying from 14 to 109 ng/L and from 53 to 250 ng/L, respectively (Table 3). The levels of E_2, the most potent endogenous estrogen, in the influent or primary effluent were much lower, ranging from 6 to 15 ng/L. These concentrations are substantially lower than those reported by Shore et al. (48–141 ng/L; 14) for raw sewage samples collected in Israel. Shore et al. used a radioimmunoassay technique, which might have measured other cross-reactive estrogens at the same time. To a smaller extent, discrepancy of the results could also be related to differences in weather (i.e., amount of precipitation) and patterns of water usage in the 2 countries.

The occurrence of E_1, E_2, and E_3 in a primary sewage effluent collected in Burlington is illustrated in Figure 4. The identity of each estrogen was confirmed by the presence of both characteristic ions at the expected retention time (± 0.04 min)

and in a similar area ratio (± 35%) compared with a standard (Figure 3). Although E_1 and E_3 were found in 2 preserved primary effluent samples (which were analyzed 1–3 months after collection), E_2 was not detected in those samples. Therefore the stability of E_2 and other estrogens in preserved sewage should be investigated.

As indicated in Table 3, the concentrations of the estrogens in the final effluents were substantially lower. In fact, in all cases, the concentration of E_2 was below the detection limit of 5 ng/L. E_1 and E_3 levels decreased by a factor of 4 between the primary and the final effluents collected on the same day from the same sewage treatment plant. The reduction is probably due to degradation of E_2 and its metabolites by activated sludge in the secondary sewage treatment processes (24). In the final effluents, concentrations varied from 5 to 19 ng/L for E_1 and from <10 to 34 ng/L for E_3. These levels are about 3 orders of magnitude lower than those found for nonylphenol ethoxylates and their degradation products in the same samples (16, 18, and unpublished results). None of the sewage effluents tested had any detectable amounts of EE_2.

The results suggest that this method is adequate for determining E_1 and E_3 in most sewage samples, as well as E_2 in the raw influent and primary sewage effluent, but it may not be sensitive enough for determining E_2 in final effluent. This procedure is not likely to be applicable to determination of EE_2 in sewage samples because the estimated environmental concentration of this estrogen is much lower than the method detection limit of 5 ng/L (15). A survey of the occurrence of E_2 and its metabolites in sewage samples collected across Canada is underway.

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RESIDUES AND TRACE ELEMENTS

Automated Method for Cleanup and Determination of Benomyl and Thiabendazole in Table-Ready Foods

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An automated solid-phase extraction (SPE) cleanup with on-line liquid chromatographic (LC) analysis was developed to determine residues of benomyl (as carbendazim) and thiabendazole in table-ready food items from the U.S. Food and Drug Administration Total Diet Study (TDS). A strong-cation-exchange cleanup of an acetone extract replaces the methylene chloride solvent partitioning steps in the procedure described in the *Pesticide Analytical Manual* (PAM). LC analysis is accomplished with a C_8 analytical column and tandem fluorescence and UV detection. Recoveries of both analytes from 32 representative TDS foods fortified at 0.05 and 0.5 μg/g were determined. Method precision was evaluated with triplicate recovery assays on 11 foods fortified at both levels. Accuracy was tested further by assaying 47 foods for incurred residues in parallel with the validated PAM procedure for comparison, and good agreement was found. The automated SPE method reduces solvent consumption, analysis time, and labor.

Since 1961, the U.S. Food and Drug Administration Total Diet Study (TDS) has monitored levels of nutrients, elements, and residues of pesticides and industrial chemicals in table-ready foods (currently 261 food items) to estimate dietary intake for various age–sex groups in the U.S. population (1). There are 4 collections per year, each composited from 3 cities within a geographical region (east, central, west, and south). In 1992, TDS was expanded to detect the fungicide benomyl in 67 TDS foods as the degradation product methyl-2-benzimidazole carbamate (carbendazim, or MBC). The *Pesticide Analytical Manual* (PAM) procedure (2, 3) used for analysis consists of a methanol extraction, liquid–liquid partitioning cleanup with methylene chloride while the pH is adjusted to cycle the analyte between neutral and cationic forms, and liquid chromatography (LC) with UV and fluorescence determination of carbendazim. Findings are reported as benomyl. TDS analysis for the fungicide thiabendazole (TBZ) started in 1989 by the organosulfur method with gas chromatography with flame photometric detection. Because the LC method also determines TBZ, the TDS began assays for TBZ by that procedure in 1994. An additional 15 food items are assayed for benomyl and TBZ for a supplemental survey of foods for infants and toddlers conducted under the auspices of the TDS.

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It is possible to reduce labor and solvent consumption in benzimidazole assays by performing the liquid–liquid partitioning on diatomaceous earth, but this still requires evaporation of methylene chloride (4, 5). Solid-phase extraction (SPE) seems to offer additional benefits. Milk assays with octadecylsiloxane-bonded (C_{18}) silica cleanup (6) and automated assays of fruits and vegetables with diol-bonded silica SPE cleanup (7) have been reported. Another approach involves the ionic nature of the analytes in ion-exchange separation schemes. For example, benzenesulfonic acid-bonded silica was used for the miticide/acaricide formetanate hydrochloride (8) and later was applied to benomyl and TBZ (9, 10). Propylsulfonic acid (PRS)-bonded silica also has been used (11–15).

To apply the SPE technique to the TDS market basket, analytical conditions had to be optimized for the wide range of foods. The desire for compatibility with an available automated workstation placed some constraints on the parameters that could be selected. For example, SPE column loading and eluting volumes could not exceed 10 mL and it was not possible to use 2 cleanup columns in series. An automated method using benzenesulfonic acid-bonded silica has been developed that allows selective retention and elution of analytes with small solvent volumes and eliminates use of methylene chloride, reducing the hazardous waste generated. Automation takes the place of repetitive and labor-intensive partitioning and rotary evaporation steps, increasing safety by minimizing contact with solvents. Automation also enhances quality with self-documenting procedures, audit trails, and gravimetric confirmation of volumetric deliveries.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Model LC-10AS LC pump (Shimadzu, Japan), Model 1050 online degasser (Hewlett-Packard, Palo Alto, CA), Model 7010 electrically actuated 6-port LC injection valve with 50 μL sample loop (Rheodyne, Cotati, CA) built into workstation, Model 486 tunable UV absorbance detector and Model 470 scanning fluorescence detector (Waters, Milford, MA) connected in series, and Model 4270 integrators (Spectra Physics, San Jose, CA). Operating condi-

tions: injection volume, 50 µL; flow rate, 1.0 mL/min; UV absorbance at 280 nm; fluorescence detection, carbendazim (benomyl) at excitation 270 nm and emission 304 nm and TBZ at excitation 290 nm and emission 388 nm; slit widths, 18 nm.

(**b**) *Analytical column.*—Platinum C_8 4.6 × 150 mm, 5 µm bonded silica, base deactivated (Alltech Associates, Deerfield, IL).

(**c**) *Automated workstation.*—BenchMate II workstation (Zymark, Hopkinton, MA) equipped for SPE, with robotic arm, liquid-transfer station, balance, Vortex mixer, membrane filtration, LC injector, 5 reagent reservoirs, one internal standard reservoir, a rack for 8 LC calibration standards, 4 racks for fifty 16 × 100 mm tubes, relays to trigger integrators, floppy-disk drive to store program sequence and sample-tracking data, and custom-made connecting cable from injector to LC detector and integrator start triggers.

(**d**) *Personal computer.*—A computer capable of running BenchMate software is used to create and edit programs that control the workstation. Quattro Pro or equivalent spreadsheet software may be used for calculations and data management.

Materials

(**a**) *Membrane filters.*—Nylon, 0.45 µm and 0.2 µm pore id, 25 mm id disk (Whatman, Clifton, NJ).

(**b**) *SPE cartridge.*—Strong cation exchange (SCX), 200 mg sorbent, 40 µm irregular silica, benzenesulfonic acid-bonded, not endcapped, 3 mL cartridge (Varian, Harbor City, CA).

(**c**) *Syringe filters.*—Nylon, 0.45 µm, 13 mm, Cat. No. 6840-1304 (Whatman), or equivalent, filter with housing diameter that fits BenchMate filter dispenser.

(**d**) *Blenders.*—Model 33AM26 1 quart glass jar blender, and Model 34BL22 1 gallon stainless steel jar blender (Waring Products Division, New Hartford, CT).

(**e**) *Choppers.*—1 gallon, Model 84145, and 2 gallon, Model 84181D (Hobart, Troy, OH).

(**f**) *Food processors.*—Model R6N, 6 quart, and Model R10, 10 quart food processors (Robot Coupe USA, Jackson, MS).

(**g**) *Containers.*—Wide-mouth, clear, colorless gallon jars with screw caps (Ryco Packing Corp, Lenexa, KS), pint and quart Mason jars with 2-piece screw caps (lids put on jars with the rubber seal not contacting contents), 32 oz. paper cups with paper lids (Sealright, Kansas City, MO).

Reagents

(**a**) *Mobile phase.*—Acetonitrile–phosphate buffer pH 4.0, 0.05M (30 + 70); mix 300 mL acetonitrile with 700 mL phosphate buffer pH 4.0, 0.05M, and filter through 0.2 µm nylon membrane filter.

(**b**) *Alternative mobile phase.*—Methanol–phosphate buffer pH 4.0, 0.05M (50 + 50); mix 500 mL methanol with 500 mL phosphate buffer pH 4.0, 0.05M, and filter through 0.2 µm nylon membrane filter.

(**c**) *Solvents.*—Acetone and methanol are distilled-in-glass, pesticide grade; acetonitrile is UV grade; deionized water is filtered with a 0.2 µm nylon membrane filter.

(**d**) *o-Phosphoric acid.*—85%, LC grade.

(**e**) *Acetic acid.*—Glacial, reagent grade.

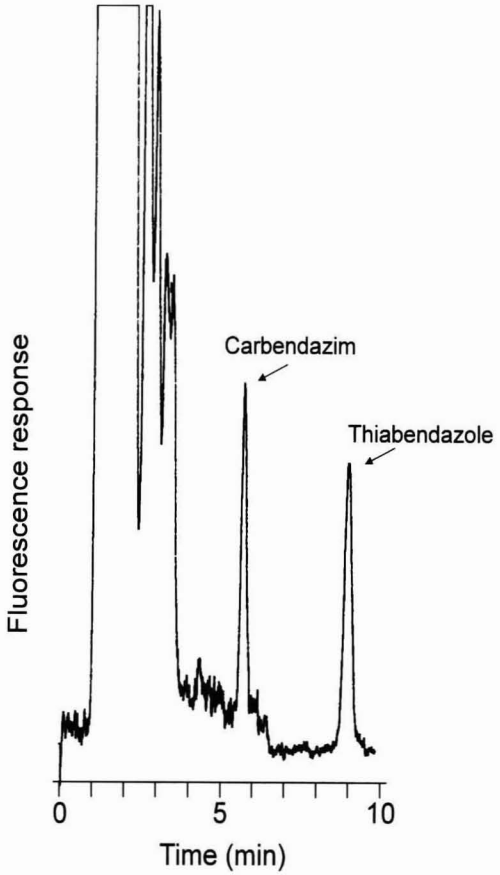

Figure 1. Chromatogram of peach sample containing incurred residues of benomyl (154 ng/g) as carbendazim and of thiabendazole (11 ng/g) after acetone extraction and SPE SCX cleanup.

(**f**) *Potassium hydroxide (KOH).*—Pellets, ACS certified (Fisher, Fair Lawn, NJ).

(**g**) *Phosphate buffer, pH 4.0, 0.05M.*—Mix 50 mL 1M mixed acetic–phosphoric acid solution with water to make 1 L and adjust to pH 4.0 with 50% (w/v) KOH solution.

(**h**) *Phosphate buffer, pH 6.0, 0.2M.*—Mix 200 mL 1M mixed acetic–phosphoric acid solution with water to make 1 L and adjust to pH 6.0 with 50% (w/v) KOH solution.

(**i**) *Extract diluent.*—1% phosphoric acid.

(**j**) *SPE wash solution.*—1% phosphoric in acetone–water (35 + 65); mix 350 mL acetone with 640 mL deionized water and 10 mL 85% *o*-phosphoric acid.

(**k**) *SPE elution solution.*—Acetonitrile–phosphate buffer pH 6.0, 0.2M (40 + 60); mix 400 mL acetonitrile with 600 mL phosphate buffer pH 6.0, 0.2M and filter through 0.45 µm nylon membrane filter.

Table 1. Analyte recoveries from foods fortified at 0.05 and 0.50 μg/g with benomyl and thiabendazole, determined by automated SCX SPE and LC with fluorescence detection

	Recovery, % (CV)[a] at indicated spike levels			
	0.05 μg/g		0.50 μg/g	
Item	Benomyl	TBZ	Benomyl	TBZ
Baby food, fruits, apricots/tapioca	76	91	90	80
Baby food, juice, apple–grape	100	82	86	76
Baby food, juice, apple–prune	73 (26)	87 (12)	84 (4)	86 (0)
Baby food, juice, grape	84	91	90	81
Baby food, juice, mixed fruit	35	97	90	81
Cantaloupe, raw	119 (7)	98 (3)	96 (7)	78 (1)
Carrot, fresh, boiled	95	89	88	79
Coffee, decaffeinated, from instant	55	113	87	80
Coffee, from ground	91	102	90	80
Dry table wine	101 (16)	82 (3)	74 (6)	80 (2)
Fruit drink, canned (e.g., Hi-C)	89	76	84	72
Grape juice, from frozen concentrate	44	81	89	78
Green beans, strained/junior	91	90	93	84
Jelly, any flavor	86 (19)	86 (3)	82 (2)	70 (0)
Mushroom soup, canned, condensed	92 (19)	90 (7)	91 (2)	74 (1)
Mushrooms, raw	58	90	90	76
Orange juice, from frozen concentrate	101	90	92	84
Peach, canned in light/medium syrup	114	86	90	82
Pear, canned in light syrup	83	85	91	77
Pineapple juice, from frozen concentrate	—[b]	71	79	70
Pineapple, canned in juice	74	86	88	84
Prune juice, bottled	73 (31)	70 (1)	83 (4)	69 (2)
Prunes, dried	73	80	97	77
Rice cereal, strained/junior	92 (29)	96 (4)	90 (4)	64 (1)
Rice infant cereal, instant	89	93	93	76
Tea, from tea bag	86	86	93	81
Tomato juice, bottled	41	93	83	80
Tomato sauce, plain, bottled	53 (5)	67 (1)	62 (3)	61 (0)
Tomato soup, canned, condensed	87	92	97	82
Tomato, stewed, canned	21	86	83	82
White potato, boiled without skin	82[c] (1)	108 (1)	122 (13)	85 (0)
White rice, cooked	65 (2)	94 (0)	90 (2)	80 (1)

[a] CV is shown in parentheses for cases where $n = 3$.
[b] Could not be determined because of interfering peaks in the chromatograms.
[c] Estimated from UV data because of interfering peaks in fluorescence chromatogram.

Standard Solutions

(**a**) *Stock standard solutions.*—Benomyl and TBZ reference standards were obtained from the U.S. Environmental Protection Agency repository. Stock solutions (1 mg/mL) were prepared by weighing 15–20 mg material into an appropriate container and adding an accurately measured volume of methanol. Benomyl standards quantitatively convert to carbendazim after standing overnight. Any benomyl residues in samples are converted to carbendazim during analyses (2). For purposes of calculations and reporting, carbendazim LC peaks are treated as if they arise from benomyl, and no conversion is necessary.

(**b**) *LC mixed standard.*—0.2 μg/mL benomyl and 0.02 μg/mL TBZ, prepared by adding an appropriate amount of each stock standard solution to a volumetric flask and diluting with acetonitrile–0.2M buffer pH 6.0 (40 + 60).

(**c**) *Spiking mixed-standard solution.*—Standard solutions for spiking were prepared by mixing appropriate amounts of each stock standard solution in a volumetric flask and diluting to volume with acetone–water (70 + 30) to obtain solutions containing each standard at 5 or 50 μg/mL.

Sample Preparation

Foods and ingredients are purchased at retail outlets in the collection cities and shipped to the TDS laboratory in Lenexa, KS. Some items go to a contract institutional kitchen for preparation and are separated from those that are prepared by the TDS group. Any items that need to be frozen are immediately

placed in the freezer, and all others are kept in refrigerator storage until their individual preparation. Foods that require preparation are treated as they would be by a typical consumer, which includes washing, peeling, discarding inedible portions, and cooking according to standard recipes with added spices and ingredients.

After cooking or any necessary preparation of the items, equal weights of samples from the 3 cities are blended, chopped, or mixed until homogenous and poured into a composite container. Individual jars and cups contain at least 300 g of each item and are frozen until analysis.

Extraction

The Luke extraction procedure (16) is used. Defrost a frozen composite, mix to homogeneity, and weigh 100 g into a labeled blender cup. Add 200 mL acetone and blend at high speed for 2 min. Place a Büchner funnel with sharkskin filter on a side-arm vacuum flask and filter acetone extract under vacuum until acetone no longer elutes from the funnel. Transfer a portion of the filtrate to a 250 mL Erlenmeyer flask.

Method Recovery Spikes

Food composites are fortified with benomyl and TBZ at 0.05 and 0.5 μg/g by adding 1 mL of the appropriate spiking solution. Recoveries are calculated after subtracting any incurred residue.

Automated SCX Cleanup

The BenchMate was programmed to prepare samples for LC analysis. Approximately 6 mL acetone filtrate is dispensed through a 0.45 μm nylon syringe filter into a test tube, and the remainder of the procedure is completed by automation. A dilution program takes 5 mL of the portion, dilutes it to 10 mL by addition of 1% phosphoric acid, and agitates the tube with a Vortex mixer. The cleanup program conditions the SCX column with 5 mL methanol followed by 5 mL 1% phosphoric acid in acetone–water (35 + 65). The 10 mL diluted extract is loaded onto the column, and the column is rinsed with 5 mL methanol and dried with N_2 gas for 60 s. The SCX is eluted with 5.25 mL acetonitrile–phosphate buffer pH 6.0, 0.2M (40 + 60); the eluate is agitated with a Vortex mixer, a 3.0 mL portion is filtered through a 0.45 μm nylon filter, and 50 μL is injected into the LC system.

Determination

The workstation injects 50 μL of each SCX eluate, with groups of 4 samples bracketed by standard injections.

Residue levels are calculated with the following formula:

$$\text{Residue, } \mu g/g = \frac{\text{Sample peak area} \times \text{Std, } \mu g/mL}{\text{Std peak area} \times \text{Sample, g}} \times (\text{Acetone, mL} + \text{Sample moisture, mL})$$

Table 2. Analysis of benomyl incurred residues (>10 ng/g), by automated SPE method and TDS PAM method

Item	Incurred residue, ng/g	
	SPE	TDS
Apple, red, raw	149	80
Applesauce, bottled	20	32
Baby food, fruits, pears, and pineapple	12	25
Baby food, juice, apple–cherry	18	26
Cantaloupe, raw	17	35
Mushrooms, raw	58	39
Peach, raw	154	147
Pineapple, canned in juice	15	ND[a]
Plums, raw	214	195
Strawberries, raw	219	179

[a] ND = none detected.

where the volume of acetone used to extract the food is adjusted for an estimated 10 mL volume contraction because of mixing with water from the sample.

Spike levels are calculated as follows:

$$\text{Spike, } \mu g/g = \frac{\text{Spiking solution, mL} \times \text{Spiking solution, } \mu g/mL}{\text{Sample weight, g}}$$

To determine recovery of a spiked item, any incurred residue in that item is subtracted from the apparent spike residue:

$$\text{Recovery, \%} = 100 \times \frac{\text{Residues, } \mu g/g - \text{Incurred residue, } \mu g/g}{\text{Spike level, } \mu g/g}$$

System Suitability

Determine optimum fluorescence detection parameters for the 2 compounds. For the detection system described above, optimum wavelengths for carbendazim were 270 nm for excitation and 304 nm for emission. For TBZ, they were 290 nm for excitation and 388 nm for emission.

Inject 50 μL LC mixed standard solution containing 0.2 μg/mL benomyl and 0.02 μg/mL TBZ and determine capacity factors and resolution for the 2 peaks. Capacity factors should be ca 2 for benomyl and ca 3.5 for TBZ and resolution between the 2 peaks should be 4 or greater. The organic/buffer ratio in the mobile phase may be adjusted to achieve the appropriate values.

Inject standard solution 6 times and determine peak area reproducibility. The coefficient of variation for each peak should be 4% or less.

Prepare a series of mixed standards in SPE eluting solution, containing from 0.1 to 1.0 μg/mL benomyl and from 0.01 to 0.1 μg/mL TBZ. Determine linearity for each compound by as-

saying 50 µL of each solution after adjusting LC to obtain conditions described above.

Results and Discussion

Method Development

For analytes such as carbendazim and TBZ that form cations at low pH, SCX cartridges based on a benzenesulfonic acid-bonded phase offer better control over the cleanup process than the more commonly used C_{18} material by making ionic interactions available as a selective retention mechanism in addition to significant nonpolar interaction with the benzene ring. Solvent and buffer variables requiring control include pH, ionic strength, solvent strength, and selectivity of counterions. Eluting an analyte of interest requires use of a high ionic strength and addition of a nonpolar solvent. Solvents that meet only one of these requirements can be used effectively as wash solvents for matrix simplification.

It was advantageous to replace the PAM methanol extraction solvent with an acetone extraction because acetone has suitable extraction properties and it is the solvent already used by TDS for extraction of organohalogen and organophosphorus residues. It is possible to use a portion of existing extracts for benomyl and TBZ analyses. Initial development focused on attaining maximum analyte recovery from a set of representative TDS items because it was impractical to vary all parameters for all foods.

SCX material was found to work better than PRS. The BenchMate limitation on column loading volume necessitates elution with the smallest possible volume to avoid unwanted dilution; 5 mL is a practical lower limit to allow for loss during filtration prior to LC and for the need to have adequate volume for multiple injections. When 5 mL of a spiked acetone extract was acidified to a concentration of 1% (v/v) phosphoric acid and loaded on a 500 mg SCX SPE column, strong retention of MBC and TBZ prevented complete analyte elution from the cartridge with 5 mL of any eluent. A 200 mg cartridge gave good recoveries.

During preliminary experiments, problems with some matrixes were encountered when the method was extended to the full variety of market basket foods (data not shown). Good recovery data demonstrated that the SPE method worked for 90% of the foods in the study but failed for the rest because of analyte breakthrough during SCX column loading. Low recoveries generally were associated with high-salt-content foods. It is likely that the high ionic strength of some matrixes provides a concentration of cations that compete with the analytes for SCX binding sites. Salt concentrations in extracts of TDS food were estimated from data in the U.S. Department of Agriculture's Agriculture Handbook Release 11 (17) for 100 g samples extracted with a mixture of 200 mL acetone plus the intrinsic moisture content. These are upper limits, based on the assumption that the elements exist entirely in the form of extractable cations. Every cation has a different selectivity for ion-exchange materials, hence these values are only rough predictors of chromatographic behavior. To test the correlation between high ionic strength and low recovery, 0.07 µg/g solutions of benomyl and TBZ in acetone–water (70 + 30, roughly the solvent composition of the extract for the foods in this study) were spiked with various levels of sodium and potassium cations. Recoveries of both analytes through the SCX procedure were reduced by approximately half when either cation was increased to 2 mg/mL. This ionic strength approximates the level expected in the saltiest TDS food items that are part of the TDS benzimidazole study. Recoveries returned to about 100% when the spiked solutions were diluted 1:2 with water before loading them onto the SCX columns. This provides some evidence that high amounts of salt, in combination with the 70% acetone, contributed to analyte breakthrough, and it was found that the method was improved by reducing the ionic strength and acetone content of the acetone–water extracts by dilution with water.

Table 3. Analysis of thiabendazole incurred residues (>8 ng/g), by automated SPE method and TDS PAM method

Item	Incurred residue, ng/g	
	SPE	TDS
Apple juice, bottled	21	29
Apple juice, strained	259	258
Apple, red, raw	456	812
Applesauce, bottled	39	32
Baby food, juice, apple-banana	52	58
Baby food, juice, mixed fruit	9	10
Baby food, juice, pear	89	84
Banana, raw	11	9
Grapefruit juice, from frozen concentrate	9	9
Grapefruit, raw	52	39
Mushroom soup, canned, condensed	21	22
Mushrooms, raw	193	325
Orange, raw	53	37
Peach, raw	11	ND[a]
Pear, raw	357	391
Plums, raw	24	17
Rice cereal, strained/junior	36	26
Rice infant cereal, instant	ND	13
Tomato soup, canned, condensed	11	ND
White potato, baked with skin	67	56

[a] ND = none detected.

Two other modifications were considered to cope with high-ionic-strength extracts. The simplest was reducing sample size from 5 mL to 1 mL, which increased recovery but is unacceptable for trace residues because of decreased sensitivity. An approach that does not decrease sample size is addition of a C_{18} SPE cleanup that desalts the Luke extract prior to the SCX procedure. This provides good recovery but is accompanied by increased cost and labor.

LC Analysis

A typical chromatogram from a peach sample containing incurred residues of both benomyl and TBZ after acetone extraction and SCX cleanup is shown in Figure 1. Fluorescence detection is more sensitive and specific than UV detection for

the analytes and was used for calculating and reporting findings. TBZ peaks can be detected at 2 ng/g, and the limit of quantitation is about 7 ng/g.

The detector is less sensitive to benomyl (as carbendazim). Benomyl generally can be detected and estimated at 23 ng/g. The limit of quantitation is about 70 ng/g. These limits can vary for different foods depending on the levels of coeluting peaks.

Concentrations are also tabulated from UV data. The UV detector is not sensitive enough for low-level incurred residues but is still useful to confirm results at higher levels. It is used for quantitation for a few matrixes where coeluting peaks in the fluorescence chromatogram make quantitation impossible.

Benomyl in foods is quantitatively converted to carbendazim before or during extraction, and the conversion occurs quickly in LC standards. Tolerances are established for the combined residues of benomyl and its metabolites containing the benzimidazole moiety, calculated as benomyl. Details of benomyl chemistry have been reported (18). There can be an overestimation of benomyl for foods that have been treated with carbendazim. The latter fungicide is not approved for use in this country and would pose a problem mainly with imported samples.

Recovery Studies

The SPE procedure for benomyl and TBZ was evaluated by fortifying 32 foods from the TDS and the infant–toddler survey to 0.05 and 0.50 μg/g with both compounds and correcting for any incurred residues (Table 1). Foods were selected to be representative for each of the TDS commodity groups. Recoveries were acceptable for most foods. At the 0.05 μg/g level, benomyl is more difficult to determine than TBZ because fluorescence detection is less sensitive to benomyl and benomyl elutes in a region of the chromatogram where some foods have coeluting peaks. A few foods gave low recoveries or could not be determined because of interfering peaks. Other than some problems with low-level benomyl, recoveries ranged from about 60 to 110%, with an average over 80%.

Also shown in Table 1 is a group of food items for which recoveries were determined in triplicate to estimate method repeatability (CV) for those matrixes. As expected, some problems were seen with benomyl at 0.05 μg/g because that level is below the limit of quantitation. Data are shown for comparison. They indicate that the method may have some utility at that level even though accuracy is reduced. Repeatabilities for TBZ and higher levels of benomyl are very good.

Incurred Residues

Determinations by SPE and PAM procedures were performed in parallel in order to compare the automated method with a validated method in their ability to detect incurred residues. PAM data were obtained as part of the TDS. A total of 47 table-ready foods were assayed by both methods. Of those, 22 foods had no detectable residue by either method: 3 infant–toddler foods (apricots/tapioca, instant rice, and green beans), 4 infant–toddler juices (apple–grape, apple–peach, apple–prune, and grape), fresh boiled carrots, instant decaffeinated coffee, ground coffee, tea, dry table wine, canned fruit drink, 3 juices from frozen concentrate (grape, pineapple, and orange), jelly, peaches canned in light or medium syrup, pears canned in light syrup, bottled tomato juice, bottled tomato sauce, and white potato boiled without skin.

There were 25 foods having at least a trace of one residue detected by one or both methods (Tables 2 and 3). For this comparison, estimated trace amounts considered to be below the limits of quantitation are included in the tabulation to compare the sensitivities and accuracies of the 2 methods for these difficult-to-detect residues.

The PAM method found that 22 foods contained a total of 27 incurred residues (9 benomyl and 18 TBZ). Qualitative agreement between the methods was very good. The only discrepancies were at levels below 15 ng/g. In 3 cases, trace residues detected by the SPE method were not confirmed by the PAM method. In one case, the SPE method did not detect a trace residue found by the PAM method. The quantitative agreement is good, with a few problems that can be noted in the data. In general, concentrations from the 2 methods are close, with a few results deviating by up to a factor of 2. The encouraging results obtained to this point will need to be validated with additional samples.

Conclusion

An automated SPE procedure coupled with LC determination was developed for analysis of benomyl (as carbendazim) and TBZ in TDS foods. This SPE cleanup replaces a procedure that uses partitioning between methylene chloride and an aqueous phase to clean extracts for determination by LC. In addition to eliminating use of about 150 L methylene chloride per year from the TDS program, the SPE method is a less labor-intensive procedure than the PAM method because much of the work is accomplished with unattended automation.

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RESIDUES AND TRACE ELEMENTS

Stability of Organochlorine and Organophosphorus Pesticides when Extracted from Solid Matrixes with Microwave Energy

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A stability study of 44 organochlorine pesticides (OCPs) and 47 organophosphorus pesticides (OPPs) was conducted. Compounds were spiked into solvent only (hexane–acetone, 1 + 1; methylene chloride–acetone, 1 + 1; methyl *tert*-butyl ether [MTBE]; and toluene–methanol, 10 + 1), solvent/dry soil suspensions, and solvent/wet soil suspensions (20% water, w/w). Spiked matrixes were heated in closed vessels with microwave energy at 2 temperatures (50° and 145°C) for 5 or 20 min. For comparison and for determination of nitrogen blowdown losses, spiked matrixes that had not been exposed to microwave energy were concentrated by using the blowdown technique and analyzed for each of the spiked compounds. For OCPs, temperature had the most significant effect on compound recovery, followed by matrix. All 3 pairwise comparisons of the 3 matrix types were statistically significant. The solvent factor was also significant, with average recoveries of 97.8% with methylene chloride–acetone, 96.3% with toluene–methanol, 92.8% with hexane–acetone, and 92.3% with MTBE. Of the 6 pairwise comparisons among the 4 solvents, all but 2—methylene chloride–acetone versus toluene–methanol and hexane–acetone versus MTBE—were statistically significant. For OPPs, temperature also had the most significant effect on recovery, followed by matrix. All 3 pairwise comparisons of the 3 matrix types were statistically significant in the same order as for OCPs. The solvent factor was also significant, but average recoveries—89.5% with hexane–acetone, 89.4% with methylene chloride–acetone, and 81.5% with either MTBE or toluene–methanol—are in a slightly different order from that for OCPs. Of the 6 pairwise comparisons among the 4 solvents, all but 2—hexane–acetone versus methylene chloride–acetone and MTBE versus toluene–methanol—were statistically significant. Compounds that appear to degrade under microwave-assisted extraction conditions include TEPP, phosphamidon, trichlorfon, naled, monocrotophos, demeton-O, and demeton-S.

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Earlier (1, 2) we reported the extractability under microwave-assisted extraction (MAE) conditions of 95 compounds listed in the U.S. Environmental Protection Agency (EPA) Method 8250, 44 organochlorine pesticides (OCPs) and polychlorinated biphenyls listed in EPA Method 8081, and 47 organophosphorus pesticides (OPPs; including 2 triazine herbicides) listed in EPA Method 8141, from freshly spiked soil samples; spiked soil samples aged for 24 h, 14 days, or 21 days; and a few standard reference materials with hexane–acetone (1 + 1) at 115°C for 10 min. In this paper, we report on the stability of the 44 OCPs and 47 OPPs under MAE conditions. A similar study for 95 semivolatile compounds listed in the EPA Method 8250 was reported elsewhere (3).

Solvents other than hexane–acetone have been used to extract some of the more polar compounds (4). However, none of the solvents recommended by EPA in Methods 3540 and 3550 (e.g., methylene chloride–acetone and toluene–methanol) has been thoroughly evaluated for use in MAE. Furthermore, the potential degradation or conversion of compounds that may occur when using microwave energy to heat the solvent/soil suspension has not been investigated. Possible ways in which compound degradation may occur include exposure to the temperature and pressure inside the microwave extraction vessel, interaction with other analytes or the solvent under these conditions, and catalysis by the matrix. To help determine whether degradation under MAE conditions may present a problem, a stability study was conducted for 44 OCPs and 47 OPPs (Table 1; atrazine and simazine were included with the OPPs, even though they are triazine herbicides, because they are listed in EPA Method 8141). These compounds were spiked into solvent only (hexane–acetone, 1 + 1; methylene chloride–acetone, 1 + 1; methyl *tert*-butyl ether [MTBE]; and toluene–methanol, 10 + 1), solvent/dry soil suspensions, and solvent/wet soil suspensions (20% water, w/w). Spiked matrixes were heated in closed vessels with microwave energy at 2 temperatures (50° and 145°C) for 5 or 20 min. For comparison and for determination of nitrogen blowdown losses, spiked matrixes that had

Table 1. Organochlorine and organophosphorus pesticides investigated in this study

Organochlorine pesticides	Organophosphorus pesticides
Alachlor	Aspon
Aldrin	Atrazine
β-BHC	Azinphos-ethyl
γ-BHC	Azinphos-methyl
δ-BHC	Bolstar
Captafol	Chlorofenvinphos
Captan	Chlorpyrifos
Chlorobenzilate	Chlorpyrifos methyl
α-Chlordane	Coumaphos
γ-Chlordane	Crotoxyphos
Chloroneb	Demeton-O
Chlorothalonil	Demeton-S
DBCP	Dichlorfenthion
DCPA	Dichlorvos
4,4′-DDD	Dicrotophos
4,4′-DDE	Dimethoate
4,4′-DDT	Dioxathion
Decachlorobiphenyl	Disulfoton
Diallate	EPN
Dichlone	Ethion
Dichloran	Ethoprop
Dieldrin	Famphur
Endosulfan I	Fenitrothion
Endosulfan II	Fensulfothion
Endosulfan sulfate	Fenthion
Endrin	Fonophos
Endrin aldehyde	HMPA
Endrin ketone	Leptophos
Etridiazole	Malathion
Heptachlor	Merphos
Heptachlor epoxide	Mevinphos
Hexachlorobenzene	Monocrotophos
Hexachlorocyclopentadiene	Naled
Isodrin	Parathion ethyl
Methoxychlor	Phorate
Mirex	Phosmet
Nitrofen	Phosphamidon
PCNB	Ronnel
Perthane	Simazine
Propachlor	Stirophos
trans-Nonachlor	Sulfotepp
cis-Permethrin	TEPP
trans-Permethrin	Thionazin
Trifluralin	TOCP
	Tokuthion
	Trichlorfon
	Trichloronate

not been exposed to microwave energy were concentrated by using the blowdown technique and analyzed for each of the spiked compounds.

Experimental

Standards

Analytical reference standards of the OCPs were purchased from Absolute Standards (Camden, CT) as one composite solution in toluene–hexane (1 + 1) containing 17 compounds (aldrin, the 3 BHCs, 4,4′-DDD, 4,4′-DDE, 4,4′-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, and methoxychlor), each at 2 mg/mL; one composite solution in isooctane containing DBCP, etridiazole, chloroneb, propachlor, PCNB, and chlorobenzilate, each at 100 μg/mL; and one composite solution in isooctane containing dichloran, chlorothalonil, DCPA, methoxychlor, and *cis*- and *trans*-permethrin, each at 1 mg/mL. Alachlor (1 mg/mL), hexachlorocyclopentadiene (5 mg/mL), and isodrin (5 mg/mL) were purchased as individual solutions in methanol from NSI Environmental Solutions (Research Triangle Park, NC). The remaining OCPs listed in Table 1 were obtained as neat materials from Chem Service (West Chester, PA), Ultra Scientific (North Kingstown, RI), or Aldrich Chemical (Milwaukee, WI). An intermediate stock solution containing each of the 44 OCPs at 2 μg/mL (Table 1) in acetone was prepared by combining the various composite stock solutions and was used for spiking soil samples and for preparing calibration standards.

Analytical reference standards of the OPPs were purchased from Absolute Standards as individual solutions in either hexane or methanol, with the exception of aspon, azinphos-ethyl, chlorofenvinphos, chlorpyrifos methyl, dichlorofenthion, ethion, and fonophos, which were obtained as neat materials from Ultra Scientific, Inc.; dicrotophos, leptophos, phosmet, trichlorfon, tri-o-cresylphosphate (TOCP), and simazine, which were obtained as neat materials from Chem Service; famphur at 1 mg/mL in acetonitrile and atrazine at 1 mg/mL in methanol, which were obtained from NSI Environmental Solutions; and thionazin at 1 mg/mL in methanol, which was obtained from Supelco (Bellefonte, PA). An intermediate stock solution containing each of the 47 OPPs at 20 μg/mL (Table 1) in hexane–methanol–acetonitrile was prepared by combining the various individual stock solutions and was used for spiking soil samples and for preparing calibration standards.

Soil

The topsoil (pH, 7.5; cation exchange capacity, 14.6 mequiv/100 g; organic carbon content, 0.1%; water content, 2.6%; sand, 57.6%; silt, 21.8%; and clay, 20.6%) used in this study was obtained from Sandoz Crop Protection (Gilroy, CA).

Solvents

All solvents used in this study were distilled-in-glass and pesticide grade and were obtained from Baxter Scientific (McGaw Park, IL).

MAE Procedure

All MAE experiments were performed with an MES-1000 microwave sample extraction system (CEM Corporation, Matthews, NC) described in reference 1.

A 5 g portion of spiked soil was accurately weighed into an aluminum dish and transferred quantitatively to the Teflon-lined extraction vessel. For wet samples, the calculated volume of water was added to the sample in the extraction vessel and allowed to equilibrate with the matrix for ca 10 min. A solution containing the test compounds was added to each sample immediately before the solvent (30 mL) was added. Spike levels were 100–1000 ng/mL for OCPs and 5–10 ng/mL for OPPs. After a new rupture membrane was secured in place, the extraction vessel was closed. Extractions were performed at 50° and 145°C for 5 or 20 min at 100% power. After extraction, the vessels were allowed to cool to room temperature for ca 20 min before they were opened. The supernatant was filtered through glass wool prewashed with solvent and then combined with the 2–3 mL solvent rinse of the extracted sample. The extract was concentrated to ca 5 mL by nitrogen blowdown evaporation and centrifuged twice for 10 min at 2300 rpm to separate the fine particulates. The extract was concentrated to 1 mL for gas chromatographic analysis with electron capture detection (GC–ECD) or nitrogen–phosphorus detection (GC–NPD).

Four solvents were used: hexane–acetone (1 + 1), methylene chloride–acetone (1 + 1), methyl *tert*-butyl ether (MTBE), and toluene–methanol (10 + 1).

Analysis of Extracts

For OCP analyses, we used a Hewlett-Packard 5890 Series II gas chromatograph equipped with 2 ECDs and a Hewlett-Packard 5973A autoinjector. Samples were introduced via a splitless injector into a retention gap (20 cm length × 0.53 mm id) connected through a fused-silica Y-shaped inlet splitter to two 30 m length × 0.32 mm id × 0.25 μm film thickness fused-silica open-tubular columns (DB-5 and DB-1701) with helium as carrier gas at flow rates of 3.7 and 4.0 mL/min, respectively. The column temperature was increased at 5°C/min from 80° to 275°C (2 min hold). The injection volume was 1 μL, and the injector temperature was 250°C. A 3-point external standard calibration using standards at 25, 50, and 100 ng/mL was performed initially to establish the GC–ECD linear range. Not all compounds were at 25, 50, and 100 ng/mL because detector response varied significantly from compound to compound. Exceptions included the following compounds: trifluralin was at 50, 100, and 200 ng/mL; chlorothalonil, DCPA, dichloran, and the 2 permethrin isomers were at 75, 150, and 300 ng/mL; methoxychlor was at 100, 200, and 400 ng/mL; chlorobenzilate, chloroneb, DBCP, etridiazole, hexachlorocyclopentadiene, nitrofen, PCNB, and propachlor were at 125, 250, and 500 ng/mL; and alachlor, captafol, captan, diallate, dichlone, hexachlorobenzene, and perthane were at 250, 500, and 1000 ng/mL. For quantitation, we used average response factors from multilevel calibration. The measurement error as established by replicate injections of calibration standards is below 5% relative standard deviation (RSD).

For OPP analyses, we used a Hewlett-Packard 5890 Series II gas chromatograph equipped with 2 NPDs and a Hewlett-Packard 5973A autoinjector. Samples were introduced via a packed-column injector connected to a fused-silica retention gap (20 cm length × 0.53 mm id) and a fused-silica Y-shaped inlet splitter connected to two 30 m length × 0.32 mm id × 0.25 μm film fused-silica open-tubular columns (DB-5 and DB-1701) with helium as carrier gas at a flow rate of 3 mL/min. The column temperature was held at 120°C for 3 min and then increased at 5°C/min to 260°C (1 min hold). The injection volume was 1 μL, and the injector temperature was 250°C. A 4-point external standard calibration using standards at 1, 2.5, 5, and 10 μg/mL was performed initially to establish the GC–NPD linear range. The only compounds that were present at concentrations different from those stated above for OPPs were mevinphos, TEPP, naled, chlorfenvinphos, crotoxyphos, phosmet, azinphos-methyl, coumaphos, and dioxathion, which were at 2, 5, 10, and 20 μg/mL; and thionazin and trichloronate, which were at 0.5, 1.25, 2.5, and 5 ng/mL. For quantitation, we used average response factors from multilevel calibration. The measurement error as established by replicate injections of calibration standards is below 5% relative standard deviation.

Safety

The microwave unit incorporates several safety features (2) and should be operated in accordance with operating safety instructions recommended by CEM. A new rupture membrane per vessel should be used for each extraction. Should the membrane rupture because of increased pressure inside individual vessels, the solvent vapor is unlikely to leak into the cavity because all vessels are connected to a containment vessel via the solvent rupture vent tube. To prevent pressure buildup inside individual vessels, wet samples should not be extracted simultaneously with dry samples. When 12 samples are extracted simultaneously (this is the maximum number that can be extracted with the CEM system), they should be either all dry or all wet. Likewise, solvent blanks should not be heated together with samples that are to be extracted by MAE because the former will heat faster than the latter.

Statistical Analysis

Recovery results from the 2 groups of compounds were analyzed by analysis of variance (ANOVA) to estimate the main effects on recovery of heating time (2 levels), temperature (2 levels), solvent (4 levels), and matrix (3 levels) and their interactions. Analyses were performed separately for OCPs and OPPs at the 95% confidence level by using the general linear model procedure (PROC GLM) of the SAS statistical software. Prior to ANOVAs, recoveries below 10% were deleted. OCP recoveries greater than 150% were excluded as well. One high OPP recovery of 182% (statistical outlier, over 3 standard deviations from the mean) was also excluded. ANOVAs were performed on the reduced set of recovery data. All 4 main effects, six 2-way interactions, four 3-way interactions, and one 4-way interaction were then included in the model. If an interaction and/or main factor was not significant at the 95% confidence level, it was excluded from the model and ANOVA was performed again. A final linear model including only significant main effects and interactions was kept for final interpretation. Adjusted (for unequal sample sizes) least-square means for recovery were then computed at each level of main effects and interactions.

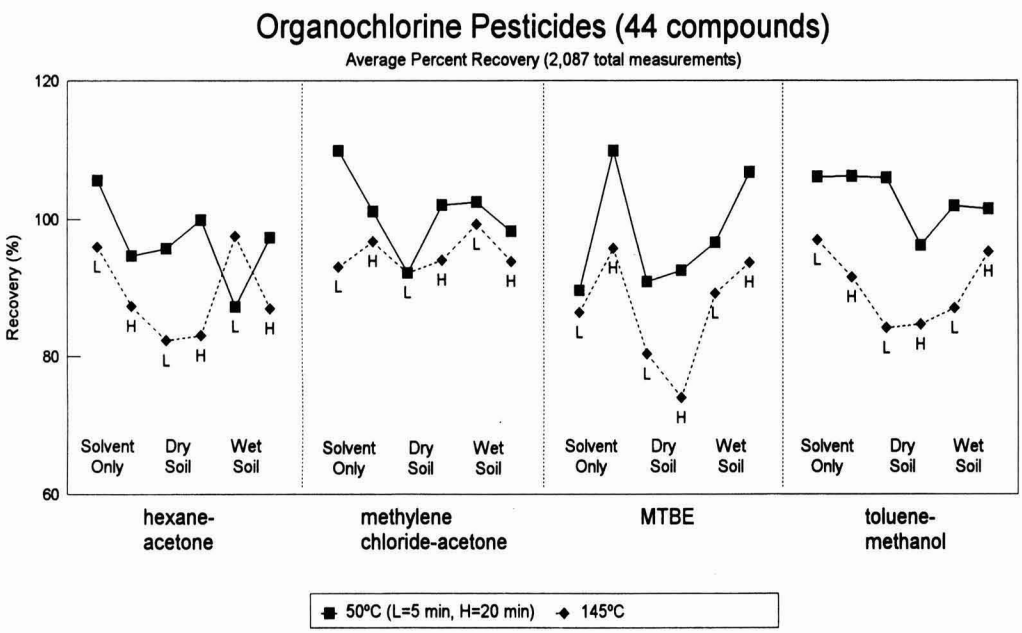

Figure 1. Effect of temperature on MAE recovery for organochlorine pesticides.

Results and Discussion

Organochlorine Pesticides

Of the initial 2112 recovery results (44 compounds × 2 heating times × 2 temperatures × 4 solvents × 3 matrixes), 28 or 1.3% (9 above 150% and 19 below 10%) were excluded as outliers. ANOVA was therefore based on the remaining 2084 recovery results ranging from 12.7 to 150%. One main factor, time ($p = 0.38$; average recoveries were 94.5% at 5 min heating and 95.2% at 20 min), two 2-way interactions (time × matrix and time × temperature), and one 3-way interaction (time × temperature × matrix) were not significant at the 95% confidence level and were thus excluded from the final analysis. On the basis of the final model, an average recovery of 94.9% with an RSD value of 16.8% was obtained across all 44 OCPs and 48 experimental conditions. The temperature was the most significant factor ($F = 193$ with 1 and 2036 degrees of freedom): average recoveries were of 99.6% at 50°C and 90.0% at 145°C. The 9.6% loss in recovery is statistically significant. The matrix was the second most significant factor ($F = 38.9$ with 2 and 2036 degrees of freedom). Average recoveries were 97.9% for solvent alone, 95.8% for solvent/wet soil suspensions, and 90.6% for solvent/dry soil suspensions. All 3 pairwise comparisons among the 3 matrix types were statistically significant. The solvent factor ($F = 14.8$ with 3 and 2036 degrees of freedom) was significant: average recoveries were 97.8% with methylene chloride–acetone, 96.3% with toluene–methanol, 92.8% with hexane–acetone, and 92.3% with MTBE. Of the 6 pairwise comparisons among the 4 solvents, all but 2—methylene chloride–acetone versus toluene–methanol and hexane–acetone versus MTBE—were statistically significant. Because the remaining interactions were all significant at the 5% level, average recoveries were plotted at the most detailed level, that is, separately by matrix, solvent, time, and temperature. Figures 1–3 show average recoveries (adjusted least-square means) for OCPs in 3 groupings. Figure 1 compares OCP recoveries at 50° and 145°C for each set defined by heating time, solvent, and matrix. Figure 2 compares OCP recoveries for solvent only, solvent/dry soil suspensions, and solvent/wet soil suspensions for each set defined by heating time, temperature, and solvent. Figure 3 compares OCP recoveries for the 4 solvents for each set defined by heating time, temperature, and matrix. Although all interactions remaining in the model were significant, there was no consistent pattern of matrix effect on recovery within solvent type. For example, in 9 of 16 cases (4 solvents × 2 heating times × 2 temperatures), best recoveries were obtained with solvent only, all other conditions being equal. In 11 of 16 cases, solvent/wet soil suspensions yielded higher recoveries than solvent/dry soil suspensions, all other conditions being equal. This lack of consistent pattern is further reflected in the significant interaction among all factors considered in the model.

Outliers for OCPs

Of the 28 outliers, 9 recoveries were in excess of 150% and 19 were below 10%. Of the compounds with low recoveries (that might have degraded under MAE conditions), hexachlorocyclopentadiene had 7 recoveries below 10% and dichlone had 5. The former is a fairly volatile compound (even the blowdown recoveries were somewhat lower for this compound), and the latter is relatively stable in methylene chloride–acetone and toluene–methanol. Low recoveries of dichlone were ob-

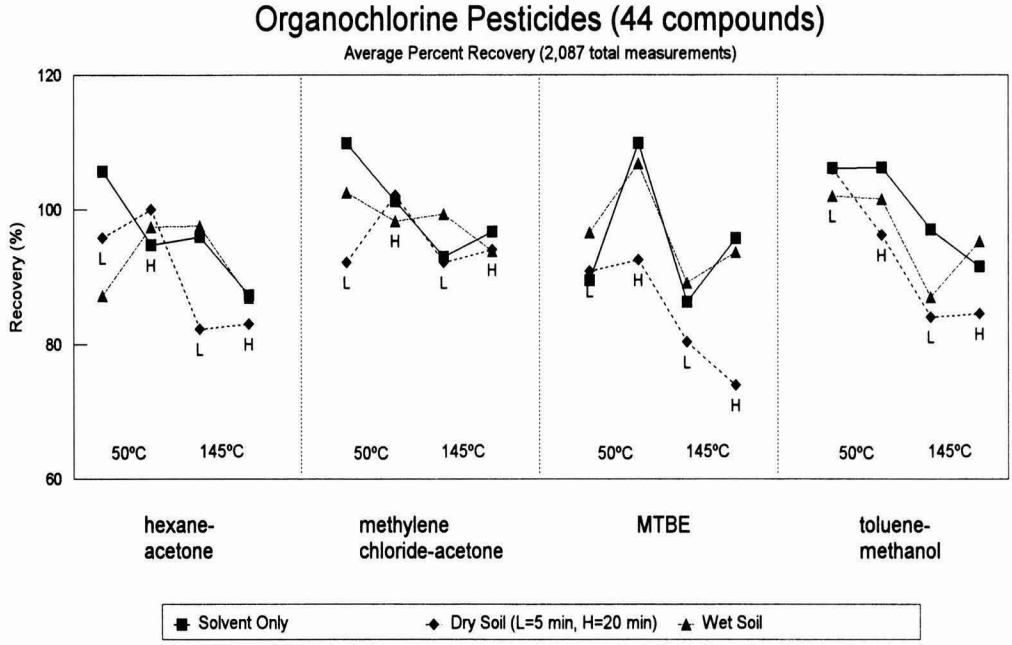

Figure 2. Effect of matrix on MAE recovery for organochlorine pesticides.

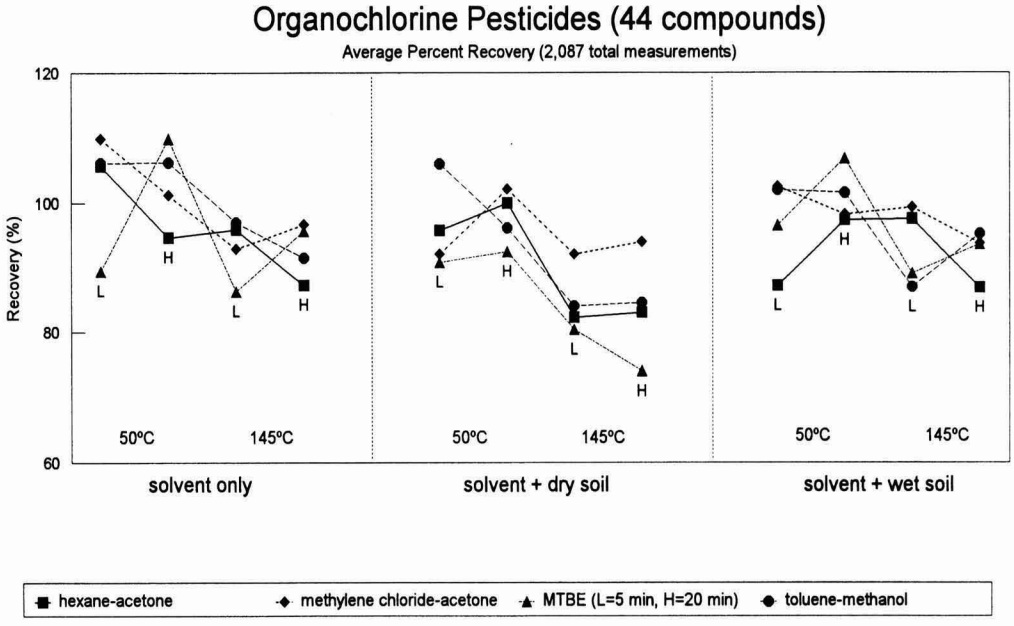

Figure 3. Effect of solvent on MAE recovery for organochlorine pesticides.

tained for hexane–acetone experiments at 145°C (at 5 and 20 min heating) and MTBE only and MTBE/dry soil suspensions (at 145°/5 min heating).

Organophosphorus Pesticides

Of the initial 2256 recovery results (47 compounds × 2 heating times × 2 temperatures × 4 solvents × 3 matrixes), 90 or 4% (1 high recovery value of 182% and 89 below 10%) were excluded as outliers. ANOVA was therefore based on the remaining 2166 recovery results ranging from 10.5 to 167%. Overall, OPP recoveries were lower and more variable than those for OCPs. As with OCPs, time ($p = 0.67$; average recoveries were 85.5% at 5 min heating and 85.2% at 20 min) and the 2-way interaction time × temperature ($p = 0.07$) were not significant at the 95% confidence level and were thus excluded from the final analysis. On the basis of the final model, an average recovery of 86.2% and an RSD value of 23.9% was obtained across all 47 OPPs and 48 experimental conditions. Temperature was the most significant factor ($F = 301$ with 1 and 2118 degrees of freedom): average recoveries were 93.2% at 50°C and 77.8% at 145°C. The 15.4% loss in recovery is statistically significant. Matrix was the second most significant factor ($F = 140$ with 2 and 2118 degrees of freedom). Average recoveries were 94.8% for solvent alone, 85.0% for solvent/wet soil suspensions, and 76.6% for solvent/dry soil suspensions. All 3 pairwise comparisons among the 3 matrix types were statistically significant, in the same order as those for OCPs. The solvent factor ($F = 26.8$ with 3 and 2118 degrees of freedom) was also significant but in a slighly different order from that observed with OCPs: Average recoveries were 89.5% with hexane–acetone, 89.4% with methylene chloride–acetone, and 81.5% with either MTBE or toluene–methanol. Of the 6 pairwise comparisons among the 4 solvents, all but 2—hexane–acetone versus methylene chloride–acetone and MTBE versus toluene–methanol—were statistically significant. Because the remaining interactions were all significant at the 5% level, average recoveries were plotted like those for OCPs. Figures 4–6 show average recoveries (adjusted least-square means) in 3 groupings. Figure 4 compares OPP recoveries at 50° and 145°C for each set defined by heating time, solvent, and matrix. Figure 5 compares OPP recoveries for solvent only, solvent/dry soil suspensions, and solvent/wet soil suspensions for each set defined by heating time, temperature, and solvent. Figure 6 compares OPP recoveries for the 4 solvents for each set defined by heating time, temperature, and matrix. Although all interactions remaining in the model were significant, there was no consistent pattern of matrix effect on recovery within solvent type, except for MTBE (Figure 5). However, the patterns here are more distinct than they were for OCPs. For example, in 13 of 16 cases (4 solvents × 2 heating times × 2 temperatures), highest recoveries were obtained with the solvent only, all other conditions being equal. In 12 of 16 cases, solvent/wet soil suspensions yielded higher recoveries than solvent/dry soil suspensions, all other conditions being equal. Finally, in 9 of 16 cases, recoveries with solvent only were always higher than those with solvent/wet soil suspensions, which in turn were always higher than those with solvent/dry soil suspensions. This pattern is especially clear for MTBE in Figure 5. In that case, the average recovery degradation from solvent only to solvent/wet soil suspensions ranged from 3.7% (50°C and 5 min) to 26.4% (145°C and 20 min), for an average recovery degradation of 11.6%. Similarly, the average degradation in recovery from solvent/wet soil suspensions to solvent/dry soil suspensions ranged from 12.8% (145°C and 20 min) to 36.1% (145°C and 5 min), for an average recovery degradation of 21.0%.

Outliers for OPPs

The 90 outliers removed from statistical analysis of OPPs were distributed as follows: one data point each for chlorfenvinphos, EPN, fensulfothion, and fonofos; 2 for dimethoate; 3 for mevinphos; 4 each for demeton-O and disulfoton; 5 each for dichlorvos and HMPA; 6 for dicrotophos, naled, and trichlorfon; 8 for demeton-S; 11 each for monocrotophos and phosphamidon; and 15 for TEPP.

Upon analysis of these data, the following conclusions can be drawn. In the case of TEPP, an unstable diphosphate that readily hydrolyzes in water and is thermally labile (e.g., decomposes at 170°C), the MAE data indicate that this compound is stable when heated up to 145°C in solvent alone. MAE recoveries dropped significantly when dry soil was present and were even lower when wet soil was added to solvent. For example, with hexane–acetone, MAE recoveries at 145°C were 102 and 95.8% for 5 and 20 min extractions, respectively. These dropped, respectively, to 9.4 and 19.2% with dry soil present and to 8.8 and 9.2% with wet soil present. With MTBE or toluene–methanol, TEPP recoveries at 145°C were 0 (except one data point of 17.9% for MTBE/wet soil suspension and 5 min heating time).

For phosphamidon, 10 of 11 outliers corresponded to MTBE/dry soil suspensions at 50° or 145°C, toluene–methanol/dry soil suspensions at 145°C, and toluene–methanol/wet soil suspensions at 50° (20 min only) or 145°C. For the other 2 solvent combinations, MAE recoveries in the presence of wet soil were still high (e.g., recoveries for hexane–acetone were 69.5 and 49% at 145°C for 5 and 20 min, respectively, and recoveries for methylene chloride–acetone were 81.5 and 67.2% at 145°C for 5 and 20 min, respectively). However, MAE recoveries dropped to 22.8 and 0% at 145°C for 5 and 20 min heating, respectively, for hexane–acetone/dry soil suspensions and to about 30% for methylene chloride–acetone/dry soil suspensions.

Trichlorfon and naled are converted to dichlorvos by dehydrochlorination and debromination, respectively (5). This reaction appears to take place even in the solvent-only experiments. For example, in the case of trichlorfon and hexane–acetone, MAE recoveries were 86% at 50°C and 5 min and 79.1% at 145°C at 5 min but dropped to 32% when heated at 145°C for 20 min. For solvent/dry soil suspensions, MAE recoveries were 11.9% (at 145°C/5 min) and 8.9% (at 145°C/20 min). In the case of naled, MAE recoveries dropped from 90.1 to 23.5% when going from heating at 50°C for 5 min to heating at 145°C for 5 min with hexane–acetone only, to 9.9% with hexane–acetone/dry soil suspensions heated at 145°C for 5 min, and to 0% recovery when heated at 145°C for 20 min.

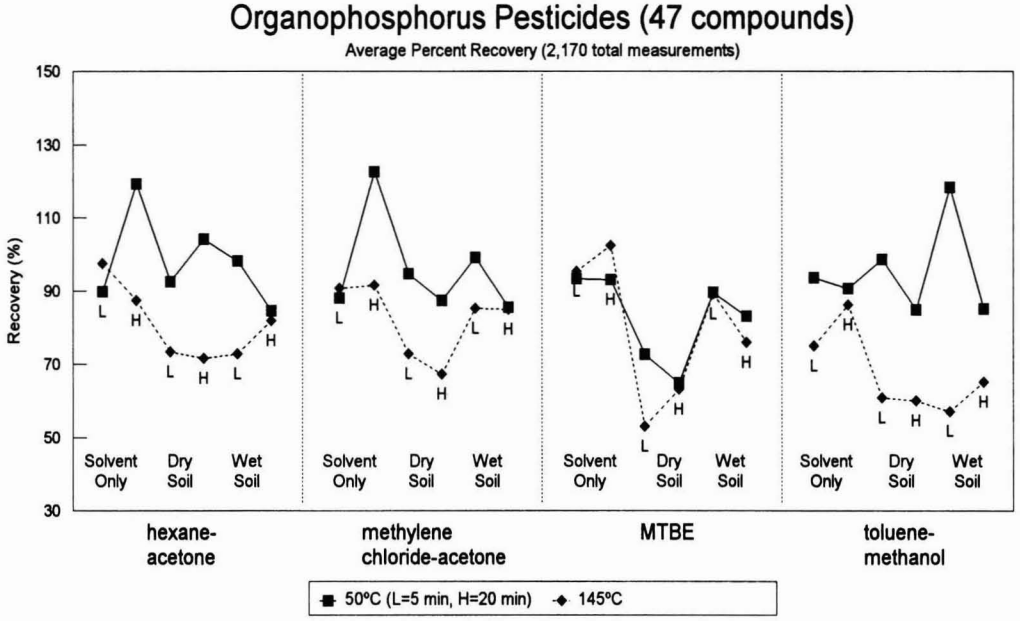

Figure 4. Effect of temperature on MAE recovery for organophosphorus pesticides.

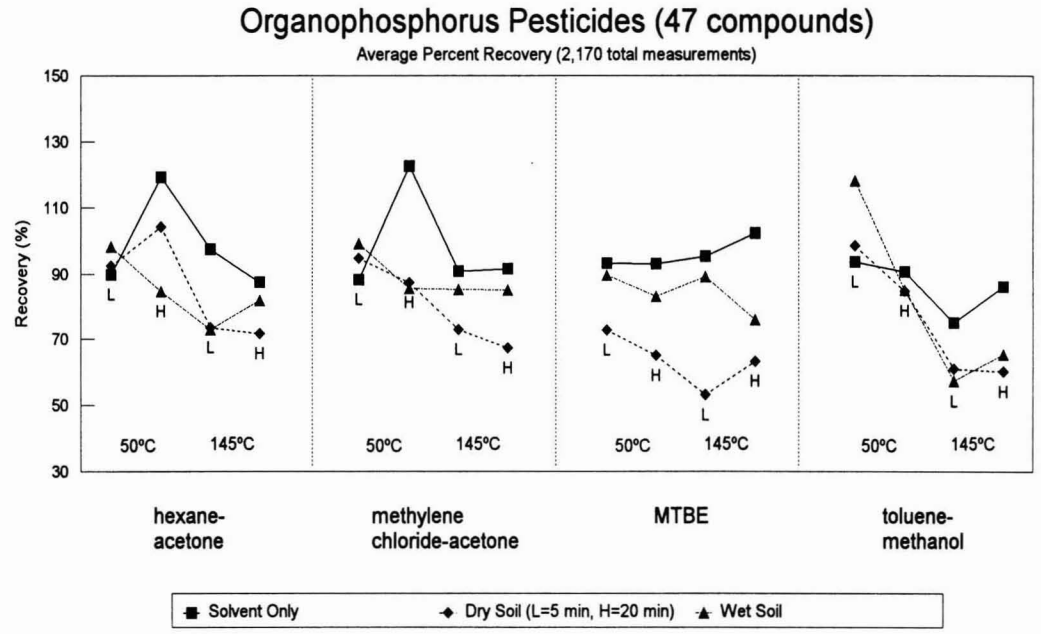

Figure 5. Effect of matrix on MAE recovery for organophosphorus pesticides.

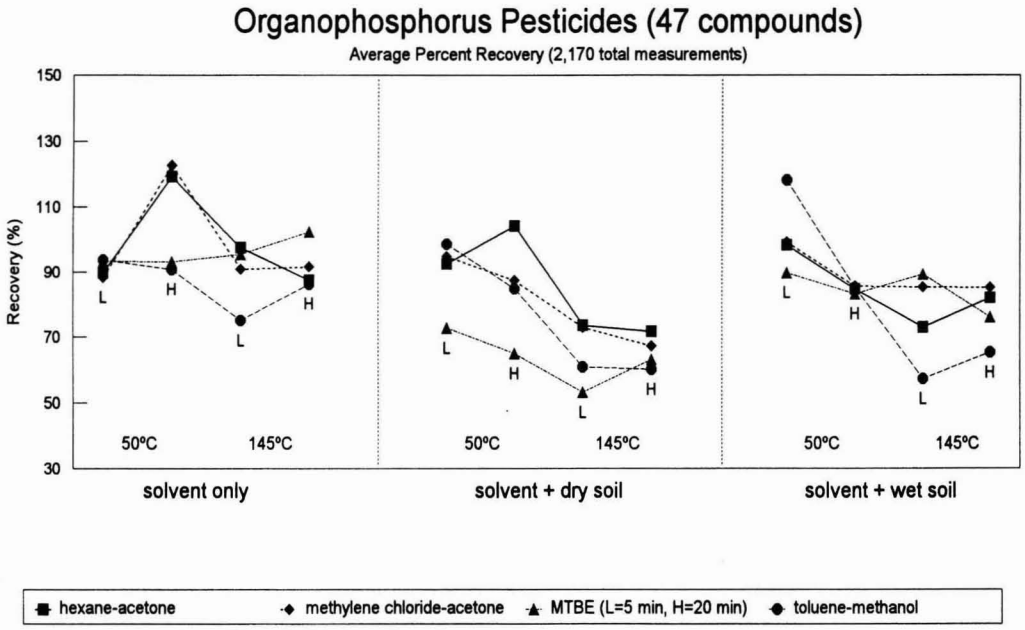

Figure 6. Effect of solvent on MAE recovery for organophosphorus pesticides.

Monocrotophos, demeton-O, and demeton-S also showed consistently lower recoveries when heated in the solvent/dry soil or solvent/wet soil suspensions and their recoveries also appeared to be a function of temperature and heating time.

Conclusions

For OCPs, temperature had the most significant effect on recovery, followed by matrix. All 3 pairwise comparisons of 3 matrix types were statistically significant. The solvent factor was significant, with average recoveries of 97.8% when using methylene chloride–acetone, 96.3% when using toluene–methanol, 92.8% when using hexane–acetone, and 92.3% when using MTBE. Of the 6 pairwise comparisons among the 4 solvents, all but 2—methylene chloride–acetone versus toluene–methanol and hexane–acetone versus MTBE—were statistically significant. Among 4 solvent combinations tested, the best solvents are hexane–acetone and methylene chloride–acetone. Both are easier to evaporate than toluene–methanol and MTBE. Nonetheless, solvent selection must be considered with matrix coextractives, which were not addressed in this study. Furthermore, optimum extraction temperature would have to be established by the user of this technology. We have demonstrated that within 50°–145°C, the OCP compounds investigated here are stable under irradiation with microwave energy, despite a 9.6% loss in recovery when temperature rises from 50° to 145°C.

For OPPs, temperature also had the most significant effect on recovery, followed by matrix. All 3 pairwise comparisons of the 3 matrix types were statistically significant, in the same order as for OCPs. The solvent factor was also significant, with average recoveries of 89.5% with hexane–acetone, 89.4% with methylene chloride–acetone, and 81.5% with either MTBE or toluene–methanol, in a slightly different order from those for OCPs. Of the 6 pairwise comparisons of the 4 solvents, all but 2—hexane–acetone versus methylene chloride–acetone and MTBE versus toluene–methanol were statistically significant. The same solvent combinations giving the best results for OCPs appear to give the best results for OPPs. However, for OPPs, several compounds appeared to degrade under MAE conditions (e.g., TEPP, phosphamidon, trichlorfon, naled, monocrotophos, demeton-O, and demeton-S). For these compounds, extraction with microwave energy would have to be performed at 50°C because of the thermal instability of the compounds.

Acknowledgment

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Notice

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development (ORD), partially funded and collaborated in the research described here. This article has not been subjected to the Agency's review and has not been approved as an EPA publication. Neither the EPA nor the ORD endorses or recommends any trade names or commercial products mentioned in this article; they are noted solely for the purpose of description and clarification. The U.S. Government has a nonexclusive royalty-free license in and to any copyright covering this article.

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RESIDUES AND TRACE ELEMENTS

Determination of Calcium by Inductively Coupled Plasma–Atomic Emission Spectrometry, and Lead by Graphite Furnace Atomic Absorption Spectrometry, in Calcium Supplements after Microwave Dissolution or Dry-Ash Digestion: Method Trial

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A 3-laboratory method trial was conducted to evaluate 2 sample digestion procedures and instrumental determination parameters for analysis of calcium and lead in Ca supplements. Calcium supplements were treated by dry-ash digestion or microwave dissolution prior to spectrometric analysis. In each case, Pb was determined by graphite furnace atomic absorption spectrometry and Ca by inductively coupled plasma–atomic emission spectrometry. Blind duplicates of 6 Ca supplement samples were analyzed after each sample treatment procedure. Matrix pairs contained dissimilar Pb levels to cover the analyte range encountered during method development. Calcium content of the Ca supplement samples also reflected the range seen during method development. Stock solutions of Ca and Pb were supplied to collaborators for preparation of quantitation standards to remove a variable external to the method. National Institute of Standards and Technology Standard Reference Material (NIST SRM) 1486, bone meal, was included to assess method accuracy and recovery at NIST certificate Ca and Pb levels for this material (26.58 ± 0.24% Ca and 1.335 ± 0.014 μg Pb/g). Analyses of the NIST SRM yielded 25.9 ± 1.1 and 27.2 ± 2.3% Ca and 1.53 ± 0.19 and 1.26 ± 0.19 μg Pb/g for dry-ash and microwave procedures, respectively. Statistical analyses of data indicated acceptable repeatability and reproducibility for determination of Pb and Ca in various Ca supplements. With either sample preparation technique, the method is appropriate for determining Pb or Ca in Ca supplements.

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A potential for lead exposure from calcium supplements exists for youth and developing fetuses. Calcium supplements often are prescribed to young children and expectant women to meet increased Ca needs. The presence of lead at elevated levels in some commonly available Ca supplements (1) is a concern because infants and young children absorb and retain a far greater percentage of consumed Pb than do adults (2). The harmful developmental and neurological effects of Pb on fetal development can occur even at slightly elevated Pb exposure levels (3). Children evaluated for neurological effects of Pb exposure as infants have significant intellectual and academic performance deficits at 10 years of age (4). Recent research indicates that infants exposed to Pb tend to display antisocial behavior as adolescents (5). In a 1991 report, the Centers for Disease Control stated that "lead poisoning is one of the most common and preventable pediatric health problems today" (6). Clearly, exposure of youth to Pb is an identifiable and preventable health risk that must continue to be addressed, and human exposure to Pb needs to be minimized.

The U.S. Food and Drug Administration (FDA) recently has begun to address calcium carbonate and other food-grade sources of Ca, proposing lower limits of Pb content than are currently in place (7). This action follows those taken by the United States Pharmacopeia (USP) and Food Chemicals Codex (FCC) to lower allowable Pb in $CaCO_3$ and a continually improving understanding of long term health risks associated with exposure to low levels of Pb. Both USP and FCC currently specify 3 mg allowable Pb/kg $CaCO_3$ (8, 9).

The method described here was developed in response to a request from FDA's Center for Food Safety and Applied Nutrition (CFSAN) for sensitive and reliable methods to quantitate

Pb in Ca supplements. The dry-ash method was developed initially to allow simultaneous preparation of many samples for analysis (10). The microwave dissolution procedure was developed to take advantage of the latest advancements in rapid sample preparation technology (Siitonen, unpublished data). Because sample mineralization occurs in closed Teflon Perfluoroalkoxy (PFA) vessels, microwave sample dissolution minimizes contamination while maximizing analyte recovery. Calcium analysis was included in the method to allow evaluation of Pb level on a Ca basis if needed for regulatory decisions. Regulatory scientists at CFSAN indicated that future Pb limits may be related to Ca content on a μg Pb/g Ca basis (11). Calcium results determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) after treatment of supplements are not significantly different from results obtained by instrumental neutron activation analysis (INAA) of intact supplements (10; Siitonen, unpublished data).

Manufacturers of Ca supplements recommend daily intakes that vary greatly for different supplements. Depending on type, commonly available Ca supplements have Ca contents varying from 9 to 40%. Manufacturers' recommended intake for achieving a recommended daily allowance for Ca can vary from as little as 2 g $CaCO_3$ to more than 11 g Ca lactate. Exposure to Pb would vary greatly if these 2 supplement types, with equivalent Pb contents, were taken as recommended. FDA uses interlaboratory method trials to validate methods with potential for regulatory use in FDA field laboratories and to comply with a recent draft guideline from the International Conference on Harmonization (12). AOAC INTERNATIONAL requires that performance characteristics of a peer-verified method be tested in one or more laboratories independent of the submitting laboratory (13). The method trial was conducted for FDA field ruggedness testing to determine whether major differences could be attributed to differences in graphite furnace atomic absorption spectrometry (GFAAS) instrumentation and whether the method meets requirements for an AOAC INTERNATIONAL peer-verified method.

Trial Conditions

Calcium supplements were purchased from area health food stores or obtained from chemical supply vendors. All Ca supplements purchased were labeled as suitable for human consumption or as USP grade. Two samples each of calcium carbonate, calcium lactate, and bone meal were chosen to represent the variety of available Ca supplements. Calcium supplements chosen for the study were those thought to be homogeneous. All gave Pb values with relative standard deviations (RSDs; $n \geq 4$) of <8% during method development and initial characterization. Supplements originating from the National Center for Toxicological Research (NCTR) gave Pb values with an average RSD of 3.9%. National Institute of Standards and Technology (NIST SRM) 1486 was included as one of the bone meal samples.

Prior to the method trial, participants received a practice Ca supplement sample to familiarize themselves with the method and to determine the suitability of their laboratory systems for applying the method. The collaborative method and background material on method development also were supplied. The practice sample characterized by GFAAS, ICP-AES, and INAA during method development was found to contain 35.3 ± 2.6% Ca and 0.711 ± 0.086 μg Pb/g at the 95% confidence limits. After participants became familiar with the method, they were provided with study samples.

Sample sets were prepared as follows: Approximately 1.5 g of each Ca supplement was distributed in duplicate to Nalgene low-density polyethylene bottles with sample code labels affixed. Two sets of 12 samples were prepared for each collaborator. Each sample set was coded and shipped separately with sample codes unknown to collaborators. Collaborators were asked to prepare one complete set of samples by each sample preparation technique. After completion of all analyses, participants were asked to return all raw data and results to originating laboratory for statistical evaluation.

For the originating laboratory to participate in the method trial without bias, 2 sets of samples were blind coded by an NCTR chemist not associated with the project. Sample codes were unknown to the participating NCTR chemist until sample data analyses were complete.

Statistical analyses for calculation of performance parameters were performed per AOAC INTERNATIONAL guidelines (14), with AOAC INTERNATIONAL-supplied computer worksheets and a LOTUS 1-2-3 macro program.

METHOD

Principle and Scope

(*Caution*: *See* safety notes on nitric acid and hydrogen peroxide.)

Calcium supplements are mineralized through either a dry-ash digestion or a microwave dissolution procedure. The sample digest matrix is modified by addition of $(NH_4)_2HPO_4$, and the resulting solution is analyzed for Pb by GFAAS and for Ca by ICP-AES. The method is applicable to Pb concentrations in supplement ranging from 0.20 to 10.0 μg/g and to Ca levels ranging from 9 to ca 40% by weight. Higher levels of Pb are addressed by dilution.

Apparatus

All labware and plasticware are washed initially with detergent, rinsed with deionized water, soaked overnight in 1N HNO_3, rinsed with deionized water, and air dried. Thereafter, thorough rinsing with 1N HNO_3 and deionized water is sufficient to avoid contamination.

(**a**) *GFAAS system.*—Capable of instrument detection limit of 2 ng Pb/mL, with background correction enabled, based on 3 × standard deviation (SD) of 3 blank H_2O atomizations; Thermo Jarrell Ash Corp. (Franklin, MA) Model Video 22 with Smith-Hieftje background correction and Model 755 furnace atomizer or equivalent.

(**b**) *Microwave digestion apparatus with Teflon-closed digestion vessels.*—CEM MDS-2000 with advanced composite vessels or equivalent (Matthews, NC).

Table 1. Method trial results: Calcium (%)

	Laboratory 1		Laboratory 2		Laboratory 3	
Sample pair[a]	Dry ash	Microwave	Dry ash	Microwave	Dry ash	Microwave
1	33.4	37.2	40.9	41.0	38.8	42.7
	35.1	36.7	40.7	40.2	38.7	42.6
2	35.0	37.8	41.5	41.7	39.8	42.8
	35.6	38.1	41.1	41.3	26.0[b]	42.8
3	12.8	13.9	14.2	14.6	13.9	13.7
	13.6	14.0	14.4	14.2	13.9	13.4
4	14.9	16.7	17.4	16.9	16.7	20.7
	15.2	16.7	17.2	16.9	16.4	20.8
5[c]	24.7	25.5	26.9	27.6	39.8[b]	31.6
	25.4	26.5	27.3	27.1	25.4	25.1
6	28.4	31.5	34.1	34.7	31.5	35.5
	30.3	31.2	33.6	34.1	31.3	32.0

[a] Sample pair types are as follows: 1 and 2, $CaCO_3$; 3 and 4, Ca lactate; 5 and 6, bone meal.
[b] *See Results and Discussion* and *Method Trial* sections of text.
[c] NIST SRM 1486 bone meal; certificate value, 26.58 ± 0.24% Ca.

(**c**) *ICP-AES system.*—Capable of calcium analysis, Thermo Jarrell Model ICAP 61E or equivalent.

(**d**) *Programmable muffle furnace.*—Lindburg type 51668 furnace with Model 59564 control console or equivalent (Watertown, WS).

(**e**) *Analytical balance.*—Calibrated, accuracy to 0.0001 g for sample weighing, Ohaus Corp. (Florham Park, NJ) Model GA200D or equivalent.

Materials

(**a**) *Calcium standard stock solution.*—10.00 mg/mL, NIST SRM 3109 or equivalent.

(**b**) *Lead standard stock solution.*—10.00 mg/mL, NIST SRM 3128 or equivalent.

(**c**) *Ammonium phosphate dibasic.*—Analytical reagent, meets ACS specifications, Mallinckrodt Chemical Works (St. Louis, MO) or equivalent.

(**d**) *Nitric acid 70.0–71.0%.*—Baker Instra-Analyzed reagent for trace metal analysis, J.T. Baker, Inc. (Phillipsburg, NJ) or equivalent.

(**e**) Hydrogen peroxide, 30%.—Baker-Analyzed reagent (stabilized), J.T. Baker or equivalent.

(**f**) Deionized water.—ASTM Type I, Barnstead/Thermolyne Corp. (Dubuque, IA) Nanopure deionized water system or equivalent.

Digestion

(**a**) Weigh duplicate 1 g samples of Ca supplement to 4 significant figures into suitable precleaned digestion vessels.

(**b**) Analyze a reagent blank—to monitor reagent contamination and limit of quantitation (LOQ)—with each batch of samples.

Dry-Ash Procedure

($\mathbf{c_1}$) Dry ash samples by using the following temperature program: heat from 200° to 450°C linearly over 4 h (to avoid ignition of sample), hold at 450°C for 8–16 h, and then cool to room temperature.

($\mathbf{d_1}$) Add 5 mL H_2O, 2 mL HNO_3 (cautiously because of foaming), and 2 mL 30% H_2O_2 to each sample and then heat sample at 125°C until nearly dry.

($\mathbf{e_1}$) Add up to 4 mL 30% H_2O_2 dropwise until all carbonaceous material is mineralized.

Microwave Dissolution Procedure

($\mathbf{c_2}$) Add 10 mL HNO_3 to each sample and allow sample to oxidize at ambient temperature for 1 h.

($\mathbf{d_2}$) Secure vessel caps and pressure monitor and heat samples through microwave program as follows: Apply 280 W for 10 min with a pressure limit of 50 psi followed by 5 min with no microwave radiation. Apply 420 W for 10 min with a pressure limit of 80 psi followed by 5 min with no heating. Apply 560 W for 10 min to complete dissolution.

($\mathbf{e_2}$) Upon completion of microwave heating, allow samples to cool for 10 min and vent pressurized vessels before removing vessel caps.

(**f**) Transfer mineralized sample to 50 mL volumetric flask with several rinses of ca 5 mL 1N HNO_3, add 1.0 mL 25% ammonium phosphate as matrix modifier, and adjust to volume with 1N HNO_3.

(**g**) Allow particulate material (MgO) to settle out of solution before diluting the digest 1:200 for Ca analysis by ICP-AES.

(**h**) Perform additional dilutions, if required for Pb analysis by GFAAS, with 1N HNO_3 containing 0.5% ammonium phosphate. *Note*: A 10:50 or 5:50 additional dilution of the original 50 mL sample digest was sufficient for measuring Pb content of supplements during method development. Ca supplements

Table 2. Method trial results: Lead (μg/g)

Sample pair[a]	Laboratory 1		Laboratory 2		Laboratory 3	
	Dry ash	Microwave	Dry ash	Microwave	Dry ash	Microwave
1	0.297	0.345	0.24	0.31	0.478[b]	0.377
	0.277	0.303	0.22	0.23	0.725[b]	<0.31[c]
2	1.36	1.34	1.22	1.29	1.33	1.11
	1.38	1.30	1.35	1.45	1.41	1.34
3	1.31	1.35	1.20	1.34	1.46	1.20
	1.27	1.11	1.24	1.27	1.21	0.99
4	0.116	<0.11	0.07	0.07	0.428[b]	<0.31
	0.138	<0.11	0.08	0.06	0.364[b]	<0.31
5[d]	1.58	1.29	1.33	1.49	1.68	1.09
	1.57	1.05	1.27	1.47	1.74	1.17
6	5.84	5.77	6.14	7.81	4.24	4.31
	5.96	5.15	8.77	6.81	5.03	3.87

[a] Sample pairs are as follows: 1 and 2, $CaCO_3$; 3 and 4, Ca lactate; 5 and 6 bone meal.
[b] *See* text.
[c] Results preceded by < represent LOQ.
[d] NIST SRM 1486 bone meal; certificate value, 1.335 ± 0.014 μg Pb/g.

with >4 μg Pb/g exceed the concentration range of the standard curve and therefore require this additional dilution.

Determination by GFAAS

The GFAAS instrument is configured and optimized for Pb analysis per manufacturer's specifications. Typically, instrument conditions for Pb analysis are wavelength = 283.3 nm, slit width = 320 μm, bandpass = 1 nm, background correction = enabled, integration time = 4 s, integration mode = peak area. A typical graphite furnace temperature program is sampling/drying = 100°–140°C/5 s, charring = 250°C/10 s, pyrolysis = 680°C/20 s, and ramp atomization = 2300°C/10 s. The amount of sample injected is adjusted such that standards containing 0–80 ng Pb/mL 0.5% ammonium phosphate in 1N HNO_3 yield a linear standard curve. The minimum fit for the standard curve is a linear regression $r \geq 0.995$. Data are acquired for standards to establish the standard curve prior to and after atomization of sample digests. At least 3 readings are taken for each sample or standard, and the peak areas are averaged for each. Using the peak area of unknown sample, the concentration of Pb in the digest is determined from the standard curve. The contribution of reagent blank and dilution factors are applied to obtain Pb concentration as μg Pb/g supplement:

$$\text{Pb concentration, } \mu g/g =$$

$$(\text{Pb } C_s \times 1\ \mu g/1000\ ng \times \text{dilution volume})/Wt_s$$

where Pb C_s = (Pb concentration in sample digest – reagent blank) and Wt_s = sample weight (g).

An instrument check standard (ICS) is read after each 10 samples to verify instrument stability. Instrument calibration and analysis of the prior 10 samples are required if ICS deviates from the actual value by more than ± 20%.

Determination by ICP-AES

The plasma is initialized and the system is allowed to equilibrate thermally over 30 min. Calibration and standardization are performed per manufacturer's specifications. Four readings are obtained for each sample or standard for signal statistics. Prior to sample analysis and after each 10 sample analyses, a calibration blank standard (CBS) and an ICS are read to evaluate instrument stability. If CBS differs by more than 3σ from the initial value or if ICS differs from the known value by more than ± 10%, analysis is terminated, the instrument is restandardized and the previous 10 samples are reanalyzed. Calcium is quantitated as follows:

$$Ca, \% = [(Ca\ C_s \times 10\ 000)/Wt_s] \times 100$$

where Ca C_s = Ca concentration in sample (mg/mL) and Wt_s = sample weight (mg).

Results and Discussion

Pretrial Practice

Laboratory 2 produced acceptable initial results from analysis of practice sample, finding Pb levels of 0.672 and 0.751 μg/g after microwave digestion and dry-ash digestion, respectively and Ca levels of 34.8 and 35.4%, respectively. The 95% confidence limit values for practice sample were 35.3 ± 2.6% Ca and 0.711 ± 0.086 μg Pb/g (determined by the originating laboratory). However, laboratory 3 could not secure Pb-free $(NH_4)_2HPO_4$ and had problems with the prescribed GFAAS furnace temperature conditions for sampling and drying. After Pb-free 25% $(NH_4)_2HPO_4$ was provided and furnace condition problems were solved, laboratory 3 found 0.879 and 0.787 μg Pb/g and 33.7 and 32.6% Ca after microwave and dry-ash digestion, respectively. These values were acceptable and the method trial phase of the study was initialized.

Table 3. Statistical treatment: Calcium (%)

Sample pair	Mean		RSD_r		RSD_R	
	Dry ash	Microwave	Dry ash	Microwave	Dry ash	Microwave
			FDA method trial statistics			
1	37.9	40.1	1.85	0.97	8.93	7.24
2	36.5	40.8	15.5	0.50	16.1	6.17
3	13.8	14.0	2.44	1.49	4.39	3.22
4	16.3	18.1	1.17	0.23	7.08	12.6
5	28.3	27.2	20.8	9.89	20.8	9.89
6	31.5	33.2	2.56	4.39	7.37	5.75
			AOAC peer-verification statistics			
1	37.5	38.8	2.28	1.22	12.5	6.71
2	38.3	39.7	0.94	0.63	11.1	6.33
3	13.8	14.2	3.00	1.45	6.04	2.47
4	16.2	16.8	1.11	0.0	9.87	0.84
5	26.1	26.7	1.55	2.10	5.67	3.87
6	31.6	32.9	3.11	1.02	10.3	6.60

Method Trial

Results of Ca and Pb analyses after microwave or dry-ash digestion are given in Tables 1 and 2. Inconsistencies in data from laboratory 3 could not be resolved through a review of the raw data or discussions with the analyst. At the suggestion of an AOAC statistician at the time, statistical treatments of the data were performed with and without this laboratory's data. Questionable data in Tables 1 and 2 are indicated. However, except for sample pairs 2 and 5 for Ca and sample pairs 1 and 4 for Pb, laboratory 3's data were similar to those from the other 2 laboratories, resulting in acceptable statistics for the method trial with the exception of statistics for Pb at LOQ.

Sample codes for Ca supplements with questionable Ca results were 6a and 6b. The questionable Ca result of each sample closely matches the remaining sample in the corresponding data set. This observation indicates that a sample switch most likely occurred.

Method trial statistical results for Pb were detrimentally affected by the elevated LOQ determined for laboratory 3. The LOQ is based on 10 × SD of the blank sample determination. Laboratories 1 and 2 obtained average LOQs of 0.1 and 0.06 μg Pb/g supplement, respectively, while laboratory 3 obtained an average LOQ of 0.31 μg Pb/g supplement. Although an LOQ of 0.31 μg Pb/g is sufficient for current method needs (10× below the current Pb limit for Ca supplements), one aim of the study was to show method applicability at Pb levels as low as possible. Thus, further discussion will concentrate on data conforming to AOAC INTERNATIONAL peer-verified method criteria but excluding data from laboratory 3. With the exception stated previously, the method trial demonstrated acceptable performance at the current Pb limit for Ca supplements.

Calcium

Statistical analyses of Ca data are given Table 3. The relative percent difference (RPD) between means from dry-ash and microwave pretreatment averaged 3.3%, with microwave dissolution giving higher values. Although Ca results tended to be slightly higher for microwave dissolution, this was not always true. Mean Ca values for NIST SRM sample pair 5 (certificate value, 26.58 ± 0.24% Ca) were within 2% of certificate value by either sample pretreatment. Intralaboratory relative standard deviation (RSD_r) values averaged 2.00 and 1.07% for dry-ash and microwave pretreatments, respectively. Interlaboratory relative standard deviation (RSD_R) values averaged 9.25 and 4.47%, respectively. This level of precision in Ca results is acceptable.

Lead

Statistical analyses of Pb results are presented in Table 4. Average RPD between means for dry-ash and microwave pretreatment was 5.8%. A bias in Pb results was not apparent. Average results of analysis of NIST bone meal for Pb were within 8% of certificate value. Harmonization guidelines state that accuracy should be between 80 and 120% of the known value. Results of analysis of the standard reference material indicate acceptable accuracy and recovery for Pb at a contaminant level one-third the current equivalent USP specification for Pb in $CaCO_3$. RSD_r values averaged 7.75 and 10.8% for dry-ash and microwave procedures, respectively. RSD_R values averaged 12.3 and 14.4%, respectively, except for data from sample pair 4, which were at the LOQs for the methods. Higher RSD_r and RSD_R values were demonstrated mainly for samples averaging ≤ 0.3 μg Pb/g, except for sample pair 6, a bone meal sample. During method development, bone meal samples frequently demonstrated the greatest lack of homogeneity in Pb content of any supplement type. An unforseen lack of homoge-

Table 4. Statistical treatment: Lead (μg/g)

	Mean		RSD_r		RSD_R	
Sample pair	Dry ash	Microwave	Dry ash	Microwave	Dry ash	Microwave
			FDA method trial statistics			
1	0.37	0.26	27.2	60.9	57.0	60.7
2	1.34	1.31	4.68	8.85	4.93	8.85
3	1.28	1.21	8.16	11.0	8.16	11.8
4	0.20	0.02	14.0	18.8	87.0	174
5	1.53	1.26	2.28	8.22	13.8	16.3
6	6.00	5.62	18.7	9.13	27.0	29.5
			AOAC peer-verification statistics			
1	0.26	0.30	5.47	15.2	16.1	16.8
2	1.33	1.35	4.95	6.13	5.72	6.13
3	1.26	1.27	2.25	9.86	4.25	9.86
4	0.10	0.03	12.0	15.4	37.4	142
5	1.44	1.33	2.12	9.09	13.6	17.8
6	6.68	6.39	19.7	9.21	21.6	21.5

neity for sample pair 6 may have negatively impacted Pb results for this sample pair.

Comments by Participants

Analysts in one laboratory commented that one reagent blank sample per analytical batch of samples was insufficient to adequately determine Pb background. They used 5–10 control blanks per series to monitor contamination and method performance by the method detection limit concept (15). Their assessment of method figures of merit was determined with 5 reagent blank samples. The originating laboratory also used this method for evaluating method characteristics. After observing consistent reagent blank Pb values (at or near the instrument detection limit), the decision was made to limit reagent blanks to approximately a 10% frequency. Inconsistent reagent blanks should alert the chemist to increase the number of reagent blanks to determine an accurate value for reagent blank contribution and to minimize sources of Pb contamination.

Instrumentation

GFAAS equipment used by the laboratories for Pb determinations were of 2 basic types. Smith-Hieftje or pulsed background correction was used in one laboratory, while Zeeman background correction was used in the 2 other laboratories. Also, discrete sample deposition was used with the Zeeman GFAAS instrument, while aerosol sample deposition was used with the other instrument. Discrete sampling dictated a modification of the initial furnace temperature to avoid sample spattering during deposition. Two laboratories used Thermo-Jarrell Ash ICAP 61E instruments for Ca analyses, while the remaining laboratory used an ARL 3580 ICP instrument. No apparent differences due to ICP instrument differences were noted.

Conclusions

A method for determining Ca and Pb in Ca supplements was developed that demonstrates acceptable accuracy and precision. Method sensitivity sufficient for a regulatory limit of 1 μg Pb/g was demonstrated by 2 of 3 laboratories. For Pb analysis, there was no apparent bias, due to either sample treatment procedure or mode of background correction during GFAAS. ICP instrumentation differences had no observable effect on Ca results. Data from microwave dissolution procedure gave slightly better statistics for Ca, but data from dry-ash digestion gave slightly better Pb statistics. The microwave procedure has advantages with respect to time savings and contamination control. Either sample treatment can be used with acceptable results.

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RESIDUES AND TRACE ELEMENTS

Monitoring Chlorinated Pesticides and Toxic Elements in Tissues of Food-Producing Animals in Yugoslavia

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According to the established monitoring program in Yugoslavia, 941 swine, 561 cattle, and 358 lamb samples collected during a 5-year period were analyzed for chlorinated pesticide residues. Less than 10% of the examined samples contained residues of HCB (hexachlorobenzene), HCH (hexachlorocyclohexane), lindane (γ-hexachlorocyclohexane), and total DDT (*p,p'*-DDT and metabolites) at concentrations greater than the lowest detectable limit. None of the swine and cattle samples exceeded the residue limit (RL). Among lamb samples, 2.5% contained lindane residues exceeding the RL, with a mean of 4.75 mg/kg. This finding suggests lindane's improper use as a veterinary pesticide, probably as a sheep-dip for destroying ectoparasites. During the same period, 849 swine, 584 cattle, and 350 lamb samples also were analyzed for toxic elements (Pb, Cd, Hg, and As). In the majority of samples, toxic elements were present at levels less than 50% of RLs. Only Cd and Pb RLs were exceeded in several cases. Differences in trace element contents of samples from different animals were not significant. The data indicate that residues in tissues of food-producing animals do not have a great potential impact on public health.

Humans today are subjected to prolonged exposure to many toxic substances at very low doses (1, 2). To evaluate human exposure to environmental contaminants and hazards and the risks associated with that exposure, monitoring of residues is very important. The primary objective of monitoring is to collect information about distribution and year-to-year variability of selected environmental pollutants.

Many toxicants are eliminated by mammals through milk. Thus, milk is the most important product through which humans are exposed to many organochlorine pesticides (3, 4). But because of the large use of meat in human nutrition and the solubility and accumulation of chlorinated pesticides and other compounds in lipids, many investigations to evaluate human exposure to different environmental contaminants are based on monitoring of animal tissues and organs (5–7). Among these environmental contaminants, pesticides and toxic elements are considered to be of particular importance because of their toxicity and cumulative properties (8–10).

Use of chlorinated hydrocarbon insecticides in crop and animal production has become very important during the past 50 years because of their effectiveness, low cost, and acute toxicity. Discovery of environmental hazards caused by their high persistence, chronic toxicity, and ability to bioaccumulate resulted in their restriction or banning in most industrialized countries (11, 12). But they remain important as pesticides and, consequently, as contaminants of food and animal feed produced in Third World countries, where their use is still permitted (13, 14).

Trace elements are another common group of environmental pollutants that are toxic to mammals, birds, and fish. Excessive concentrations of these materials are present in water, air, or soil as a result of natural deposits, and their use can lead to accumulation in the environment (15–17). The presence of toxic elements in soil, water, and air contributes to the contamination of food chains (18). To evaluate human exposure to diverse toxic elements, animals are used as indicator organisms (19). Because of their toxicity and bioconcentrations, mercury, cadmium, and lead have been the most extensively studied metals (15–18).

Arsenic is another widespread element in the environment. Apart from its presence in some minerals—mainly as arsenides of copper, nickel, and iron or as arsenic sulfides or oxides—it has some applications in metallurgy for hardening Cu, Pb, or alloys. Small amounts are used in the glass and ceramics industries. Arsenic compounds were used in agriculture and forestry mainly as pesticides. At present, because of legislation, As compounds cannot be used as crop-protecting agents (20). Because of its widespread presence in the environment and its acute and chronic toxicity, permanent monitoring of As residues in foodstuffs and in food-producing animals is required (21, 22).

Exposure of animals to environmental contaminants or inappropriate use of pesticides in animal husbandry can leave high levels of residues in animal tissues. To maintain the safety of meat and meat products, a national system of residue control was established in Yugoslavia in 1972. Under the supervision and on request from the U.S. Department of Agriculture (USDA), Foreign Agricultural Service, Office of Agricultural Affairs, the residue control program in Yugoslavia was set up

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Table 1. Residues of chlorinated hydrocarbons detected in adipose tissue of food-producing animals in Yugoslavia, 1991–1996

Animal	Pesticide detected	Total No. of samples	<LDL[a]		LDL–50% RL[b]		50–100% RL		>RL	
			No.	%	No.	%	No.	%	No.	%
Swine	HCB	941	936	99.5	5	0.5	0	—	0	—
	α + β + δ-HCH	941	938	99.7	3	0.3	0	—	0	—
	Lindane	941	891	94.6	41	4.4	9	1.0	0	—
	Total DDT	941	937	99.6	4	0.4	0	—	0	—
Cattle	HCB	561	556	99.1	3	0.5	2	0.4	0	—
	α + β + δ-HCH	561	556	99.1	2	0.4	1	0.2	0	—
	Lindane	561	535	95.4	17	3.0	9	1.6	0	—
	Total DDT	561	561	100.0	0	—	0	—	0	—
Lamb	HCB	358	356	99.4	2	0.6	0	—	0	—
	α + β + δ-HCH	358	356	99.4	1	0.3	1	0.3	0	—
	Lindane	358	333	93.0	13	3.6	3	0.9	9	2.5
	Total DDT	358	352	98.3	2	0.6	4	1.1	0	—

[a] LDL = lowest detectable limit: 0.01 mg/kg for HCH, HCB, and lindane; 0.04 mg/kg for total DDT.
[b] RL = residue limit: 0.1 mg/kg for HCH, HCB, and lindane; 1.00 mg/kg for total DDT.

with the task of avoiding the exposure of the domestic human population and its food supply—as well as consumers in the United States who buy meat products imported from Yugoslavia—to unexpectedly high residue levels. The first official guidelines for veterinary inspection of slaughtered animals, meat, and meat products to detect residues was set in 1985 (23).

Experimental

Apparatus

(**a**) *Gas chromatograph.*—Varian Model 3400 (Walnut Creek, CA), equipped with ^{63}Ni electron capture detector and J&W DB-17, 30 m long Megabore column (J&W Scientific); 0.53 mm id and 1.0 μm film thickness. Operating conditions: injector temperature, 250°C; detector temperature, 300°C; and programmed column temperature, 160°C with 2 min hold, ramp from 160° to 180°C at 2°C/min, ramp from 180° to 230°C at 5°C/min, 10 min hold at 230°C. Carrier gas flow (highly purified nitrogen) was 16 mL/min.

(**b**) *Data acquisition.*—Varian integrator, Model 4400.

(**c**) *Atomic absorption spectrophotometer.*—Pye Unicam Model SP-9 (Cambridge, UK), equipped with Pb, Cd, and Hg hollow cathode lamps, acetylene/air burner, and deuterium background correction.

(**d**) *UV/visible spectrophotometer.*—Pye Unicam Model SP8-500, with deuterium lamp and 1 cm near-infrared silica cell.

(**e**) *Microcolumn.*—12 × 300 mm fritted glass column with Teflon stopcock.

Reagents

(**a**) *Reagents and solvents.*—E. Merck, Darmstadt, Germany; graded for residue analysis.

(**b**) *Alumina.*—ICN Biomedicals (Eschwege, Germany) neutral, Brockman Activity I, 80/200 mesh, treated according to determinative method procedure.

(**c**) *Pesticide reference standards.*—HCB, α + β + γ + δ-HCH, lindane, *p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, aldrin, dieldrin, heptachlor, heptachlor epoxide, metoxichlor, endrin, and chlordane were obtained from Supelco (Gland, Switzerland). Concentrations of standard solutions were appropriate for instrumentation used and samples tested.

(**d**) *Standard solutions.*—Solutions for atomic absorption spectrophotometric (AAS) determination of Cd, Pb, and Hg, as well as for spectrophotometric determination of As (stock and calibration standards), were made from commercially available reference standard solution (Serva, Feinbiochemica, D-6900, Heidelberg, Germany).

Sample Collection

Testing of slaughtered animals for residue content comprises 2 phases: monitoring and surveillance. Monitoring provides general information on occurrence of residue violations in specified animal populations on an annual national basis. Results are used to identify producers that deliver animals with violative concentrations of residues. Subsequent offer of animals for slaughter from those producers is subjected to surveillance sampling and testing, until accordance with regulation is demonstrated.

Data presented in this paper originated from the monitoring and surveillance phases of Yugoslavia's national residue control program. Samples were collected from registered slaughterhouses throughout the country. Monitoring information was obtained from a statistically based collection of random samples from healthy appearing animals under inspection at the slaughter line. The number of samples was chosen to provide 95% probability of detecting at least one violative sample when 1% of the total animal population is violative. When residues were detected at higher than tolerance levels, the surveillance program was applied. On the basis of monitoring information, sampling would be directed to a particular area, animal species,

Table 2. Residues of arsenic and heavy metals in liver and kidney of food-producing animals in Yugoslavia, 1991–1996

Animal	Toxic element detected	Total No. of samples	<LDL[a]		LDL–50% RL[b]		50–100% RL		>RL	
			No.	%	No.	%	No.	%	No.	%
Swine	Arsenic	849	207	24.4	369	43.5	273	32.1	0	—
	Cadmium	849	125	14.7	576	67.8	143	16.9	5	0.6
	Lead	849	468	55.1	322	37.9	55	6.5	4	0.5
	Mercury	849	354	41.7	456	53.7	39	4.6	0	—
Cattle	Arsenic	584	122	20.9	322	55.1	140	24.0	0	—
	Cadmium	584	84	14.4	427	73.1	63	10.8	10	1.7
	Lead	584	290	49.7	236	40.4	56	9.6	2	0.3
	Mercury	584	175	30.0	397	68.0	12	2.0	0	—
Lamb	Arsenic	350	73	20.9	185	52.9	92	26.2	0	—
	Cadmium	350	110	31.4	212	60.6	25	7.1	3	0.9
	Lead	350	181	51.7	125	35.7	41	11.7	3	0.9
	Mercury	350	235	67.1	110	31.5	5	1.4	0	—

[a] LDL = lowest detectable limit: 0.01 mg/kg for As, Cd, and Pb; 0.001 mg/kg for Hg.
[b] RL = residue limit: As, 0.5 mg/kg; Cd and Pb, 1.0 mg/kg; Hg, 0.1 mg/kg.

and compound, if warranted. In such a case, carcasses were retained until laboratory analyses were received. Samples were taken at random, unforeseen and unexpected by veterinary inspection at the slaughterhouses and at no fixed time or particular day of the week. Collected tissues were placed separately in plastic bags to prevent transfer of residues from tissue to tissue, marked, and sent to a laboratory in a frozen state.

Target tissues were tissues containing 80–90% fat for chlorinated pesticides, kidney for heavy metals (Cd, Pb, and Hg), and liver for As. Target tissues were seleected according to data from literature or available analytical methods (7, 24–26).

Analysis

Samples were treated according to USDA's *Guidebook* (24). Chlorinated hydrocarbons were extracted and separated by elution from fat in small glass columns filled with partially deactivated alumina. The eluate was evaporated to a small volume and transferred to a volumetric flask. A portion was injected into a gas chromatograph for detection and quantitation. Residues were expressed on a fat basis (24).

To analyze for trace elements, the matrix was completely destroyed by heating (600°C). The ash residue was dissolved in hydrochloric acid, and trace elements were determined by AAS. Mercury was determined by cold-vapor technique after wet digestion. Arsenic was determined spectrophotometrically at 840 nm as a molybdenum blue complex.

Results were evaluated against national residue limits established in regulations (27).

Accuracy

Data accuracy was estimated from recovery of compounds from fortified samples. For each sample, one reagent blank sample and one spiked sample were analyzed. Blank samples were spiked with single or a group of contaminants in the range of concentrations expected for analyzed samples. Results for analyzed samples were corrected for recovery of the corresponding compound from the spiked blank sample. Acceptable recoveries ranged 70 to 110%, depending on compound. When recoveries were out of range, results were rejected and the reasons for the deviation had to be determined and eliminated.

Results and Discussion

Residues of chlorinated pesticides detected in different animal species during the 5-year period 1991–1996 are shown in Table 1. Adipose tissues from swine (941 samples), cattle (561 samples), and lamb (358 samples) were analyzed. Organochlorine pesticide residues detected were HBC (hexachlorobenzene), HCH (hexachlorocyclohexane), lindane (γ-hexachlorocyclohexane), and *p,p′*-DDT and its metabolites, presented as total DDT. Other chlorinated pesticides such as aldrin, dieldrin heptachlor and its epoxide, and chlordane were not detected. Use of chlorinated pesticides in agriculture (except lindane) and forestry (except DDT) was banned in Yugoslavia in 1972. Pesticide residues in more than 90% of samples examined were at levels below the lowest detectable limit (LDL) of the method, in agreement with our previous results (28). Lindane was the most frequently detected pesticide at levels above LDL. None of the pork and beef samples contained residues in excess of the residue limits (27). Among lamb samples, 2.5% contained lindane in excess of the residue limit, with a mean lindane concentration 4.75 mg/kg. The residue limit for lindane is 0.1 mg/kg.

Literature data indicate that the banning or restricted use of many organochlorine insecticides in agriculture was followed by a decrease in levels of pesticide residues in animal feed and, consequently, in tissues of slaughtered animals (12, 29). Lindane still is widely used in agriculture and animal husbandry. Thus, the source of lindane in tissue samples could be contaminated feed or improperly used veterinary pesticides. Direct use

Table 3. Concentrations of cadmium and lead in samples exceeding residue limits (RL)

Toxic element	Animal	No. of samples exceeding RL	Range of concentrations, mg/kg	Average concn, mg/kg
Cadmium	Swine	5	1.12–1.85	1.36
	Cattle	10	1.24–2.02	1.57
	Lamb	3	1.20–1.76	1.42
Lead	Swine	4	1.14–1.68	1.31
	Cattle	2	1.55–2.14	1.84
	Lamb	3	1.34–2.28	1.61

of lindane to control ectoparasites in livestock can leave high residue levels in the animal body (30, 31). The data for lambs suggest improper use of lindane as a veterinary pesticide, probably as a sheep-dip for destroying ectoparasites. The pesticide might enter through the skin into the animal body and then penetrate to the fetus through the placenta. Milk is the most effective way of eliminating persistent organohalogen compounds from the body (32, 33). So, milk is another way that lambs may be contaminated with lindane. Our previous results (34) and other literature data (30) support this conclusion.

Residues of toxic elements detected in tissue samples are shown in Table 2. During the 5-year period, 849 swine, 584 cattle, and 350 lamb samples were analyzed. For the majority of examined samples, residues of toxic elements were detected at levels <50% of residue limits. None of the samples contained As or Hg at a level above the residue limit. The Cd residue limit was exceeded in 5 pork, 10 beef, and 3 lamb samples. The Pb residue limit was exceeded by 4 swine, 2 cattle, and 3 lamb samples. The range of concentrations and average values for Cd and Pb in samples exceeding residue limits are shown in Table 3.

The Cd and Pb found in pork and beef kidneys may be due to contaminated feed. Phosphate fertilizers, rainfall, and increased industrialization and motorization continually increase the contamination of the environment and, consequently, of tissues of food-producing animals (18, 26). Our results are very similar to previous data (28, 35, 36). Differences in trace element contents of samples from different animals were not significant.

The data for organochlorine pesticides and trace element residues indicate that the occurrence of residues in tissues of food-producing animals in Yugoslavia has no great potential impact on public health. For samples exceeding tolerance levels, the monitoring program allows identification and, consequently, elimination of the source of animal contamination.

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RESIDUES AND TRACE ELEMENTS

Determination of (+/–) Elution Orders of Chiral Organochlorines by Liquid Chromatography with a Chiral Detector and by Enantioselective Gas Chromatography

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Enantioselective gas chromatography (GC) with 5 modified cyclodextrins was applied to chiral organochlorines. A prerequisite for determining GC elution orders of enantiomers is the availability of enantioenriched standard solutions. In addition to compounds reported before (e.g., α-HCH, PCB 174, oxychlordane), we determined the sign of optical rotation of enantioenriched solutions of e-aeee-pentachlorocyclohexene-1 (β-PCCH), perdeuterated α-HCH (α-PDHCH), perdeuterated β-PCCH, and the persistent compound of technical toxaphene—2-*exo*,3-*endo*,5-*exo*,9,9,10,10-heptachlorobornane (B7-1453)—by liquid chromatography (LC) with a chiral detector. An enantioenriched solution of β-PCCH was obtained by enantioselective degradation of α-HCH with (–)-brucine. In addition to forming an enantiomeric excess of (–)-α-HCH, we formed enantioenriched (+)-β-PCCH. In a similar study, α-PDHCH showed the same behavior with respect to enantioselectivity. Dextrorotation of an enantioenriched solution of B7-1453 was also confirmed by LC with a chiral detector. Enantioseparation of chiral organochlorines on 5 chiral stationary phases resulted in several reversed elution orders. These results indicate that a careful check of elution orders of organochlorine enantiomers is necessary prior to comparison of literature data for the study of enantioselective processes in the environment.

Since the 1940s, organochlorines (e.g., hexachlorocyclohexanes, DDT, chlordane, toxaphene, and polychlorinated biphenyls) have been applied at million-ton scales all over the world (1). Because of their persistence and lipophilicity, these xenobiotics are accumulated at parts-per-trillion to parts-per-million levels, particularly in adipose tissues of species at higher trophic levels. Many organochlorines are chiral, and technical products contain both enantiomers. However, optical antipodes often have different effects on living organisms. For example, McBlain (2) showed that (–)-*o,p'*-DDT possesses estrogenic activity while (+)-*o,p'*-DDT has none.

In 1989, König et al. (3) succeeded in gas chromatographic (GC) enantioseparation of an aaeeee-1,2,3,4,5,6-hexachlorocyclohexane (α-HCH) standard solution and stated that this technique may be used to study enantioselective degradation of α-HCH in the environment. In the past few years, GC enantioseparations of chiral organochlorines have attracted growing attention in environmental chemistry (4). Although no chiral stationary phase (CSP) could enantioseparate all chiral compounds, different CSPs based on alkyl- or silyl-terminated cyclodextrins allow resolution of enantiomers of most chiral organochlorines and collection of data on enantioselective accumulation in some matrixes (4).

Enantiomeric ratios (ER) usually are defined as the ratio of the dextrorotary enantiomer to the levorotary enantiomer. If the signs of the specific rotations of individual enantiomers are unknown, ER is based on the ratio of the first eluted enantiomer to the second eluted enantiomer under defined chromatographic conditions (4). However, elution orders of enantiomers frequently are reversed on different CSPs (5, 6) and in one case even on the "same" CSP synthesized by different manufacturers (7). Therefore, knowing the (+/–) elution order of enantiomers is necessary to evaluate enantioselective accumulation of chiral organochlorines in environmental samples. This determination has been done for some materials, for example through chiroptical investigations of enantiopure or enantioenriched α-HCH (8), chlordane-related compounds (8, 9), and some atropisomeric polychlorinated biphenyls (PCBs; 10).

We have now established the sign of optical rotation of e-aeee-pentachlorocyclohexene-1 (β-PCCH), the first metabolite of α-HCH (11). We also determined the signs of the optical rotations of perdeuterated α-HCH (α-PDHCH), perdeuterated β-PCCH (β-PDPCCH), and an enantioenriched compound of technical toxaphene (CTT). And we determined elution orders

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of the enantiomers of these and other organochlorines on 5 CSPs.

Experimental

Materials and Reference Compounds

Standard solutions of α-HCH and oxychlordane (10 ng/μL each) were from Promochem (Wesel, Germany). Solid *o,p'*-DDT was from Dr. Ehrenstorfer (Augsburg, Germany), solid α-HCH was from Riedel-de-Haen (Seelze, Germany), and solid PCB 132 and PCB 174 were from Promochem. α-PDHCH was synthesized and purified in our laboratory (12) but is available from Promochem. Enantioenriched oxychlordane was from Dr. Ehrenstorfer, and enantiopure PCB 132 and PCB 174 were prepared (10) and kindly donated by P. Haglund (Umeå, Sweden). Enantiopure PCB standards were mixed with racemic PCB solutions to obtain enantioenriched solutions that allow simpler determination of (+/–) elution orders. 2-*exo*,3-*endo*,5-*exo*,9,9,10,10-Heptachlorobornane (B7-1453) was isolated from the technical product Melipax (13). This persistent heptachlorobornane is present in Melipax in nonracemic composition (14). 1,4-Dioxane (chromatographic grade) was from Merck (Darmstadt, Germany), and *n*-hexane (residue analysis grade) was from Promochem. (–)-Brucine (2,3-dimethoxystrychnidin-10-one) was from Merck. The natural product brucine exists only in the levorotary form.

Silica gel 60, extra pure for column chromatography (particle size, 0.063–0.200 mm; Merck), was activated for 16 h at 130°C before use.

Preparation of Enantioenriched Standards

Organochlorines (α-HCH, α-PDHCH, PCB 174, and *o,p'*-DDT) were treated with (–)-brucine as described by Cristol for α-HCH (15). Forty milligrams organochlorine (except 1 mg for PCB 174) and 200 mg (–)-brucine were added to 10 mL 1,4-dioxane. Solutions were stored for several days in closed 50 mL flasks and then filtered through 1 g silica gel to remove (–)-brucine. The α-HCH reaction solution was chromatographed on 60 g silica gel with *n*-hexane as mobile phase. The parent compound (1000–1450 mL) eluted prior to the dehydrochlorinated product β-PCCH (1550–2150 mL), allowing quantitative separation of the compounds. The same procedure was applied to α-PDHCH and β-PDPCCH. A reaction between ca 20 μg B7-1453 with 40 mg (–)-brucine was performed in 1,4-dioxane. In addition, we tested the reaction of B7-1453 with (–)-brucine at 95°C in a sealed reaction vial (Reacti-Vial, Pierce, The Netherlands).

LC with a Chiral Detector

The LC system consisted of a degassing system, a PU-980 pump, a UV-975 detector (operated at 210 nm), and an OR990 chiral detector (all Jasco, Japan). The LC column (200 mm length × 4 mm id, silica; ET 200/4 Nucleosil 100-5) was from Macherey-Nagel (Düren, Germany). The mobile phase was *n*-hexane at a flow rate of 0.7 or 0.5 mL/min. About 20 μL was injected into a 20 μL sample loop. Data treatment was performed with Borwin software (Jasco).

GC with Electron Capture Detection

Five CSPs were tested. β-PMCD (10% immobilized heptakis[2,3,6-tri-*O*-methyl]-β-cyclodextrin bonded to polysiloxane) and γ-PMCD (10% immobilized octakis[2,3,6-tri-*O*-methyl]-γ-cyclodextrin bonded to polysiloxane) columns, distributed with the tradename Chirasil-Dex, were from Chrompack (Middelburg, The Netherlands). Columns were 25 m long, 0.25 mm id, 0.25 μm film thickness (d_f).

β-BSCD (20% *tert*-butyldimethylsilylated-β-cyclodextrin in 85% dimethyl-, 15% diphenylpolysiloxane [PS086]) was a prototype similar to a column available under the tradename BGB-172 (BGB Analytik, Adliswil, Switzerland). Column was 30 m long, 0.25 mm id, and 0.18 μm d_f.

The β-TBDM (35% heptakis[6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl]-β-cyclodextrin in 85% methyl-, 7% phenyl-,

Table 1. Elution order and elution temperatures of organochlorine enantiomers on 5 chiral stationary phases

Organochlorine	Elution order (reference); temperature, °C on indicated stationary phase				
	β-PMCD	γ-PMCD	β-TBDM	β-BSCD	β-PPCD
α-HCH	(+) < (–) (5, 6, 17); 130	(–) < (+) (5, 6, 17); 150	—[a]; 150	—[b]; 160	(+) < (–); 145
α-PDHCH	(+) < (–); 130	(–) < (+); 150	—[a]; 150	—[b]; 160	(+) < (–); 145
β-PCCH	(+) < (–); 130	(–) < (+); 140	(+) < (–); 150	n.s.[c]	(+) < (–); 135
β-PDPCCH	(+) < (–); 130	(–) < (+); 140	(+) < (–); 150	n.s.	(+) < (–); 135
B7-1453	n.s.	n.s.	(+) < (–); 140	(+) < (–); 180	n.s.
Oxychlordane	n.s.	n.s.	(–) < (+) (6); 165	(+) < (–); 200	n.s.
PCB 132	(–) < (+) (10); 190	(–) < (+)[d]; 160	(–) < (+); 180	(–) < (+); 180	n.s.
PCB 174	(+) < (–) (10); 190	n.s.	(–) < (+); 190	(–) < (+); 180	n.s.

[a] The β-TBDM used in the study eluted (+)-α-HCH prior to (–)-α-HCH, which is the reverse of the elution order determined recently on another β-TBDM phase of different purity (27). Elution order of enantiomers on β-TBDM must be carefully checked.

[b] The β-BSCD used in the study eluted (–)-α-HCH prior to (+)-α-HCH, which is the reverse of the elution order obtained recently on a β-BSCD phase from a different synthesis batch of the same manufacturer (7). Elution order of α-HCH enantiomers on β-BSCD must be carefully checked.

[c] n.s. = not separated.

[d] Only a shoulder.

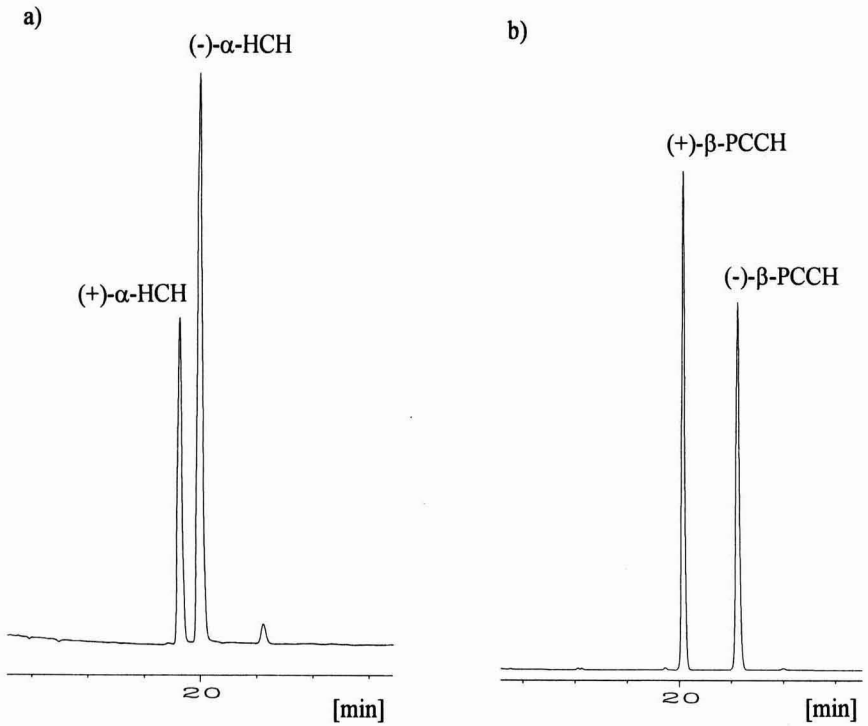

Figure 1. Enantioselective GC–ECD chromatograms (β-TBDM) of (a) enantioenriched (–)-α-HCH and (b) enantioenriched (+)-β-PCCH obtained after separation of products of reaction of α-HCH with (–)-brucine.

7% cyanopropyl-, 1% vinyl-polysiloxane [OV1701]) phase was from M. Müller (Eidgenössische Forschungsanstalt Wädenswil, Switzerland; 6). Column was 20 m long, 0.25 mm id, and 0.15 μm d_f.

Undiluted β-PPCD (heptakis[2,3,6-tri-*O*-*n*-pentyl]-β-cyclodextrin) column is available under the tradename Lipodex C (Macherey-Nagel). Column was 25 m long and 0.25 mm id.

Achiral separations were performed on an HP 5890 gas chromatograph (Hewlett-Packard, Ratingen, Germany) equipped with 2 fused-silica columns (CP-Sil 2 and CP-Sil 8/20% C_{18}, both Chrompack) and 2 electron capture detectors (ECDs) in parallel, as recently reported (6, 7).

Enantioseparations were performed on HP 5890 and HP 5840 gas chromatographs, each equipped with an ECD (7). Samples were injected splitless at 240°C injector temperature; detector temperature was 300°C. Carrier gas was helium (column head pressure was 1.0 bar, except for β-TBDM for which column head pressure was 0.8 bar).

GC oven programs started at 60°C for 2 min. Then the oven was heated at 10° or 15°C/min to the required separation temperature (Table 1).

Results and Discussion

Reaction of Organochlorines with (–)-Brucine

The reaction of racemic α-HCH with (–)-brucine has been the subject of several studies (5, 15–19). Degradation of α-HCH is accompanied by formation of the chiral degradation product β-PCCH. The second eluted α-HCH peak (Figure 1a) was significantly more abundant than the first eluted enantiomer. The peak ratio of α-HCH enantiomers was about 1:2. Möller et al. (18) found a significant optical rotation of pure α-HCH enantiomers in *n*-hexane.

Enantioenriched α-HCH was injected into the LC system. The chiral detector was used in the + mode. Thus, a signal exceeding the baseline (positive signal) corresponds with dextrorotation, and a negative signal indicates levorotation. The LC system was used with an achiral stationary phase. With this technique, signals in the chiral detector are obtained only when one enantiomer predominates. Racemic compounds neutralize the opposite optical rotation of the enantiomers, and only the surplus of one enantiomer gives rise to a signal. In the case of a 1:2 ratio of enantiomers such as with α-HCH, only one third of the injected solution is responsible for the detector signal. Because of this fact and the poor sensitivity of the chiral detec-

tor for organochlorines, 10–100 µg enantioenriched compounds was required to obtain a significant signal in the chiral detector.

α-HCH gave a negative signal in the chiral detector (Figure 2a). The corresponding peak was fractionated and, after dilution (about 200 pg/µL), analyzed by GC–ECD. The GC–ECD chromatogram of the LC fraction showed only 2 peaks at the retention times of the α-HCH enantiomers and reproduced the enantiomeric ratio measured prior to LC analysis. These results confirm that degradation of α-HCH with (–)-brucine leads to enantioenriched (–)-α-HCH (15), which can be read as $ER_{(+/-)} = 0.57$. The enantiomeric excess was only slightly lower than that reported by Hühnerfuss et al. (20).

Along with formation of enantioenriched (–)-α-HCH, dehydrochlorination with (–)-brucine yielded nonracemic β-PCCH. Although formation of β-PCCH from α-HCH after

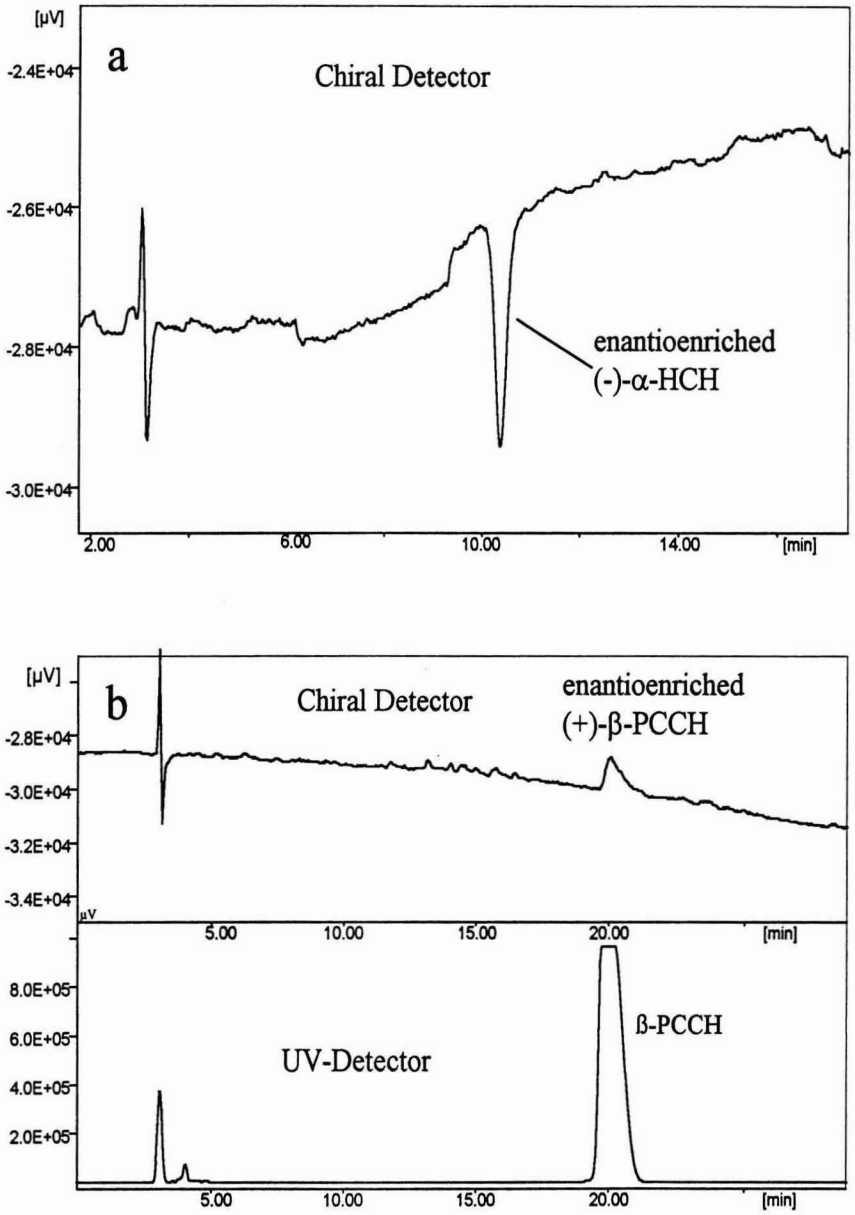

Figure 2. Liquid chromatograms of organochlorines: (a) LC/chiral detector chromatogram of enantioenriched (–)-α-HCH, (b) LC/chiral detector (top) and LC/UV detector (bottom) chromatograms of enantioenriched (+)-β-PCCH.

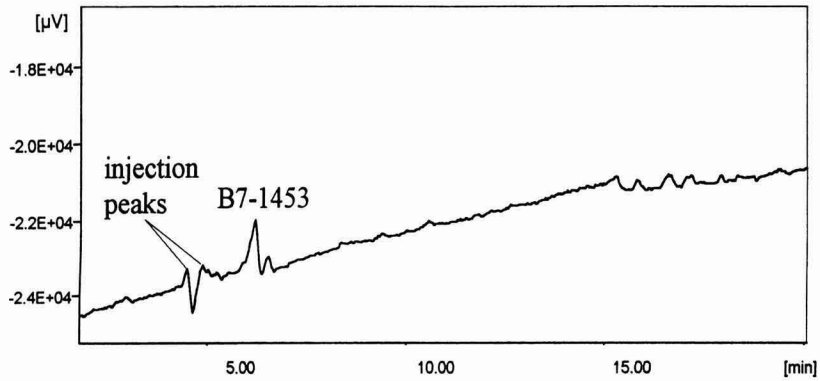

Figure 3. LC/chiral detector chromatogram of enantioenriched (+)-B7-1453 isolated from Melipax (positive signals are caused by dextrorotation).

treatment with brucine has been described (5, 15–19, 21), the sign of rotation of β-PCCH hitherto has not been established. Enantioenrichment of β-PCCH was less pronounced than that of α-HCH (Figures 1a and 1b). Only a small enantiomeric excess of the first eluted β-PCCH enantiomer (ER = 1.36) was observed. However, the β-PCCH isolate caused a significant positive signal in the chiral detector, corresponding to greater abundance of the dextrorotary enantiomer (Figure 2b). Therefore, reaction of α-HCH with (–)-brucine resulted in enantioenriched (–)-α-HCH and enantioenriched (+)-β-PCCH. β-PCCH also caused a signal in the UV detector (210 nm), which was not the case with α-HCH because α-HCH lacks a double bond.

α-PDHCH was synthesized recently (12). It has been predicted that α-HCH and the isotope-labeled analogue α-PDHCH will behave in the same way with respect to enantioselectivity (6). To test this prediction, we treated α-PDHCH with (–)-brucine, separated the reaction products on silica, and studied the enantiomeric excess by LC with a chiral detector. Reaction with brucine led to enantioenriched (–)-α-PDHCH and enantioenriched β-PDPCCH, in agreement with results for nonlabeled compounds. In contrast to α-HCH and α-PDHCH, *o,p′*-DDT and PCB 174 did not react with (–)-brucine, and enantioselective GC confirmed that these compounds exist in racemic composition (data not shown).

Next we determined the sign of the rotation of B7-1453, an abundant CTT in fish (22) and seal blubber (23), isolated recently from the technical product Melipax (13). With enantioselective GC on β-BSCD and electron-caption negative ionization-mass spectrometry (ECNI-MS) and electron impact-MS (EI-MS) detection, we established an ER of 1.26 ± 0.03 for the B7-1453 isolate from Melipax (14). The nonracemic composition of B7-1453 confirmed that toxaphene mixtures contain enantioenriched compounds (24). Reaction of (–)-brucine with B7-1453 failed when performed both at ambient temperature and at 95°C in a sealed vial. Consequently, we studied the enantioenriched B7-1453 isolated from Melipax by LC with a chiral detector. Because of the small surplus (about 11.5%) of one B7-1453 enantiomer, the Melipax isolate was concentrated to about 50 μL, and 20 μL was injected into the LC system. Two significant positive signals were observed in the chiral detector (Figure 3). Eluates were separately collected at the respective retention times and analyzed by GC–ECD. The GC–ECD chromatogram of the LC cut of the major positive peak in the chiral detector was identified as B7-1453. The fraction was concentrated and reinjected into the LC system. This time, we recorded only the one positive signal in the chiral detector. GC–ECD analysis on nonchiral and chiral stationary phases confirmed that the peak originated from B7-1453 (Figure 4). Although the abundance of the positive signal in the chiral detector was low, we concluded that the isolate from Melipax contained enantioenriched (+)-B7-1453. Enantiomers of polychlorinated bornanes are obtained by mirroring the substituents on C-2, C-3, and C-9 with those on C-6, C-5, and C-8, respectively (25). Consequently, both enantiomers of B7-1453 must be labeled with different chemical names (26), that is, 2-*exo*,3-*endo*,5-*exo*,9,9,10,10-heptachlorobornane and 3-*exo*, 5-*endo*,6-*exo*,8,8,10,10-heptachlorobornane (13). At the moment, it is still not clear which structure corresponds to (+)-B7-1453.

Elution Orders of Organochlorines on Chiral GC Stationary Phases

Elution orders and separation temperatures of organochlorine enantiomers on 5 different CSPs are listed in Table 1. Enantioseparations of organochlorines are enthalpy controlled, and best chiral resolutions are obtained with isothermal elution (4). A decrease in separation temperature increases chiral resolution but increases retention times. Note that separation temperatures are optimized not to obtain the best chiral resolution or separation factor but to achieve a reasonable compromise between resolution and GC run time. Coelutions with other compounds in samples might require other conditions and optimization procedures. Consequently, these temperatures are recommended as initial values to start with in similar studies.

Elution orders of labeled and nonlabeled α-HCH and β-PCCH were the same on all CSPs. Only α-HCH and its perdeuterated analogue were enantioseparated on all CSPs. α-PDHCH and β-PDPCCH had shorter retention times than nonlabeled α-HCH and β-PCCH, respectively. β-PPCD enan-

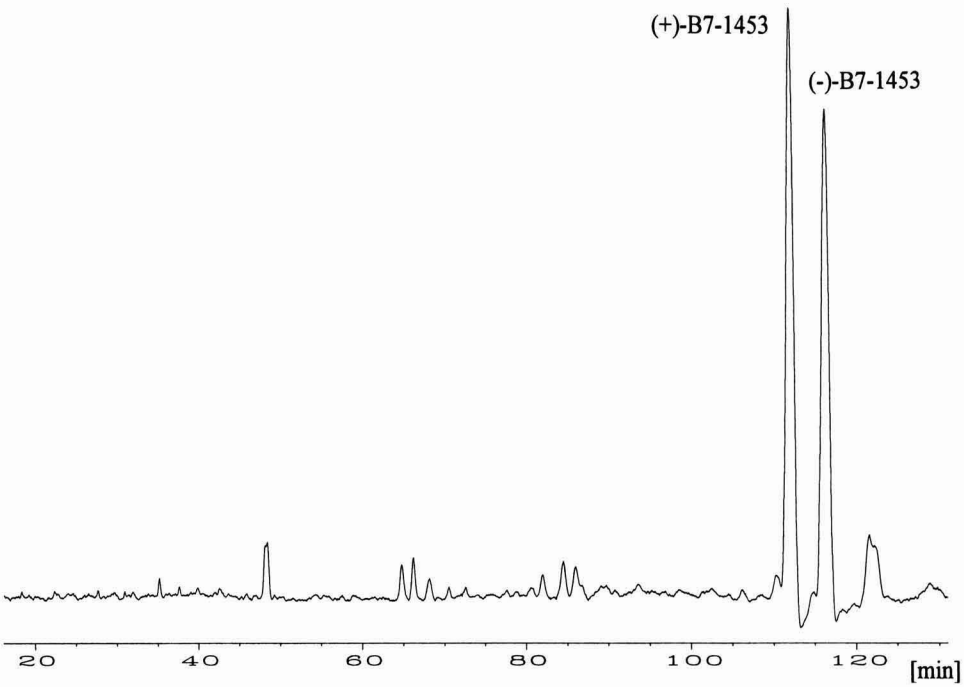

Figure 4. GC–ECD chromatogram of enantioseparation of B7-1453 on β-BSCD (LC cut).

tioseparated only α-HCH, β-PCCH, and their perdeuterated analogues. Therefore, this CSP, which is reported to enantioseparate many chiral compounds (28), is suitable only for a few chiral organochlorines.

(+)-α-HCH eluted earlier than the levorotary enantiomer on all CSPs except γ-PMCD and the β-BSCD phases. However, we have found evidence for reversed elution orders on β-BSCD phases depending on the synthesis procedure (7). β-BSCD, introduced by Blum and Aichholz (29), is synthesized by silylation of terminal -OH groups on C-2, C-3, and C-6 of the cyclodextrins. Because the low stability of trimethylsilyl ethers, *tert*-butyldimethylsilyl groups were used to prepare suitable CSPs (29). However, these bulky substituents prevent persilylation of the free -OH groups on the cyclodextrin, and β-BSCD consists of several products that may vary from batch to batch (7, 28–30). Some of these products elute the dextrorotary enantiomer before the levorotary enantiomer, while others give a reversed elution order (7). For example, the elution order of α-HCH enantiomers we obtained on the β-BSCD phase is the reverse of the elution order obtained with a β-BSCD phase tested earlier from the same manufacturer (7). Although reversal of elution order of enantiomers on β-BSCD has been shown only for α-HCH, we cannot exclude the possibility that it can occur with other compounds. Thus, an entire testing of the elution order of organochlorine enantiomers is strongly recommended. The same effect was observed on β-TBDM, which also contains *tert*-butyldimethyl groups on the cyclodextrin. But in contrast to β-BSCD, preparation of well-defined β-TBDM (purity, >99%) has been described (31). However, these purified β-TBDM phases enantioseparate only a limited number of organochlorines while the randomly silylated β-TBDM (as with β-BSCD) enantioseparate a large number of organochlorines (32). Despite the disadvantages of randomly silylated β-BSCD and β-TBDM phases in terms of being not well-defined CSPs, at present they are indispensable for enantioselective analysis of chiral organochlorines. For example, B7-1453 and oxychlordane can be enantioseparated only with randomly silylated β-BSCD and β-TBDM and not with β-PMCD, γ-PMCD, and β-PPCD. Consequently, working with these CSPs requires an entire study of the elution order of enantiomers. Note that the β-BSCD and β-TBDM phases we used for this study elute oxychlordane enantiomers in reversed order.

Reversal of elution order also was observed for PCB 174 enantiomers on β-PMCD and β-TBDM. β-PMCD and γ-PMCD eluted α-HCH (5, 6) and β-PCCH in reversed order (Table 1), and this reversal is expected for other chiral organochlorines. Although (–)-PCB 132 eluted prior to (+)-PCB 132 on β-PMCD (10), enantioseparation on γ-PMCD resulted only in a broad peak with low abundance. Because (–)-PCB 132 is more abundant in the solution, (–)-PCB 132 eluted earlier than (+)-PCB 132, confirming the same elution order on β-PMCD and on γ-PMCD. Because enantioseparations of organochlorines are enthalpy controlled, crossing of the isoenantioselective temperature that leads to reversal of the elution order of enantiomers on the respective CSP can be excluded (4). As we said at the outset, elution order of enantiomers barely can be predicted.

Recently, the elution order of β-PCCH enantiomers was determined on a chiral LC phase (ChiraDex, Merck). Now we confirm that the first eluted enantiomer (β_f-PCCH) corresponds to (+)-β-PCCH and the second eluted enantiomer (β_s-PCCH) corresponds to (–)-β-PCCH. Hühnerfuss et al. (20) used β-PPCD to enantioseparate β-PCCH enantiomers. On the basis of their data and the data we obtained on our β-PPCD phase (Table 1), it is now clear that the enantiomer labeled β_1 (20) corresponds to (+)-β-PCCH. Hühnerfuss et al. (20) found preferential microbial and photochemical decomposition of β_1-PCCH, which now can be read as (+)-β-PCCH. However, all effort to identify β-PCCH enantiomers in seal blubber failed, perhaps because of the low persistence of this first metabolite of α-HCH (33).

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RESIDUES AND TRACE ELEMENTS

Determination of Bitertanol Residues in Strawberries by Liquid Chromatography with Fluorescence Detection and Confirmation by Gas Chromatography/Mass Spectrometry

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A simple and rapid method was developed for determining bitertanol residues in strawberries. Bitertanol was extracted from samples with ethyl acetate. Bitertanol acetate was added prior to extraction as a surrogate standard. The ethyl acetate extract was cleaned up by passing through tandem solid-phase extraction columns consisting of anion-exchange (SAX) and aminopropyl (NH_2) bonded silica. The eluate was evaporated to dryness and reconstituted with methanol. Bitertanol residues were determined by liquid chromatography with fluorescence detection. Recoveries at 4 fortified levels (0.05, 0.25, 0.5, and 1.0 μg/g), calculated by the internal standard method, ranged from 92.1 to 99.1%, with coefficients of variation ranging from 0.3 to 4.0%. The detection limit was 0.01 μg/g. Of 25 commercial strawberry samples analyzed for bitertanol residues, 5 contained bitertanol residues at concentrations ranging from 0.02 to 0.51 μg/g. Positive samples were confirmed by gas chromatography/mass spectrometry with mass-selective detection (*m/z* 170 and 168).

Bitertanol [1-(biphenyl-4-yloxy)-3,3-dimethyl-1-(1H-1,2,4,-triazol-1-yl)butan-2-ol; Figure 1] is a fungicide widely used on fruit and vegetable crops. It is commercially available as Baycol or Baycoral. Because strawberries are directly sprayed with the fungicide, bitertanol residues are likely to be left on the edible portion. In Japan, the tolerance level for bitertanol residues in strawberries is 1.0 μg/g (1). Hence, there is a requirement to measure bitertanol residues.

Bitertanol residues in crops have been determined by gas chromatography (GC) with nitrogen–phosphorus detection (NPD; 2). Multiresidue methods have been developed based on GC with flame thermionic detection (3) and GC/mass spectrometry with mass-selective detection (GC/MS-SIM; 4, 5). A multiresidue method for analysis of wine also has been developed based on GC with electron capture detection and NPD (6). However, these methods do not address the issues of simple extraction and cleanup for fruit samples and short instrumental analysis time.

Recently, a method for determining bitertanol in bananas was developed, using liquid chromatography (LC) with fluorescence detection (7). We attempted to develop an LC method that is more rapid, simpler, and more suitable for strawberry samples than the method for bananas. Here, we report a method based on a simple solvent extraction, subsequent solid-phase extraction (SPE) cleanup, and determination by LC with fluorescence detection. Bitertanol acetate (Figure 1) is used as a surrogate standard for quality assurance. Positive samples are confirmed by GC/MS-SIM.

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METHOD

Apparatus

(a) *Blender.*—Model MX-C20G (Toshiba, Tokyo, Japan).

(b) *Homogenizer.*—Physcotron (Nichion, Tokyo, Japan).

(c) *SPE vacuum manifold.*—Vac Elut 24 (Varian Sample Preparation Products, Harbor City, CA).

(d) *Rotary vacuum evaporator.*—RE 47 (Yamato, Tokyo, Japan).

(e) *Glass filter.*—Buchner funnel with fused-in fritted glass disk (Shibata Scientific Technology, Tokyo, Japan).

(f) *SPE cartridges.*—Bond Elut SAX, 500 mg, and Bond Elut NH2, 500 mg (Varian). Attach SAX cartridge below NH_2 cartridge by using Bond Elut cartridge adapter (Varian).

(g) *LC column.*—Mightysil RP-18 (particle size 5 μm, 250 × 4.6 mm id; Kanto Chemical, Tokyo, Japan) with TSK gel 80 guard column (particle size 5 μm, 15 × 3.2 mm id; Tosoh, Tokyo, Japan).

(h) *Liquid chromatograph.*—Model PU-980 pump (Jasco, Tokyo, Japan); Model 7125 injector (Rheodyne, Cotati, CA); Model 821-FP fluorescence detector (Jasco); Model 807 IT integrator (Jasco); flow rate, 1.0 mL/min for isocratic elution with acetonitrile–water (7 + 3); injection volume, 10 μL; column temperature, 40°C; detector, 265 nm excitation and 325 nm emission.

(i) *Gas chromatograph/mass spectrometer.*—Shimadzu GC 14A (Shimadzu Corp., Kyoto, Japan) equipped with GC/MS QP2000GF (Shimadzu) and DB-1 capillary column, 30 m × 0.25 mm id, 0.25 μm film thickness (J&W Scientific, Folsom, CA). Gas chromatograph operating conditions: oven

temperature program, 1 min at 60°C, 20°C/min to 260°C, hold 20 min; carrier gas flow, helium 0.4 kg/cm^2 head pressure; injector, 260°C; splitless injection; splitless time, 2 min; injection volume, 2 μL. *Mass spectrometer operating conditions:* electron ionization, 70 eV; ion source, 280°C; selected-ion monitoring, *m/z* 170 and 168.

Reagents

(a) *Acetic anhydride.*—Reagent grade (Wako Pure Chemical Industries, Osaka, Japan).

(b) *Pyridine.*—Reagent grade (Wako Pure Chemical Industries).

(c) *Chloroform.*—Reagent grade (Wako Pure Chemical Industries).

(d) *Ethyl acetate.*—For pesticide residue analysis (Wako Pure Chemical Industries).

(e) *Methanol.*—For pesticide residue analysis (Wako Pure Chemical Industries).

(f) *Anhydrous sodium sulfate.*—For pesticide residue analysis (Wako Pure Chemical Industries).

(g) *Acetonitrile.*—LC grade (Wako Pure Chemical Industries).

(h) *Bitertanol standard.*—For pesticide residue analysis, >99.0% (Wako Pure Chemical Industries).

Standard Solutions

(a) *Bitertanol stock solution (100 μg/mL).*—Dissolve 10 mg bitertanol standard in 100 mL methanol. Store at 4°C.

(b) *Bitertanol intermediate solution (10 μg/mL).*—Dilute 10 mL bitertanol stock solution to 100 mL with methanol.

(c) *Surrogate and internal (IS) solution (200 μg/mL as bitertanol).*—Dilute 2 mL bitertanol acetate solution (10 mg/mL as bitertanol; *see Preparation of Bitertanol Acetate*) to 100 mL with methanol.

(d) *Bitertanol working standards for LC (0.1, 0.5, 1.0, and 2.0 μg/mL).*—Transfer 1, 5, 10, and 20 mL bitertanol intermediate solution (10 μg/mL) to individual 100 mL volumetric flasks. Add 1 mL IS solution to each flask. Dilute to 100 mL with methanol.

(e) *Bitertanol working standards for GC/MS (0.1, 0.5, 1.0, and 2.0 μg/mL in control strawberry matrix).*—Transfer 1, 5, 10, and 20 μL bitertanol stock solution (100 μg/mL) to individual calibrated glass tubes. Add 10 μL IS solution to each tube. Dilute contents of each tube to 1 mL with strawberry extract prepared as described in *Sample Extraction and Cleanup*.

Preparation of Bitertanol Acetate

The method described by Candida et al. (2) was used: Dissolve 100 mg bitertanol in 1 mL pyridine and 1 mL acetic anhydride in a 20 mL screw-capped vial with Teflon-lined cap. Allow mixture to stand at room temperature for 72 h in the dark. Add 15 mL water and transfer mixture to a 100 mL separatory funnel. Extract successively with three 25 mL portions of chloroform. Collect chloroform layer. Rinse chloroform layer once with 20 mL concentrated HCl–water (2 + 1) and twice with 25 mL water. Pass chloroform layer through filter paper containing ca 2 g anhydrous sodium sulfate, and evaporate filtrate to dryness in 200 mL round-bottom flask with rotary vacuum evaporator at 40°C. Dissolve residue in 10 mL methanol (10 mg/mL as bitertanol).

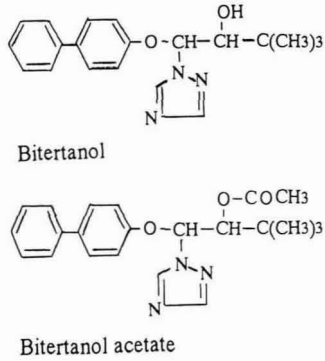

Figure 1. Structures of bitertanol and bitertanol acetate (IS).

Sample Preparation

Remove calyxes of strawberries. Cut edible portion into small pieces. Store at –18°C in polyethylene bags if not used immediately.

Sample Extraction and Cleanup

Weigh 10 g chopped strawberries in 300 mL glass beaker. Add 50 μL IS solution, 100 mL ethyl acetate, and 40 g anhydrous sodium sulfate. Homogenize mixture for 3 min. Pass homogenate through glass filter under vacuum. Rinse solid residue on glass filter with 5 mL ethyl acetate. Transfer solid residue to 300 mL glass beaker and repeat homogenization with 100 mL ethyl acetate and filtration step. Combine ethyl acetate extracts and evaporate to ca 1 mL in 300 mL round-bottom flask with rotary vacuum evaporator at 40°C. Precondition tandem SPE cartridges with 3 mL ethyl acetate. With a Pasteur pipet, apply concentrated ethyl acetate extracts to tandem SPE cartridges on SPE vacuum manifold. Rinse flask with 3 mL ethyl acetate. Add rinses to cartridges. Pass through cartridges additional 6 mL ethyl acetate. (Let each solution pass through cartridges at ca 2 mL/min by vacuum.) Collect ethyl acetate eluate in 10 mL calibrated centrifuge tube. Evaporate eluate to dryness in 100 mL round-bottom flask with rotary vacuum evaporator at 40°C. Reconstitute residue in 5 mL methanol.

Fortification

Fortify 10 g finely chopped control strawberry samples (previously determined not to contain bitertanol) with 5, 25, 50, and 100 μL bitertanol stock solution, corresponding to concentrations of 0.05, 0.25, 0.5, and 1.0 μg/g, respectively.

Follow procedure described above.

LC Determination

Run working standard solutions and sample extracts on LC system. Quantitate bitertanol residues based on the internal

standard method and the calibration curve constructed by plotting bitertanol concentration vs ratio of bitertanol and bitertanol acetate peak areas. Use bitertanol acetate peak area as IS. Check linearity of standard curve. Calculate bitertanol concentration as follows:

$$\text{Bitertanol, } \mu g/g = C/R(\text{std}) \times R(\text{samp}) \times V/W$$

where C = concentration of standard solution (μg/mL), R(std) = peak area of standard solution/peak area of IS, R(samp) = peak area of bitertanol in sample extract/peak area of IS in sample extract, V = volume of extract (5 mL), and W = sample weight (10 g).

Confirmation by GC/MS-SIM

Inject 2 μL sample extract and an appropriate bitertanol working standard in control strawberry matrix (0.1, 0.5, 1.0, or 2.0 μg/mL) into GC/MS system. Use peak retention times and relative abundances of 2 ions (*m/z* 170 and 168) within 5% of those obtained for the bitertanol standard to confirm identity.

Results and Discussion

The proposed method is both simple and rapid. Despite the double extraction, sample extraction was not time-consuming. Filtration with glass filter was fast. And the procedure did not need a partition step. Cleanup was performed by using tandem SPE cartridges.

Cairns et al. (8) described successful use of C_{18}, anion-exchange (QMA), and aminopropyl bonded silica (NH_2) Sep Pak cartridges for cleanup of pesticide residues in fruits and vegetables. The C_{18} Sep Pak cartridge removed long-chain fats and waxes, the QMA Sep Pak cartridge removed colored compounds and flavors, and the NH_2 Sep Pak cartridge removed sugars.

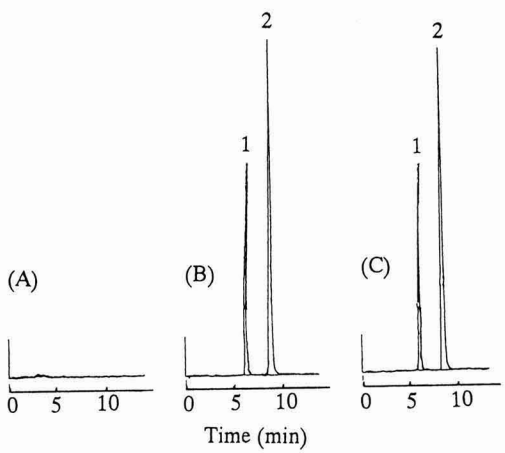

Figure 2. Liquid chromatograms of (A) control strawberry, (B) bitertanol standard (1.0 μg/mL), and (C) bitertanol-fortified strawberry (0.5 μg/g). Peak 1, bitertanol; peak 2, bitertanol acetate (IS).

Table 1. Recovery of bitertanol from fortified strawberries

Fortification level, μg/g	Absolute recovery, %[a]	CV, %	Recovery corrected for IS, %	CV, %
0.05	80.5	3.0	92.1	4.0
0.25	87.6	0.7	96.1	1.6
0.50	89.0	3.7	98.7	0.3
1.00	92.4	2.0	99.1	0.9

[a] $n = 3$ at each fortification level.

In our proposed method, we used anion exchange (SAX) and NH_2 cartridges. Bitertanol was eluted through these SPE cartridges with ethyl acetate. Although we did not use the C_{18} cartridge, the combination of SAX and NH_2 cartridges provided adequate cleanup.

After cleanup, the sample was reconstituted with methanol. In the methanol solution, a small amount of oily precipitate appeared. By pipetting the clear supernatant, a filtration step was not needed before injection into LC system or GC/MS system.

For quality assurance, a surrogate and internal standard (IS) was needed to monitor extraction, cleanup, and determination steps. The criteria for the selection of the IS were as follows: *(1)* Its recovery should be similar to that of bitertanol, *(2)* it should be detectable by LC with fluorescence detection, *(3)* its retention time should be distinct from that of bitertanol, and *(4)* it should elute in <15 min.

Bitertanol acetate meets these criteria. It is prepared easily by acetylation of bitertanol. It is stable in methanol solution for at least 3 months, and its recovery from strawberry samples is similar to that of bitertanol.

Figure 2 shows liquid chromatograms for bitertanol standard, control strawberry, and bitertanol-fortified strawberry. No chromatographic interference from control strawberry was observed at the retention times of bitertanol (6.8 min) and bitertanol acetate (9.5 min). Other triazol fungicides, such as triadimenol and traidimefon, were not expected to interfere under the LC conditions used.

Table 2. Determination of bitertanol residues in 25 commercial strawberry samples

Strawberry sample No.	Bitertanol, μg/g[a]	
	LC	GC/MS
1	0.13	0.15
2	0.02	0.02
3	0.06	0.06
4	0.04	0.05
5	0.51	0.58

[a] Duplicate determinations. Bitertanol was found only in the 5 samples presented here. Bitertanol was not detected (<0.01 μg/g) in the 20 remaining samples.

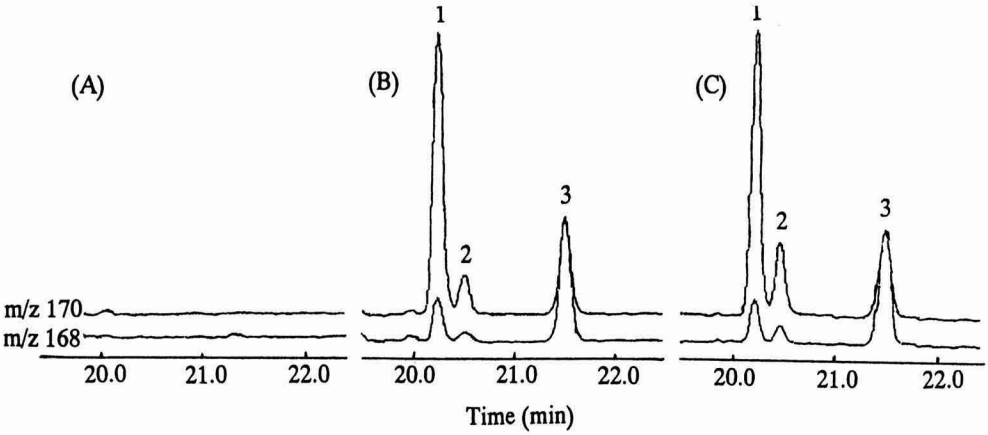

Figure 3. Selected ion chromatograms of (A) control strawberry, (B) bitertanol standard in control strawberry matrix (1.0 μg/mL), and (C) strawberry sample 5 in Table 2. Peaks 1 and 2, bitertanol; peak 3, bitertanol acetate (IS).

The LC conditions were similar to those reported by Nagayama et al. (7), except for the LC column and the acetonitrile/water ratio of the mobile phase. Under our LC conditions, bitertanol appeared as a single peak. Bitertanol can be separated into 2 diastereoisomeric peaks by increasing the water content of the mobile phase (acetonitrile–water) or by using a mobile phase consisting of methanol, tetrahydrofuran, and water (7).

Table 1 gives recoveries of bitertanol from strawberries fortified at 0.05, 0.25, and 0.5 μg/g and at the Japanese tolerance level of 1.0 μg/g. Absolute recoveries without correction for IS ranged from 80.5 to 92.4%, with coefficients of variation (CVs) ranging from 0.7 to 3.7%. Recoveries corrected for IS ranged from 92.1 to 99.1%, with CVs ranging from 0.3 to 4.0%. Absolute recovery of bitertanol acetate (IS) from strawberry samples was 96.4%. Recoveries of bitertanol were improved by the IS method. CVs for corrected recoveries were not significantly different from those of absolute recoveries. The method provides acceptable recoveries and precision.

The detection limit (signal-to-noise ratio, S/N = 3) was 0.01 μg/g. The standard calibration curve was linear over the range 0.05–1.0 μg/mL. The correlation coefficient was 0.999.

Table 2 gives bitertanol residues in 25 strawberry samples purchased from local supermarkets determined by the proposed method. Bitertanol residues were detected in only 5 samples at concentrations ranging from 0.02 to 0.51 μg/g.

Positive samples were confirmed by GC/MS-SIM. No false-positive results were observed. Figure 3 shows the selected ion chromatograms for control strawberry, bitertanol standard in control strawberry matrix, and strawberry sample 5 in Table 2. The selected ions at *m/z* 170 and 168 were used to confirm the identity of bitertanol. Bitertanol acetate was also monitored at *m/z* 170 and 168. Under the GC/MS conditions, bitertanol was separated into 2 diastereoisomeric peaks. Retention times of bitertanol diastereoisomers and bitertanol acetate were 20.2, 20.5, and 21.5 min, respectively. At bitertanol peak 1 (Figure 3), the relative abundances of ions at *m/z* 170 and 168 were 100 and 15%, respectively. At bitertanol peak 2, the relative abundances of the ions were 100 and 20%, respectively. Abundances of bitertanol peaks tended to be affected by the strawberry matrix extract.

To compensate for the matrix effect, standards for GC/MS were prepared in residue-free strawberry matrix extract (9). For reference, bitertanol residues in positive samples were quantitated under the GC/MS conditions (peak areas at *m/z* 170) by the IS method. Results were similar to those obtained by LC, as shown in Table 2.

Bitertanol could be also monitored at *m/z* 171, but interferences from strawberry matrix were observed in this region at the retention time of bitertanol. Other adequate ions were not found. The GC/MS conditions did not meet the guidelines for confirmation; that is, confirmation should be based on the presence of at least 3 ions (10, 11). However, the combination of 2 analytical techniques, LC with fluorescent detection and GC/MS, provides reliable identification of bitertanol residues.

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STATISTICAL ANALYSIS

A Simple Method for Evaluating Data from an Interlaboratory Study

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Large-scale laboratory- and method-performance studies involving more than about 30 laboratories may be evaluated by calculating the HORRAT ratio for each test sample (HORRAT = [experimentally found among-laboratories relative standard deviation] divided by [relative standard deviation calculated from the Horwitz formula]). The chemical analytical method is deemed acceptable per se if HORRAT ≈ 1.0 (± 0.5). If HORRAT is ≥2.0, the most extreme values are removed successively until an "acceptable" ratio is obtained. The laboratories responsible for the extreme values that are removed should examine their technique and procedures. If ≥15% of the values have to be removed, the instructions and the methods should be examined. This suggested computation procedure is simple and does not require statistical outlier tables. Proposed action limits may be adjusted according to experience. Data supporting U.S. Environmental Protection Agency method 245.1 for mercury in waters (manual cold-vapor atomic absorption spectrometry), supplemented by subsequent laboratory-performance data, were reexamined in this manner. Method-performance parameters (means and among-laboratories relative standard deviations) were comparable with results from the original statistical analysis that used a robust biweight procedure for outlier removal. The precision of the current controlled performance is better by a factor of 4 than that of estimates resulting from the original method-performance study, at the expense of rejecting more experimental values as outliers.

The International Union of Pure and Applied Chemistry (IUPAC) classifies interlaboratory studies into 3 major self-defining categories: method performance, material performance, and laboratory performance (1). Although the procedure for statistical evaluation of data from these 3 types of interlaboratory studies may be identical, the use and interpretation of the resulting statistical estimates will be determined by the primary purpose of the study. The purpose of a method-performance study is to characterize the performance of a method within the context of the materials analyzed in the study. The purpose of the material-performance study is to assign a value and an associated uncertainty (or reliability) to a characteristic (usually a concentration) of a material. The purpose of a laboratory-performance study is to provide data that will permit evaluation of each participant against preset criteria or criteria estimated directly from the study itself.

Method-performance studies serve to determine the bias and precision of analytical methods. General protocols are available from AOAC INTERNATIONAL (2), International Organization for Standardization (3), and IUPAC (4). The American Society for Testing and Materials has a protocol for methods for water analysis (5). The protocols differ somewhat in requirements for number of laboratories, matrixes, and levels, as well as in details of outlier removal. For studies not requiring outlier removal, the protocols return the same values for the mean and repeatability and reproducibility standard deviations from the same set of data, but they differ with respect to the confidence that can be ascribed to the derived method parameters. The protocol based on the largest number of laboratories, matrixes, and levels, in general, would have the highest degree of validation. Method-performance studies require that all laboratories use the same method and follow all details of the method description and study instructions exactly so that no uncontrolled variable is introduced.

Material-performance studies determine the properties of a material, usually analyte concentration, with a stated uncertainty. Assigned values are evaluated on a case-by-case basis by the organization supplying or certifying the reference material according to a critical evaluation of the bias and precision exhibited by the results. Typically, the certifying organization prefers to use a variety of methods and multiple laboratories so that local biases from these sources have the opportunity to appear and to cancel out or be minimized by randomization.

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Laboratory performance is evaluated from the point of view of producing results that are satisfactory for the intended purpose (6) or of producing results according to previously set specifications. A standard based on the desired reliability or what is "fit for the purpose" may not necessarily correspond to what is practically attainable. Laboratories are expected to use their routine method for this type of study.

Theoretically, interlaboratory studies are sampling exercises. These studies involve a sample of laboratories that are expected to use the method or material in the future. In some sectors, as in clinical chemistry and some environmental analyses, the entire population of laboratories must participate. The test materials used in all interlaboratory studies must be homogeneous.

Many sets of experimentally derived data are marred by the presence of outliers—values that do not seem to belong to the bulk of the data. But how distant does a data point have to be before it should be rejected? The chemist usually turns the data over to a statistician, who applies standard statistical tests whose validity depends upon several assumptions. Two such assumptions are (*1*) that the submitted data follow a known distribution usually assumed to be "normal" and (*2*) that the values are independent. The criterion of independence among laboratories is easily met in interlaboratory studies as long as there is no consultation among them. Data from simultaneous or consecutive replicate analyses within a laboratory, however, are inherently nonindependent.

The IUPAC internal quality control document (7) and proficiency-testing protocol (8) are based upon an examination of *z* scores. This score is the deviation of an individual value from the mean, expressed in standard deviation units. The probability that a laboratory is more than 2 standard deviation units away from the mean of a normally distributed set of data by chance alone is only about 5%; the chance of being more than 3 units away is less than 1%. If the set contains fewer than 30 laboratories, the critical statistics should be further modified by use of Student's *t* factors to account for the uncertainty of standard deviation estimates made from small samples.

We present here a simple method for evaluating large data sets such as those that are frequently available from laboratory-proficiency studies and those that are occasionally obtained in method- and material-evaluation studies. This proposed protocol does not require statistical tables. The test is based entirely upon the thesis that interlaboratory data that are in statistical control will follow a consistent historical pattern. Laboratories that produce values that distort this pattern are not performing in an acceptable manner and should investigate the cause of their discrepancies. The historical, empirical Horwitz curve can be used as the initial screen for a distorted pattern, but final decisions are based on the data themselves or on the pattern of related data. Laboratories that cause the statistical distribution to exhibit relative standard deviations consistently greater than twice the value predicted from the Horwitz formula are not operating according to historical performance standards (9).

The Horwitz curve is an empirically derived exponential equation that relates the relative standard deviation among-laboratories, RSD_R, in %, to concentration, C, expressed in mass/mass or volume units (e.g., μg/g or μg/mL = 10^{-6}). Thus, RSD_R, % = $2C^{-0.1505}$. The relationship is more or less independent of analyte, matrix, method, and time when the interlaboratory study was conducted. It was originally derived from a review of several hundred interlaboratory studies conducted primarily by the Association of Official Analytical Chemists (10). The relationship is now supported by the examination of almost 10 000 data sets from method-performance studies that typically use 8 laboratories analyzing 5 individual test samples of commodities ranging from agricultural products to geological specimens, with analytes ranging from chlorinated industrial contaminants to nutrients, at concentration levels from pure compounds ($C = 1$) to ultratrace contaminants ($C = 10^{-12}$). The equation recently was derived by assuming that a fractional change in concentration is proportional to a fractional change in standard deviation (11).

METHOD

A spreadsheet or a calculator with statistical functions is convenient and sufficient for calculations. The estimated mean concentration, C, and the standard deviation, s, are calculated from all reported values from individual laboratories, material by material. If all laboratories report replicates, laboratory means are used or the following procedure is applied: Calculate the mean, C_1, and the standard deviation, s_1, from the first value reported by each laboratory, C_2 and s_2 from the second values reported, etc., and then average the C_i estimates and pool the s_i estimates to obtain overall estimates of C and s for each analyte in each material studied. Multiple values from an occasional laboratory in a large study may be treated as separate laboratories or a single value may be chosen at random from the group. From the mean and s, calculate the RSD_R, in percent:

$$RSD_R, \% = 100\ (s/C)$$

Also calculate the predicted RSD_R, in percent, from the Horwitz formula (11):

$$PRSD_R, \% = 2C^{-0.1505}$$

where C is expressed in mass/mass or volume (fractional) units, e.g., g/g or mL (1 ppm = 10^{-6} g/g; at $C = 10^{-6}$, $PRSD_R$ = 16%). Then calculate the HORRAT value:

$$HORRAT = RSD_R/PRSD_R.$$

Interpretation

If HORRAT is ca 1.0 (± 0.5), the data may be accepted directly as corresponding to the equation representing historical performance.

If HORRAT is ≥1.5, remove the most extreme value(s) without regard to direction (i.e., high or low) and recalculate HORRAT. Most outliers are at the high end of the distribution, but also check the lowest value to determine if its removal makes a meaningful contribution to convergence toward the targeted HORRAT of unity. Continue removal of extreme data until either HORRAT is ≤1.5 or further removal changes the

HORRAT very little (≲0.2). If several extreme values are close together or if they are present as a cluster an order of magnitude distant from their neighbors, they can be removed as a group. The critical value to use for the decision to stop data point removal is arbitrary and is a function of the number of participating laboratories. For method-performance studies, where data often are available from only 8 laboratories, the need to remove 2 laboratories (i.e., >22%) is considered excessive for method approval (2, 4). For large studies with the number of participants approaching or exceeding 100, failure by 15 laboratories suggests the potential existence of misinterpretation of the protocol; problems with study sample homogeneity, or standard procurement or preparation; or a need to improve the method. Current experience with large-scale interlaboratory studied involving analyses of water suggests that on the average ca 8% of the data represents out-of-control performance.

An initial HORRAT value of ≲0.5 suggests either lack of independence or use of a superior, compatible quality assurance program on the part of most laboratories. Lack of independence may take the form of unreported consultation, replication, averaging, self-censoring, or excessive rounding of reported data, thereby artificially reducing within-laboratory and among-laboratories variabilities. Low HORRAT values also can indicate that poorly performing laboratories have discovered and corrected their errors, that they have abandoned the analysis, or that they no longer participate in the proficiency studies.

Although these recommended critical HORRAT values are the integrated result of experience with numerous analytes, matrixes, methods, and experimental designs, now approaching 10 000 interlaboratory data sets, they may be modified to conform to specific situations and experience. Exclusive application to specialized areas may result in development of other specific parameters applicable to limited matrixes, analytes, or commodity areas. A specific example is determination of polychlorinated contaminants (biphenyls, dioxins, and furans). These compounds exhibit better than predicted performance (12). This achievement is ascribed to use of labeled internal standards and adherence to reference performance parameters at practically every step of the complicated separation and measurement procedure. As demonstrated in the current study, continuous operation of a laboratory proficiency control program for almost 2 decades has shifted the initial, empirically determined HORRAT value of ca 1.5 to a relatively stable 0.4. Nevertheless, a recent independent statistical examination of then-current published databases containing 7502 data sets (13) affirmed the original conclusions with regard to the overall behavior of variability as a function of concentration and time in the examination of initial method-performance studies of numerous procedures.

Application of a HORRAT-like ratio to within-laboratory designs (within-runs, among-analysts, among-test portions, and similar replications), however, should be approached with caution because of the difficulty of meeting the independence criterion. Strict adherence to the requirement for independence calls for all results to be completely uninfluenced by other measurements. Theoretically, this would even require a new calibration curve for every measurement—an impractical requirement. The influence of this factor, however, may be automatically incorporated into internal quality control limits and used as the basis for an internal HORRAT interpretation factor by using a consistent number of randomly placed replicates in a run.

In this proposed data-outlier-purging procedure, extreme values may be selected for removal "by eye," by plotting the data on a linear scale, by use of scatter plots or histograms, by use of the most extreme z score, and by invoking the critical value of any of the conventional outlier tests, as well as on the basis of reports of method deviation, instrument instability, or any desired unbiased criterion. In statistical phraseology, this robust, practical procedure would be called "successive trimming."

Example

The original method-evaluation data supporting use of U.S. Environmental Protection Agency (EPA) cold-vapor method 245.1 (manual) for mercury in waters (14) are used as an example, supplemented by subsequent, recent performance data. Although the cold-vapor, flameless, atomic absorption method is in its fourth edition, it remains fundamentally the same as originally accepted. The first study (14) provided the data for method-performance assessment, which was used as the basis for acceptance of the method by EPA. Known quantities of Hg from 0.2 to 10 μg/L ($C = 0.2–10 \times 10^{-9}$) were added to 2 matrixes—distilled and natural water—arranged as Youden pairs. The Youden pair design, also known as split levels, consists of 2 test samples that differ only "slightly" in concentration or in matrix to avoid the tendency of analysts to match replicates (5).

The second group of data consists of the most recent sets of test samples used for monitoring ongoing EPA and state laboratory performance.

The data are presented in groups as "jitterplots" in Figures 1–3, with concentrations in μg/L as the y-axis and the identified individual test samples as the x-axis. Results are summarized in Tables 1–3. Individual values for each test sample are displaced by small, random noise for visibility (15). The original data will be available from the correspondence author for a reasonable length of time after publication. This presentation conceals the identity of most laboratories but shows the main features of the distribution. The rectangles overlying the data give the mean ± 1 (final) standard deviation with the identification numbers of the most extreme, off-scale outliers placed at the top with their reported concentrations in parentheses. Tables 4 and 5 are presented to show the pattern of consistency of producing extreme, biased values by the same laboratories across many of the test samples in a series.

Few cases of multiple analysts from the same laboratory were present so that each reported value was treated as a separate laboratory. The original values from each test sample were sorted from lowest to highest and were reassigned consecutive laboratory numbers, with some gaps for the shorter series. The means ($\bar{x}$), standard deviations among laboratories (s_R), RSD_R, and HORRAT values were first calculated from all the data without outlier removal. Then the most extreme value(s) was removed, and performance parameters were recalculated from

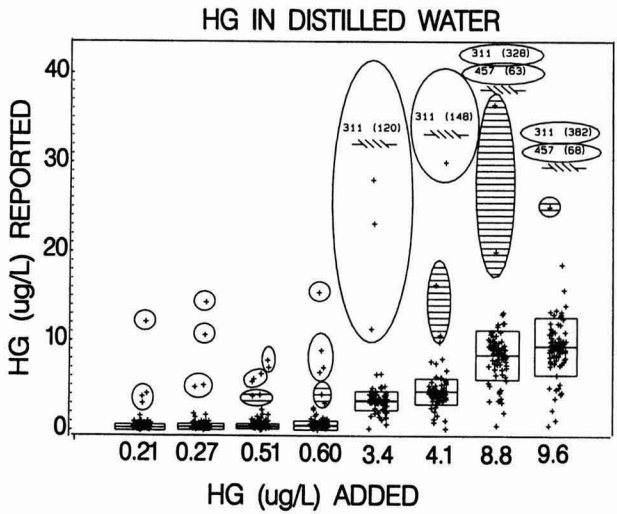

Figure 1. Reported data for method-performance study of mercury in distilled water. Values above the hatched marks, as well as those in the solid circular figures, were removed to provide acceptable HORRAT values. Removal of values in the filled circular figures is optional. Rectangles indicate the final mean ± 1 final standard deviation.

the new set. A data point was removed as a single value when it was isolated, but if several values were of about the same magnitude, they were removed together. All outliers were on the high side, but occasionally the lowest value was removed to verify that this point made no significant contribution to excessive variability. Data removal was continued until the calculated statistic converged to the acceptable region of HORRAT ≤1.5–2.

The progressive calculated statistics from spiked distilled water are presented in Table 1; from spiked natural water, in Table 2; and from the recent performance data studies on reagent water, in Table 3. Tables 4 and 5 show the consistency of the data from outlying laboratories across all test samples for distilled water and natural waters, respectively. In the last 2 tables, the original assignment of laboratory numbers was retained to permit laboratories to identify themselves.

The original data of Tables 1 and 2 exhibited extreme variability, with RSD_R values of 140–290%, an excessive out-of-control condition. These corresponded to unacceptable HOR-

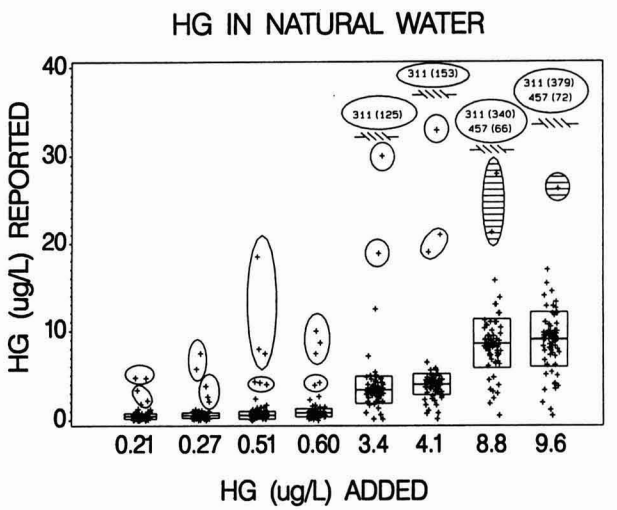

Figure 2. Reported data for method-performance study of mercury in natural water. Values above the hatched marks, as well as those in the solid circular figures, were removed to provide acceptable HORRAT values. Removal of values in the broken circular figures is optional. Rectangles indicate the final mean ± 1 final standard deviation.

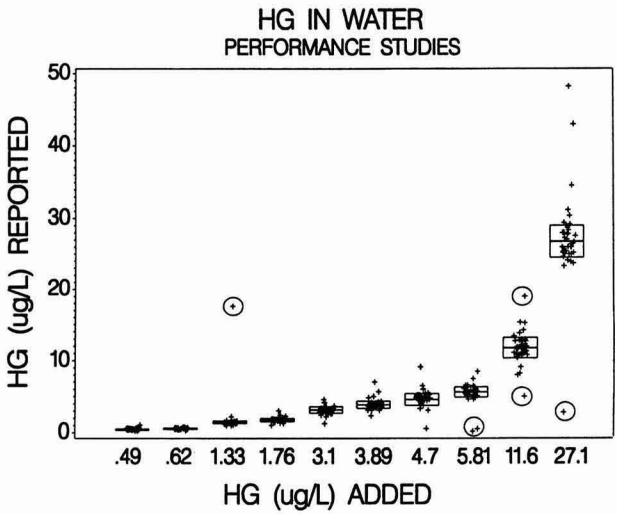

Figure 3. Reported data for subsequent laboratory-performance studies of mercury in waters. Circled values were removed to provide acceptable HORRAT values. Rectangles indicate the final mean ± 1 final standard deviation.

RAT values of 3.1–9.6. In 4 of 16 test samples, removal of 2–4 laboratories (2–4%) in a single cycle was sufficient to reduce extreme RSD_R (>200%) and HORRAT (≥9) values to acceptable ranges (ca 40%; <1.5). For the 7 remaining cases, a maximum of 2 cycles of laboratory removal were required to bring HORRAT values to the acceptable range of 1.0 ± 0.5, and for only 5 test samples were 3 cycles needed. Even so, an occasional breach of the limits might be accepted, as in the case of the HORRAT of 1.8 with the fourth set of the natural waters.

The same few laboratories are producing most of the outlying values. In almost all cases in Tables 4 and 5, where the performance of the laboratories across the 16 test samples is shown, the most extreme values are consistently produced by the same laboratories and usually on the high side, often by a factor of 10, indicating that the method is not in control in these laboratories. In the more than 1000 values, only a few isolated single-laboratory outliers appear. Some laboratories are unable to measure levels below ca 10 μg/L. After the most extreme outliers are excluded, further elimination of values result only in marginal additional reductions in the variability estimates. In the figures, values enclosed by a circle or ellipsoid are those extreme values. The values within the shaded figures are marginal outliers whose removal would result in further improvement of the HORRAT value but by just a few tenths. Removal of these values is optional. In almost all cases, means are little affected because of the large number of acceptable values.

After the method is established and laboratories have had experience in its performance, the overall performance of the method improves considerably. Ten current examples are presented in Table 3, performed almost 2 decades after adoption of method 245.1. Acceptable RSD_R values for method performance are obtained initially in all but 2 test samples. In these cases, one high value (test sample 27.1) and one low value (test sample 1.33) are off by factors of 10, suggesting misplaced decimal points rather than an intrinsic flaw in method performance.

Discussion

It is difficult to understand how some laboratories consistently report outlandish results (laboratories 311 and 457 of the original data), sometimes a deviation of 2 orders of magnitude. A few laboratories reported high results only for test samples containing the lowest levels. The laboratories responsible for outliers were fairly consistent across the test samples, suggesting an incorrectly prepared standard calibration solution, a consistent calculation error, or an insufficiently sensitive measuring instrument.

Many chemists do not understand that for a method to be suitable for use, the characteristic among-chemists variability of the results they obtain on the same test samples must be held within specified limits. Often, they also do not realize that much arbitrary judgment enters into the statistical evaluation of their data or that when they report a value it is merely an estimate of a target value, in this case an added, known quantity. Measurement of multiple, independent tests (not just repetitious instrument readings of the same solution) results in the typical, normal distribution of results, with most values near the central peak but occasional values near the extremes. Therefore, occasional (e.g., 5%) extreme values not too distant from the central cluster in large data sets (about 100) can be tolerated as being part of the parent distribution. However, strange values, differing by an order of magnitude or more, show a lack of control in the laboratories that fail to discover and remove the aberrations. Such values must be ignored when the objective is to estimate the characteristic method statistics; it should provoke an investigation within the responsible laboratory(ies) when the objective is laboratory performance.

Table 1. Progression of HORRAT values with removal of extreme values: distilled water set

	Added, µg/L							
Statistic	0.21	0.27	0.51	0.60	3.4	4.1	8.8	9.6
Original No. of labs	91	92	94	93	93	92	90	92
Original mean, µg/L	0.65	0.81	1.0	1.2	5.1	6.3	13	14
Original RSD_R, %	212	237	139	173	244	244	266	280
HORRAT	4.4	5.1	3.1	3.9	6.9	7.1	8.6	9.2
With labs removed								
Lab No./value removed	93/12.1	37/10.6			91/23			
		93/14.3	92/7.0		52/28	52/30		
			36/7.8	33/15.4	36/120	36/148	36/328	36/328
No. of labs remaining	90	90	92	92	90	90	89	91
Mean, µg/L	0.52	0.55	0.89	1.0	3.4	4.4	9.4	10
RSD_R, %	123	134	122	134	38	45	75	69
HORRAT	2.5	2.7	2.7	3.0	1.0	1.2	2.3	2.2
Additional labs removed								
Lab No./value removed	38/3.0		38/6.28	36/8.90		90/10.6		
	92/3.75	92/5.0	37/5.40	92/7.00	92/11.3	92/16.3		
	95/4.10	95/4.8	95/5.70				52/63.0	52/68.0
No. of labs remaining (% labs removed)	87 (4.4%)	88 (4.4%)	89	90	89 (4.3%)	88 (4.4%)	88	90
Mean, µg/L	0.42	0.45	0.72	0.84	3.3	4.2	8.8	9.4
RSD_R, %	68	73	84	99	30	33	46	35
HORRAT	1.3	1.4	1.8	2.1	0.8	0.9	1.4	1.1
Additional labs removed								
Lab No./value removed				95/3.1				
			52/3.8	52/6.46				94/0.58[a]
			91/3.9	51/4.0			91/36.4[a]	
No. of labs remaining (% labs removed)			87 (7.4%)	87 (6.4%)			87 (3.3%)	89 (3.3%)
Mean, µg/L			0.65	0.72			8.4	9.5
RSD_R, %			48	57			32	33
HORRAT			1.2	1.2			1.0[a]	1.0[a]

[a] Additional values removed to show effect of eliminating next largest value.

On average, in the examples given here, 5% of the values were removed by this proposed procedure, coincidental with arbitrary selection by statisticians of the 5% point as a reasonable dividing line between acceptable and unacceptable risk of being wrong. Unfortunately, chemists and many data users tend to take a reported value as fixed, not making allowances for the inherent variability of estimates. A statement that 5% of values are removed, on the average, means not 5.00% but a reasonable range of values that may extend from say 2.5 to 10%. Removal is stopped when a reasonable, acceptable HORRAT value is attained, or the HORRAT values approximate the equivalent values in a series of similar test samples, or they converge to a value approximating 1.0. If too many values are removed, the analytical method should be reconsidered. The process forces precision estimates to match roughly predicted precision values but also serves to highlight maverick laboratories.

At the lowest concentration levels, the average value obtained by the successive-removal procedure does not converge to the "true value" but to a higher value. Reagent blanks were not reported; therefore, it cannot be determined if the method is biased at the lowest levels or if the substrate or reagents contained trace quantities of mercury.

In the tables, we occasionally have removed more values than necessary to show the effect of the removal of the next candidate. Elimination of the lower values that may appear to be outliers usually has little effect on the HORRAT value because of asymmetry. In large studies, removal of extreme values usually has little effect on the mean because of dilution with acceptable values.

Once a method has been in operation for a number of years, outliers become rare. Inept laboratories no longer appear as participants in proficiency exercises, through voluntary or forced attrition. The target HORRAT value also shifts from the initial 1.0 to a superior 0.5, reflecting improved performance gained through experience and elimination of poor performers.

Table 2. Progression of HORRAT values with removal of extreme values: natural water set

	Added, µg/L							
Statistic	0.21	0.27	0.51	0.60	3.4	4.1	8.8	9.6
Original No. of labs	78	81	83	80	83	80	80	79
Original mean, µg/L	0.54	0.66	1.2	1.1	5.4	6.4	14	15
Original RSD_R, %	157	169	204	150	256	266	277	290
HORRAT	3.2	3.5	4.6	3.4	7.3	7.8	9.1	9.6
With labs removed								
Lab No./value removed			80/18.48	31/8.70				
	81/4.75	32/5.75	81/7.50	81/7.50			46/66	46/72
	84/4.70	81/5.75	31/8.00	28/10.0	31/12.5	31/153	31/340	31/379
No. of labs remaining	76	79	80	77	82	79	78	77
Mean, µg/L	0.43	0.51	0.74	0.82	3.9	4.6	8.8	9.1
RSD_R, %	119	111	103	86	94	63	42	40
HORRAT	2.3	2.2	2.2	1.8	2.6	2.6	1.3	1.2
Additional labs removed								
Lab No./value removed		84/3.80	84/4.30					
	80/3.35	80/2.09	32/4.20	84/4.20			81/21.3	81/26.3
	32/2.20	82/2.60	46/4.00	46/3.90	46/30.0	46/33.0	80/28.0	
No. of labs remaining (% labs removed)	74	76 (6.2%)	77 (7.2%)	75 (6.2%)	81	78	76 (5.0%)	76 (3.8%)
Mean, µg/L	0.37	0.42	0.63	0.73	3.6	4.2	8.4	8.9
RSD_R, %	88	69	64	62	63	67	32	34
HORRAT	1.7	1.3	1.3	1.3	1.7	1.8	1.0	1.0
Additional labs removed								
Lab No./value removed						79/21.0		
	82/1.8				80/18.86	80/19.0		
No. labs remaining (% labs removed)	73 (6.8%)				80 (3.8%)	76 (5.0%)		
Mean, µg/L	0.35				3.4	3.8		
RSD_R, %	81				44	30		
HORRAT	1.5				1.2	0.8		

Table 3 also presents results by the robust statistical procedure ("biweight"; 16) used routinely by EPA to examine results of their interlaboratory studies. This procedure assigns a weight from 0 to 1.000 to various values, depending upon their distance from the median. A weight of 0 indicates sufficient deviation to warrant rejection. Although the programming to assign weights is relatively difficult, it needs to be done once. The advantages are that a conclusion is obtained in a single pass through the data and that all values are used. The disadvantage is that, depending on the amount of outlier data present, final standard deviations may not be rigorously compared with those obtained by the classical procedure. When the relative standard deviations obtained by the biweight procedure are expressed in terms of HORRAT values, the biweight values are about 2/3 of those obtained by the classical procedure. EPA believes that about 7–8% of the data submitted by the water laboratories currently participating in performance-evaluation studies are outlier data, that is, data produced by laboratories operating out-of-control as determined by followup observations and comparison with true values prepared by addition of known quantities of analytes. This value is also supported by the number of outliers that must be removed from method-performance studies to obtain reasonable analytical estimates, as recently summarized by Thompson and Lowthian (13). Such a high rate of outlier data presents a difficult challenge to classical procedures, which can be expected to fail to identify and remove all outliers present.

Conclusion

An extremely simple technique of successive data removal is proposed for examining method- and laboratory-performance studies based on the ratio of the relative standard deviation to the corresponding value predicted from the Horwitz function. The procedure is simple and straightforward and can be accomplished with ordinary spreadsheet or electronic calculator arithmetic and without statistical tables or difficult computations. Outliers are removed until the ratio converges to about 1.0 ± 0.5. The EPA cold-vapor method for mercury is used as an example of the application of the technique. After removal

Table 3. Progression of HORRAT values with removal of extreme values: performance data on mercury in ASTM Type 1 reagent-grade water[a]

	Added, µg/L									
Statistic	0.494	0.615	1.33	1.76	3.1	3.89	4.7	5.81	11.6	27.1
Original No. of labs	57	51	51	51	47	51	55	41	47	41
Original mean, µg/L	0.512	0.60	1.69	1.80	3.08	3.94	4.64	5.52	11.7	27.15
Original RSD_R, %	23.8	16.6	135	15.6	16.9	16.6	16.2	25.0	17.2	22.4
HORRAT	**0.48**	**0.34**	3.22	**0.38**	**0.44**	**0.45**	**0.45**	0.71	0.55	0.81
With labs removed										
Lab No./value removed	57/1.09	1/0.31	51/17.6	1/1.03	1/1.26	51/7.01	1/0.59	1/0.20	1/5.0	1/2.8
				51/3.03				2/0.56	47/19.0	
No. labs remaining (% labs removed)	56 (2%)	50 (2%)	50 (2%)	49 (4%)	46 (2%)	50 (2%)	54 (2%)	39 (5%)	45 (4%)	40 (2%)
Mean, µg/L	0.502	0.61	1.38	1.79	3.12	3.88	4.72	5.80	11.7	27.8
RSD_R, %	18.9	15.1	13.3	10.9	14.4	12.65	10.2	12.6	12.2	17.1
HORRAT	0.38	0.31	0.31	0.26	0.38	0.34	0.29	**0.36**	**0.39**	**0.62**
Lab No./value removed							54/9.12[b]			43/43.0[b]
							55/9.14[b]			44/48.2[b]
No. of labs remaining							53			39
Mean, µg/L							4.70			26.2
RSD_R, %							17.0			17.0
HORRAT							0.48[b]			0.61[b]
Lab No./value removed										1/2.8[c]
										43/43.0[c]
										44/48.2[c]
No. of labs remaining										38
Mean, µg/L										26.8
RSD_R, %										8.49
HORRAT										0.31[b]
Biweight results[d]										
Mean, µg/L	0.50	0.60	1.36	1.78	3.05	3.86	4.77	5.69	11.7	26.5
RSD_R, %	15.4	17.2	9.5	7.7	12.3	8.2	6.5	10.5	9.5	8.1
Equivalent HORRAT	0.31	0.35	0.22	0.19	0.32	0.21	0.18	0.30	0.30	0.29
Limits	0.30–0.70	0.321–0.881	1.01–1.71	1.41–2.16	2.03–4.07	3.01–4.72	3.93–5.61	4.04–7.33	8.65–14.7	20.6–32.4
No. values (%) assigned 0 weight	2/57 (4%)	0/51 (0%)	3/51 (6%)	3/51 (6%)	2/47 (4%)	3/51 (6%)	7/55 (13%)	3/41 (7%)	2/47 (4%)	3/41 (7%)

[a] Values rounded to 2 decimal places. Accepted values are in bold.
[b] Additional values removed to show effect of eliminating next largest value.
[c] Additional removals are given to illustrate effects.
[d] Average HORRAT = 0.27; classical = 0.39.

Table 4. Relationship of removal of laboratories as outliers across test samples of distilled water (D)[a]

D 0.21	D 0.27	D 0.51	D 0.60	D 3.4	D 4.1	D 8.8	D 9.6
329 (3.0)		329 (6.2)					
	324 (10.6)	324 (5.4)					
532 (3.7)	532 (5.0)	532 (7.0)	532 (7.0)	532 (11)	532 (16)		
		311 (7.8)	311 (8.9)	311 (120)	311 (148)	311 (328)	311 (382)
		457 (3.8)	457 (4.0)	457 (30)	457 (30)	457 (63)	457 (68)
		531 (3.9)		531 (23)		531 (36)	
533 (12.1)	533 (14.3)						
	535 (4.8)	535 (5.7)	535 (3.0)				
			261 (15.4)				
			467 (6.4)		530 (10)		

[a] Mercury concentrations (μg/L) added are listed in the first row. Numbers in parentheses are concentrations (μg/L) reported by the numbered laboratories (original identification number assignment).

Table 5. Relationship of removal of laboratories as outliers across test samples of natural water (N)[a]

N 0.21	N 0.27	N 0.51	N 0.60	N 3.4	N 4.1	N 8.8	N 9.6
	533 (2.6)						
		311 (8.0)	311 (8.7)	311 (125)	311 (153)	311 (340)	311 (379)
324 (2.2)	324 (7.5)	324 (4.2)					
		457 (4.0)	457 (3.9)	457 (30)	457 (33)	457 (66)	457 (72)
531 (3.3)	531 (2.0)	531 (18.4)		531 (18.86)	531 (19)	531 (28)	
532 (4.7)	532 (5.7)	532 (7.5)	532 (7.5)			532 (21.3)	532 (26.3)
535 (4.7)	535 (3.8)	535 (4.3)	535 (4.2)				
			261 (10.0)		530 (21)		

[a] Mercury concentrations (μg/L) added are listed in the first row. Numbers in parentheses are concentrations (μg/L) reported by the numbered laboratories (original identification number assignment).

of about 5% of the laboratories, the HORRAT ratio converged to an acceptable value of 1.5 in the initial method-performance studies. Later laboratory-performance studies showed that the HORRAT ratio had improved to a value of about 0.4 with a negligible outlier rate.

Acknowledgment

An inquisitive, thought-provoking editorial by Gabor Levy in *American Laboratory* was responsible for this reexamination of the support for the method for determining mercury contamination of environmental media.

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TECHNICAL COMMUNICATIONS

Improvement in the Multiresidue Liquid Chromatographic Analysis of Residues of Mono- and Dibasic Penicillins in Bovine Muscle Tissues

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A recently developed multiresidue method using gradient liquid chromatographic conditions for analysis of residues of β-lactam drugs in bovine muscle tissues after precolumn derivatization with acetic anhydride was modified to permit isocratic analysis. The modification included replacing the acylating reagent, acetic anhydride, with benzoic anhydride and increasing sample size for extraction from 2 to 3 g. The modifications have reduced analysis time per sample from >1 h to about 22 min without loss in detection sensititivities for the β-lactams, resulting in a significant increase in the number of samples that can be analyzed in 1 day from 1 to 8. The modifications make the multiresidue LC method more suitable than the original method for use in regulatory programs for routine analysis of these veterinary drug residues in bovine muscle tissues.

In 1991, we developed a sensitive method for determining penicillin G residues in animal tissues. It was based on formation of stable mercaptide complexes of penicillin G and penicillin V by reaction with mercuric chloride (1). This method was suitable for analysis of monobasic penicillins only. To make it applicable for dibasic penicillins such as amoxicillin and ampicillin, which are also used very frequently in food animal production, we used acetic anhydride to preferrentially convert the free amino groups in the dibasic penicillins into their corresponding acetamidopenicillin derivatives. Both the acetylated dibasic penicillins and the monobasic penicillins react to form stable mercaptide derivatives. However, because the retention characteristics of the derivatized penicillins on an analytical C_{18} column are very different, we had to use gradient liquid chromatographic (LC) techniques to resolve them from each other and from other endogenous and exogenous components in the tissue matrix.

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We recently developed a sensitive multiresidue method based on this concept for determining residues of 4 penicillins in bovine muscle tissue at concentrations of interest to regulatory agencies (2). The procedure enabled 12 samples, including 4 calibration standards, to be prepared for analyses in 2.5 h. However, the chromatographic conditions permitted analysis of only one sample per hour. In addition, retention parameters were not always repeatable from run to run. Although the method had sufficient sensitivity for the indicated β-lactams at concentrations of interest to regulatory agencies, we think the chromatographic conditions were not suitable for routine analyses. Here, we report a modification to the chemical derivatization reaction that has enabled us to chromatographically resolve these β-lactams under isocratic conditions without loss of sensitivity. The modification renders the method more suitable for routine use in regulatory programs.

METHOD

Materials and Reagents

(**a**) *Derivatization reagent, mobile phase phosphate buffer, and extraction solutions.*—Preparations of these have been described in detail elsewhere (1).

(**b**) *0.2M Benzoic anhydride.*—Weigh 4.524 g benzoic anhydride (Sigma Chemical Co., St. Louis, MO) into ca 80 mL acetonitrile and mix to dissolve. Transfer quantitatively into a 100 mL volumetric flask and dilute to volume with acetonitrile. Store at room temperature in amber glass vials.

(**c**) *Mobile phase.*—Acetonitrile–0.1M phosphate buffer containing 0.0157M thiosulfate (26 + 74, v/v), filtered through 0.45 μm filters and degassed.

(**d**) *Dissolving solution.*—60% 0.1M phosphate buffer (pH 6.5)–40% acetonitrile (v/v). Weigh 0.497 g dibasic anhydrous sodium phosphate and 0.897 g monobasic sodium phosphate (monohydrate). Dissolve in 100 mL water. Add 6 mL phosphate buffer to 4 mL acetonitrile and mix well.

All other reagents and solutions were prepared as detailed previously (1).

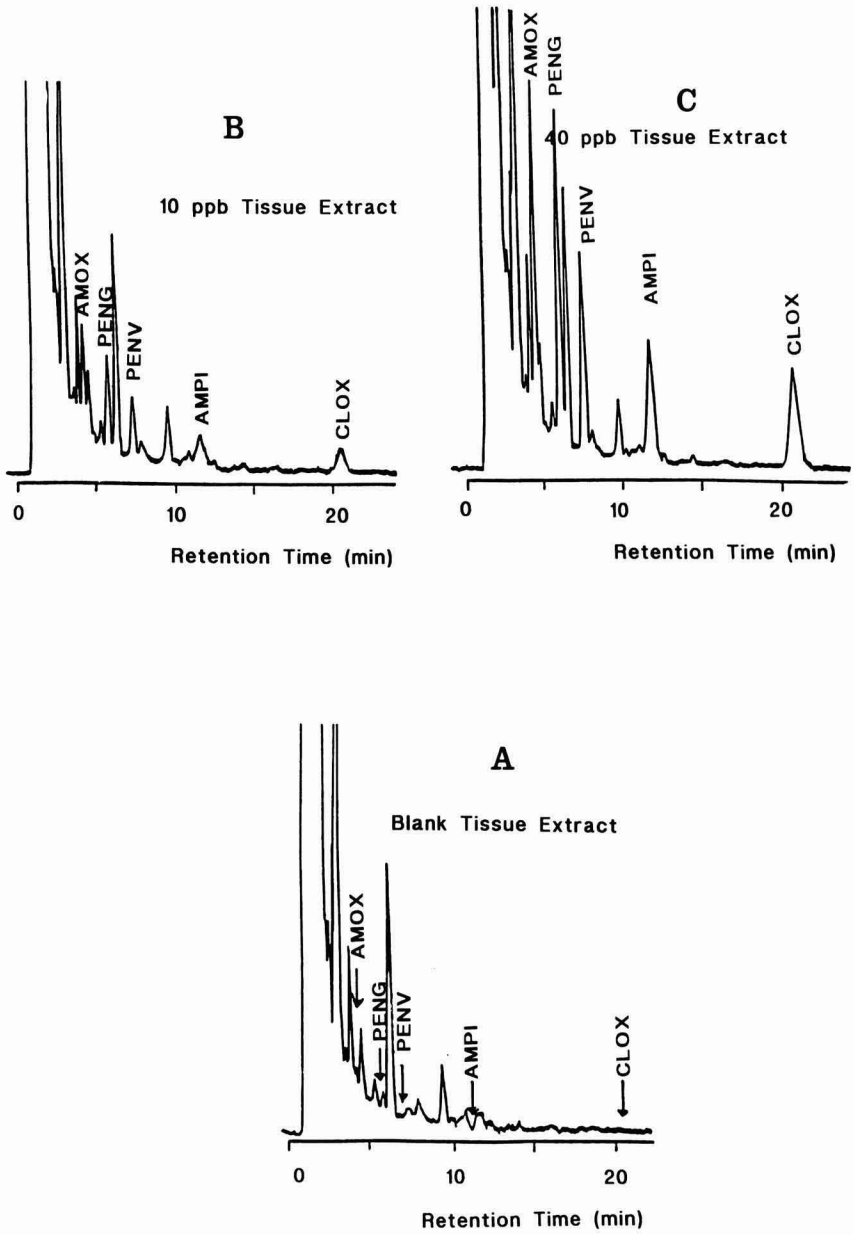

Figure 1. Liquid chromatogram of a drug-free bovine muscle tissue extract (A). Typical chromatograms of a drug-free bovine muscle tissue fortified with amoxicillin, penicillin G, ampicillin, and cloxacillin each at a concentration of 10 (B) or 40 ppb (C). Penicillin V is the internal standard.

Apparatus

(a) *Solid-phase extraction (SPE) cartridges.*—t-C_{18} 500 mg capacity, 3 cc vacuum type, 17% carbon loading; from Waters Chromatography Ltd., Mississauga, ON, Canada.

(b) *Vortex mixers, SPE vacuum manifold, solvent evaporator, and centrifuge.*—The same as previously described (1).

(c) *LC-UV equipment.*—Waters 501 pump, Waters 712 WISP autosampler, Waters 490 programmable wavelength detector set at 325 nm and a sensitivity of 0.003 absorption unit full scale, and an analog strip chart recorder (0.5 cm/min). The components in the tissue extracts were separated on a 3.9 × 150 mm Waters C_8 Symmetry column (5 μm) at a mobile phase flow rate of 1.2 mL/min.

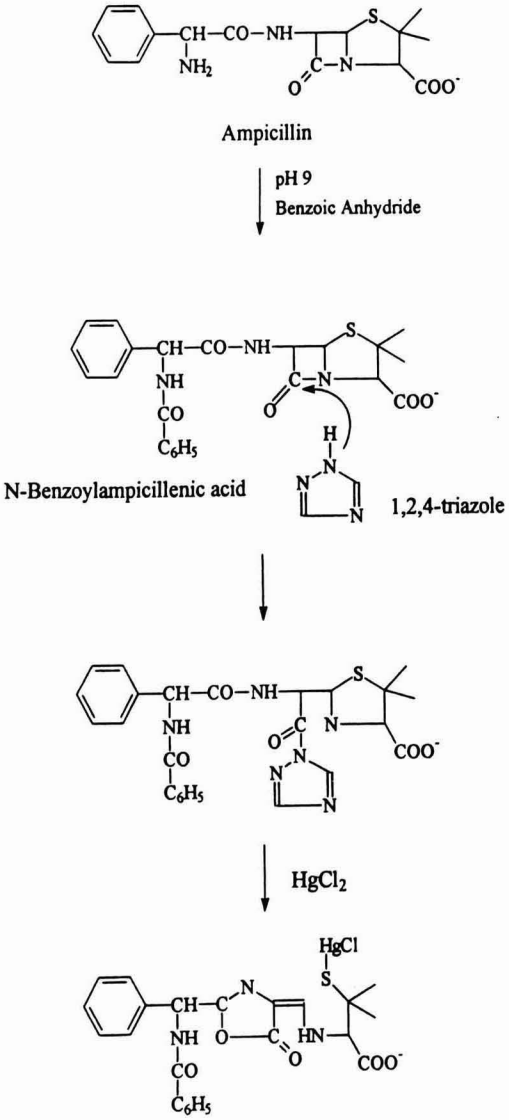

Figure 2. Suggested mechanism for precolumn derivatization reaction of benzoic anhydride with dibasic penicillins (ampicillin and amoxicillin) prior to reaction with mercuric chloride to form mercaptide derivatives.

Extraction of β-Lactams from Muscle Tissue

Accurately weigh 3 g homogenized control (drug-free) muscle tissue into each of five 50 mL polypropylene centrifuge tubes. Fortify with 15, 30, and 75 µL 2 ppm standard working solution and 15 and 30 µL 20 µg/mL standard to provide calibration standards containing 10, 20, 50, 100, and 200 ppb tissue equivalencies of ampicillin, amoxicillin, penicillin G, and cloxacillin.

Accurately weigh 3 g homogenized test sample into a 50 mL polypropylene centrifige tube. Add 15 mL 0.1M NaH_2PO_4 (pH 8.5) and agitate on a Vortex mixer at high speed for 1 min. Add 2 mL 0.17M sulfuric acid to the centrifuge tube, hand shake contents for ca 10–20 s, and centrifuge at 2500 × *g* for 10 min. Transfer supernatant into a clean 50 mL polypropylene centrifuge tube. Repeat extraction with 10 mL 0.1M NaH_2PO_4 (pH 8.5) by agitating on a Vortex mixer at high speed for 1 min followed by centrifugation at 2500 × *g* for 10 min. Combine supernatant from the second extraction with that from the previous extraction, add 180 µL 5M NaOH to the combined extract, and centrifuge at 2500 × *g* for 10 min. *Note*: To minimize analyte losses and eliminate clogging of Sep-Pak cartridges, the pH of the combined extract must be between 8.3 and 8.5 at this point.

Cleanup of Tissue Extracts on t-C_{18} Sep-Pak Cartridges

Condition a t-C_{18} Sep-Pak cartridge equipped with a 20 mL solvent reservoir with 20 mL methanol followed by 20 mL water, 10 mL 2% sodium chloride, and 10 mL 0.1M NaH_2PO_4 (pH 8.5) buffer. Load extract onto Sep-Pak cartridge. Rinse the centrifuge tube with 5 mL 0.1M phosphate (pH 8.5) buffer and add the rinse to the t-C_{18} cartridge. Continue evacuation of cartridge for another 2 min. Remove solvent reservoir and adaptor and elute retained β-lactams with 2 mL acetonitrile into a labeled Kimax 12 × 75 mm borosilicate glass tube. Evaporate acetonitrile in the eluate to dryness at 50°C with prepurified nitrogen and a Zymark Turbo LV evaporator unit. *Note*: To prevent analyte losses and degradation of penicillins, do not substitute other evaporators for this stage of the procedure. Add 200 µL dissolving solution to the residue, ultrasonicate sample for 2 min, and agitate on a Vortex mixer at high speed for 10–20 s.

Derivatization of β-Lactams in Cleaned-Up Sample Extract

Add 115 µL 0.2M benzoic anhydride reagent to each sample, agitate on a Vortex mixer at high speed for 10–20 s and allow samples to react for 5 min in a 50°C water bath. Add 500 µL derivatizing reagent to each sample, agitate on a Vortex mixer at high speed for 10–20 s, and incubate in a 65°C water bath for 30 min. Cool reaction mixture at 4°C (refrigerator) for 10 min, filter through 0.45 µm polyvinyledene difloride (PVDF) Acro disc filter, and inject 20–50 µL of each sample into the LC system. *Note*: Mercuric chloride in the derivatizing reagent is highly toxic. Avoid all contact. Consult Material Safety Data Sheets for mercuric chloride for its handling and disposal.

LC Analysis

Measure the peak height of the peak responses of calibration and test samples. Using regression analysis, construct a calibration curve of peak height vs concentration of each penicillin added to blank muscle tissue. Using the measured peak response of each penicillin in the test sample, calculate the concentration of the penicillin in the test sample from the calibration curve.

Table 1. Intra-assay (within-day) precision

β-Lactam	Concentration added to tissue, ppb	Mean concentration found[a] (n = 4), ppb	Standard deviation	Coefficient of variation, %	Accuracy, %[b]
Amoxicillin	15.0	15.6	1.2	7.7	4
	50.0	50.5	3.5	6.9	1
	180.0	180.6	6.0	3.3	0
Ampicillin	15.0	13.3	0.6	4.5	11
	50.0	49.4	3.4	6.9	1
	180.0	182.3	7.8	4.3	1
Penicillin G	15.0	15.1	0.9	5.9	1
	50.0	51.0	4.2	8.2	2
	180.0	180.1	5.9	3.3	0
Cloxacillin	15.0	15.0	0.9	5.7	0
	50.0	50.3	3.6	7.2	1
	180.0	183.0	4.6	2.5	2

[a] Concentrations in blind-fortified samples were estimated by interpolation from tissue calibration curves plotted over the concentration range 10–200 ppb, prepared under similar conditions.
[b] Accuracy is defined as the percentage deviation from the added concentration.

Validation of Analytical Method with Fortified Tissue Samples

Interassay (between-day) precision was determined by duplicate assays on 4 successive days of blank (drug-free) muscle tissue samples fortified with each drug at 15 and 180 ppb. Intra-assay (within-day) precision was determined by replicate analyses (n = 4) of blank (drug-free) tissue samples fortified with each β-lactam at 15, 50, and 180 ppb. Method accuracy was determined by calculating from the calibration curve the amounts of each analyte found in the fortified samples. Recoveries were calculated by comparing the UV detector responses obtained for each analyte in the fortified samples with those of equivalent external standards. Because the chemical standards have limited stability in the dissolving solution (1–2 h) in the absence of a tissue matrix, quantitative analyses must be based on calibration curves generated from drug-free tissue samples fortified with chemical standards.

The validated method was used to determine the concentrations of β-lactam residues in 2 sets of unknown samples, one consisting of 5 muscle tissue samples prepared and coded by our quality assurance and quality control officer and the other consisting of 9 presumptive positive bovine muscle tissues previously screened with an immunoassay test kit (LacTek β-lactam enzyme-linked immunosorbent assay, Idexx Laboratories, Westbrook, ME). These are real samples collected from slaughter animals from Canadian federally-inspected abattoirs as part of the Canadian Veterinary Drug Residue Surveillance and Monitoring Programs. These 9 samples were also analyzed independently with our previously published method for determining penicillin G residues in animal tissues (1).

Results and Discussion

Figure 1A shows a typical chromatogram of a control (drug-free) muscle tissue extract obtained by the modified procedure. Figures 1B and 1C show chromatograms of control (drug-free) muscle tissue extracts fortified with amoxicillin, ampicillin, penicillin G, penicillin V, and cloxacillin at 10 and 40 ppb, respectively. All the β-lactams were separated from one another and from other components of the tissue extracts. Because all the penicillins eluted from the LC column in less than 25 min,

Table 2. Interassay (between-day) precision and accuracy

β-Lactam	Concn added, ppb	Concentration of drug found[a] on indicated day, ppb				Mean concn found, ppb	CV, %	Accuracy, %
		Day 1	Day 2	Day 3	Day 4			
Amoxicillin	15.0	15.7	15.8	15.9	14.8	15.6	3.2	4
	180.0	181	177	182	183	181	2	1
Ampicillin	15.0	13.8	13.2	13.9	15.0	14.0	5.4	7
	180.0	188	175	182	183	182	3	1
Penicillin G	15.0	14.4	16.3	15.2	15.7	15.4	5.2	3
	180.0	183	174	184	190	183	4	2
Cloxacillin	15.0	13.9	15.1	13.4	15.3	14.4	6.4	4
	180.0	189	182	185	189	186	2	3

[a] These concentrations were interpolated from tissue calibration curves prepared on each day of analysis.

Table 3. Verification of method accuracy[a]

β-Lactam drug detected	Concentration of identified analyte found in indicated coded unknown bovine muscle tissue sample, ppb				
	600	601	602	603	604
Ampicillin	198 (200)	0 (ND[b])	43 (40)	ND (0)	203 (200)
Amoxicillin	187 (200)	68 (60)	ND (0)	71 (60)	199 (200)
Penicillin G	186 (200)	65 (60)	ND (0)	64 (60)	201 (200)
Cloxacillin	188 (200)	ND (0)	41 (40)	ND (0)	204 (200)

[a] Samples were prepared by our quality assurance chemist, coded, and given to us for analysis. Values in parentheses represent the level at which samples were fortified.
[b] ND, not detected.

at least 12 samples, plus 4 calibration standards, could be analyzed in 1 day. By contrast, the original gradient method permitted analysis of only 1 sample and 4 calibration standards in 1 day. For a multiresidue analysis, the modified method offers speed in deciding identities and concentrations of penicillins confirmed in presumptive positive tissue samples from carcasses being held in the abattoirs, pending confirmation of results of rapid test kits.

The heart of the original method for multiresidue analyses of monobasic and dibasic penicillins is the reaction with acetic anhydride to deactivate the amino groups in the dibasic penicillins, amoxicillin and ampicillin (3). In the modified method, the amino-group-protecting agent for aminopenicillins is benzoic anhydride rather than acetic anhydride. Figure 2 shows the mechanism we suggest for the formation of the benzoylamido mercaptide derivatives of aminopenicillins. Once the activity of the amino side chain in these dibasic penicillins has been removed by acetylation or benzoylation, the penicillins are able to form stable mercaptide derivatives, just like the monobasic penicillins.

Table 4. Comparison of the test responses obtained with original penicillin method (1) and with improved multiresidue method on presumptive positive (LacTek β-lactam test kit) bovine muscle tissue samples[a]

Sample No.	Concentration of β-lactam drug residues detected in presumptive positive tissues, ppb			
	Amoxicillin	Ampicillin	Penicillin G[b]	Cloxacillin
L118-3560	ND[c]	ND	353 (350)	ND
L1507-3999	ND	ND	397 (399)	ND
L1626-4131	ND	ND	5.1 (6.2)	ND
L529-2774	ND	ND	78 (81)	ND
L990-3427	ND	ND	25 (29)	ND
L1993-4523	ND	ND	638 (650)	ND
L575-2844	ND	ND	448 (459)	ND
L990-3429	ND	ND	1673 (1720)	ND
L2030-4565	ND	ND	65 (72)	ND

[a] Samples were taken from beef cows slaughtered in Canadian federally-inspected abattoirs and tested for the presence of veterinary drug residues with a screening test as part of the Canadian Surveillance and Monitoring Program for Residue Avoidance.
[b] Values in parentheses are concentrations determined by the original method (1).
[c] ND, not detected.

Table 5. Comparison of operational parameters of 3 methods for determining penicillin residues in bovine muscle tissues

Method and derivatization condition	Limit of detection for applicable analytes, ppb[a]	LC conditions	Sample preparation time, h	No. of samples analyzed per hour	Suitability for routine LC analysis	Reference
Method 1, mercuric chloride	Pen G, 2; Pen V	Isocratic	2.5	5	Yes	1
Method 2, acetic anhydride, mercuric chloride	Pen G, 2; Pen V; Ampi, 5; Amox, 2; Clox, 5	Gradient	2.5	1	No	Unpublished data
Method 3, benzoic anhydride, mercuric chloride	Pen G, 2; Pen V; Ampi, 5; Amox, 2; Clox, 5	Isocratic	2.5	3	Yes	This study

[a] Pen G, penicillin G; Pen V, penicillin V; Ampi, ampicillin; Amox, amoxicillin; clox, cloxacillin.

Table 6. Detection limit of multiresidue method and maximum residue limits (MRLs) for 4 penicillins in bovine muscle tissues

Penicillin	Detection limit, ppb	Maximum residue limit, ppb			
		Canada[a]	United States[a]	European Union[b]	Codex Alimentarius[c]
Amoxicillin	2	—	10	50	—
Ampicillin	5	10	10	50	—
Penicillin G	2	50	50	50	50
Cloxacillin	5	—	10	300	—

[a] Reference 3; —, no MRLs have been assigned to these compounds.
[b] Reference 4.
[c] Reference 5; —, no MRLs have been assigned to these compounds.

When dibasic penicillins are acetylated prior to formation of mercaptide derivatives, the acetylamido penicillins still have significantly different elution characteristics on a reversed-phase column compared with the monobasic analogues. Thus, it was necessary to use gradient elution conditions to resolve them from one another and from other components of the tissue extract. Under such conditions, the order of elution of mercaptide derivatives is amoxicillin, ampicillin, penicillin G, penicillin V, and cloxacillin.

The benzoylamido mercaptide derivatives, however, have similar elution characteristics, thus permitting use of isocratic elution conditions to analyze all the penicillins within a very short period. Under these conditions, the elution order for the penicillins is amoxicillin, penicillin G, penicillin V, ampicillin, and cloxacillin.

Tables 1 and 2 indicate that the modified method is sensitive, accurate, and precise. Tables 3 and 4 demonstrate that the modified method can be used to determine accurately the concentrations of β-lactams in bovine muscle tissue samples containing physiologically induced penicillins, that is, penicillins that have been physically added to drug-free muscle tissue. Table 5 shows that the method is relatively easy to perform and requires no extensive sample preparation procedures or instrumentation. Like the original multiresidue method, the modified method requires about 2.5 h to complete extraction, cleanup, and derivatization.

Table 6 shows that with detection sensitivities of 2, 5, 2, and 5 ppb respectively, for amoxicillin, ampicillin, penicillin G, and cloxacillin in bovine muscle tissues, the method can be used in regulatory programs for analyses of these veterinary drug residues in tissue samples. Table 6 also lists the Canadian and international maximum residue limits for 4 of the 5 penicillins under study in this project (4–6); penicillin V is not approved for use in food animals in North America.

Conclusion

By modifying the precolumn derivatization reaction, we improved our previous multiresidue LC method for simultaneous determination of monobasic and dibasic penicillins in bovine muscle tissues. With this modification, chromatographic analyses of the isolated penicillins can be conducted under isocratic instead of gradient conditions without loss in sensitivity, thus making the method more suitable for routine analyses.

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TECHNICAL COMMUNICATIONS

Determination of Small Amounts of Niacin in Vinegar: Comparison of Liquid Chromatographic Method with Microbiological Methods

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An LC method using a new cleanup technique was compared with 2 microbiological methods for determining small amounts of niacin in vinegar. Coefficients of variation for grain vinegar and rice vinegar were 8.33 and 5.3%, respectively, with the LC method, 5.26 and 2.0% with the titrimetric method, and 5.55 and 7.11% with the turbidimetric method. Among the 3 methods, titrimetry was the most precise. The turbidimetric method tended to yield greater positive error than the titrimetric method. On the other hand, the titrimetric method requires a longer incubation period (72 h) than the turbidimetric method. The LC method using the new cleanup procedure may be useful for routine analysis of small amounts of niacin in vinegars.

Niacin in foodstuffs generally has been determined by colorimetric or microbiological methods (1). The colorimetric method requires cyanogen bromide (2-4), which is difficult to separate from interferences. Thus the method generally is not preferred by analysts (5). For this reason, microbiological methods have become accepted as official methods for determining niacin in foodstuffs.

The 2 AOAC microbiological methods are the titrimetric method and the turbidimetric method. The titrimetric method is more precise, but it requires an incubation period of 72 h compared with 16–22 h for the turbidimetric method. Loy et al. (6) reported that when certain laboratory precautions are observed, the turbidimetric method may replace the more time-consuming titrimetric method for determining various standard vitamins.

Recently, liquid chromatographic (LC) methods have been reported for determination of niacin in foodstuffs as alternatives to the more cumbersome microbiological methods. Most apply to determinations of high concentrations of niacin in food additives and strengtheners (5, 7), grains (5, 8, 9), and meats (10). Hirayama and Maruyama (11) reported determination of a small amount of niacin (0.01 mg/100 g) in vinegars and jams, cleaned up by using an anion-exchange column and a cation-exchange cartridge and separated from interferences on an amino phase column. Tyler and Genzale (12) used an LC method to analyze low to high levels of total niacin in unfortified foods, cleaned up by using a C_{18} cartridge column and analyzed with a C_{18} analytical column. These researchers reported that LC methods may yield recoveries higher than those obtained by the microbiological method for certain foods and may not be appropriate as a general method for foods. The purpose of the present study was to compare the LC method of Hirayama and Maruyama (11) with the AOAC (1) turbidimetric and titrimetric microbiological methods for determining small amounts of niacin in vinegar.

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METHOD

LC System

(**a**) *Pump.*—Waters Model 590 pump with U6K injector (Waters Associates, Milford, MA).

(**b**) *Detector.*—Waters Model 490 UV/visible detector and Model 840 data system.

(**c**) *LC column.*—5 mm Asahipak NH2P-50, 4.6 × 250 mm; guard column NH2P, 4.6 × 50 mm (Asahi Kasei Industries, Tokyo, Japan).

(**d**) *Glass column.*—27 × 150 mm (Nikkyo Co., Tokyo, Japan), equipped with a stopcock.

(**e**) *Analytical workstation.*—HP 79994A workstation equipped with Model 1090 pump and autoinjector with diode-array detector (Hewlett-Packard GmbH, Waldbronn, Germany).

Reagents

(**a**) *Solvents.*—Acetonitrile was LC grade (Kanto Chemical Co., Inc., Tokyo, Japan). Methanol and ethanol were reagent grade (Kanto).

(**b**) *LC elution solvent.*—Acetonitrile–water (60 + 40, v/v) containing 0.075M sodium acetate; filtered through 0.45 μm filter.

(**c**) *Standard niacin.*—Purchased from Wako Pure Chemical Industries (Tokyo, Japan).

(**d**) *Niacin stock solution.*—Weigh 10.0 mg niacin in 100 mL volumetric flask, dilute with 25% aqueous ethanol, and store in a refrigerator.

(**e**) *LC standard solution.*—Prepare a 1 μg/mL standard solution fresh for each analysis by taking 1 mL niacin stock solution and diluting with water in 100 mL volumetric flask.

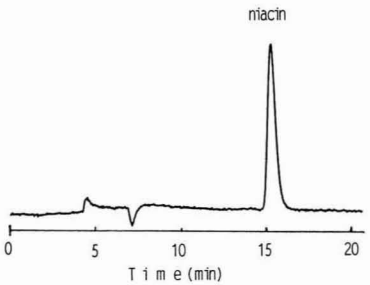

Figure 1. Chromatogram of standard niacin.

(**f**) *Microbiological standard solutions.*—Prepare 2 μg/mL standard fresh daily in 100 mL volumetric flask from stock solution; prepare 20 ng/mL standard by diluting 2 μg/mL standard in 100 mL volumetric flask.

(**g**) *Sodium hydroxide, sodium acetate, and hydrochloric acid.*—Reagent grade (Kanto).

(**h**) *Niacin assay medium.*—Nissui Pharmaceutical Co., Ltd., Tokyo, Japan.

(**i**) *Anion-exchange resin.*—AGI-X8, acetate form, 100–200 mesh (Bio-Rad Laboratories, Hercules, CA).

(**j**) *Cation-exchange resin.*—Toyopak IC-SP M (Toso Co., Tokyo, Japan).

LC Method

(**a**) *Extraction.*—Accurately weigh 10 g sample into 100 mL beaker, add 4 mL 40% aqueous NaOH, heat on steam bath for 20 min, and cover with watchglass. After mixture has cooled to room temperature, adjust to ca pH 7 with 25% HCl and add the same volume of methanol. Filter through Toyo No. 5 filter paper into 100 mL volumetric flask. Rinse residue with 50% methanol–water and make up volume to 100 mL with 50% methanol. Transfer 30 mL of this solution into 200 mL flask and add 50 mL methanol. Dry solution by rotary evaporation at 45°C. Add ca 20 mL water to residue.

(**b**) *Cleanup.*—(*1*) *Anion-exchange column.*—Pack 9 g anion exchange resin in column and wash with 50 mL pure water. Add 20 mL sample solution at 1 mL/min. Rinse with 50 mL water at the same rate and then elute niacin with 50 mL 13% aqueous acetic acid into 50 mL volumetric flask. Evaporate 30 mL of fractionated solution to dryness by rotary evaporation at 45°C. Take up residue in 5 mL water. (*2*) *Cation-exchange cartridge.*—Condition Toyopak cation-exchange cartridge with 4 mL 1N HCl and rinse with 10 mL water. Pass 5 mL sample solution through and then elute from cartridge with 15 mL 1N HCl. Add 50 mL methanol to fraction and dry this fraction by rotary evaporation at 50°C until free from HCl odor. Accurately take up residues in exactly 2 mL water. Pass sample solution through a 0.45 μm filter and inject 5–25 μL into LC system.

(**c**) *Analysis.*—The LC mobile phase consisted of acetonitrile–water (60 + 40, v/v) containing 0.075M sodium acetate. Assay conditions were as follows: temperature, ambient; flow rate, 0.5 mL/min; UV detection wavelength, 261 nm. The standard curve was prepared by injecting between 10 and 100 μL LC standard solution.

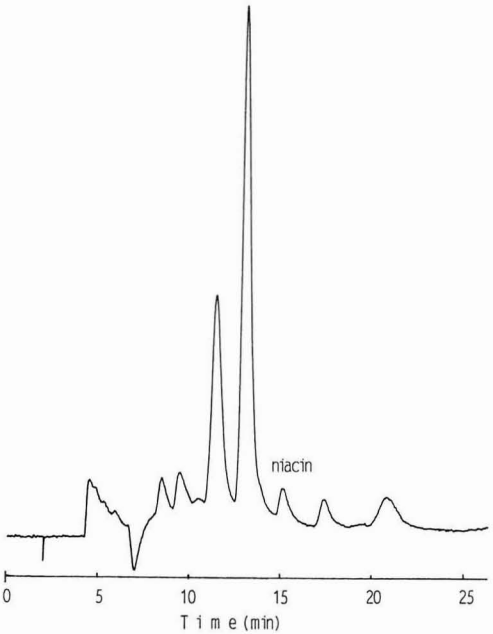

Figure 2. Chromatogram of niacin extracted from grain vinegar.

(**d**) *Recovery test.*—Weigh 600 mg niacin in 20 mL volumetric flask and make up to volume with 0.5N NaOH. Dilute 1 mL of this niacin solution (30 mg/mL) with water in 10 mL volumetric flask to yield a 3 mg/mL solution. Make further dilutions to yield solutions containing 300 μg/mL and 30 μg/mL. Take 1 mL of each of these niacin solutions and add to 10 g grain vinegar. Analyze solution as described previously.

Microbiological Assay

Weigh 10 g sample into 100 mL beaker, add 4 mL 40% NaOH and 10 mL water, and cover with watchglass. Hydrolyze mixture on a steam bath for 20 min. After mixture has cooled to room temperature, adjust to ca pH 8–9 with 25% HCl and then adjust accurately to pH 6.8 ± 0.1 with 10% phosphoric acid. Make up volume to 100 mL with water. Estimate biological activity according to AOAC Methods **960.46** and **944.13** (12), using *Lactobacillus plantarum* (ATCC 8014).

(**1**) *Turbidimetric method.*—Prepare duplicate tubes containing 0, 10, 25, 50, 75, and 100 ng niacin by adding 0, 0.5, 1.25, 2.5, 3.75, and 5 mL working standard to the appropriate tube. Prepare 3 levels of each sample such that each contains between 10–100 ng niacin.

To each tube of standard solution and sample solution add water to make up the volume to 5.0 mL. Next, add 5 mL of appropriate basal medium stock solution. Cover tubes with silicon plugs to prevent bacterial contamination, autoclave at 121°–123°C for 5 min, and cool.

Table 1. Niacin found (mg/100 g) in grain vinegar

Experiment No.	Turbidimetry	Titrimetry	LC
1	0.058	0.036	0.040
2	0.055	0.037	0.037
3	0.052	0.039	0.037
4	0.056	0.040	0.035
5	0.050	0.038	0.033
Mean	0.054	0.038	0.036
SD	0.003	0.002	0.003
CV, %	5.55	5.26	8.33

Table 2. Niacin found (mg/100 g) in rice vinegar

Experiment No.	Turbidimetry	Titrimetry	LC
1	0.42	0.30	0.29
2	0.36	0.29	0.29
3	0.38	0.30	0.30
4	0.38	0.29	0.30
5	0.35	0.30	0.33
Mean	0.38	0.30	0.30
SD	0.03	0.01	0.02
CV, %	7.11	2.00	5.30

Inoculate each tube with 1 drop of appropriate inoculum and incubate for 16–22 h at 37°C.

(**2**) *Titrimetric method.*—Procedures for preparing calibration curve and sample solutions are similar to those for the turbidimetric method. Incubate at 37°C for 72 h. Titrate contents of each tube with 0.05N NaOH, using phenolphthalein indicator.

Results and Discussion

Figure 1 shows a standard chromatogram for 40 ng niacin, and Figure 2 shows the chromatogram for niacin extracted from grain vinegar. The peak purity of niacin in grain vinegar (Figure 2) was 90%, determined by using the Hewlett-Packard HPLC ChemStation system. Results of determinations of niacin in grain vinegar and rice vinegar are shown in Tables 1 and 2, respectively.

The titrimetric method was the more precise of the 3 methods. Coefficients of variation obtained by titrimetry were not more than 5% for either grain vinegar or rice vinegar. The coefficient of variation for the LC method was 8.33% for the lower niacin content of grain vinegar but decreased to 5.3% for the higher niacin content of rice vinegar. In determining lower niacin contents, the coefficient of variation is likely to be high because niacin tends to be adsorbed on the preparative column.

The coefficient of variations for the turbidimetric method were 6.2% for grain vinegar and 7.11% for rice vinegar. Niacin contents determined by the turbidimetric method were higher than those obtained by the 2 other methods. Calibration curves for the range 10–100 ng were linear and passed through the origin for the titrimetric and LC methods. The turbidimetric method produced only approximately linear calibration curves for the same range and failed to pass through the origin.

Recoveries were determined by spiking grain vinegar samples with niacin at 3 μg/10 g. The titrimetric method was the most accurate of the 3 methods, achieving a mean recovery of about 99% (Table 3). In addition, the quantity of niacin correlated well with the lactic acid made from cultures ($r = 0.998$). Recovery by the LC method was about 90%. By contrast, recoveries by the turbidimetric method were consistently greater than 100%.

Kamei et al. (13) obtained high recoveries of vitamin B_{12} by turbidimetry but suggested the method may lack reproducibility because of the short incubation time, initial cell concentration of test culture, and the different growth rates from the preincubation time. Loy et al. (6) suggested that the incubation period for the turbidimetric method should be 18–24 h, because maximum turbidity is obtained after 16 h.

The high recovery of niacin from grain vinegar by the turbidimetric method suggests that the growth of reference standard may be too slow for the potency of the material. It may be necessary to extend the incubation period beyond 24 h.

Numerous researchers have improved LC methods so these can replace the more time-consuming microbiological methods. Until now, most LC methods have been successful only at detecting high contents of niacin in foodstuffs. Tyler and Genzale (12) used an LC method to determine niacin levels at about

Table 3. Recovery of niacin from grain vinegar spiked with 3 μg niacin/10 g

	Titrimetry		Turbidimetry		LC	
Experiment No.	Amount, μg	Recovery, %	Amount, μg	Recovery, %	Amount, μg	Recovery, %
1	2.93	97.7	3.74	124.7	2.61	87.0
2	2.95	98.8	4.14	138.0	2.70	90.0
3	3.00	100.0	4.24	141.3	2.86	95.3
4	2.85	95.0	4.24	141.3	2.79	93.0
5	3.10	103.3	3.79	126.3	2.54	84.6
Mean	2.96	98.9	4.03	134.3	2.70	90.0
SD	0.09		0.25		0.13	
CV, %	3.11		6.20		4.81	

Table 4. Recovery of niacin spiked at 4 levels from grain vinegar by LC method

Experiment No.	30 μg spike		300 μg spike		3 mg spike		30 mg spike	
	Amount, μg	Recovery, %	Amount, μg	Recovery, %	Amount, mg	Recovery, %	Amount, mg	Recovery, %
1	29.7	99.0	303	101.0	2.80	93.6	28.8	96.0
2	27.3	91.1	300	100.0	2.98	99.3	27.8	92.7
3	29.0	96.7	289	96.4	2.92	97.4	28.3	94.3
4	29.0	96.7	289	96.6	2.81	93.8	29.0	96.0
5	29.0	96.7	296	98.8	2.79	93.1	28.0	93.3
Mean	28.8	96.0	295	98.6	2.86	95.4	28.4	94.5
SD	0.9		6		0.09		0.5	

0.1 mg/100 g in unfortified foods. However, with foods containing lower contents of niacin, the coefficient of variation of their method rose to 10%. With the present LC method with ion-exchange resins for cleanup and an amino column for separation, it was possible to analyze niacin with the same sensitivity as microbiological methods. Table 4 shows recoveries of 4 levels of fortification in grain vinegar. Recoveries at all levels were >90%.

The LC method has several advantages. Niacin is analyzed directly and in a short time. The method does not require stock cultures of test organisms and their transfer into new media. It may be used efficiently and reliably for foods with a wide range of niacin contents and may be adopted as a routine method for determining niacin in foodstuffs.

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TECHNICAL COMMUNICATIONS

Comparison of Microwave Oven and Convection Oven for Acid Hydrolysis of Dietary Fiber Polysaccharides

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Hydrolysis of dietary fiber polysaccharides (DFP) is an integral part of any enzymatic–chemical method for dietary fiber analysis. Residues obtained after enzyme treatments of fiber-containing foods are usually suspended in 12M sulfuric acid and kept at or slightly above ambient temperature for at least 1 h, and then the mixtures are diluted with deionized water to a final concentration of 1M or 2M acid, followed by heating at 100°C in a water bath or convection oven for 1 or 2 h. Under these hydrolytic conditions, some degradation of the released monosaccharides generally takes place over the duration of hydrolysis. We investigated the feasibility of using microwave energy as a heat source to reduce time and minimize degradation. Preliminary tests were done on the well-characterized soy polysaccharide Fibrim. With a microwave digestion system equipped with temperature and pressure monitors and control lines, optimum settings of power (5%, 75%), time (up to 3 min and 30 s), temperature (35°–55°C), and pressure (45–65 psi) were determined for different foods depending on the residue weight and volume of acid. Results were comparable for microwave oven and convection oven hydrolysis of DFP from 5 foods with good correlations for neutral sugar values; r^2 = 0.997 for arabinose, 0.925 for galactose, 0.981 for glucose, 0.969 for mannose, and 0.990 for xylose.

Dietary fiber methods that have been developed or modified during the past 20 years and that have been given official status by AOAC INTERNATIONAL generally fall into 2 major classes: enzymatic–gravimetric methods as represented by Official Methods **985.29** and **991.43** and the enzymatic–chemical method as represented by Official Method **994.13**. The main difference between these 2 classes is in further characterization of fiber residues isolated after various enzyme treatments.

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Compositional analysis of nonstarch or dietary fiber polysaccharides (DFP) usually requires acid hydrolysis to release constituent monosaccharides as neutral sugars, which can then be determined quantitatively by gas–liquid chromatography (GLC; 1, 2) or high performance liquid chromatography (3, 4). Acid hydrolysis typically is performed in 2 stages involving different concentrations of H_2SO_4. The original conditions described by Saeman et al. (5) have been modified by many analysts (1–4, 6–8) intending to maximize hydrolysis and minimize degradation of neutral sugars. Regardless of the temperature and the concentration of acid used, all methods call for reaction times of at least 1 h at or slightly above ambient temperature for the first stage and 1–2 h at 100°C in water bath or oven or 125°C in an autoclave for the second stage. One laboratory has estimated the loss of sugars during the second stage of hydrolysis to be 5–10% (1). Another laboratory has reported losses ranging from 25% for mannose to 38% for xylose, based on heating standard monosaccharides (3).

To reduce hydrolysis time and loss of released monosaccharides, we investigated the use of microwave energy as a heat source. Microwave technology has been used for rapid moisture determination, acid digestion for inorganic analyses, sample extraction, hydrolysis of protein and peptides, dry ashing, and organic syntheses among others. However, only a few reports have been published on the use of microwave energy for acid hydrolysis of organic and biological samples other than proteins (9, 10).

Experimental

Materials

A collection of freeze-dried samples of selected high-consumption foods, identified by the Nutrient Data Laboratory at the Agricultural Research Service of the U.S. Department of Agriculture, has been kept at –20°C in our laboratory since late 1993. We chose one from each of 5 classes of foods and isolated their respective dietary fiber residues according to an enzymatic–gravimetric method described earlier (11). The frozen samples were thawed and dried in a vacuum oven at 40°C before use. Fibrim 1450 (Protein Technologies International, St. Louis, MO), a soy fiber isolated from dehulled and defatted soybean cotyledon with well-characterized physicochemical

Table 1. Comparison of microwave oven and convection oven for acid hydrolysis of Fibrim

Sample	Sugar, g/100 g dry weight							
	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total neutral sugars
Microwave oven								
1	1.70	1.87	13.70	5.35	1.12	24.37	12.95	54.35
2	1.54	2.24	14.12	5.06	1.26	27.15	12.43	56.78
3	1.92	1.94	12.39	4.32	1.16	25.93	12.77	53.79
4	1.60	1.98	12.92	4.48	1.24	25.93	12.68	54.13
5	1.79	2.24	13.04	5.00	1.04	25.98	11.95	54.33
Mean ± SD	1.71 ± 0.16	2.05 ± 0.17	13.24 ± 0.68	4.84 ± 0.43	1.16 ± 0.09	25.87 ± 0.99	12.56 ± 0.39	54.68 ± 1.20
Convection oven								
A	1.75	2.13	13.44	4.86	1.28	26.57	13.61	56.63
B	1.74	2.11	13.15	4.73	1.29	26.39	13.56	56.04
C	1.08	2.05	13.40	4.69	1.26	26.00	12.60	54.35
D	1.03	2.06	13.76	4.85	1.22	26.07	12.63	54.84
E	1.04	2.07	13.83	4.91	1.22	26.21	12.71	55.17
Mean ± SD	1.33 ± 0.38	2.08 ± 0.03	13.52 ± 0.28	4.81 ± 0.10	1.25 ± 0.03	26.25 ± 0.23	13.02 ± 0.52	55.41 ± 0.92

properties, as described by Lo (12) was used as an in-house reference material for all our earlier acid hydrolysis studies using a convection oven and for subsequent derivatization and chromatographic determination of neutral sugars as their alditol acetates. For the present study, Fibrim was the control sample for determining optimum microwave hydrolysis conditions before they were tested on the food samples.

Apparatus

A microwave digestion system (Model MDS 2100; CEM Corporation, Matthews, NC) equipped with temperature and pressure monitors from control lines placed inside one of the advanced composite vessels and a digital computer programmable for 30 multistep programs was used.

Reaction vessels consist of the outer body and the cap made of microwave-transparent polyetherimide and the inner liner, the liner cover, and the safety rupture membrane made of perfluoroalkoxy Teflon. The system delivers ca 950 watts of microwave energy at a frequency of 2450 MHz at full power. The power is programmable in 1% increments of duty cycle. The fluoropolymer-coated microwave cavity is equipped with an exhaust fan with programmable speed in 10% increments, a direct-drive alternating turntable, and 3 door-safety interlocks. Domestic microwave ovens should never be used for the acid hydrolysis reactions described in this study.

Hydrolysis

Fibrim or dietary fiber residue (100–250 mg) was suspended in 2.5 or 3.5 mL 12M H_2SO_4 (72%) depending on the weight of the residues, in advanced composite vessels. Samples were heated at 5% power so that final temperatures ranging from 35° to 55°C are reached within 40 s to 2 min and 20 s. After samples had been cooled to room temperature, 5 volumes of deionized water was added, and the mixture was heated at 75% power for 2 min and 30 s to 3 min and 30 s until final pressure reached 45–65 psi, resulting in final temperatures ranging from 90° to 155°C. Different settings were required depending on residue weight and volume of acid used. For hydrolysis with a convection oven, samples and acid mixtures similar to those used for microwave digestion were suspended in glass tubes (18 × 150 mm) with Teflon-lined screw caps and kept at 35°C for 1 h. After dilution with water, they were heated at 100°C for 2 h.

Determination of Neutral Sugars

Portions containing neutral sugars from either microwave oven or convection oven hydrolysis were neutralized with ammonium hydroxide, and the neutral sugars derivatized to alditol acetates and quantitated by GLC according to a procedure described by Blakeney (13), as modified by Englyst (4), and adapted for our analyses (14). Total neutral sugars were calculated as the sum of individual neutral sugars multiplied by 0.89.

Statistical Analysis

Arithmetic mean and standard deviation for each set of triplicate analyses of microwave hydrolyzates and linear correlation between microwave oven and convection oven hydrolysis for each individual sugar and total neutral sugars were calculated by using a computer spreadsheet program (Microsoft Excel version 4.0) on a Power Macintosh.

Results and Discussion

Our microwave acid hydrolysis studies began several years ago with a CEM MDS-81 microwave digestion system and with Fibrim as test sample. By varying the power setting and

Table 2. Microwave digestion system settings for acid hydrolysis of dietary fiber polysaccharides

		First stage[a]			Second stage[b]			
Sample	Total neutral sugars, g/100 g	Power, %	Temp., °C	Time, min:s	Power, %	Time, min:s	Pressure, psi	Temp., °C
Bread, white, reduced calorie, firm crumb	12.23	5	48	2:20	75	2:30	45	118
	11.71	5	45	2:15	75	2:30	45	127
	12.02	5	48	1:50	75	2:30	45	117
Oatmeal, instant, cooked	8.46	5	55	1:55	75	3:15	56	150
	8.32	5	55	2:02	75	3:05	54	155
	7.79	5	55	2:10	75	3:00	65	150
Pears, raw, ripe	8.21	5	45	1:55	75	2:30	45	110
	8.53	5	45	1:10	75	2:30	50	128
	8.69	5	43	1:40	75	2:30	50	113
Peas, green, frozen, microwaved	14.40	5	50	2:30	75	3:30	55	110
	14.37	5	50	2:30	75	3:30	55	110
	13.17	5	50	1:49	75	3:30	50	90
Red kidney beans, canned, drained	16.95	5	50	2:10	75	3:30	55	125
	19.39	5	50	1:50	75	3:30	55	110
	19.03	5	50	1:28	75	3:30	55	110
Fibrim (200 mg, 3.5 mL acid)	54.35	5	50	1:22	75	3:30	50	120
Fibrim (150 mg, 2.5 mL acid)	56.78	5	45	0:50	75	2:30	45	125
Fibrim (150 mg, 2.5 mL acid)	53.79	5	40	0:55	75	2:30	55	135
Fibrim (150 mg, 2.5 mL acid)	54.13	5	35	0:50	75	2:30	50	120
Fibrim (150 mg, 2.5 mL acid)	54.33	5	35	0:40	75	2:30	55	125

[a] Power and final temperature were preset, and resulting time was recorded.
[b] Power time and final pressure were preset, and final temperature was recorded.

the time of heating, our analysis of neutral sugar composition of this soy polysaccharide gave results similar to those from convection oven hydrolysis, which has been optimized in both our laboratory and others. However, poor precision and uncertainty of the final temperature and pressure prevented us from continuing the studies. For example, when we used 10% power for 2 min 30 s for first-stage heating and 80% power for 2 min for the second stage, total neutral sugar contents varied between 48 and 57% on different trials.

Recently, we purchased the later model and better equipped CEM MDS-2100. This digestion system enabled us to monitor the temperature and pressure of the reaction mixture during hydrolysis. It also allowed us to optimize hydrolysis conditions more systematically than we could with the earlier model. After a series of preliminary runs, we finally narrowed the power settings to 5% for the first stage of hydrolysis with 12M H_2SO_4 and 75% for the second stage with 2M H_2SO_4. The newer system delivers higher microwave energy (950 versus 590 watts). A setting of 10% power for the first stage led to charring of the samples, and hence the power was lowered to 5 or 6%. For different combinations of time and final temperature for the first stage and time ramp to pressure for the second stage, values for individual neutral sugars were quite repeatable, as shown in Table 1. For many years, we always included Fibrim as a control with any set of test samples to be hydrolyzed and derivatized to alditol acetates. Consequently, we have an extensive collection of neutral sugar composition data on this material. Those listed in Table 1 under the heading "Convection" represent typical results from the past year. Results from the 2 hydrolysis methods gave a regression coefficient of 0.98.

Once we found the proper conditions for microwave hydrolysis of Fibrim, we were ready to test them on some foods. From a collection of freeze-dried high-consumption (or frequently consumed) foods, we chose 5: reduced-calorie white bread, cooked instant oatmeal, raw pears, microwave-cooked frozen green peas, and canned red kidney beans. Four samples of dietary fiber residues were isolated from each food. One sample was hydrolyzed with a convection oven and the remaining 3 samples were hydrolyzed in a microwave digestion system according to conditions given in Table 2. Overall, good repeatability was found for total neutral sugars from triplicate analyses using microwave hydrolysis, and values were comparable within the range of various settings. With the power constant at 5% for the first stage, the temperature setting was varied depending on the weight of residue and the volume of acid. Power was set at 75% for the second stage, and the time for ramping to the required pressure varied between 2 min 30 s and 3 min 30 s. Final pressures were between 45 and 65 psi, and resulting temperatures were in the range 90°–155°C. Samples were hydrolyzed individually. A second advance composite vessel containing 15 mL deionized water was placed on the carousel for balance. Table 3 gives the means and standard deviations for individual neutral sugars and their sum as total neutral sugars for each food as obtained from microwave hydrolysis, along with the value for a single determination using a convection oven. The 2 sets of data show good correlation: r^2 =

Table 3. Neutral sugar contents of dietary fiber polysaccharides of selected foods as determined by microwave acid hydrolysis[a]

Sample	Sugar, g/100 g dry weight							
	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total neutral sugars
Bread, white, reduced calorie, firm crumb	ND[b] [ND][c]	ND [ND]	0.59 ± 0.05 [0.68]	1.04 ± 0.02 [0.97]	0.38 ± 0.02 [0.47]	0.18 ± 0.05 [0.41]	11.28 ± 0.22 [13.02]	11.99 ± 0.26 [13.85]
Oatmeal, instant, cooked	ND [ND]	ND [ND]	0.87 ± 0.06 [1.00]	0.94 ± 0.02 [1.16]	0.59 ± 0.04 [0.63]	0.48 ± 0.05 [0.48]	6.32 ± 0.54 [6.21]	8.19 ± 0.35 [8.45]
Pears, raw, ripe	0.11 ± 0.01 [0.25]	<0.10 [ND]	2.39 ± 0.09 [2.72]	2.43 ± 0.05 [2.82]	0.21 ± 0.01 [0.27]	0.77 ± 0.01 [0.96]	3.59 ± 0.24 [5.19]	8.48 ± 0.24 [10.86]
Peas, green, frozen, microwaved	ND [0.30]	ND [ND]	2.24 ± 0.45 [2.70]	0.70 ± 0.08 [0.69]	0.18 ± 0.02 [0.28]	0.52 ± 0.03 [0.57]	12.07 ± 1.37 [12.18]	13.98 ± 0.70 [14.88]
Red kidney beans, canned, drained	<0.10 [0.20]	0.19 ± 0.02 [0.20]	2.44 ± 0.24 [2.74]	1.39 ± 0.11 [1.46]	0.54 ± 0.13 [0.71]	0.76 ± 0.08 [0.91]	15.35 ± 2.16 [15.24]	18.45 ± 1.32 [19.11]

[a] Values are means ± SD of triplicate analyses.
[b] ND = not detectable.
[c] Values in brackets are those from acid hydrolysis using a convection oven.

0.997 for arabinose, 0.925 for galactose, 0.981 for glucose, 0.969 for mannose, 0.990 for xylose, and 0.995 for total neutral sugars. Rhamnose and fucose were present in insignificant amounts and were not detectable in some of the foods tested.

The primary objective of this study was to compare the extent of acid hydrolysis of DFPs in either a microwave oven or a convection oven by monitoring the individual neutral sugar constituents of DFP by GLC. Preliminary results indicate that hydrolyzates from these 2 methods of heating give similar results for uronic acids (part of DFP in most fruits and vegetables). Thus, uronic acids subjected to microwave heating under the conditions described here do not behave differently from those heated in a convection oven.

Conclusion

Acid hydrolysis of DFPs can be accomplished in a much shorter time—about 6 min versus 3 h—by using the equipment and conditions specified in this paper. Further studies will be conducted to hydrolyze multiple samples simultaneously and to show actual reduction in the loss of monosaccharides during microwave hydrolysis. *Note*: Any attempt to reproduce our results should begin with preliminary tests on a control sample (such as Fibrim) to determine the heating characteristics of a particular microwave digestion system and the appropriate settings for optimum results.

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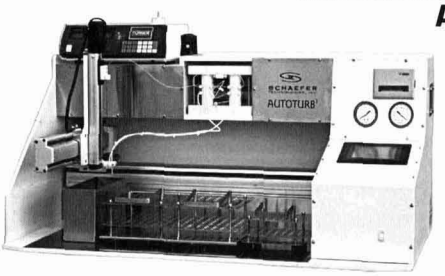

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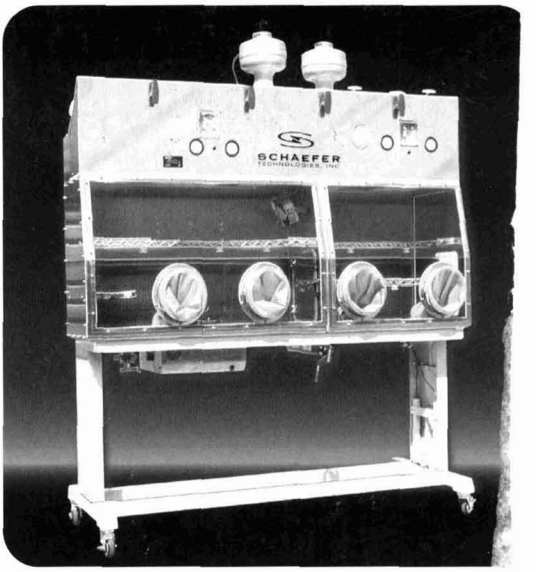

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