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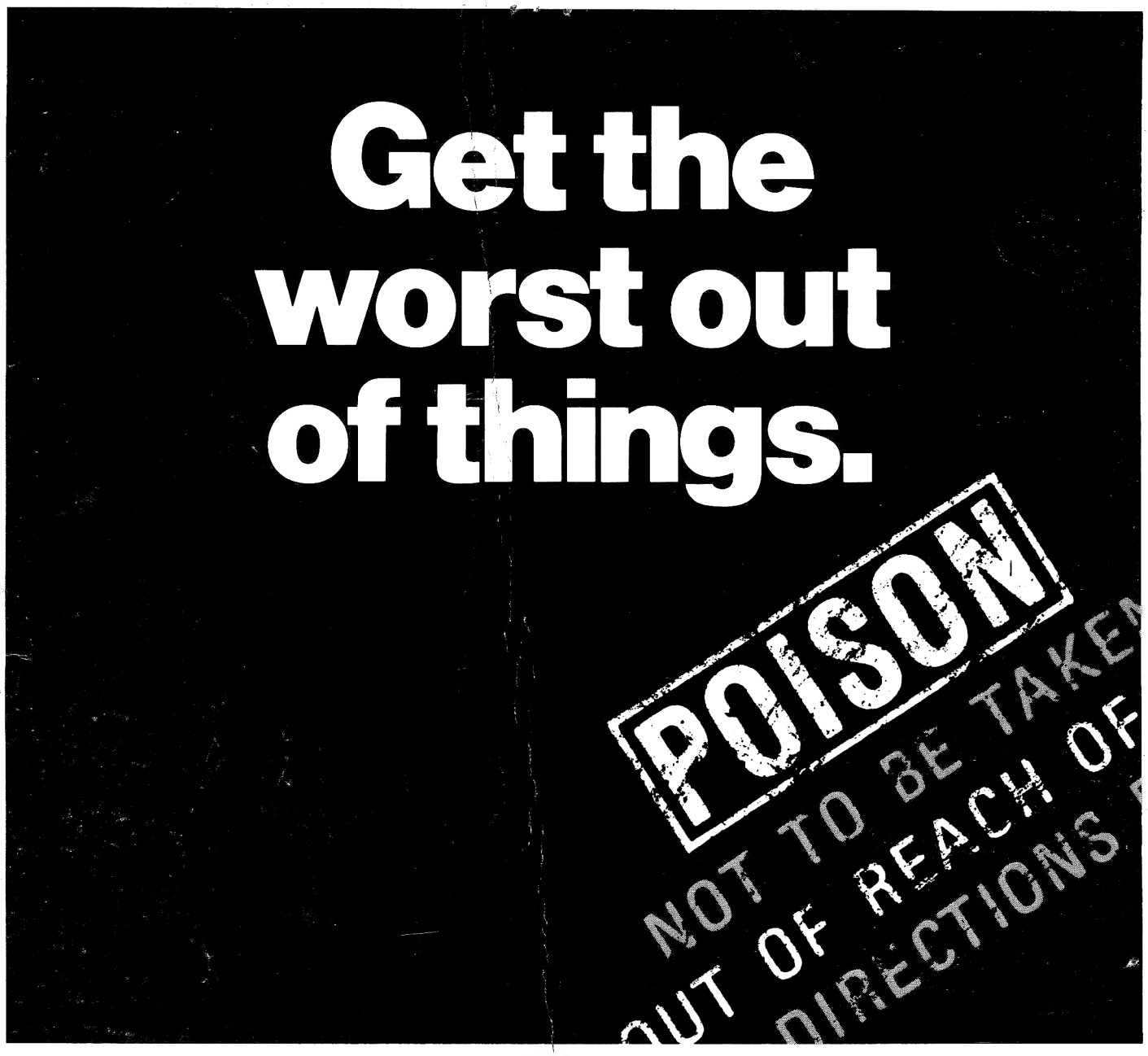

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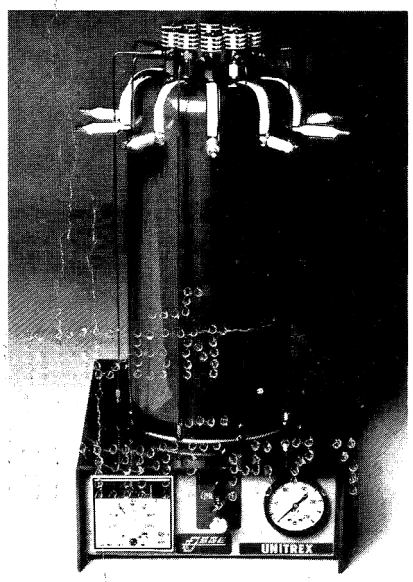

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Higher Speed, Higher Volume Centrifuges

Four compact new centrifuges that spin more sample at higher speeds and forces than other general purpose models are being introduced by Beckman Instruments, Inc. Called the "GP" series, the centrifuges will be available with or without refrigeration in tabletop models and in portable floor models. Beckman Instruments, Inc.

Circle No. 103 on reader service card.

Ultracentrifuge

The new Model L7-65 ultracentrifuge from Beckman Instruments, Inc., spins samples at 65 000 rpm and 485 000 *g*. It is useful in laboratories isolating plasmids, DNA, or RNA by using cesium chloride gradients. Beckman Instruments, Inc.

Circle No. 104 on reader service card.

Biotechnology Systems

Five different multicomponent systems for biotechnology projects have been introduced by Savant Instruments, Inc. Each system contains all components and accessories needed for vacuum drying and concentrating biochemical materials for a specific type of application. Savant Instruments, Inc.

Circle No. 105 on reader service card.

Concentrator and Dryer for DNA Preparations

"Gene Jockey's Delight" is the name given by Savant Instruments, Inc., for an equipment arrangement that concentrates and dries natural and synthetic preparations of DNA. The system consists of a SpeedVac concentrator, a slab gel dryer, mechanically refrigerated trap, and vacuum pump, all mounted on a 2-shelf cart with 4 electrical outlets. Savant Instruments, Inc.

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Sample Preparation System

Each new Waters Sep-Pak sample preparation system contains sample reservoirs, reservoir supports, Sep-Pak C_{18} cartridges, and an air pressure pump. The assembly functions as a cartridge holder, sample/solvent reservoir, and positive pressure flow adapter for either sorbent extraction or membrane filtration. Waters Chromatography Division.

Circle No. 107 on reader service card.

Sulfite Analyzer for Foods and Beverages

Food and beverage producers need to equip their laboratories with more sensitive, accurate test methods for analyzing true levels of sulfites to meet new, more stringent U.S. Food and Drug Administration regulations. The Waters sulfite analyzer cuts analysis time to 5 min, and it uses the Waters Model 460 electrochemical detector to provide detection limits of low parts per billion as opposed to 10 parts per million by conventional methods. Waters Chromatography Division.

Circle No. 108 on reader service card.

Purification Kit

Antibodies can be purified and concentrated in 90 min with a new kit that uses rProtein A (a trademark of Repligen Corp.) introduced by Beckman Instruments. Beckman Instruments, Inc.

Circle No. 109 on reader service card.

Multiple Sample Processing Station

Vac Elut SPS 24 multiple sample processing station processes as many as 24 Bond Elut bonded silica extraction columns simultaneously. The deluxe vacuum manifold features time savings for complex methods, complete flow control, and flexible operation for elution into a variety of collecting devices. Analytichem International, Inc.

Circle No. 110 on reader service card.

Robot-Compatible Columns

Bond Elut LRC (laboratory robot compatible) extraction columns can be used with any commercially available laboratory robot, allowing the automation of repetitive tasks in the laboratory. Analytichem International, Inc.

Circle No. 111 on reader service card.

New Kjeldahl Apparatus

With the fourth generation of Kjeltec, the Kjeltec Auto Plus, a sample is weighed, distilled, recorded, and evaluated automatically, all in about 2 min. Tecator, Inc.

Circle No. 112 on reader service card.

Automatic System for Water Analysis

The new Aquatec System water analysis system from Tecator is based on the flow injection analysis technique. The system analyzes ammonium, nitrites, nitrates, and phosphates; switches rapidly from one kind of analysis to another; conducts analyses in accordance with standardized methods; is sensitive to ppb levels; operates at speeds of 30–40 s/sample; and handles 40 or 100 samples. Tecator, Inc.

Circle No. 113 on reader service card.

Measurement of Polyphenol in Beverages

Total phenol content in beer, wine, and fruit juices can be measured by a method developed on the Auto-Analyzer II from Technicon Industrial Systems Corp. The same instrument can also be used to analyze for bitterness, diacetyl, air oxidizable precursors, and acetoin. Bran + Luebbe, Inc.

Circle No. 114 on reader service card.

Automated DNA Scanner System

The automatic Gene-Master DNA scanner system has been developed in cooperation with E. M. Southern and J. Elder of Oxford University. The system includes an optical scanner based on a linear CCD array detector, pattern recognition and sequence assignment software, and an IBM AT or compatible computer. Bio-Rad Laboratories.

Circle No. 115 on reader service card.

Fraction Collector

The Model 2100 fraction collector from Bio-Rad Laboratories collects fractions on either a time or a volume basis—the switch on the front panel may be set to the collection mode wanted. Bio-Rad Laboratories.

Circle No. 116 on reader service card.

Low-Cost LC Pump

The SM-909 isocractic LC pump from Anspec has features usually found only in higher priced pumps: automatic or manually set compressibility; 1½ piston design with one set of check valves; integral purge valve; digital readout of flow, pressure, or compressibility; optional microbore or preparative capabilities. The Anspec Co., Inc.

Circle No. 117 on reader service card.

Nitrogen-Specific GC Detector

Applications on the Model 705 nitrogen-specific GC detector from Antek Instruments range from detection of nitrogenous compounds in air,

water, or soil samples for environmental testing to the analysis of petroleum fractions for quality control or product development. The instrument measures only nitrogen—without the sulfur, phosphorous, halogens, or hydrocarbons that have complicated this type of work in the past. Antek Instruments, Inc.
Circle No. 118 on reader service card.

Gas Cell for FT-IR Spectrometers

A new, variable, long-pathlength gas cell is designed to provide the reported advantages (over previous methods of analysis) of gas and vapor analysis with FT-IR spectrometers, i.e., increased sensitivity, better resolution, and more rapid data acquisition. Spectra-Tech, Inc.
Circle No. 119 on reader service card.

Microwave Digestion Bombs

Parr Instrument Co. has introduced entirely new microwave acid digestion bombs that speed the digestion of laboratory samples at higher temperatures and pressures. These new bombs are Teflon-lined and combine the advantages of closed high pressure and high temperature digestion with the special requirements of faster microwave heating. Parr Instrument Co.
Circle No. 120 on reader service card.

Automatic Preparative Gas Chromatograph

Varex Corp. offers the PSGC 10/40 preparative gas chromatograph. This fully automated system consists of an oven module that accommodates up to eight 1 cm × 1 m columns (four 4 cm × 1 m columns), a module for fingertip control of all operating parameters, and a microcomputer for fully automated, unattended repetitive processing of samples. Varex Corp.
Circle No. 121 on reader service card.

Centrifugal Partition Chromatography

Centrifugal partition chromatography is a new liquid chromatographic technique which uses liquid-liquid partition—counter-current distribution to fractionate complex mixtures of chemical substances. It offers advantages for the isolation of materials of biological origin, e.g., natural products, biopolymers, antibodies, and genetically engineered molecules. Sanki Laboratories, Inc.
Circle No. 122 on reader service card.

Miniature Pressure Regulator

The Model 4000 pressure regulator from Porter Instrument Co., Inc., measures only 1⅛ in. in diameter and features 15 turn adjustment for 0–10, 0–30, 0–60, 0–100 psig range. Its compact design makes it suitable for panel mounting or use in small instruments. Porter Instrument Co., Inc.
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Liquid Delivery System

A liquid dispenser from Penn Systems, the Compu-Feed, allows for the control of up to 4 accurately metered streams at the same time. The programmable microprocessor-based controller can operate 1, 2, or 4 pumps continuously at fixed rates or can make them deliver precise batch volumes or can meter liquids as a function of temperature, pressure, pH, liquid level, viscosity, conductivity, RPM, mechanical coordinates, or other analog signal. Penn Systems, Inc.
Circle No. 124 on reader service card.

Audio Courses

"Effective Management of Chemical Analysis Laboratories" is the title of an audio course produced by the American Chemical Society. This course gives an in-depth understanding of the problems and solutions involved in analytical laboratory mangement. Instructors are John H. Taylor, Jr. Analytical Technologies, Inc., and Mary M. Routson, Bechtel Corp. Includes 4 cassettes (3.8 h playing time) and 120 page manual. American Chemical Society.
Circle No. 125 on reader service card.

James Kaufman, director of the Laboratory Safety Workshop of Curry College, Milton, MA, is the instructor on an audio course, "Laboratory Safety and Health," which offers an understanding of the guidelines for dealing with accidents in the laboratory and for making health and safety concerns an integral part of effective laboratory practices. American Chemical Society.
Circle No. 126 on reader service card.

Computer Bulletin Board Service

ChemSoftware BBS, the first computer bulletin board service for members of the paint and chemical industries, allows callers to locate sources of raw materials and equipment, read and leave messages, and monitor news of interest to the chemical community. Membership is open to industry members who have an IBM computer or compatible and a 300–1200 baud modem. ChemSoftware BBS.
Circle No. 127 on reader service card.

BOOKS IN BRIEF

Foodborne Microorganisms and Their Toxins. Developing Methodology. Edited by Merle D. Pierson and Norman J. Stern. *IFT Basic Symposium Series.* Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1986. 496 pp. Price: $59.75 United States and Canada; $71.50 all other countries. ISBN 0-8247-7607-0.

This volume presents the proceedings of the Ninth Basic Symposium sponsored by the Institute of Food Technologists and the International Union of Food Science and Technology. Information offered includes the current status of instrumental techniques, spoilage indicators, predictive modeling, and indicator organisms, and applications of DNA hybridization, immunology, and hybridoma technology to the detection of foodborne microorganisms and toxins.

Water Activity: Theory and Applications to Food. Edited by Louis B. Rockland and Larry R. Beuchat. *IFT Basic Symposium Series.* Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1987. 424 pp. Price: $59.75 United States and Canada; $71.50 all other countries. ISBN 0-8247-7759-X.

This book offers an up-to-date, integrated, and comprehensive review of both basic research and practical applications concerning water activity and its influence on food. It comprises the Tenth Basic Symposium of the Institute of Food Technologists and the International Union of Food Science and Technology.

Biotechnology: Applications and Research. Edited by Paul N. Cheremisinoff and Robert P. Ouellette. Published by Technomic Publishing Co., PO Box 3535, Lancaster, PA 17604. 716 pp. Price: $95.00.

The new information and reference data in the 49 chapters of this volume are intended to provide an authoritative reference work for all those involved in the research, development, and use of biotechnology.

Food Product-Package Compatibility. Edited by B. R. Harte, J. Ian Gray, and J. Miltz. Proceedings of seminar, School of Packaging, Michigan State University, July 14–16, 1986. Published by Technomic Publishing Co., Inc., PO Box 3535, Lancaster, PA 17604, 1987. Approx. 300 pp, softcover. Price: $75.00. ISBN 0-87762-520-4.

Interested readers will find reports on major topics in food packaging materials, performance, and safety; examinations of today's important food packaging materials, with test and performance data; special reports on tamper-proof packaging, FDA testing of materials, and packaging for microwave food products.

Practical Statistics for Engineers and Scientists. By Nicholas P. Cheremisinoff. Published by Technomic Publishing Co., Inc., PO Box 3535, Lancaster, PA 17604, 1986. 211 pp. Price: $29.00.

This monograph is intended as a reference of standard statistical analyses and data regression techniques. Process engineers, chemists, and researchers should find it to be a handy guide in designing experiments and analyzing data.

Organic Pollutants in Water: Sampling, Analysis, and Toxicity Testing. Edited by I. H. Suffet and Murrugan Malaiyandi. *Advances in Chemistry Series No. 214.* Published by American Chemical Society, 1155 Sixteenth St, NW, Washington, DC 20036, 1987. 816 pp. Price: $109.95 United States and Canada; $131.95 export. ISBN 0-8412-0951-0.

This book offers several schemes in its 36 chapters for isolating and concentrating trace contaminants from water. All of the analytical methods discussed are based on phase-transfer processes in which the compound is isolated by a second phase or separated by a membrane phase.

Separation, Recovery, and Purification in Biotechnology: Recent Advances and Mathematical Modeling. Edited by Juan A. Asenjo and Juan Hong. *ACS Symposium Series No. 314.* Published by American Chemical Society, 1155 Sixteenth St, NW, Washington, DC 20036, 1986. 226 pp. Price: $54.95 United States and Canada; $65.95 export. ISBN 0-8412-0978-2.

Successful production of biotechnology products hinges on their separation and recovery from the production site and subsequent purification. This new work discusses the most recent advances and latest developments in operations used for bioproduct recovery in biotechnology and fermentation systems and includes a mathematical analysis and modeling of such operations.

Citrus Fruits and Their Products: Analysis and Technology. By S. V. Ting and Russell L. Rouseff. *Food Science and Technology Series/18.* Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1986. 312 pp. Price: $59.75 United States and Canada; $71.50 all other countries. ISBN 0-8247-7414-0.

Methods of citrus analysis are brought together in this volume, including those to determine quality and nutritional value and those to detect and control adulteration in citrus juices.

Laboratory Safety: Principles and Practices. Edited by Brinton M. Miller, Dieter H. M. Gröschel, John H. Richardson, Donald Vesley, Joseph R. Songer, Riley D. Housewright, and W. Emmett Barkley. Published by American Society for Microbiology, 1931 I St, NW, Washington, DC 20006, 1986. 374 pp. Price: $38.00 member; $55.00 nonmember. ISBN 0-914826-77-8.

Managers and other personnel in laboratories dealing with biohazards are the intended audience for this reference. It includes discussions of safety and training programs, hazard assessment (several hazardous microorganisms are detailed), containment and personal protective equipment and techniques, and a range of accidents and medical emergencies.

Quantitative Analysis Using Chromatographic Techniques. By E. Katz. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1987. 446 pp. Price: $69.95.

The publishers describe this as the "most comprehensive manual available on quantitative techniques in all areas of chromatography."

How to Find Chemical Information: A Guide for Practicing Chemists, Educators, and Students. 2nd edition. By Robert E. Maizell. Published by John Wiley & Sons, Inc., One Wiley Dr,

Somerset, NJ 08873, 1987. 402 pp. Price $44.95.

This second edition updates and expands by 40% the material of the first edition, including new information on computer-based and on-line database systems.

Chromatographic Theory and Basic Principles. Edited by Jan Åke Jönsson. *Chromatographic Sciences Series/38.* Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1987. 408 pp. Price: $79.95 United States and Canada; $95.50 all other countries. ISBN 0-8247-7673-9.

This book surveys the basic terminology and common concepts that tie together the different facets of chromatography, covers peak-broadening and peak-shaping mechanisms operating in all chromatographic methods, focuses on the use of chromatography for chemical analysis, provides a detailed discussion of chromatographic fundamentals, and helps chromatographers optimize and customize their work.

Silent Spring Revisited. Edited by Gino J. Marco, Robert M. Hollingworth, and William Durham. Published by American Chemical Society, 1155 Sixteenth St, NW, Washington, DC 20036, 1987. 214 pp. Price: $29.95 United States and Canada; $35.95 export. ISBN 0-8412-0981-2.

Where are we some 25 years after Rachel Carson's *Silent Spring* was published? The major result of her book was the establishment of the Environmental Protection Agency, and virtually all the problems she explored are in some stage of correction. This 12 chapter book addresses the issues that Rachel Carson raised and focuses on their pertinence then and now.

Applications of New Mass Spectrometry Techniques in Pesticide Chemistry. Edited by Joseph D. Rosen. Volume 91 in *Chemical Analysis: A Series of Monographs on Analytical Chemistry and its Applications.* Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1987. 264 pp. ISBN 0-471-83280-4.

The purpose of this volume is to bring the researcher up to date on the developments in the field of mass spectrometry that are of interest to pesticide chemists. Techniques covered include, among others: gas chromatography/mass spectrometry, selected ion monitoring, negative ion chemical ionization, and liquid chromatography/mass spectrometry.

Standard Methods for the Analysis of Oils, Fats and Derivatives. 7th Revised and Enlarged Edition. Compiled by C. Paquot and A. Hautfenne for International Union of Pure and Applied Chemistry, Applied Chemistry Division, Commission on Oils, Fats and Derivatives. Published by Blackwell Scientific Publications, Osney Mead, Oxford OX2 0EL, England; distributed in the United States and Canada by Blackwell Scientific Publications, PO Box 50009, Palo Alto, CA 94303, 1987. 347 pp. Price: $96.00. ISBN 0-632-01586-1.

This volume is the first revision of IUPAC's book of methods since 1979. It enlarges on the 6th edition and incorporates some organizational amendments.

Laboratory Robotics: A Guide to Planning, Programming, and Applications. By W. Jeffrey Hurst and James W. Mortimer. Published by VCH Publishers, Inc., 220 East 23rd St, New York, NY 10010-4606, 1987. 129 pp. ISBN 0-89573-322-6.

Laboratory robotics is an extension of programmable computers which permits computers to do physical work as well as process data. This guide introduces the reader to the concepts, the language, and the reasoning behind the purchase of a laboratory robot and tells the practicing laboratory scientist whether such a robot may relieve some of the work load.

COME TO

THE 102nd AOAC ANNUAL

INTERNATIONAL MEETING AND EXPOSITION

AT THE BREAKERS, PALM BEACH, FLORIDA

AUGUST 29–SEPTEMBER 1, 1988

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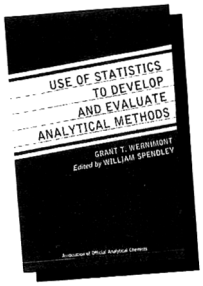

☐ **Use of Statistics to Develop and Evaluate Analytical Methods**

By G.T. Wernimont. Ed. by W. Spendley. 1985. 199 pp. Index. Figures. Tables. Glossary. Softbound. ISBN 0-935584-31-5.
Members: $47.55 in U.S., $50.55 outside U.S.
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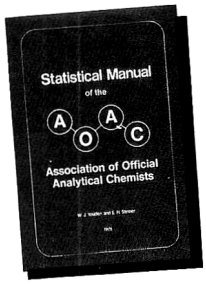
A basic reference for evaluating collaborative studies and a natural extension to the *Statistical Manual of the AOAC*.



☐ **Statistical Manual of the AOAC**

By W.J. Youden and E.H. Steiner. 1975. 96 pp. Softbound. Illustrations. ISBN 0-935584-15-3.
Members: $18.55 in U.S., $19.55 outside U.S.
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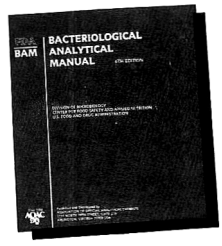
A do-it-yourself manual for statistical analysis of interlaboratory collaborative tests.



☐ **FDA Bacteriological Analytical Manual, 6th Ed.**

1984. 448 pp. Looseleaf. ISBN 0-935584-29-3.
Members: $44.85 in U.S., $47.85 outside U.S.
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Provides regulatory and industry laboratories with methods for detection of microorganisms. Includes one *Classification of Visible Can Defects* poster, 24" × 36", in color, with photographs.

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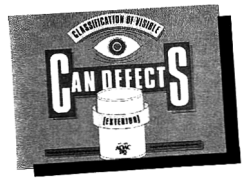

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1984. 24" × 36" chart. In color with photographs. Minimum order, 1 package of 10 charts, $40.00 + $3.00 postage; 2nd package of 10, $30.00 + $3.00 postage; each additional package, $25.00 + $3.00 postage.

☐ **Principles of Food Analysis for Filth, Decomposition, and Foreign Matter— FDA Technical Bulletin No. 1**

1981. 286 pp. 2nd printing 1985. Illustrated. Softbound.
Members: $42.60 in U.S., $45.60 outside U.S.
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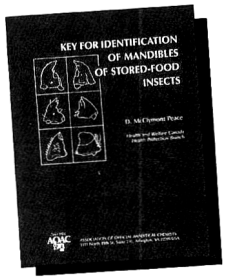
Comprehensive laboratory manual/text on basic concepts of food sanitation analysis.



☐ **Key for Identification of Mandibles of Stored-Food Insects**

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FOR YOUR INFORMATION

Meetings

October 11-14, 1987: Western Agricultural Chemicals Association, 58th Annual Meeting, Hotel del Coronado, San Diego, CA. Contact: Lisa M. K. Zetterberg, Director of Public Affairs, 6650 Belleau Wood Lane, Suite 209, Sacramento, CA 95822, telephone 916/393-4050.

October 20–21, 1987: AOAC Europe VI/AFSILAB joint meeting, "Advanced Techniques for Vitamin and Microbiological Determinations," Hoffmann-La Roche Amphitheatre, Neuilly sur Seine, France. Contact: Margreet Lauwaars, PO Box 153, 6720 AD Bennekom, The Netherlands, telephone 011 + 31-8389-18725.

October 20–21, 1987: Mid-Canada AOAC Regional Section Meeting. Contact: Joe Hilliard, Manitoba Research Council, Canadian Food Products, Development Centre, 810 Phillips St, PO Box 1240, Portage la Prairie, MB R1N 3J9, Canada, telephone 204/857-7861.

November 12, 1987: New York/New Jersey AOAC Regional Section Meeting, Sheraton Hotel, Newark Airport, Newark, NJ. Contact: George Boone, FDA, 850 Third Ave, Brooklyn, NY 11232, telephone 718/965-5033.

November 1987: Eastern Ontario/Quebec AOAC Regional Section Meeting. Contact: Milan Ihnat, Agriculture Canada, Land Resource Research Centre, Ottawa, Ontario K1A 0C6, Canada, telephone 613/995-5011.

December 8, 1987: 9th Annual Midwest Conference on Environmental Laboratory Technology, University of Minnesota, St. Paul, MN. Contact: John Vollum, Department of Professional Development, Continuing Education and Extension, University of Minnesota, 315 Pillsbury Dr SE, Minneapolis, MN 55455, telephone 612/625-1534.

February 10–14, 1988: Instrumentation Turkey '88, Istanbul Hilton Convention & Exhibition Centre, Istanbul, Turkey. Contact: Gerald G. Kallman, Kallman Associates, 5 Maple Ct, Ridgewood, NJ 07450-4431, telephone 201/652-7070, telex 264 715 GDWD UR, telefax 201/652-3898.

March 22–25, 1988: 7th Symposium on the Transfer and Utilization of Particulate Control Technology, Stouffer Nashville Hotel, Nashville, TN. Contact: Claudia Runge, Symposium Coordinator, Electric Power Research Institute, PO Box 10412, Palo Alto, CA 94303.

April 19–22, 1988: Analytica '88, Munich Trade Fair Centre, Munich, Federal Republic of Germany. Contact: Gerald G. Kallman, Kallman Associates, 5 Maple Ct, Ridgewood, NJ 07450-4431, telephone 201/652-7070, telex 264 715 GDWD UR, telefax 201/652-3898.

April 26–28, 1988: AOAC Infant Formula Conference II, Radisson Francis Marion Hotel, Charleston, SC. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

May 16–19, 1988: International Symposium on the Analysis of Anabolizing and Doping Agents in Biosamples, Ghent, Belgium. Contact: C. Van Peteghem, Pharmaceutical Institute, State University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium, telephone 091/21-89-51, ext. 235.

Spring 1988: Northeast AOAC Regional Section Meeting. Contact: Gerald Roach, FDA, 599 Delaware Ave, Buffalo, NY 14202, telephone 716/846-4494.

June 1988: Pacific Northwest AOAC Regional Section Meeting. Contact: John Neilson, Neilson Research Corp., 446 Highland Dr, Medford, OR 97504, telephone 503/770-5678.

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

June 1988: Midwest Regional AOAC Section Meeting. Contact: Howard Casper, North Dakota State University, Veterinary Diagnostic Laboratory, Fargo, ND 58102, telephone 701/237-7529.

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

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

September 9–13, 1990: 104th AOAC Annual International Meeting and Exposition, The Clarion Hotel, New Orleans, LA. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

Workshops and Short Courses

Brigham Young University and the State of Utah Center for Excellence in Supercritical Fluid Separation Technologies are joint sponsors of the 1988 Workshop on Supercritical Fluid Chromatography to be held at the Prospector Square Hotel, Park City, UT, January 12–14, 1988. Supercritical fluid chromatography (SFC) is a novel method for the analysis of foods, drugs, pesticides, fragrances, polymers, household compounds, and industrial mixtures. The BYU workshop, which will include oral presentations, invited lectures, informal discussions, and hands-on sessions using SFC equipment, will provide a forum in which to discuss uses of the technique. Registration fee (after September 1, 1987) is $400.00. Contact: Milton Lee, Department of Chemistry, Brigham Young University, Provo, UT 84602.

For the fifth consecutive year, "Fundamentals of Chromatographic Analysis," an overview of chemical separations via chromatographic methods sponsored by Nicolet Instrument Corp. and by Kent State University's Chemistry Department and University Conference Bureau, will be presented at Kent State University. The dates will be December 7–11, 1987. The course is different from other similar programs in that gas, liquid, and thin-layer chromatography are presented as complementary rather than competing processes. Contact: Carl J. Knauss, Chromatographic Course Coordinator, Chemistry Department, Kent State University, Kent, OH 44242, telephone 216/672-2327.

The Center for Professional Advancement, East Brunswick, NJ, will offer courses in analytical chemistry in February and March 1988. February courses will include: "Electrophoresis: Principles and Applications in the Biotechnology and Pharmaceutical Industries" and "Fourier Transform Infrared Spectroscopy." Those scheduled for March are: "Automated Instrumentation for the Analytical Laboratory," "Mass Spectrometry: Recent Advances and New Developments," and "Thin

Layer Chromatrography: Techniques and Applications." For course descriptions, specific dates, costs, and enrollment information, contact the Center for Professional Advancement, PO Box H, East Brunswick, NJ 08816-0257, telephone 201/238-1600, telex 139303 (CENPRO EBRW).

Jo Yeargin Wins Wiley Scholarship

A career in cancer research is the goal of this year's Harvey W. Wiley Scholarship winner, Jo Yeargin, a widowed mother of 2 whose husband died of cancer. Yeargin is a student at the University of California at San Diego, where she is a junior majoring in biochemistry. Until recently, she has worked nearly full-time, has pursued her college course work (maintaining a 3.95 GPA), and has been single parent to her 2 active children. Her decision to quit her job so as to commit herself full time to her education spurred her to apply for the Wiley Scholarship. When she isn't studying or parenting, Yeargin plays and has coached soccer, plays tennis, reads, and attends local theatrical productions.

AOAC will contribute $1000 over the next 2 years to help Yeargin complete the course work for a bachelor's degree in biochemistry. The Wiley Scholarship is awarded annually to a college sophomore planning to major in a scientific area of interest to AOAC—food, agriculture, the environment, or public health. Other qualifications include maintenance of at least a "B" average through the sophomore year and demonstration of need of financial aid.

Nominations for the 1988 award must be received by May 1, 1988. For information on how to submit a nomination, write to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209.

1987 Fellows of the AOAC

In recognition of at least 10 years of meritorious service, the following individuals will receive honors as 1987 Fellows of the AOAC at the 101st Annual International Meeting and Exposition in San Francisco, CA:

Patricia Bulhack, FDA. Associate Referee: 1966–1970, subsidiary colors in water-soluble AZO color additives; 1970–1979, arsenic and heavy metals; 1979–1981, intermediates, uncombined, in other certifiable colors. Member, 1980–1985, secretary, 1982–1984, Committee on Feeds, Fertilizers, and Related Materials. Member, 1985, Committee on Foods I. Member, 1985–present, Committee on Foods II. Member, 1983–1984, Centennial Committee.

David W. Fink, Merck Sharp & Dohme, Inc. Associate Referee (3 methods adopted): 1973–1977, ronidazole; 1973–present, sulfaquinoxaline; 1979–present, arprinocid; Associate Referee of the Year Award, 1980. Member, 1985–present, Committee on Feeds, Fertilizers, and Related Materials. Member, 1983–present, Interlaboratory Studies Committee, subcommittee on Guidelines for Statistical Analysis.

Robert A. Isaac, University of Georgia. General Referee: 1981–1986, plants. Associate Referee (2 methods adopted): 1967–1968, sulfur in fertilizers; 1968–1969, boron in fertilizers; 1969–present, atomic absorption methods for plants; 1981–present, plasma emission spectroscopy. Member, 1973, Committee on Ashing Methods. Member, 1982–present, Committee on Instrumental Methods and Data Handling.

Join an AOAC Regional Section or Start One in Your Area!

Current AOAC Regional Sections

Mid-Canada Regional Section
President: Joe Hilliard
Manitoba Research Council
Canadian Food Products
Development Center
810 Phillips St. PO Box 1240
Portage la Prairie, MB R1N 3J9
Canada
(204) 857-7861 ext. 292

- Next Meeting: October 20-21, 1987

New York — New Jersey Regional Section
Chairman: George Boone
Food & Drug Administration
HFR-2160, 850 Third Ave.
Brooklyn, NY 11232
(718) 965-5033

- Next Meeting: November 12, 1987
Sheraton-Newark Airport
Newark, New Jersey

Eastern Ontario — Quebec Regional Section
Chairman: Milan Ihnat
Agriculture Canada
Land Resource Research Centre
Ottawa, Ontario, K1A 0C6
Canada
(613) 995-5011 ext. 7846

- Next Meeting: November 1987

Northeast Regional Section
President George Willkens
Agway, Inc.
777 Warren Road
Ithaca, NY 14850
(607) 257-2346

- Next Meeting: Spring 1988

Southeast Regional Section
President: Frank Allen
Environmental Protection Agency
Reg. 4 ESD
College Station Rd.
Athens, GA 20613
(404) 546-3387

- Next Meeting: June 1988

Midwest Regional Section
General Chairman: George Rottinghaus
University of Missouri
Vet Medical Diagnostic Lab
Columbia, MO 65211
(314) 882-6811

- Next Meeting: June 20-22, 1988
Columbia, Missouri

Northwest Regional Section
Chairman: John Neilson
Neilson Research Corporation
446 Highland Drive
Medford, OR 97504
(503) 770-5678

- Next Meeting: June 1988

For information on starting a section, contact:
Marilyn J. Siddall, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA, 22209, USA, Phone: (703) 522-3032

James J. Karr, Pennwalt. Associate Referee (2 methods adopted): 1974–1982, encapsulated methyl parathion; 1974–present, encapsulated organophosphorus pesticides. Member, 1985–present, Committee on Pesticides and Disinfectants. Member, 1982–1983, Long Range Planning Committee. Member, 1985-present, Committee on Meetings, Symposia, and Educational Programs. Cochairman, 1984 Spring Workshop. Coordinator, pesticides poster session, 1984 Annual International Meeting. Organizer and chairman, Symposium on Industrial Analytical Chemistry, 1987 Annual International Meeting.

Dick H. Kleyn, Rutgers University. Associate Referee (3 methods adopted): 1967–present, rapid method for phosphates in dairy products; 1980–present, enzymatic determination of lactose in dairy products; 1986–present, fat in milk.

Gerald R. Myrdal, Wisconsin Department of Agriculture. Associate Referee: 1969–1977, galactose-oxidase method for meat products. Member, 1979–1982, Committee on Gas and Liquid Chromatography for Pesticide Formulations. Member, 1981–present, chairman, 1984–1986, Committee on Residues. Member, 1984–1986, Official Methods Board. Member, 1982–1984, Committee on Instrumental Methods and Data Handling. Member, 1983–1986, Midwest AOAC Regional Section Executive Committee, representative of Wisconsin.

Douglas L. Park, FDA. Associate Referee (2 methods adopted): 1974–1976, neutralizing value of sodium aluminum phosphate; 1982–present, aflatoxin methods. Secretary, 1986–present, Committee on Foods I. Member, 1973–1978, Long Range Planning Committee. Member, 1973–1978, Committee on Meeting Arrangements. Member, 1973–present, Committee on International Cooperation. Member, 1985–present, Committee on Membership. AOAC representative, 1983–present, Joint Mycotoxin Committee. Assistant editor, *Official Methods of Analysis, 12th Ed.* ISO correspondence coordinator, 1973–1976.

Nominations for these awards were made by AOAC members, were reviewed and recommended by the Committee on Fellows, and finally were approved by the Board of Directors.

Methods Committee Associate Referee Awards for 1987

Newly created in 1986, the Methods Committee Associate Referee Awards recognize the best Associate Referee in a committee for a given year. Those named for 1987 are: David F. Tomkins, Committee on Pesticide Formulations and Disinfectants; Elaine A. Bunch, Committee on Drugs and Related Topics; Douglas L. Park, Committee on Foods I; James T. Tanner and Stephen A. Barnett, Committee on Foods II; Leon D. Sawyer, Committee on Residues; Russell S. Flowers, Committee on Microbiology; and David L. Osheim, Committee on Feeds, Fertilizers, and Related Topics.

ISO Standards Published

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

ISO 949-1987: Cauliflowers—Guide to cold storage and refrigerated transport. $13.00.

ISO 5764-1987: Milk—Determination of freezing point—Thermistor cryoscope method. $18.00.

ISO 6883-1987: Animal and vegetable fats and oils—Determination of mass per unit volume ("litre weight") in air. $15.00.

ISO 7366-1987: Animal and vegetable fats and oils—Determination of 1-monoglycerides and free glycerol contents. $15.00.

ISO 7700/2-1987: Check of the calibration of moisture meters—Part 2: Moisture meters for oilseeds. $18.00.

ISO 8460-1987: Instant coffee—Determination of free-flow and compacted bulk densities. $18.00.

ISO 8588-1987: Sensory analysis—Methodology—"A"-"not A" test. $18.00.

ISO 8892-1987: Oilseed residues—Determination of total residual hexane. $15.00.

IDF Standards Published

The following standards of the International Dairy Federation (IDF) were published between September 1986 and June 1987. To inquire about cost or to obtain copies, contact: in the United States, USNAC, 464 Central Ave, Northfield, IL 60093; in Canada, FIL-IDF, Agriculture Canada, Sir John Carling Bldg, Ottawa, Ontario K1A 0C5, Canada; in the United Kingdom, Giggs Hill Green, Thames Ditton KT7 0EL, United Kingdom; and worldwide, IDF, Square Vergote, 41, 1040 Brussels, Belgium.

IDF 20A: Milk—Nitrogen content (Kjeldahl).

IDF 59A: Whey cheese—Fat content (Röse Gottlieb).

IDF 73A: Milk and milk products—Coliforms.

IDF 98A: Milk—Nitrogen (protein) content (dye-binding-amido-black).

IDF 103A: Milk and milk products—Iron content.

IDF 108A: Milk—Freezing point (thermistor cryoscope).

IDF 137: Butter—Water content (routine method).

New Journal Announced

The International Network of Food Data Systems (INFOODS) has announced a new journal, *Journal of Food Composition and Analysis.* The journal, devoted to all aspects of the chemical composition of human foods, will emphasize new methods of analysis; data on composition of foods; studies on the manipulation, storage, distribution, and use of food composition data; and studies on the statistics and distribution of such data and data systems. Target audience for the journal includes analytical chemists, food scientists, nutritionists, dietitians, epidemiologists, and public health planners. The editor is Kent K. Stewart of Virginia Polytechnic Institute and State University. Associate editors are George H. Beaton, Ricardo Bressani, Osman Galal, William M. Rand, David Southgate, and Kyoden Yasumoto.

For more information or to subscribe, contact: Academic Press, Inc., Journal Promotion Department, 1250 Sixth Ave, San Diego, CA 92101, telephone 619/230-1840.

Special Issue of TrAC Available

TrAC, *Trends in Analytical Chemistry,* published a special issue devoted to liquid chromatography to coincide with the 11th International Symposium

on Column Liquid Chromatography, which was held in Amsterdam, The Netherlands, June 28–July 3, 1987. Copies are available for $10.00 prepaid (Dfl. 20) from Derek Coleman, Coordinating Staff Editor, PO Box 330, 1000 AH Amsterdam, The Netherlands.

Database Expo

Five major scientific and technical information producers will join together to host Database Expo, a day-long product update, at the Disneyland Hotel in Anaheim, CA, on October 19, 1987. In addition to introducing the newest scientific and technical bibliographic resources available, the update will be a forum for discussing cost-effective searching techniques, vendor differences, and the unique applications of and relationships among selected databases. Participants are: Biosis; Engineering Information, Inc.; IFI/Plenum Data Co.; Institute for Scientific Information; and PsycINFO.

Fee for the program is $25.00 and includes lunch and all activities. For more information or to register, call Engineering Information, Inc., 800/221-1044 (in NY and outside the United States, call 212/705-7635).

Database Announcements

STN International (the Scientific & Technical Information Network) announces availability of the DIPPR data compilation file of physical property data for 766 commercially important chemical substances. Each substance in the file is identified by its CAS registry number, complete name, molecular formula, and compound family name. For more information, contact: in Europe, STN International, Postfach 2465, D-7500 Karlsruhe 1, Federal Republic of Germany; in Japan, STN International, % Japan Association for International Chemical Information, Gakkai Center Bldg, 2-4-16 Yayoi, Bunkyo-ku, Tokyo 113, Japan; in North America, STN International, 2540 Olentangy River Rd, PO Box 02228, Columbus, OH 43202.

Three new CD-ROM databases are available from John Wiley & Sons: the *Kirk-Othmer Encyclopedia of Chemical Technology,* the *Mark Encyclopedia of Polymer Science and Engineering,* and the *International Dictionary of Medicine and Biology.* These are in addition to the *1987 Registry of Mass Spectral Data,* which became available in CD-ROM format in March. Purchasing information for these products can be obtained from Patricia Howe, John Wiley & Sons, 605 Third Ave, New York, NY 10158, telephone 212/850-6189.

New Private Sustaining Members

The AOAC welcomes the following new private sustaining members: Haarmann & Reimer Corp., Springfield, NJ; Technological Institute, Copenhagen, Denmark; and Schenley Distillers, Lawrenceburg, NJ.

Interim Methods

The following methods have been approved interim official first action by the respective committees and by the Chairman of the Official Methods Board and will be submitted for adoption as official first action at the 101st AOAC Annual International Meeting and Exposition, Sept. 14–17, 1987, at San Francisco, CA: by the Methods Committee on Foods I—(*1*) Manual Determination of Minced Fish Flesh in Mixed Fillet-Minced Cod Blocks, by J. P. Lane and R. J. Learson (submitted by F. J. King, National Marine Fisheries Service, Gloucester, MA) and (*2*) Semiquantitative Determination of Soy Protein in Meat Products by an ELISA Procedure, submitted by C. H. S. Hitchcock (Unilever Research, Sharnbrook, Bedford, England); by the Methods Committee on Foods II—Determination of Specific Gravity of Beer and Wort Using a Digital Density Meter, AOAC-ASBC Method, submitted by P. Gales (Anheuser-Busch, Inc., St. Louis, MO); by the Methods Committee on Feeds, Fertilizers, and Related Topics—Rapid Detection and Identification of Seven Families of Antimicrobial Drugs in Milk with a Microbial Receptor Assay, submitted by S. E. Charm (Penicillin Assays, Inc., Malden, MA) and R. Chi (Food and Drug Administration, Division of Mathematics, Washington, DC).

Copies of these methods are available from the AOAC office.

AOAC Publication Wins Youden Award

Use of Statistics to Develop and Evaluate Analytical Methods, written by Grant T. Wernimont, edited by William Spendley, and published by AOAC, is the 1987 winner of the American Statistical Association's prestigious annual W. J. Youden Award in Interlaboratory Testing. The award recognizes a publication that makes an outstanding contribution to the design and/or analysis of interlaboratory tests or that describes ingenious applications of statistics to the evaluation of data from such tests. Eligible publications must have been professionally refereed as journal articles or monographs. AOAC is proud to have been the publisher of this book and extends its sincere congratulations to Mr. Spendley and Dr. Wernimont's widow.

A newly updated compilation of over 300 methods for pesticide formulation analysis

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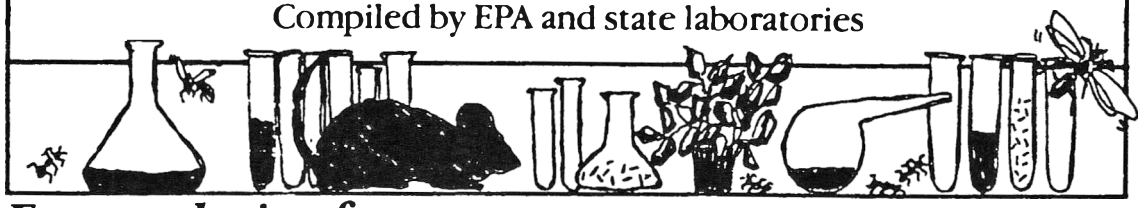

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Contains 200 methods published in 1976, plus supplements which added 20 methods in 1977, 30 in 1979, and 55 in 1982

1363 pages. With spectra. 1983. Includes 3 supplements and binder. ISBN 0-935584-23-4. Price—Members: $61.95 in U.S., $64.95 outside U.S., Nonmembers: $68.50 in U.S., $71.50 outside U.S. 1982 Supplement can be purchased separately: Price—Members: $15.40 in U.S., $16.40 outside U.S., Nonmembers: $17.00 in U.S., $18.00 outside U.S.

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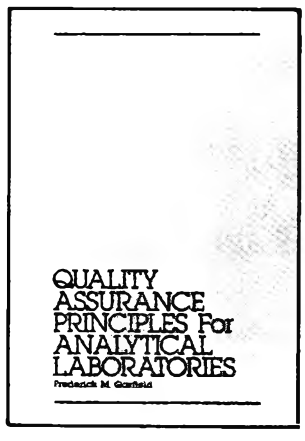

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   (3) Siegel, S. (1956) *Nonparametric Statistics for the Behavioral Sciences,* McGraw-Hill Book Co., New York, NY

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- Regulatory Perspective of Pesticide Analytical Methodology in the United States—*M. F. Kovacs, Jr, and C. L. Trichilo*
- Importance of Quality Assurance in Canadian Pesticide Analysis—*H. B. S. Conacher*

SPECIAL REPORTS

Listeria monocytogenes—A Current Dilemma

H. MICHAEL WEHR
Oregon Department of Agriculture, 635 Capitol St NE, Salem, OR 97310

Since 1985, *Listeria monocytogenes* has gone from being an organism known to only a handful of microbiologists to being a fully recognized food-borne pathogen of concern. With nearly unprecedented speed and vigor, food processors, particularly in the dairy industry, and regulatory agencies have reacted in concert to resolve unanswered questions about *Listeria* so as to assure the safety of the food supply. This report summarizes what has been learned and what is being done.

Two years ago, *Listeria monocytogenes* was an organism virtually unknown to all but a handful of microbiologists. Since then, this microorganism has gone through the stage of an emerging pathogen to a fully recognized food-borne pathogen of concern. Indeed, the term *Listeria* can evoke a feeling of near panic within the dairy industry and may reach that stage for other food products.

Listeria today represents the classical dilemma for food processors and regulatory agencies serving the food and dairy industries. Both segments are forced to deal with this microorganism with many unknowns. The food processor must contend with an organism that is probably ubiquitous, but without a clear knowledge of its biology and its behavior in the environment and without good analytical methods. Similarly, the regulatory agencies not only must deal with these limitations but also must make decisions on food safety and product recalls without a clear knowledge of the organism's behavior in foods, its survivability through processing, and the dose level needed to cause illness.

Were it not for a single incident in 1985 that involved multiple illnesses and deaths due to the presence of *Listeria monocytogenes* in Mexican-style soft cheeses, the food industry would probably be proceeding still today as if *Listeria* did not exist. What is *Listeria*? What is its history? What occurred to create today's problem? What is the status of the biology and analytical methodology of *Listeria*? What is the future for the food and dairy industry with respect to *Listeria*? This paper will attempt to answer these questions.

What is Listeria*? . . . What occurred to create today's problem? . . . What is the future for the food and dairy industry with respect to* Listeria*?*

Received April 28, 1987.

Characteristics and History of Listeria

The *Listeria* organism is a small gram-positive coccoid rod that occurs in short chains (1). It is a motile, nonspore-forming, facultative anaerobe. Characterization after isolation (see below) relies on colony morphology and gram staining, tumbling motility, a positive catalase reaction, beta hemolysis on blood agar, and typical biochemical reactions. The organism grows at refrigeration temperatures; indeed, cold enrichment is used as a selective enrichment procedure to isolate the bacteria (2). The pH growth range of the organism is 5.0–9.6, but the organism can survive at lower pH values. *Listeria* is an intracellular pathogen capable of residing within macrophages and neutrophils.

Listeria monocytogenes is widespread in the environment (3). It has been isolated from soil, animals, birds, fish, insects, green plants, silage, water, and food products including milk, cheeses, meat products, fruits, and vegetables.

Listeria causes a food-borne disease named listeriosis (4). Primary manifestations of the disease in humans are meningitis, abortion, and perinatal septicemia. Listeric meningitis can result in death. Development of the disease in pregnant women results in abortion and the delivery of stillborn or acutely ill infants. The mortality rate can be high, and pregnant women and newborns are at greatest risk. Immunocompromised individuals or persons with underlying illnesses such as malignancy and cirrhosis also are at substantially higher risk than healthy individuals.

Listeria is not a new organism. It was probably first seen in tissue sections from patients as early as 1891 and was first isolated in 1911 from rabbit liver in Sweden. The disease syndrome, now referred to as listeriosis, was first recognized in sheep in 1925; the first confirmed human listeriosis was reported in 1929. Subsequently, *Listeria* was found as the cause of abortion in cattle and sheep, septicemia in chickens and fowls, encephalitis in sheep, and bovine mastitis.

Epidemiologically, *Listeria* has been regarded as zoonotic. Routes of infection in humans can involve the handling of

Table 1. Incidents of food-borne illness in which *Listeria monocytogenes* has been implicated

Year	Place	No. of cases	Fatality rate, %	Probable source	Probable cause
1978	Massachusetts	23	—	raw vegetables	—
1981	Nova Scotia	51	44	cole slaw	cabbage grown in field fertilized with sheep manure
1983	Boston, MA	49	29	pasteurized milk	—
1985	Los Angeles, CA	85	33	Mexican-style soft cheese	poor pasteurization and poor plant sanitation

newborn animals, contact with infected animals, contact with infected feces, or ingestion of contaminated food.

The presence of *Listeria* in food is probably the result of multiple causes, both direct and indirect. A circular route can occur involving the shedding of *Listeria* from infected animals, subsequent contamination of soil and grass, contamination of silage produced from the grass, and reintroduction into cows or meat animals, leading to contamination of milk and meat. Processing plant contamination can lead to indirect contamination of food products.

Why the Concern with Listeria?

Today's concern with *Listeria* is due to an increased awareness of the organism's ability to cause food-borne disease and the isolation of *Listeria* from foods. In particular, a single incident in 1985 involving Mexican-style soft cheeses led to followup investigations and multiple *Listeria* isolations in dairy products.

Food-borne outbreaks implicating *Listeria* as the causative agent have been relatively few. Only 5 such incidents have been identified since 1978, as shown in Table 1 (5–7). Following the 1985 incident, investigations and analyses conducted by the U.S. Food and Drug Administration (FDA) and the dairy industry resulted in the isolation of *Listeria* from numerous soft-style cheese products, from ice cream products, and from processing plant environments. These isolations, coupled with a lack of information about the organism and lack of ability to control it, have created today's concern with *Listeria*.

What Are the Problems Associated with Listeria?

Numerous problems with *Listeria* plague the industry and regulatory agencies. Specific difficulties include: (*1*) inadequate analytical methodology; (*2*) lack of knowledge on susceptible dose; (*3*) lack of knowledge on how to control the organism in the processing plant environment, including questions involving its survivability through the pasteurization process; (*4*) lack of knowledge on the biology of the organism, including its behavior in sensitive food products.

Status of analytical methodology.—As with most current microbiological procedures, *Listeria* methodology relies on classic cultural procedures for its isolation and identification. Procedures of this type rely on enrichment and/or selective enrichment procedures, followed by isolation using selective plating techniques, with confirmation by biochemical and serological means.

Isolation procedures for *Listeria* utilize a cold (4°C) enrichment procedure—an approach that creates an inherent problem with the method. The *Listeria* methodology used in the Massachusetts and California outbreaks (3) utilized cold enrichment over a period of several weeks. Such an approach obviously is inadequate to assure public health. Efforts to date have resulted in modifications to available methodology to make the procedure somewhat more timely. The method currently used most frequently is that of the FDA (8), which utilizes a 1- and 7-day cold enrichment step followed by isolation on modified McBride's agar and confirmation through biochemical, serological, and mouse pathogenicity testing.

The FDA method, while better than earlier isolation procedures, still takes longer than desirable. Food processors, especially dairy and meat processors, cannot hold a product awaiting *Listeria* results. This creates the first dilemma. Processors must ship and sell without knowing whether *Listeria* is present.

Significant efforts are under way to try to remedy the methodology problem. FDA has funded research comparing selective plating procedures to identify the most beneficial isolation medium for a variety of product types (D. Archer, FDA). It has also committed significant resources to develop a DNA probe technique for *Listeria*; such a technique would provide a much more rapid approach to *Listeria* identification. Other work funded by the Dairy Research Foundation involves research to improve the enrichment procedures for *Listeria*. Additional research in the field seeks to develop enzyme-linked immunosorbent assays and other techniques for *Listeria* isolation/identification. Although confirmatory procedures for *Listeria* may always remain somewhat time-consuming, it is hoped that research and progress will lead quickly to rapid and reliable screening techniques to resolve the dilemma faced by industry: that of having to ship potential problem products without knowing *Listeria* test results.

> *. . . it is hoped that research . . . will lead . . . to . . . screening techniques to resolve the dilemma: having to ship potential problem products without knowing* Listeria *test results.*

Dose response.—At the present time, there is no clear picture as to the actual dose of *Listeria* needed to cause illness. This lack presents a real dilemma to both industry and regulatory agencies. Without such data, regulators are forced to specify a zero tolerance in processed items. Industry, on the other hand, is forced to accept such a tolerance knowing that it may be difficult to provide completely *Listeria*-free product and having insufficient information about *Listeria* to predict its characteristics in product or process environments. Research is under way, again funded by the Dairy Research Foundation, to obtain dose response information. The Centers for Disease Control have also implemented a program to monitor closely *Listeria*-caused illnesses, not only to document the epidemiology of the organism, but also to attempt to determine dose response relationships. Meanwhile, we live with the dilemma.

Survivability through pasteurization.—Of current concern is the possibility that *Listeria monocytogenes* may survive

the milk pasteurization process. Such a situation would present a true dilemma for the dairy industry—one in which the organism survives pasteurization, yet the industry is forced to adhere to a zero tolerance because susceptible dose information is lacking. While it appears likely that the current pasteurization process guidelines of the pasteurized milk ordinance are adequate to destroy *Listeria monocytogenes* in whole milk (9), some researchers report findings that suggest *Listeria monocytogenes* may survive pasteurization (M. P. Doyle, University of Wisconsin). Of importance is the fact that *Listeria monocytogenes* is an intracellular pathogen existing within somatic cells and in this state may be able to survive. Significant current research is in progress by FDA and other investigators to clarify the survivability issue.

Survivability is a critical issue, but also important are the environmental aspects of the organism. While FDA has indicated (10) that postpasteurization contamination is a significant factor which leads to finished product contamination, the behavior of *Listeria monocytogenes* in the processing plant environment is not clear. Even though the presence of *Listeria* in finished dairy products may be infrequent, its presence in the dairy processing plant, on equipment, in drains, air, etc. is more commonplace. Although its presence can be documented, the cause of its presence is difficult to determine. More frustrating is the fact that *Listeria* frequently remains in the processing plant (or returns to it) despite the industry's best efforts to eliminate it through vigorous sanitation efforts. Significant research is under way to examine the environmental sources of *Listeria* in dairy processing plant operations to identify reservoirs, minimal growth conditions, and modes of transportation of *Listeria* (Dairy Research Foundation). Work is also under way to evaluate the effect of sanitizers on *Listeria*. It is hoped that such work can lead to a better understanding of how this organism responds to the environment and how the presence of *Listeria* in processing plants can be minimized or eliminated.

Behavior in sensitive food products.—Following the 1983 and 1985 *Listeria* outbreaks, it became clear that knowledge of the behavior of *Listeria* in dairy and other food products was lacking. Information on such items as the effects of pH and salt or on competitive effects of lactic starter culture or other microorganisms on *Listeria* was not available. Work initiated since that time by the University of Wisconsin (11, 12) has shown the ability of *Listeria* under certain conditions to survive in a variety of cheese types (cottage, cheddar, camembert). The work also indicated that potentially *Listeria* could grow during storage of certain cheeses such as camembert. Extensive additional effort is under way to clarify the behavior of *Listeria* under a variety of conditions (Dairy Research Foundation). Its behavior in Mexican-style cheeses is under study. Also, projects have been undertaken to determine the effects of competitive lactic starter cultures on *Listeria* and the effects of ice cream components on *Listeria*. It is anticipated that substantial additional work will occur in this area.

Positive Steps to Resolve the Listeria Crisis

Several steps have been, or are being taken, to resolve the concerns and problems associated with *Listeria*.

Dairy safety initiative program.—In April 1986, FDA began a program of intensified surveillance efforts for dairy processing plants (J. Kozak, FDA). The program incorporated check ratings (audits of interstate milk plants), FDA inspections, and microbiological testing for *Listeria* (10). Between the spring and fall of 1986, over 350 check ratings and inspections were conducted. The program yielded 9 processing plants (2.5%) with products containing *Listeria*. According to FDA (10), extensive followup efforts in each plant demonstrated that postpasteurization contamination is a significant factor leading to finished product contamination. Although defects in pasteurization equipment and plant design were noted, environmental sampling revealed plant conditions that more likely contributed to postpasteurization contamination. FDA noted that survivability through the pasteurization process in the investigated plants was unlikely. FDA has also drawn up recommended guidelines for controlling environmental microbiological contamination in dairy plants. Recommendations have been made in the areas of pasteurization adequacy; postpasteurization contamination; cross connections; the use of returned milk and reclaiming operations; airborne contamination; plant operation, including traffic patterns and personnel cleanliness; and quality assurance sampling and testing programs. It is important to note that most state dairy regulatory agencies have initiated similar intensive investigation efforts on their own, using FDA guidelines and related information.

Education and training.—The dairy industry, primarily through its industry associations, including the Milk Industry Foundation, the National Milk Producers Federation, and the International Association of Ice Cream Manufacturers and in conjunction with FDA, has sponsored a series of meetings for processing plant operators to review procedures for controlling and detecting *Listeria*. FDA recommendations noted above have been a key part in these education/training seminars. Programs offered through state extension specialists and direct company-sponsored educational efforts have also been an important element.

> *The response to the* Listeria *problem may well be unparalleled in the dairy industry.*

Intensified research efforts.—As noted throughout the discussion above, significant research on *Listeria* is under way to learn more about the organism and how to control and/or eliminate it from foods. The focus of this effort has been the Dairy Research Foundation. Through the Foundation and related dairy research funding organizations, including the Wisconsin Milk Marketing Board, the National Milk Marketing and Promotion Board, and the California Milk Advisory Board, a true initiative has been undertaken to resolve the knowledge gap on *Listeria*. The Dairy Research Foundation, using a blue ribbon advisory group of scientific experts, identified 4 basic areas of needed research. These included: (a) analytical methodology, (b) dose response, (c) survivability through product pasteurization, and (d) environmental and behavioral aspects of *Listeria*. Research proposals were requested, and on a rapid review/response basis specific proposals in each of the 4 areas were approved for funding through the Dairy Research Foundation and the other organizations noted above. The research program is essentially on a 2-year completion basis to help assure a rapid increase in our knowledge of *Listeria*.

The Future

The response to the *Listeria* problem may well be unparalleled in the dairy industry. Both industry and regulators alike have reacted promptly and intensively to assure the safety of the food supply and to resolve unanswered questions

about *Listeria*. While many of the dilemmas concerning the organism still exist, the level of activity in plant sanitation control, analytical testing programs, education, and research has allowed the dairy industry to resolve some of its immediate concerns and to anticipate a full resolution of the *Listeria* problem.

While the dairy industry has been the center for *Listeria* concern to date, other food product areas, particularly meats, also present the potential for *Listeria* food-borne disease. It is this area that will most probably be the next focus for *Listeria* research and control efforts.

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History of the Food and Drug Administration's Total Diet Study—1961 to 1987

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The Total Diet Study provides the Food and Drug Administration with baseline information on the levels of pesticide residues, contaminants, and nutrient elements in the food supply and in the diets of specific age-sex groups. The study also identifies trends and changes in the levels of these substances in the food supply and in diets over time and thereby assists in identifying potential public health problems. This paper describes the evolution of the Total Diet Study from 1961 to 1987. Food collections, sites of analysis, diets, food commodity groups, analytes, analytical methodologies, data transfer, publication of results, notable results, resources, and advantages of the study are discussed.

The U.S. Food and Drug Administration's (FDA) Total Diet Study is a continuing program that determines the levels of various pesticide residues, contaminants, and nutrient elements in foods and estimates the intakes of these substances in representative diets of specific age–sex groups. These assessments, which require the purchase, preparation, and analysis of typically consumed foods, allow for the identification of changes and trends in the pesticide residue, contaminant, and nutrient element content of the food supply and for identification of potential public health problems. Continuous monitoring of the food supply through the Total Diet Study is necessary because the pesticide residue, contaminant, and nutrient element levels of foods may be altered by changes in agricultural or manufacturing practices, processing technology, or methods of food packaging or by environmental or industrial contamination.

Although the primary purposes of the Total Diet Study are to provide FDA with baseline information on the levels of pesticide residues, contaminants, and nutrient elements in diets, to identify trends and changes in intakes of these substances over time, and to identify potential public health problems concerning these substances, the Total Diet Study has proved to be of value beyond these purposes. Results have been used by other government agencies and by academic and other nongovernmental organizations, both in the United States and abroad. The program has been recognized as a model system. Moreover, in addition to the purposes stated above, the Total Diet Study also serves to: (*1*) assist in determining the dietary sources of pesticide residues, contaminants, and nutritional elements; (*2*) guide FDA programs by identifying analytes of potential public health significance; (*3*) alert FDA about potential needs for action (e.g., increased surveillance, regulation, or communication with industry); and (*4*) monitor the effects and effectiveness of other FDA regulatory activities.

The FDA initiated the Total Diet Study in May 1961 primarily in response to concern about levels of radioactive contamination in foods from atmospheric nuclear testing. Although government agencies had done fairly extensive monitoring of milk and limited monitoring of other foods for strontium-90, there was no nationwide government-sponsored program designed to estimate total dietary intake

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Table 1. FDA regions and district offices

Region	States/territories within regions	District offices within regions
I	Maine,[a] New Hampshire,[a] Vermont,[a] Massachusetts,[a] Connecticut,[a] Rhode Island[a]	Boston
II	New York,[a] New Jersey,[a] Puerto Rico[a]	Buffalo, Newark, New York,[b] San Juan
III	Pennsylvania,[a] Delaware,[c] Maryland,[c] West Virginia,[c] Virginia,[c] District of Columbia[c]	Baltimore, Philadelphia
IV	Kentucky,[c] Tennessee,[c] South Carolina,[c] North Carolina,[c] Georgia,[c] Florida,[c] Alabama,[c] Mississippi[c]	Atlanta, Nashville, Orlando
V	Minnesota,[d] Wisconsin,[d] Michigan,[d] Ohio,[d] Indiana,[d] Illinois[d]	Chicago, Cincinnati, Detroit, Minneapolis
VI	Oklahoma,[c] Texas,[c] Arkansas,[c] Louisiana,[c] New Mexico[e]	Dallas, New Orleans
VII	Nebraska,[d] Iowa,[d] Kansas,[d] Missouri[d]	Kansas City, St. Louis[f]
VIII	North Dakota,[d] South Dakota,[d] Utah,[e] Montana,[e] Wyoming,[e] Colorado[e]	Denver
IX	California,[e] Nevada,[e] Arizona,[e] Hawaii[e]	Los Angeles, San Francisco
X	Washington,[e] Oregon,[e] Idaho,[e] Alaska[e]	Seattle

[a] Northeastern/eastern states/territories.
[b] Name changed to Brooklyn District Office in 1983.
[c] Southern states/territories.
[d] North central/central states.
[e] Western states.
[f] Previously a district office, currently a station office.

Table 2. Total Diet Study collections (cols)

Year (FY)	Col. type[a]	No. of col. districts	No. of cols/ district	Total no. of cols	Analysis location[b]
1961/62	A	1	4	4	collecting district & Wash., DC
1962/63	A	5	8/12	44	collecting district
1964	A	9	4/8	40	collecting district
1965	A	3	6	18	collecting district
1966	A	5	5/6	28	collecting district
1967	A	5	6	30	collecting district
1968	A	5	6	30	collecting district
1969	A	5	6	30	collecting district
1970	A	5	6	30	collecting district
1971	A	5	6	30	Kansas City, MO
1972	A	5	7	35	Kansas City, MO
1973	A	18	1/2/3	30	Kansas City, MO
1974	A	19	1/2/3	30	Kansas City, MO
1975	A	20	1	20	Kansas City, MO
	I&T	10	1	10	Kansas City, MO
1976	A	20	1	20	Kansas City, MO
	I&T	10	1	10	Kansas City, MO
1977	A	20	1/2	25	Kansas City, MO
	I&T	12	1	12	Kansas City, MO
1978	A	14	1/2/3	20	Kansas City, MO
	I&T	9	1/2	10	Kansas City, MO
1979	A	14	1/2/3	20	Kansas City, MO
	I&T	9	1/2	10	Kansas City, MO
1980	A	15	1/2	20	Kansas City, MO
	I&T	10	1	10	Kansas City, MO
1981	A	14	1/2	18	Kansas City, MO
	I&T	10	1	10	Kansas City, MO
1982	A	8	1/2	9	Kansas City, MO
	I&T	3	1	3	Kansas City, MO
1982/83	IND	12	1	12	Kansas City, MO
1983/84	IND	12	1	12	Kansas City, MO
1984/85	IND	12	1	12	Kansas City, MO
1985/86	IND	12	1	12	Kansas City, MO
1986/87	IND	12	1	12	Kansas City, MO

[a] A = adult male collections; I&T = infant and toddler collections; IND = individual food collections.
[b] Radionuclide analyses for Cs-137 were done at FDA Headquarters in Washington, DC, from FY 1961/62 to FY 1966. During this time Sr-90 analyses were done in the collecting districts with duplicate check samples analyzed in the Washington, DC, laboratory. There were no radionuclide analyses between FY 1967 and FY 1972. Analyses since FY 1973 have been done at WEAC in Boston, MA.

of radioactive material. Consumers Union advocated such a program and conducted limited studies in the late 1950s and early 1960s (1).

The first Total Diet Study estimated the levels of strontium-90 and cesium-137 as well as organochlorine pesticide residues, organophosphorus pesticide residues, and selected nutrients in the diets of young men (2). The Total Diet Study program has continued on a yearly basis since 1961. This program has monitored the levels of radionuclides (2–11), pesticide residues, toxic elements, industrial chemicals (2, 12–59), and selected nutrients (2, 37, 60–73) in the United States' food supply and in the diets of selected age–sex groups. There have been many changes, refinements, and expansions in the Total Diet Study program since 1961 regarding collection sites, foods collected, analytes, and analytical methodologies. Many FDA employees have been involved with the Total Diet Study including those in the Total Diet Study laboratory in Kansas City, MO, in the FDA Center for Food Safety and Applied Nutrition (CFSAN) in Washington, DC, in the Office of Regulatory Affairs (ORA) in Rockville, MD, and in FDA district offices throughout the United States.

Numerous reports (2–73) have been published on Total Diet Study results over the years; however, a chronological description of the Total Diet Study has not yet been published. Individuals requesting information about the Total Diet Study must attempt to piece together various reports or speak at length with FDA employees currently involved with the study. An FDA contract report issued by CDP Associates (74) in 1983 evaluating the Total Diet Study indicated that the study was important and valid for its purposes, but that some non-FDA professionals interviewed by CDP Associates were misinformed about the study and unaware of its 1982 revision. The purpose of this paper is to describe briefly the evolution of the Total Diet Study, to summarize the activities, changes, and expansions of the program from its beginning in 1961 to the present, to provide references for the results of the study, and to provide references for the analytical methodologies used in the study.

Food Collections

The foods for the Total Diet Study program are collected by inspectors from FDA district offices. The number and location of FDA district offices have varied over the years of the Total Diet Study. Currently, there are 21 district offices as shown in Table 1. Table 2 indicates the number of collection sites for the Total Diet Study from Fiscal Year (FY) 1961/62 through FY 1986/87, and Appendix A[1] specifies the cities from which foods were collected and the districts and geographical regions—northeast (NE), south (S), north central (NC), or west (W) between FY 1961/62 and FY 1982 and east (E), south (S), central (C), or west (W) from FY 1982/83 to FY 1986/87—to which these cities are assigned.

For the first Total Diet Study, 82 foods were purchased quarterly between May 1961 and February 1962 from gro-

[1] Appendixes for this paper are available from the National Technical Information Service, Springfield, VA, Doc. No. PB87-151676/AS.

Table 3. Total Diet Study diets

Years used (FY)	Diet basis	Age–sex group	Diet type
1961/62	1955 USDA HFCS	16–19-year male, 82 foods, 4200 kcal/day, 3.76 kg food/day, 14 day diet	national diet
1962/63–1970	1955 USDA HFCS	16–19-year male, 82 foods, 4200 kcal/day, 3.76 kg food/day, 14 day diet	national diet with adjustments for regional food patterns for other years
1971–1974	1965 USDA HFCS	15–20-year male, 120 foods, 3900 kcal/day, 2.9 kg food/day, 14 day diet for FY 71-73, 28 day diet for FY 74	regional diets for northeast, south, north central, and west
1975–mid 1982	1965 USDA HFCS	15–20-year male, 120 foods, 3900 kcal/day, 2.9 kg food/day, 28 day diet	regional diets for northeast, south, north central, and west
		6-month infant, 50 foods, 880 kcal/day, 1.4 kg food/day, 14 day diet	
		2-year child, 110 foods, 1300 kcal/day, 1.5 kg food/day, 14 day diet	
1982/83–1986/87	1977–1978 USDA NFCS & NHANES II	6–11-month infant, 230 foods, 871 kcal/day, 1.2 kg food/day	national diets
		2-year child, 224 foods, 1295 kcal/day, 1.5 kg food/day	
		14–16-year female, 201 foods, 1714 kcal/day, 2.0 kg food/day	
		14–16-year male, 201 foods, 2520 kcal/day, 2.7 kg food/day	
		25–30-year female, 201 foods, 1576 kcal/day, 2.2 kg food/day	
		25–30-year male, 201 foods, 2541 kcal/day, 3.1 kg food/day	
		60–65-year female, 201 foods, 1355 kcal/day, 2.3 kg food/day	
		60–65-year male, 201 foods, 1944 kcal/day, 2.7 kg food/day	

cery stores in the metropolitan area of the District of Columbia. Quarterly food collections and analyses were completed by 5 FDA district offices (Atlanta, Baltimore, Minneapolis, San Francisco, and St. Louis) in the second year (FY 1962/63), by 9 district offices (Atlanta, Baltimore, Boston, Dallas, Denver, Minneapolis, St. Louis, San Francisco, and Seattle) in FY 1964, and by 3 FDA district offices (Boston, Kansas City, and Los Angeles) in FY 1965. From FY 1966 through FY 1972, 5–7 collections per year were made from 5 FDA district offices (Baltimore, Boston, Kansas City, Los Angeles, and Minneapolis) for 28–35 yearly collections. Between FY 1973 and mid-FY 1982, all FDA district offices were involved in food collections for the Total Diet Study. A sampling scheme rotated the collections among the FDA districts in the 4 geographical areas. States within these 4 geographical areas are indicated in Table 1. Total Diet Study collections have been obtained from all states except Alaska and from the District of Columbia and Puerto Rico.

Before FY 1975, the food collections represented the diet of the teenage or young adult male (adult diet) (Table 2). Between FY 1975 and mid-FY 1982, approximately two-thirds of the collections represented adult diets and about one-third represented infant and children's (toddler's) diets. Between FY 1967 and FY 1981, approximately 30 collections per year were made. In FY 1977, the fiscal year was 18 months long (due to the conversion from the July–June Fiscal Year to the October–September Fiscal Year) allowing for 25 adult and 12 infant and toddler collections. In FY 1981, 2 adult collections were omitted to allow a pilot collection for the revised Total Diet Study program which began in mid-FY 1982. Twelve collections, 9 adult and 3 infant and toddler, were made in FY 1982 before the new program began.

For the revised program, which began in mid-FY 1982, foods are collected 4 times per year, once from each of the 4 geographical areas of the United States. Each collection consists of the purchase of identical foods from grocery stores in 3 cities within a geographical area. The 3 subsamples of each food (from the 3 cities) are combined to form a sample for analysis. Because each city is in a different FDA district, the year's collections involve 12 FDA districts (4 collections × 3 districts/collection).

Location of Food Preparation and Analyses

Foods of the first Total Diet Study (FY 1961/62) were prepared in an institutional kitchen in the Baltimore district and analyzed in the FDA Baltimore District laboratory and the FDA Washington, DC, laboratory. Between FY 1962/63 and FY 1970, the foods of the Total Diet Study were prepared in institutional kitchens within each FDA collecting district and analyzed in the FDA collecting district laboratory (except for cesium-137 analysis, which was done in the FDA Washington, DC, laboratory). Beginning in FY 1971, the analytical work was centralized at the FDA Total Diet Laboratory in Kansas City, MO, for greater uniformity and efficiency in the use of analytical equipment and staff. This necessitated that the foods purchased in the other districts be shipped to Kansas City for preparation and analysis. Food preparation was contracted to the Home Economics Department of St. Mary College in Leavenworth, KS, from FY 1971 until FY 1985, when St. Mary College lost its Home Economics Department. Foods of the first collection in FY 1986 were prepared by Johnson County Community College in Overland Park, KS; foods in subsequent collections have been prepared by Canteen Corp., which operates in a federal building in Kansas City, MO. Radionuclide analysis, which was discontinued between FY 1967 and FY 1972, has been performed at the FDA Winchester Engineering Analytical Center (WEAC) in Boston, MA, since January 1973. Food samples are sent to WEAC from the Total Diet Laboratory in Kansas City.

Diets

Table 3 describes the age–sex group diets used in the Total Diet Study from FY 1961/62 to the present, and Appendix B lists the individual foods collected for analysis. Food con-

Table 4. Total Diet Study food commodity group composites

FY 1961/62–1964[a] adult 1955 HFCS diet	FY 1965–mid FY 1982 adult 1955 & 1965 HFCS diets	FY 1975–mid FY 1982 infant & toddler 1965 HFCS diets
1. dairy products	1. dairy products	1. water
2. meat and eggs	2. meat, fish, poultry	2. whole, fresh milk
3. grain products	3. grains and cereal products	3. other dairy products and substitutes
4. potatoes	4. potatoes	4. meat, fish, poultry
5. leafy vegetables	5. leafy vegetables	5. grains and cereal products
6. dried beans	6. legume vegetables	6. potatoes
7. root vegetables	7. root vegetables	7. vegetables
8. smooth vegetables	8. other vegetables	8. fruits and juices
9. fruits	9. fruits	9. fats and oils
10. sugars, fats, and oils	10. fats, oils, and shortenings	10. sugars and adjuncts
11. beverages	11. sugars and adjuncts	11. beverages
	12. beverages	

[a] Baltimore District only.

Table 5. Radionuclides—years analyzed and methods used[a,b]

Year	Sr-90	Cs-137	I-131	Ru-106	K-40	Ra-226
1961/62	BC	GRS				
1962/63	BC	GRS				
1964	BC	GRS				
1965	BC	GRS				
1966	BC	GRS				
1967						
1968						
1969						
1970						
1971						
1972						
1973[c]	BC	GRS	GRS	GRS	GRS	
1974	BC	GRS	GRS	GRS	GRS	
1975	BC	GRS	GRS	GRS	GRS	AS[d]
1976	BC	GRS	GRS	GRS	GRS	AS[d]
1977	BC	GRS	GRS	GRS	GRS	AS[d]
1978	BC	GRS	GRS	GRS	GRS	
1979	BC	GRS	GRS	GRS	GRS	
1980	BC	GRS	GRS	GRS	GRS	
1981	BC	GRS	GRS	GRS	GRS	
1982	BC	GRS	GRS	GRS	GRS	
1982/83	BC	GRS	GRS	GRS	GRS	
1983/84	BC	GRS	GRS	GRS	GRS	
1984/85	BC	GRS	GRS	GRS	GRS	
1985/86	BC	GRS	GRS	GRS	GRS	
1986/87	BC	GRS	GRS	GRS	GRS	

[a] Analysis for Pu-239 (84) has been intermittent; no Pu-239 has yet been detected.

[b] BC = beta counting of daughter product yttrium-90 (85–87); GRS = gamma-ray spectroscopy (88–90); AS = alpha spectroscopy (85).

[c] Partial yearly collection (8 composites in 6 months beginning January 1974).

[d] Only selected samples were analyzed.

sumption data from the U.S. Department of Agriculture's (USDA) 1955 Household Food Consumption Survey (75) were used to develop the diet for the first Total Diet Study. The diet was based on the quantities of 11 food groups suggested for males 16 to 19 years old in the USDA's moderate cost food plan (76) and followed the general dietary patterns of moderate income families but with some modifications to meet nutritional goals. The diet, which contained 82 foods in quantities for a 14 day intake, was excessive in energy intake (4200 kcal/day) to assess maximum exposure to dietary contaminants. This same diet was used between FY 1962/63 and FY 1970, with adjustments for regional food patterns, to collect foods from other U.S. locations. Some adjustments were made to the diets in the late 1960s as preliminary data from USDA's 1965 Household Food Consumption Survey (77) became available.

In FY 1971, the diet was revised according to data from the USDA's 1965 Household Food Consumption Survey (77) and according to the 1964 moderate cost food plan for 15–20-year-old males (78). Four diets for geographical areas (northeast, south, north central, and west) were developed for the 15–20-year-old male. The foods and quantities of foods in these 4 diets varied among the geographical areas. Each diet contained approximately 120 foods and provided approximately 3900 kcal/day. The estimates of essential element intake were extrapolated to a more realistic caloric base of 2850 kcal/day so that the daily intake of elements would not be overestimated.

In FY 1975, regional diets for 6-month-old infants and 2-year-old toddlers were added to the Total Diet Study program. The diets for these 2 age groups were developed from individual data of the spring quarter of USDA's 1965 Household Food Consumption Survey (79). The infant and toddler diets contained approximately 50 and 110 foods, respectively, in quantities sufficient for a 14-day period. Caloric content of these diets represented typical intakes for these age groups. To accommodate the 10 yearly collections of the infant and toddler diets, the number of adult collections was reduced from 30 to 20 per year.

The mid-FY 1982 revision of the Total Diet Study program allowed for updated diets, expanded coverage of age–sex groups, and analysis of individual foods. Data from the USDA's 1977–1978 Nationwide Food Consumption Survey (80) and the National Center for Health Statistics' (NCHS) Second National Health and Nutrition Examination Survey of 1976–1980 (81) were used to select a group of commonly consumed foods and to develop nationally representative diets for 8 age–sex groups based on these foods (82, 83). The caloric content of these diets represents typical intakes according to age and sex. It is anticipated that the food list and diets of the Total Diet Study will be revised when newer food consumption data become available from USDA or NCHS. (The next Nationwide Food Consumption Survey is scheduled for 1987, and the Third National Health and Nutrition Examination Survey is scheduled to begin in 1988.)

Food Commodity Groups

For the first 3 years of the study, the foods were analyzed as a single total diet food composite; however, the foods collected from the Baltimore District during these 3 years were also analyzed in 11 food commodity group composites (Table 4). Beginning in the fourth year (FY 1965), the 82 foods from each collection were divided among 12 commodity group composites before analysis. The approximately 120 foods of the adult diet used between FY 1971 and FY 1982/82 were also analyzed in 12 food commodity group composites. The foods of the infant and toddler diets used between FY 1975 and FY 1981/82 were analyzed in 11 food commodity group composites. Beginning in FY 1982/83, the 234 foods of the Total Diet Study were analyzed individually, and food commodity group composites were no longer used.

Analytes

Analytes have been and may be added to the Total Diet Study according to the needs and concerns of the FDA. Tables 5–7 show the yearly analytical scheme for radionuclides, for pesticide residues, industrial chemicals, and toxic ele-

Table 6. Pesticide residues, industrial chemicals, and toxic elements—years analyzed and methods used

	Analyte class[a]																	
FY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1961/62	X		X							X								
1962/63	X		X							X								
1964	X		X	X		X				X				X	X			
1965	X		X	X		X	X			X				X	X			
1966	X		X	X		X	X			X				X	X			
1967	X		X	X		X	X			X				X	X	X		
1968	X		X	X		X	X			X				X	X	X		X
1969	X		X	X		X	X			X				X	X	X		
1970	X		X	X		X	X			X				X	X	X		
1971	X	X	X	X	X	X	X			X				X		X		X
1972	X	X	X	X	X					X				X		X		X
1973	X	X	X	X	X					X				X		X	X	X
1974	X	X	X	X	X					X				X		X	X	X
1975	X	X	X	X	X					X		X		X		X	X	X
1976	X	X	X	X	X					X				X		X	X	X
1977	X	X	X	X	X					X	X			X		X	X	X
1978	X	X	X	X	X				X	X	X			X		X	X	X
1979	X	X	X	X	X				X	X	X			X		X	X	X
1980	X	X	X	X	X				X	X	X			X		X	X	X
1981	X	X	X	X	X				X	X	X		X	X		X	X	X
1982	X	X	X	X	X				X	X	X		X	X		X	X	X
1982/83	X	X	X	X[1]					X	X	X		X	X		X	X	X
1983/84	X	X	X	X[1]				X	X	X	X		X	X		X	X	X
1984/85	X	X	X	X[1]				X	X	X	X		X	X		X	X	X
1985/86	X	X	X	X[1]					X	X	X		X	X		X	X	X
1986/87	X	X	X	X[1]					X	X	X		X	X		X	X	X

[a] Analyte class, methodology reference:

1. organohalogen pesticides/metabolites, 91–103
2. polychlorinated biphenyls, 97, 98
3. organophosphorus pesticides/metabolites, 104–112
4. carbaryl (X) and other (X[1]) *N*-methyl carbamates, 113–119
5. *o*-phenylphenol, 120
6. dithiocarbamates, 121
7. amitrole, 122
8. ethylene dibromide, 123
9. early eluting industrial chemicals, 124
10. chlorophenoxy acids and pentachlorophenol, 99, 125–129
11. chloroethyl esters of fatty acids, 130, 131
12. nitrate/nitrite, 132
13. higher chlorinated dioxins (6–8 chlorines), 133
14. arsenic, 134–137
15. bromide, 138
16. cadmium, 139–146
17. lead, 144–148
18. mercury, 149–153

ments, and for nutritional elements, respectively, from FY 1961/62 through FY 1986/87.

As indicated in Table 5, strontium-90 and cesium-137 were determined between FY 1961/62 and FY 1966. Because radioactivity levels were declining as a result of the 1963 Limited Test Ban Treaty, FDA discontinued monitoring radionuclides in foods in 1967. The radionuclide portion of the Total Diet Study was reinstated in January 1973 to retain FDA's radiochemical analytical capability in case of a radiological incident. The new program included routine analysis for strontium-90, cesium-137, iodine-131, ruthenium-106, and potassium-40 and intermittent analyses for radium-226 and plutonium-239. Between January 1973 and mid-FY 1982, several (8–10) adult, infant, or toddler market baskets were analyzed (by commodity groups) for radionuclides each year; since the mid-FY 1982 revision, one complete collection (234 individual foods) yearly has been analyzed for radionuclides.

Table 8 identifies the types of residues and contaminants included in the Total Diet Study; Appendix C[1] provides an alphabetical listing of the organic residues and contaminants that are known to be detectable by the Total Diet Study. The methods used are also capable of detecting other organic compounds for which analytical behavior is not yet known. Table 6 indicates the years of analyses for these substances. Analyses have been performed routinely for residues of organohalogen, organophosphorus, and chlorophenoxy acid pesticide residues and for pentachlorophenol. Carbaryl or *N*-methyl carbamates have been routinely determined since 1964, and polychlorinated biphenyls have been routinely determined since 1971. Other organic residues or contaminants have been selectively determined. Arsenic has been routinely determined since FY 1964; total bromides were determined between FY 1964 and FY 1970. Cadmium analysis began in FY 1967, lead analysis began in FY 1973, and routine mercury analyses began in FY 1971.

The nutritional elements (Table 7) have been determined as part of the Selected Minerals in Foods Survey since FY 1973, beginning with selenium and zinc and expanding to other elements in FY 1974. Between FY 1974 and FY 1982 the nutritional elements were analyzed in periodic cycles of 6–9 elements per year for the 3 age–sex groups. Beginning in FY 1982/83, all 11 essential elements were analyzed each year.

Table 7. Nutritional elements—years analyzed and methods used (the Selected Minerals in Food Surveys)[a]

FY	Na	K	Ca	P	Mg	Fe	Zn	Cu	Mn	Se	I	Diet
1973							AAS			SPF		adult male
1974			AAS	C		AAS	AAS			SPF	CI	adult male
1975			AAS	C		AAS	AAS			SPF	CI	3 age–sex groups
1976			AAS[b]	C[b]	AAS[c]	AAS	AAS	AAS[c]	AAS[c]	RHE	CI[b]	3 age–sex groups
1977	AAS	AAS			AAS	AAS	AAS	AAS	AAS	RHE		3 age–sex groups
1978	AAS	AAS	AAS	C		AAS	AAS			RHE	CI	3 age–sex groups
1979			AAS	C		AAS	AAS			RHE	CI	3 age–sex groups
1980	AAS	AAS			AAS	AAS	AAS	AAS	AAS	RHE	CI[d]	3 age–sex groups
1981	AAS	AAS			AAS	AAS	AAS	AAS	AAS	RHE	CI	3 age–sex groups
1982	AAS	AAS			AAS	AAS	AAS	AAS	AAS	RHE	CI	3 age–sex groups
1982/83	ICP	ICP	ICP	ICP	ICP	ICP	ICP	ICP	ICP	RHE	CI	8 age–sex groups
1983/84	ICP	ICP	ICP	ICP	ICP	ICP	ICP	ICP	ICP	RHE	CI	8 age–sex groups
1984/85	ICP	ICP	ICP	ICP	ICP	ICP	ICP	ICP	ICP	RHE	CI	8 age–sex groups

[a] AAS = atomic absorption spectrometry (154–156); C = colorimetric method for phosphorus (157); CI = colorimetric method for iodine (158); ICP = sequential inductively coupled plasma spectroscopy (159); RHE = atomic absorption spectrometry with rapid hydride evolution (137); SPF = spectrophotofluorometric analysis (160, 161).

[b] Infant and toddler diets only.

[c] Adult diet only.

[d] Also analyzed by neutron activation analysis (162).

Analytical Methodologies

Tables 5–7 also indicate or give reference to the methodologies used to analyze the foods for the various analytes covered by the Total Diet Study. Although various methods have been used to determine the radionuclides (Table 5) in foods, the most consistently used methods have been beta-counting of the daughter product, yttrium-90 (85–87) for strontium-90; gamma-ray spectroscopy (88–90) for cesium-137, iodine-131, ruthenium-106, and potassium-40; and alpha spectroscopy for radium-226 (85).

The methodologies used to analyze for organohalogen, organophosphorus, and other pesticides and organic contaminants (91–133) have advanced throughout the history of the Total Diet Study, closely paralleling developments in analytical chemistry (Table 6). Early work used paper and thin-layer chromatography, polarography, colorimetry, and microcoulometric gas chromatography. For many years, organic residues have been determined principally by various gas chromatographic techniques. Currently, electron-capture and/or element specific detectors (i.e., halogen, nitrogen, phosphorus, sulfur) are used. Liquid chromatography is used to determine the *N*-methyl carbamates. Several different techniques to confirm substance identity, including mass spectrometry, are utilized. Because the purpose of this study is to determine intake levels and because many of the levels present are quite low, the analytical methods have been modified to achieve quantitative limits one-fifth to one-tenth of those used for regulatory monitoring by FDA.

The analytical methods used for determination of the toxic elements arsenic, lead, cadmium, and mercury (Table 6) have also reflected advances in analytical techniques (134–153). Cadmium was initially determined in FY 1967 by oscillographic polarography (139). Currently, either dry ash-graphite furnace atomic absorption (145) or dry ash-anodic stripping voltammetric procedures (144) are used, as they are for lead, which was originally determined (FY 1973) by atomic absorption spectrometry (AAS) (147, 148). Colorimetric arsenic analyses were initiated in FY 1964 (134, 135), and AAS analysis with rapid hydride evolution was adopted in FY 1976 (137). Mercury analyses originally used a colorimetric determinative procedure (149); a flameless atomic absorption technique has been used for over a decade (150–153).

Between FY 1973 and FY 1982, zinc, calcium, iron, magnesium, copper, manganese, sodium, and potassium were determined by AAS (154–156), and a colorimetric method was used for phosphorus (157) (Table 7). Beginning in FY 1982/83, inductively coupled plasma spectroscopy (159) was used for these 9 nutritional elements. Through FY 1975, spectrophotofluorometric analysis was used for selenium (160, 161), and from FY 1976 onward, selenium was determined by AAS analysis with rapid hydride evolution (137). A colorimetric method has been used for iodine (158) throughout the Total Diet Study; however, the FY 1980 Total Diet Study samples were also analyzed for iodine by neutron activation analysis by FDA personnel at the National Bureau of Standards in Gaithersburg, MD (70, 162). In addition, scientists in the Department of Biochemistry at the University of Min-

Table 8. Pesticide residues, industrial chemicals, and toxic elements detected in the Total Diet Study[a]

Chemical type	Residue type
Incidental additives	
organohalogens	pesticide
organophosphates	pesticide
N-methyl carbamates	insecticide
carbaryl (a carbamate)	insecticide
o-phenylphenol	fungicide
amitrole	herbicide
ethylene dibromide	fumigant, insecticide, nematicide
dithiocarbamates	fungicide
gas chromatographic early eluters	pesticide
chlorophenoxy acids	herbicide
other herbicides (e.g., triazines)	herbicide
pentachlorophenol	fungicide, wood preservative
plant growth regulators	limiter or increaser of plant growth
Intentional additives	
nitrites/nitrates	botulism preventive
Incidental/accidental additives	
polychlorinated biphenyls	industrial chemical
other industrial chemicals	industrial chemical
gas chromatographic early eluters	environmental contaminant
arsenic	environmental/industrial/agricultural contaminants
bromide	
cadmium	
lead	
mercury	
higher chlorinated dioxins (Cl_6–Cl_8)	

[a] See Table 6 for years of residue determinations.

Table 9. References for results of the Total Diet Study

FY	Radionuclides	Pesticide residues, industrial chemicals, toxic elements	Nutritional elements
1961/62	2, 4, 9	2, 14	60
1962/63	2, 4, 5, 9	2, 12, 14	
1964	5, 9	12–14, 22, 26, 28	
1965	9	14, 15, 16, 19, 22, 26, 28	
1966	9	16, 18, 19, 22, 26, 28	
1967		21, 22, 24, 26, 28	
1968		23, 24, 26, 28	
1969		25, 26, 28, 30	
1970		29, 30, 45	
1971		31, 45	
1972		33, 34, 37, 41	37
1973	9, 10	35, 36, 37, 45, 59	37
1974	9, 10	38, 45, 59	62, 63, 67, 71, 72
1975	9, 10	39, 42, 45, 59	62, 63, 64, 67, 71, 72
1976	9, 10	43–45, 59	63, 67, 71, 72
1977	10	46, 47, 59	64–68, 71, 72
1978	10	48, 49, 59	63, 65–68, 71, 72
1979	11	53, 54, 59	67, 68, 71, 72
1980	11	55, 56	70–72
1981/82	11	57, 58	71, 72
1982/83			73
1983/84			73
1984/85			
1985/86			
1986/87			

nesota (64–66) determined fluoride in Total Diet Study composites using an ion selective electrode.

Data Transfer

Since the 1982 revision of the Total Diet Study, analytical data have been transferred from the Total Diet Laboratory in Kansas City, MO, via the FDA Parklawn Computer Center in Rockville, MD, to CFSAN in Washington, DC, by way of the ORA computerized Laboratory Management System. The Total Diet Laboratory has a VAX 11/750 computer into which the data are entered. Before this computerized system was instituted, hard copies of data were mailed to CFSAN from the Kansas City or other district laboratories and entered into CFSAN's computer system for analysis. Hard copies of radionuclide data are sent from WEAC to CFSAN.

Publication of Results

Table 9 indicates the primary references for results of the Total Diet Study by fiscal year. This list does not include secondary references (those that quote or retabulate Total Diet Study results or that describe the study for the lay person). Some of the results for radionuclides, pesticide residues, toxic elements, industrial chemicals, and nutritional elements have been released in the FDA Compliance Program Evaluations (10, 59, 72). Some of these evaluations (59, 72) are available from the National Technical Information Service. Pesticide residue and contaminant results for FY 1966 through FY 1976 have been published in the *Pesticides Monitoring Journal* (17, 18, 21–25, 27–31, 33, 36, 38, 39, 42–44). Since the discontinuation of this journal in 1981, the results have been published in the *Journal of the Association of Official Analytical Chemists* (46–49, 51, 53–58). Results for the nutritional elements (the Selected Minerals in Foods Survey) have generally been published in the *Journal of the American Dietetic Association* (62, 63, 68, 70, 71, 73).

Notable Results

Because the number of organic pesticides and industrial chemicals included in the current Total Diet Study (Appendix C[1]) is much larger than the number of chemicals measured in the earlier program, the number of these substances detected in each collection has greatly increased from about a dozen initially to over 60 currently. Calculated dietary intake levels of pesticides have generally been less than 1% of the Acceptable Daily Intakes (ADIs) established by expert committees of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations. An exception to this is that intakes of some persistent chlorinated pesticides were much closer to their ADIs in the 1960s and 1970s. It is apparent from current data, however, that intakes of these persistent materials have declined steadily since cessation of their agricultural uses over a decade ago. This is best typified by dieldrin, the only pesticide ever to approach its ADI. Today, dieldrin intakes are about one-twentieth of their level 2 decades ago. Although the intakes of persistent chlorinated pesticides have declined dramatically in the last 20 years, residues continue to occur frequently at low levels. This is particularly true for foods of animal origin.

In the 1971 Total Diet Study, polychlorinated biphenyl (PCB) residues were found in a ready-to-eat breakfast cereal. Followup investigations revealed that the chemical migrated from the paperboard package which had been produced from PCB-contaminated recycled paper. This finding ultimately led to regulations limiting the PCB content of paperboard intended for food contact use. In the 1975 Total Diet Study, a residue of the preservative and fungicide pentachlorophenol (PCP) was detected in unflavored gelatin. PCP had been used to treat hides in slaughterhouses, and many of these hides were ultimately shipped to gelatin manufacturers. This use of PCP had been discontinued by the U.S. industry several years prior to the finding; investigation revealed that the sample in question was a mixture of domestic and Mexican gelatin. The Mexican gelatin was found to contain the PCP and was ultimately diverted from food use.

Among the results for the nutritional elements, the most notable has been the high levels of iodine in U.S. diets (71, 72). Intake levels of iodine have ranged from 1.7 to over 10 times the Recommended Dietary Allowances (RDA) for this element. The major sources of this element have been dairy products, grain products, and foods containing the iodine-containing red food color FD&C No. 3.

Resources

Currently, the Total Diet Study requires 29 person-years of effort (the equivalent of 29 full-time employees per year). This time is divided among the staff at CFSAN, the Total Diet Analytical and Research Laboratories, the WEAC Laboratory, the district offices, and ORA. The cost of the program (including the salaries of the FDA employees, but excluding the overhead costs of facilities) is about 2 million dollars per year. These costs include the purchase and transportation of the foods; laboratory chemicals; equipment maintenance; new equipment; inspector transportation expenses for collecting foods; periodic Total Diet Study meetings in Kansas City; computer hardware, software, and maintenance; and the contract for food preparation. Appendix D[1] provides an overview of the activities and interaction of FDA employees involved with the Total Diet Study.

Program Advantages

The Total Diet Study provides for annual monitoring of a broad range of pesticide residues, contaminants, and nutrient elements in foods in their table-ready forms, and it allows for yearly estimates of intake of pesticide residues,

contaminants, and nutrient elements of selected age–sex groups based on laboratory analyses. This type of information, which is available from no other source, is essential to monitor the safety and quality of the U.S. food supply and to identify potential public health problems. The Total Diet Study played a major role in early identification of unusual findings (e.g., polychlorinated biphenyls, high levels of dieldrin, and excessive levels of iodine in the food supply), and it provides a check on the effectiveness of current regulations and surveillance activities in protecting the public from harmful levels of pesticide residues, contaminants, or nutrient elements in foods. The demonstration of very low levels or absence of residues or contaminants in foods and the demonstration of adequate nutrient element intake by the Total Diet Study are also of importance as they point out the continuing safety of the U.S. food supply. The Total Diet Study also provides an invaluable baseline reference for determining the impact on the food supply of environmental contamination accidents, e.g., the April 1986 nuclear reactor accident in Chernobyl, Ukraine, U.S.S.R.

Data from the Total Diet Study have been widely used and accepted by other government agencies, industry, and other professional groups. The Total Diet Study program, which has been operating for 25 years, is a well-organized, efficient system offering on-site consistency of analysis, analytical methodology, and personnel. Without this program, many expensive, individual surveys would have to be instituted to respond to congressional, consumer, and other requests made to FDA.

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METHOD PERFORMANCE

More Powerful Peroxide Kjeldahl Digestion Method

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Enhanced ammonia recovery and a simplified method are described for a rapid Kjeldahl digestion using sulfuric acid and hydrogen peroxide as the sole digestion reagents. This micro procedure uses a Vigreux fractionating head fitted to a 100 mL volumetric flask and a hot plate with a solid-state controller. Continuous-flow peroxide addition is controlled by a capillary funnel, and fumes are evacuated through a side-arm vent leading to a water aspirator. Complete recovery of nitrogen from the refractory compound, nicotinic acid, is obtained with less than 10 min digestion. The described method reduces digestion time by 25-50% over the open-manifold peroxy method. A digestibility index (DI), scaled 0-10, establishes the difficulty of digestion for each sample and assigns values to compounds. A useful tool for determining the minimal amount of reagent and digestion time required, the DI assigns zero for compounds not needing digestion and 10 for nicotinic acid. Digested samples obtained from the described method are suitable for direct colorimetric analysis of many elements in addition to Kjeldahl nitrogen. Distillation of the digested sample is not required.

In the conventional determination of Kjeldahl nitrogen, a sample is heated in concentrated sulfuric acid with salt and metal catalysts added to speed the release of ammonia from organic matter. Distillation into an acid solution followed by titration completes the test.

Because large amounts of equipment, reagents, and time are consumed by traditional Kjeldahl methods, alternative methods have received extensive study. Several researchers have proposed use of hydrogen peroxide as a digestion reagent. Kleemann (1) was able to speed the reaction by adding hydrogen peroxide to the sample followed by digestion with sulfuric acid and potassium sulfate. Koch and McMeekin (2) obtained rapid oxidation with complete retention of ammonia–nitrogen by carbonizing the sample in sulfuric acid prior to the dropwise addition of hydrogen peroxide with heating. Others have reported the importance of precarbonization and multiple additions of peroxide (3) and the need to heat the digestion mixture after initial clearing (4).

We have observed that the effective use of hydrogen peroxide requires a high residual concentration of peroxide at high temperature for sufficient time to decompose organic material (5). By carbonizing the sample in concentrated sulfuric acid before the continuous-flow addition of a hydrogen peroxide–sulfuric acid reagent ("peroxy" reagent), we are able to achieve rapid digestion (as much as 25 times faster than conventional procedures) for a variety of samples without the use of metal catalysts. Digestion time for the difficult-to-digest, refractory compound, nicotinic acid, is 17.5 min (5 min carbonization + 12.5 min flow digestion)—far less than the 3 h that Shirley and Becker (6) required for 100% recovery of nitrogen from nicotinic acid when using the best of the various Kjeldahl catalysts available in their laboratory (Figure 1).

Peroxy digests, prepared without mercury or other metal catalysts, are suitable for direct colorimetric determinations of phosphorus, calcium, magnesium, iron, copper, manganese, and zinc as well as Kjeldahl nitrogen (7, 8).

Despite wide acceptance of the peroxy method by many analysts (8, 9), we were concerned about occasional low nitrogen values obtained from standard test samples. A thorough check of the system was undertaken to determine if nitrogen losses were occurring, and if so, the source of such losses. Slight nitrogen loss was identified as spray carry-over in the ventilation air flow of the digestion apparatus. Therefore, one of our goals was to redesign the digestion system to achieve complete nitrogen recovery 100% of the time.

We also hoped to simplify use of digestion reagents and to standardize the analytical procedure. As we described earlier (5), the "peroxy" method requires analysts to prepare a 4:1 hydrogen peroxide–sulfuric acid digestion reagent by adding 400 mL of 50% hydrogen peroxide to a 500 mL graduated cylinder followed by the slow addition of 100 mL sulfuric acid. This reagent is a strong oxidizer requiring careful handling and storage in a vented container. For this reason, we sought a procedure by which the acid and peroxide could be used with greater ease and safety.

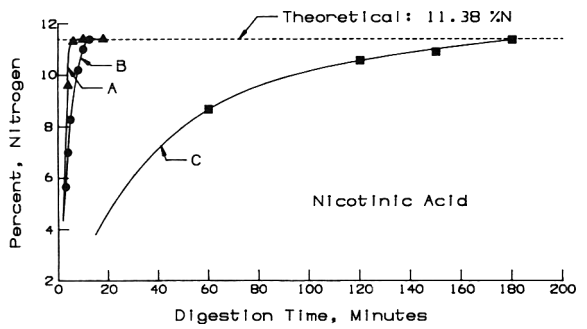

Figure 1. Nitrogen recovery curves for nicotinic acid digested by conventional Kjeldahl method and by 2 peroxide–sulfuric acid (peroxy) methods. A, Vigreux manifold, peroxy method; B, open manifold, peroxy method; C, conventional Kjeldahl method (6).

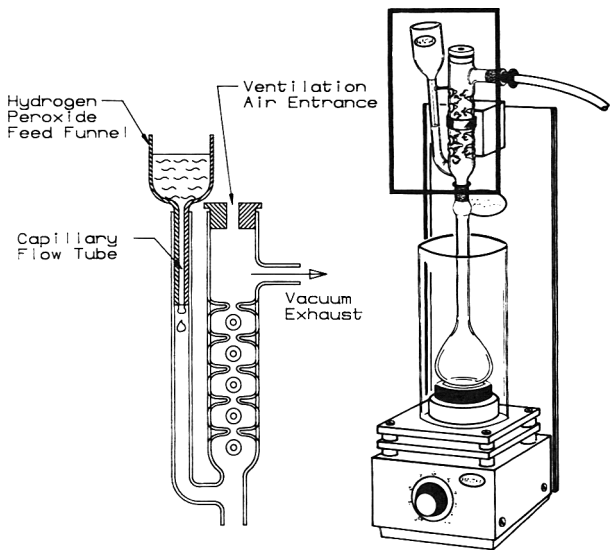

Figure 2. Vigreux capillary-flow digestion manifold, digestion flask, and heater with details of manifold design.

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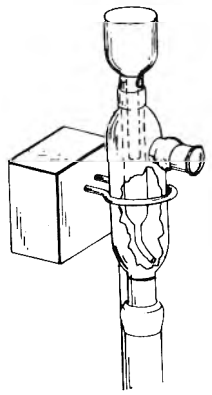

Figure 3. Open-head capillary-flow digestion manifold.

Still another aim was to simplify the actual digestion procedure. During our investigations, it became obvious that an easy-to-use, reliable index of sample digestibility, one applicable to all methods of digestion, would be useful in forecasting the degree of treatment (i.e., heating time, volume of reagents) needed to bring about complete digestion for a variety of samples.

Experimental

Reagents

All organic compounds such as amines, amino acids, nitrogen heterocycles, and porphyrins are reagent grade, used as received.

(a) *Digestion.*—Concentrated sulfuric acid for carbonization, and hydrogen peroxide (50%) and concentrated sulfuric acid for digestion. Where 50% peroxide is unavailable, 30% solutions may be used, but with ca 50% more digestion time. (*Caution:* Hydrogen peroxide and concentrated sulfuric acid are strong oxidizing agents. Wear protective coverings, especially on hands, face, and eyes.)

(b) *Colorimetric assay for nitrogen.*—Nessler reagent (Hach 21194), treated during manufacture to remove any mercurous ion impurity. Dilute sample with 0.1 g/L solution of polyvinyl alcohol (av. mol wt 10 000).

(c) *Amino acids.*—Purity 99% (Crescent Chemical Co., Hauppauge, NY 11788, and Calbiochem-Behring, San Diego, CA 92112).

(d) *Nicotinic acid.*—Recrystallize before use.

(e) *Reference materials.*—Bovine Liver (NBS 1577); Orchard Leaves (NBS 1571); and fish meal, feather meal, and soybean meal previously tested (8) by AOAC methods (10).

Table 1. Digestion indices obtained using open-head and Vigreux-head methods

Material	Open head, H_2O_2 flow time, min	Vigreux head, 0–10 scale
Ammonium chloride	0	0
Ammonium sulfate	0	0
Ammonium perchlorate	0	0
Ammonium *p*-toluenesulfonate	0	0
Aspartic acid	1.0	0.8
Soybean meal	3.3	3.3
Feather meal	3.5	7.5
Tryptophan	4.7	6.7
Fish meal	10.0	10.0
Lysine HCl	15.0	8.3
Nicotinic acid	12.5	10.0

Apparatus

(a) *Digestion.*—100 mL volumetric flasks with ground glass joints; 25–250 W disk element heater with solid-state controller (Hach Model 21400 Digesdahl apparatus) (Figure 2); glass digestion manifold designed in the form of a Vigreux fractionating column with outer manifold containing sidearm vent leading to water aspirator for fume removal and capillary tube/funnel for controlling reagent addition at 3 mL/min (Hach Model 22873 manifold) (Figure 2).

(b) *Ammonia measurement.*—Diluter/dispenser for withdrawing aliquots of digest, diluting with polyvinyl alcohol solution, and adding Nessler reagent for ammonia determination; single-beam spectrophotometer set at 460 nm with a 2.5 cm pathlength pour-through cell for colorimetric assay.

Sample Preparation

Grind all grain, cereal, and feed samples in a chopper mill to a fine powder (less than 1 mm or 20 mesh) before digestion. Before milling coarse samples such as hay, grind first in a food blender.

Procedure

(a) *Sample digestion.*—Weigh 0.25 g sample on weighing paper or in tared container. Fold sample into paper and transfer entire packet to 100 mL volumetric (digestion) flask. If using tared container, transfer with funnel, making sure all particles are transferred quantitatively.

Add 3 mL concentrated H_2SO_4 to digestion flask. Preheat hot plate for ca 10 min and place flask on preheated plate. (Samples were digested in a 2 in. diam. ceramic heater with 150 W input.) Connect fractionating head to flask and start vent system (aspirator).

Heat ca 4 min to char sample and bring H_2SO_4 to boiling. At this point, acid will reflux part way up the inside of the flask. Continue heating and add desired volume of 50% H_2O_2 (see *Note* following *Colorimetric assay*) to capillary flow funnel. Make sure capillary tube is not air bound and peroxide is flowing. After addition of peroxide is complete, continue heating ca 1 min. (Allowing 1 min additional heating after peroxide flow has stopped is a safety precaution that provides sufficient time for excess peroxide to decompose.) Remove flask from hot plate, cool, and dilute to 100 mL with deionized water; mix.

This sample may be analyzed directly for protein nitrogen. The lack of interfering substances such as heavy metal catalysts also allows analysis of digested samples for phosphorus, calcium, magnesium, potassium, iron, copper, manganese, zinc, and other metals (7, 8).

(b) *Nitrogen recovery curves.*—Repeat digestion and analysis for each sample, using different volumes of peroxide (varying digestion times). Graph nitrogen recovery vs digestion (peroxide flow) time to determine minimum time required for quantitative recovery. (The time needed to obtain 99% recovery is used in this study.) Although helpful for minimizing digestion time, it is not necessary to determine nitrogen recovery curves for routine analysis.

(c) *Colorimetric calibration.*—Prepare ammonium chloride standards equivalent to 0–100% protein (% protein = % N × 6.25) and analyze by Nesslerization using procedure of Hach et al. (5). Determine best-fit linear calibration line.

(d) *Colorimetric assay.*—Use either semi-automated (diluter/dispenser) or manual procedure for Nesslerization given by Hach et al. (5). Read results in spectrophotometer at 460 nm. On the basis of linear calibration curve, convert absorbance readings to concentration (% N or % protein).

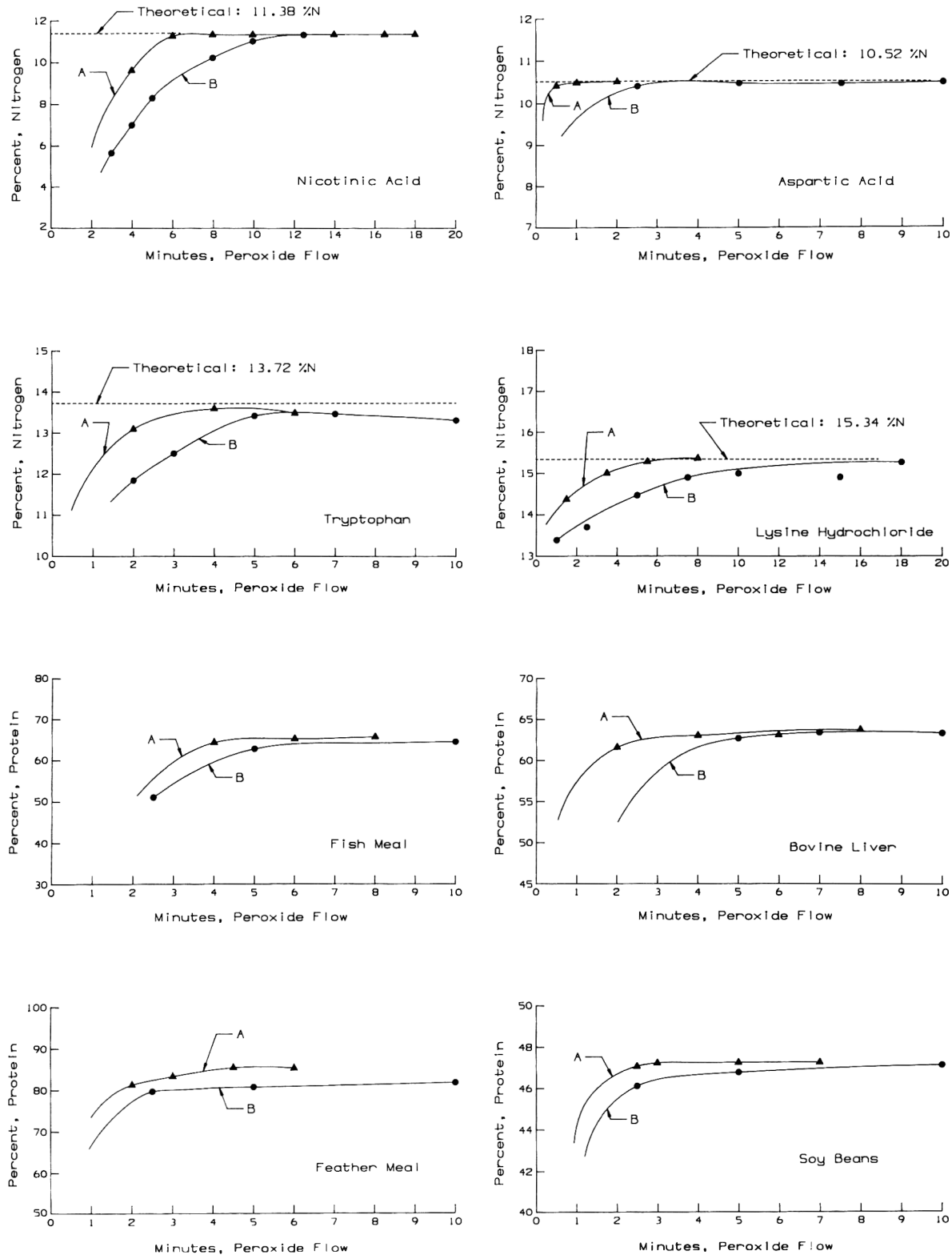

Figure 4. Nitrogen recovery curves obtained using open-head and Vigreux digestion manifolds and peroxide–sulfuric acid methods. A, Vigreux manifold; B, open-head manifold.

Note: Add as little as 6 mL or as much as 20 mL H_2O_2, depending on sample susceptibility to digestion (Digestibility Index, Table 1). Laboratories running sporadic TKN measurements or those testing a variety of samples can ensure complete digestion of unknown samples by adding 20 mL peroxide. Using slightly more peroxide than necessary does not cause problems. However, laboratories regularly testing similar samples will save considerable time by determining the DI and using the minimal amount of reagent (and time) suggested by the DI.

Results and Discussion

To determine if the ventilating air stream was carrying spray out of the open-headed digestion flask (Figure 3), the stream was passed through an all-glass, water-cooled condenser; the condensate was collected and analyzed for am-

Table 2. Comparison of protein values obtained using open-head, Vigreux, and AOAC Kjeldahl methods

Sample	Crude protein ($N \times 6.25$), %: H_2O_2–H_2SO_4 methods, Peroxy[a,b]	Vigreux[c]	AOAC Kjeldahl[a,d]
Soybean meal	47.2	47.3	46.4
Fish meal	65.5	65.9	66.1
Feather meal	85.3	85.3	84.8
Bovine Liver (NBS 1577)	67.1	63.8	66.3 ± 3.8 (NBS certified)
Orchard Leaves (NBS 1571)	17.3	17.3	17.3 ± 0.3 (NBS certified)
Nicotinic acid	71.2	71.1	71.1 (calcd)
Tryptophan	85.2	85.4	85.7 (calcd)

[a] Brayton (8).
[b] Open head, 4:1 peroxide reagent (50% H_2O_2–H_2SO_4).
[c] Vigreux fractionating head, 50% peroxide.
[d] Sec. 7.015 (10).

monia. Results showed that significant amounts of ammonia were carried over when hot plate operating wattage was much above 200 W. This was particularly evident when longer peroxide flows were used for digesting resistant materials. Carry-over losses for very refractory substances amounted to as much as 0.4–0.8% absolute loss. For example, losses were as high as 0.29% protein for a sample containing 35% protein and 0.46% for one containing 57% protein.

In an attempt to eliminate nitrogen carry-over, a new head was designed in the form of a Vigreux fractionating column (Figure 2). Ammonia determinations on the condensate from the airstream vent showed that this column is able to trap all escaping ammonia and consistently reduce carry-over losses below 0.3% (absolute). Thus a sample containing 50% protein will register 49.85% or better.

The Vigreux column produces an additional benefit: digestion speed is doubled. During digestion, we believe the column head retains hydrogen peroxide by fractional distillation while allowing water vapor to pass to vent. The boiling points of hydrogen peroxide and water are 150°C and 100°C, respectively, so water is removed readily during fractionation; concentrated peroxide returns to the digestion flask, thus allowing an increase in digestion efficiency.

Studies with the Vigreux fractionating head showed the 4:1 peroxide–sulfuric acid reagent used in earlier work could be replaced by a "straight" 50% hydrogen peroxide without loss of efficiency or performance (Figure 4). Being able to use peroxide in place of the mixed reagent is a decided advantage because of the precautions needed for premixing peroxide with concentrated sulfuric acid.

When straight 50% peroxide was used with the open-head apparatus, it flowed into the hot digest and partially volatilized before fully reacting with the sample. Under these conditions, fume-removal air flow carried off some of the unreacted peroxy species. Therefore, peroxide was premixed with sulfuric acid to raise the boiling point of the digestion mixture and thus help retain peroxide in the hot digest. In contrast, the Vigreux head allows use of 50% hydrogen peroxide because the fractional distillation function of the column retains evaporating peroxide and returns it to the digestion flask. Peroxide builds up to a higher concentration in the boiling acid and, for this reason, increases the rate (speed) of sample digestion.

A comparison of protein values obtained using Vigreux fractionating head (50% peroxide), open-head (peroxy), and AOAC Kjeldahl methods is presented in Table 2. The data were obtained from single sample digestions. Except for the Bovine Liver sample which we believe deteriorated, results obtained with the Vigreux fractionating method show equal or enhanced recovery over those obtained with the previous open-head peroxy procedure. Results from both methods are comparable to those obtained using AOAC methodology. Watkins et al. (9) also have reported good agreement between peroxy and AOAC Kjeldahl methods.

A series of curves (Figure 4) compares the digestion of several substances using both open- and fractionating-head methods. Heat input (hot-plate setting) was identical for all tests. The curves show the time required to achieve 99% protein recovery with the fractionating head is about 50–75% of the time needed when the open head is used.

A successful digestion requires 3 conditions: suitably high sulfuric acid temperature, adequate concentration of hydrogen peroxide in sulfuric acid, and maintenance of these conditions for sufficient time to allow complete digestion.

The duration of digestion is determined by the volume of peroxide fed to the system, optimal temperature of the hot plate, and rate and time of peroxide flow. Optimal flow rate for peroxide with the Vigreux manifold is 3 mL/min; time of flow (volume of peroxide used) is determined by the digestibility of the sample.

Using the optimal flow rate, the amount of time required for digestion depends on the digestibility of the sample. Decolorization and clearing of the charred sample usually occurs about 1 min after peroxide flow is initiated. In a careful study of the minimal time required to digest a variety of substances, complete nitrogen recovery was obtained for many samples immediately upon clearing. However, resistant materials such as nicotinic acid required ca 5 min continued peroxide digestion after clearing to obtain 100% nitrogen recovery.

A digestibility index (DI) has been proposed (5) to compare the ease or difficulty of digesting various materials by the open-manifold, peroxy method. Defined as the number of minutes of reagent flow required to secure 99% protein recovery under described conditions, the index correlates poorly with data obtained using the Vigreux fractionating head and method of peroxide addition described in this paper. For this reason, we propose establishment of an arbitrary but enduring DI with a scale of 0–10. Zero is reserved for ammonium compounds not requiring digestion (e.g., ammonium chloride) and 10 is arbitrarily assigned to the refractory compound, nicotinic acid. (Although more resistant compounds exist, nicotinic acid is the most digestion-resistant compound normally encountered.) The DI for several substances is presented in Table 1. For comparison, the "old" index (based on peroxide flow time) is presented in the same table.

The use of a fractionating reflux head for the peroxide Kjeldahl digestion achieves 3 significant improvements over the previously described open manifold. Digestion speed is doubled; spray loss of ammonia is eliminated, resulting in more accurate protein measurement; and digestion is accomplished with aqueous 50% hydrogen peroxide solution rather than a hydrogen peroxide–sulfuric acid mixture.

The overall system—peroxide digestion and direct colorimetric analysis—is highly accurate for all forms of nitrogen determinable by the Kjeldahl method. Protein content of most materials can be measured in 9 to 12 min from the time the sample is weighed to completed ammonia determination. Because the digest is not contaminated by salts and metal catalysts, it also is suitable for the determination of other elements (7).

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ANTIBIOTICS

Evaluation of AOAC Microbial Diffusion Procedure for Analysis of Chlortetracycline in High Mineral Feeds

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The performance of the AOAC microbial diffusion assay procedure for the analysis of chlortetracycline was evaluated in mixed feeds and premixes as well as laboratory-prepared feeds, all with various mineral contents. In 100 mixed feeds with a calcium content ranging from <1 to >22%, no relationship was shown between calcium content and incidence of chlortetracycline deficiency. In 106 premixes, a relationship was shown between chlortetracycline content and adherence to guarantee: below 10 g/lb, the incidence of deficiency was high; in premixes containing 20 g chlortetracycline and above, the incidences of deficiency were less than 5%. In laboratory-prepared feeds containing 12-15% calcium, grinding and storage at both 4°C and at room temperature (20°C) caused no decrease in the chlortetracycline concentration. Only storage of the ground feed at 37°C caused a distinct loss of activity. The temperature-related loss was attributed to the epimerization of chlortetracycline.

For at least 10 years, considerable concern has been voiced over the capability of the official AOAC diffusion methods (1) to assay for chlortetracycline and oxytetracycline in feeds having a high mineral content. It has been quite common to question the results obtained with the official diffusion procedures in so-called "high mineral feeds" when the assayed potency was below guaranteed tolerance levels. Conversely, it has been a modest heresy to defend any low results obtained in the analysis of these feeds by the official diffusion methods. To resolve the "high mineral feed" problem, a modification of the official assay procedure was suggested, such as the use of EDTA as a chelating agent to overcome the binding of the tetracyclines to Group II and Group III cations. However, the use of EDTA did not resolve the problem; the results of an AAFCO check sample assay that included the modification were essentially the same as those from the official AOAC microbial method (E. Glocker, private communication, 1983).

There is little doubt that the tetracyclines can chelate with Group II and Group III cations (2). However, the concept that such chelations occur in animal feeds between solid materials is tenuous, at best. In addition, if such chelation reactions occurred readily in the solid phase, it would be completely illogical for manufacturers of tetracycline-containing premixes to formulate with calcium carbonate as a carrier; yet, this is a common practice.

It was the purpose of the study reported here to investigate the capability of the official AOAC microbiological assay procedures (1) to determine the tetracycline content in feeds containing high levels of minerals. Chlortetracycline was chosen as the pilot antibiotic because of its ease of assay by different analytical techniques, thus allowing for greater insight into any reactions that could occur.

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Experimental

Methods

(a) Microbiological assay procedure for chlortetracycline in feeds and premix, **42.232-42.235** (1).

(b) Fluorometric assay procedure for chlortetracycline in feeds (3).

Materials

(a) *Premixes.*—Containing label guarantee of 50 g chlortetracycline (CTC)/lb.

(b) *High mineral feed.*—Manufactured by Furst-McNess Co., Freeport, IL: Beef Balancer 50, containing ingredients and guarantees listed in Table 1.

Results and Discussion

Because of the recurring concerns of control officials and industry representatives that the official AOAC microbiological methods for chlortetracycline and oxytetracycline (1) could not measure accurately the potency of these antibiotics in animal feeds containing high mineral levels, the authors undertook a review of the performance of the diffusion methods. The literature provided little or no insight into the problem because of a paucity of published information on the subject. Thus, it appeared that a retrospective review of the analytical results obtained in 1984 in the laboratories of the

Table 1. Contents of high mineral feed

Guaranteed analysis, %:

Crude protein, min. (this includes not >48.0% equiv. protein from nonprotein N)	50.0
Crude fat, min.	0.0
Crude fiber, max.	3.0

Minerals, %:

Calcium, min.	12.8	Zinc	0.044
Calcium, max.	15.3	Iron from iron sulfate	0.033
Phosphorus, min.	1.0	Manganese	0.014
Salt, min.	7.3	Copper	0.013
Salt, max.	8.7	Iodine	0.013
Potassium	12.7	Cobalt	0.0008
Sulfur	1.1	Selenium	0.00036
Magnesium	0.5		

Vitamin potency (min. USP units/16):

Vitamin A	50 000
Vitamin D-3	5 000
Vitamin E	50

Ingredients:

Calcium carbonate, potassium chloride, urea, salt, soybean meal (dehulled, solvent-extracted), monoammonium phosphate, magnesium sulfate, potassium sulfate, mineral oil, vitamin A supplement, D-activated animal sterol (source of vitamin D-3), vitamin E supplement, iron sulfate, zinc oxide, manganous oxide, copper oxide, ethylenediamine dihydriodide, cobalt carbonate, sodium selenite, artificial flavor

Table 2. Relationship of assay results for CTC in mixed feeds with the guaranteed analysis and calcium content (CTC as the sole drug)

Calcium content, %	Frequency of occurrence at percent of guaranteed analysis						
	>100	90–100	80–90	70–80	60–70	50–60	<50
<1	2	2	3	0	4	1	1
1–2	2	1	4	1	1	2	—
2–3	0	0	1	3	1	0	—
3–4	0	0	2	1	1	2	—
4–5	0	0	1	2	1	1	—
6–10	1	0	1	1	1	1	—
>10	3	0	0	0	2	0	—

Indiana State Chemist for feeds containing CTC and varying mineral content could provide the analytical performance and product-related information necessary to assess the problem. Because of the volume of data, CTC was used as the pilot compound.

For the review, mixed feeds with a guaranteed analysis were used; no special mixes were included. Calcium levels were available for all but one of the feeds reviewed. Premixes were also reviewed. The criteria used to examine the data were as follows: (a) deficiency of CTC for mixed feeds was defined as less than 70% of the guaranteed analysis; (b) premixes were deemed deficient in CTC when the potency was below 95% of guarantee. The mixed feeds were organized into 3 categories: (*1*) feeds that contained CTC as the only medication, (*2*) feeds that contained CTC and a sulfonamide, and (*3*) feeds having a combination of CTC, a sulfonamide, and penicillin.

Table 2 shows the relationship of the potencies measured to the guaranteed analysis as a function of the calcium content of the feeds in category 1 (containing only CTC). Of the 50 feeds in this category, 31 were acceptable and 19 were deemed deficient. There was neither a pattern nor any correlation between deficiency and calcium content. Of the 19 feeds deemed deficient, 10 had calcium levels less than 3%; of the 31 feeds "meeting" the guarantee, 19 had a calcium content less than 3%; of the 19 feeds deemed deficient, 9 contained calcium levels greater than 3%. If there was an analytical "failure" related to the mineral content, i.e., calcium content, some pattern should have been noted; none was.

Table 3 shows the relationship between the concentration of CTC, feed type, and frequency of deficiency. Neither the feed type (category 1) nor the concentration was a factor. Of the feeds deemed deficient, excluding one formulated to contain 50 g CTC/ton and found to contain 5 g/ton, the measured potencies ranged from 51.0 to 67.6% of the guarantee. The coefficient of variation for this group of feeds was 9.7%. This was a remarkably uniform level of deficiency; no explanation based on fact was available to describe this observation. There exists the possibility that some feeds are being formulated to meet the permissible analytical value of 70% of guarantee.

Table 3. Relationship of concentration and feed type to deficiency

Feed type	Frequency of occurrence at g/ton guaranteed analysis			
	<50	50–100	100–200	>200
Swine	—	4	—	2
Sheep	2	2	—	1
Cattle	2	—	3	2

Table 4. Relationship of assay results for CTC in mixed feeds with guaranteed analysis (CTC and a sulfonamide as the drugs)

Calcium content, %	Frequency of occurrence at percent of guaranteed analysis				
	>100	90–100	80–90	70–80	<70[a]
<1.00	2	4	5	3	1
1–2	3	5	6	3	4
2–3	1	2	1	—	—

[a] Calcium content of the 5 feeds below 70% level ranged from 0.86 to 1.80%. All samples were deficient; one additional sample was deficient in the sulfonamide.

Table 4 summarizes the distribution of analytical results for the 40 feeds containing CTC and a sulfonamide (category 2). Thirty-three of the feeds guaranteed 100 g CTC/ton and a constant sulfonamide level, indicating an abundance of a single species feed (swine feed). The calcium content of feeds in this category ranged from 0.21 to 2.20%. The 5 deficient feeds had a calcium content ranging from 0.86 to 1.80%; the 6 feeds with assayed potencies above 100% of guarantee contained calcium levels of 0.80 to 1.30%. The distribution of results, although not classical Gaussian, did demonstrate what might be the expectation of meeting guarantee: a small percentage above 100%, a small percentage below the tolerance, and the majority of feeds within tolerances.

The third category was feeds that contained the combination of CTC, a sulfonamide, and penicillin; this group was represented by only 9 feeds. The calcium levels in this category ranged from 1 to 15%. Two of the 9 feeds were deemed deficient, and one feed assayed above 100%. Table 5 shows the distribution of the assay results as related to the calcium content. As noted previously, no relationship between CTC deficiencies and calcium content could be seen.

This overview of the results indicated no relationships between calcium content and the incidence of deficient chlortetracycline assays. If a relationship existed between calcium content and low assays, the greatest number of low assays would occur in feeds having calcium levels above 2–3%. Neither a relationship nor a trend toward such a relationship was found.

The analytical results for 106 premixes were reviewed to determine if any analytical patterns could be noted. Of the 106 premixes, 51 premixes contained CTC, a sulfonamide, and penicillin, 36 premixes contained only CTC, and 19 contained CTC and a sulfonamide. Table 6 shows the results of the microbial assays and the relationship between adherence to guarantee and the potency of the premix. In the 106 premixes, 28 had CTC potencies greater than 105%; 30 were greater than 100% but less than 105%; 26 were in the 95–

Table 5. Relationship of assay results for CTC in mixed feeds with guaranteed analysis (CTC, sulfonamide, penicillin as the drugs)

Calcium content, %	Frequency of occurrence at percent of guaranteed analysis				
	>100	90–100	80–90	70–80	<70
<1	—	—	—	—	—
1–2	—	—	—	1	1
2–3	—	—	—	—	—
3–4	—	2	1	—	—
4–5	—	—	—	—	—
5–6	1	1	—	—	—
6–10	—	—	—	—	1
10	—	1	—	—	—
>10	—	—	—	—	—

Table 6. Summary of the analytical results for all premixes, and relationship of product concentration to guaranteed analysis

Potency CTC, g/lb	Frequency of occurrence at percent of guaranteed analysis					
	>105	100–105	95–100	90–95	85–90	<85
≤2–0	2	7	11	—	5	8
2–7	2	2	1	—	2	4
10	1	1	—	—	—	1
20	14	14	10	—	—	1
40	—	2	—	—	—	—
50	8	4	4	—	1	—
70	1	—	—	—	—	—

100% of the guarantee range, and 22 premixes were below the 95% potency level. In the 106 premixes, 18 were deficient in sulfonamide content, and 3 were deficient in penicillin. One premix showed a deficiency in all 3 drugs; another premix showed a deficiency in both sulfonamide and penicillin. Based solely on the deficiency parameters previously stated, 20.8% of all premixes were deficient in CTC content while 25.7% of the sulfonamide-containing premixes were deficient in sulfonamide. For the 2 g or less CTC/lb premixes, 39.4% of the premixes were deficient; 54.5% of the premixes in the 2–7 g CTC/lb premixes were deficient, and 1 of 3 of the premixes containing 10 g CTC/lb was deficient. In contrast, premixes containing 20 and 50 g CTC/lb had deficiency incidences of 2.5 and 5.9%, respectively. For all products containing 20 g CTC or greater, the deficiency incidence was 3.4%.

It appears that the problem exists in those premixes where CTC concentrations are below 10 g/lb. This brings into question the tolerances for meeting guarantee, namely, what tolerances should be assigned to what concentrations? Admittedly, this is a regulatory function and not an analytical problem; but, since these procedures are used for product quality control, this aspect cannot be ignored. Should a ±30% or even ±25% tolerance be assigned to feeds regardless of label potency? If we are to expect that at 20 g/ton, the potency will be within 5 or 6 g/ton, why should a 200 g/ton feed be allowed to have an acceptable level of 140 or 150 g/ton? It would appear that the concept of tolerances being a straight percentage of label potency should be reexamined.

Table 7. Stability of laboratory-prepared high mineral feeds at different temperatures

Day	Feed A[a]			Feed B[b]		
	Initial assay		Total CTC,	Initial assay		Total CTC,
	μg/g	%	μg/g	μg/g	%	μg/g
			37°C			
0	1006.4	100.0	—	941.6	100.0	—
4	1048.5	104.2	—	1001.2	106.3	—
17	902.9	89.7	—	798.8	84.8	—
22	896.6	89.1	943.4	750.8	79.7	894.7
29	859.9	85.4	936.9	672.6	71.4	866.1
			20°C			
0	1006.4	100.0	—	941.6	100.0	—
4	1121.3	111.4	—	889.8	94.5	—
17	992.6	98.6	—	875.9	93.0	—
22	1135.0	112.8	1057.0	917.6	97.5	906.1
29	1125.6	111.8	1040.6	879.6	93.4	899.3
			4°C			
0	1006.4	100.0	—	941.6	100.0	—
4	1043.6	103.7	—	950.7	100.9	—
17	1000.2	99.4	—	883.5	93.8	—
22	1047.7	104.1	954.0	919.9	97.7	909.8
29	1052.5	104.6	959.0	926.7	98.4	921.4

[a] Premix for Feed A assayed 49.95 g CTC/lb. The feed was prepared by adding 2.5 g premix to 250 g mineral mix. Theoretical concentration 1089.3 μg CTC/g. Feed was 92.4% of theoretical.

[b] Premix for Feed B assayed 45.29 g CTC/lb. The feed was prepared by adding 2.5 g premix to 250 g mineral mix. Theoretical concentration 987.7 μg CTC/g. Feed was 95.33% of theoretical.

Table 8. Assay results, microbiological and fluorometric, in commercial feeds of high mineral content

Feed No.	Chlortetracycline, g/ton			Calcium guarantee, %
	Guarantee	Found		
		Micro.	Fluor.	
85-0637	1667	1548.7	1608.0	21.0–23.0
85-0638	3333	3421.9	4540.8[a]	20.5–22.5
85-0639	6667	5641.8	6742.6[b]	20.5–22.5
85-0640	1000	942.5	941.8	7.8–8.2
85-0642	2000	1773.3	2000.0	9.5–11.5
84-1995	1000	528.3[c]	669.4	7.5–8.0
84-2001	500	287.6[c]	279.8	3.5–4.5
84-2198	250	131.0[c]	145.4	3.5–4.0
84-2404	500	375.7	500.0	2.8–3.8
84-2525	140	90.7[c]	87.9	6.0–7.2
84-2526	140	69.7[c]	99.9	5.0–6.0
84-2623	400	383.2	363.6	2.0–3.0
84-2650	234	127.7[c]	119.1	12.8–15.3
84-2659	140	90.5[c]	115.4	3.75–4.75

[a] Contains 24.6% epimer (presumed).

[b] Contains 24.2% epimer (presumed).

[c] Deficient official AOAC procedures.

Retrospective studies were useful, but direct experimentation to create the purported problem would allow for better definition. Hence, laboratory-prepared feeds with a calcium content of approximately 12–15% and also containing other Group II and III cations were prepared using 50 g CTC/lb premixes (manufactured by 2 large pharmaceutical companies). The feeds were prepared to contain approximately 1000 μg CTC/g. The feeds were blended and finely ground to maximize any interactions in the feeds; they were assayed immediately after grinding and prior to being divided into 3 parts for storage at 37°C, 20°C, and 4°C. The feeds were assayed microbiologically (1) for potency over a 29 day period and fluorometrically (3) for total chlortetracyclines after 22 days of storage. The fluorometric procedure determines total CTCs, both epimer and active, since both are converted to the fluorophane isochlortetracycline. If the total CTC level remains constant and the microbial activity decreases significantly, epimerization has occurred. Other degradation products do not affect the fluorometric assay.

Table 7 shows the relative stability of the feeds at different temperatures. At both 20° and 4°C, the feeds were stable, indicating either that no complexing of CTC with the minerals occurred or, if it did occur, that such binding was broken by the conventional acid-acetone extraction. Fluorometric assays on days 22 and 29 confirmed the potency and indicated little if any epimerization.

Feeds A and B showed a loss of CTC activity after storage at 37°C for 29 days. Feed A lost 14.6% of the initial assay potency; activity in feed B declined 28.6%. The total CTC content of feed A (active and epimer) was 8–9% greater than the microbially measured activity after 29 days; feed B had a 12–13% greater total CTC content. There was significant loss of chlortetracycline activity in feed B, stored at 37°C, approaching 30% of potency. At 20° and 4°C, there was little

or no loss of potency in either feed. The hypothetical binding reaction between CTC and calcium and/or other trace minerals resulting in a significant loss of activity could not be produced; even grinding to enhance the potential of such reactions did not succeed. From these studies, it appeared that the purported mineral–tetracycline interactions in feeds did not occur or, if they did occur, the normal extraction system of acetone–acid–water extractant and attention to the pH of the extraction was capable of breaking the binding and allowing for a reasonably accurate measurement of potency.

Another aspect of the problem was examined in commercial feeds containing high mineral content, namely, the relationship between the total tetracyclines, active and epimer, and the microbially measured activity (Table 8). Where the microbial potency was low, below the 30% tolerance level, the total chlortetracyclines were low; where the microbially measured potency was in an acceptable range, the total chlortetracyclines were equivalent to or greater than the guarantee. Again, there was no correlation between the calcium content and assay results.

As early as 1959, Tanguay et al. (4) reported that feed extractives "protect chlortetracycline from inactivation by high mineral concentrations." Collaizzi and Klink (5) reported that a pH of 1.0 or less was required for the dissociation of calcium–tetracycline complexes. Klothen (6) reported adding calcium hydroxide and increasing the pH to 8.5–11.5 stabilized chlortetracycline-containing premixes through the formation of the calcium salt. Thus, it appears that the presently used solvent extraction system is capable of extracting chlortetracycline since the pH is low enough to dissociate the calcium–tetracycline complex. In addition, it would be both illogical and totally self-defeating to formulate tetracycline-containing premixes in such a manner that the materials would assay low and have the potential of not being as efficacious because of the complexing reactions.

From the 1984 assay data examined and the laboratory data developed, it was concluded that if chlortetracycline were added to feeds at the proper level and mixed adequately, the present AOAC microbial diffusion assay procedure (1) would measure the chlortetracycline activity, reasonably accurately, in feeds including those that are high in mineral content. This conclusion was based on the following: (a) the lack of correlation between the calcium content and the incidence of deficiency in feeds, (b) the inability to create feeds that reflect an interaction between minerals and chlortetracycline that would result in low assay results, and (c) commercial feeds having a high mineral content and found deficient by the microbial diffusion assay also were low in potency when assayed by a fluorometric procedure that measures total chlortetracyclines; conversely, feeds that met the guarantee when assayed by the diffusion assay also met the guarantee when assayed fluorometrically.

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PESTICIDE FORMULATIONS

Determination of *N*-Nitrosodiethanolamine in Dinoseb Formulations by Mass Spectrometry and Thermal Energy Analyzer Detection

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A new method is described to determine trace quantities of *N*-nitrosodiethanolamine (NDEIA) in aqueous diethanolamine (DEIA) formulations and in oil solutions of dinoseb. A formate anion-exchange column is used in series with a cation-exchange column if there is DEIA in the formulation. The eluate is then passed through a Clin Elut® column. Depending on the concentration of NDEIA in the sample, a packed silica-gel column is used to purify the extract further. This extract is analyzed on a liquid chromatograph coupled with a thermal energy analyzer (LC/TEA), using a mixture of methanol–hexane–methylene chloride containing 0.1% acetic acid (8 + 56 + 35) as the mobile phase. This solvent system gives good separation of NDEIA from trace quantities of dinoseb remaining in the extract. The NDEIA is also converted to the trimethylsilyl derivative and analyzed by gas chromatograph coupled with a mass spectrometer (GC/MS). Analyses of 11 commercial samples of dinoseb diethanolamine salt showed NDEIA levels of 116–2409 ppm expressed relative to the weight of dinoseb. In contrast, analyses of 2 samples of organic solutions of technical dinoseb showed NDEIA levels to be nondetectable and 0.3 ppm, respectively. Limit of detection by LC/TEA is 6.5 ng (0.5 ppm), and by GC/MS it is 0.02 ng (0.15 ppm). Recoveries from samples spiked at 0.514–1664 ppm range from 92.2 to 105.2%.

Recently, we reported a sensitive, specific, and reproducible method for the isolation, detection, and quantitation of *N*-nitrosodiethanolamine (NDEIA) in 2,4-D diethanolamine formulations (1). Trace levels of NDEIA were found in samples of this formulation. The presence of NDEIA in pesticide formulations is a matter of concern because it is carcinogenic to laboratory animals (2).

2-*Sec*-butyl-4,6-dinitrophenol (dinoseb) is a contact herbicide formulated as the diethanolamine salt and as a solution in oil for postemergent weed control, preemergent control of annual weeds, and as a preharvest desiccant of crops (3). It is produced by reacting 2-(1-methylpropyl)phenol with sulfuric acid and then with nitric acid at 60–80°C (4). Diethanolamine has been reported to be a precursor of NDEIA (5). Therefore, as a continuing study of NDEIA in pesticide formulations, different dinoseb formulations produced by several pesticide manufacturers were analyzed for NDEIA contamination. The method developed previously (1) was found to be unsuitable for dinoseb formulations and, thus, a modified method, reported here, has been developed.

METHOD

Apparatus

(a) *Liquid chromatograph/thermal energy analyzer.*—Spectra-Physics (SP) (San Jose, CA 95134-1995) Model 8100 liquid chromatograph equipped with 25 cm × 4.6 mm Spheri-5® cyano normal-phase column (Brownlee Labs, Santa Clara, CA 95050); SP Model 8110 autosampler; SP Model 8440 variable-wavelength detector connected in series by pneumatic 3-way valve (Rheodyne Model 5302), which was controlled by Autochrom® solenoid interface, to thermal energy analyzer (TEA) detector Model 502A (Thermedics, Inc., Woburn, MA 01888-1799); and SP Model 4200 dual-channel computer integrator equipped with SP Model 9600 Labnet® computer. Operating conditions: mobile phase flow rate, 1 mL/min; UV wavelength, 234 nm; sensitivity, 0.02 AUFS; carrier gas, high-purity argon, 100 mL/min; high-purity oxygen, 45 mL/min; pyrolizer temperature, 600°C; vacuum, 1.2 torr; cold trap, ethanol maintained at −72°C using a Neslab (Newington, NH 03801) Model CC-100 II Cryocool immersion cooler; attenuation, 8; chart speed, 0.5 cm/min.

(b) *Gas chromatograph/mass spectrometer.*—Finnigan MAT (San Jose, CA 95134) Model 9610 gas chromatograph with J & W Scientific (Rancho Cordova, CA 95670) Model II capillary on-column injector and Finnigan MAT Model 4500 mass spectrometer with pulsed positive-ion, negative-ion chemical ionization and Townsend discharge ionization. GC column: 15 m × 0.32 mm id DB-1 (J & W Scientific). Data acquisition and MS control: Finnigan MAT 2300 Incos data system. GC operating conditions: column temperature, 85°C for 0.1 min, then 5°/min to 150°C; head pressure, 3 psi; carrier gas, helium UHP. Mass spectrometer source pressure: 0.48 torr. Oxygen (UHP) negative chemical ionization (ONCI) with Townsend discharge mode: temperature, 100°C; discharge current 70 μA, voltage 1.5 kV.

(c) *Anion-exchange columns.*—5 mL of anion-exchange material AG1-X8 (100–200 mesh) formate form packed with water in disposable 11 mL polypropylene Econo-Columns (Bio-Rad Laboratories, Richmond, CA 94804).

(d) *Absorbent column.*—Glass column (27 cm × 1.5 cm od) packed with 3 in. of silica gel 60 (70–230 mesh ASTM) in methylene chloride.

(e) *Extraction tube.*—Clin Elut® columns unbuffered (part No. 1020, Analytichem International, Harbor City, CA 90710).

(f) All other apparatus were as reported in the previous study (1).

Reagents

(a) *Analytical standard.*—Dilute appropriate aliquots of stock solution to 10 mL with 10% methanol in methylene chloride to give final concentrations of 0.1, 0.3, 0.5, and 1.0 μg/mL. Handle standards and samples in fumehoods with all appropriate safety precautions. Handle under red fluorescent lighting to avoid photodegradation of nitrosamines.

(b) *Mobile phase.*—Methanol–hexane–methylene chloride containing 0.1% acetic acid (8 + 56 + 35).

(c) All other reagents as previously reported (1).

Extraction and Cleanup

Diethanolamine formulations.—Weigh 1 g dinoseb diethanolamine formulation in 25 mL (or 100 mL, depending on concentration of NDEIA) volumetric flask and dilute to mark with water. Measure 1 mL diluted formulation and mix in a test tube with 1 mL sodium azide solution, an *N*-nitrosation inhibitor that prevents artifactual formation of nitrosamines. Quantitatively transfer mixture with 15 mL water to drained

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anion exchanger in series with cation exchanger column, prerinsed with 2 mL water, and elute directly into extraction tube. Wait 3 min for eluted sample to be adsorbed onto dry column. Place 500 mL round-bottom flask under column. Elute NDElA with 20 mL 10% acetone in ethyl acetate, wait 1 min to ensure efficient extraction of NDElA between 2 aliquots, then elute again with 230 mL same solution. Add 0.5 mL butanol to eluate to prevent solvent from going dry under evaporation and concentrate to 0.5–1 mL on rotary evaporator at 36°C. Quantitatively transfer concentrated solution to 10 mL volumetric flask with 10% methanol in methylene chloride. Transfer aliquots to sealed vials for LC/TEA analysis or prepare trimethylsilyl (TMS) derivative for GC/MS determination.

Oil-based formulation.—Weigh 1 g dinoseb formulation in 15 mL centrifuge tube, add 1 mL sodium azide solution, mix, and dilute to 6 mL with water. Quantitatively transfer mixture with 12 mL water to drained anion exchanger column which elutes directly into extraction tube. Proceed in same manner as for diethanolamine formulations up to point at which solution is concentrated in 500 mL round-bottom flask by rotary evaporation. Prepare silica gel column using slurry of methylene chloride as follows: (a) plug bottom of column with glass wool and top with 2 mm sand, (b) fill column with 3 in. silica gel, and (c) top with 3 mm anhydrous sodium sulfate. Quantitatively transfer concentrated solution to this column with 10 mL methylene chloride. Elute with 120 mL 10% methanol in chloroform and collect eluate in 2 fractions, discarding first 50 mL, and collecting following 70 mL in 125 mL round-bottom flask. Add 0.5 mL butanol to flask and concentrate to 0.5–1 mL on rotary evaporator at 36°C. Quantitatively transfer concentrated solution to 15 mL centrifuge tube with 8 mL 10% methanol in methylene chloride. Evaporate to 2 mL under nitrogen. Transfer aliquots to sealed vials for LC/TEA analysis or prepare TMS derivative for GC/MS analysis.

LC Determination

Using the autosampler, inject 50 μL NDElA aliquots into LC/TEA system. Record peak areas and retention times with SP Model 4200 computing integrator. Quantitate NDElA concentrations with an external standard. Calculate NDElA as follows:

$$\text{NDElA (ppm a.i.)} = (\text{A sam/A std}) \times (\text{W std/W sam}) \times \text{purity of std}$$

where A sam = area response of sample injected, A std = area response of standard injected, W std = weight (μg) NDElA standard injected, and W sam = weight (g) dinoseb calculated from the amount of dinoseb (% w/w) contained in dinoseb diethanolamine sample as determined by LC–UV analysis.

Preparation of TMS Derivative

Evaporate 0.5 mL NDElA extract from oil-based sample or 1 mL from all others in 15 mL centrifuge tube under nitrogen to dryness. Add 0.5 mL *N,O*-bis(trimethysilyl) acetamide (BSA), shake, stopper, and heat 30 min at 70°C (6). Cool to room temperature and dilute to 1 mL with acetone. To prepare standard for GC quantitation, transfer 1 mL appropriate diluted standard solution into 15 mL centrifuge tube, evaporate to dryness, and prepare silyl derivative as above. Proceed to GC/MS determination within 24 h.

UV Confirmation Test

Transfer 1 mL NDElA extract to quartz cell and place within 7 inches of UV source (1). After 2 h exposure, reanalyze extract by LC/TEA. Disappearance of peak corresponding to NDElA is good evidence of its identity.

GC/MS Determination

Inject 1 μL aliquot NDElA silyl derivative into GC/MS. Quantitate NDElA concentrations using external standard and calculate as described in *LC Determination.*

Results and Discussion

Several cleanup methods to isolate NDElA from the dinoseb samples were attempted. For the dinoseb DElA formulation, the best method incorporates the following sequential steps: (a) dilution of the sample with distilled water; (b) addition of sodium azide solution to prevent artifactual formation of nitrosamine; (c) use of an anion-exchange column in the formate form to retain the dinoseb anion, (d) use of a cation-exchange column to retain the diethanolammonium cation, and (e) use of a Clin Elut extraction tube to extract the NDElA into the organic phase. The resulting solution is analyzed using an LC/TEA detection system. This extract may also be converted into the trimethylsilyl derivative and then analyzed using a GC/MS detection system. Telling and Dunnett have chromatographed NDElA directly using a GC/TEA detection system (7). They also described the difficulties of using this system, which generated NDElA peaks with bad tailing. Our attempt to repeat and improve their chromatographic procedures gave the same results. Therefore, we used an LC/TEA detection system for NDElA and a GC/MS system for TMS derivative of NDElA. In contrast to GC/TEA, the 2 systems reported in the present work produced sharp responses for NDElA and the TMS derivative.

For the oil-based formulation, the cleanup method involves using the same anion exchange column and extraction tube followed by a packed silica gel column to purify the extract further and to improve the limit of detection for the method. A cation-exchange column is not used because amine is not present in this formulation.

In the dinoseb DElA formulation, the active ingredient reacts with amine to form a quaternary ammonium salt. It was expected that the previously developed method (1) for NDElA in 2,4-D DElA formulations using an anion-exchange column followed by a cation-exchange column would be a suitable cleanup method for the dinoseb amine samples. However when the dinoseb sample was added to the chloride form of an AG1 anion-exchange column, a milky eluate containing a fine powdery solid was observed. The same effect was observed using the AG1 resin in the acetate and hydroxide forms. Attempts to use Bio-Beads® (Bio-Rad Laboratories) to retain the dinoseb anion were also unsuccessful. However, when the AG1 resin in the formate form was used, the dinoseb anion was successfully retained, and the eluate was a clear solution.

Because the relative selectivity of the formate counterion is very low—4.6 compared with 22 for the chloride counterion (8)—it was suspected that NDElA, being a very polar chemical, might also be retained by this column. Recovery studies of the samples of the amine formulations spiked at 333, 832, and 1664 ppm, relative to the active ingredient weight, gave 94.0, 99.9, and 105.2%, respectively. Recoveries of the oil-based samples spiked at 0.514 to 6.74 ppm ranged from 92.2 to 100.0%. These results, shown in Table 1, indicate that NDElA was not retained by this anion-exchange column.

For the organic oil-based formulations, although dinoseb is present as a neutral molecule in the solution, an anion-exchange column was useful in removing the dinoseb from

Table 1. Percent recoveries of NDEIA from fortified formulations, using GC/MS

Original level, ppm	Fortified level, ppm	Found, ppm	Level found − original level, ppm	Recovery, %
571	333	884	313	94.0
571	832	1402	831	99.9
571	1664	2322	1751	105.2
ND[a]	0.51	0.47	0.47	92.2
ND	1.03	0.99	0.99	96.1
ND	2.06	2.06	2.06	100.0
0.3	6.74	6.95	6.65	98.7
Av.				98.01
SD[b]				4.34

[a] ND = nondetectable with a detection limit of 0.15 ppm.
[b] SD = standard deviation.

the solution due to the addition of the sodium azide solution. Dinoseb has a pKa value of 4.62 (3). The addition of sodium azide solution changed the pH value of the dinoseb sample from 4 to over 6 and thus enhanced the retention of dinoseb by the column.

Before NDEIA was eluted from the extraction tube, the tube was prewashed with hexane. However, this procedure caused interference with the subsequent eluting solvent, 10% acetone in ethyl acetate. The use of methyl ethyl ketone as an eluting solvent also was unsuccessful in that it produced a yellow extract.

Samples of amine formulations were diluted 25 to 100 times before cleanup because of the high concentration of NDEIA present in these samples. Initially, cleanup was conducted on undiluted samples. In these cases, the extract from the extraction tube was further purified in various ways: *viz* a Baker-10 SPE® silica gel disposable column (J. T. Baker Chemical Co., Phillipsburg, NJ 08865); a Baker-10 column packed with an additional equal volume of silica gel; a disposable column packed with 5 mL silica gel; a silica gel Sep-Pak disposable cartridge (Waters Chromatography, Milford, MA 01757); and a Baker-10 SPE cyano (CN) disposable column, all eluted with 10% methanol in chloroform; and a silica gel Sep-Pak column eluted with 50% methanol in methylene chloride. These different cleanup procedures produced similar yellow extracts, showing the presence of interferences in the LC/TEA chromatograms. Those samples that contained relatively high levels of NDEIA were diluted with water before column cleanup because of the sensitivity of the detection system used and the toxicity of this contaminant. Further cleanup of the extract from the extraction tube was unnecessary.

Due to the presence of two nitro groups in the molecule, dinoseb shows a substantial response to the LC/TEA detection system. Therefore, trace quantities of dinoseb in the final aliquot will interfere with the analysis for NDEIA. However, the mobile phase chosen was able to separate the dinoseb peak from the NDEIA peak in a well-resolved manner, with dinoseb responding at 3.21 min and NDEIA at 5.86 min as indicated in Figure 1A. The most effective mobile phase to separate these 2 peaks was a mixture of methanol–hexane–methylene chloride (8 + 56 + 35) containing 0.1% acetic acid. Two other methanol–hexane–methylene chloride solvent systems—5 + 60 + 35 or 2 + 63 + 35 for 14 min and then changed to 6 + 59 + 35—were also used as a mobile phase but were less satisfactory. The first system generated poor separation with a tailing dinoseb response. The second system showed good separation, although dinoseb still responded in a tailing fashion.

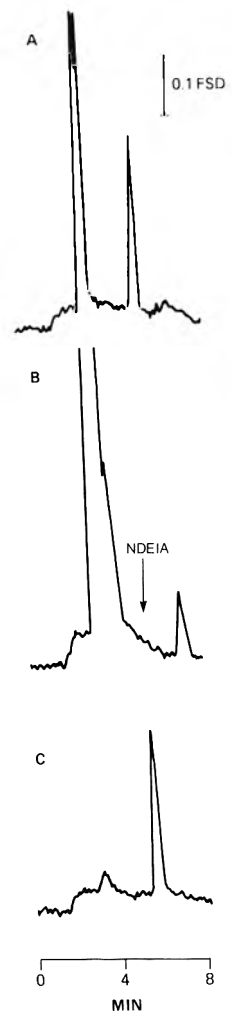

Figure 1. Typical LC/TEA chromatogram of: A, sample 3; B, sample 12; C, 52 ng (758 ppm) NDEIA standard.

For samples of the oil-based formulations, because of the very low levels of NDEIA present in the samples, it was necessary to analyze the samples without dilution to achieve a low detection limit. Thus, a silica-gel column was used to further purify the extract obtained from the extraction tube. The eluate after concentration gave satisfactory LC/TEA chromatograms as shown in Figure 1B.

The identity of the NDEIA response was confirmed by (a) its LC retention time, which is identical to that of a standard solution of NDEIA (see Figure 1C), (b) the disappearance of the response at 5.86 min after UV irradiation of the aliquot, and (c) the chemical ionization mass spectrum of its TMS-derivatized sample which is identical to that of a standard NDEIA–TMS solution (1). The identity of the dinoseb response was confirmed by its LC retention time, which is identical to that of a standard solution of dinoseb, and by its persistence after UV irradiation of the aliquot.

All NDEIA extracts were converted to TMS derivatives by reaction with BSA followed by GC/MS determination using single-ion monitoring at m/z 248.074 ± 0.500 and 310.093 ± 0.500. The former m/z value represents the ion resulting from the loss of an NO group from the molecular ion (M − 30). The latter m/z value represents the oxygen adduct of the molecular ion (M + 32). Figure 2 shows the GC/MS chromatograms of the sample, the sample spiked with NDEIA and the standard solution of NDEIA–TMS derivative.

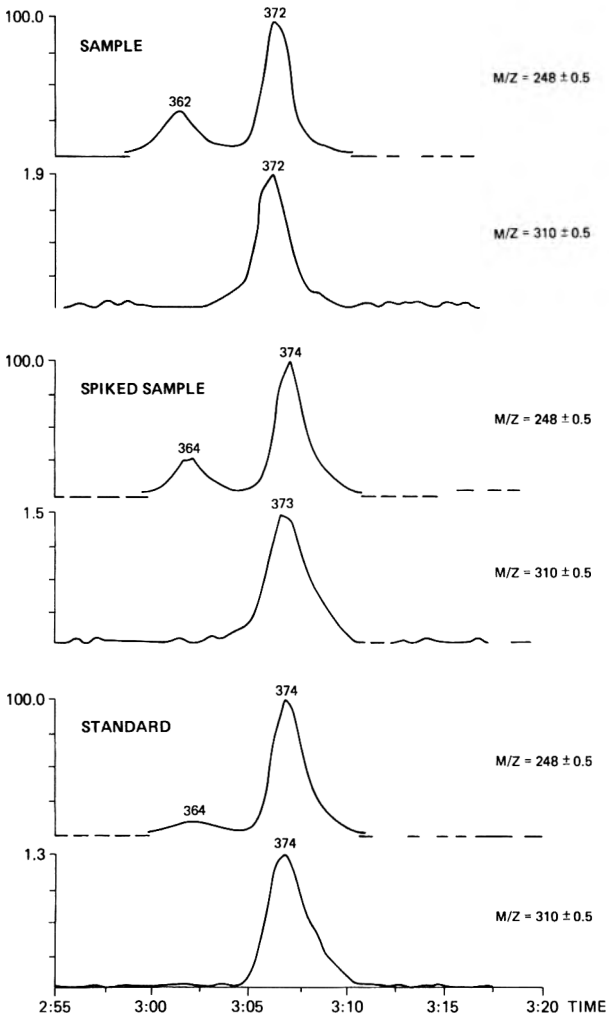

Figure 2. Typical GC/MS (ONCI) chromatogram of: sample 8; sample 8 spiked with 0.26 ng (832 ppm) NDEIA; 0.52 ng (1664 ppm) NDEIA standard.

Samples 1 to 11 in Table 2 of dinoseb diethanolamine formulation and samples 12 and 13 in Table 2 of oil-based formulations were analyzed for NDEIA contamination. Results for these samples from the GC/MS and the LC/TEA systems, expressed in ppm relative to the active ingredient weight, are shown in Table 2. Using GC/MS detection, the NDEIA levels in samples of amine formulation ranged from 116.0 to 2409.0 ppm, whereas those in oil solutions were nondetectable and 0.3 ppm, respectively. Analysis of reagent blanks using the same cleanup procedures gave NDEIA levels below the limit of detection.

On the basis of the criterion that the minimum detectable amount (MDA) of a compound gives a response equal to twice the background noise, the MDA for LC/TEA was 6.5 ng. The MDA for GC/MS was 0.02 ng and was based on a signal to noise ratio of 10:1. These values correspond to 0.5 ppm and 0.15 ppm, respectively, when they are expressed relative to the active ingredient weight. The comparable results for the same sample produced from 2 different detection systems demonstrate that NDEIA was not artifactually generated in the hot GC injection port and that the TMS derivatization reaction was completed.

When LC/TEA was used to analyze the samples, the results were less repeatable and the detection system was less sensitive than when GC/MS was used. These undesirable effects are presumably due to the need to freeze out the solvent

Table 2. Level (ppm) of NDEIA relative to active ingredient found in dinoseb formulations

	NDEIA, ppm	
Sample	GC/MS	LC/TEA
1	1006.0	959.0
2	1112.0	1074.0
3	768.0	765.0
4	2230.0	2049.0
5	2409.0	2392.0
6	703.0	733.4
7	300.0	308.0
8	626.0	694.0
9	791.0	858.0
10	116.0	99.4
11	1684.0	1668.0
12	ND[a]	—
13	0.3	—

[a] ND = nondetectable with a detection limit of 0.15 ppm.

before the eluate reaches the TEA detector and to the subsequent venting of the solvent from the system after several analyses. Thus, GC/MS detection is preferred for its sensitivity and repeatability. In the absence of a GC/MS detection system, the NDEIA–TMS derivative could be analyzed using GC/TEA (1) if more sensitive detection is required.

Technical-grade dinoseb may contain traces of nitrosating agents due to the chemical process used to produce this product (4). When dinoseb is formulated with an aqueous solution of diethanolamine, these impurities react with the amine to form NDEIA. Because the rate of nitrosation reaction is usually rapid (9), the level of nitrosamine produced will depend on the concentration of nitrosating agents in the dinoseb; high levels may be observed depending on the purity of the herbicide used in the formulation. However, the formation of nitrosamine requires the simultaneous presence of both precursors in a mixture. Therefore, in an oil solution of dinoseb where amine is not present, nitrosamine is not detectable despite the presence of nitrosating agents. Table 2 shows the widely contrasting NDEIA results obtained with these 2 formulations and supports the rationale discussed. The high levels of NDEIA detected in the samples of the amine formulation are probably indicative of the presence of sufficient quantities of the nitrosating impurities in the technical grade of this herbicide.

Sample No. 13 showed low but definite contamination by NDEIA. In a reagent blank, analyzed simultaneously with this sample, NDEIA was nondetectable. The cause of this contamination is not evident.

Similar results for NDEIA in dinoseb DEIA formulations have previously been reported, using the LC/TEA and LC/UV systems (10). The detection limits for these systems were 10 and 3 ppm, respectively. However, the details of the sample cleanup procedures and recovery results were not reported. This information is essential since the active ingredient (dinoseb) responds to both detection systems. Dinoseb products are usually dark brown, viscous liquids. In the present study, the concentrations of dinoseb in different products ranged from 30 to 50%. Therefore, it is important to separate dinoseb from NDEIA before analysis so it will not interfere with the detection and quantitation of trace levels of NDEIA. The procedures described here provide a method to isolate and separate NDEIA from dinoseb and to detect NDEIA as low as 0.5 or 0.15 ppm, depending on the system used.

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Determination of Ethylene Oxide in Ethoxylated Surfactants and Demulsifiers by Headspace Gas Chromatography

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A headspace gas chromatographic method for the determination of traces of ethylene oxide in ethoxylated surfactants and demulsifiers was developed. Samples are analyzed directly by the technique to a 1.0 ppm (w/w) quantitation limit. The procedure also performs well for propylene oxide, acetaldehyde, and 1,4-dioxane. It is simple, sensitive, and linear. The percent relative standard deviations for 0.5 and 30 ppm ethylene oxide in the surfactant were 2.8 and 8.3%, respectively.

Recent studies and a review of the scientific evidence has convinced the Occupational Safety and Health Administration that ethylene oxide is carcinogenic in laboratory animals and that a significant cancer risk exists for workers exposed to it (1). This report has resulted in a search for an analytical approach to determine trace quantities of ethylene oxide in ethoxylated surfactants and demulsifiers. Headspace gas chromatographic techniques have proved useful in several instances (1–3) to increase detection limits over methods that require solvent extraction or dilution (4–6).

Trace ethylene oxide has been determined in sterilized medical devices, polymeric materials, workplace environment, and foods, using headspace gas chromatography (GC). No determination or methods have been described, however, for determining ethylene oxide in ethoxylated surfactants and demulsifiers. Most of these types of organic compounds are viscous liquids and lend themselves nicely to headspace analysis with no sample preparation required.

This report describes a procedure for determining traces of ethylene oxide in ethoxylated surfactants and demulsifiers. In developing the methodology, one of our goals was to make the test simple yet accurate. In the proposed procedure, the surfactant is weighed into a headspace vial and analyzed by headspace gas chromatography.

METHOD

Apparatus

(a) *Gas chromatograph.*—Perkin-Elmer Sigma 2000, or equivalent, equipped with 8 ft × ⅛ in. stainless steel column packed with 80–100 mesh Chromosorb 102 (Supelco, Inc., Bellefonte, PA) and fitted with an HS-100 headspace sampler and a flame ionization detector. Operating conditions: detector, 200°C; helium carrier at 30 mL/min; column 120°C for 5 min, then programmed at 8°/min to 190°C and held for 10 min; retention time for ethylene oxide, 6–7 min. Data collection, Perkin-Elmer LSI-100 integrator. Headspace sampler conditions: sample temperature, 100°C; transfer temperature, 130°C; thermostating time, 30 min; pressurization time, 0.7 min; injection time, 0.08 min; withdrawal time, 0.2 min; cycle time, 30 min. Headspace vials should not be vented to laboratory atmosphere.

(b) *Gas-tight syringes.*—25 μL, 50 μL, 100 μL, and 1.0 mL syringes for manipulating standards (Ohio Valley Specialty Chemicals, Inc., Marietta, OH).

(c) *Vials.*—10 mL hypovials with Tuf-Bond disks (septa) (Ohio Valley Specialty Chemicals, Inc.) and aluminum crimp seals for preparation of standards.

Reagents and Materials

Caution: Ethylene oxide is very volatile, explosive, highly toxic, and a potential carcinogen and reproductive hazard (7). All work in preparation of standards using this material should be performed in a well-ventilated hood. All metal containers and connections should be well grounded to avoid static discharges. Avoid skin contact with this and all solvents.

(a) *Ethylene oxide.*—(Matheson Gas Products, Secaucus, NJ.)

(b) *Propylene oxide, acetaldehyde, and 1,4-dioxane.*—(Aldrich Chemical Co., Milwaukee, WI.)

(c) *Standard solutions.*—*(1) Ethylene oxide solution.*—Dry LC grade hexane, using anhydrous sodium sulfate or 3A molecular sieves (8–12 mesh). (Refer to Figure 1A and 1B.) Connect an ethylene oxide cylinder to 10 mL hypovial (sealed with a Teflon-faced silicone septa) using short piece of Tygon tubing and hypodermic needle inserted nearly to bottom of vial. Connect a second piece of Tygon tubing, with hypodermic needle attached to each end, between top of vial and beaker of water (Figure 1A). Slowly purge (1–2 bubbles/s) ethylene oxide through beaker of water for 15 min to remove air from tubing. At this point, increase flow of ethylene oxide to several bubbles/s. Attach dry needle to end of second piece of tubing and insert into a 10 mL sealed (Teflon-faced silicone septum) and tared vial containing 10 mL dry hexane (Figure 1B). Add 40–80 mg ethylene oxide to headspace above hexane to prepare primary standard. Cool primary standard to ca 0°C and place 5 min on a shaker. Prepare additional standards by diluting primary standard. Store all standards at ca −10°C when not in use. *(2) Acetaldehyde, propylene oxide, and 1,4-dioxane standards.*—Prepare gravimetrically.

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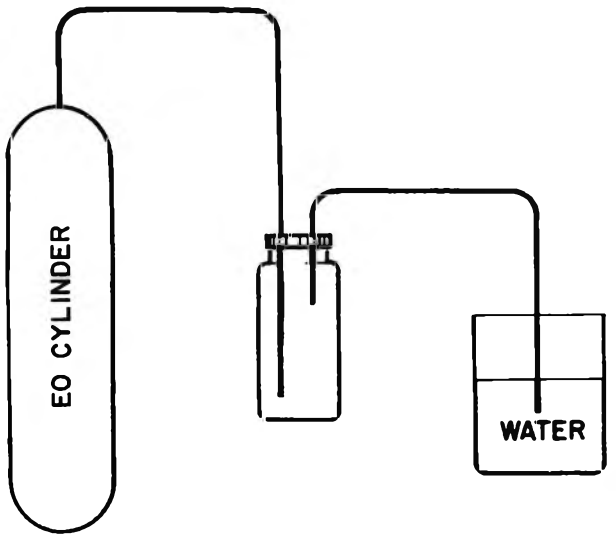

Figure 1A. Purging gas lines for ethylene oxide standard preparation.

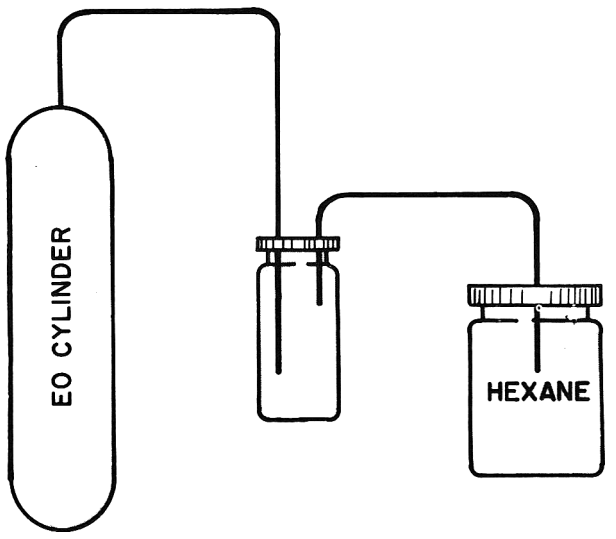

Figure 1B. Addition of ethylene oxide to hexane.

(d) *Calibration standards.*—Vacuum-strip sample of surfactant material at 50–60°C on rotary evaporator to remove any residual ethylene oxide. Prepare standards by weighing 2.00 g (±0.002 g) into headspace vials. Seal vials with Teflon-faced silicone septa and inject known amounts of ethylene oxide in hexane into sealed vials. Prepare standards at concentrations such that amount of hexane injected into each vial (10 μL) is the same. This ensures identical sample matrix effects between the standards and samples (8). The calibration standards are analyzed, and a plot of the area versus amount of ethylene oxide in the surfactant is made.

Preparation of Samples

Weigh 2.00 g (±0.002 g) into headspace vials and seal as with standards. Inject 10 μL dry hexane into vial through septa to ensure that samples and standards are of an identical matrix. Let vials stand 1 h before analysis.

Calibration

With LCI-100 integrator properly calibrated, results are calculated and reported in μg/g ethylene oxide in the surfactant.

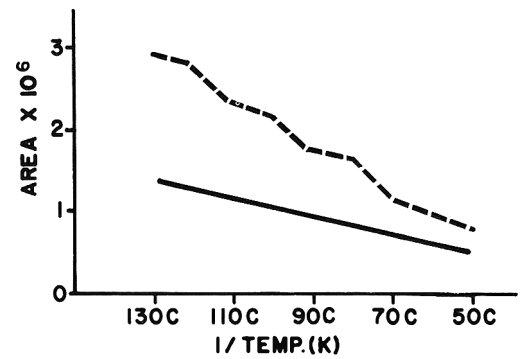

Figure 2. Plot of ethylene oxide peak area vs thermostating temperature for various surfactants.

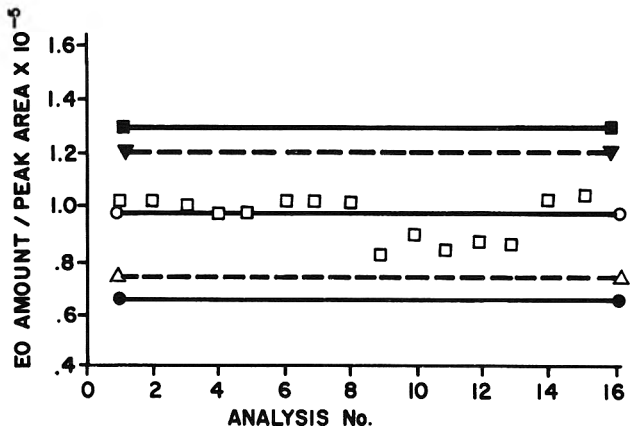

Figure 3. Shewart chart of ethylene oxide response from standards prepared over a period of time.

Results and Discussion

Initial studies in the development of suitable methodology for ethylene oxide in surfactants were conducted by diluting the surfactant with acetone and directly injecting the samples onto the gas chromatograph. This procedure could not provide the sensitivity needed to meet the detection limit set for worker safety. With the acquisition of a headspace analyzer, a large difference could be seen in the sensitivity for detection of ethylene oxide. The headspace analyzer also offered better reproducibility and accuracy for measuring concentrations at 1.0 ppm.

Our analytical goal was a simple yet accurate method. Preliminary investigation included comparison of direct injection data with headspace data. As the amount of ethylene oxide in the surfactants was reduced, the detection of ethylene oxide by the direct injection procedure was not possible. Several parameters of the headspace method were examined, and comparisons were made to previous data wherever possible.

Initial investigation into the multiplicity of variables (8–10) affecting headspace analysis began with temperature and time effects on the sensitivity of the analysis. Numerous samples were run at various temperatures to determine if an optimum thermostating temperature existed. Figure 2 details the results of these analyses performed on 2 dissimilar surfactant materials.

On the basis of the data collected, 100°C was chosen as the thermostating temperature. At this temperature, sufficient sensitivity for ethylene oxide existed under the operating conditions and results were approximately 50% below ethylene oxide peak areas at 130°C. Also, at the lower temperature, less of the starting materials (having boiling points

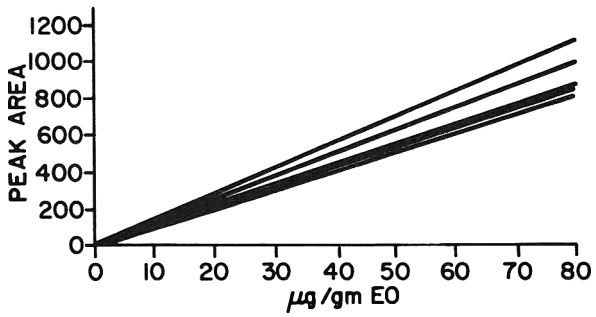

Figure 4. Calibration curves of several different types of surfactants.

above ethylene oxide) would be injected onto the column, which would mean less interference. An examination of the thermostating time at 100°C showed slight difference among 10, 20, and 30 min. Recalling that the GC run time was 30 min, it was decided to allow samples to equilibrate for 30 min. These conditions proved to be excellent to give detection limits and quantitation limits within satisfactory ranges (0.5 and 1.0 ppm, respectively).

Repeatability of the headspace technique was examined throughout the range from 0.5 to 75 ppm. This range was used to cover the amount of ethylene oxide originally found in some of the surfactant materials. The repeatability at 0.5 ppm (2.8% RSD) and 30 ppm (8.3% RSD) assured that acceptable performance could be maintained. The large percent relative standard deviation (RSD) at the 30 ppm level was attributed to the variety of surfactants used to make the standards. When only one type of surfactant was used to make standards, the RSD was lowered to 1.3%. This indicated, as expected, that the surfactant used in the standardization should be of a similar nature to avoid inaccuracies due to sample composition.

For this study, numerous standards were prepared using various surfactant types. The continuing analysis of the 30 ppm calibration standard (individually prepared at various times) ensures the repeatability of the overall scheme of the analysis. Figure 3 is a Shewart (11–13) chart which is used to show the response of ethylene oxide over a period of time. The Shewart chart is generally associated with percent recovery in environmental chemistry, but is useful in tracking standard preparation and instrument performance. The chart can be used to inform the analyst quickly when a prepared standard is "out-of-control"—beyond the 2.8 times the standard deviation (dashed lines).

Various surfactants were examined in the standard preparation scheme to determine the effects of the materials on the calibration curve. Several products were repeatedly spiked at levels from 1.0 to 75 ppm and analyzed at various levels and the calibration curves plotted. Figure 4 is a plot of the calibration curves from some of the products tested and exhibits the range of "slopes" of the lines seen. Although the slopes of the lines of the various surfactants are slightly different, the points along the line remain within the limits of reproducibility seen at the 1.0 and 30 ppm levels (4–8% RSD). The various slopes noted demonstrate the variability in the numerous types of surfactants investigated. At the high end of the calibration curves (75 ppm) the variation is 10.6% RSD, which is high but within reasonable limits when considering the large variation in surfactant types studied.

Table 1. Quantitation of ethylene oxide in surfactants by direct injection and headspace analysis

Direct injection method, ppm	Headspace method, ppm[a]
29	25.7/24.7
78	75.8/79.3
<10	6.9/7.0
24	23.3/25.2
<10	3.4/2.7
<10	3.5/2.7

[a] Duplicate analyses.

Table 2. Analysis of samples for ethylene oxide by standard additions method

Headspace single analysis, ppm	Headspace standard additions, ppm
3.5	4.2
0.5	0.1
3.9	3.3
17.0	19.3
2.5	2.9
<1	0.5

Several sample results were compared to results from other analytical methods. These results are displayed in Table 1. Table 2 compares data collected from single headspace analysis of some samples and data from the same samples analyzed by the method of standard additions. The data collected are from single observations and agree very well within the expected ranges of variation.

Although our main interest lay in developing a method for ethylene oxide in surfactants, it was observed throughout the analyses that the procedure could be applied to propylene oxide (for propoxylated surfactants), acetaldehyde, and 1,4-dioxane (byproducts of ethyoxylation), with similar expected results in variation.

Despite the variability in the materials tested, the described procedure provides an approach for quantitating residual ethylene oxide in surfactants and demulsifiers. Further studies have indicated that ethylene oxide can be detected in the surfactants at levels as low as 0.1 ppm with an RSD of 6.1%.

Acknowledgment

The authors thank Victoria Morris of Air Products for her input, via private communications, into the chromatographic procedure used in this study.

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VITAMINS AND OTHER NUTRIENTS

Liquid Chromatographic Determination of Taurine in Vitamin Premix Formulations

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A liquid chromatographic (LC) method is described for the determination of taurine in vitamin and vitamin-mineral premix formulations. The method involves extraction of taurine with 0.1M bicarbonate buffer, followed by precolumn derivatization with dansyl chloride and LC using fluorescence detection. 6-Aminocaproic acid is used as an internal standard. A reverse phase analytical column and a mobile phase of 0.1M acetate buffer solution (pH 7.2)-acetonitrile (75 + 25) are used. Vitamins, minerals, and other excipients in the premix formulations do not interfere in the determination. The method is simple, precise, and accurate.

Taurine (2-aminoethanesulfonic acid), abundant in human milk, is recognized as a conditionally essential infant nutrient—one whose absence from the diet does not produce immediate deficiency diseases but may cause long-term developmental problems. Taurine is currently added to many vitamin premix formulations used in infant nutritional preparations to provide the same measure of safety that human milk provides.

In recent years, liquid chromatographic (LC) methods to assay taurine in biological samples have been described (1–6). Among the LC methods are those using precolumn derivatization with *o*-phthalaldehyde, dabsyl chloride, or dansyl chloride and fluorescence detection. Ion exchange chromatography (IEC) based on the ninhydrin reaction and the principle of postcolumn derivatization have been used for the analysis of taurine in biological samples (7–9) and in vitamin preparations (10). However, IEC methods require specialized equipment, thereby increasing the cost per sample for routine analysis.

There are, as yet, no published LC methods to determine taurine in vitamin premix formulations. A simple, precise, and specific assay method for taurine in vitamin-mineral premix formulations and their mineral-free counterparts has been developed for quality control purposes and for use in accelerated stability studies. The method is based on reverse phase LC, with precolumn derivatization and fluorescence detection, and is stability-indicating.

METHOD

Apparatus

(a) *Shaker.*—Burrell wrist-action or equivalent.

(b) *LC apparatus.*—Waters Associates Model 6000A pump and Model 710B WISP Autoinjector, Schoeffel Model FS-970 spectrofluorometer, Spectra-Physics SP-4100 Computing Integrator, or equivalent system. Typical operating conditions: ambient temperature; flow rate, 1.3 mL/min; injection volume, 20 μL; detector range, 1 μA; excitation wavelength, 360 nm; excitation filter, Corning 7-51; emission filter, KV 418 nm cutoff filter.

(c) *Analytical column.*—15 cm × 4.6 mm id column packed with 5 μm reverse phase C_{18} material (Alltech Nucleosil, Cat. No. 89162), or equivalent; 3 cm × 4.6 mm id C_{18} guard column (Brownlee, Cat. No. 18 GNU), or equivalent, is inserted between injector and analytical column.

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Reagents

(a) *Solvents.*—LC grade or equivalent.

(b) *Chemicals.*—Reagent grade.

(c) *Acetate buffer solution (pH 7.2).*—0.1M. Dissolve 13.6 g sodium acetate trihydrate in 900 mL water, adjust pH to 7.2 ± 0.5 with dilute acetic acid (1 in 100), and dilute with water to 1 L. Mix well.

(d) *Mobile phase.*—Mix 0.1M acetate buffer solution (pH 7.2) and acetonitrile (75 ± 25). Filter solution through solvent-resistant membrane filter having a porosity of 0.45 μm or less and degas using a house vacuum for 5–10 min.

(e) *Sample solvent—bicarbonate buffer solution.*—0.1M. Dissolve 8.4 g sodium bicarbonate in 950 mL water, adjust pH to 9.5 with 10N sodium hydroxide solution, and dilute with water to 1 L. Mix well.

(f) *Derivatization reagent.*—5 mg/mL. Dissolve 125 mg dansyl chloride (Pierce Chemicals, Cat. No. 21755, or equivalent) in 25 mL acetone. Filter, if necessary, through medium-porosity sintered-glass funnel to remove any insoluble material. This reagent is stable for up to 3–4 months when refrigerated in a tightly stoppered bottle under a blanket of nitrogen.

(g) *Internal standard solution.*—Accurately weigh ca 50 mg 6-aminocaproic acid (Aldrich Chemical Co., Cat. No. A4460-6, or equivalent) and transfer to a 50 mL volumetric flask. Dissolve and dilute to volume with sample solvent. Mix well.

(h) *Standard preparation.*—Accurately weigh ca 50 mg taurine standard (in-house standard) and transfer to a 50 mL volumetric flask. Dissolve, dilute to volume with sample solvent, and mix. Mix 5.0 mL of this solution with 5.0 mL internal standard solution.

Sample Preparation

Accurately weigh a portion of the premix formulation equivalent to ca 50 mg taurine and transfer to a 50 mL volumetric flask. Add 30 mL sample solvent, shake mechanically 30 min, dilute with sample solvent to volume, and mix. Mix 5.0 mL of this solution with 5.0 mL internal standard solution and filter a portion of the solution through a 0.45 μm solvent-resistant membrane filter.

Derivatization Procedure

Transfer 1.0 mL each of the standard and sample preparations to separate 4 mL screw-cap (with Teflon septum) WISP vials. Add 1.0 mL derivatization reagent to each vial, stopper, and mix the contents. Place vials in an oven at 55°C for 1 h. Remove vials from the oven and cool to room temperature.

System Suitability Test

Allow LC system to equilibrate before beginning the system suitability test. Inject 20 μL of derivatized standard preparation. Elution order is taurine followed by internal standard. A retention time of 6.4 min or greater is optimal for taurine. If taurine elutes in less than 6.4 min, adjust mobile phase composition with additional 0.1M acetate buffer so-

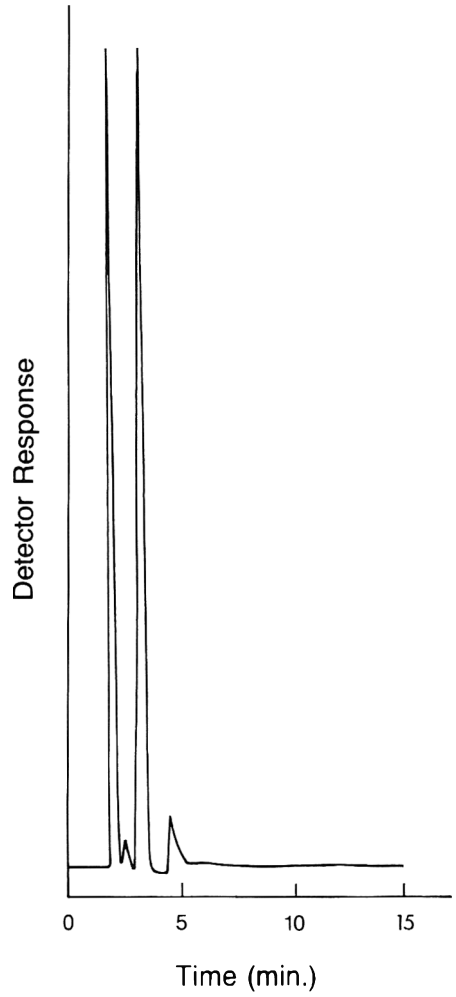

Figure 1. Typical LC chromatogram of a placebo (LC conditions as stated in text).

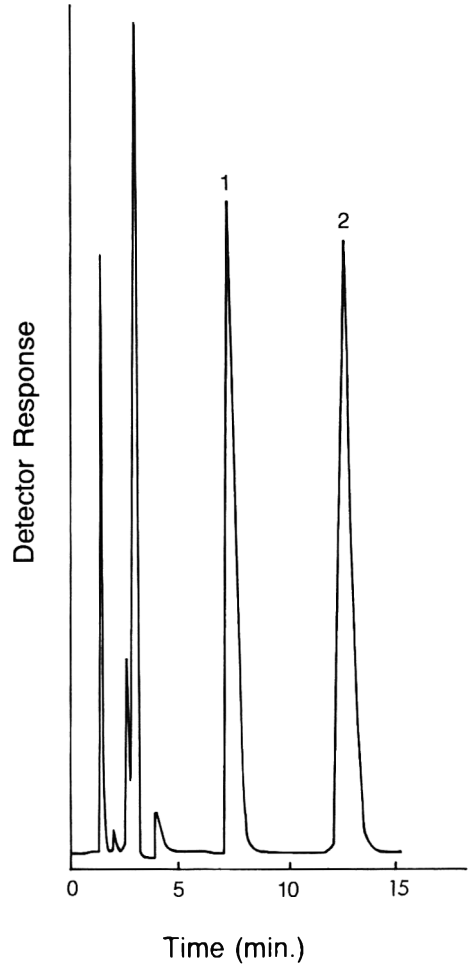

Figure 2. Typical LC chromatogram of a sample preparation (LC conditions as stated in text): 1, taurine; 2, internal standard.

lution (pH 7.2) until retention time is 6.4 min or greater. The resolution factor, R, between taurine and the internal standard is greater than 3. (For a particular column, resolution may be increased by increasing the amount of acetate buffer solution in the mobile phase.)

Analysis

Inject duplicate aliquots of the derivatized standard preparation. Calculate response ratio by dividing the area of taurine peak by that of the internal standard peak. Response ratios should agree within 2%. Average the duplicate response ratios.

Inject duplicate aliquots of the derivatized sample preparation. Average the duplicate response ratios, which should agree within 2%. *Note:* After the first injection of any sample, let the instrument run ≥20 minutes after emergence of the taurine peak to elute any peaks due to excipients in the premix formulation. Subsequent injections should be timed so that late-eluting peaks from sample injections do not interfere with taurine and internal standard peaks of subsequent samples.

Calculations

Calculate the amount of taurine in the samples, using response ratios according to the following equation:

$$\text{Taurine, mg/g} = R/R' \times W'/W,$$

where R and R' = response ratios for sample and standard, respectively; W and W' = g and mg taken for sample and standard preparations, respectively.

Results and Discussion

In the described methodology, taurine and 6-aminocaproic acid (internal standard) were subjected to precolumn deriva-

Table 1. Optimization of derivatization reaction time at 55°C

Time, min	Response ratio, taurine/internal standard
0	0.434
15	0.830
30	0.833
60[a]	0.832
90	0.830

[a] Reaction time used in this method.

Table 2. Optimization of derivatization reagent concentration

Dns-Cl (approx. concn), mg/mL	Mole ratio, Dns-Cl/ (taurine + int. std)	Response ratio
2.50	1.2	0.597
3.25	1.5	0.806
4.0	1.9	0.803
5.0[a]	2.4	0.810
7.5	3.6	0.812

[a] Concentration used in this method.

Table 3. Optimization of derivatized sample stability

Time, h	Response ratio
1.0	0.829
2.0	0.827
3.4	0.831
22.6	0.830
25.2	0.828
Mean	0.829
RSD	±0.2%

tization with dansyl chloride (Dns-Cl) to give dansyl amino acids, which are fluorescent and readily detected after LC separation. The excess derivatization reagent and its by-products are well resolved from taurine and the internal standard in the described LC system. Vitamins, minerals, and other formulation excipients do not interfere in the determination.

Typical chromatograms of a placebo and sample preparations are shown in Figures 1 and 2, respectively.

The derivatization reaction was optimized for reaction time, reagent ratios, and stability of the derivatized sample. As shown in Table 1, the reaction is complete within 15 min with essentially no change up to 90 min. The effect of Dns-Cl concentration on the peak response ratio is shown in Table 2. The amount of Dns-Cl used in the described procedure is approximately 2.4 times the amount needed to derivatize both the taurine and 6-aminocaproic acid. Table 2 shows that is more than ample. The stability of the derivatized sample solution is important for assays run overnight using an automated system. There was essentially no degradation over a 25 h period (Table 3), making this method viable for long runs.

Linearity of detector response was established for taurine in the range of 0.04 to 1.6 mg/mL (correlation coefficient = 1.000, y-intercept = 0.008). The taurine content of premix formulations ranged from 28 to 62% by weight.

Standard addition-recovery studies were performed by adding known amounts of taurine standard at 250–750 mg/g levels to 3 placebo formulations. Recoveries ranged from 97.9 to 102.4%. Results are shown in Table 4.

In the validation of methodology, a premix sample representing the most complex formulation from an analytical point of view was selected to generate the precision and stress stability data.

The validity of the method was tested by subjecting a sample of premix formulation to thermal stress, high-intensity ultraviolet lamp, and hydrolytic conditions. The stressed samples were analyzed by the described methodology. The results of the analyses are summarized in Table 5. In the chromatograms of the stressed placebo samples, no interfering peaks were observed to elute with the same retention time as either taurine or the internal standard. The data from

Table 4. Standard addition-recovery of taurine from placebos[a]

Approx. addition level, %	Recovery, %		
	Placebo I	Placebo II	Placebo III
50	101.0	102.0	102.4
100	98.4	99.8	99.7
150	98.8	98.8	97.9
Mean	99.4	100.2	100.0
RSD	±1.4%	±1.6%	±2.3%

[a] Placebo I, 7 vitamins and 4 minerals; placebo II, 7 vitamins; placebo III, 8 vitamins.

Table 5. Analysis of premix formulation for taurine subjected to various stress conditions

Stress conditions	% Label claim
Initial[a] (unstressed)	100.0
Thermal (100°C, 88 h)	96.4
High-intensity UV lamp (7 h)	98.3
pH 3.0 buffer (3 h reflux)	99.1
pH 7.0 buffer (3 h reflux)	97.0
pH 10.0 buffer (3 h reflux)	99.3

[a] Initial value normalized to 100% and the rest of the data calculated relative to the initial.

Table 6. Precision data for the analysis of taurine in premix formulation

Run	Analyst	Day	% Label claim
1	A	1	100.1
2	A	2	98.3
3	A	2	97.5
4	A	3	97.2
5	A	4	100.1
6	B	5	100.9
7	B	6	95.5
8	B	6	100.7
Mean			98.8
SD			±2.0
RSD			±2.0%

Table 5 show only minor degradation of taurine in the formulated product under the indicated stress conditions. Taurine is expected to be a very stable compound. If there is any degradation, it is the amine function of the molecule that is expected to degrade. The derivatization reaction (in the described methodology) is selective to the amine function of the taurine molecule. Based on this selectivity of derivatization, coupled with the powerful LC separation capability and specificity of fluorescence detection, the method is expected to be stability-indicating for taurine.

Precision of the method was determined by analyzing a sample of premix formulation 8 times by 2 analysts on 6 different days. The results are shown in Table 6. The method has a relative standard deviation of ±2%.

In conclusion, liquid chromatography provides a simple, stability-indicating, and precise method for the quantitative determination of taurine in vitamin premix formulations.

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Sample Preparation and Liquid Chromatographic Determination of Vitamin D in Food Products

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Vitamin D in different fortified foods is determined by using liquid chromatography (LC). Sample preparation is described for fortified skim milk, infant formulas, chocolate drink powder, and diet food. The procedure involves 2 main steps: saponification of the sample followed by extraction, and quantitation by LC analysis. Depending on the sample matrix, additional steps are necessary, i.e., enzymatic digestion for hydrolyzing the starch in the sample and cartridge purification before LC injection. An isocratic system consisting of 0.5% water in methanol (v/v) on two 5 μm ODS Hypersil, 12 × 0.4 cm id columns is used. Recovery of vitamin D added to unfortified skim milk is 98%. The results of vitamin D determination in homogenized skim milk, fortified milk powder, fortified milk powder with soybean, chocolate drink powder, and sports diet food are given.

Inherent problems of vitamin D determination in food are the low levels of the vitamin in samples and the complexity and variety of the sample matrix. Biological assays and classical physicochemical methods are usually lengthy and tedious. A gas chromatographic method for the determination of vitamin D in dried milk has been reported (1). The method demands a time-consuming cleanup procedure involving several column chromatographic steps. Recently, several publications have reported liquid chromatographic (LC) techniques for the determination of vitamin D in fortified milk (2–14). Thompson et al. (2) have used normal-phase silica column liquid chromatography to determine vitamin D in fortified milk following saponification and purification on a modified Sephadex column. Henderson and Wickroski (3) reported the use of an alumina cleanup column before determining vitamin D in fortified milk by reverse phase liquid chromatography. Mankel (4) determined vitamins A, E, D, and β-carotene in margarines, powdered foodstuffs for infants, and fortified milk powder directly by reverse phase chromatography after a saponification step. Cohen and Wakeford (5) reported the determination of vitamin D in fortified instant nonfat dried milk on a normal-phase amino column after extraction and purification using a silica Sep-Pak cartridge and Partisil-10 PAC column. Barnett et al. (6) developed reverse phase liquid chromatography for the simultaneous determination of vitamins A, D_2 or D_3, E, and K_1 in milk, infant formulas, and dairy products after enzymatic hydrolysis of lipids. Nabholz and Herforth (7) described the LC determination of vitamin D in infant formulas by normal phase silica column without preliminary purification after fat extraction and saponification.

The procedure reported here involves 2 main steps: saponification of the sample followed by extraction, and quantification by LC analysis with UV detection at 265 nm. Depending on the nature of each food product, additional steps are necessary, i.e., enzymatic treatment and cartridge purification before LC injection. The method is used to assay vitamin D in fortified skim milk, infant formulas, chocolate drink powder, and diet food.

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METHOD

Apparatus

(a) *LC system.*—Bio-Rad (Richmond, CA) liquid chromatograph, Waters Associates (Milford, MA) Model 710 B autosampler with 30–50 μL injection volume, Perkin-Elmer (Norwalk, CT) LC-75 variable wavelength UV detector with autocontrol, and Perkin-Elmer LC I-100 integrator. Operating conditions: absorbance 265 nm, chart speed 5–10 mm/min, ambient temperature.

(b) *LC columns.*—Two ODS Hypersil, 5 μm (Shandon Southern Products, Runcorn, UK), 120 × 4 mm id columns (Knauer, Berlin, FRG); RP-18, 7 μm, guard column (Brownlee, Santa Clara, CA), 15 × 3.2 mm id.

(c) *LC mobile phase.*—0.5% (v/v) water in methanol, flow rate 1 mL/min.

(d) *Disposable cleanup cartridges.*—Silica, Sep-Pak (Waters Associates).

Reagents

Use low-actinic glassware for all preparations.

(a) *Solvents.*—LC grade 95% ethanol and petroleum ether (bp 40–60°C) (May and Baker, Dagenham, UK).

(b) *Vitamin D_3 standard solutions.*—Dissolve 50 mg vitamin D_3 (Merck, Darmstadt, FRG) in 100 mL volumetric flask with hexane (stock solution). Dilute 2 mL stock solution in 100 mL volumetric flask with hexane. Determine exact concentration from absorption at 265 nm, using $\epsilon^{1\%}_{1cm}$ = 18 200 (solution I). Evaporate hexane from 5 mL solution I to dryness with stream of nitrogen in 100 mL volumetric flask, and dilute to 100 mL with methanol (chromatographic standard solution, 40 IU/mL, 1 μg/mL).

Samples

(a) *Fortified skim milk.*—Pasteurized and ultrahigh temperature (UHT) fortified homogenized skim milk were purchased from a market. Both products claimed values for vitamin D_3 of 220 IU (5.5 μg)/500 mL milk.

(b) *Other foods.*—Infant formulas, 200–400 IU (5–10 μg) vitamin D_3/100 g; chocolate drink powder, 100–500 IU (2.4–12.5 μg)/40 g; and diet food, 100–400 IU (2.4–10 μg)/100 g were supplied by different regional control laboratories.

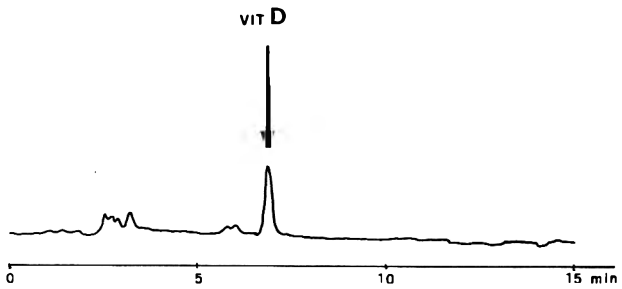

Figure 1. Chromatogram of vitamin D_3 standard, 40 IU/mL (1 μg/mL).

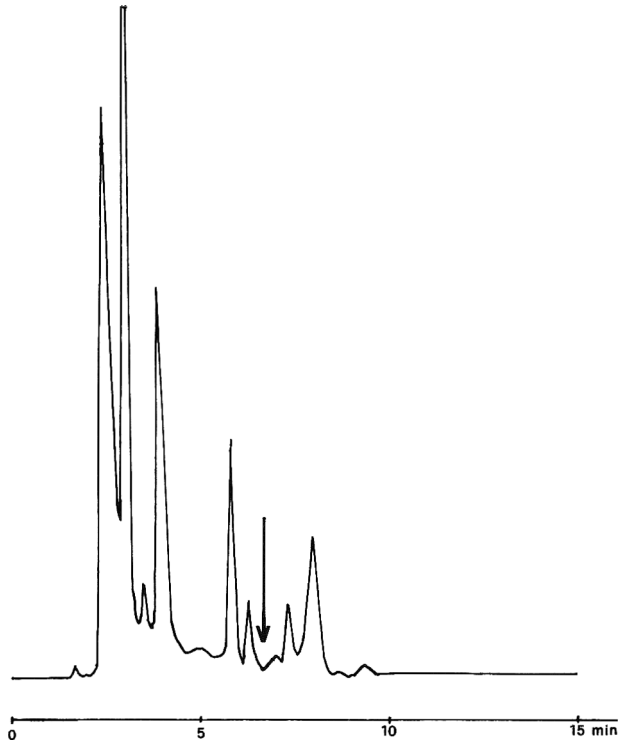

Figure 2. Chromatogram of unfortified sample of reconstituted skim milk. Arrow indicates position of vitamin D.

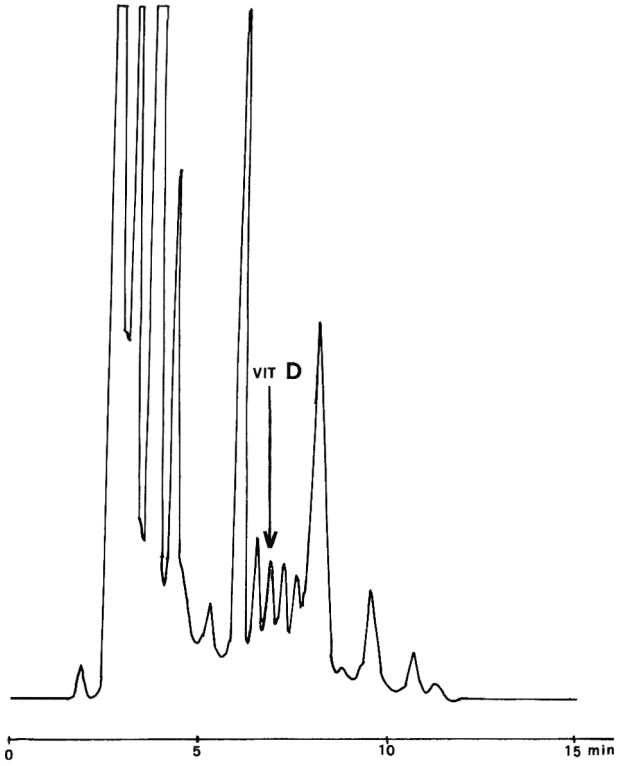

Figure 4. Chromatogram of fortified milk powder.

(c) *Nonfortified skim milk.*—Purchased from Oxoid (Basingstoke, UK), used as a control for recovery of the vitamin.

Procedure

The procedure largely depends on the nature of the sample, although saponification, extraction, and quantitation are, in general, the principal steps. For samples containing starch, digestion with an enzyme such as takadiastase is required before saponification so as to avoid formation of lumps in solution. Additional purification steps on a cartridge prior to analytical chromatography is necessary for high-fat preparations.

(a) *Fortified homogenized skim milk.*—For saponification and extraction, place magnetic stirring bar in 250 mL round-bottom flask, add 40 mL milk sample, 30 mL freshly prepared 1% ethanolic pyrogallol solution, and 10 g of 100%

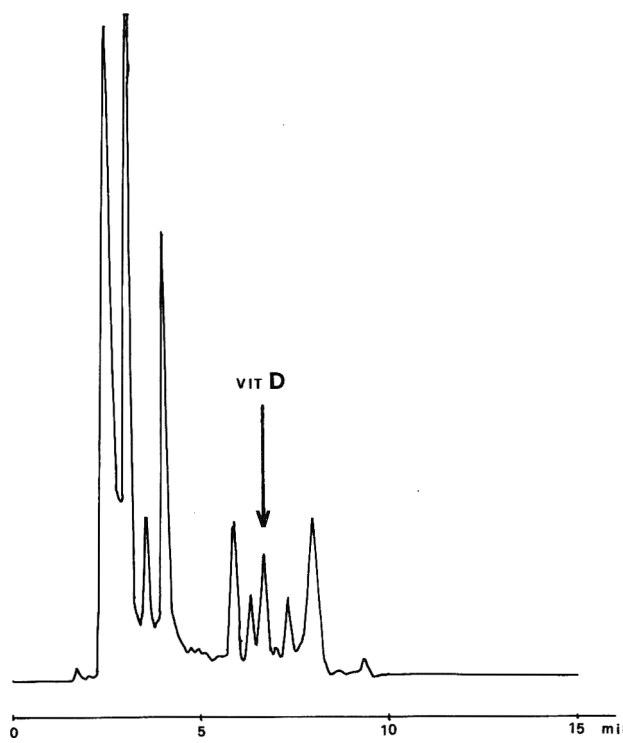

Figure 3. Chromatogram of skim milk fortified with 400 IU vitamin D/100 g.

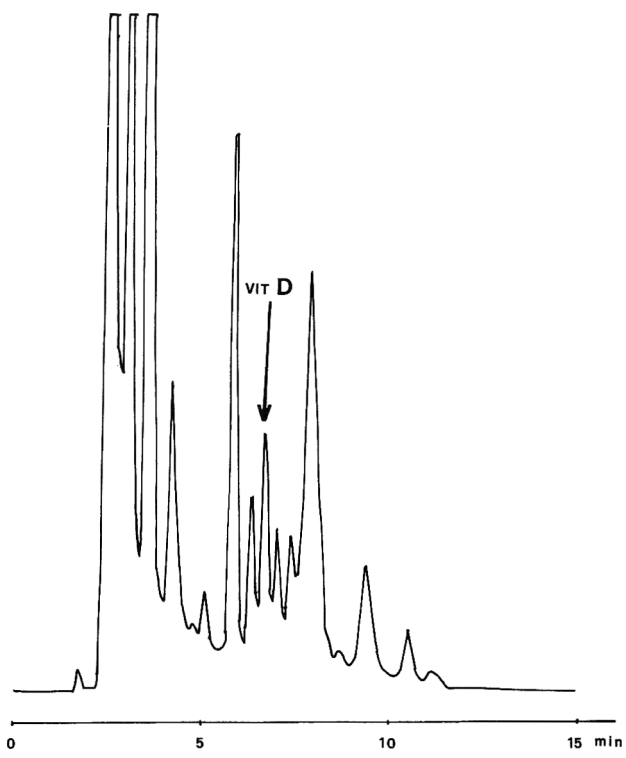

Figure 5. Chromatogram of fortified milk powder spiked with 400 IU vitamin D/100 g.

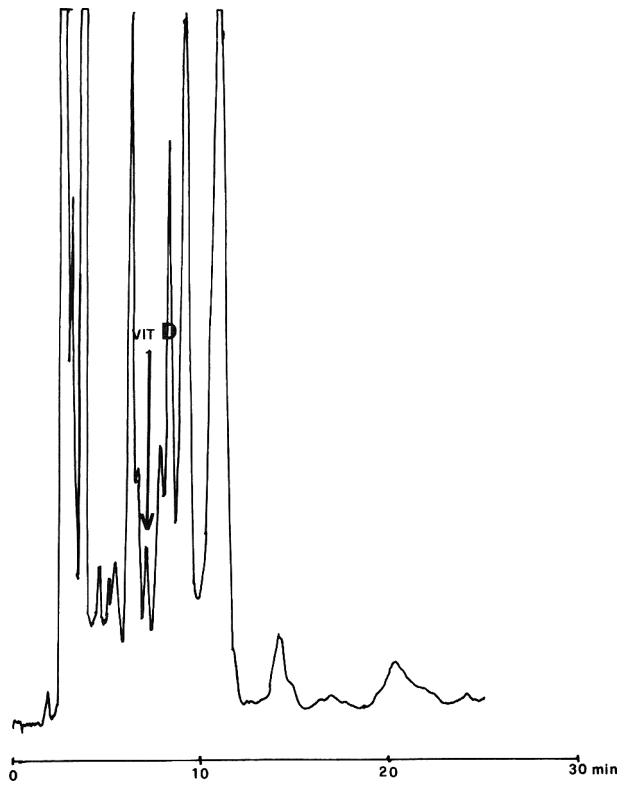

Figure 6. Chromatogram of fortified milk powder with soy, using 50 μL injection volume.

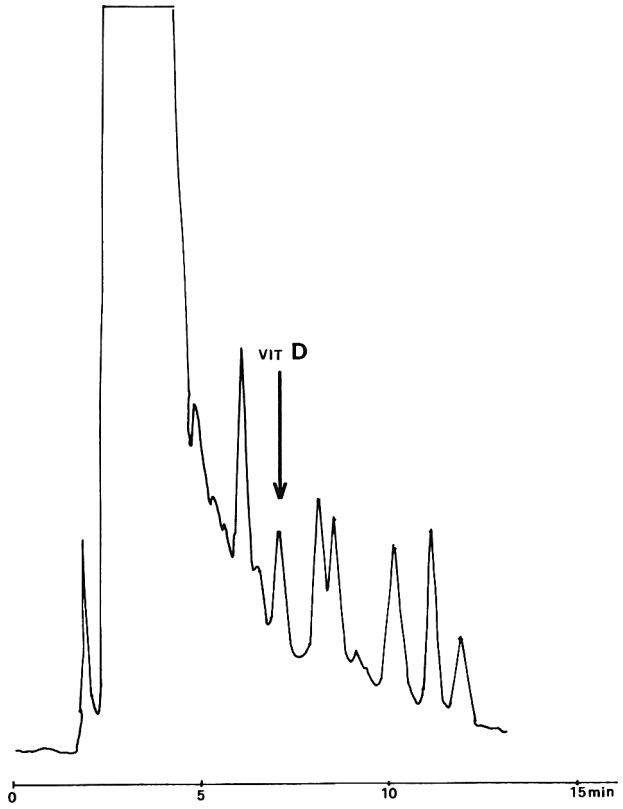

Figure 7. Chromatogram of chocolate powder obtained without cartridge purification step.

KOH. Let mixture saponify under reflux in water bath at 70°C under stream of nitrogen for 30 min with constant stirring. Cool bulb in ice bath. Transfer saponified mixture to 250 mL separatory funnel. Rinse flask with two 10 mL portions of water, 10 mL ethanol, and two 25 mL portions of petroleum ether; add rinses to separatory funnel and shake. Drain lower layer into second separatory funnel and reextract with 4 additional 50 mL portions of petroleum ether. Pour petroleum ether extracts into 500 mL separatory funnel. Wash combined extracts with 50 mL of 5% (w/v) aqueous KOH solution. Draw off KOH layer, reextract with 50 mL petroleum ether, and add extract to previous combined extracts. Wash combined petroleum ether extracts with 50 mL portions of water until there is no reaction to phenolphthalein. Filter washed extract through anhydrous sodium sulfate layer to eliminate residual water. Collect extract in 500 mL round-bottom flask. Rinse separating funnel with petroleum ether. Add 1 mL of 0.1% BHT hexane. Evaporate to dryness under reduced pressure at 40°C. Cool flask in an ice bath. Add 3 mL hexane and swirl flask to dissolve residue.

To purify cartridge, prewet silica cartridge attached to 5 mL syringe with 2 mL hexane. Load exactly 2 mL sample extract into syringe barrel. Insert plunger and pump solution through cartridge. Remove interfering compounds by washing with 3 mL of hexane–ethyl acetate (85 + 15 v/v). Elute vitamin D with 5 mL hexane–ethyl acetate (80 + 20 v/v) into 10 mL conical flask. Evaporate to dryness under stream of nitrogen. Immediately add 500 μL of 1% tetrahydrofuran in methanol (v/v), swirl flask, and proceed with LC analysis for vitamin D.

Table 1. Results of determination of vitamin D in fortified foods

Sample	Declared	Found (±SD)[a]
Homogenized skim milk	220 IU/500 mL	251 ± 4 4
Fortified milk powder	300 IU/100 g	310 ± 6 0
Fortified milk with soy	300 IU/100 g	292 ± 7 2
Chocolate drink powder	100–500 IU/40 g	325 ± 5 2
Sports diet food	100–400 IU/40 g	90 ± 6 7

[a] Each value represents the mean of 3 replicate determinations.

(b) *Fortified infant milk powder.*—(Values of vitamin D in fortified infant milk powder were about 300 IU (7.5 μg)/100 g powder.) Dissolve 10 g fortified milk powder in 20 mL water at 45°C to obtain homogenized suspension. Perform saponification, extraction, and cartridge purification as described under procedure **(a)**.

(c) *Fortified infant milk powder containing cereals.*—Weigh 10 g sample into 250 mL round-bottom flask. Add 20 mL water at 45°C and 0.3 g takadiastase (from *Aspergillus orizae,* Serva Heidelberg, FRG) and incubate 1 h at 45°C. Perform saponification, extraction, cartridge purification, and liquid chromatography as described under procedure **(a)**.

(d) *Chocolate drink powder.*—(Soluble chocolate drink powders are usually fortified with ca 200 IU [5 μg] vitamin D/40 g.) Dissolve 5 g powder in 20 mL water at 45°C. Proceed with saponification, extraction, and LC analysis as described above without cartridge purification step.

(e) *Adult diet food.*—(These are essentially sports diet foods with high levels of carbohydrates such as glucose and starch, and weight-reducing diet food with polysaccharides and fiber. They contain 100–200 IU (2.5–5 μg) vitamin D/40 g.) Weigh 10 g sample into 250 mL round-bottom flask. Add 40 mL water at 45°C, 0.3 g takadiastase, and incubate 1 h at 45°C. Proceed with saponification, extraction, and LC analysis as described above, omitting cartridge purification step.

Results and Discussion

Vitamin D is identified on the chromatogram on the basis of its retention behavior and cochromatography with the

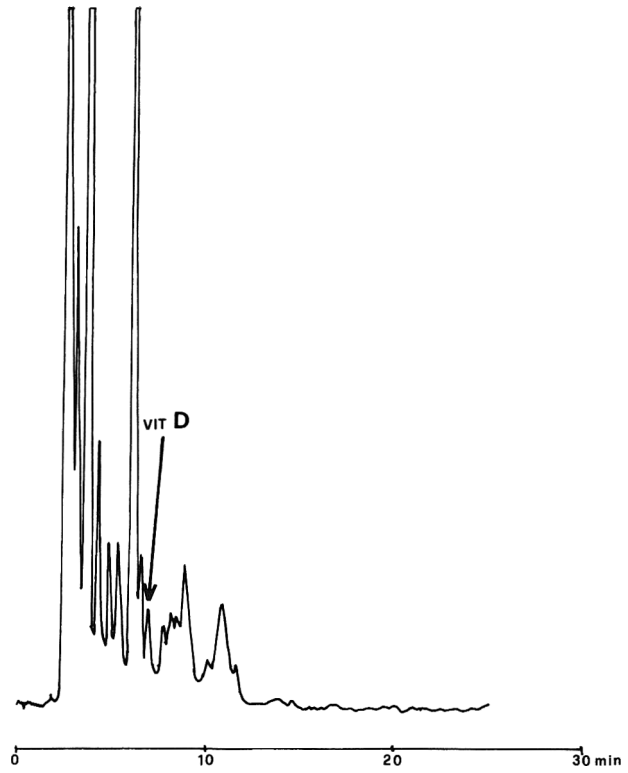

Figure 8. Chromatogram of sports diet food, obtained without cartridge purification step, but using takadiastase to remove starch.

reference compound. It is quantitated on the basis of peak height, using an external standard. A linear response of UV detector was observed from 0.25 to 5 μg/mL (10–200 IU/mL) of vitamin D at 265 nm. The limit of detection was 10 ng.

Reconstituted unfortified nonfat skim milk was fortified with 400 IU (10 μg) vitamin D/100 g product to determine recoveries after saponification, extraction, and cartridge purification. Typical results were 394, 401, 369, 417, and 389 IU/100 g; recoveries were 98.5, 100.3, 92.3, 104.3, and 97.3%. Mean recovery was 98%. Loss of vitamin D during the complete assay was negligible.

Figure 1 shows a chromatogram of the vitamin D_3 standard. Figure 2 represents a chromatogram of an unfortified sample of reconstituted skim milk: the absence of interfering peaks for vitamin D_3 is evident. Figure 3 shows a chromatogram of skim milk fortified with 400 IU vitamin D/100 g milk powder.

Results of vitamin D determination in homogenized skim milk, fortified milk powder, fortified milk powder with soybean, chocolate drink powder, and sports diet food are summarized in Table 1.

Figure 4 is a chromatogram of a fortified milk powder, and Figure 5 is the same product spiked with 400 IU vitamin D/100 g. Figure 6 shows a chromatogram of a milk powder fortified with soybean. Figure 7 is a chromatogram of a chocolate powder obtained without a cartridge purification step. Finally, Figure 8 is a chromatogram of sports diet food also obtained without a cartridge purification step but with takadiastase treatment to remove starch.

Using this procedure over 1 year, we have analyzed 100 samples containing vitamin D without changing the analytical columns. The guard column has been changed once, when a high increase in pressure occurred. When back pressure occurred, the columns were reconditioned by injecting four times with 200 μL portions of DMSO at a flow rate of 1 mL/min followed by water over a 30 min period, and then washing with methanol for 30 min. This procedure considerably extended column life. It has been found that resolution of vitamin D_3 and *dl*-α-tocopherol (vitamin E) with reverse-phase chromatography was superior when using spherical particles rather than irregular ones. The 5 μm ODS Hypersil was selected because of its even particle size distribution and its high carbon content. Recovery of vitamin D_3 following silica Sep-Pak cartridge purification was 98%. The major advantages of the silica Sep-Pak purification step are its simplicity and reproducibility, the disposability of cartridges, and the saving of time in column preparation and standardization. The cartridge removes most unsaponifiable sterols in milk powder remaining after saponification and increases reverse-phase column life. The residue was completely soluble in 1% tetrahydrofuran in methanol for LC analysis, thus avoiding losses of vitamin D.

One sample of fortified milk powder was analyzed by the rat bioassay, yielding 300 IU vitamin D_3/100 g. The proposed procedure gave a vitamin D_3 content of 310 IU/100 g for the same sample. The method described above has been used to analyze more than 200 samples and is simple, rapid, and reliable for survey and routine analyses.

Acknowledgments

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Stabilization of Ascorbic Acid and Its Measurement by Liquid Chromatography in Nonfat Dry Milk

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The determination of ascorbic acid by liquid chromatography (LC) was improved by performing the analysis in the presence of solvents that had been purged with argon to reduce the concentration of oxygen. This methodological modification eliminated the oxidation of ascorbic acid during the chromatographic procedure and reduced the minimum detection level to 1 μg. Solutions of ascorbic acid have been successfully stabilized for 67 days by addition of dithiothreitol to a deaerated solution of water-acetonitrile (25 + 75 v/v), sealed under argon in amber vials and stored at −20°C. In a second independent study, a procedure for the extraction of ascorbic acid from nonfat dry milk in a single step was developed. The ascorbic acid content of Nonfat Dry Milk (SRM 1549) was determined by LC, using the method of standard additions. The mean ascorbic acid content was 54 ± 5 μg/g of sample. Analysis of variance of the analytical results indicates that there is a significant continual increase in the content of the ascorbic acid in each bottle from first to last sample.

The accurate measurement of ascorbic acid has been limited by the inherent instability of this readily oxidizable, photosensitive material. Preparation of dilute standard solutions that are stable for long periods is difficult. Usually, standards are made daily in freshly prepared solutions containing metaphosphoric acid. Antioxidants and chelating agents have also been used to stabilize ascorbic acid (1). A similar approach has been used in the development of methods to stabilize ascorbic acid in biological samples. Ascorbic acid is measured in biological samples after protein precipitation either by forming a stable derivative that can be measured spectrophotometrically (2) or by chromatographically separating the analyte and measuring it spectrophotometrically or electrochemically (1).

The objectives of this study were (a) the development of a procedure for preparation of a stable, dilute solution of ascorbic acid to be used as a reference material in an interlaboratory proficiency testing study; (b) development of an extraction procedure that precipitates proteins and preserves the integrity of ascorbic acid; and (c) extraction and measurement of ascorbic acid in a nonfat dry milk sample which is an NBS Standard Reference Material (SRM) with certified concentrations of selected trace elements.

Experimental

Reagents and Apparatus

(a) *Ascorbic acid solutions.*—Use with dithiothreitol (DTT) (Sigma Chemical Co., St. Louis, MO) in varying amounts.

(b) *Acetonitrile.*—LC grade, 1 mg/mL water–acetonitrile (1 + 3 v/v).

(c) *Liquid chromatograph (method 1).*—Varian Model 5560, equipped with Model 402 data station (Varian Associates, Sunnyvale, CA). Column, Zorbax amine, 30 × 0.4 cm (Du Pont Chemical Co., Wilmington, DE).

(d) *Liquid chromatograph (method 2).*—Described previously (3). Column, μ-Bondapak amine, 30 × 0.78 cm (Waters Associates, Milford, MA).

Evaluation of Stability of Ascorbic Acid Solutions for Use as Standards

Long-term stability studies on ascorbic acid solutions were performed on a Varian Model 5500 liquid chromatograph equipped with a Model 402 data system and a Model 200 detector, using a modification of the method of Doner and Hicks (4). Ascorbic acid was chromatographed isocratically on the Zorbax amine column which was equilibrated with 0.005 mol/L KH_2PO_4–acetonitrile (30 + 70 v/v). The solvent was purged with argon. Pressure on the solvent vessel was maintained at 0.2 psi above ambient, and the temperature was maintained at 30°C; the flow rate was 1 mL/min. Ascorbic acid was detected at 268 nm. No additional reducing agents were used in this procedure.

Determination of Ascorbic Acid in Nonfat Dry Milk (SRM 1549)

Ascorbic acid was determined in nonfat dry milk by a method independent of the one described in the previous paragraph. This measurement was performed on an instrument that we described previously (3) according to the method of Doner and Hicks (4) with the following modifications: Ascorbic acid content of nonfat dry milk was determined isocratically on the μ-Bondapak amine column equilibrated with 0.005 mol/L KH_2PO_4–acetonitrile (30 + 70) to which mercaptoethanol was added at a concentration of 0.125 mL/L solvent. Temperature was 21°C and flow rate was 4 mL/min. Ascorbic acid was detected at 268 nm.

Ascorbic acid concentration in nonfat dry milk was measured by LC, using the standard additions method. Four samples were selected randomly from the samples prepared for SRM 1549, Nonfat Dry Milk. Each sample was analyzed on 2 separate days. The LC standard additions measurement was performed by adding 0, 53, 105, or 158 μg ascorbic acid to a 1 g sample of nonfat dry milk that had been dissolved in 2 mL distilled water containing 1 mg/mL of dithiothreitol (DTT) and stirred 30 s on a vortex mixer. A 4 mL portion of freshly prepared metaphosphoric acid (12% w/v) was added and each sample was again stirred on a vortex mixer for 15 s. Acetonitrile (2 mL) was then added, and each sample was stirred on a vortex mixer for 15 s, and then centrifuged at 1000 × *g* for 1 h. An aliquot of 40 μL was then evaluated by LC.

Results

The ability to measure ascorbic acid accurately depends on the development of extraction and analytical methods that do not permit its oxidation and photodecomposition. The photooxidation can be minimized by preparing and storing the samples in amber vials or by covering clear vials with

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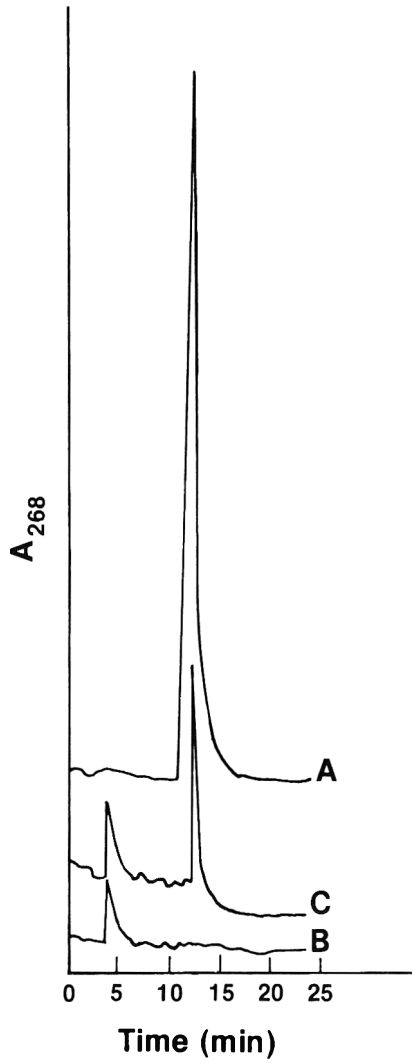

Figure 1. Chromatograms of ascorbic acid and dithiothreitol on Zorbax NH_2 (method 1). Ascorbic acid and/or DTT was applied to column and eluted isocratically with argon-sparged solvent, acetonitrile–5 mmol/L KH_2PO_4 (70 + 30 v/v), temperature 30°C, flow rate 1 mL/min. A, ascorbic acid; B, dithiothreitol; C, mixture of ascorbic acid and dithiothreitol.

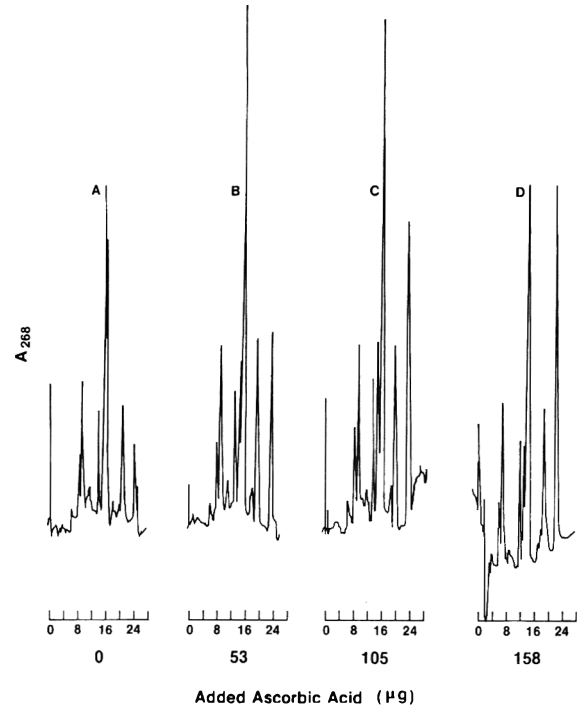

Figure 2. Chromatograms from determination of ascorbic acid by standard additions method. Samples were analyzed isocratically with acetonitrile–5 mmol/L KH_2PO_4 (70 + 30 v/v) containing mercaptoethanol 125 µL/L of solvent; temperature 21°C, flow rate 3 mL/min. A–D, nonfat dry milk with added amounts of ascorbic acid.

aluminum foil. The observation that DTT would reduce dehydroascorbic acid (DHA) (5) suggested that it might be the reagent of choice for conversion of trace levels of DHA to ascorbic acid as well as inhibition of the oxidation of ascorbic acid in aqueous solutions. Since DTT absorbs UV light at 268 nm as does ascorbic acid, it is essential that DTT be resolved from ascorbic acid. Figure 1 demonstrates that DTT elutes near the solvent front (retention time = 4.3 min) whereas ascorbic acid has a retention time of 12 min. Thus DTT does not interfere with the analysis of ascorbic acid by the method used in the studies described here.

Conditions that can affect the stability of ascorbic acid solutions have been evaluated, including solvent composition, DTT concentration, temperature, and atmosphere inside the ampule. Preservation of the integrity of the ascorbic acid was directly dependent on the presence of DTT. When samples were stored in vials that had not been flushed with argon, the ascorbic acid started to oxidize after the DTT was oxidized. In the presence of air, storage at −20°C retarded the oxidation of DTT and ascorbic acid. When the ampules were flushed with argon, sealed, and stored at −20°C, neither the DTT nor the ascorbic acid was oxidized for the duration of the experiment. Solvent composition was not critical. Therefore, water–acetonitrile (30 + 70 v/v) was chosen to minimize the magnitude of the solvent peak. Table 1 shows that ascorbic acid in a water–acetonitrile (30 + 70 v/v) solution stored at −20°C and sealed in an amber vial under an argon atmosphere degrades very little over 67 days.

Ascorbic acid is not particularly stable on the column. The addition of mercaptoethanol to the solvent (method 2) partially prevented the decomposition of ascorbic acid, but the graph of peak height vs concentration in µg/sample, (Y = 1.02X − 12.14, r^2 = 0.992, X intercept = 12.57) does not pass through the origin, indicating that the protection was not complete; therefore, the ascorbic acid content was determined in nonfat dry milk by using the standard additions method. In subsequent studies (method 1) designed to develop a stable, standard reference solution of ascorbic acid, it became necessary to evaluate the conditions needed to completely stabilize ascorbic acid on the column. To achieve this, stabilization was evaluated after the purging of solvents with argon. When the solvents were thoroughly purged with argon, the graph of peak area vs concentration in µg/mL (Y = 10.04X − 3.47, r^2 = 0.998, X intercept = 0.34) passed closer to the origin than in the absence of solvent purging (Y = 7.39X − 15.74, r^2 = 0.999, X intercept = 2.13), demonstrating that solvent purging reduced most of the oxidation of

Table 1. Stability of ascorbic acid solutions

Soln	Concentration of ascorbic acid, µg/mL ± SD[a]		
	0 days	30 days	67 days
11	4.04 ± 0.21	3.64	3.88
12	8.09 ± 0.23	8.16	ND[b]
13	29.01 ± 0.14	29.29	27.52

[a] n = 10.
[b] ND = not determined.

Table 2. Concentration of ascorbic acid in nonfat dry milk

Day	Sample 1-1	5-1	9-1	12-2	1-1
	Concentration of ascorbic acid, mg/g non-fat dry milk (correlation coefficient)				
1	48.3 (0.999)	51.7 (0.999)	61.3 (0.996)	60.7 (0.997)	46.3 (0.998)
3	48.2 (0.999)	53.7 (0.999)	52.3 (0.992)	57.4 (0.999)	

Analysis of variance	DF	SS	MS
Between-sample variation	3	0.137	0.046
Within-sample variation	4	0.047	0.012
Total variation	7	0.185	0.023

ascorbic acid that occurred during the chromatographic procedure.

The procedure for extracting ascorbic acid from nonfat dry milk was developed using the following rationale. Several authors (3, 5) had previously demonstrated that DTT effectively reduced dehydroascorbic acid to ascorbic acid and prevented the oxidation of ascorbic acid. The classical method (2) for extracting ascorbic acid from foods utilized metaphosphoric acid as the stabilizer for ascorbic acid and as a protein precipitant. To convert all of the dehydroascorbic acid to ascorbic acid and preserve the ascorbic acid, the milk powder was dissolved directly in 0.1% DTT. The proteins and other large molecules were precipitated with metaphosphoric acid, and acetonitrile was added to improve the precipitation and to minimize the precipitation of material on the top of the column when the extract came in contact with the chromatographic solvent. Examination of the various extraction procedures indicated that the time intervals between the addition of each of the 3 extraction solvents was not critical. However, recovery of ascorbic acid was not complete at lower concentrations of metaphosphoric acid. Metaphosphoric acid concentrations of 4 to 12% were equally effective in extracting ascorbic acid, and a concentration of 6% metaphosphoric acid was selected to ensure complete extraction. This procedure also permitted the entire extraction to be accomplished in a single tube.

The ascorbic acid concentration in Nonfat Dry Milk SRM 1549 was determined by the standard additions method. Chromatograms of a typical experiment are illustrated in Figure 2. The ascorbic acid had a retention time of 23.6 min. It was completely resolved from the other peaks and was the last peak to elute under the conditions of the analysis. Four randomly selected samples were analyzed on 2 separate days with the exception of sample 1-1 which was analyzed in duplicate on the first day, once at the beginning and once at the end of the experiment (Table 2). Mean concentration of ascorbic acid was 54 ± 5 μg/g of sample (n = 8). Correlation coefficient (r^2) for the linear regression analysis of the standard addition data for the ascorbic acid determination for each nonfat dry milk sample was greater than 0.997 except for one determination which was 0.992. These results suggest that the regression curves generally fit a linear model well and that the variation in the concentration of ascorbic acid was due to the inhomogeneity between samples as well as some inhomogeneity in a single sample, especially sample 9-1. The value for sample 9-1, day 3, shows the greatest deviation from linearity, and some of this variation may be due to a less accurate determination of the intercept of the second set of data for this sample. There was a decided trend toward higher values from sample 1-1 to 12-2, which reflects variation in concentration as a function of the sequence of the sample distribution into the bottles. This trend is confirmed by the magnitude of the between-sample variance in the data summarized in the lower part of Table 2. This inhomogeneity is probably not the result of bias due to methodological error because the values for sample 1-1 at the beginning and at the end of the first day as well as on the second day vary much less than the sample-to-sample variation on either day.

Discussion

Preparation of solutions of ascorbic acid that are stable for extended periods of time (i.e., several months) represents a significant step in our ability to distribute reference samples as dilute solutions. The critical parameters for obtaining this stability are the use of a mixture of acetonitrile and water, the addition of dithiothreitol, the use of amber glass vials, storage under argon in a sealed vial, and storage at −20°C.

The need to measure the concentration of ascorbic acid accurately has also led to the development of methodology for the prevention of the degradation of the ascorbic acid during its residence time on the propylamine column. The critical parameters for achieving this level of stability are purging of the LC solvents with argon and addition of DTT to the sample during its preparation. The purging process removes the majority of the oxygen from the solvents, and DTT prevents oxidation of the ascorbic acid sample while it is in the sample vial awaiting analysis.

The ascorbic acid content of Nonfat Dry Milk (SRM 1549) was determined by use of the standard additions method to eliminate the need to consider the possibility of the slightest amount of oxidation of ascorbic acid during the analysis procedure. However, oxidative degradation followed by further chemical transformation (6) during the preparation and packaging steps is not ruled out. Since the ascorbic acid was added to the samples at the same time as the first solvent, the variations in the ascorbic acid content of the samples can be attributed to either inter- or intra-sample heterogeneity. Both types of variation were observed. In sample 9-1, intrasample variation was large and there was a significant trend toward higher values from sample 1-1 to 12-2. This variation was not due to a systematic error in the analysis. In support of this conclusion, the ascorbic acid was measured in sample 1-1 at the beginning and at the end of the first series of measurements. The similarity of the values for the 2 estimations of sample 1-1 indicates that this trend is not related to the order of sample analysis. These analytical results demonstrate the necessity of performing multiple analyses on a single sample and of assessing the content of a specific vitamin in samples that are taken at known intervals during the packaging process.

Finally, several samples from this same lot of nonfat dry milk were analyzed by Tanner et al. (7). The range of ascorbic acid content in this study was 43–55 μg/g of nonfat dry milk. This range of ascorbic acid content, which was determined by a totally independent method, is consistent with our results. In the absence of measurable systematic errors, these values represent the actual range of ascorbic acid concentration in this SRM.

Acknowledgments

We thank Robert Schaffer for his advice and encouragement, John Mandel for his advice on the statistical analysis

of the standard additions data, and James Tanner for sharing his data with us during the early stages of this project.

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Determination of Depletion of Bithionol Sulfoxide and Its Two Major Metabolites in Bovine Milk

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A liquid chromatographic method is described for determining bithionol sulfoxide and its metabolites, bithionol and bithionol sulfone, in milk. Samples are treated with HCl to precipitate proteins and to permit extraction of bithionol sulfoxide in nonionized form. Tetrahydrofuran is added to the organic phase to facilitate extraction in diethyl ether; the dried residue is dissolved in chloroform, hexane, and sodium hydroxide and subjected to LC analysis. Residues of bithionol sulfoxide and its 2 metabolites were determined in milk of lactating cows. Holstein-Friesian dairy cows were administered a single oral dose of bithionol sulfoxide (50 mg/kg). Milk samples were analyzed with a reliable detection level of 0.025 μg/mL for each compound. Residues of bithionol sulfoxide and bithionol were detected during 30 and 16 milkings, respectively; bithionol sulfone was never present at detectable levels.

The anthelmintic bithionol sulfoxide [2,2'-sulfinyl-bis (4,6-dichlorophenol)] (Figure 1) is widely used in veterinary medicine as a fasciolicide, especially in bovine species (1). The metabolic fate of this compound has been investigated in rats (2) and goats (3). Bithionol and bithionol sulfone (Figure 1) are metabolites of bithionol sulfoxide, occurring via reduction and oxidation pathways, respectively. Plasma kinetics in lactating cows has shown that bithionol sulfoxide is also converted to bithionol and bithionol sulfone. A method was developed for determining bithionol sulfoxide and its metabolites (4) but was unsuitable for milk.

For this reason, a liquid chromatographic method was developed with a reliable detection level of 0.025 ppm in milk samples. Data on depletion of bithionol sulfoxide and its 2 metabolites in milk of treated lactating cows are also presented.

METHOD

Apparatus and Reagents

(a) *Shaker.*—Rotary, capable of 35 rpm.

(b) *Liquid chromatograph.*—Varian Model 5060 gradient system equipped with 200 μL sample loop, Varian UV 100 variable wavelength detector set at 303 nm, 10 cm × 4.6 mm id column packed with 5 μm Lichrosorb RP-8, and Vista Model 402 computer (Varian) for quantitation. Use acetonitrile–0.2N sulfuric acid (55 + 45) as mobile phase at 1 mL/min.

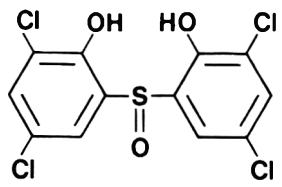
(c) *Solvents.*—Acetonitrile, residue analysis grade; all other chemicals, reagent grade (Merck, GFR).

(d) *Standard solutions.*—(*1*) *Stock solutions.*—In 100 mL volumetric flasks, separately dissolve 15 mg bithionol sulfoxide (Sanofi Santé Animale, Paris, France), bithionol (Vétoquinol, Lure, France), and bithionol sulfone (IRCHA, Paris, France) in 0.5 mL 10N sodium hydroxide. Dilute to volume with water. Dilute aliquot of each stock solution with water to give (1 + 1) intermediate solution. (*2*) *Working solutions*—Further dilute intermediate solutions with water as needed.

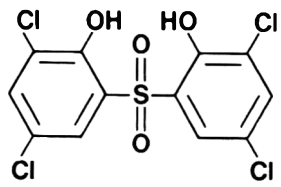
Extraction and Cleanup

Add 20 drops of 12N HCl and 30 mL tetrahydrofuran to 50 mL milk in 250 mL screw-cap centrifuge tube. Shake vigorously. Add 100 mL diethyl ether and agitate for 20 min on rotary shaker. Centrifuge 5 min at 1000 rpm. Pour aqueous phase into 150 mL beaker. Add 1 g Na_2CO_3 for drying and shake gently. Let settle (10 min) and transfer 65 mL extract to 100 mL round-bottom flask. Evaporate to dryness, under reduced pressure, in 40°C water bath.

Dissolve residue in 3 mL chloroform, 3 mL hexane, and 2.5 mL of 0.02N sodium hydroxide with 3 drops of phenolphthalein. Transfer mixture to 10 mL centrifuge tube and centrifuge 5 min at 5000 rpm. Inject 200 μL supernatant liquid into chromatograph.



Bithionol sulfoxide



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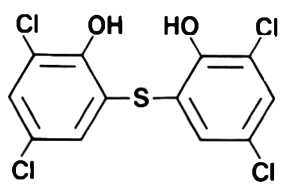

Bithionol

Figure 1. Structures of bithionol sulfoxide and its 2 metabolites.

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Table 1. Recoveries of bithionol sulfoxide and its metabolites from spiked milk samples

Amount added, ppm	% Rec., mean ± SD (*n*)		
	Bithionol sulfoxide	Bithionol	Bithionol sulfone
0.025	89.7 ± 1.3 (4)	80.1 ± 3.7(4)	79.8 ± 3.2 (4)
0.05	83.1 ± 2.4 (8)	82.9 ± 1.9 (8)	84.7 ± 3.9 (8)
0.1	99.9 ± 1.7 (4)	91.5 ± 1.9 (4)	94.1 ± 4.6 (4)
Mean rec., % ± SD	90.9 ± 8.5 (16)	84.8 ± 5.9 (16)	86.2 ± 7.3 (16)
	$y = 15.33x - 103.73$ $r = 0.998$	$y = 19.25x - 170.90$ $r = 0.999$	$y = 15.98x - 154.55$ $r = 0.998$

Results and Discussion

Bithionol sulfoxide appeared to be a dibasic acid compound. The 2 ionization constants (5) showed that, at the pH of the bovine milk (pH 6.5), bithionol sulfoxide was present in ionized forms. Treatment of milk with hydrochloric acid was effective in precipitating proteins and in permitting extraction of bithionol sulfoxide by organic solvents as a nonionized form; tetrahydrofuran was added to the organic phase to facilitate extraction into diethyl ether.

To obtain the final sample in water, lipids were dissolved in hexane and chloroform, and bithionol sulfoxide was then reextracted into a small volume of 0.02N sodium hydroxide. Under these conditions, bithionol sulfoxide was converted to its dibasic form and easily recovered in the water phase.

Table 1 summarizes recoveries from spiked milk samples. Recoveries for bithionol sulfoxide were 83–100%, but recoveries for its 2 metabolites were generally much lower. Mean recoveries were also calculated for use in the residue study. Detector response to the 3 compounds, based on peak area, is linear over concentrations of 0.025–0.1 ppm.

Figure 2 shows chromatograms of a blank and milk samples spiked with 0.1 and 0.025 ppm each of the trial compounds. Bithionol sulfoxide and its 2 metabolites were well separated from a minor interfering peak (Figure 2A) eluting between bithionol and bithionol sulfone. The limit of detection is about 0.01 ppm for bithionol sulfoxide and 0.02 ppm for bithionol and bithionol sulfone.

Recovery Experiment

Spike bovine milk with 0.025, 0.05, and 0.1 ppm bithionol sulfoxide and its 2 metabolites. Proceed with extraction described above. Compare peak areas obtained with those of appropriate analytical standards.

Bovine Treatment Protocol

Four clinically healthy Holstein-Friesian cows received a single oral dose of 0.5 mL/kg of Nilzan FN (Rhône-Mérieux, France), a veterinary suspension containing 10% bithionol sulfoxide and 1.5% levamisole hydrochloride. Milk samples (100 mL) were taken before drug administration and then at every milking (9 am and 5 pm) for 15 days.

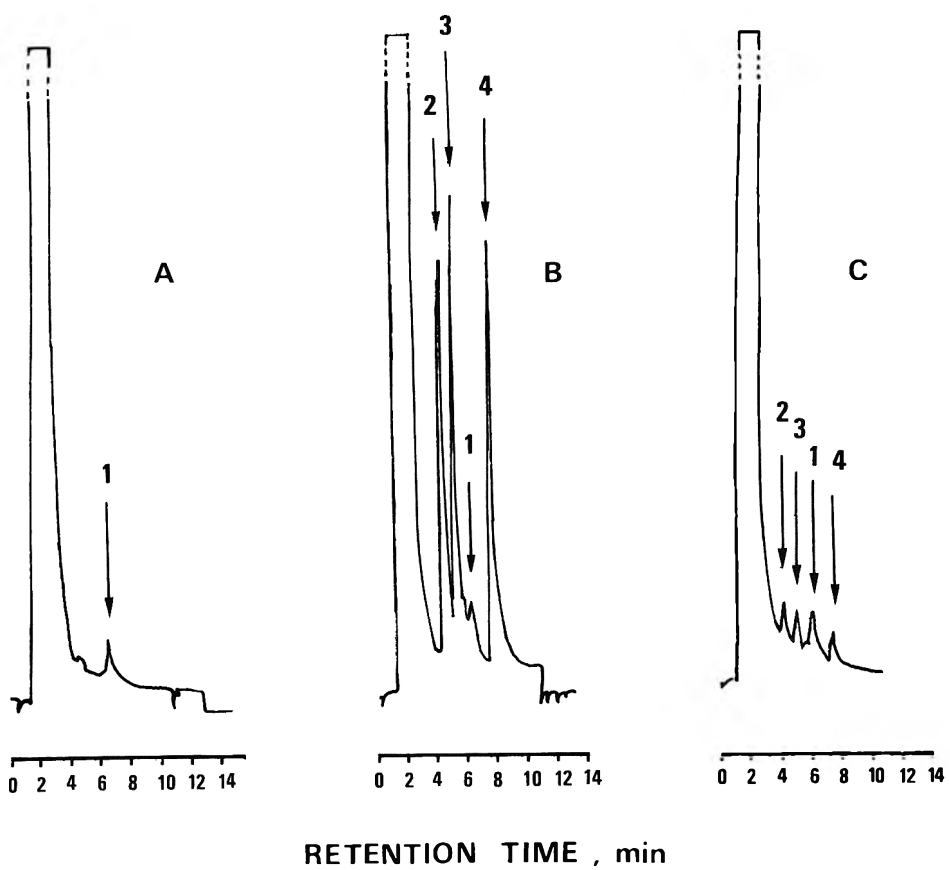

Figure 2. Chromatograms of bithionol sulfoxide (2), bithionol (3), and bithionol sulfone (4) in milk extracts: A, milk extract blank (1 = unidentified peak); B, milk fortified with 0.1 ppm of each compound; and C, milk fortified with 0.025 ppm.

Table 2. Bithionol sulfoxide residues in bovine milk after a single oral dose of 0.5 mL/kg bithionol sulfoxide suspension

Time after treatment (h)	Residues, ppm				
	No. 4[a]	No. 8	No. 13	No. 14	Mean ± SD
T_1 (8)	0.07	0.08	0.10	0.09	0.09 ± 0.01
T_2 (24)	0.10	0.25	0.23	0.21	0.20 ± 0.07
T_3 (32)	0.10	0.13	0.20	0.22	0.16 ± 0.06
T_4 (48)	0.06	0.13	0.13	0 14	0.12 ± 0.04
T_5 (56)	0.08	0.13	0.17	0 19	0.14 ± 0.05
T_6 (72)	0.04	0.11	0.13	0 27	0.14 ± 0.10
T_7 (80)	0.05	0.08	0.10	0.15	0.10 ± 0.04
T_8 (96)	0.05	0.08	0.10	0.14	0.09 ± 0.04
T_9 (104)	0.06	0.11	0.08	0.14	0.10 ± 0.04
T_{10} (120)	0.06	0.14	0.07	0.10	0.09 ± 0.04
T_{11} (128)	0.04	0.05	0.06	0.10	0.06 ± 0.03
T_{12} (144)	0.04	0.07	0.06	0.10	0.07 ± 0.03
T_{13} (152)	0.04	0.06	0.08	0.09	0.07 ± 0.02
T_{14} (168)	0.03	0.07	0.05	0.09	0.06 ± 0.03
T_{15} (176)	0.05	0.06	0.06	0.08	0.06 ± 0.01
T_{16} (192)	0.02	0.07	0.06	0.09	0.06 ± 0.03
T_{17} (200)	0.03	0.09	0.05	0.07	0.06 ± 0.03
T_{18} (216)	0.03	0.08	0.04	0.07	0.06 ± 0.02
T_{19} (224)	0.03	ND	0.03	0.08	0.04 ± 0.03
T_{20} (240)	0.04	0.07	0.04	0.07	0.06 ± 0.02
T_{30} (248)	ND[b]	0.03	ND	0.05	0.03 ± 0.01

[a] Animal identification.
[b] <0.025 ppm.

Table 3. Bithionol residues in bovine milk after a single oral dose of 0.5 mL/kg bithionol sulfoxide suspension

Time after treatment (h)	Residues, ppm				
	No. 4[a]	No. 8	No. 13	No. 14	Mean ± SD
T_1 (8)	ND[b]	ND	ND	ND	—
T_2 (24)	0.20	0.20	0.31	0.26	0.24 ± 0.05
T_3 (32)	0.19	0.38	0.35	0.43	0.34 ± 0.10
T_4 (48)	0.25	0.28	0.19	0.35	0.27 ± 0.07
T_5 (56)	0.17	0.36	0.16	0.38	0.27 ± 0.12
T_6 (72)	0.10	0.28	0.07	0.15	0.15 ± 0.09
T_7 (80)	0.06	0.10	0.05	0.18	0.10 ± 0.06
T_8 (96)	0.04	0.07	0.04	0.13	0.07 ± 0.04
T_9 (104)	0.04	0.09	0.04	0.10	0.07 ± 0.03
T_{10} (120)	0.03	0.08	0.03	0.09	0.06 ± 0.03
T_{11} (128)	ND	0.05	ND	0.05	0.04 ± 0.01
T_{12} (144)	ND	0.04	ND	0.04	0.03 ± 0.01
T_{13} (154)	0.04	0.07	ND	0.07	0.05 ± 0.02
T_{14} (168)	ND	0.04	ND	0.04	0.03 ± 0.01
T_{15} (176)	ND	ND	ND	ND	—
T_{16} (192)	ND	ND	ND	ND	—
T_{17} (200)	ND	ND	ND	ND	—
T_{18} (216)	ND	ND	ND	ND	—
T_{19} (224)	ND	ND	ND	ND	—
T_{20} (240)	ND	ND	ND	ND	—
T_{30} (248)	ND	ND	ND	ND	—

[a] Animal identification.
[b] <0.025 ppm.

Tables 2 and 3 summarize results of residue analysis of treated lactating cows. For bithionol sulfoxide the highest concentration seen after 24 h (i.e., on the T_2 samples) was 0.2 ppm. Levels of bithionol were much higher (0.34 ppm) but were observed after 36 h. At 7 days after treatment, bithionol was undetectable; however, bithionol sulfoxide was present at significant levels until 10 days after treatment. No residues of bithionol sulfone were detected during the residue study. Excretion of bithionol sulfoxide in bovine milk appeared to be a minor route of elimination, probably because bithionol sulfoxide was bound to plasma protein to a high extent. In lactating cows (4), the plasma half life of bithionol sulfoxide calculated during the elimination phase was 173.38 ± 22.2 h (n = 5).

There are no published reports on quantitative depletion of bithionol sulfoxide in milk after oral administration to lactating cows. Our results show that, at therapeutic dosage, it may be necessary to discard milk for more than 15 days to ensure that it is free of detectable residues (<0.025 ppm) of the parent drug.

The sample cleanup procedure was selective enough to permit determination of bithionol sulfoxide and its 2 metabolites at low residue levels; recoveries from milk were consistently high for the 3 compounds.

Acknowledgment

The authors thank J. Manceau for technical assistance.

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Spectrofluorometric Determination of BAY Vp 2674 Residues in Poultry Tissues

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A spectrofluorometric (SPF) method is described for determination of residues of BAY Vp 2674 in chicken and turkey tissues. The drug is extracted from tissues with dichloromethane–methanol. The organic extract is concentrated to near dryness and cleaned up by a series of partitionings with *n*-hexane, then dichloromethane against pH 2 buffer and dichloromethane against pH 12 buffer. The drug is partitioned into dichloromethane from pH 7 buffer and concentrated to dryness. The residue is dissolved in pH 3.5 buffer for SPF analysis at 282 nm (excitation) and 445 nm (emission). Recoveries of BAY Vp 2674 added to chicken and turkey tissues at levels of 0.05, 0.1, and 0.2 ppm range from 86 to 92% with a coefficient of variation of 3.4-10.1%. Detection limit is 0.02 ppm. A liquid chromatographic confirmatory procedure is also described, with ultraviolet and fluorescence detection.

BAY Vp 2674, (common name, enrofloxacin) 1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxonb3-quinolinecarboxylic acid, is a new derivative of 3-quinolinecarboxylic acids having a broad spectrum of antimicrobial activity. Its structure is shown in Figure 1. To monitor drug residues, an analytical residue procedure was developed to determine levels of BAY Vp 2674 in liver, muscle, and skin of turkeys and chickens. Nalidixic acid, a 1,8-naphthyridine-3-carboxylic acid which has a similar structure and also has antimicrobial activity, has been determined in chicken muscle and fat by a fluorescence method (1). Tissue methods have been described for other 3-quinolinecarboxylic acids for determining pefloxacin in rats and dogs (2) and norfloxacin in human prostate and liver (3). Early methods consisted of extraction/partitioning of the sample with chloroform or methylene chloride and subsequent fluorescence measurements in acidic aqueous media (4). Analytical methods for this class of drugs have been reviewed (5). Recent methods include liquid chromatographic (LC) determination of the following drugs in animal body fluids: rosoxacin (6), miloxacin (7), norfloxacin (8,9), ciprofloxacin (10), and amifloxacin (11).

The procedure reported here represents the first tissue monitoring method for a 3-quinolinecarboxylic acid residue in poultry. A simple cleanup of the initial extract by partitionings alone and subsequent direct measurement by fluorescence allow a rapid analysis. Further cleanup and measurement by LC with either ultraviolet (UV) or fluorescence detection provide versatility and specificity to the method.

METHOD

Reagents

Use distilled water and analytical LC grade chemicals and solvents unless otherwise specified.

(a) *Solvents.*—Dichloromethane, methanol, and *n*-hexane (Fisher Scientific).

(b) *Phosphate buffers.*—Prepare 1M KH_2PO_4 stock solution and adjust to pH 2.0 and 3.5 with orthophosphoric acid or to pH 7.0 and 10.0 with 5M NaOH. Dilute stock solutions 10-fold to 0.1M as working solutions.

(c) *Triethylamine (TEA), H_3PO_4, KH_2PO_4, and NaOH.*—(Fisher Scientific.)

(d) *BAY Vp 2674 standard solution.*—Prepare stock solution at 500 μg/mL, using 50 mg BAY Vp 2674 (Mobay Corp., Animal Health Division, Lot no. R84-335-79, 99.2% a.i.) in 100 mL CH_2Cl_2. *Methanol solution.*—Dilute 0.4 mL stock solution to 100 mL CH_3OH. *Phosphate buffer solution.*—Evaporate 0.1 mL stock solution to dryness and redissolve residue in 100 mL of 0.1M phosphate buffer (pH 3.5). *Mobile phase working standards.*—Evaporate 0.1 mL stock solution and redissolve residue in mobile phase to give 0.25, 0.5, and 1.0 μg/mL.

(e) *"Keeper" solution.*—Prepare 5% solution of diethylene glycol in CH_2Cl_2 (v/v). Addition of several drops to sample solution prior to evaporation minimizes loss of low levels of BAY Vp 2674.

Apparatus

(a) *Spectrophotofluorometer.*—Aminco-Bowman equipped with 1P21 phototube (American Instrument Co., Silver Spring, MD 20901).

All fluorescence measurements of BAY Vp 2674 were at 282 nm (excitation) and 445 nm (emission) in 1 cm square cells (Figure 2). Maximum relative intensity (RI) from 0.5 μg/mL BAY Vp 2674 in 0.1M phosphate buffer occurs at pH 3.5 (Figure 3). A 0.1M buffer concentration was chosen because it provides good buffering capacity with maximum fluorescence intensity.

Measurements of RI at various levels of concentration showed a linear response from 0.001 to 1.0 μg/mL in 0.1M phosphate buffer at pH 3.5, but at increasing concentrations of BAY Vp 2674 RI decreased, Figure 4.

(b) *Liquid chromatograph.*—Applied Chromatography Systems (State College, PA). Column: 4.6 mm × 25 cm, Supelcosil, 5 μm LC-18 DB reverse phase (BelleFonte, PA). Detectors: UV detector (Applied Chromatography Systems), 280 nm, range setting 0.03, and gain adjusted to give peak height of 60% full scale for 10 ng BAY Vp 2674; fluorescence detector, Model Mark I fluorometer (Farrand, Valhalla, NY), 282 nm (excitation) and 445 nm (emission), equipped with 10 μL flow cell. Fluorescence detector is operated in tandem with UV detector. Operating parameters: Mobile phase, acetonitrile–water–TEA (20 + 75 + 5) adjusted to pH 3.5 with orthophosphoric acid; pressure, 1500 psi; flow rate, 1 mL/min; injection volume, 10 μL. Under these conditions, retention time (t_R) of BAY Vp 2674 is 5.6 min.

(c) *Centrifuge.*—IEC Model 5BR (International Equipment Co., Boston, MA).

(d) *Soxhlet extractor.*—400 mm id (Fisher Scientific).

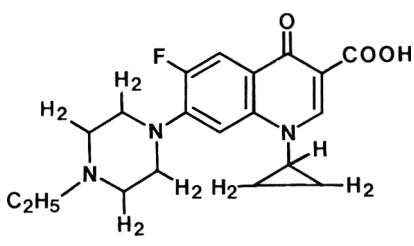

Figure 1. Structure of BAY Vp 2674.

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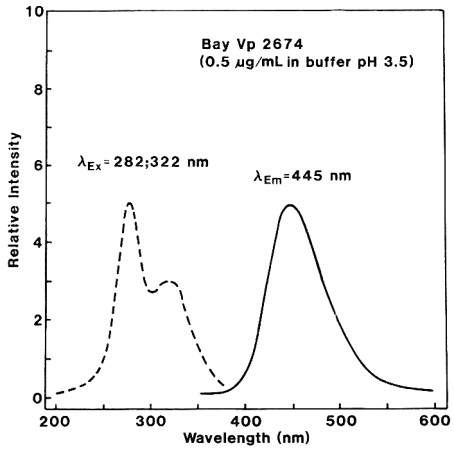

Figure 2. Fluorescence spectra of BAY Vp 2674.

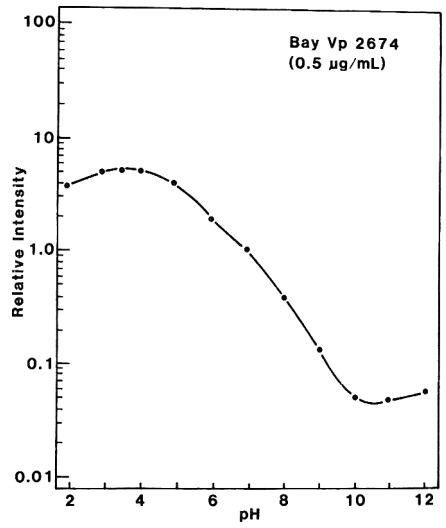

Figure 3. Effect of pH on relative intensity of 0.5 µg/mL BAY Vp 2674 in 0.1M phosphate buffer (pH 2–12).

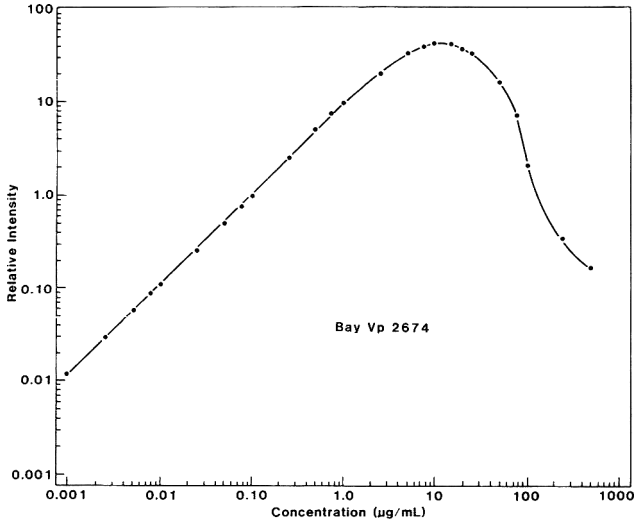

Figure 4. Effect of concentration on relative intensity of BAY Vp 2674 in 0.1M phosphate buffer (pH 3.5).

(e) *Culture tubes.*—All culture tubes are borosilicate glass equipped with Teflon-lined screw caps.

(f) *Hypodermic syringes.*—Fitted with cannulas. Use for transfers of all layers in cleanup stage.

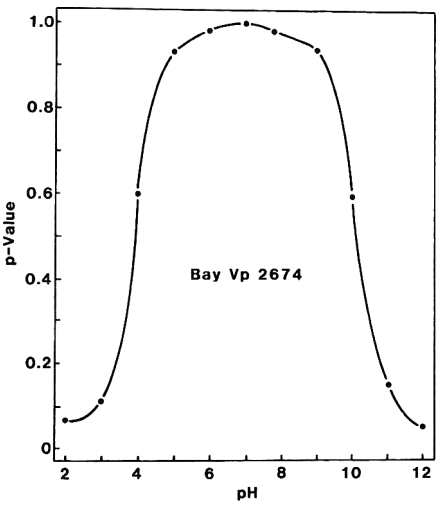

Figure 5. Distribution of *p*-values of BAY Vp 2674 between dichloromethane and phosphate buffer, pH 2–12.

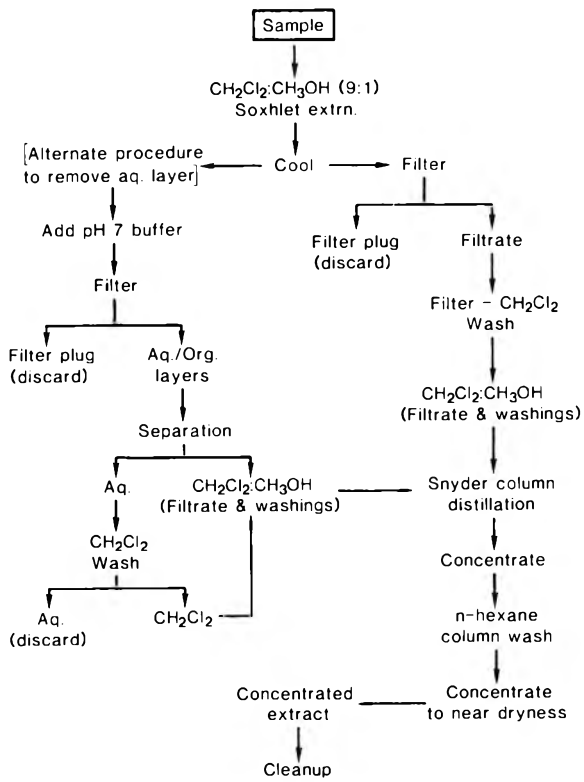

Figure 6. Flow scheme for extraction of BAY Vp 2674 from poultry tissue.

Fortification of Tissue Samples

To 20 g homogenized tissue, add 0.5, 1.0, and 2.0 mL of 2 µg/mL methanolic solution of BAY Vp 2674 to give 50, 100, or 200 parts per billion (ppb) in tissue. Let stand at room temperature 1 h prior to addition of extraction solvent.

Calculations

Standardize spectrophotofluorometer to give intensity reading of 5.0 with 0.5 µg/mL quinine sulfate dihydrate in 0.1N H_2SO_4 at 350 nm (excitation) and 450 nm (emission). Then, determine maximum relative intensity (RI) for 0.1M pH 3.5 phosphate buffer (background) and 50, 100, and 200 µg/mL solutions of BAY Vp 2674 in the same buffer from meter readings and the multiplier setting: RI = meter ×

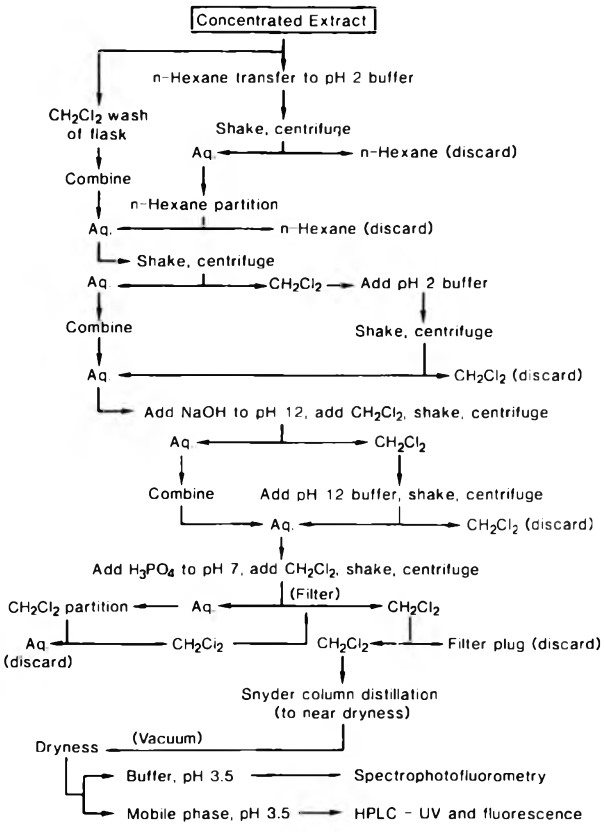

Figure 7. Flow scheme for cleanup of extract of BAY Vp 2674 from poultry tissue.

multiplier. Determine corrected RI by subtracting RI (background) from uncorrected RI.

For determining corrected RI for all control and fortified tissue samples, set RI value at 2.00 for 100 ppb standard solution of BAY Vp 2674 in 0.1M pH 3.5 phosphate buffer.

Tissue controls (unfortified):

$$\text{Corrected RI (control)} = \text{RI} - 0.02 \text{ buffer (background)}$$

Fortified tissues:

$$\text{Corrected RI} = \text{RI} - \text{uncorrected RI (control)}$$

$$\text{Residue, ppb} = \frac{\text{corrected RI (sample)}}{\text{corrected RI (standard)}} \times \text{ppb (standard)}$$

Extraction

Place 20 g finely chopped sample (ca 20 mesh or finer) into Soxhlet extractor containing glass wool plug to prevent siphoning of sample particles into extraction flask. Extract sample 15 h with 130 mL dichloromethane–10% MeOH (v/v) at rate of 5 solvent exchanges/h. Let extract cool and examine it for presence of distinct aqueous layer. If none is present, filter extract through glass wool plug (ca 25 × 10 mm thick), wash flask, and plug with 20 mL and then 10 mL dichloromethane. Reserve combined filtrates in 250 mL boiling flask.

If distinct aqueous layer is present, add 1 mL of 1M pH 7 buffer, mix contents well, and filter through glass wool plug into 250 mL separatory funnel. After layers separate, drain organic layer into 250 mL boiling flask, wash extraction flask and filter plug, and extract aqueous layer in a separatory funnel with additional 20 mL and then 10 mL dichloromethane. Reserve combined extracts.

Table 1. Spectrofluorometric analysis of chicken tissue fortified with BAY Vp 2674

Tissue	Added, ppb	Recovered ($\bar{x}$ ± SE)[a] ppb	%
Liver	control	9 ± 1	—
	50	43 ± 3	86 ± 6
	100	89 ± 5	88 ± 5
	200	176 ± 12	88 ± 6
Muscle	control	7 ± 2	—
	50	44 ± 3	88 ± 6
	100	89 ± 6	89 ± 6
	200	178 ± 6	89 ± 3
Skin	control	7 ± 1	—
	50	43 ± 2	86 ± 4
	100	89 ± 9	89 ± 9
	200	185 ± 8	92 ± 4

[a] Triplicate samples.

Table 2. Spectrofluorometric analysis of turkey tissue fortified with BAY Vp 2674

Tissue	Added, ppb	Recovered ($\bar{x}$ ± SE)[a] ppb	%
Liver	control	5 ± 1	—
	50	47 ± 3	93 ± 6
	100	89 ± 8	89 ± 8
	200	168 ± 8	84 ± 4
Muscle	control	7 ± 1	—
	50	43 ± 3	86 ± 6
	100	90 ± 5	90 ± 5
	200	182 ± 8	91 ± 4
Skin	control	7 ± 1	—
	50	45 ± 4	90 ± 8
	100	82 ± 4	82 ± 4
	200	179 ± 8	90 ± 4

[a] Triplicate samples.

Table 3. Recovery of BAY Vp 2674 from chicken and turkey skin fortified at 50 ppb as determined by SPF and LC analysis[a]

Method of detection	Found, ppb		Mean recovery, %	
	Chicken	Turkey	Chicken	Turkey
SPF	43–49	43–46	89.5	88.5
LC-UV	47–49	46–49	97.6	95.8
LC-fluorescence	45–46	44–46	91.1	92.6

[a] Each value represents a mean of 4 separate determinations.

Carefully concentrate extracts to near dryness by distillation under 3-ball Snyder column; remove distillation flask and Snyder column, wash column with 10 mL hexane, and again concentrate contents to near dryness. Reserve residue for cleanup.

Cleanup

Transfer concentrated extract (containing 1 mL or less of hexane) to culture tube (150 mm × 25 mm; ca 53 mL capacity) containing 10 mL of 0.1M pH 2 buffer by using three 10 mL portions of hexane. Wash inner walls of flask with 10 mL dichloromethane and gently warm and swirl contents to dissolve any additional residue. Transfer contents to second culture tube of the same size. Again wash flask with 10 mL dichloromethane and reserve combined washings (20 mL) for use in subsequent extraction. Shake hexane and pH 2 buffer phases in first tube vigorously by hand for 2 min. Centrifuge and, using hypodermic syringe, remove and dis-

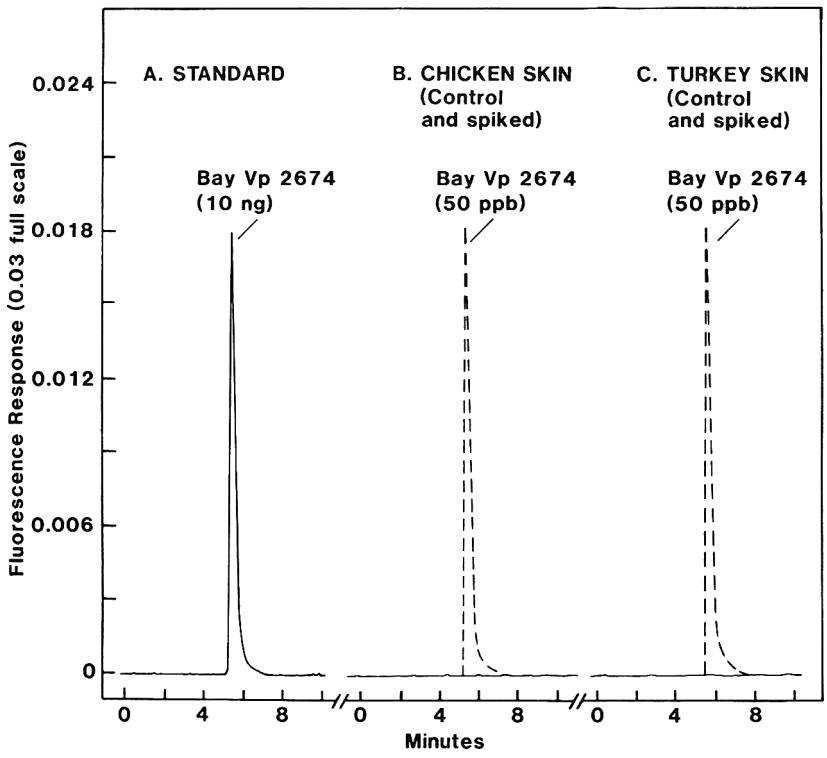

Figure 8. LC fluorescence response to BAY Vp 2674 standard alone and in presence of chicken and turkey skin controls.

card hexane layer, taking care not to remove any aqueous layer. Extract aqueous layer with additional 10 mL portion of hexane. Centrifuge and again carefully remove hexane layer and discard it.

Add 20 mL dichloromethane washings reserved in second tube to buffer layer in first tube; shake contents vigorously 1 min, centrifuge, and transfer organic layer back to second tube. Add 10 mL of 0.1M pH 2 buffer to second tube, shake contents vigorously 1 min, centrifuge, and transfer aqueous layer to first tube (now contains 20 mL of pH 2 buffer); discard second tube and contents. Adjust aqueous phase to pH 12 by adding 1.7 mL of 5M sodium hydroxide. Add 20 mL dichloromethane, shake vigorously 1 min, centrifuge, and transfer aqueous layer (20 mL) to larger culture tube (200 mm × 25 mm; ca 73 mL capacity). Add 10 mL of 0.1M pH 12 buffer to dichloromethane in first tube, shake contents vigorously 1 min, centrifuge, and transfer aqueous layer to larger tube (now contains 30 mL); discard first tube and contents. Add 0.6 mL of 30% H_3PO_4 tc aqueous layer (30 mL) and measure pH to ensure that aqueous solution is exactly pH 7.0. Then add 30 mL dichloromethane. Shake contents vigorously 1 min and let layers separate (no centrifugation is required). Transfer organic layer to 100 mL boiling flask by filtering it through small glass wool plug. Extract aqueous layer with additional 15 mL portion of dichloromethane in the same manner.

If entire dichloromethane extract (45 mL) is to be analyzed by spectrofluorometric (SPF) or LC analysis, add 1.0 mL keeper solution prior to concentrating sample to dryness. For simultaneous quantitation of BAY Vp 2674 by SPF and LC analysis, adjust combined dichloromethane extracts to 50 mL in volumetric flask and then divide into two 25 mL aliquots. To one 25 mL aliquot add 0.5 mL keeper solution and concentrate to near dryness by distillation as described in extraction procedure. Then, evaporate residue completely to dryness by using vacuum and 50°C water bath. Add 5 mL of 0.1M pH 3.5 buffer to flask. Warm contents in 50°C water bath while swirling to dissolve residue; then, alternate flask between ultrasonic bath and vortex mixer for 2–3 15 s intervals to ensure complete dissolution of residue (contains 2 g equivalent sample/mL). If solution is not optically clear, remove suspended particles by forcing solution through 0.45 μm filtering cartridge using hypodermic syringe. Cover flask and, after contents have equilibrated to room temperature, quantify residues of BAY Vp 2674 by SPF analysis.

Add 0.5 mL keeper solution to other 25 mL aliquot, concentrate to dryness, and dissolve residue in 1.0 mL mobile phase.

Table 4. Amount of coextractives and relative intensity (RI) from untreated tissues of chickens and turkeys[a]

Tissue	Coextractives wt, g[b]	RI[c]	Apparent BAY Vp 2674, ppb
Chicken:			
Liver	0.12	0.16	9 ± 1
Muscle	0.21	0.13	7 ± 2
Skin	4.64	0.14	7 ± 1
Turkey:			
Liver	0.06	0.10	5 ± 1
Muscle	0.22	0.14	7 ± 1
Skin	6.01	0.13	7 ± 1

[a] Average mean ± SE for 4 replicate samples/tissue.
[b] 20 g sample size.
[c] RI = RI (observed) − 0.02 (buffer background). Reading of 0.2 μg/mL BAY Vp 2674 standard = 2.00 RI units.

Confirmation by LC Analysis

Inject 10 μL working standard and sample solution into liquid chromatograph, measure peak heights, and calculate concentration by the following formula:

$$\text{BAY Vp 2674, ppb} = R \times V_w \times C_w \times 100/W$$

where R is ratio of sample to working standard peak heights; V_w = volume (μL) of working standard injected; C_w = con-

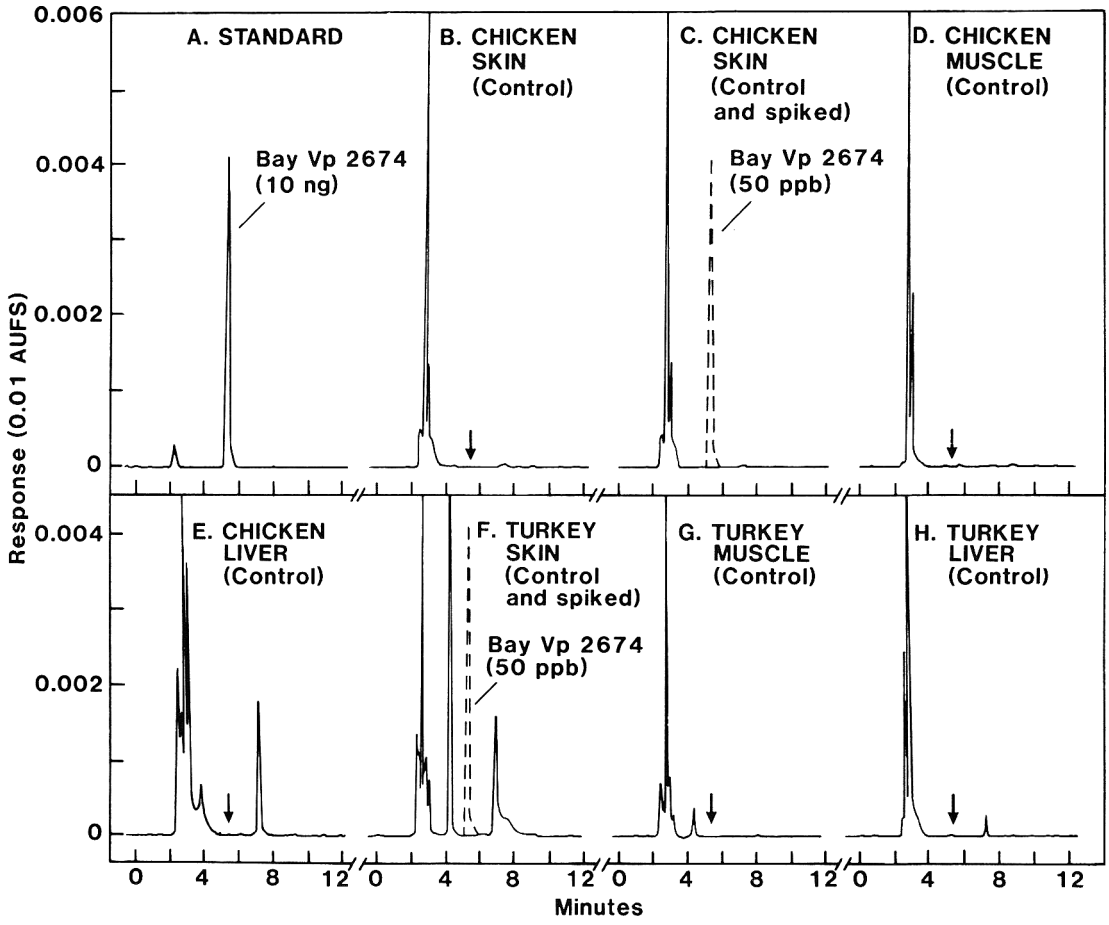

Figure 9. Liquid chromatographic UV response to BAY Vp 2674 standard alone and in presence of chicken and turkey liver, muscle, and skin controls.

centration (ng/μL) of working standard; and W = total weight of sample extracted.

Results and Discussion

The sample extract and dichloromethane washings were combined and concentrated to near dryness. Hexane was then added before the final concentration step to help eliminate the last traces of dichloromethane and methanol before cleanup.

Cleanup was accomplished by taking advantage of the partitioning characteristics of BAY Vp 2674 between hexane or dichloromethane and various phosphate buffers at pH range 2–12. The best conditions favoring the organic phase were found by determining *p*-values (12, 13) for BAY Vp 2674. A *p*-value, determined by distributing a solute between equal volumes of 2 immiscible phases, is defined as the fraction of the total solute partitioning into the upper phase. The *p*-values were calculated from absorbancy measurements at 282 nm of a standard solution of 10 μg drug/mL dichloromethane before and after shaking against an equivalent volume of 0.1M phosphate buffer. Results for dichloromethane are shown in Figure 5. Values from hexane and buffer over a pH 2–12 range were zero.

Flow schemes for extraction and cleanup procedures are given in Figures 6 and 7, respectively. Losses may occur at 2 critical steps during the cleanup procedure. Transfer of BAY Vp 2674 to pH 2 buffer may be incomplete due to its insolubility in hexane and the low amount of coextractives present. Losses are minimized by washing the flask with dichloromethane. Unless complete equilibrium is established after partitioning hexane against pH 2 buffer, losses could occur when the hexane is discarded.

Recoveries of BAY Vp 2674 as determined by SPF analysis from fortified liver, muscle, and skin of chickens and turkeys ranged from 82 to 93% (Tables 1 and 2, respectively). Recoveries determined by LC analysis, as shown in Table 3, were comparable.

Incurred residues in skin from chickens and turkeys receiving single oral doses of [^{14}C] BAY Vp 2674 per day for 7 consecutive days were analyzed by the LC procedure utilizing both UV and fluorescence detection. For samples collected 6 and 24 h after the last dose, residues of BAY Vp 2674 from the LC procedure were in agreement with levels found by radioassay utilizing thin-layer chromatography.

The level of coextractives removed by the dichloromethane–methanol Soxhlet extraction procedure from a 20 g sample represented approximately 1% or less for muscle and liver and 23–30% for skin (Table 4). Maximum background levels were 7 ppb for muscle and skin and 9 ppb for liver (Table 4). Lowest detection limit is approximately 0.02 ppm, based on twice the maximum background level for liver.

Response of 50 ppb BAY Vp 2674 by LC analysis utilizing fluorescence detection is shown in Figure 8A. Essentially flat baselines were observed for skin controls from chicken and turkey (Figure 8B and C). Responses of 50 ppb BAY Vp 2674, utilizing UV detection, are shown in Figure 9A for the drug alone and in C and F for chicken and turkey skin, respectively. For skin, muscle, and liver of chicken and turkey, response due to interferences at the retention time of BAY Vp 2674 was absent (Figure 9B, D–H).

Poultry tissues may be monitored routinely by an SPF procedure. Levels of BAY Vp 2674 may then be confirmed by LC analysis utilizing UV and/or fluorescence detection.

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Liquid Chromatographic Method for Determining the Macrolide Antibiotic Sedecamycin and Its Major Metabolites in Swine Plasma and Tissues

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A liquid chromatographic (LC) method was developed to determine sedecamycin, a 17-membered macrolide antibiotic used for treating swine dysentery, and its major metabolites (lankacidin C, lankacidinol A, and lankacidinol) in swine plasma and tissues. Plasma is directly extracted with ethyl acetate and analyzed by liquid chromatography without purification. Tissues are homogenized in a phosphate buffer containing sodium chloride, and then extracted with ethyl acetate. The extracts are subjected to silica gel-Florisil, double-layered column chromatography to remove endogenous interfering substances. The LC determination uses silica gel and ODS-silica as a stationary phase. The detection limits for sedecamycin and its metabolites were ≤0.05 ppm, and average recoveries and coefficients of variation (0.2–1 ppm range) were >75% and <10%, respectively.

Sedecamycin (lankacidin A) is derived enzymatically from lankacidin C, a 17-membered macrolide antibiotic produced by *Streptomyces rochei* var. *volubilis* (1, 2). The antibiotic, antibacterially active against Gram-positive organisms (3), is preeminently effective against *Treponema hyodysenteriae* (4), the pathogenic organism for swine dysentery (4, 5). In Japan, sedecamycin, at a concentration of 25–75 ppm in feed, was approved in June 1985 for treating swine dysentery.

As is the case with macrolide antibiotics in general, sedecamycin is extensively metabolized by swine to approximately 20 metabolites (S. Kondo & J. Okada, unpublished data). Three of these metabolites, lankacidin C, lankacidinol A, and lankacidinol, are active against *T. hyodysenteriae*; the remainder have hardly any antibacterial properties (T. Yamazaki et al., Takeda Chemical Industries, unpublished data). The present report deals with a method to determine sedecamycin and its 3 major metabolites in swine plasma and tissues with cleanup on a silica gel–Florisil, double-layered column followed by liquid chromatography (LC).

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METHOD

Reagents

All reagents are reagent grade unless otherwise stated. Water is deionized and distilled.

(a) *Solvents for LC.*—*n*-Hexane, isopropanol, and acetonitrile (LC grade, Wako Pure Chemical Industries, Ltd, Osaka, Japan).

(b) *Standards.*—Sedecamycin (purity 99.2%), lankacidin C (purity 99.7%), lankacidinol A (purity 94.2%), and lankacidinol (purity 93.1%) (Figure 1).

(c) *Standard solutions.*—Store in cold, dark place when not in use. *(1) Stock solutions.*—100 μg/mL. Dissolve sedecamycin, lankacidin C, and lankacidinol A in ethyl acetate, and lankacidinol in methanol. *(2) Working solution 1.*—0.5, 2, and 10 μg/mL of each standard. Dilute each stock solution with ethyl acetate or methanol (for recovery studies). *(3) Working solution 2.*—0.25, 0.5, 1, and 2 μg/mL. Combine aliquots of working solution 1 of each compound, evaporate, and dissolve with ethyl acetate as appropriate for normal phase LC. Evaporate aliquots of sedecamycin working solution 1 and dissolve with acetonitrile for reverse phase LC.

(d) *Silica gel.*—Silica gel 60, 70–230 mesh (E. Merck, Darmstadt, FRG). Activate 6 h at 130°C.

(e) *Florisil.*—100–200 mesh (Floridin Co., obtained from Wako Pure Chemical Industries). Activate 6 h at 130°C.

(f) *0.1M Phosphate buffer containing sodium chloride for extraction (pH 4.5).*—Dissolve 13.6 g KH_2PO_4 and 100 g NaCl in 1 L water. Adjust to pH 4.5 with KOH or phosphoric acid.

(g) *Mobile phase.*—(A) *n*-Hexane–isopropanol (80 + 20); (B) *n*-hexane–isopropanol–acetic acid (75 + 25 + 0.2) for normal phase LC; (C) 0.01M phosphate buffer (pH 8.2)–acetonitrile (60 + 40) for reverse phase LC. Dissolve 1.32 g Na_2HPO_4 and 0.091 g KH_2PO_4 in 1 L water. Adjust to pH 8.2 with NaOH or phosphoric acid and filter through 0.55

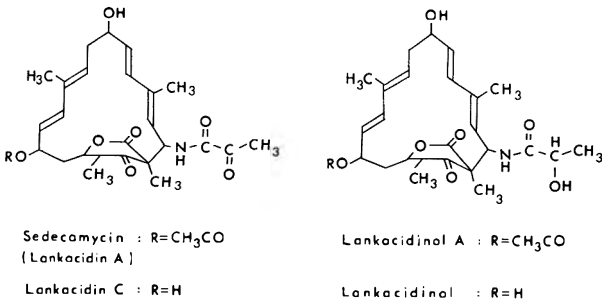

Figure 1. Chemical structures of sedecamycin and its metabolites.

μm glass microfiber filter (Type SM 13400) over 0.45 μm membrane filter (Type SM 11306, Sartorius GmbH, Goettingen, FRG). Add acetonitrile to buffer solution.

Apparatus

(a) *Liquid chromatograph.*—Model 6000A pump and Model U6K injector (Waters Associates, Milford, MA 01757) connected with Model SPD-2A variable wavelength ultraviolet detector (Shimadzu Seisakusho Ltd, Kyoto, Japan).

(b) *Analytical columns.*—(A) Stainless steel, 3.9 mm id × 30 cm, containing μPorasil, 10 μm particle size, and equipped with guard column, 3.9 mm id × 2.5 cm, packed with Porasil. (B) Stainless steel, 3.9 mm id × 30 cm, containing μBondapak C_{18}, 10 μm particle size, and equipped with guard column packed with Bondapak C_{18}/Corasil (Waters Associates).

(c) *High-speed homogenizer.*—Ultra-Turrax TP 18/2 (Janke & Kunkel GmbH, Staufen, FRG).

(d) *Centrifuge.*—Model 05P-2 (Hitachi Koki Co., Ltd, Tokyo, Japan).

(e) *Ultrasonic generator.*—Sono Cleaner MINI (Kaijo Denki Co., Ltd, Tokyo, Japan).

(f) *Chromatographic column.*—Glass column with fritted glass disc (pore size 100–120 μm, 60–80 mesh) and stopcock, 1.1 cm id × 25 cm length.

Extraction

Plasma.—Place 5 mL plasma in 25 mL glass centrifuge tube. Add 10 mL ethyl acetate and blend 20 s at maximum speed with Ultra-Turrax homogenizer at room temperature. Centrifuge 5 min at 3000 rpm and collect organic layer. Extract aqueous layer twice with 10 mL portions of ethyl acetate. Evaporate combined extracts to dryness by rotary evaporation and dissolve with 1 mL ethyl acetate for LC determination.

Tissue.—Weigh 10 g chopped tissue into 50 mL glass centrifuge tube and homogenize 30 s with 10 mL 0.1M phosphate buffer (pH 4.5) containing 10% NaCl in ice bath. Add 15 mL ethyl acetate and blend 20 s with homogenizer at room temperature. Centrifuge 5 min at 3000 rpm and collect organic layer. Extract aqueous layer twice with 10 mL portions of ethyl acetate. Evaporate combined extract to dryness and dissolve residue with ca 5 mL dichloromethane, using ultrasonic generator for cleanup procedure.

Column Cleanup for Tissue Extract

Suspend silica gel and Florisil in dichloromethane in separate flasks. Prepare cleanup column by wet-packing 3 cm silica gel at the bottom and 1.5 cm Florisil in the middle, and placing 2 cm Na_2SO_4 on the top. Apply dichloromethane solution to silica gel–Florisil column. Wash column with 75 mL dichloromethane–ethyl acetate (90 + 10) and elute with ethyl acetate–acetone (80 + 20). Accurately collect 20 mL and then 30 mL eluates, separately. Evaporate an aliquot of the first eluate to dryness and dissolve residue with 1 mL ethyl acetate per g tissue to quantitate sedecamycin, lankacidin C, and lankacidinol A. Combine aliquots of first and second eluates, evaporate to dryness, and dissolve residue with 1 mL ethyl acetate per g tissue to quantitate lankacidinol.

Table 1. Partition coefficients of sedecamycin and its metabolites between organic solvents and water

Compound	Organic solvent			
	n-Hexane	Chloroform	Ethyl acetate	*n*-Octanol
Sedecamycin	0.033	>400	>400	120
Lankacidin C	0.006	33.6	17.0	6.22
Lankacidinol A	0.016	28.9	47.5	17.3
Lankacidinol	<0.002	0.20	1.21	1.40

Chromatographic Determination

Run LC analysis under conditions described below. Determine sedecamycin, lankacidin C, and lankacidinol A under condition A, and lankacidinol under condition B. Use condition C only when identification of sedecamycin in a kidney sample is ambiguous because of interfering substances. Evaporate aliquot of sample solution of kidney to dryness and dissolve with acetonitrile.

Monitor at 227 nm with detection sensitivity of 0.02 AUFS and quantitate each compound by comparing its peak height with calibration curve obtained from peak heights of working solution 2.

Condition A: column A; mobile phase A; flow rate 1.5 mL/min; column pressure 600–1000 psi; injection volume 20 μL.

Condition B: column A; mobile phase B; flow rate 1.5 mL/min; column pressure 700–1000 psi; injection volume 40 μL.

Condition C: column B; mobile phase C; flow rate 1.0 mL/min; column pressure 700–1000 psi; injection volume 20 μL.

Recovery Studies

Add appropriate aliquots of working solution 1 to centrifuge tube and evaporate under stream of nitrogen. Add to tube plasma and tissues obtained from pigs fed unmedicated feed (hereafter referred to as control sample) and mix using vortex mixer. Determine compounds added by method described above.

Results and Discussion

Extraction

Table 1 shows the partition coefficients of sedecamycin and its metabolites (hereafter referred to as the compounds, collectively) between organic solvents and water. The coefficients indicate that these compounds have different solvent extractabilities. Three methods were tried to extract the compounds simultaneously from the tissues with organic solvents. Method 1 (Table 2)—in which the tissues were deproteinized by homogenizing them with such hydrophilic solvents as alcohols, saturated sodium chloride solution was added to the homogenate, and then the mixture was extracted with less polar solvents—gave unsatisfactory recoveries because the compounds were partitioned too much into the aqueous layer because of the alcohols. In Method 2, or the adopted method, the tissues, without deproteinizing, were homogenized in a buffer and extracted with hydrophobic solvents. This method gave excellent recoveries only when the samples were extracted with ethyl acetate.

The addition of sodium chloride to the buffer improved

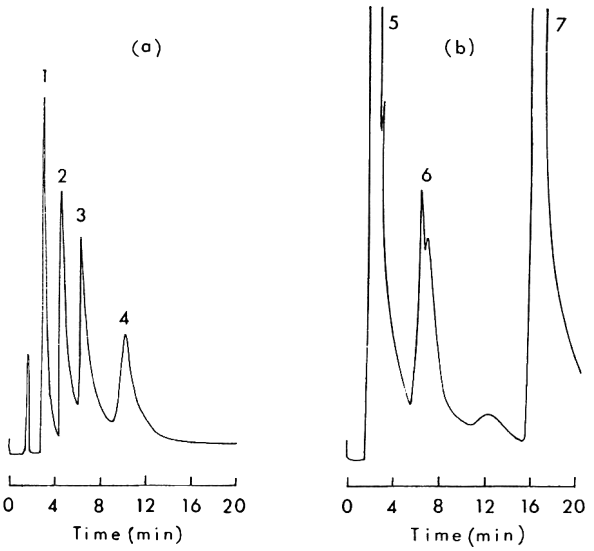

Figure 2. Liquid chromatograms of standards and control swine tissue extract. a, mixture of standards; b, control swine liver extract before column cleanup. 1, sedecamycin; 2, lankacidinol A; 3, lankacidin C; 4, lankacidinol; 5, substance I; 6, substance II; 7, substance III. Conditions: column μPorasil; mobile phase *n*-hexane–isopropanol–acetic acid (75 + 25 + 0.2).

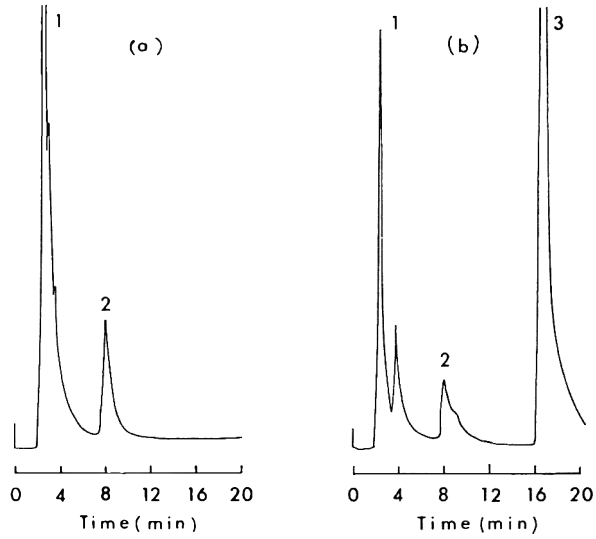

Figure 3. Liquid chromatograms of eluates from silica gel column (1.1 × 4.5 cm) of control liver extract. a, first 20 mL eluate; b, second 30 mL eluate. 1, substance I; 2, substance II; 3, substance III. LC conditions same as Figure 2.

the separation of the phases and increased the recoveries of lankacidin C and lankacidinol. In view of the stability of the compounds in aqueous solution (3), a phosphate buffer of pH 4.5 was selected. It appears that no significant differences were obtained in recoveries between pH 4.5 and 6.0.

Ice-cooled homogenization was used because of the stability of the compounds. However, the aqueous homogenate was extracted with ethyl acetate at room temperature to improve recoveries. Direct extraction with a hydrophobic solvent was unsuccessful because the tissues could not be thoroughly crushed in the solvent and consequently low recoveries were obtained (data not shown).

The direct extraction of a fat sample with methanol (Method 3) gave fairly good recoveries; however, Method 2 was also utilized for fat extraction because less of the interfering substances appeared on the chromatogram obtained by this method.

Direct extraction of a plasma specimen with ethyl acetate gave excellent recoveries (see later discussion).

Cleanup of Extract

The control tissue extracts contained at least 3 kinds of endogenous interfering substances (Figure 2b). The first (hereafter referred to as substance I) consisted of nonpolar substances eluting between the solvent front and sedecamycin in the normal phase LC. The second (substance II) was moderately polar, eluting around lankacidin C and lankacidinol. The last (substance III) was strongly polar and eluted long after lankacidinol. Tissue extracts containing these substances were subjected to chromatographic cleanup.

An ethyl acetate extract of control tissue only or tissue spiked with the compounds was obtained as described under *Extraction*. The extract was evaporated to dryness, dissolved with dichloromethane, and applied to a column with silica

Table 2. Extraction efficiency of variable solvent systems[a]

Method	Tissue	Solvent		Recovery, %			
		1st	2nd	Sedecamycin	Lankacidin C	Lankacidinol A	Lankacidinol
1[b]	Kidney	MeOH	AcOEt	76	72	71	68
		MeOH	CH_2Cl_2	70	86	NA[c]	41
		MeOH	MIBK[d]	NA	57	74	70
		EtOH	AcOEt	60	70	86	58
2	Kidney	NaCl–buffer[e] (pH 4.5)	AcOEt	90	90	85	82
		NaCl–buffer (pH 6.0)	AcOEt	92	87	88	80
		Buffer[f] (pH 4.5)	AcOEt	86	74	86	68
		Buffer (pH 6.0)	AcOEt	80	68	88	62
		NaCl-buffer (pH 4.5)	CH_2Cl_2	4	13	14	0
		NaCl–buffer (pH 6.0)	$CHCl_3$	7	14	25	9
		NaCl–buffer (pH 4.5)	MIBK	55	33	73	85
		NaCl–buffer (pH 4.5)	*n*-BuOH	77	75	70	74
	Fat	NaCl–buffer (pH 4.5)	AcOEt	88	88	75	78
3	Fat	MeOH	none	72	74	84	76

[a] Control tissue (10 g) was spiked with 10 μg each of sedecamycin and its 3 metabolites.
[b] Saturated sodium chloride solution was added to the tissue extract of the first solvent before extraction with the second solvent.
[c] Not assayed.
[d] Methyl isobutyl ketone.
[e] 0.1M phosphate buffer containing 10% NaCl.
[f] 0.1M phosphate buffer.

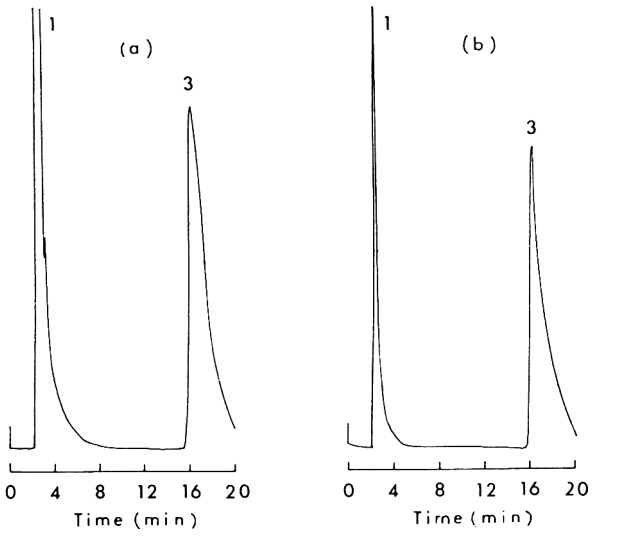

Figure 4. Liquid chromatograms of eluates from Florisil column (1.1 × 4.5 cm) of control liver extract. a, first 20 mL eluate; b, second 30 mL eluate. See Figure 3 for peak identification. LC conditions same as Figure 2.

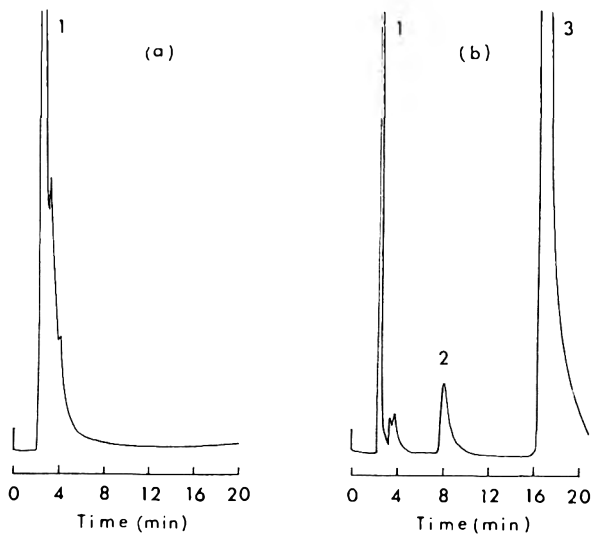

Figure 5. Liquid chromatograms of eluates from silica gel (3 cm, lower) plus Florisil (1.5 cm, upper) column of control liver extract. a, first 20 mL eluate; b, second 30 mL eluate. See Figure 3 for peak identification. LC conditions same as Figure 2.

gel or Florisil alone, or with combinations of each. The column was washed with 75 mL dichloromethane–ethyl acetate (90 + 10), and the compounds were eluted with ethyl acetate–acetone (80 + 20). The first 20 mL and the subsequent 30 mL of the eluate were collected separately, and each fraction was injected into the LC apparatus after being evaporated and reconstituted with ethyl acetate.

The silica gel column eliminated substances I and III from the first 20 mL eluate, recovering most of the sedecamycin, lankacidin C, and lankacidinol A in this eluate and lankacidinol in the whole (first + second) eluate. However, it failed to remove substance II, and thus it was difficult to determine lankacidin C (Figure 3 and Table 3).

On the other hand, the Florisil column eliminated substances I and II, but it did not remove substance III. Furthermore, recoveries of lankacidinol A and lankacidinol from the column were low when more than 2 cm of Florisil was used (Figure 4 and Table 3).

The double-layered column of silica gel (3 cm, lower) and Florisil (1.5 cm, upper), which combined the different properties of the 2 adsorbents, resulted in a satisfactory cleanup. First, substance I was removed by the washing process. Second, sedecamycin, lankacidin C, and lankacidinol A were mostly recovered in the first eluate, leaving substances II and III on the column. Third, some lankacidinol was recovered in the first eluate, but most of it was recovered in the second together with some portions of substances II and III that could be separated from lankacidinol by liquid chromatography (Figure 5 and Table 3).

Another double-layered column with 1 cm Florisil in the upper layer did not remove substance II sufficiently from the first eluate. Neither did the reverse double-layered column (3 cm silica gel, upper and 1.5 cm Florisil, lower) (data not shown).

As shown in Table 3, lankacidinol A, which had a fairly strong affinity for Florisil, was poorly recovered from the double-layered column in the first eluate when only a standard solution was applied to the column. However, when it was applied together with the tissue extracts, more than 70% of the compound was recovered in the first eluate. On the

Table 3. Recovery of sedecamycin and its metabolites from columns

Column packing, cm				Recovery,[b] %			
Florisil	Silica gel	Tissue	Eluate[a]	Sedecamycin	Lankacidin C	Lankacidinol A	Lankacidinol
0	4.5	none	1	99	104	78	31
			2	3	0	22	72
1.5 (Upper)	3.0 (Lower)	none	1	82	87	38	24
			2	7	9	40	66
		liver	1	90	91	81	82[d]
			2	NA[c]	NA	NA	
		kidney	1	88	92	74	71[d]
			2	NA	NA	NA	
		muscle	1	90	89	73	67[d]
			2	NA	NA	NA	
		fat	1	88	88	75	78[d]
			2	NA	NA	NA	
4.5	0	none	1	67	90	8	0
			2	10	6	62	52

[a] 1, first 20 mL; 2, second 30 mL.
[b] Neither sedecamycin nor its metabolites were found in the dichloromethane–ethyl acetate washing.
[c] Not assayed.
[d] The first and second eluates were combined to determine.

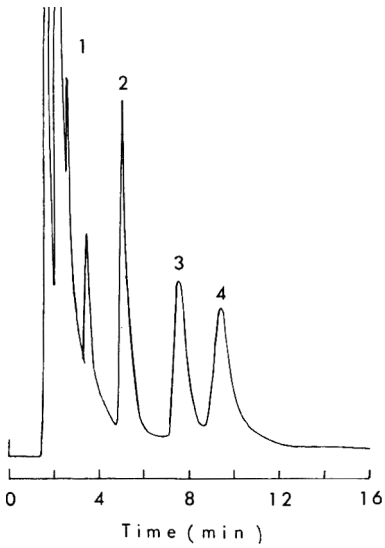

Figure 6. Liquid chromatogram of sedecamycin, lankacidin C, and lankacidinol A recovered from swine liver. 1, substance I; 2, sedecamycin; 3, lankacidinol A; 4, lankacidin C. Conditions: column μPorasil; mobile phase *n*-hexane–isopropanol (80 + 20).

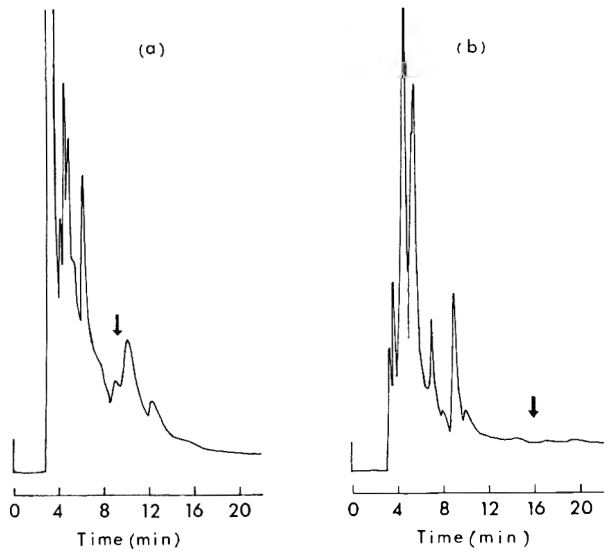

Figure 8. Liquid chromatograms of control swine kidney extract after silica gel–Florisil column cleanup. Conditions: a, column μPorasil; mobile phase *n*-hexane–isopropanol–acetic acid (75 + 25 + 0.2); b, column μBondapak C_{18}; mobile phase 0.01M phosphate buffer (pH 8.2)–acetonitrile (60 + 40). Arrow indicates elution position of sedecamycin.

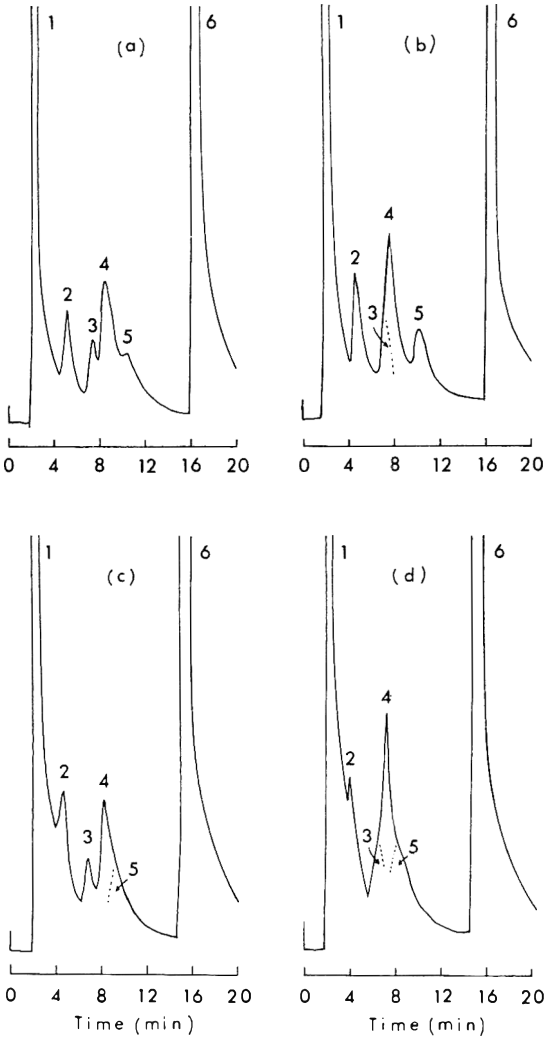

Figure 7. Liquid chromatograms of combined eluate from silica gel–Florisil column of swine liver extract spiked with a mixture of standards. 1, substance I; 2, lankacidinol A; 3, lankacidin C; 4, substance II; 5, lankacidinol; 6, substance III. Column μPorasil; mobile phase a, *n*-hexane–isopropanol (75 + 25); b, *n*-hexane–isopropanol–acetic acid (75 + 25 + 0.2); c, *n*-hexane–isopropanol (70 + 30); d, *n*-hexane–isopropanol–acetic acid (70 + 30 + 0.2).

other hand, the recoveries of the other compounds were hardly affected by the existence of the tissue extracts.

It should be specially noted that satisfactory cleanups were achieved by delicate combinations of the sequence and depth of the layers of silica gel and Florisil and of solvents for washing and eluting.

The plasma extract contained nonpolar substances, but it did not require any purification.

LC Conditions

Sedecamycin, lankacidin C, and lankacidinol A could be determined on the first eluate by using mobile phase A in the normal phase LC analysis with no interference from the endogenous substances (Figure 6). On the other hand, the first and second eluates had to be combined and used to recover lankacidinol at a rate higher than 70%. Several solvent systems were investigated to adequately separate lankacidinol from substance II and minimize the elution time for substance III (Figure 7). Although *n*-hexane–isopropanol (70 + 30) did not separate lankacidinol from substance II, changing the solvent ratio to 75 + 25 improved the separability somewhat. The addition of acetic acid to the latter mobile phase improved the separability to an acceptable level. Eventually, mobile phase B was selected to determine lankacidinol.

Sedecamycin and its 3 metabolites, which have strong absorption maxima around 227 nm, were detected with 2.5 to 3 times greater sensitivity at 227 nm rather than the commonly used 254 nm.

Normal phase liquid chromatography identified and quantitated the compounds in all the tissues except kidney. The kidney extract, despite purification on the silica gel–Florisil column, occasionally exhibited some minor peaks close to the peak of sedecamycin (Figure 8a), making the identification and quantification of sedecamycin ambiguous. Eventually, reverse phase LC was adopted to assay sedecamycin in kidney when the results were ambiguous in the normal phase LC. The chromatographic conditions clearly distinguished sedecamycin from endogenous substances in the kidney (Figure 8b).

Table 4. Recovery of sedecamycin and its metabolites added to swine plasma and tissues

Sample	Added, ppm	N	Recovery (mean ± SD), %			
			Sedecamycin	Lankacidin C	Lankacidinol A	Lankacidinol
Plasma	1.0[a]	4	87.2 ± 0.5	94.2 ± 1.2	97.1 ± 5.6	99.6 ± 5.5
	0.2[a]	4	91.2 ± 8.7	96.0 ± 2.8	95.2 ± 2.1	101.9 ± 9.0
	0.05[a]	2	79.0 ± 2.5	91.7 ± 0.7	92.9 ± 0.7	95.0 ± 4.2
Plasma av.	0.2–1.0[a]	8	89.2 ± 6.12	95.1 ± 2.23	96.2 ± 4.03	100.7 ± 6.97
Liver	1.0	4	92.5 ± 1.3	92.0 ± 3.4	87.5 ± 3.8	81.8 ± 7.6
	0.2	5	89.6 ± 7.2	81.4 ± 8.5	84.4 ± 7.0	78.4 ± 6.5
	0.05	3	85.0 ± 11.5	85.6 ± 5.1	75.3 ± 4.5	86.7 ± 11.2
Kidney	1.0	4	87.2 ± 5.0	88.8 ± 3.0	85.5 ± 3.9	79.2 ± 5.5
	0.2	4	88.2 ± 3.5	89.8 ± 3.0	80.2 ± 5.5	75.0 ± 8.3
	0.05	2	76.5 ± 16.3	84.0 ± 6.1	68.5 ± 12.0	82.0 ± 5.7
Muscle	1.0	5	95.0 ± 1.7	91.4 ± 5.5	88.6 ± 5.2	74.4 ± 5.5
	0.2	4	88.2 ± 1.5	85.0 ± 2.7	77.8 ± 3.2	71.8 ± 4.9
	0.05	2	84.0 ± 12.7	86.0 ± 2.8	69.0 ± 2.8	78.5 ± 13.4
Fat	1.0	4	82.8 ± 3.9	80.2 ± 4.1	79.5 ± 5.1	73.2 ± 7.1
	0.2	4	85.5 ± 4.8	80.5 ± 7.6	74.8 ± 4.0	71.5 ± 5.4
	0.05	3	82.3 ± 13.6	78.0 ± 8.2	75.7 ± 15.9	78.0 ± 4.4
Tissue av.	0.2–1.0	34	88.9 ± 5.24	86.2 ± 6.70	82.5 ± 6.45	75.7 ± 6.68

[a] μg per mL.

Table 5. Regression analysis on the linear relation between sedecamycin and its metabolites added and their peak heights[a]

Statistic	Sedecamycin	Lankacidin C	Lankacidinol A	Lankacidinol
	None added			
N	27	27	27	27
Range: max, mm	3.0	1.0	1.0	0.7
min, mm	0.0	0.0	0.0	0.0
Mean (X_{bl}), mm	0.49	0.16	0.13	0.10
SD (S_{bl}), mm	0.86	0.44	0.30	0.22
Mean + 3 S_{bl} (X_L), mm	3.07	1.47	1.02	0.75
C_L eq. to X_L, ppm[b]	0.014	0.019	0.015	0.016
	0.05 ppm added			
N	12	12	12	12
Range: max, mm	12.8	4.4	5.8	3.0
min, mm	8.0	2.8	2.8	1.8
Mean, mm	9.23	3.43	4.16	2.46
SD, mm	1.50	0.64	0.99	0.37
	0.2 ppm added			
N	21	21	21	21
Range: max, mm	48.3	18.7	23.0	14.0
min, mm	29.9	11.5	14.0	6.3
Mean, mm	37.48	15.03	17.63	9.41
SD, mm	4.02	2.05	2.38	2.15
	1.0 ppm added			
N	21	21	21	21
Range: max, mm	203.9	86.9	118.3	69.0
min, mm	166.4	64.2	78.7	32.3
Mean, mm	185.11	75.20	93.48	48.29
SD, mm	9.60	7.61	11.26	12.38
Slope	184.72	75.18	93.71	48.26
SD	1.43	1.08	1.58	1.35
Intercept (a), mm	0.40	0.01	−0.38	−0.01
SD (S_a), mm	0.74	0.56	0.82	0.70
a + 3 S_a (X_{La}), mm	2.62	1.69	2.08	2.09
C_{La} eq. to X_{La}, ppm[b]	0.012	0.022	0.026	0.044
Correlation coefficient	0.9976	0.9920	0.9890	0.9537

[a] Peak heights of sedecamycin obtained by reverse phase LC were omitted.
[b] Calculated using slopes and intercepts.

Recoveries and Detection Limits

Table 4 summarizes the recovery studies on plasma, liver, kidney, muscle, and fat spiked with sedecamycin and its metabolites at 0.05–1.0 ppm. Because the peak heights of the samples spiked with the compounds at 0.05 ppm, close to the detection limits described below, were under 10 mm and far from quantitative, these data were omitted from the calculation of the average recoveries and their variances. Since the purification procedures differed between plasma and tissues, the data from each were separately analyzed. The results of the statistical treatment are shown in Table 4. The mean recovery was highest for sedecamycin and lowest for lankacidinol. The recoveries from plasma were higher than those from tissues for every compound, because column cleanup was not involved in the plasma extraction. The accuracy and precision, indicated by recoveries >75% and coefficients of variation <10%, respectively, are satisfactory for residue analysis.

The detection limits of the present method were statistically defined. According to the IUPAC recommendation (6), the detection limit (C_L) is defined as the quantity equivalent to X_L in the following equation:

$$X_L = X_{bl} + 3S_{bl}$$

where X_{bl} and S_{bl} are the mean and standard deviation (SD) of normally distributed background signals, respectively. The background signals obtained from control samples were too small to estimate the detection limits (C_L in Table 5), so the intercept (a) and its standard deviation (S_a) of the regression curves obtained from the relation of the quantities of the compounds added vs the peak heights in the recovery samples were used in place of X_{bl} and S_{bl}, respectively. All recovery data (including control samples) were lumped for the analysis. The detection limits were estimated to be 0.012 ppm for sedecamycin, 0.022 ppm for lankacidin C, 0.026 ppm for lankacidinol A, and 0.044 ppm for lankacidinol (see C_{La} in Table 5). The detection limit for lankacidin C, however, should be regarded as being around 0.028 ppm (equivalent to 2 mm) because a peak height of at least 2 mm is required to identify the peak distinctively. The detection limits defined here are also satisfactory for residue analysis.

The present method is appropriate for monitoring plasma concentrations and tissue residues of sedecamycin and its metabolites. Its application will be reported elsewhere.

Acknowledgments

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DRUGS

Gas Chromatographic, Liquid Chromatographic, and Titrimetric Procedures for Determination of Glycerin in Allergenic Extracts and Diagnostic Antigens: Comparative Study

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Three methods for the determination of glycerin are examined as applied to several allergenic extracts and diagnostic antigens. The liquid chromatographic procedure uses a sulfonic acid functional PSDVB resin (Aminex HPX-87H), a mobile phase of 0.013N H_2SO_4; and refractive index detection. The titrimetric procedure involves oxidation of glycerin with sodium metaperiodate followed by potentiometric titration of the resulting formic acid with sodium hydroxide. Samples are quantitated by comparing the equivalence point obtained from the sample to those obtained from a series of standards. The gas chromatographic procedure includes a column of 5% Carbowax 20 M on 80-100 mesh Chromosorb WHP; *p*-cresol was used as an internal standard. The 3 procedures are shown to be valid for the majority of product types examined. A positive interference was encountered in the titrimetric analysis of a tuberculin purified protein derivative that contained simple sugars. Recoveries of added glycerin ranged from 95.0 to 100.2% by the liquid chromatographic method, from 98.7 to 101.4% by the gas chromatographic method, and from 99.8 to 101.6% by the metaperiodate oxidation method when interference from simple sugars was not present. Coefficients of variation determined from 8 replicates of samples that contained glycerin were 2.2% or less for the liquid chromatographic method, 2.3% or less for the GC method, and 3.6% or less for the metaperiodate oxidation method.

Glycerin is commonly used as a stabilizing agent and antimicrobial preservative in a variety of biological products; it is used alone or in conjunction with phenol or other antimicrobial agents. Products that may contain glycerin include allergenic extracts, tuberculin purified protein derivative for Heaf testing, Old Tuberculin, and bacterial and fungal antigens.

Glycerin in 50% (v/v) concentration has been shown to maintain sterility effectively in various biologicals; that concentration has been shown to restrain the growth of mold and bacteria, while molds grew in solutions of 49% glycerin or less (1). Pollen antigens have been shown to remain sterile when preserved in 46% (v/v) glycerin, while solutions of 40% or less showed bacterial growth (2). Allergenic extracts containing 50% or more glycerin are exempt from the requirement stipulated by the U.S. *Code of Federal Regulations* (3) that products in multiple dose containers contain an antimicrobial preservative; glycerin serves this purpose.

The ability of glycerin to stabilize allergenic extracts, thus enabling them to retain potency during extended storage, has been well established (4). The *Code of Federal Regulations* allows a dating period of 3 years for allergenic extracts preserved in 50% or more glycerin, while extracts containing less than 50% glycerin are allowed a dating period of 18 months (5).

Concentrated solutions of glycerin, when injected, may cause pain or local irritation, caused by dehydration of tissue cells. This local tissue reaction may interfere with diagnostic observations of skin or intradermal tests. For this reason, it is desirable that glycerin content in the original product not be excessive.

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Several general methods for the determination of glycerin are in use. Most compendial methods involve the oxidation of glycerin by periodate or periodic acid to 2 equivalents of formaldehyde and one of formic acid. The current USP procedure involves the reaction of glycerin with potassium periodate followed by reduction of the iodate reaction product to iodine and titration with potassium arsenite (6). Other approaches have involved the determination of formaldehyde (7, 8) or formic acid (9, 10) produced by periodate oxidation. Oxidative procedures are nonspecific in that a diol with a terminal hydroxyl will oxidize to produce formaldehyde, and any triol-containing compound will produce aldehydes and formic acid.

Chromatographic methods have been used to determine glycerin. Gas chromatography has been used with a variety of stationary phases (11–13). Liquid chromatography (LC) has utilized a column of sulfonated polystyrene–divinyl benzene (PSDVB), a dilute sulfuric acid mobile phase, and detection by differential refractometry (14, 15).

In this study, methods based on periodate oxidation followed by potentiometric titrimetry, liquid chromatography, and gas chromatography (GC) are described and evaluated for use in the quantitation of glycerin in commercial biologicals.

METHOD

General Reagents

(a) *Glycerin.*—Anhydrous, ≥99.5% (J. T. Baker Chemical Co.), density = 1.257 g/mL at 25°C.

(b) *Standard solutions.*—Weigh 5 quantities of glycerin, from 0.4 to 2.0 g, in ca 0.4 g increments, directly into 5 separate 100 mL volumetric flasks. Add ca 20 mL water and mix thoroughly. Add water to volume, mix thoroughly, and let equilibrate overnight. Mix again before use.

Reference Materials and Samples

(a) *Center for Drugs and Biologics Phenol/Glycerin Reference, Lot G-1.*—Solution of phenol and glycerin in water, packaged in glass ampoules. Phenol content: 0.399% w/v determined by gas chromatographic method (16). Glycerin content: 61.0% w/v, average value determined by the 3 methods described here.

(b) *Samples.*—Allergenic extracts and diagnostic antigens, commercial products manufactured in the United States and Europe.

General Sample Preparation

(a) *Samples of ca 50% glycerin.*—Pipet 2 mL sample into 100 mL volumetric flask. Add ca 20 mL water and mix thoroughly. Add water to volume, mix thoroughly, and let equilibrate overnight. Mix again before use.

(b) *Samples of high viscosity.*—Accurately weigh ca 1–2 mL into 100 mL volumetric flask. Add ca 20 mL water and mix thoroughly. Add water to volume and mix again.

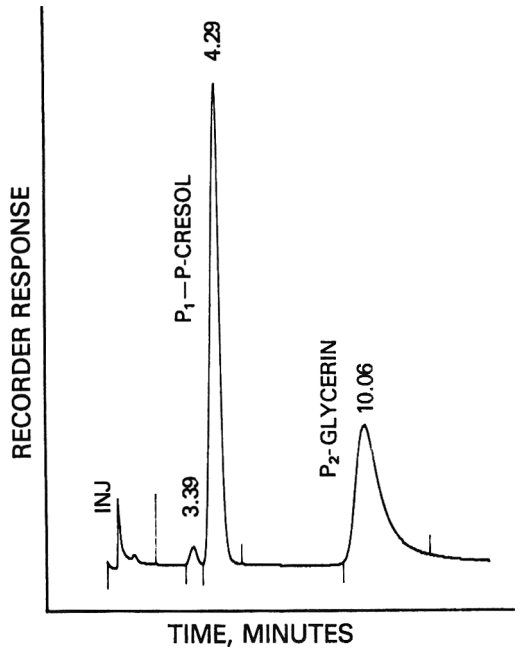

Figure 1. Gas chromatogram of cherry allergenic extract. Original concentration of glycerin, ca 50% (v/v).

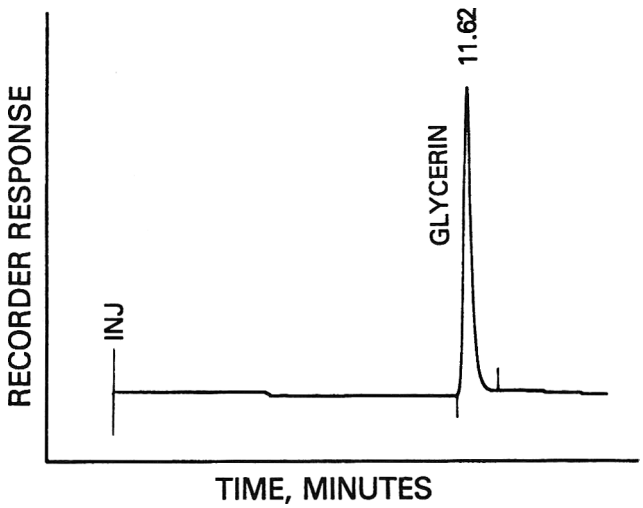

Figure 2. Liquid chromatogram of house dust allergenic extract. Original glycerin concentration, ca 50% (v/v).

Gas Chromatographic Apparatus

(a) *Gas chromatograph.*—Perkin-Elmer Sigma 2. Equipped with flame ionization detector and glass-lined, off-column injection system (Perkin-Elmer Corp., Norwalk, CT 06856). Injection liner must be free of visible sample residue. If necessary, clean liner in concentrated sulfuric acid overnight. Rinse well with water and dry before replacing.

(b) *Column.*—Silanized glass, 6 ft × ¼ in. od, 4 mm id, packed with 5% Carbowax 20 M on 80–100 mesh Chromosorb WHP (Alltech Associates, Deerfield, IL 60015). Fill column by suction, tapping, and rotation. Avoid use of mechanical vibrator in packing column.

(c) *GC conditions.*—Column, 175°C; injection port, 185°C; detector, 270°C; helium carrier gas, 30 mL/min.

(d) *Integrator.*—Spectra-Physics System 1. Slope sensitivity, 1000; peak width, 30 s; attenuation, 256.

(e) *Recorder.*—Perkin-Elmer Model 56.

GC Standard and Sample Preparations

(a) *Internal standard.*—*p*-Cresol, Aldrich Gold Label, 99+% (Aldrich Chemical Co., Milwaukee, WI 53233).

(b) *Internal standard solution.*—Pipet 0.5 mL *p*-cresol into 200 mL volumetric flask, add methanol to volume, and mix.

(c) *Standard preparations.*—Pipet 2 mL of each of the 5 standard solutions along with 2 mL internal standard solution into separate 10 mL volumetric flasks. Add water to volume and mix.

(d) *Sample preparations.*—Pipet 2 mL of the 1/50 sample dilution along with 2 mL internal standard solution into 10 mL volumetric flask. Add water to volume and mix.

GC Analysis

With 10 μL gas chromatographic syringe, inject 3 μL of each standard preparation. Record chromatograms and areas of peak 1, *p*-cresol, and peak 2, glycerin. Similarly inject 3 μL of each sample in duplicate. Calculate peak area ratio, glycerin/*p*-cresol (P_2/P_1), for each standard and sample chromatograph. Do not include a blank in the standard curve for the GC determination.

Glycerin peak must not be skewed on its frontal side. Some tailing of the peak is permissible.

Liquid Chromatographic Apparatus

(a) *Mobile phase.*—0.013N H_2SO_4, filtered and degassed. Flow rate, 0.8 mL/min. While in place in LC reservoir, heat and stir mobile phase at ca 50°C over stirring hot plate.

(b) *Liquid chromatograph.*—Spectra-Physics 3500, equipped with forced air oven and Valco C6U injector with 25 μL loop.

(c) *Detector.*—Laboratory Data Control (Riviera Beach, FL) Model 1107 Refractomonitor; range, 32 × 10^{-5} RIU full scale.

(d) *Integrator.*—Hewlett-Packard 3380A; slope sensitivity, 0.3 mV/s.

(e) *LC column.*—300 × 7.8 mm Aminex HPX-87H with Micro-Guard ion exclusion cartridge (Bio-Rad Laboratories, Richmond, CA 94804). Column temperature, 65°C.

LC Determination

Flush injection loop with 200 μL of one of the standard solutions. Inject full loop volume of 25 μL. Record chromatogram and peak areas. Similarly inject each of the remaining standards plus a water blank. Inject each sample in duplicate.

Titrimetric Apparatus and Reagents

(a) *Titration system.*—Mettler DK14 Electrode Potential Amplifier, Mettler DV11 Autoburette (10 mL), Ingold combination pH electrode, Mettler GA14 stepping motor recorder (Mettler Instrument Corp., Hightstown, NJ 08520). Configure system so that recorder x-axis will display titrant volume and y-axis will display electrode potential. Conditions: recorder range, 500 mV; recorder speed, 40 cm/10 000 pulses (4 cm/mL).

(b) *Sodium metaperiodate.*—Crystalline or powder.

(c) *0.1N H_2SO_4.*

(d) *Oxidizing reagent.*—0.28N $NaIO_4$. Weigh ca 12 g sodium metaperiodate and transfer to dark glass bottle. Add 12 mL 0.1N H_2SO_4 and 188 mL water. Mix thoroughly.

(e) *Titrant.*—0.12N NaOH.

Titrimetric Analysis

Pipet 2 mL standard into titration vessel containing ca 50 mL water. Place vessel in titrator and stir at moderate speed.

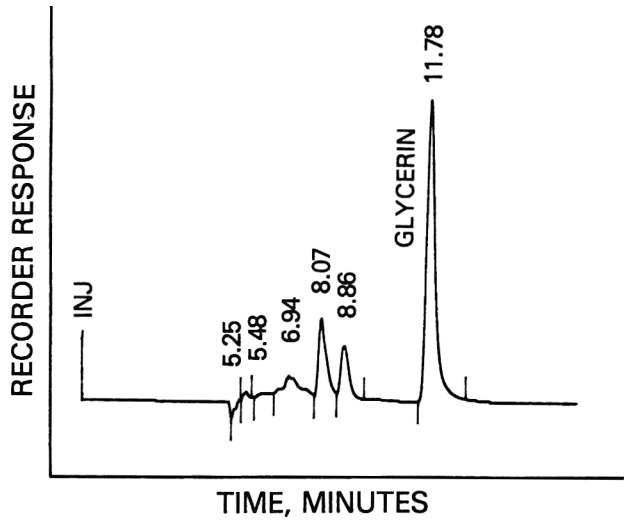

Figure 3. Liquid chromatogram of unglycerinated tuberculin purified protein derivative with 56.4% (w/v) added glycerin.

Pipet 5 mL oxidizing reagent into titration vessel. After 3 min, begin titrating and continue until potential change, as displayed on recorder, passes through a maximum and decreases. Similarly react and titrate 2 mL remaining standards plus water blank. React and titrate each sample in duplicate.

Determine equivalence volume in milliliters for each titration. This is measured as total volume of titrant dispensed at maximum rate of potential change (inflection point) displayed on chart recording.

General Calculations

Perform least squares analysis of data obtained from standards where x = glycerin concentration in g/mL, y = analytical response, a = y-intercept, and b = slope. For liquid chromatography, y = peak area in integration units. For gas chromatography, y = peak area ratio, glycerin/*p*-cresol. For titrimetry, y = mL titrant.

Calculate percent glycerin (w/v) by:

$$\text{Glycerin, g/mL} = [(y - a)/b] \times 50 \text{ (dilution factor)}$$
$$\text{Glycerin, \% (w/v)} = (\text{g glycerin/mL}) \times 100$$

For samples of high viscosity that cannot be dispensed by volumetric pipet, calculate percent glycerin (w/w) by:

$$\text{Glycerin, g/g} = [(y - a)/b] \times (100 \text{ mL/g sample})$$
$$\text{Glycerin, \% (w/w)} = (\text{g glycerin/g}) \times 100$$

Results

Figure 1 shows the gas chromatogram of a glycerinated cherry allergenic extract. Original concentration of glycerin in this sample was approximately 50% (v/v). Under the conditions used here, *p*-cresol, the internal standard, is retained 4.3 min, and glycerin is retained 10.1 min. A relatively small peak representing phenol, present as an antimicrobial preservative in an original concentration of 0.4% (w/v), is observed just before the internal standard.

Figure 2 illustrates the liquid chromatogram of a glycerinated house dust allergenic extract. Figure 3 shows the liquid chromatogram of an unglycerinated tuberculin purified protein derivative (PPD) used in a recovery study to which a quantity of glycerin equivalent to 56.4% (w/v) had been added. Nonglycerin peaks in the tuberculin PPD sample are believed to correspond to simple saccharides that are components of this product.

Typical correlation coefficients obtained by least squares analysis of the data obtained from calibration curves constructed from glycerin standards in the range of 4 to 20 mg/mL for each of the 3 methods were r = 0.999. Therefore, acceptable linearity was obtained by each procedure.

Table 1. Glycerin content of various products determined by gas chromatography, liquid chromatography, and potentiometric titration

Sample	Method	Glycerin % (w/v)[a]	CV, %	Diff. rel. to LC, %
Glycerin Reference G-1	GC	61.4 ± 0.9	1.5	1.3
	LC	60.6 ± 0.5	0.8	—
	PT	60.9 ± 2.2	3.6	0.5
House dust allergenic extract	GC	52.8 ± 1.1	2.0	2.7
	LC	51.4 ± 0.8	1.6	—
	PT	53.7 ± 1.6	2.9	4.5
Cocoa allergenic extract	GC	54.8 ± 1.0	1.8	4.8
	LC	52.3 ± 1.1	2.2	—
	PT	54.9 ± 1.6	3.0	5.0
Peanut allergenic extract	GC	51.3 ± 1.0	1.9	5.3
	LC	48.7 ± 0.8	1.7	—
	PT	52.0 ± 1.4	2.8	6.8
Candida antigen	GC	68.6 ± 1.2	1.8	3.6
	LC	66.2 ± 1.3	1.9	—
	PT	66.9 ± 1.5	2.2	1.1
Diphtheria antigen	GC	68.7 ± 1.3	1.8	1.2
	LC	67.9 ± 1.1	1.6	—
	PT	68.7 ± 1.6	2.3	1.2
Tuberculin purified protein derivative	GC	49.8 ± 0.8	1.6	6.6
	LC	46.7 ± 0.9	2.0	—
	PT	48.8 ± 1.0	2.0	4.5
Old Tuberculin	GC	78.8 ± 1.8	2.3	4.0
	LC	75.8 ± 1.3	1.8	—
	PT	75.9 ± 1.2	1.6	0.1

[a] Units are percent (w/v) except for units for Old Tuberculin which are percent (w/w). Average of 8 determinations ± standard deviation. 50.0% (v/v) glycerin is equivalent to 63.0% (w/v) glycerin.

Values obtained in the testing of representative samples of a variety of products are summarized in Table 1. Eight determinations were performed on each sample.

Coefficients of variation were 2.2% or less for the LC method, 2.3% or less for the GC method, and 3.6% or less for the titrimetric method.

Percent differences among the results range from 1.2 to 6.6% for GC vs LC and from 0.1 to 6.8% for the titrimetric vs LC method, showing good agreement among the 3 methods.

Recovery results from a variety of samples to which measured amounts of glycerin were added are shown in Table 2. Glycerin was added in quantities that would correspond to concentrations of 46.4% (w/v) to 68.8% (w/v). Recoveries ranged from 95.0 to 116.8%. The titrimetric procedure showed a significantly high recovery of 116.8% for Tuberculin PPD, Manufacturer B. Recoveries of 101.4 and 100.2% were obtained from this sample by gas chromatography and by liquid chromatography, respectively.

Results of testing on some additional allergenic extract products are summarized in Table 3.

Discussion

Some difficulty was experienced in obtaining satisfactory conditions for the determination of glycerin by gas chromatography. This was not surprising given glycerin's low vapor pressure, strong hydrogen bonding properties, and tendency toward thermal decomposition. The use of one particular batch of packing material resulted in a skewing of the frontal part of the glycerin peak, indicating probable on-column decomposition. In other cases, severe tailing of the glycerin peak was experienced, indicating possible hydrogen

Table 2. Recovery of added glycerin from products as determined by gas chromatography, liquid chromatography, and potentiometric titration

Sample	Method	Recovery, %[a]	CV, %
House dust allergenic extract	GC	99.4 ± 1.8	1.8
	LC	95.0 ± 1.4	1.5
	PT	99.8 ± 3.2	3.2
Peanut allergenic extract	GC	100.4 ± 1.4	1.4
	LC	98.1 ± 1.7	1.7
	PT	100.2 ± 2.9	2.9
Giant and short ragweed allergenic extract	GC	99.4 ± 1.2	1.2
	LC	97.4 ± 1.7	1.7
	PT	101.5 ± 2.3	2.3
Alternaria tenuis allergenic extract	GC	98.7 ± 3.6	3.6
	LC	98.6 ± 1.7	1.7
	PT	101.6 ± 1.8	1.8
Tuberculin PPD, Manufacturer A	GC	99.1 ± 3.2	3.2
	LC	98.2 ± 2.8	2.8
	PT	100.9 ± 2.3	2.3
Tuberculin PPD, Manufacturer B	GC	101.4 ± 2.3	2.2
	LC	100.2 ± 2.0	2.0
	PT	116.8 ± 1.6	1.4

[a] Percent recovery ± standard deviation. Average of 8 determinations.

Table 3. Liquid chromatographic, gas chromatographic, and potentiometric titrimetric results for glycerin content of various allergenic extract samples

Sample	Glycerin, % (w/v)[a]		
	LC	GC	PT
Lemon allergenic extract	48.2 ± 1.2	48.0 ± 1.1	50.4 ± 1.0
Honey allergenic extract	60.5 ± 0.8	61.3 ± 0.8	63.2 ± 1.2
Rice allergenic extract	62.8 ± 0.2	62.5 ± 1.3	63.5 ± 1.5
Cockroach allergenic extract	60.3 ± 0.1	59.9 ± 0.6	60.5 ± 1.3
Kentucky Blue Grass allergenic extract	61.7 ± 0.7	62.5 ± 1.2	62.3 ± 2.9
Mixed feathers allergenic extract	61.7 ± 0.3	62.5 ± 0.6	62.3 ± 0.8
Aspergillus flavus allergenic extract	60.1 ± 0.8	60.4 ± 1.1	62.0 ± 2.6
Chicken feathers allergenic extract	59.8 ± 0.8	62.8 ± 2.1	62.5 ± 3.1
Johnsongrass allergenic extract	62.7 ± 1.2	65.3 ± 3.3	65.0 ± 0.9
Cat hair allergenic extract	58.3 ± 1.1	60.3 ± 1.3	59.4 ± 1.0
Junegrass allergenic extract	56.7 ± 1.2	57.7 ± 3.0	59.0 ± 0.8
Horse epithelium allergenic extract	59.5 ± 0.7	60.9 ± 0.9	60.5 ± 2.0
Candida albicans allergenic extract	59.4 ± 0.7	57.7 ± 2.5	60.4 ± 1.8

[a] Average of 3 determinations ± standard deviation.

bonding interactions with active sites on the support material.

GC columns were packed without the use of mechanical vibration in order to avoid fracturing the support material. Conditioning was done at a relatively low temperature, 195°C, to avoid the bleeding of excessive amounts of stationary phase. Under these conditions, a minimally satisfactory peak shape for glycerin was obtained.

No significant problems were encountered in implementing the liquid chromatographic or titrimetric procedures. In the LC procedure, an elevated column temperature of 65°C was used to improve stationary phase mass transfer and reduce peak broadening.

The high recovery of 116.8% of added glycerin for Tuberculin PPD, Manufacturer B, by titrimetry may be related to the presence of simple saccharides in this product. Compounds containing 3 adjacent hydroxyls, such as simple sugars, will oxidize in the presence of periodate to produce formic acid. This would be expected to produce a positive interference in the titrimetric procedure. The application of a standard addition (17) technique to the analysis of such a sample by titrimetry might overcome the interference. This sample of Tuberculin PPD was used in this study but does not normally contain glycerin and in the usual course of events would not be assayed for glycerin.

Conclusion

The three described procedures have been evaluated as valid for the determination of glycerin in the glycerin-containing biological products reported in this study.

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Reverse Phase Liquid Chromatographic Determination of Dexamethasone Acetate and Cortisone Acetate in Bulk Drug Substances and Dosage Forms: Method Development

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The determination of the steroid acetates was evaluated for ruggedness of the method by using an octyldecylsilane column, 254 nm detection, and acetonitrile-water as mobile phase. Mobile phase pH, oven temperature, and columns from various manufacturers had no dramatic effect on the chromatography. The method was then optimized for dexamethasone acetate and cortisone acetate bulk drug and dosage forms. For dexamethasone acetate, the bulk drug substance should be dried at 105°C before use, and the sample should be dissolved in 50% acetonitrile–buffer pH 6 for stability. Cortisone acetate, on the other hand, was found to be nonhygroscopic and hence could be used as received. For stability, the sample should be stored in 50% acetonitrile-buffer pH 4.

The steroid acetates are extensively available to the consumer in pharmaceutical formulations in the form of suspensions, tablets, creams, ointments, lotions, aerosols, pastes, and powders (1–3). A review of the literature from 1967 to 1986 (CAS Online, 1967–1986) indicated that numerous methods have been published for the analysis of this class of compounds. At least 18 studies used LC methods.

The 2 official USP general methods for steroids use a blue tetrazolium procedure following multiple extraction, or a thin-layer chromatography/blue tetrazolium combination technique (4). The actetates of the bulk drug substance and dosage forms included in these methods are hydrocortisone (suspension), cortisone (tablet content uniformity), prednisolone, paramethasone, betamethasone, triamcinolone diacetate, and desoxycorticosterone.

The current USP method for dexamethasone acetate bulk drug substance and suspension uses reverse phase chromatography. The sample preparation for the suspension requires extraction with methylene chloride, drying, and redissolving in methanol (5). Cortisone acetate bulk drug substance, suspension, and composite tablet preparations are analyzed by normal phase chromatography with minimal sample preparation. However, content uniformity for tablets necessitates multiple extraction in chloroform and assay by the blue tetrazolium method (6).

The present study supports a reverse phase chromatographic technique (7) for the determination of steroid acetates. The method is an improvement over the USP general methods because of minimal sample preparation, fast equilibration of mobile phase, and stabilized sample solutions, and because it avoids the use of carcinogenic solvents. The linearity, precision, recovery, and specificity of the method have been discussed (8, 9).

Further supporting information on the ruggedness of the method, stabilization of the sample solutions, and handling of the bulk drug substance for the determination of dexamethasone acetate and cortisone acetate in bulk material and commercial formulations is provided in this study.

Experimental

Apparatus and Reagents

(a) *Liquid chromatograph.*—Isocratic pump system, UV detector capable of monitoring at 254 nm, and suitable recorder.

(b) *Chromatographic column.*—Reverse phase octyldecylsilane column.

(c) *Ultrasonic generator.*

(d) *Acetonitrile.*—LC grade or equivalent.

(e) *Chemicals.*—Potassium chloride, sodium acetate, potassium phosphate monobasic, chloroacetic acid, sodium hydroxide (1N), and hydrochloric acid (1N). Reagant grade and used as received.

(f) *Reference standards.*—USP or equivalent reference standards.

(g) *Chloroacetic acid buffer.*—0.025M, pH 3. Mix 17 mL 1N NaOH, 166 mL 0.5N KCl, and 50 mL 0.5M chloroacetic acid in 1 L volumetric flask. Dilute to mark with water.

(h) *Acetate buffer.*—0.025M, pH 4. Mix 20 mL 1N HCl, 150 mL 0.5N KCl, and 50 mL 0.5M sodium acetate in 1 L volumetric flask. Dilute to mark with water.

(i) *Phosphate buffer.*—0.025M, pH 6. Mix 3 mL 1N NaOH, 138 mL 0.5N KCl, and 50 mL 0.5M KH_2PO_4 in 1 L volumetric flask. Dilute to mark with water.

(j) *Phosphate buffer.*—0.025M, pH 7. Mix 15 mL 1N NaOH, 90 mL 0.5N KCl, and 50 mL 0.5M KH_2PO_4 in 1 L volumetric flask. Dilute to mark with water.

General Chromatographic Method

Dexamethasone acetate and prednisolone acetate with their respective alcohols were selected as test compounds.

(a) *Column-to-column reproducibility.*—Columns from 4 manufacturers were evaluated. For one manufacturer, 5 old and new columns were tested by keeping the mobile phase and temperature constant. The performance specifications for the peaks were calculated for comparison.

(b) *Effect of temperature on chromatography.*—The mobile phase was equilibrated at oven temperatures of 30, 40, and 50°C, and the 4 compounds were evaluated under isocratic conditions. All other LC conditions were kept constant.

(c) *Effect of pH on chromatography.*—The pH of the aqueous phase was adjusted to 2.2 (0.1% phosphoric acid), 4.3 (0.01M phosphate buffer), and 5.8 (water used as received). The ratio of organic modifier to aqueous phase and the oven temperature remained the same on the one column tested.

Analysis of Dexamethasone Acetate and Cortisone Acetate

(a) *Hygroscopicity.*—About 100 mg neat drug, accurately weighed, was placed in an aluminum boat. The sample was placed in various humidity stations beginning from the lowest. At each station, the weights were noted until duplicate weights or equilibrated weight were recorded.

(b) *Solubility in acetonitrile–buffer solvent.*—An accurate amount of drug substance was weighed and transferred to a graduated centrifuge tube. Small increments of solvent were added with vortex mixing until the sample was completely dissolved. The approximate or dynamic solubility was calculated by amount weighed per volume of solvent. The aqueous phases for evaluating the solubility of dexamethasone acetate and cortisone acetate were pH 6 and 4 buffers, respectively.

(c) *Stabilization of the drug in solution.*—Dexamethasone acetate and cortisone acetate in buffers (0.025M) at pH of 3,

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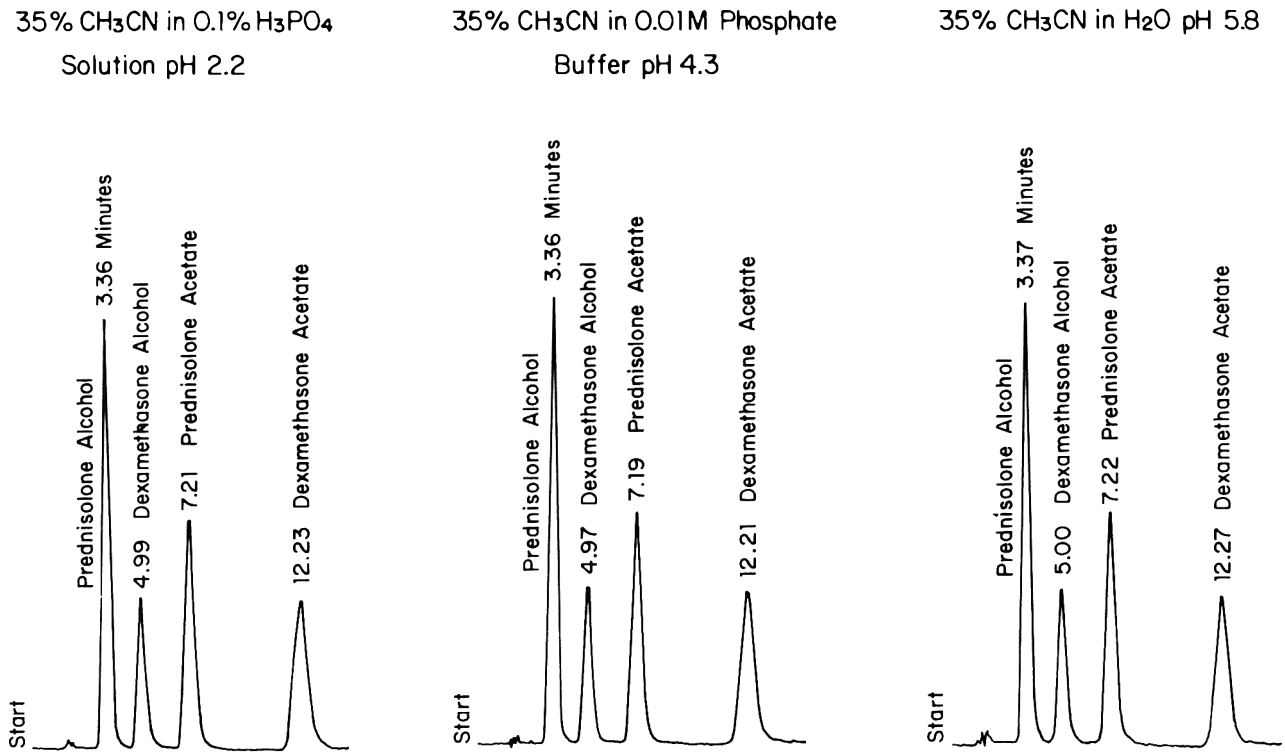

Figure 1. Effect of pH on chromatographic analysis of acetate and alcohol of dexamethasone and prednisolone.

4, 6, and 7 were evaluated. Samples were stored in volumetric flasks under ambient temperature and laboratory conditions. Samples were assayed at 24, 48, and 72 h for the selection of the most stable solution for each steroid acetate.

Results and Discussion

The ruggedness of the liquid chromatographic technique for steroid acetates was evaluated. The method requires a mobile phase of acetonitrile in water at ambient column temperature. Linearity, precision, recovery, and specificity were excellent as reported by Van Dame (7) and Tymes (8). However, the samples were not very stable in the diluent. The present study reports further supporting evidence for the general methodology and provides detailed enhancement of the specific methods for the analysis of dexamethasone acetate and cortisone acetate in bulk drug and dosage forms.

Chromatography of Steroid Acetates

Dexamethasone acetate and prednisolone acetate with their respective alcohols were selected as test materials. The ste-

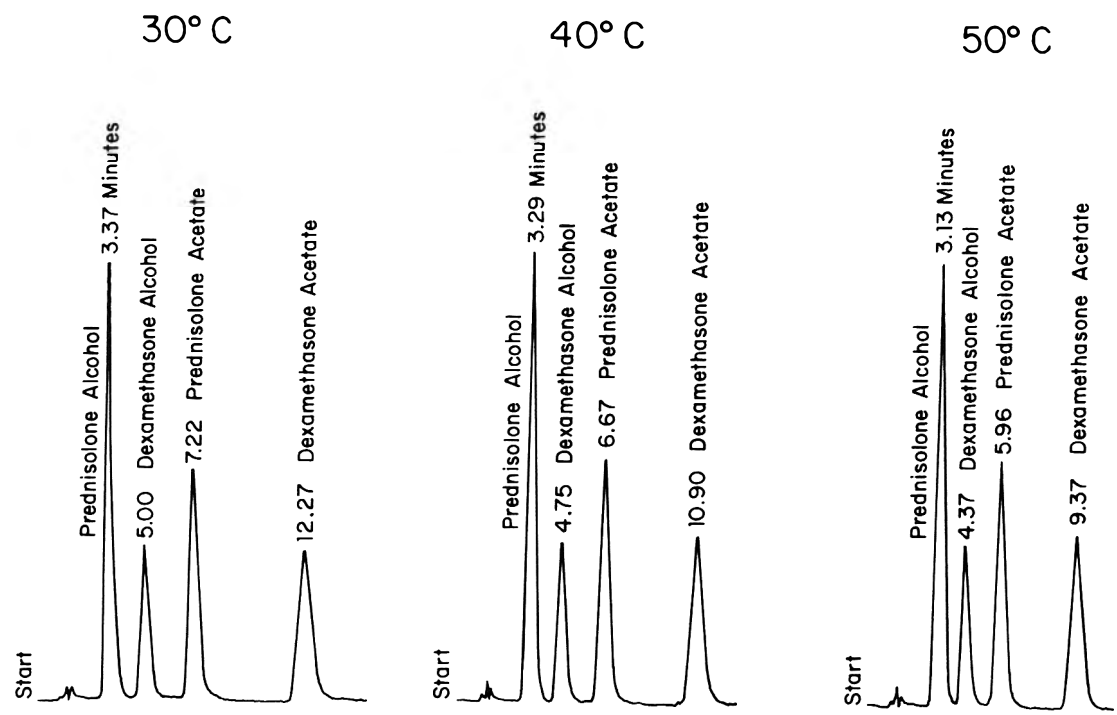

Figure 2. Effect of temperature on chromatographic analysis of acetate and alcohol of dexamethasone and prednisolone.

Table 1. Hygroscopicity measurements of dexamethasone acetate and cortisone acetate

Relative humidity	Weight change, %	
	Dexamethasone acetate	Cortisone acetate
Ambient to 11%	−1.8	−0.02
11 to 58%	+0.4	0.0
58 to 76%	+1.8	+0.1

roid alcohol is a potential hydrolysis degradate of the steroid acetate. The LC conditions of 35% acetonitrile in water, 30°C oven temperature, and 2 mL/min flow rate were constant for the testing of columns from various manufacturers.

The octyldecyl 10 μm columns from 4 manufacturers were used and performance parameters for each peak were noted. Five new and used (less than 2 years old) columns from one manufacturer were also tested. The tailing, k′, and theoretical plates of all peaks were acceptable and within specification. Better than baseline resolution (R = >3) of the alcohol from the parent acetate was also observed.

The effect of pH on mobile phase and the temperature of the column environment were explored. Since water was chosen as the aqueous phase and slight differences in pH of water can be expected, chromatography was tested by using various aqueous phases covering a large pH range. Figure 1 illustrates that no noticeable difference in chromatography was observed with pH 2.2, 4.3, and 5.8 as monitored by the performance parameters of the peaks.

The samples were assayed at oven temperatures of 30, 40, and 50°C. Some sharpening of the peaks and slight decrease of retention time with increased temperature were observed as indicated on Figure 2. However, the oven temperature had no dramatic influence on the retention times of the peaks. Hence, C_{18} columns with ambient temperature and mobile phase of acetonitrile in water are acceptable for the determination of steroid acetates.

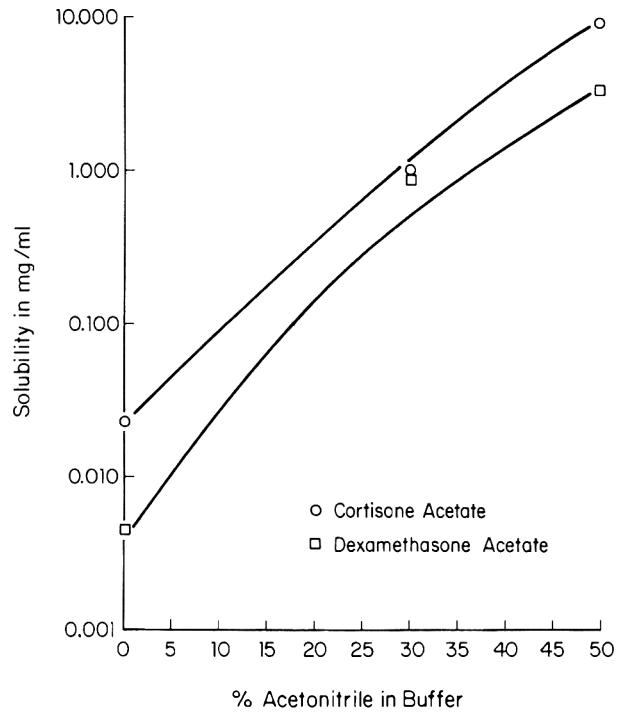

Figure 3. Dynamic solubility of dexamethasone acetate in 50% acetonitrile/0.025M phosphate buffer pH 6, and cortisone acetate in 50% acetonitrile/0.025M acetate buffer pH 4.

Analysis of Dexamethasone Acetate and Cortisone Acetate Bulk Drug and Dosage Forms

The bulk drug substances of both dexamethasone acetate and cortisone acetate were evaluated for their hygroscopicity.

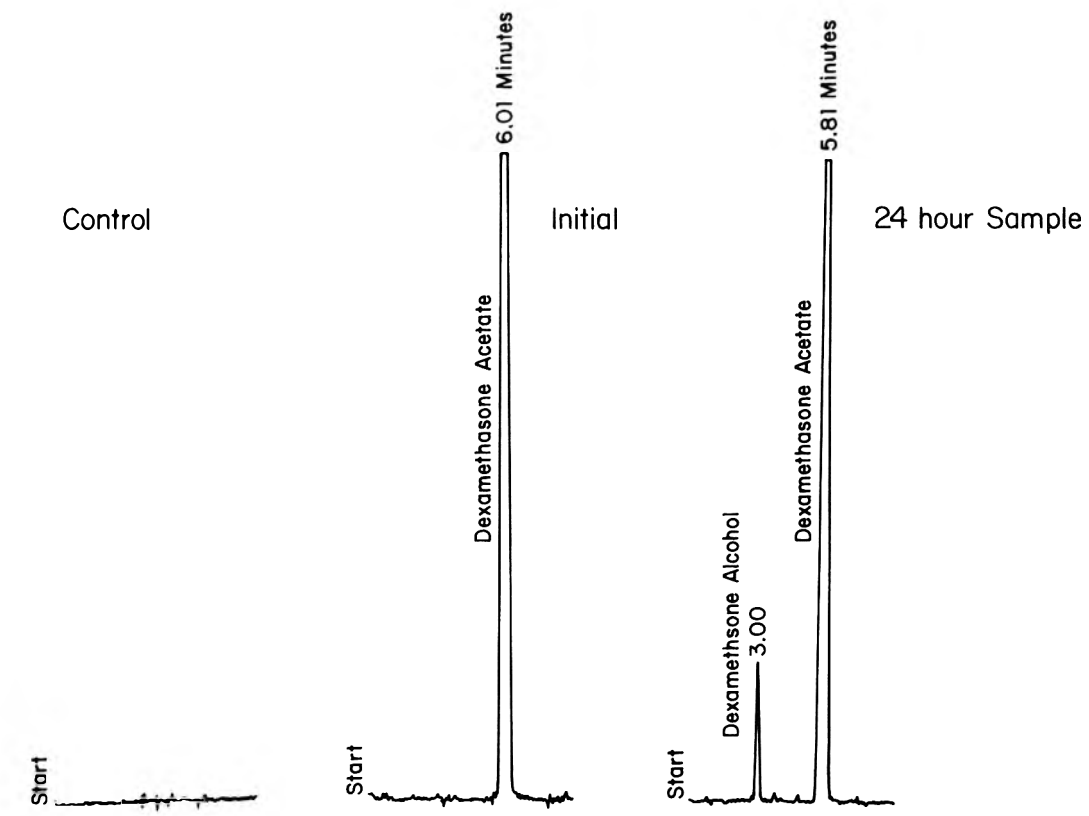

Figure 4. Stability profile of dexamethasone acetate in 50% acetonitrile in water.

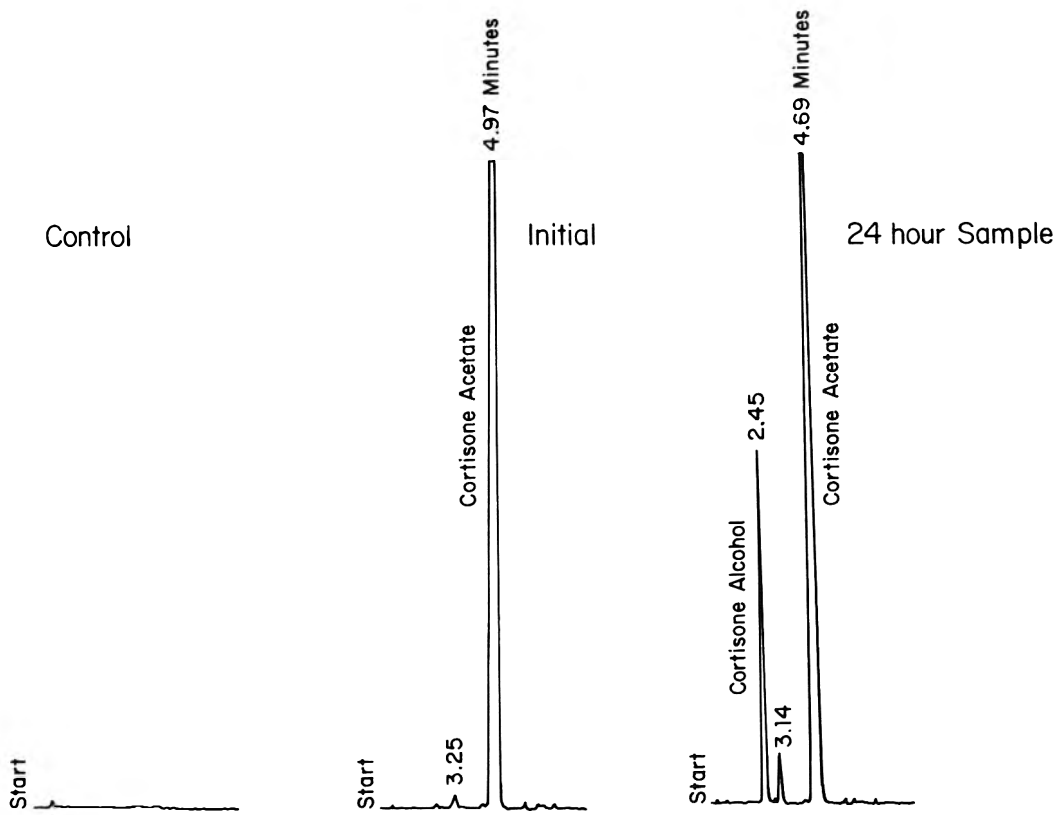

Figure 5. Stability profile of cortisone acetate in 50% acetonitrile in water.

For the neat drugs at each humidity station, the equilibrated weights were noted. As indicated in Table 1, weight variability of about 2% with respect to relative humidity conditions occurred for dexamethasone acetate. However, negligible weight difference was observed for cortisone acetate under similar conditions. Hence, dexamethasone acetate should be dried before use.

The dynamic solubility profiles of dexamethasone and cor-

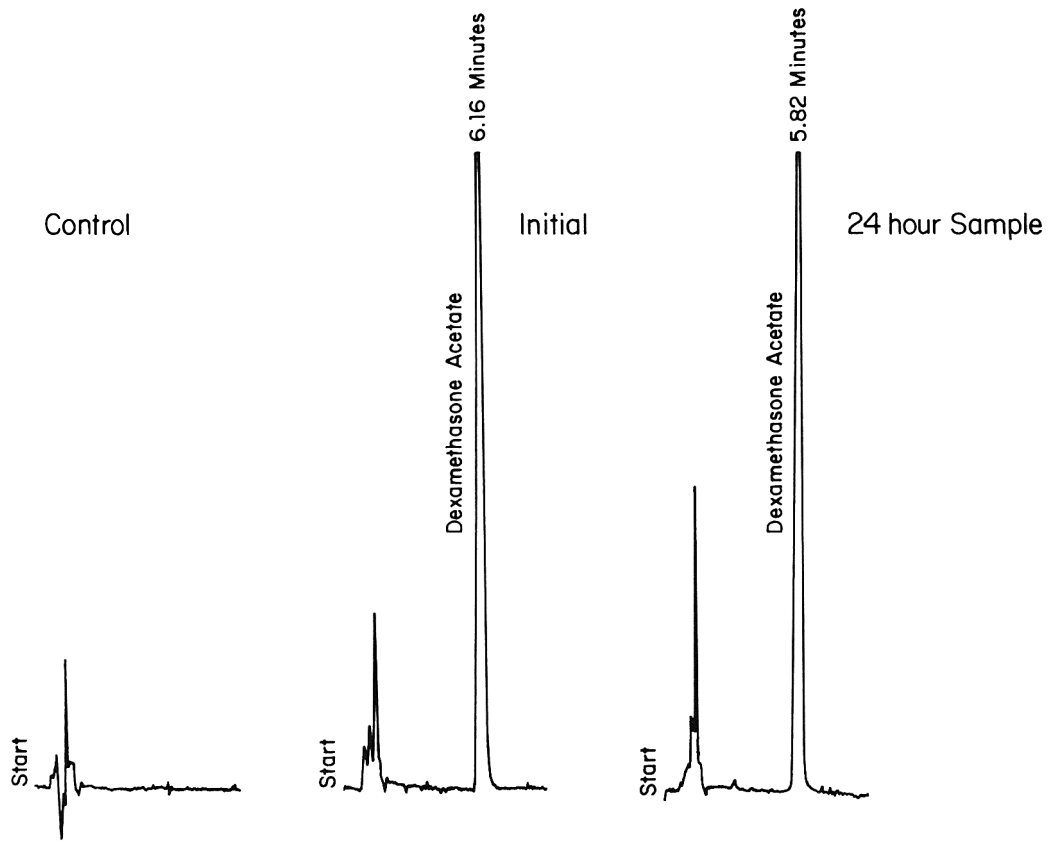

Figure 6. Stability profile of dexamethasone acetate in 50% acetonitrile in buffer pH 6.

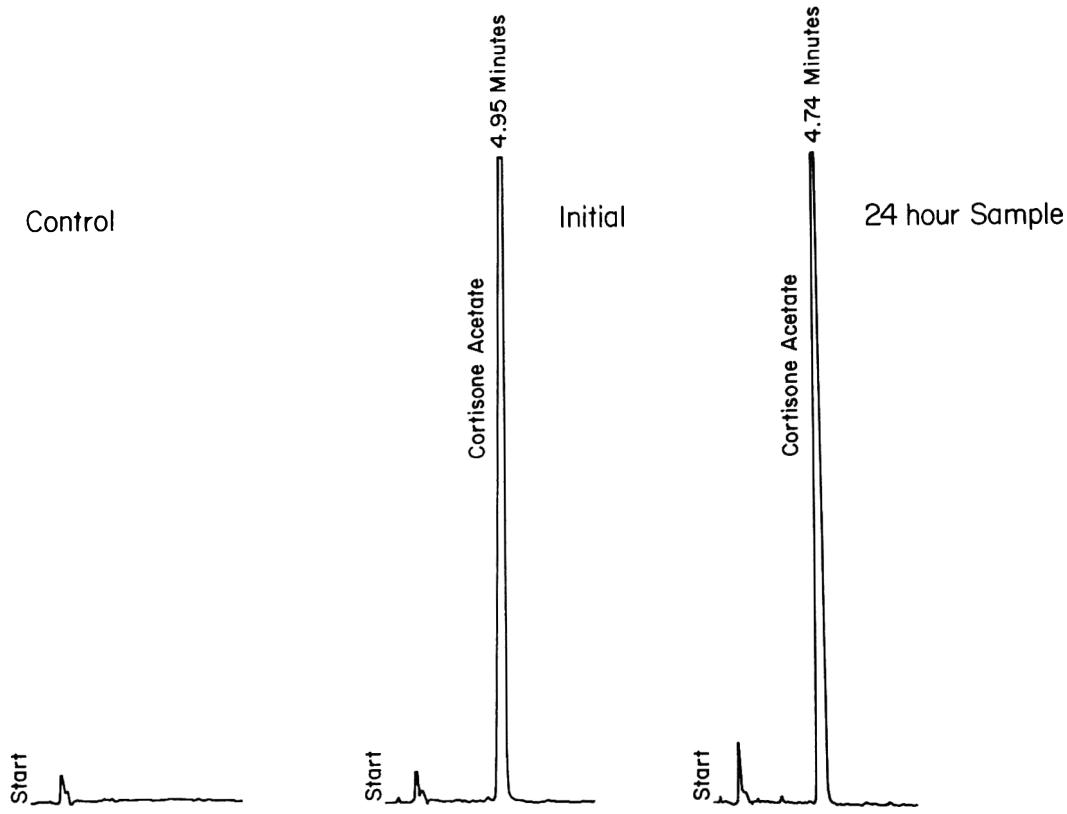

Figure 7. Stability profile of cortisone acetate in 50% acetonitrile in buffer pH 4.

tisone acetates are shown in Figure 3. Dexamethasone acetate and cortisone acetate are slow to equilibrate in aqueous solvents. Hence, the equilibrium solubility should be expected to be higher than the dynamic solubility. On the basis of the dynamic solubility of the bulk drug substance, a solvent mix of 50% acetonitrile in buffer was selected as the diluent. The solubility is at least 5-fold greater than the solubility in the sample solution described in the method selected for collaborative study. The diluent selected had no effect on the peak symmetry.

Acetonitrile instead of methanol was selected as the organic modifier because of potential transesterification of the acetate with the methanol. The steroid alcohol was observed to be a hydrolysis product in an acetonitrile–water mixture. To stabilize the sample solution, dexamethasone acetate and cortisone acetate in buffers of pH 3, 4, 6, and 7 were evaluated after storage for 24, 48, and 72 h at room temperature. The results showed that dexamethasone acetate was most stable at pH 6 as indicated by the slow appearance of degradates. Cortisone acetate was found to be most stable at pH 4. Representative liquid chromatograms for the control, initial, and 24 h solution using acetonitrile–water (1 + 1) are illustrated in Figure 4 for dexamethasone acetate and Figure 5 for cortisone acetate. The stabilized solutions of the 2 acetates in buffers are shown in Figures 6 and 7.

For the analysis of cortisone acetate bulk drug and dosage forms, the bulk material can be used as received. Sample stability can be achieved by storage in diluent of 50% acetonitrile in pH 4 buffer. Ambient temperature reverse phase chromatography and acetonitrile–water mobile phase is used. However, for dexamethasone acetate, the bulk drug substance should be dried at 105°C before analysis and the sample should be dissolved in 50% acetonitrile in pH 6 buffer for stability.

Acknowledgment

The assistance of J. P. Draper for preparation of the buffers is acknowledged.

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Liquid Chromatographic Determination of Coumarin Anticoagulants in Tablets: Collaborative Study

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A liquid chromatographic method for the determination of coumarin anticoagulants in tablets was collaboratively studied by 7 laboratories. The method uses an octadecylsilane-bonded microparticulate column, tetrahydrofuran-methanol-water-acetic acid mobile phase, and photometric detection at 311 nm. Each collaborator received samples of warfarin sodium, phenprocoumon, and dicumarol as a synthetic composite and as commercial individual and composited tablets. Pooled average assay values for synthetic and commercial tablet samples of warfarin sodium were 101.6 and 99.5%, respectively, with a combined reproducibility SD of 2.38% (CV = 2.37%) and combined repeatability SD of 1.49% (CV = 1.49%). Pooled average (SD) assay values for dicumarol and phenprocoumon commercial samples were 98.0 (2.27) and 101.3% (4.00), respectively. The content uniformity determinations of 2 mg warfarin sodium and 25 mg dicumarol tablets indicated average tablet contents (range) of 99.5% (91.0-116.0) and 98.0% (89.8-108.8), respectively. The method has been approved interim official first action.

A previous paper from this laboratory described the development of a rapid, specific, accurate liquid chromatographic (LC) method for the identification and quantitative determination of those coumarin anticoagulants of current clinical interest, in both bulk and commercial tablet samples (Moore, E., & Lau-Cam, C. [1986] *J. Assoc. Off. Anal. Chem.* **69**, 629–632). This method, which is based on a reverse phase (RP) separation on a C_{18} column with a quaternary, slightly acidic mobile phase and detection in the ultraviolet spectral region, was submitted for a collaborative study. The results of the study are the subject of this report.

Collaborative Study

Seven collaborators were supplied with 6 samples (labeled A–F). Four samples were to be used for identification and assay, and 2 samples for determination of content uniformity. The samples included a synthetic formulation that simulated a commercial tablet of warfarin sodium (sample D); individual 2 mg warfarin sodium commercial tablets (sample C) and 25 mg dicumarol (sample F) commercial tablets; the corresponding powdered composites of these latter 2 commercial formulations (samples B and E); and a powdered composite of commercial 3 mg phenprocoumon tablets (sample A). The collaborators were given an instruction sheet, a copy of the method, vials containing the samples, and reporting forms. The collaborators were informed of the average tablet weights and label declarations of the composited tablet samples. Collaborators were instructed to weigh duplicate portions of each composite for the quantitative determinations. Each assay preparation was injected in duplicate, and the average peak response was used in the calculations.

Submitted for publication March 31, 1987.
This report of the Associate Referee was presented at the 99th AOAC Annual International Meeting, Oct. 27–31, 1985, at Washington, DC.
The recommendation of the Associate Referee was approved interim official first action by the General Referee, the Committee on Drugs and Related Topics, and the Chairman of the Official Methods Board. The method will be submitted for adoption official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) **71**, January/February issue.

Dicumarol, Phenprocoumon, and Warfarin Sodium in Drug Tablets Liquid Chromatographic Method Interim First Action

Principle

Coumarin anticoagulants dicumarol, phenprocoumon, and warfarin Na are identified and quant. detd in com. tablets by reverse phase LC on C_{18} column with tetrahydrofuran–MeOH–H_2O–HOAc mobile phase, and photometric detection at 311 nm.

Apparatus

(a) *Liquid chromatograph.*—Equipped with Du Pont Model 8800 solv. pump, variable wavelength detector, and strip chart recorder (E. I. du Pont de Nemours & Co.), or equiv.; and Rheodyne Model 7125 injection valve with 20 μL sample loop (Rheodyne Inc., PO Box 996, Cotati, CA 94928), or equiv. Operating conditions: column temp. ambient; solv. flow rate 1.5 mL/min; detector wavelength 311 nm; detector attenuation 16 AUFS; recorder range 1 mV; chart speed 0.5 cm/min.

(b) *Chromatographic column.*—Stainless steel, 30 cm × 3.9 mm id, packed with 10 μm μBondapak C_{18} (Waters Associates, Inc.), or equiv.

Reagents

(a) *Solvents.*—LC grade MeOH and reagent grade glacial HOAc (Fisher Scientific Co.); tetrahydrofuran (Mallinckrodt, Inc.); and H_2O double distd in glass.

(b) *Mobile phase.*—Tetrahydrofuran–MeOH–H_2O–HOAc (35 + 10 + 65 + 0.1 v/v/v/v). Filter thru 0.45 μm membrane and degas under vac.

(c) *Dicumarol std soln.*—0.05 mg/mL. Accurately weigh ca 25 mg USP Ref. Std Dicumarol into 100 mL vol. flask, dissolve in and dil. to vol. with 0.01N NaOH, and mix. Pipet 5 mL of this soln into 25 mL vol. flask, dil. to vol. with mobile phase, and mix.

(d) *Warfarin Na std soln.*—0.2 mg/mL. Accurately weigh ca 10 mg USP Ref. Std Warfarin Na into 50 mL vol. flask, and dissolve in mobile phase. Dil. to vol. with mobile phase, and mix.

Table 1. Collaborative results for LC analysis of 25 mg dicumarol tablets[a]

Coll.	Found, % of declared		
	Run 1	Run 2	Av.
1	98.8	96.4	97.6
2	100.8	100.4	100.6
3	97.6	101.2	99.2
4	93.6	96.4	95.0
5	95.8	98.1	96.9
6	97.6	99.6	98.4
7[b]	88.9	87.8	88.3
Av.			98.0
SD			2.27
CV, %			2.32

[a] Average tablet weight = 77.1 mg.
[b] Procedural outlier. Not included in the statistical evaluation. See text.

Table 2. Collaborative results for LC analysis of 3 mg phenprocoumon tablets[a]

Coll.	Found, % of declared		
	Run 1	Run 2	Av.
1	101.0	101.3	101.3
2	109.3	108.3	108.7
3	98.3	101.7	100.0
4	101.3	99.0	100.2
5	96.3	97.3	96.8
6	97.0	99.0	98.0
7	104.9	103.0	103.9
Av.			101.3
SD			4.00
CV, %			3.95

[a] Average tablet weight = 198.2 mg.

(e) *Phenprocoumon std soln.*—0.12 mg/mL. Accurately weigh ca 3 mg USP Ref. Std Phenprocoumon into 25 mL vol. flask, and dissolve in mobile phase. Dil. to vol. with mobile phase, and mix.

Preparation of Sample

(a) *Tablet composites.*—Weigh and finely powder ≥20 tablets. Transfer accurately weighed amt of powder to suitable vol. flask and quant. dissolve in mobile phase with aid of ultrasonic bath. Dil. to vol. with mobile phase to prep. soln contg ca 0.12 mg/mL of phenprocoumon or 0.2 mg/mL of warfarin Na. For dicumarol samples, first dissolve powder in 0.01N NaOH with aid of ultrasonic bath to obtain soln contg 0.25 mg/mL; then quant. dil. 5.0 mL aliquot of soln with mobile phase to final dicumarol concn of ca 0.05 mg/mL. Filter all sample prepns prior to injection into LC system.

(b) *Single tablets.*—Place 1 powdered tablet in suitable vol. flask, and proceed as described for tablet composites.

Determination

Equilibrate system with mobile phase at 1.5 mL/min until baseline is steady. Use sampling valve to inject measured vol. of std soln into LC system. Adjust injection vol. and operating parameters so std soln gives peak ht ca 60% full scale. Under these conditions, 3 replicate injections of a std soln should give coeff. of var. ≤3% and tailing factor ≤2.0. Make alternate injections of equal vols of std and sample solns. Measure peak responses in sample and std solns.

Calculations

Calc. amt coumarin anticoagulant in sample as follows:

Tablet composite sample:

$$\text{mg/tablet} = (H/H') \times (W'/D') \times (D/W) \times A$$

Table 3. Collaborative results for LC determination of warfarin sodium in 2 mg tablets and 2 mg synthetic tablet samples[a]

Coll.	Found, % of declared					
	Commercial tablet			Synthetic tablet		
	Run 1	Run 2	Av.	Run 1	Run 2	Av.
1	97.5	96.5	97.0	99.5	98.5	99.0
2	102.0	102.0	102.0	102.5	101.5	102.0
3	99.5	102.0	101.0	106.0	103.5	104.8
4	103.0	99.5	101.3	101.0	101.0	101.0
5	97.5	93.5	95.5	103.5	101.5	102.5
6	99.5	102.0	101.0	100.5	103.5	102.0
7	98.7	99.7	99.2	100.0	100.1	100.1
Av.			99.5			101.6
SD			2.64			2.00
CV, %			2.66			1.96
Reproducibility SD			2.38			
Reproducibility CV, %			2.37			
Repeatability SD			1.49			
Repeatability CV, %			1.49			

[a] Average tablet weight = 222.0 mg.

Table 4. Collaborative results for LC analysis of individual 2 mg warfarin sodium tablets

Tablet No.	Found, % of declared						
	Coll. 1	Coll. 2	Coll. 3	Coll. 4	Coll. 5	Coll. 6	Coll. 7
1	98.0	100.5	96.0	100.0	98.0	109.9	104.4
2	95.5	103.0	96.5	98.5	106.0	104.6	97.7
3	99.5	101.0	107.5	100.0	101.0	101.6	105.2
4	91.0	101.5	97.0	100.0	103.0	104.5	98.4
5	95.5	102.5	105.0	103.0	102.0	105.9	98.2
6	94.5	97.0	97.5	95.5	96.5	97.2	99.2
7	93.0	99.0	97.5	99.5	90.0	99.4	97.3
8	100.5	101.0	100.5	116.0	97.5	100.9	96.8
9	97.0	99.0	96.5	99.5	91.0	98.0	96.7
10	97.0	105.5	99.5	99.5	91.0	96.2	100.0
Av.	96.0	101.0	99.5	101.2	97.6	101.8	99.4
Range	91.0–100.5	97.0–105.5	96.0–107.5	95.5–116.0	90.0–106.0	96.2–109.9	96.7–105.2

Single tablet sample:

$$\text{mg/tablet} = (H/H') \times (W'D') \times D$$

where H and H' = peak responses of sample and std solns, resp.; W and W' = mg sample and std taken, resp.; D and D' = diln factors for sample and std solns, resp.; and A = av. tablet wt, mg. To calc. amt warfarin Na in either tablet composites or individual tablets, use 1.071 as multiplier in above equations (1.071 = ratio of MW of warfarin Na/MW of warfarin).

CAS-66-76-2 (dicumarol)

CAS-435-97-2 (phenprocoumon)

CAS-81-81-2 (warfarin)

CAS-129-06-6 (warfarin Na)

Results and Discussion

Collaborative assay results for the synthetic and commercial tablets samples are given in Tables 1–3. Commercial tablets of dicumarol (Table 1), phenprocoumon (Table 2), and warfarin sodium (Table 3) were found to contain on the average (SD; CV; n = 7 for phenprocoumon and warfarin sodium, n = 6 for dicumarol) 98.0% (2.27; 2.32%), 101.3% (4.00; 3.95%), and 99.5% (2.64; 2.66%) of the declared amount. The synthetic tablet of warfarin sodium was found to contain on the average (SD; CV) 101.6% (2.00; 1.96%) of the formulated amount (Table 3). Collaborative assay results for the content uniformity determination of 2 mg warfarin sodium and 25 g dicumarol tablets are presented in Tables

Table 5. Collaborative results for LC analysis of individual 25 mg dicumarol tablets

Tablet No.	Found, % of declared						
	Coll. 1	Coll. 2	Coll. 3	Coll. 4	Coll. 5	Coll. 6	Coll. 7[a]
1	93.6	95.6	95.6	94.0	94.3	99.7	87.4
2	100.2	102.0	94.8	95.4	98.1	91.6	84.6
3	102.6	100.0	97.6	98.0	92.0	96.4	94.2
4	99.5	103.6	103.2	92.4	100.4	98.2	85.5
5	100.6	106.8	103.6	93.6	95.8	97.2	80.3
6	90.1	102.0	102.0	106.0	97.4	104.6	81.4
7	92.5	103.2	104.8	101.2	90.7	90.8	77.4
8	100.1	108.4	94.8	104.4	91.4	90.2	80.2
9	100.2	95.6	103.6	108.8	92.9	92.9	67.1
10	95.9	97.2	93.6	102.8	93.7	89.8	72.4
Av.	97.5	101.4	99.4	99.7	94.7	95.1	81.0
Range	90.1–102.6	95.6–108.4	93.6–104.8	92.4–108.8	90.7–100.4	89.8–104.6	67.1–94.2

[a] Values reported by this collaborator were procedural outliers and were excluded from the statistical evaluations. See text.

4 and 5, respectively. Although 7 laboratories participated in the study, the results from one of the collaborators were found to be procedural outliers (Tables 1 and 5) because of failure to use an ultrasonic bath for preparation of the sample. These results were excluded from statistical treatment.

No difficulties with the reverse phase LC method were reported nor were procedural modifications suggested by any of the collaborators.

Recommendation

It is recommended that the proposed reverse phase LC method for the determination of coumarin anticoagulants in tablets be adopted official first action.

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Liquid Chromatographic Methods for Assay of Carbamazepine, 10,11-Dihydrocarbamazepine, and Related Compounds in Carbamazepine Drug Substance and Tablets

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Liquid chromatographic (LC) methods have been developed for the determination of carbamazepine, the impurity 10,11-dihydrocarbamazepine, and related compounds in carbamazepine drug substance and tablets. The LC methods specify a 5 μm diol column and a mobile phase of acetonitrile-methanol-0.05% aqueous acetic acid (5 + 5 + 90). Iminodibenzyl and iminostilbene, starting materials for some routes of synthesis, elute late in the LC system; therefore, a thin-layer chromatographic method for their detection at the 0.05% level has been developed. Eight tablet and 13 raw material samples from several sources were examined. The impurities most frequently found were 10,11-dihydrocarbamazepine and a compound identified as 10-bromocarbamazepine at levels up to 1.3 and 0.5%, respectively; minimum detectable amounts were about 0.01 and 0.03%, respectively.

Carbamazepine (5*H*-dibenz[b,f]azepine-5-carboxamide), used in the treatment of trigeminal neuralgia and as an anticonvulsant, is manufactured by several companies. The drug and tablets are listed in the *U.S. Pharmacopeia* (USP) (1) and the *British Pharmacopoeia* (BP) (2). No specific restrictions on related substances are listed in USP, but a proposed general test and a limit for ordinary impurities are included (3). The *British Pharmacopoeia* requires a thin-layer chromatographic (TLC) test for related substances in drug raw material and tablet formulations that limits impurities to no more than 0.2 wt% of the drug; iminodibenzyl is used as a standard.

Typical syntheses of carbamazepine proceed via the dehydrogenation of an iminodibenzyl derivative, either directly or through an intermediate 10-halogenated compound, to an iminostilbene, followed by deprotection of the nitrogen, carbonylation with an equivalent of phosgene, and finally amidation (4, 5). The major metabolites of carbamazepine are reported to be 10,11-epoxycarbamazepine and *trans*-10,11-dihydro-10,11-dihydroxycarbamazepine (6). Early methods for determination of carbamazepine-related compounds in blood used quantitative TLC (6, 7), but these have been supplanted by liquid chromatographic (LC) methods for 10,11-epoxycarbamazepine in body fluids (8, 9). Gas chromatographic (GC) methods for carbamazepine have also been reported but the drug is unstable at the temperature required for vaporization (10).

The purpose of the present work was to develop liquid chromatographic (LC) methods for the determination of carbamazepine, the impurity 10,11-dihydrocarbamazepine, and other related impurities in drug substance and tablets. Also described are a thin-layer chromatographic (TLC) method for iminodibenzyl and iminostilbene in carbamazepine drug substance and tablets and a procedure primarily developed to facilitate identification of trace impurities by gas chromatography with an electron capture detector and a mass spectrometer.

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Experimental

Reagents

Acetic acid, acetonitrile, and methanol were LC grade and ethanol was USP grade. Antipyrine, iminostilbene, iminodibenzyl, and 10,11-dihydrocarbamazepine were obtained from Aldrich Chemical Co., Milwaukee, WI. *Trans*-10,11-dihydro-10,11-dihydroxycarbamazepine and 10,11-epoxycarbamazepine were gifts from Ciba-Geigy, Basel, Switzerland. All other chemicals were ACS reagent grade.

Apparatus

Two Hewlett-Packard gas chromatographs were used: a Model 5840 equipped with a flame ionization detector (FID) and a Chromalytic Technology Model SPH4 septum purge

head, and a Model 5730A equipped with an electron-capture detector (ECD). The same bonded dimethylpolysiloxane column, 30 m × 0.53 mm, film thickness 1.5 μm, was used in both chromatographs. Operating conditions were as follows: ultra pure grade hydrogen carrier gas, 10 mL/min; nitrogen makeup gas, 40 mL/min; hydrogen FID gas, 30 mL/min; air, 300 mL/min; injection port, 210 ± 10°C; detector, 300°C; column temperature program, 210°C for 10 min, increase 4°/min to 290°C, and hold for 10 min. A Finnigan MAT automated gas chromatograph–electron impact mass spectrometer and a Bruker Model WP-80 nuclear magnetic resonance spectrometer were also used. The liquid chromatograph was a Varian Model 5000 with a CDS 401 data station.

Identification of 10-Bromocarbamazepine

During development of the LC methods for related substances in carbamazepine, unidentified impurities were observed in some drug raw materials and tablets. Therefore, a gas chromatographic (GC) procedure was developed to resolve these impurities from the drug and subsequently to obtain their mass spectra.

Gas Chromatography.—Carbamazepine and related compounds were injected on-column as solutions, 2 mg/mL, in glass-distilled ethyl acetate. The relative retention times are given in Table 1. The response of the peak at a relative retention time of 1.35 was several orders of magnitude greater on the electron-capture detector compared to the flame ionization detector, indicating a possible halogen-containing compound.

Mass spectroscopy.—The mass spectrum of the peak at a relative retention time of 1.35 (Table 1) had molecular ions at m/z 314 (36%) and 316 (34%), indicating the presence of one bromine atom in the molecule. Other major fragments include: m/z = 273 (78%), 271 (80%), 192 (38%), 191 (60%), 190 (73%), and 165 (100%).

Synthesis of 10-bromocarbamazepine (11).—Carbamazepine was treated with bromine in chloroform to yield a precipitate of 10,11-dibromo-10,11-dihydrocarbamazepine which was filtered, dried, dissolved in tetrahydrofuran, and dehydrobrominated with 1,8-diazobicyclo(5,4,0)undec-7-ene. The 10-bromocarbamazepine thus obtained had a mass spectrum and LC and GC retention times identical with the brominated impurity found in some carbamazepine samples. The NMR spectrum was consistent with that of 10-bromocarbamazepine.

GC assay of carbamazepine-related compounds—A powdered tablet composite equivalent to 10 mg carbamazepine was tumbled with 5 mL ethyl acetate for 30 min and centrifuged, and a 1 μL aliquot was injected. Quantitation was done by the external standard method.

METHODS

I. LC Method for 10,11-Dihydrocarbamazepine and Other Related Substances in Carbamazepine Drug Substance and Tablets

Apparatus and Reagents

(a) *Equipment.*—Use liquid chromatograph equipped with detector set at 230 nm and 5 μm diol bonded column, 250 × 4.6 mm (Merck).

(b) *Solutions.*—Acetic acid solution: 0.05% in double-distilled water (v/v). Standard solution I: 0.003 mg/mL of 10,11-dihydrocarbamazepine and 0.001 mg/mL of carbamazepine in ethanol–methanol (1 + 9) (v/v). Standard solution II: 0.03 mg/mL of 10,11-dihydrocarbamazepine and 0.01 mg/mL of carbamazepine in ethanol–methanol (1 + 9) (v/v).

Table 1. Relative retention times by LC and GC of carbamazepine-related substances

Compound	LC[a]	GC
Carbamazepine[b]	1.0	1.0
Trans-10,11-dihydro-10,11-dihydroxycarbamazepine	0.58	1.36
10,11-Epoxycarbamazepine	0.72	1.25
10,11-Dihydrocarbamazepine	0.83	0.95
10-Bromocarbamazepine	1.52	1.35
Iminostilbene	2.76	0.47
Iminodibenzyl	3.00	0.43

[a] Flow rate 2 mL/min.
[b] Absolute retention times by LC and GC are 3.14 and 16.71 min, respectively.

(c) *Mobile phase.*—Acetonitrile–methanol–acetic acid solution (5 + 5 + 90) (v/v/v), adjusted to obtain required resolution. Flow rate ca 1 mL/min.

System Suitability

Condition column until stable baseline is obtained. Inject five 10 μL aliquots of standard solution II. Resolution between carbamazepine and 10,11-dihydrocarbamazepine should be greater than 1. Relative standard deviation of peak responses should be no more than 3%.

Calibration

Chromatograph duplicate 10 μL aliquots of standard solutions I and II and calculate response factors for carbamazepine and 10,11-dihydrocarbamazepine.

Procedure

Drug substance.—Prepare solution containing ca 3.5 mg/mL drug substance, accurately weighed, in ethanol–methanol (1 + 9).

Tablets.—Transfer amount of powdered tablet composite, accurately weighed, equivalent to ca 35 mg carbamazepine to tube fitted with Teflon-lined screw cap, add 10.0 mL ethanol–methanol (1 + 9), tumble for 30 min, and centrifuge. Inject duplicate 10 μL aliquots of standard and test solutions into chromatograph and record responses. Estimate amount of 10,11-dihydrocarbamazepine by interpolation on standard curve for this substance. Estimate amount of any other impurity by interpolation on standard curve for carbamazepine.

II. LC Procedure for Assay of Carbamazepine Drug Substance and Tablets

Apparatus and Reagents

(a) *Equipment and mobile phase.*—Described in Method I.

(b) *Solutions.*—Internal standard solution: 0.015 mg/mL of antipyrine in ethanol–methanol (1 + 9). Standard solution: 0.02 mg/mL of carbamazepine in internal standard solution.

System suitability

Condition column until stable baseline is obtained. Inject five 10 μL aliquots of standard solution. Resolution between carbamazepine and antipyrine should be greater than 3.5. Relative standard deviation of ratio of carbamazepine to antipyrine responses should be less than 2%.

Procedure

Drug substance.—Prepare solution containing ca 0.02 mg/mL of carbamazepine, accurately weighed, in internal standard solution.

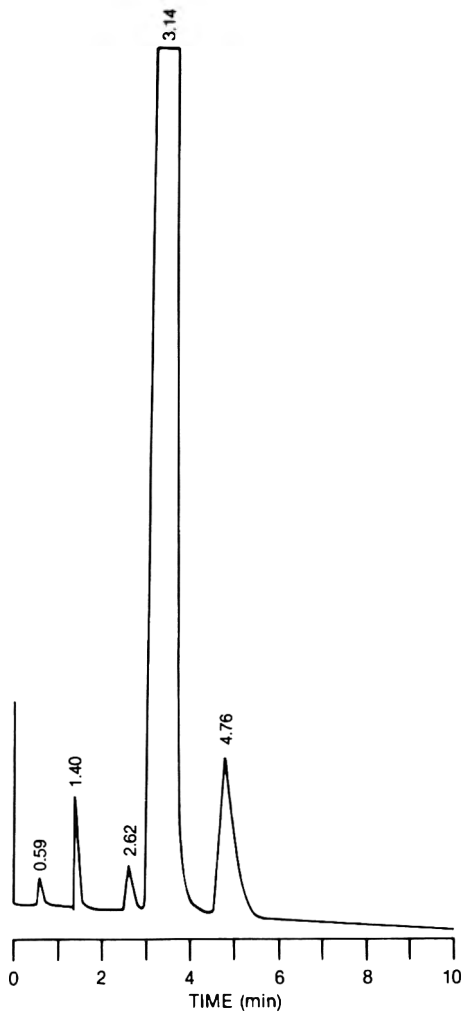

Figure 1. Liquid chromatogram of carbamazepine raw material (Manufacturer II, Lot B, 4.93 mg/mL, rt = 3.14 min) spiked with 10,11-dihydrocarbamazepine (Aldrich, 0.493 mg/mL, rt = 2.62 min) and 10-bromocarbamazepine (synthetic, 0.555 mg/mL, rt = 4.76 min) in ethyl acetate (rt = 1.40 min), 5.0 μL injection, flow 2 mL/min, 16 mV full scale deflection. Peak at 0.59 min is due to methanol–ethanol (9 + 1) solution used to rinse syringe.

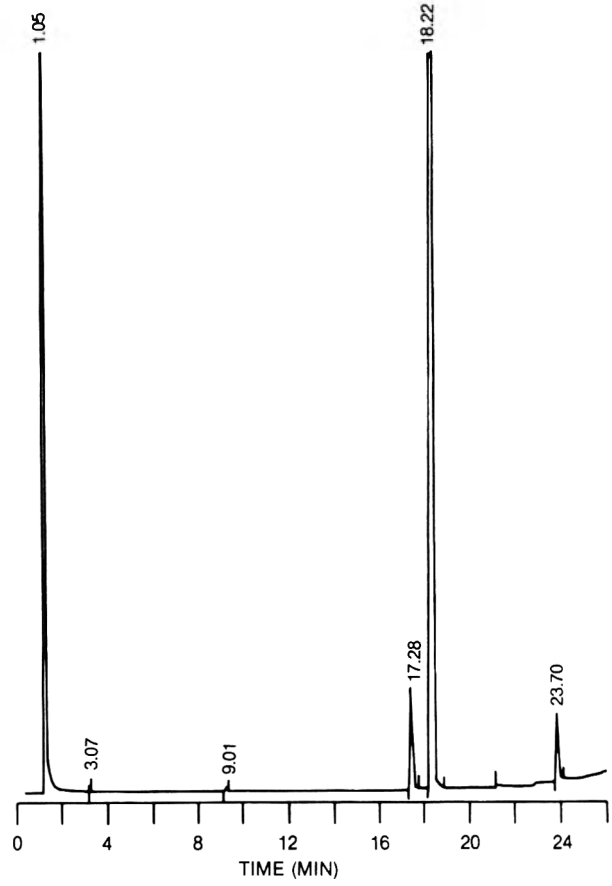

Figure 2. Capillary gas chromatogram of carbamazepine raw material (Manufacturer II, Lot B, 4.93 mg/mL, rt = 18.22 min) spiked with 10,11-dihydrocarbamazepine (Aldrich, 0.493 mg/mL, rt = 17.28 min) and 10-bromocarbamazepine (synthetic, 0.555 mg/mL, rt = 23.70 min) in ethyl acetate (rt = 1.05 min), 0.4 μL injection on-column, attenuation 32.

Tablets.—Transfer amount of powdered tablet composite, equivalent to ca 20 mg carbamazepine, accurately weighed, to tube fitted with Teflon-lined screw cap, add 10 mL internal standard solution, tumble 30 min, and centrifuge. Transfer 1 mL of this solution to 100 mL volumetric flask, dilute to volume with internal standard solution, and mix. Inject duplicate 10 μL aliquots of standard and test solutions into chromatograph and record responses. Calculate concentration of carbamazepine in mg/mL in test solution by C(Ru/Rs), where C = concentration in mg/mL of carbamazepine in standard solution, and Ru and Rs = ratios of response of carbamazepine peak to internal standard peak obtained from test solution and standard solution, respectively.

III. TLC Method for Iminodibenzyl and Iminostilbene in Carbamazepine Drug Substance and Tablets

Apparatus and Reagents

(a) *TLC system.*—Precoated Merck 0.25 mm silica gel 60 F-254 plates, toluene mobile solvent.

(b) *Detecting agents.*—(*1*) Short wavelength ultraviolet (254 nm); (*2*) 0.5% potassium dichromate (w/v) in 20% aqueous sulfuric acid (v/v).

(c) *Solutions.*—Standard solution I: 0.0025% iminodibenzyl in chloroform (w/v). Standard solution II: 0.0025% iminostilbene in chloroform (w/v). Test solution: 5% carbamazepine in chloroform (w/v).

Procedure

Separately apply 10 μL aliquots of standard and test solutions. Let plate develop (15 cm) in chamber previously equilibrated with mobile solvent for ca 1 h. Remove plate and let it dry in air to remove toluene. Observe under ultraviolet light, then spray with the dichromate solution and observe under ordinary light. Spots in the test solution corresponding to iminodibenzyl or iminostilbene that are larger than spots in the standard solution indicate the presence of these compounds at levels greater than 0.05%. Use dichromate detection for iminodibenzyl and ultraviolet for iminostilbene.

Results and Discussion

Liquid Chromatographic Methods

An LC procedure was developed to resolve and quantitate a series of carbamazepine-related substances (Figure 1). These compounds and their retention times relative to carbamazepine are listed in Table 1. The method has been used to assay 8 tablet and 13 raw material samples of carbamazepine for related substances. Some of the samples were also assayed for drug content (Table 2). Levels of 10-bromocarbamazepine and 10,11-dihydrocarbamazepine in drug raw material and tablets were also assayed by gas chromatography (Figure 2) to confirm the LC results (Table 2). Results obtained by

Table 2. Concentration of carbamazepine and related substances in drug raw materials and tablets by LC and GC[a,b]

Manufacturer (lot)	Assay, %	10,11-Dihydrocarbamazepine, %	10-Bromo carbamazepine, %
		Drug raw materials	
I (A)	101.4	0.12 (0.08)	nd[c] (0.01)
(B)	—	0.12 (0.12)	0.05 (0.09)
(C)	—	0.54 (0.59)	0.06 (0.05)
(D)	—	0.10 (0.11)	nd (tr)[d]
II (A)	—	nd (nd)	nd (nd)
(B)	—	0.01 (nd)	tr (nd)
(C)	—	0.01 (nd)	tr (nd)
(D)	101.2	nd (nd)	nd (nd)
III (A)	100.5	0.28 (0.29)	0.09 (0.12)
IV (A)[e]	101.0[f]	0.04[g](0.04)	nd (nd)
(B)	—	1.2 (1.3)	0.06 (0.06)
(C)	—	1.3 (1.3)	tr (0.08)
V (A)	—	nd (nd)	nd (nd)
		Tablets	
I (A)	95.8	1.4 (1.6)	0.10 (0.12)
(B)	95.2[h]	0.94[i](0.93)	0.05[j](0.05)
(C)	97.1	0.12 (0.09)	nd (tr)
(D)	99.5	0.13 (0.12)	nd (0.02)
II (A)	100.3	tr (nd)	nd (nd)
(B)	96.5	tr (0.02)	nd (nd)
III (A)	98.7	0.03 (0.02)	0.53 (0.62)
VI (A)[k]	97.4	0.85 (0.96)	0.09 (0.15)

[a] Values are the means of duplicate analyses, unless otherwise noted. None of the samples analyzed contained detectable amounts of trans-10,11-dihydro-10,11-dihydroxycarbamazepine, 10,11-epoxycarbamazepine, iminostilbene, or iminodibenzyl.
[b] Results in parentheses were obtained by GC.
[c] None detected.
[d] Trace; minimum detectable amount of 10,11-dihydrocarbamazepine is 0.01% (0.01%), of 10-bromocarbamazepine, 0.03% (0.02%).
[e] Mass spectroscopy showed this sample to contain a monobrominated impurity: LC RRT = 1.52 (0.09%), GC RRT = 1.84 (0.07%).
[f] Mean of 6 determinations, RSD = 0.5%.
[g] Mean of 6 determinations, RSD = 14.5%.
[h] Mean of 4 determinations, RSD = 2.1%.
[i] Mean of 4 determinations, RSD = 12.4%.
[j] Mean of 4 determinations, RSD = 13.2%.
[k] This sample contained an unidentified impurity: LC RRT = 1.33 (0.03%).

the 2 methods are in good agreement. The gas chromatographic method lacks robustness and is not recommended for routine use.

The LC responses to carbamazepine and 10,11-dihydrocarbamazepine were linear up to 0.32 and 0.36 μg on-column, respectively, with what were essentially zero intercepts. The minimum detectable amounts of 10,11-dihydrocarbamazepine and 10-bromocarbamazepine are about 0.01 and 0.03%, respectively (3.5 and 10.5 ng, 35 μg carbamazepine on-column). Levels of 10-bromocarbamazepine were determined relative to carbamazepine. The molar absorptivities of carbamazepine, 10-bromocarbamazepine, and 10,11-dihydrocarbamazepine at 230 nm are 13830, 17010, and 5950 L/mol cm, respectively.

The extraction procedure was verified by shaking powdered tablet composites equivalent to 9–27 mg carbamazepine with 6 mL internal standard solution. The amount of drug extracted in each case was directly proportional to the amount of tablet matrix present. Shaking for periods of up to 45 min made no difference in the amount of drug extracted. Reproducibilities for the related substances method and assay are less than 15% and 3%, respectively (Table 2).

TLC Methods for Iminodibenzyl and Iminostilbene

None of the samples analyzed contained detectable amounts of iminodibenzyl or iminostilbene. These compounds are starting materials in some routes of carbamazepine synthesis, and their detection forms the cornerstone of the BP test for related substances. They elute at about 15 min in the LC method, but the peak shape makes quantitation unreliable. For this reason, several TLC systems were investigated as alternatives to LC quantitation. The systems evaluated and the R_f values observed are presented in Table 3. The TLC systems are all based on silica gel 60 plates, and all call for virtually the same detection agents.

Table 3. TLC R_f values for carbamazepine and related compounds

Compound	TLC method BP[a]	IP[b]	USP[c]	BDR[d]
Carbamazepine	0.15	0.27	0.63	0
10,11-Dihydrocarbamazepine	0.16	0.27	0.67	0
10,11-Dihydro-10,11-*trans*-dihydroxycarbamazepine	0.04	0.13	0.64	0
10,11-Epoxycarbamazepine	0.11	0.24	0.62	0
Iminodibenzyl	0.69	0.71	0.75	0.51
Iminostilbene	0.65	0.68	0.75	0.31
10-Bromocarbamazepine	0.12	—	—	0

[a] Official in BP (2). Mobile solvent: toluene–methanol (95 + 5).
[b] Proposed for inclusion in *International Pharmacopoeia*, 3rd Edition. Mobile solvent: toluene–methanol (86 + 14).
[c] Proposed USP method for Ordinary Impurities (3). Mobile solvent: ethanol–acetic acid (90 + 10).
[d] Bureau of Drug Research method described in this paper. Mobile solvent: toluene.

Only the method described in this paper resolves iminostilbene and iminodibenzyl from each other and from carbamazepine. Minimum detectable amounts of iminodibenzyl and iminostilbene under shortwave ultraviolet light are 0.2 and 0.05 μg, respectively; after treatment with dichromate spray, the minimum detectable amounts of iminodibenzyl (blue spot) and iminostilbene (blue-green spot) were 0.05 and 0.2 μg, respectively. None of these systems could be used to obtain a complete profile of impurities in carbamazepine.

Gas Chromatography

Carbamazepine is reported to be unstable under some gas chromatographic conditions (12). In our laboratory, decomposition to iminostilbene and 9-methylacridine was decreased from 15 to 4.5% by decreasing the injection port temperature from 290 to 210°C. Decomposition was further reduced by avoiding any packing material in the injection port liner and using on-column injection after attachment of a septum purge head. A lower injection port temperature would have been preferred because decomposition was virtually eliminated at 160°C, but recycling of the injection port temperature of the gas chromatograph used in this work was very inconvenient. The best results were obtained with cool, on-column, injection.

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Analysis of Piperazine Drug Formulations for *N*-Nitrosamines

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A quantitative method is described for the measurement of *N*-mononitrosopiperazine (NPIP) and *N,N'*-dinitrosopiperazine (DNPIP) in drug formulations containing piperazine, using a gas chromatograph interfaced to a thermal energy analyzer (GC/TEA). The method has detection limits of 20 ppb for NPIP and 12 ppb for DNPIP. In a survey of 6 products available on the Canadian market, all contained NPIP at levels of 0.38–15.3 μg NPIP/g piperazine and none contained any detectable amount of DNPIP.

It is well known that many *N*-nitroso compounds are mutagenic and/or carcinogenic. Many drugs contain primary or secondary amine functional groups, which could possibly nitrosate in vivo or in vitro. Piperazine (PIP) (an anthelmintic) contains 2 secondary nitrogens and has been shown to undergo nitrosation in vitro as well as in experimental animals, yielding both *N*-mononitrosopiperazine (NPIP) and *N,N'*-dinitrosopiperazine (DNPIP) (1). Some controversy appears in the literature concerning whether or not NPIP is mutagenic (2, 3) and/or carcinogenic (4, 5), but DNPIP has been shown to be both mutagenic (3) and carcinogenic (6). It has been reported (7, 8) that different batches of a piperazine drug formulation sold in Sweden contained 3–20 μg NPIP/g PIP, but no detectable amounts of DNPIP. In the present report a method for the determination of NPIP and DNPIP in drug formulations containing piperazine, or its citrate or adipate salts, is described, and results are reported for products on the Canadian market.

METHOD

Apparatus

(a) *Gas chromatograph/thermal energy analyzer (GC/TEA).*—Hewlett-Packard Model 5840A reporting gas chromatograph interfaced to Model 502 thermal energy analyzer (Thermo Electron Instruments, Inc., Hopkinton, MA 01748). Operating conditions: 3.05 m × 4.0 mm id coiled glass column packed with 10% OV-101 on 80–100 mesh Supelcoport; temperatures: injection and detector ports 250°C, oven 150°C, TEA furnace 475°C; carrier gas (argon) 20 mL/min; pressure 1.1 torr.

(b) *Rotary evaporator.*—Buchi Rotovapor R attached to a circulating pump containing ice water.

(c) *Evaporative concentrator.*—10 mL round-bottom flask (14/23 neck) with 1 mL cone, graduated in 0.1 mL increments, on the bottom.

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Reagents

(a) *Piperazine formulations.*—Piperazine adipate suspension was purchased locally. All others were obtained from manufacturers.

(b) *Dichloromethane.*—Distilled in glass (Caledon Laboratories Ltd, Georgetown, Ontario, Canada).

(c) *L-Ascorbic acid.*—(BDH Chemicals Ltd, Poole, UK.)

(d) *Sodium sulfate.*—Anhydrous (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(e) *Sodium hydroxide.*—(BDH Chemicals Ltd, Toronto, Ontario, Canada.)

(f) *Piperazine.*—(Aldrich Chemical Co., Inc., Milwaukee, WI 53201.)

(g) *Sodium nitrate.*—(BDH Chemicals Ltd.)

(h) *NPIP and DNPIP stock solutions.*—Slowly add solution of 8.4 mg piperazine in 1 mL 4M HCl to solution of 6.67 mg sodium nitrate in 1 mL water. After 15 min, make this solution alkaline by adding 5 mL 5M NaOH, then extract with dichloromethane (3 × 33.3 mL). Dry combined dichloromethane extracts over anhydrous sodium sulfate and then decant into bottle. Crimp-seal bottle and wrap in aluminum foil. Store in refrigerator when not in use.

(i) *Standard solution.*—Pipet 0.5 mL stock solution into 10 mL volumetric flask and dilute to volume with dichloromethane.

(j) *Spiking solution.*—Pipet 15 mL stock solution into 500 mL volumetric flask and dilute to volume with dichloromethane.

Procedure

Pipet 10.0 mL liquid piperazine formulation into 125 mL separatory funnel. Add 100 mg L-ascorbic acid and 7 mL water. (For solid formulations, dissolve 2 g granules and 100 mg L-ascorbic acid in 17 mL water.) If sample is to be spiked to obtain estimate of nitrosamine recoveries, add 1.0 mL spiking solution and shake funnel vigorously for a few minutes. Add 3 mL 5M sodium hydroxide and extract solution with dichloromethane (5 × 30 mL). Dry combined extract over anhydrous sodium sulfate and reduce volume to ca 5 mL on rotary evaporator. Quantitatively transfer solution to evaporative concentrator and reduce volume to 1.0 mL. Analyze this solution with GC/TEA by comparing average areas for 5 injections of 5 μL each with average area for 5 injections of standard solution (also 5 μL each). Figure 1 shows typical chromatograms for standard solution and for one of the samples ("C" in Table 1), both unspiked and spiked.

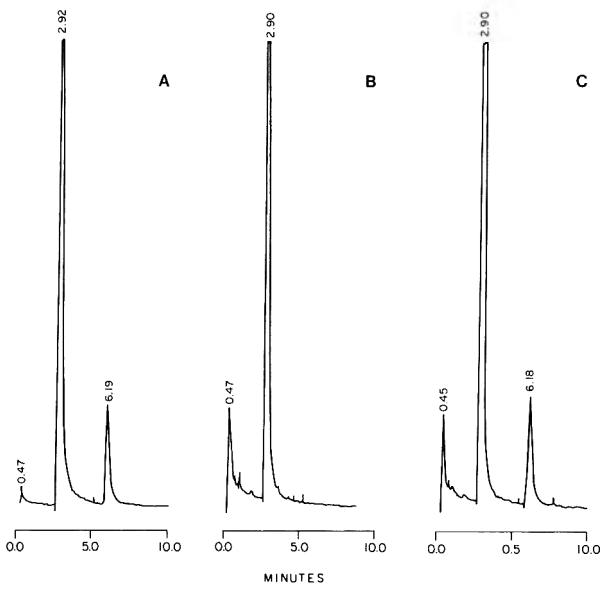

Figure 1. Typical gas chromatograms. A, Standard solution; B and C, unspiked and spiked extracts for formulation C in Table 1.

Results and Discussion

After the stock solution of NPIP was prepared, a 10 mL aliquot was concentrated to 0.2 mL and analyzed by GC-flame ionization detection and by GC/mass spectrometry (GC/MS). Comparison of the peak areas showed that the solution contained 0.4% PIP, 95.7% NPIP, and 3.9% DNPIP. Repeating the preparation 2 more times gave comparable percentages of these components (identified by GC/MS).

A thermal energy analyzer was used for the nitrosamine analysis because of its well-known sensitivity and selectivity in the detection of *N*-nitroso compounds (9). To calibrate the TEA instrument, a series of solutions covering the range of 100–10 000 ppb NPIP was prepared by appropriate dilutions of the stock solution. Since no suitable nitrosamine could be found to serve as an internal standard, absolute injections were used to determine the levels of NPIP. To check the precision of this procedure, each of the standard solutions was injected several times and the percent standard deviation for each was calculated. At concentrations of 10 000, 5000, 3000, 1000, 500, and 100 ppb, the standard deviations were 1.05, 1.01, 0.85, 4.78, 6.37, and 12.96, respectively. TEA response to NPIP was linear (slope = 3470; intercept = −24 500; correlation coefficient = 0.9998).

The analytical procedure described above calls for the dichloromethane extraction of the nitrosamines from an aqueous solution. To verify complete extraction, this part of the procedure was repeated using five 30 mL portions of dichloromethane for representative samples of each type of product. In each case, no detectable levels of either nitrosamine were found in the second extract. This not only shows that the first extraction is essentially complete, but also that no appreciable artifactual formation of either nitrosamine occurs during this procedure.

To check the reproducibility of the method, one of the samples containing piperazine citrate was analyzed 5 times, giving 11.58 μg NPIP/g PIP ± 4.3%. To check the reproducibility of the recovery experiments, replicate analyses were conducted with 3 different types of formulations, each spiked with 1 mL of 3000 ppb NPIP. The results were 87.4 ± 1.2% (5 determinations) for the solid preparation (A), 98.0 ± 2.3% (5 determinations) for the suspension (B), and 91.5 ± 3.2% (4 determinations) for the liquid formulation (C).

Table 1. Levels and percent recoveries of NPIP in piperazine formulations, using GC/TEA

Formulation	Piperazine derivative	NPIP found		
		ppb	μg/g PIP	Recovery, %
A	adipate	84	0.38	87.4 ± 1.2
B	adipate	164	3.69	98.0 ± 2.3
C	citrate	456	10.26	91.5 ± 3.2
D	citrate	501	11.27	99.3, 98.4
E	citrate	515	11.58 ± 4.3%	97.4, 95.5
F	hexahydrate	681	15.32	95.0, 101.2

The results for all the piperazine formulations analyzed are given in Table 1. The levels of NPIP found vary from 84 to 681 ppb of the formulation, which corresponds to 0.38 to 15.32 μg/g PIP. This is in good agreement with the results reported by Bellander et al. (8), who found values in the range 3–20 μg/g PIP for 3 batches of a piperazine formulation sold in Sweden. None of the formulations contained any detectable levels of DNPIP.

Conclusion

The GC/TEA method described here is suitable for the analysis of piperazine formulations for *N*-mononitrosopiperazine and *N,N'*-dinitrosopiperazine with detection limits of about 20 ppb and 12 ppb, respectively.

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MYCOTOXINS

Thin-Layer Chromatographic Determination of Sterigmatocystin in Cheese: Interlaboratory Study

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A collaborative study of a method for the determination of sterigmatocystin in cheese was conducted by 10 laboratories. The study included control samples and samples spiked at levels of 5, 10, and 25 ppb, in coded blind pairs. Recoveries were 60.0, 90.7, and 59.3%, outliers excluded, for the respective levels. The mean reproducibilities, outliers excluded, were 81.97, 17.13, and 52.77%, respectively. Mean repeatabilities, outliers excluded, were 77.66, 17.13, and 46.40%, respectively. Results of this collaborative study indicate that the method, modified as described in this report, is applicable to the determination of sterigmatocystin in cheese at low levels (5–50 ppb) for the purpose of surveys. With regard to the difficulty with thin-layer chromatography in this study, it is recommended that a more satisfactory determinative step be developed. Recommendation for official first action status is deferred.

The study reported here was initiated to evaluate the method of Francis et al. (1) for the determination of sterigmatocystin in cheese. Cheese is extracted with acetonitrile–4% KCl (85 + 15). A simplified liquid–liquid partition cleanup is used, and the sample extract is passed through a cupric carbonate–Celite column for final purification. Sterigmatocystin is quantitated using one-dimensional thin-layer chromatography (TLC) after the plate is treated with the fluorescence-enhancing sprays consisting of 15% $AlCl_3$ in EtOH and silicone–ether (18 + 82). Experience in our laboratory has shown this method to be satisfactory and efficient. Due to the favorable performance characteristics exhibited during those trials and to the need for further evaluation of this method for the determination of sterigmatocystin in cheese, a collaborative study was initiated.

Collaborative Study

Description of Samples

The gouda cheese sample selected for use in the study, as the control and for spiking, contained no detectable sterigmatocystin. The sample was prepared for analysis by size reduction in a meat grinder followed by homogenization in a planetary mixer. All analytical samples were weighed into wide-mouth glass jars:

(*1*) Each collaborator's practice sample contained about 115 g cheese. Analysts were instructed to analyze a 36 g portion of the sample as a control, and to spike 2 additional 36 g portions of sample with 10 and 25 ppb of the provided standard spiking solution. This scheme was designed to determine if a collaborator's system was suited for this analysis. In the event of problems, a method monitor/consultant was provided.

(*2*) Control and spiked samples were provided in coded blind pair sets, containing 0, 5, 10, and 25 ppb sterigmatocystin. Spiked samples were prepared by adding known amounts of sterigmatocystin in benzene to the weighed sample from the control lot of cheese. The solvent was allowed to evaporate ca 60 min, and the containers were tightly sealed.

(*3*) Samples and standards were frozen prior to packaging. Frozen materials were placed into Styrofoam ice chests, along with frozen wet-packs, and shipped via express mail.

Participants were instructed to use the entire contents of each jar (36 g) for each analysis.

Description of Study

Fourteen laboratories originally agreed to participate in the study, and each was provided with the previously described, coded samples.

Each collaborator's package included sample and standard materials, standard data sheet, storage information for samples and standards, instructions describing the quantitative procedure of transferring sample materials from jars to extraction containers, and a copy of the method.

Sterigmatocystin Standards

(a) *Practice sample spiking standard.*—Approximately 450 μL sterigmatocystin solution (10.8 μg/mL) dissolved in benzene and contained in sealed glass ampules.

(b) *Working standard.*—Approximately 1.5 mL sterigmatocystin solution (1.0 μg/mL) dissolved in benzene and contained in sealed glass ampules. Collaborators were instructed to report any deviations in standard weight from that recorded on the accompanying data sheet. They were also instructed to transfer the ampule contents without rinsing to a small (2–5 mL) volumetric flask or vial, stopper the container tightly, and store in a freezer. Samples were to be brought to room temperature and mixed prior to being used.

Data Requested

Each collaborator was provided a report sheet that requested his or her name, address, type of TLC plate used, and other parameters such as grades of solvents used. In addition, a tabulated format was designed for listing analytical results. Remarks (comments, suggestions, criticisms, and/or descriptions of difficulties) were requested. Instructions also stated that if method modifications were tried, collaborators were to report the findings to the monitor, but to keep those data separate from the study report.

METHOD

The method has been published (1).

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Table 1. Collaborative results for TLC determination of sterigmatocystin in spiked cheese samples

	Spike levels, μg/kg										
	Control								Practice samples		
Coll.	0		5		10		25		0	10	25
1[a]	18	2	3	9	(36)[b]	(27)	36	13	—	—	—
2[c]	0	0	—[d]	—	9	—	—	2	—	—	—
3	0	1	5	2	11	9	33	17	0	9	26
4	0	0	7	2	11	7	13	18	0	9	20
6	4	0	4	7	9	11	22	22	0	9	27
7	0	0	0	T[e]	4	4	9	17	0	9	22
9	0	0	(28)	(31)	(19)	(14)	9	7	0	9	25
12	0	0	0	4	9	9	22	0	0	9	16
13	0	0	4	0	7	9	18	18	0	11	22
14	0	0	4	3	9	8	20	18	0	10	17

[a] Data submitted by this collaborator omitted from calculations.
[b] Values in parentheses are statistical outliers by Dixon or Grubbs tests.
[c] Incomplete data submitted; values were not used in calculations.
[d] No data submitted.
[e] This result was reported as a trace, but was interpreted to be 0 because the value is below the detection limit of the method.

Results and Discussion

The results reported by each collaborator for the determination of sterigmatocystin in cheese are presented in Table 1. Results were reported by 10 of the 14 laboratories to which samples were issued.

Of the 10 reporting laboratories, 8 submitted the results of their practice samples. Those results show no false-negative or false-positive spots for the controls, and they showed mean recoveries for the spikes of 9.4 and 21.9 ppb, with RSDs of 7.9 and 18.5%, for the 10 and 25 ppb levels, respectively.

With respect to the coded, blind spikes, 3 of the 9 collaborators whose results were statistically included reported a total of 4 (22.2%) false-positive spots for the control samples. In the case of collaborator 1, all results were eliminated from further statistical evaluation for the following reasons: results of practice samples were not reported; both controls were reported as false positives, including the exceptionally high value of 18 ppb; and results reported at the 10 ppb spike level were classified as statistical outliers by both Dixon and Grubbs tests (2, 3).

The result of 1 ppb in the control sample reported by collaborator 3 was interpreted as 0 because the value is less than the detection limit of the method. Subsequently, the evaluation of data for the control samples showed there to be 1 false-positive result (collaborator 6) of the 16 samples from 8 collaborators considered (6.25%).

Results reported by collaborator 9 for the 5 and 10 ppb spike levels were determined to be statistical outliers by the Dixon test; however, the collaborator's results for the 25 ppb spike level are included in the calculations. A cursory evaluation of those spiked sample results may indicate a problem with crossed-up samples.

Table 2. Precision measurements of TLC method to determine the presence of sterigmatocystin in cheese

	Added, ppb		
Statistic	5	10	25
Mean, ppb	6.31 (3.00)[a]	9.33 (9.07)	14.83
Recovery, %	60.0	90.7	59.3
RSD_r, %	153.01 (81.97)	38.05 (17.13)	52.77
S_r	9.659 (2.459)	3.559 (1.555)	8.226
RSD_o, %	36.51 (77.66)	19.64 (17.13)	46.40
S_o	2.305 (2.330)	1.837 (1.555)	7.233
Outliers	Lab. 9—Grubbs, Dixon	Lab. 9—Dixon Lab. 7—Grubbs	none
Expected RSD_r, %	38.36	32.47	30.16

[a] Values in parentheses are quantities after outlier rejection.

Table 3. Results of postcollaborative study to determine if product or analyte degradation occurred between the times of sample preparation and collaborator analyses

	Spike levels, μg/kg			
	Control			
Sample set	0	5	10	25
1	0	3.4	9.6	22.2
	0	4.1	11.0	17.2
2	0	4.5	10.2	18.4
	0	4.8	8.9	21.6
Range: low	—	3.4	8.9	17.2
high	—	4.8	11.0	22.2
$\bar{x}$	—	4.2	9.9	19.9
SD	—	0.61	0.89	2.43
RSD, %	—	14.4	8.99	12.2
Rec., %	—	84.0	99.3	79.4
Overall rec., %			87.6	

In Table 2, the overall precision estimates for sterigmatocystin found in the spiked samples, outliers excluded, showed mean values of 3.00, 9.07, and 14.83, representing recoveries of 60.0, 90.7, and 59.3% for the levels of 5, 10, and 25 ppb, respectively. A one-way analysis of variance was applied to each duplicate sample set and was used to calculate the reproducibility relative standard deviation (variation between laboratories), RSD_r, and the repeatability relative standard deviation (within laboratory variation), RSD_o, for the spiked samples. RSD, values for spiking levels 5, 10, and 25 ppb were 81.97, 17.13, and 52.77%. RSD_o values for spiking levels 5, 10, and 25 ppb were 77.66, 17.13, and 46.40%. RSDs for reproducibility and repeatability were satisfactory at the 10 ppb spike level but were much higher for the 5 and 25 ppb spike levels, compared to their calculated expected RSDs (4).

Results obtained at the 10 ppb level agreed with results obtained during method development and with results obtained by collaborators for practice samples.

The unexpected results obtained at the 5 and 25 ppb level spikes were investigated for the possibilities of product or analyte degradation; 2 extra sample sets were analyzed after completion of the collaborative study to resolve this matter. As shown in Table 3, the obtained results eliminate those possibilities.

Feedback received from collaborators 2, 4, 13, and 14 indicated that some problems existed with the thin-layer chromatographic determinative step in that some background streaking made spot estimation difficult. This concern was also investigated during the postcollaborative study experiments; we concur that streaking did occur, but not to the point where it interfered with quantitation. Coincidental to the matter of TLC quantitation, collaborator 7 was not able to obtain the recommended type of TLC plates and, as a result, experimented with other types of available TLC supports. The collaborator reports that very acceptable results were obtained using E. Merck HPTLC plates, with no background streaking. We evaluated the collaborator's findings and concur. Additional TLC experiments yielded very satisfactory TLC results for Whatman KC_2 reverse phase TLC plates and development with hexane–methanol (95 + 5) for

45 min to 1 h, followed by the previously described spray enhancers.

Collaborators 3 and 4 commented on problems with the glass wool filtration technique, indicating that filtration was unusually slow. Since this is a minor procedural detail, it was suggested that the filtration surface area be increased by passing the material through a larger-diameter glass wool-packed tube such as a Pyrex No. 3700 butt tube.

Recommendations

Results of the collaborative study indicate that the method, modified as described in this report, is applicable to the determination of sterigmatocystin in cheese, if we take into account that the purpose of this work is to provide a simple, economical, rapid method for hazard assessment surveys that are intended to determine levels and incidence of contamination in cheese below the 50 ppb level of current methods. Statistically, the analytical variance of results at the 10 ppb level is acceptable. However, because of the high variability at the 5 and 25 ppb levels, a recommendation for official first action status is deferred pending development of a more satisfactory determinative step.

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Determination of Cytotoxic Trichothecenes in Corn by Cell Culture Toxicity Assay

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The great sensitivity of some cell species to toxins has been adapted to a direct biological determination of trichothecene contamination of food and feeds. The murine spleen lymphocyte stimulated by PHA (*Phaseolus vulgaris* phytohaemagglutinin) appeared to be the most convenient cells because of their particular sensitivity to cytotoxic trichothecenes and the opportunity to translate this cytotoxicity to immunosuppressive hazard, one of the most important concerns for trichothecenes. In this paper, the use of cell cultures was adapted for a survey of corn. The toxins were extracted by aqueous methanol, and the extract was defatted with hexane and purified on a silica gel/Florisil column. This extract was then used for a gas chromatographic (GC) determination and the biological test. The growth of cells was measured by the incorporation of tritiated thymidine (^{3}H Tdr), and the inhibition was expressed by the IC_{50}: concentration of corn extract inhibiting by half the ^{3}H Tdr incorporation. We have tested pure toxins, control corn, corn spiked with T-2 toxin, corn experimentally inoculated with toxigenic *Fusarium* strains, and naturally contaminated corn. A good correlation exists between IC_{50} and the T-2 toxin concentration as determined by GC analysis. The response is not affected by the presence of zearalenone or by small amounts of deoxynivalenol. A quantitative evaluation of cytotoxic trichothecenes in corn is valuable in the range of 100 ppb to 10 ppm, expressed as T-2 toxin equivalents. The result is obtained in 48 h.

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Trichothecenes compose a large group of mycotoxins, of which more than 75 compounds have been identified. They are mostly produced by microfungi such as *Fusarium,* also responsible for the production of zearalenone, butenolide, moniliformin, and other less toxic compounds. They constitute an important problem in public health and in animal feeding because of their possible occurrence in food and feed and their implication in numerous human and animal diseases. Trichothecenes belong to the scirpen group; their activity is highly modulated by the number and size of substituents to the central nucleus. Lethal doses (LD_{50}) range from 0.5 mg/kg for verrucarine A to 810 mg/kg for crotocin (1).

Because of the lack of fluorescence, the UV absorption in a limited range of the spectrum (*A* to 210 nm), and the immunosuppressive characteristics that make antibody production difficult (2), quantitative analysis of trichothecenes is particularly challenging. Thin-layer chromatography (TLC), either with nonspecific sulfuric acid development or with other more specific reactions (3, 4) is not accurate enough to determine levels in agricultural commodities, except for particular compounds like deoxynivalenol (DON) that can be detected by TLC through $AlCl_3$ development (5). Because of higher specificity, gas chromatography (GC) (6) and GC/mass

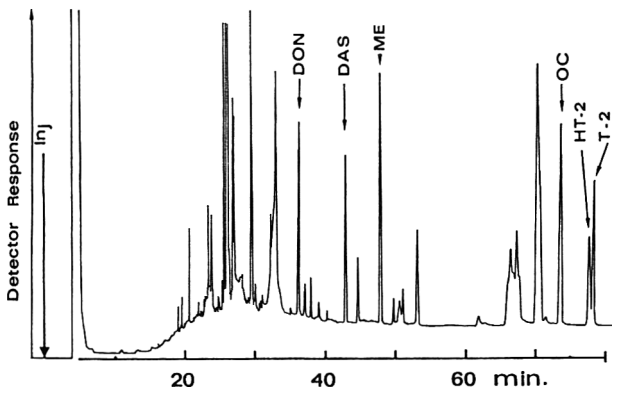

Figure 1. Example of GC separation of trichothecenes extracted from corn spiked with 0.5 μg/g of DON, DAS, HT-2, and T-2 toxin. (ME and OC are added as internal standards, see text.)

spectrometric analyses (7) are now being used in numerous laboratories.

Physicochemical determination of a few particular mycotoxins that could contain numerous molecules with various activities cannot realistically portray the measure and significance of contamination in foodstuffs. Therefore, it would be useful to perform a global evaluation of contamination from mycotoxins on the basis of their toxicological characteristics. For example, detection using the necrotizing power on animal skin (8) or the lethal effect of most trichothecenes on *Artemia salina* larvae has been studied (9).

Most trichothecenes show a strong cytotoxic activity in some cell lines; therefore, tests with cell cultures have proved to be good methods for evaluating the potential toxic contamination of a food product. Several cells even show a selective sensitivity to trichothecenes, particularly lymphoid cells, epithelial cells of intestinal mucosa, and some tumor cells (10, 11). A first method based on the use of cell cultures has been published (12).

In the present paper, a method suitable for surveying cytotoxic mycotoxins in contaminated agricultural products is proposed and applied to corn samples. The presence in vegetable products of stimulating lectins and inhibitory alkaloids required the evaluation of possible interference from the corn itself and the development of a procedure for cleanup and dilution of extracts. Two kinds of cells were used: murine splenic lymphocytes stimulated by *Phaseolus vulgaris* phytohaemagglutinin (PHA) and a rat hepatoma cell line (LF cells). In addition to their suitability for in vitro culture, the lymphocytes permit the estimation of one of the main toxicological effects of trichothecenes—their immunosuppressive potency.

Experimental

Materials

Pure mycotoxins were purchased from Makor (PO Box 6570, Jerusalem, Israel) except for deoxynivalenol which was a gift from H. Cohen (Ottawa, Ontario, Canada). All products and solvents were analytical grade. Some batches of DMSO appear to be significantly toxic to cells, so this solvent must be tested before use.

Two media were used for cell cultures: Roswell Park Memorial Institute Medium 1640 (RPMI 1640) and Minimum Essential Medium (MEM) (Eurobio).

Preparation of Corn Extracts and GC Analysis

Two methods were necessary for trichothecene determination in naturally or artificially contaminated corn samples.

(a) *DON determination.*—The first method concerns polar trichothecenes (i.e., DON). A 50 g sample of ground corn is extracted with 200 mL acetonitrile–water (84 + 16 v/v) according to the procedure proposed by Trucksess et al. (5). After filtration through paper, 20 mL organic phase is defatted by hexane and evaporated to dryness. The residue is dissolved in 5 mL chloroform, and 5 mL hexane is added. This extract is then purified on a Florisil column, previously deactivated with 10% water, by eluting with 40 mL chloroform. DON is then eluted with 60 mL chloroform–acetone (50 + 50 v/v). After evaporation to dryness, 20 μg *N*-methyldecosanoate and *N*-octacosane are added as internal standards.

Prior to GC analysis, TMS–DON derivative is prepared by heating with 100 μL Tri-Sil/TBT (Pierce, Rockford, IL 61105) for 10 min at 60°C. The resulting derivative and solvent are used for GC analysis.

(b) *T-2, HT-2, DAS, and zearalenone determination.*—For cytotoxic trichothecenes and zearalenone, extraction is more efficient with pure methanol (200 mL for 50 g grain). Nevertheless, it is necessary to change slightly the polarity and ionic strength by adding 80 mL water with 2% NaCl before the extract is washed with 100 mL hexane. Toxins are then reextracted from aqueous phase by 100 mL chloroform. One half is used for LC determination of zearalenone after purification according to a procedure adapted from Mirocha et al. (13).

The other half is evaporated to dryness and then purified on a column of silica gel/Florisil/10% water, for T-2, HT-2, and diacetoxyscirpenol (DAS) determination. Nonpolar impurities are first removed with 20 mL benzene and toxins are collected with 50 mL benzene–acetone (50 + 50). An aliquot of this extract is evaporated to dryness and used for the cell culture test. TMS derivatives are prepared using BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide) for 60 min at 60°C.

(c) *Gas chromatography.*—GC separation is performed on a glass capillary column (50 m × 0.3 mm id) coated with SE-52 as stationary phase. Oven temperature is maintained at 100°C for 10 min after splitless injection and increased to 250°C at 10°/min. With the flame ionization detector, the average sensitivity is better than 100 μg/kg; 1 μL injection represents 100 mg corn.

Cell Cultures

After silica gel/Florisil column purification, dry extracts were then dissolved in DMSO in increasing dilutions, to contain between 10^{-5} and 0.5 g equivalent corn per volume of DMSO (10 μL), which is applied to 1 mL cell culture medium. Cell growth is estimated by incorporation of tritiated thymidine (TMM199B, 25 Ci/mM, CEA, Saclay, France).

(a) *Splenic lymphocytes.*—Preparations were obtained from spleen of Balb/c mice as described elsewhere (14). Spleens were aseptically removed, minced with scissors, and filtered through a 200-mesh stainless steel sieve. Cell suspensions were collected and centrifuged 10 min at 2000 rpm. The pellet was resuspended in a known volume of RPMI 1640. A 50 μL aliquot (diluted 1:10 with trypan blue) was counted with a hemocytometer.

Cells (500 000) were seeded in microplate wells in 150 μL RPMI supplemented with 2 mM glutamine, 10% fetal calf serum inactivated by heating 30 min at 56°C, and antibiotics (100 IU penicillin/mL and 100 μg streptomycin/mL). Then 50 μL PHA (HA 16, Wellcome, 1 μg/mL cell culture) and 2 μL dilutions in DMSO of test extract were added to each

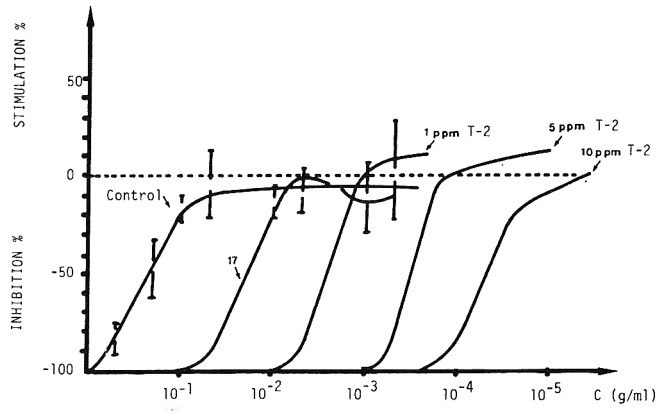

Figure 2a. Tests on lymphocytes. Control and T-2 toxin spiked corn.

Inhibition is calculated by comparing ^{3}H Tdr incorporation in cells treated by dilutions of corn extract with that in control cells. Each point was made in triplicate. For each extract, a curve is plotted from the dilution range of the extract; the percent variation compared to controls is expressed as a function of "equivalent corn" concentration in g/mL cell culture.

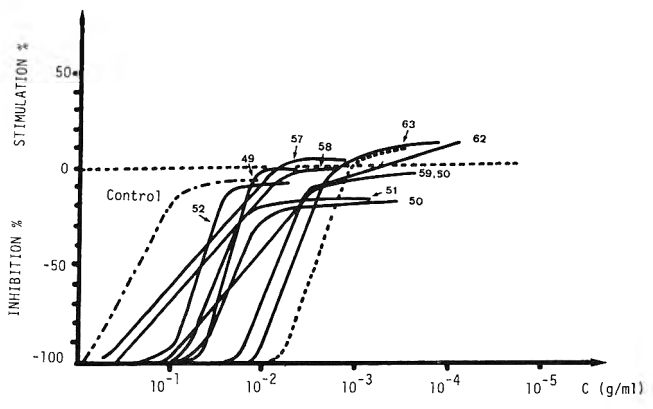

Figure 2b. Tests on lymphocytes. Corn used as substrate for *F. lateritium* culture. (-----) 1 ppm T-2 toxin. See Figure 2a for conditions.

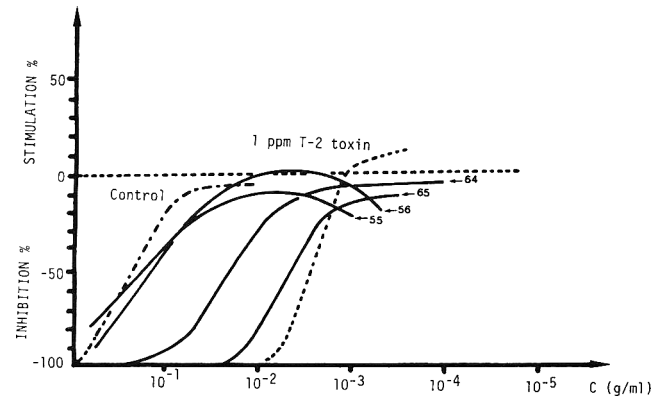

Figure 2c. Tests on lymphocytes. Corn used as substrate for *F. graminearum* culture. See Figure 2a for conditions.

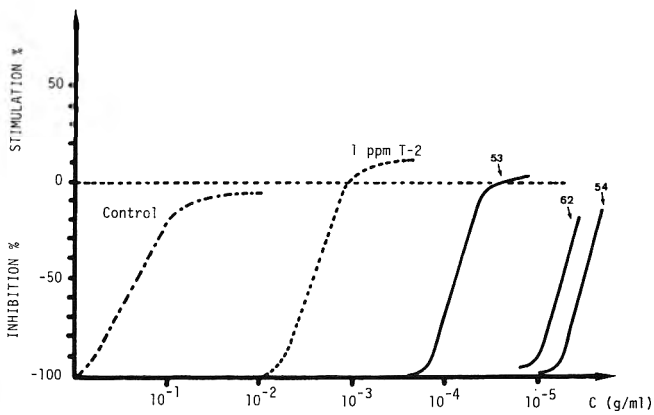

Figure 2d. Tests on lymphocytes. Corn used as substrate for *F. sporotrichioides*. See Figuer 2a for conditions.

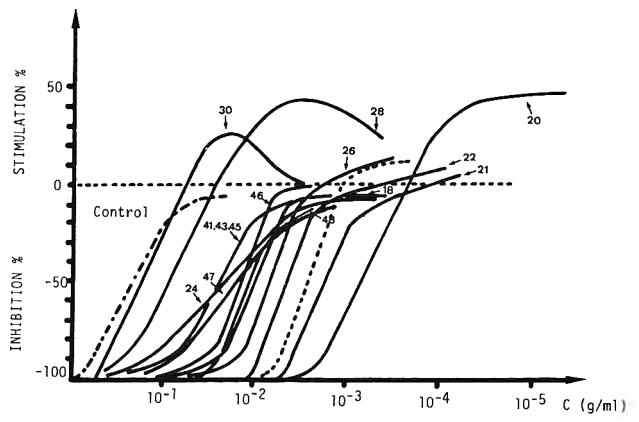

Figure 2e. Tests on lymphocytes. Naturally contaminated corn. (-----) 1 ppm T-2 toxin. See Figure 2a for conditions.

well. Cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 48 h.

Five hours before cells were harvested, 1 μCi tritiated thymidine was added to each well. Cultures were harvested with an automated sample harvester (Skatron) on glass fiber filters. The radioactivity was counted in Omnifluor (1 mL) in a liquid scintillation spectrometer.

(b) *LF cells.*—LF hepatoma cells were taken from a transplantable rat hepatoma obtained from a primary cancer induced by paradimethylaminoazobenzene. The cell line was established in the laboratory and subcultured twice a week in MEM containing 10% fetal calf serum, glutamine, and antibiotics (100 IU penicillin and 100 μg streptomycin/mL). LF cells were seeded at 20 000 cells per well in 200 μL MEM. The dilutions of extract to be tested were added in 2 μL DMSO. Cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 24 h. Tritiated thymidine was added at 1 μCi/well, 32 h before the end of culture. Just before harvesting, the cells were trypsinized for 10 min at 37°C. The results were expressed in percent variation compared to the controls which had received only 2 μL pure DMSO.

(c) *Zearalenone interference.*—To characterize the interference of zearalenone, a limited number of tests in culture were done before column purification of the extracts. The

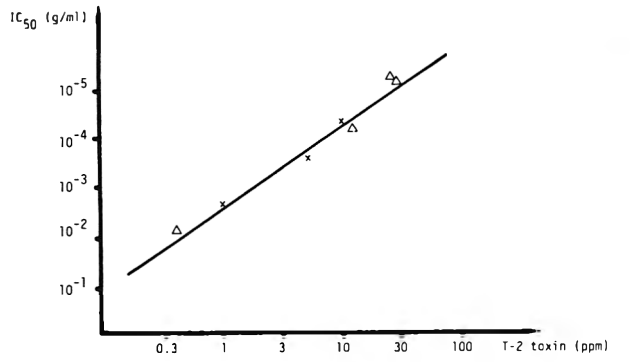

Figure 3. Correlation between IC_{50} on lymphocytes and T-2 toxin level of corn sample: × = spiked corns; △ = corns contaminated with *F. sporotrichioides* (T-2 toxin is estimated by GC).

level of zearalenone was determined by LC. The test was done on corn samples inoculated with *F. lateritium* (No. 62 and 63) and *F. graminearum* (No. 55, 56, 64, and 65). The crude extracts dissolved in small volumes of DMSO gave a sticky solution, very difficult to handle; usually the test must be performed with purified extracts.

From the results obtained, a curve could be plotted and the extract concentration that inhibits cell growth by half (IC_{50}) compared with control cells could be estimated.

Results

Effect of Cleanup

A survey of GC patterns showed that the charcoal–alumina cleanup columns proposed by Trucksess et al. (5) for TLC analysis of DON gave recoveries significantly lower than the Florisil columns used in this study. For the proposed procedures, recoveries from spiked samples were better than 85% for DON, 95% for T-2 toxin, 69% for HT-2, 98% for DAS, and about 90% for zearalenone. Figure 1 is an example of chromatographic separation.

Effect of Cell Lines

Results obtained on lymphocytes (Figures 2 and 3) and LF cells (Figure 4) can be compared. All contaminated extracts could be clearly separated from controls. Sensitivity for a given toxin frequently varied between different cell lines, and could explain the slight differences between tests on spleen lymphocytes and LF cells. For example, the corn sample No. 20 seemed much more inhibitory for lymphocytes than for LF cells (Figure 2).

Tests with Pure Toxins

Inhibitory activity of pure toxins is given in Table 1, distributed into 2 groups: T-2, HT-2, and DAS were active at concentrations of ng/mL and should be detected by the proposed cytotoxicity test; in contrast, DON and zearalenone were very less active and were hardly detected. We investigated whether combinations of several trichothecenes would have additive or synergistic effects by testing 5 toxins (T-2, HT-2, DAS, DON, and zearalenone) 2 by 2 on spleen lymphocytes (data not shown). The inhibition of lymphocytes stimulated with PHA was evaluated as previously indicated. Generally, no significant synergy was shown; in particular, no effect was shown for zearalenone. Slightly increased lymphocyte response was observed when DAS was associated with the 3 other trichothecenes at low concentration.

Table 1. IC_{50} obtained with standard toxins for murine splenic lymphocytes stimulated by PHA and for LF hepatoma cells

Toxin	Lymphocytes, ng/mL[a]	LF cells, ng/mL[a]
T-2 toxin	1.1 ± 0.24	1.5 ± 0.06[b]
HT-2 toxin	3.1 ± 0.56	3.9 ± 0.4
DAS	5.9 ± 0.8	10 ± 0.13[b]
DON	115 ± 23	200 ± 12[b]
Zearalenone	3200 ± 800	5450 ± 2000

[a] Mean value ± SD for 3 or more analyses each.
[b] According to Robbana-Barnat et al. (11).

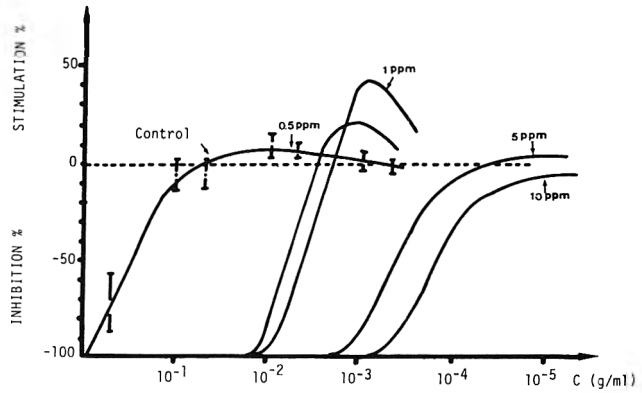

Figure 4a. Tests on LF cells. Control and T-2 toxin spiked corns. A curve is plotted from the dilution range of each extract; the percent variation compared to controls is expressed as a function of "equivalent corn" concentration in g/mL cell culture.

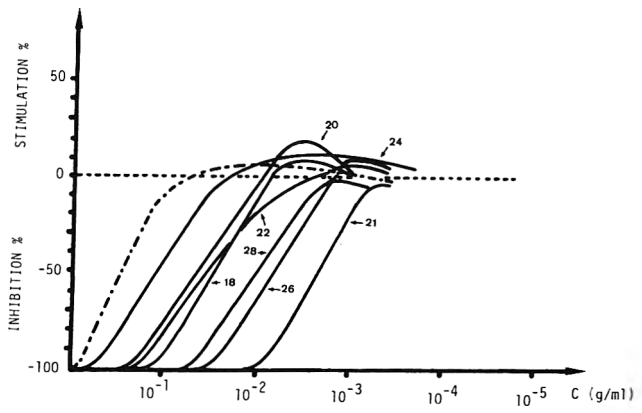

Figure 4b. Tests on LF cells. Naturally contaminated corn. (–·–·–) control. See Figure 4a for conditions.

Test Survey

We used the cell culture test to survey the following samples: corn considered sound; corn spiked with T-2 toxin; corn used as substrate for the following *Fusarium* cultures; *F. lateritium, F. graminearum, F. sporotrichioides, F. moniliforme;* and naturally contaminated corn. Results are summarized in Figure 2 and Table 2.

(a) *Sound corn (control) and corn spiked with T-2 toxin (Figure 2a).*—Control corn has a slight inhibitory action on lymphocytes in culture, but this phenomenon is regular and reproducible. The IC_{50} value is equivalent to 0.2 ± 0.05 g corn per mL cell culture. There is no effect at concentration equivalents less than 0.05 g/mL. No hypothesis can be made on the nature of inhibitory substances extracted with the polar solvents used.

Among the control corn tests, one of 10 samples tested showed a clear inhibitory effect on both types of cells. This suggests the presence of a not-yet-identified contaminant.

Corn was spiked with increasing concentrations of T-2 toxin (1, 5, and 10 ppm), added to the solvent used for

extraction. These curves were distinct from the corn controls. For example, on the 1 ppm curve, IC_{50} equaled 0.003 g corn equivalent/mL, whereas on the control curve IC_{50} equaled 0.2 g/mL, with a coefficient of 70 between the 2 values. The correlation between IC_{50} and T-2 toxin concentration is good, which enables quantitative evaluation of contamination (Figure 3).

(b) *Artificially contaminated corn.*—Pure *Fusarium* strains were inoculated in sterilized control corn with water activity adjusted to 0.98; samples were taken at 7, 14, 25, and 47 days of culture at 22°C.

DON and zearalenone producers (*F. lateritium* and *F. graminearum,* Figure 2b and 2c): LC analysis confirmed the presence of zearalenone at concentrations ranging from 0.8 to 355 ppm, and GC analysis showed quantities of DON up to 4 ppm in *F. lateritium* samples. Contaminated samples were distinct from controls but did not reach the levels of corn spiked with T-2 toxin (1 ppm). Thus, the presence of zearalenone did not interfere with the detection of trichothecenes.

Cytotoxic trichothecene producers (*F. sporotrichioides,* Figure 2d): All samples reached T-2 toxin levels up to 29 ppm. Inhibitory curves were distinct from controls, and confirmed previous observations for spiked corn. Good correlation was shown among levels of T-2 toxin determined by GC, observed IC_{50} values, and results for spiked corn (Figure 3).

(c) *Naturally contaminated corn (Figure 2e).*—Corn contaminated with low quantities of zearalenone (No. 30 = 170 ppb) shows curves near controls. Most naturally contaminated corn cannot be confused with control corn. Some extracts had an inhibitory effect (up to 1 ppm T-2 toxin) that cannot be explained by the toxins detected. Because no enhancing effects were found with pure toxins, different toxins not tested may be present. Some extracts showed significant stimulation of toxicity when they were sufficiently diluted.

Tests on LF Cells

We used the LF cell culture procedure to test sound corn, corn spiked with T-2 toxin (Figure 4a), and naturally contaminated corn (Figure 4b). Results confirmed those previously obtained with lymphocytes.

Discussion

Many biological tests concerning trichothecene detection have been proposed. The lymphocyte test was more suitable than the phytotoxic and skin tests because it could be more easily quantitated and extrapolated than, for example, the *Artemia salina* test (9).

Cells in culture that show a selective sensitivity to cytotoxic trichothecenes enable a global evaluation of contamination by toxins for which no standards are yet available and for which physicochemical detection would therefore be difficult. Lymphocyte tests were simple and easy to reproduce and codify. They exhibited great sensitivity toward cytotoxic trichothecenes and results could be obtained in less than 48 h. In our experiment, the use of an established line (LF hepatoma) gave reproducible results from one test to another. The sensitivity, although slightly lower than that observed for lymphocytes, enabled a satisfactory detection of contaminants. The incorporation of tritiated thymidine is a good marker of cytotoxicity for both cell types used. By contrast with lymphocytes, LF cells require permanent maintenance; the test on lymphocytes is therefore more convenient.

Corn.—Noncontaminated corn always contains small quantities of inhibitory substances that are difficult to char-

Table 2. Characteristics of tested corn

Corn	Period of *Fusarium* culture, days	Toxins detected by GC or LC, ppm	Lymphocyte tests, IC_{50} (g/mL)	T-2 toxin equivalent, ppm
Control corn:				
1–6, 8, 34, 39		0	0.23 ± 0.05	
17		0	$2.09.10^{-2}$	
T-2 spiked corn:				
7, 10–13		T-2: 1	$2.75.10^{-3} \pm 0.23.10^{-3}$	
15		T-2: 5	$3.31.10^{-4} \pm 0.09.10^{-4}$	
16		T-2: 10	$5.25.10^{-5} \pm 0.5.10^{-5}$	
Corn inoculated with *F. lateritium*:				
49	7	DON: 0.3	$2.51.10^{-1}$	0
50	7	DON: 0.3	$1.91.10^{-2}$	0.35
51	14	DON: 0.55	$2.88.10^{-2}$	0.27
52	14	DON: 0.6	$4.78.10^{-2}$	0.2
57	7	Z: 0.3	$6.61.10^{-2}$	0.16
58	14	Z: 11	$4.78.10^{-2}$	0.2
59, 60	25	DON: 4	$1.94.10^{-3}$	1.4
62	25	Z: 170	$1.44.10^{-2}$	0.4
63	47	Z: 355	$5.01.10^{-3}$	0.8
Corn inoculated with *F. graminearum*:				
55	7	Z: 0.8	$1.73.10^{-1}$	0
56	14	Z: 8	$1.38.10^{-1}$	0
64	25	Z: 103	$2.19.10^{-2}$	0.3
65	47	Z: 288	$4.57.10^{-3}$	0.5
Corn inoculated with *F. sporotrichioides*:				
53	7	T-2: 12	$6.31.10^{-5}$	10.5
54	14	T-2: 27	$5.12.10^{-6}$	39
61	25	T-2: 29	$8.31.10^{-6}$	35
Corn inoculated with *F. moniliforme*:				
67	25	T-2: 0.4; DON: 0.1; DAS: 0.1; Z: 1.2	$7.24.10^{-3}$	0.63
71	47	DON: 0.17	$5.01.10^{-3}$	0.8
Naturally contaminated corn:				
18		Z: 6.4 + DON: 0.5	10^{-2}	0.5
20		Z: 6.4 + DON: 0.5 + T-2, HT-2: traces	$6.92.10^{-4}$	2.6
21		Z: 41 + DON: 20	$1.82.10^{-3}$	1.4
22		Z: 3 + DON: 1	$4.79.10^{-3}$	0.8
24		Z: 3 + DON: 1	$2.17.10^{-2}$	0.3
26		Z: 41 + DON: 20	$7.24.10^{-3}$	0.6
28		Z: 1 + DON: 0.17	$9.12.10^{-2}$	0.1
30		Z: 1 + DON: 0.17	$1.82.10^{-1}$	0
32		Z: 0.15 + DON: traces	$1.40.10^{-1}$	0
41, 42		Z: 0.8 + DON: 3	$2.6.10^{-3}$	1.2
43, 45		Z: 2.8 + DON: 5	$2.3.10^{-3}$	1.2
46		Z: 0.8 + DON: 3	$1.38.10^{-2}$	0.4
47		Z: 0.8 + DON: 3	$1.38.10^{-2}$	0.4
48		Z: 2.8 + DON: 5	$1.15.10^{-2}$	0.5

acterize. Reproducibility of results from one test to another suggests natural constituents of corn. Among the 10 samples tested, 9 gave a homogeneous response; one showed a more inhibitory effect that was repeated in the 2 cell strains and is apparently due to a contaminant that is not detected through the usual methods.

Toxins.—Many toxins can be found in corn: zearalenone, DON, cytotoxic trichothecenes, etc. The use of corn spiked with T-2 toxin allowed standardization of the response and showed the repeatability of the test for quantitative analysis. Inhibition due to T-2 toxin even at low levels was apparent over that observed for sound corn. The limit of the method sensitivity is about 50–100 ppb T-2 toxin; a quantitative evaluation can be performed in a range of concentration of 0.1 to 10 ppm T-2 toxin. The test eliminates interference by zearalenone, a frequent corn contaminant that is easily detected by physicochemical methods. Corn contaminated with up to 8 ppm zearalenone does not differ from controls; even at 300 ppm, the contaminated corn shows less inhibition than 1 ppm T-2 toxin.

DON is not easily detected; its presence (4 ppm) has no major effect on cell growth curves. Although DON is a trichothecene, it exhibits a rather low toxicity; IC_{50} = about 100–150 ng/mL in both cell tests. Contaminated corn extracts do not reach such concentrations. DON is easily detected by TLC or GC analysis.

In some naturally contaminated corn or corn samples inoculated with *F. lateritium,* the unexplained toxicity (Table 2) must be due to undetected toxins or unknown synergism.

In 3 cases we observed a stimulating effect with low concentrations of contaminated corn extracts (Figure 2c). Although this effect has been observed with pure toxins (14), we suspect the presence of stimulating factors in the extract. These factors may interfere with the quantitation of results in terms of T-2 equivalents, but the result itself represents the toxic potency of the corn, as indicated by the cellular response.

Conclusion

The biological test based on the use of murine splenic lymphocytes gives results in agreement with those obtained with physicochemical methods. The cell response is regular; although not specific, it is selective enough to detect primarily contamination by cytotoxic trichothecenes. The simple test using cells in culture can process a great many samples to give results in 48 h. All samples significantly contaminated with T-2, HT-2, or DAS can be distinguished from controls; therefore, the test is useful for determining safety of agricultural products.

Acknowledgments

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Liquid Chromatographic Determination and Stability of the *Fusarium* Mycotoxin Moniliformin in Cereal Grains

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Moniliformin is a mycotoxin produced by *Fusarium subglutinans* and other *Fusarium* species. A rapid, liquid chromatographic method for its determination in corn and wheat is described. Samples are extracted in acetonitrile–water (95 + 5); following defatting with *n*-hexane, an aliquot of the extract is evaporated and cleaned up on small C_{18} and neutral alumina columns successively. Reverse-phase liquid chromatography (LC) is conducted on a C_{18} column with 10 or 15% methanol or acetonitrile in aqueous ion-pair reagent as mobile phase, with detection by ultraviolet absorption at 229 and 254 nm. Average recoveries of moniliformin (potassium salt) added to ground corn and wheat at levels of 0.05–1.0 μg/g were 80% (n = 20) and 85% (n = 12), respectively, and the limit of detection was ca 0.01–0.18 μg/g, depending on LC conditions. Analysis of 24 samples of wheat, 4 samples of rye, and 12 samples of corn showed moniliformin in only 2 corn samples (0.06 and 0.2 μg/g). Moniliformin was also detected in a sample of artificially damaged (slashed) corn (0.2 μg/g) and selected kernels of corn that were field-inoculated with *F. subglutinans* and *F. moniliforme* (50 μg/g and 0.5 μg/g, respectively). In stability studies, moniliformin (potassium salt, 1 μg/g) in ground corn and ground wheat heated at 50, 100, and 150°C for 0.5–2 h decomposed moderately, e.g., 55% remained in corn after 0.5 h at 100°C.

Moniliformin was discovered as a mycotoxin from *Fusarium moniliforme* by Cole et al. (1) and was structurally characterized as the sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione (2). The free acid had previously been chemically synthesized (3, 4). Moniliformin is now known to be produced by several other species of *Fusarium*, of which *F. moniliforme* var. *subglutinans* (*F. subglutinans*) and *F. graminearum* are, together with *F. moniliforme*, particularly important pathogens of corn and other cereal crops (5–7). Yields as high as 34 mg/g were reported in cultures of *F. moniliforme* on autoclaved corn, whereas up to 0.65 mg/g was found in field-inoculated corn ears (6). Moniliformin is acutely toxic to cockerels (1), chicks (8), and ducklings (9), with oral LD_{50} values of 3.7–5.4 mg/kg; in rats and mice the oral LD_{50} values ranged from 42 to 50 mg/kg (9, 10). Lack of mutagenicity of moniliformin in the Ames test was reported by Wehner et al. (11).

Natural occurrence of moniliformin in ears of corn visibly infected with *Fusarium* (up to 45 μg/g, mean 10.9 μg/g) and in ears of corn intended for human consumption (up to 12 μg/g, mean 2.1 μg/g) in Transkei was demonstrated by Thiel et al. (12, 13), who used ion-pair reverse-phase and ion-exchange liquid chromatography (LC) for the determinations. However, overall recovery data were not given and were stated to be low and to vary with the cleanup step (12). Thalmann et al. (14) similarly used ion-pair reverse-phase LC analysis to detect moniliformin in 25 of 58 German corn samples (24 of these contained ≤0.65 μg/g). Recently another LC method for moniliformin has been published by Shepherd and Gilbert (15). Densitometric thin-layer chromatography (TLC) has been used in 2 other methods for determining moniliformin in grains (16, 17). It was apparent that further method development for moniliformin was needed, particularly since preliminary studies in this laboratory had indicated recovery problems (18). Because the free acid of moniliformin has a very low pKa of 0.0–1.7 (19–21), it would not be expected to occur as such in grains; therefore, all work was carried out using the potassium salt. In addition to the simple method described here for determining moniliformin in corn and wheat, a limited survey of Canadian grains for moniliformin and a study of toxin stability in ground corn and wheat at different temperatures are included in this paper.

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METHOD

Moniliformin should be handled with caution and treated as a toxic substance.

Apparatus

Equipment specified is not restrictive and other suitable equipment may be substituted.

(a) *Liquid chromatographic apparatus.*—Rheodyne Model 7125 syringe-loading sample injector (20 μL loop); Altex Model 110A pump; 2.1 mm id × 6 cm guard column packed with 30–38 μm Whatman Co:Pell ODS; 4.6 mm id × 250 mm LC column packed with 5 μm Ultra Techsphere ODS; Waters Model 440 absorbance detector with 229 nm and 254 nm filters; recorder with 10 mV input, 2 mm/min chart speed.

(b) *Blender.*—Osterizer, with 400 mL Mason jar.

(c) *Filter tube.*—Glass, 4.6 cm diameter, medium-porosity glass frit.

(d) *Rotary evaporator.*—Buchi Rotavapor R.

(e) *Solid-phase extraction columns.*—Baker-10 SPE® C_{18} disposable columns, 3 mL, Cat. No. 7020-3 (J. T. Baker Chemical Co., Phillipsburg, NJ 08865); filtration rack (Analytichem Int., Harbor City, CA 90710). Use with 2.5 mL Plastipak disposable plastic syringe (Becton, Dickinson and Co., Canada, Ltd, Mississauga, Ontario).

(f) *Alumina filter column.*—1 mL BD Tuberculin syringe fitted with Millex HV_4 filter unit, 0.45 μm, 4 mm diameter (Millipore No. SJHV004NS) and packed with 0.15 g neutral alumina (100–200 mesh Bio-Rad AG 7, grade I).

(g) *Screw-cap vials.*—4 mL and 2 mL.

(h) *Filter unit.*—For solvents; Millipore with attached 1 L flask.

(i) *Syringes.*—Glass, 10, 25, and 500 μL.

Reagents

(a) *Solvents.*—Distilled-in-glass acetonitrile, hexane, and methanol.

(b) *Ion-pair solution.*—Tetrabutylammonium hydroxide, 40% (w/w) in water (Aldrich Chemical Co., Inc., Milwaukee, WI). Mix 48 mL with 100 mL 1.1M KH_2PO_4 in deionized, distilled water (final pH ca 7.3).

(c) *LC mobile phases.*—Dilute 10 mL ion-pair solution to 1 L with 10–15% (v/v) methanol in water or 10–15% (v/v) acetonitrile in water. Use deionized, distilled water filtered through 47 mm diameter Millipore type HA disc, 0.45 μm. Filter methanol through 47 mm diameter Millipore type FG disc, 0.2 μm and mobile phase containing acetonitrile through

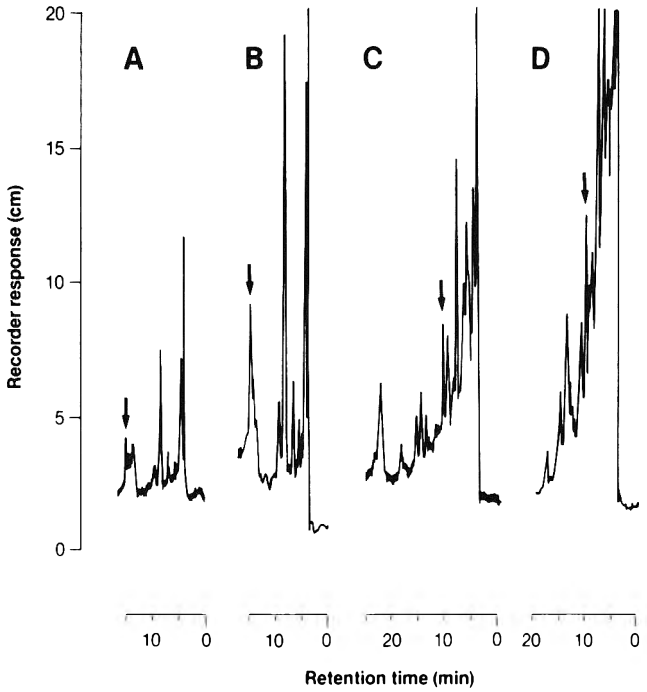

Figure 1. Chromatograms of corn sample naturally contaminated with moniliformin (arrows).
(A) 10% methanol in aqueous ion-pair reagent, 254 nm, 0.02 AUFS, 25 mg corn equiv. injected, 0.18 μg moniliformin/g estd concentration. (B) 10% methanol in aqueous ion-pair reagent, 229 nm, 0.01 AUFS, 25 mg corn equiv. injected, 0.22 μg moniliformin/g estd concentration. (C) 10% acetonitrile in aqueous ion-pair reagent, 254 nm, 0.02 AUFS, 50 mg corn equiv. injected, 0.15 μg moniliformin/g estd concentration. (D) 10% acetonitrile in aqueous ion-pair reagent, 229 nm, 0.01 AUFS, 25 mg corn equiv. injected, 0.20 μg moniliformin/g estd concentration.

type HA disc, 0.45 μm, or filter either solvent through 47 mm diameter Ultipor Nylon-66 disc, 0.20 μm (Chromatography Sciences Co., Montreal, cat. No. 120-3). Degas aqueous methanol mobile phase by stirring under reduced pressure.

(d) *Standard solutions.*—Potassium salt of moniliformin was donated by H.-D. Scharf, Rheinisch-Westfälischen Technischen Hochschule Aachen, FRG. Prepare stock solution in methanol (500 μg/mL). Dilute to 0.0625–0.25 μg/mL in mobile phase. Solutions are stable at least 1 week at 22°C. (Note: Sodium salt of moniliformin is available from Sigma Chemical Co., St. Louis, MO.)

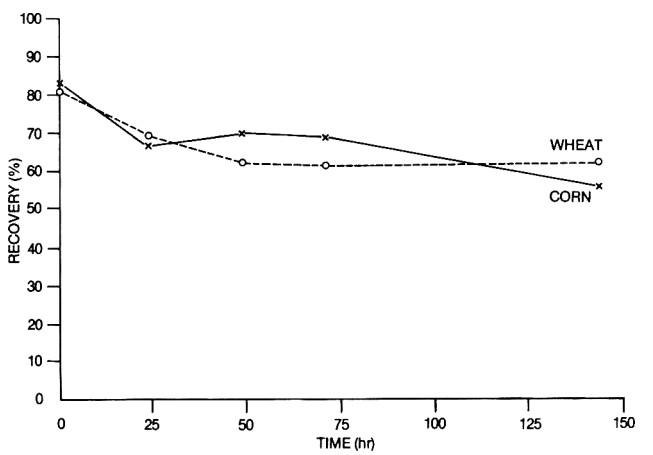

Figure 2. Stability of 0.2 μg moniliformin (potassium salt)/g in single samples of ground corn and wheat at 22°C.

Table 1. Recovery of moniliformin (potassium salt) added to ground corn and wheat

Sample	Moniliformin added, μg/g	Rec. ± SD, %
Corn	0.05	74.2 ± 4.7 (n = 4)
	0.125	70.3, 78.8 (n = 2)
	0.2	83.3 (n = 1)
	0.25	82.7 ± 9.1 (n = 6)
	0.5	81.1, 91.3 (n = 2)
	1.0	79.7 ± 7.1 (n = 5)
Wheat	0.05	72.7, 92.0 (n = 2)
	0.1	83.1 ± 13.7 (n = 5)
	0.2	81.4, 84.5 (n = 2)
	1.0	89.7 ± 15.2 (n = 3)

Extraction

Weigh 10 g ground and mixed sample into blender jar, add 100 mL acetonitrile–water (95 ± 5), and blend 5 min at "purée" setting. Filter through medium-porosity glass frit under reduced pressure. Shake filtrate (for corn only) with 150 mL n-hexane in 250 mL separatory funnel; let phases separate and drain lower layer. Discard hexane layer. Evaporate 50 mL extract to dryness under reduced pressure on rotary evaporator at ≤50°C and dissolve residue in 2.0–2.5 mL methanol.

Cleanup

Condition Baker-10 SPE C_{18} disposable column by forcing 2 mL water through column with plastic syringe (Waters C_{18} Sep-Pak may also be used in the same way). Add 200–250 μL extract solution onto top of column by syringe pressure, but do not force through column. Elute moniliformin with 2.0 mL water and collect eluate in 25 mL round-bottom flask. Evaporate to dryness using rotary evaporator at ≤50°C.

Dissolve residue in 0.5 mL LC mobile phase. Pass solution, without rinsing flask, through small alumina column (apparatus f) by syringe pressure and collect eluate in 2 mL vial.

LC Determination

Successively inject 20 μL standard solution, 20 μL each of 2 sample solutions, and again 20 μL standard solution. Use flow rate of 0.8 or 1.0 mL/min and attenuation of 0.02, 0.01, or 0.005 AUFS. Dilute sample solution if necessary with mobile phase. Compare peak height of moniliformin (retention time ca 10 min in 10% acetonitrile system) with average of the standard peak heights. Calculate moniliformin concentration as follows:

$$\text{Moniliformin, } \mu g/g = (\text{sample peak ht/std peak ht}) \times (\text{std concn, } \mu g/mL/w) \times 20$$

where w = mg equiv. sample injected (e.g., 25 mg based on initial extract volume of 2.0 mL and 250 μL added to C_{18} column). Standard curve is linear (1.25–5.0 ng moniliformin injected).

Confirm presence of moniliformin with alternative solvent system and wavelength. At end of each day, wash LC column with deionized, distilled water flowing for at least 30 min at 1 mL/min, then with methanol at the same flow rate for ca 10 min; before the first LC run of the day, wash column with deionized, distilled water for ca 15 min at 1 mL/min.

Stability Studies

Ground corn and wheat were spiked with 0.2 μg moniliformin/g and analyzed after storage at 22°C for periods of

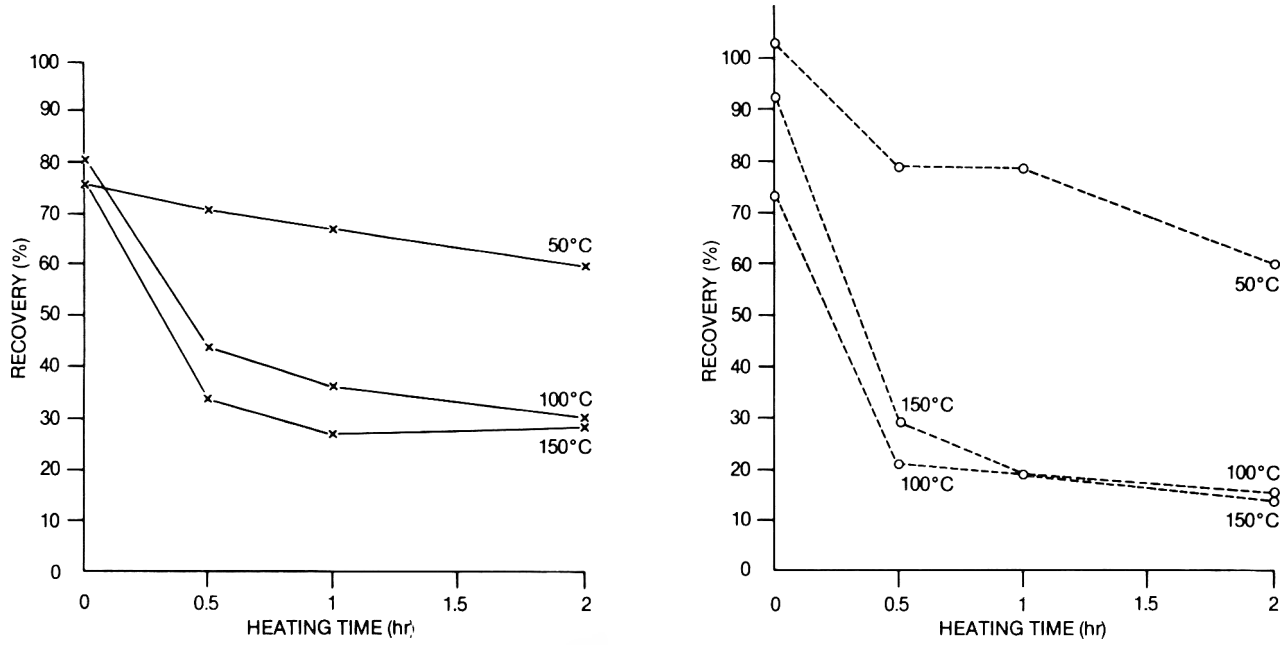

Figure 3. Stability of moniliformin (potassium salt) in ground corn (1 μg/g) (at left) and ground wheat (1 μg/g) (at right) heated at 50, 100, and 150°C. Recoveries at ≥30 min are means of duplicate samples analyzed.

up to 150 h. They were also spiked with 1 μg moniliformin/g and analyzed after being heated at 50, 100, and 150°C in a constant-temperature cabinet (Stabil. Therm., Blue M Electric Co., Blue Island, IL) for times up to 2 h.

Stability studies were also conducted on moniliformin itself heated at temperatures of up to 150°C for 15 min. As ancillary information for the analytical method, the stability of moniliformin was determined in corn and wheat extracts (≡0.25 μg moniliformin/g) kept in 10% acetonitrile LC mobile phase for 7 days at 22°C.

Mass Spectrometric Confirmation

Add 2 drops (ca 50 μL) HCl to 0.5 mL extract residue in LC mobile phase and extract with two 0.5 mL portions of methylene chloride. Evaporate solvent under N_2. Carry out negative chemical ionization (NCI) mass spectrometry (MS) using a VG 7070 EQ mass spectrometer (or equivalent) operated in the conventional sector only, with methane as reagent gas and sample introduction by direct probe heated from ambient temperature to 250°C. A prominent signal at m/z 97 provides confirmatory evidence of the presence of moniliformin. Run controls on blank corn and wheat extracts taken through the procedure described above. Use free-acid form of moniliformin for reference mass spectrum (m/z 97 = 100%, m/z 98 = 13%, and m/z 195 = 10%).

Results and Discussion

The method developed for determining moniliformin in corn and wheat is rapid and sensitive. Recoveries averaged 80% for corn and 85% for wheat, at spiking levels of 0.05 to 1.0 μg/g, respectively (Table 1). The 229 nm wavelength was preferred over 254 nm because detectability was 3 times greater. Thiel et al. (12) used 229 nm for paired ion LC of moniliformin in corn extracts. Acetonitrile was favored over methanol as the organic solvent component of the LC mobile phase because sensitivity was greater and interferences separated better (Figure 1). Detection limits of the method using a 229 nm wavelength and 10% acetonitrile in the mobile

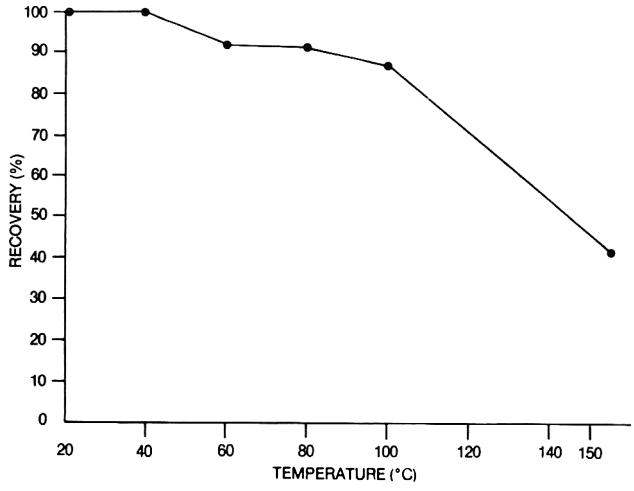

Figure 4. Stability of moniliformin (potassium salt) heated as thin film (1 μg) at various temperatures for 15 min. Recoveries are means of duplicate experiments.

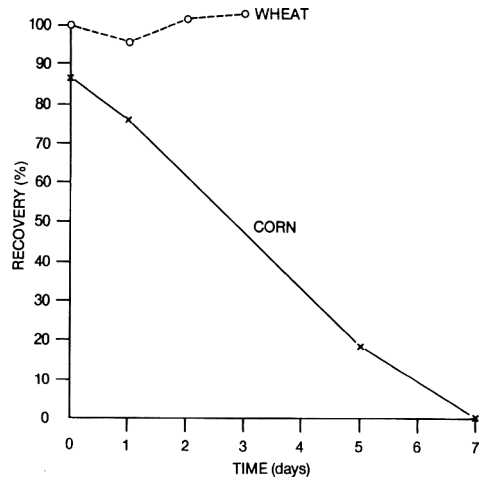

Figure 5. Stability of moniliformin (potassium salt) in extracts of corn and wheat in LC mobile phase (10% acetonitrile) at 22°C over 7 days.

phase were 0.01 μg/g for corn and 0.02 μg/g for wheat (signal-to-noise ratio, 3:1). At 254 nm, detection limits were 0.11 μg/g for corn and 0.12 μg/g for wheat. Changing the organic solvent component of the mobile phase to methanol resulted in detection limits for corn of 0.02 μg/g at 229 nm and 0.18 μg/g at 254 nm. In spite of these differences in detection limits, it is advisable to use the alternative detection wavelength and mobile phase for confirmatory purposes.

The proposed method was used to analyze 20 Canadian soft wheat samples from Ontario and Nova Scotia, 4 western Canadian hard wheat samples, 4 Canadian rye samples, and 12 Ontario corn samples for moniliformin. Ten of the wheat samples and all 12 of the corn samples had previously been shown to contain vomitoxin and thus can reasonably be assumed to have been contaminated with *F. graminearum* in the field. Moniliformin was found in only 2 corn samples. One was contaminated with about 0.2 μg moniliformin/g and the good agreement for the 4 LC measurement conditions is illustrated in Figure 1. A second sample contained 0.05–0.06 μg/g as determined at 229 nm. Moniliformin was confirmed in the first sample extract by NCI mass spectrometry, showing a prominent signal at m/z 97 (twice background intensity); however, 0.2 μg/g is close to the limit for confirmation of moniliformin in corn by this technique. In addition to the survey samples, 3 other corn samples were analyzed for moniliformin by LC: 0.23–0.52 μg/g was found in an artificially damaged (slashed) corn sample from an experimental plot, 50–54 μg/g was found in hand-selected kernels of corn that had been inoculated in the field with *F. subglutinans*, and 0.49–0.69 μg/g was detected in selected kernels of corn field inoculated with *F. moniliforme*.

The potassium salt of moniliformin was moderately stable in ground corn and wheat kept at room temperature. After 6 days, about 68–77% remained after correction for method recovery (Figure 2). However, heating at 50°C produced similar losses of moniliformin after only 2 h, and at higher temperatures (100 and 150°C) only 38% (corrected) remained in the ground corn and 15–22% (corrected) in the wheat after 2 h (Figure 3). The potassium salt of moniliformin was itself unstable when heated at 150°C (Figure 4).

Experiments to determine the stability of moniliformin in the LC mobile phase in the presence of grain extracts showed that the wheat extract had no effect on moniliformin; however, it would not be advisable to keep moniliformin-containing corn extracts in mobile phase beyond 1 day (Figure 5). It should also be pointed out that the free acid of moniliformin must not be used as a standard because of its instability in both methanol and water (18).

Acknowledgments

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Improved Enzyme-Linked Immunosorbent Assay for Aflatoxin B_1 in Agricultural Commodities

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An improved enzyme-linked immunosorbent assay (ELISA) for aflatoxin B_1 in cornmeal and peanut butter was developed. Aflatoxin B_1 in cornmeal and peanut butter samples was extracted with 70% methanol in water containing 1% dimethylformamide diluted with assay buffer to a final concentration of 7.0% methanol, and directly subjected to an ELISA procedure that took less than 1 h for quantitative analysis and less than 30 min for screening tests. Analytical recoveries for 5–100 ppb B_1 added to the cornmeal and peanut butter were 91 and 95.4%, respectively. The interwell and interassay coefficient of variation was 10% or less at the 20 ppb level and above. Agreement for B_1 levels in more than 30 naturally contaminated corn, mixed feed, and peanut butter samples was excellent between the ELISA data and the data obtained from different independent laboratories using TLC or other analytical methods.

Aflatoxins are a group of toxic secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* (1). Aflatoxin B_1, the most toxic compound in this series, has been found to be one of the most potent carcinogens occurring naturally (1). Because of frequent contamination of B_1 in agricultural commodities such as peanuts, corn, and animal feedstuffs, aflatoxin problems become a potential hazard to human and animal health (1). To control the aflatoxin problem, considerable efforts have been made to develop a simple, sensitive, and specific method for the analysis of aflatoxin in different commodities (2–4). However, because of interfering substances in the sample matrixes, most of the analytical methods need a cleanup step before actual analysis (2–4). Thus, different commodities need a different procedure (2–4). Recent investigations in our laboratory (5–11) and others (12–14) have led to a simple, rapid, and specific enzyme-linked immunosorbent assay (ELISA) for aflatoxins B_1 and M_1 in different commodities. Detection limits for B_1 in corn and peanuts were around 3–5 ppb (10, 11, 14, 15). Aflatoxin after extraction from samples without column chromatography cleanup was directly subjected to ELISA. Milk samples without any treatment were also directly used in ELISA (16). The detection limit of ELISA for M_1 without cleanup was 0.25–0.5 ppb (16) and with cleanup was between 0.01 and 0.05 ppb (17, 18). Although ELISA is sensitive and simple, the assay is still prone to some interference; thus, the coefficients of variation in some ELISAs were still relatively high (5–7). In addition, the overall time for ELISA was also long, taking approximately 2 h to finish the assay after the sample was extracted (10, 11, 14, 15). In view of these problems, efforts to optimize the ELISA conditions and to improve the ELISA protocol were made in our laboratories. We have shortened the assay time to less than 1 h by optimizing the hyperimmune serum coating and incorporating a more sensitive substrate. The improved protocol has been tested for the analysis of corn and peanut butter spiked with a known amount of B_1 as well as for a number of naturally contaminated samples. Details of the improved protocol as well as results obtained from such analyses are presented in this paper.

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Experimental

Apparatus

(a) *Blender.*—Explosion-proof Waring blendor or tissue homogenizer.

(b) *ELISA washer.*—Automatic ELISA washer, such as Dynatech (Alexandria, VA) Model B Miniwasher, or equivalent.

(c) *ELISA reader.*—Dynatech MR 600, or equivalent.

(d) *ELISA plate.*—Dynatech Immunlon I, or equivalent.

(e) *Dispenser.*—Oyster Bay, NY.

(f) *Automatic pipet.*—Titertek (Flow Laboratories, McLean, VA) 8 channel automatic pipet (50–200 μL) and single-channel automatic pipet such as a Gilson automatic pipet (Rainin Instrument Co., Woburn, MA) (50 and 100 μL sizes).

Reagents

(a) *Phosphate-buffered saline (PBS).*—0.01 mol/L sodium phosphate buffer containing 0.15 mol/L of NaCl, pH 7.4.

(b) *Carbonate–bicarbonate buffer.*—0.05 mol/L, pH 9.6.

(c) *Tween–PBS.*—0.05% (v/v) Tween 20 in PBS.

(d) *BSA–PBS solution.*—0.05%. Dissolve 0.05 g bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) in 100 mL 0.01M PBS (pH 7.4).

(e) *Sample extraction solvent (SES).*—Similar to that of Whitaker and Dickens (19) as modified by Ram et al. (14). Mix 70 mL methanol with 29 mL water and 1 mL dimethylformamide (DMF).

(f) *Sample and standard dilution buffer (SDB).*—Mix 7 mL methanol with 92 mL PBS and 1 mL DMF.

(g) *Substrate buffer.*—0.05 mol/L of citric acid plus 0.1 mol/L of Na_2HPO_4, pH 5.0.

(h) *Enzyme substrate solution (ESS).*—Dissolve 40 mg *o*-phenylenediamine and 0.04 mL 30% H_2O_2 in 100 mL substrate buffer. Make fresh daily and use within 60 min after preparation.

(i) *Stopping solution (SS).*—0.1N HCl.

(j) *Stock solutions.*—(*Note*: Because aflatoxins are sensitive to light, all the standards should be kept in the dark.) Prepare B_1 stock solution according to AOAC secs **26.006**–**26.011** (2). Store in benzene–acetonitrile (9.8 + 0.2, v/v) at a concentration of 1 μg/mL in the dark. To prepare primary working stock solution, withdraw 0.2 mL stock solution and dilute to 1 mL with methanol (200 ng/mL). Store methanolic solution in the dark and use within 1 week.

(k) *Working solution.*—Prepare fresh working solution daily. Withdraw 0.5 mL primary working stock solution and dilute to 2 mL with dilution buffer (SDB), which gives a concentration of 50 ng/mL, and then make a series of dilutions with dilution buffer to give final concentrations of 0, 0.1, 0.25, 0.5, 1, 2.5, and 5 ng/mL.

(l) *Antisera and antisera coating.*—Produce antisera against B_1 in rabbits according to procedure described by Chu and Ueno (8). Hyperimmune serum is obtained 1 year after initial immunization and booster injections with car-

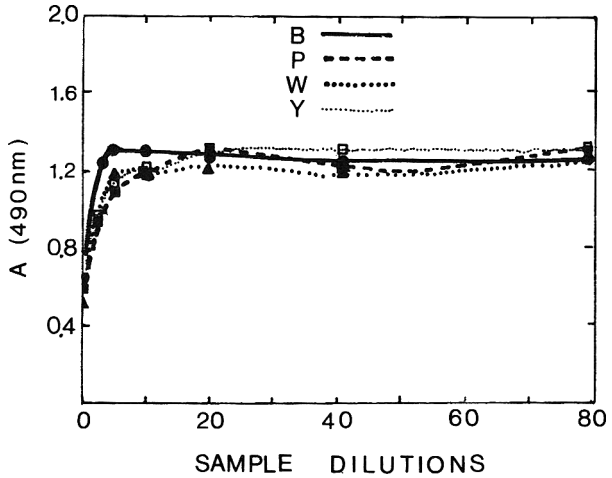

Figure 1. Effect of sample matrix on ELISA of B_1. Samples (50 g) were extracted with 250 mL extraction solvent, diluted in PBS, and subjected to ELISA directly. B, buffer control; P, peanut extract; W, white cornmeal extract; and Y, yellow cornmeal extract. Numbers in *x*-axis represent times of dilution. Thus, at 1:20 dilution, sample extract contained 10 mg sample in each assay.

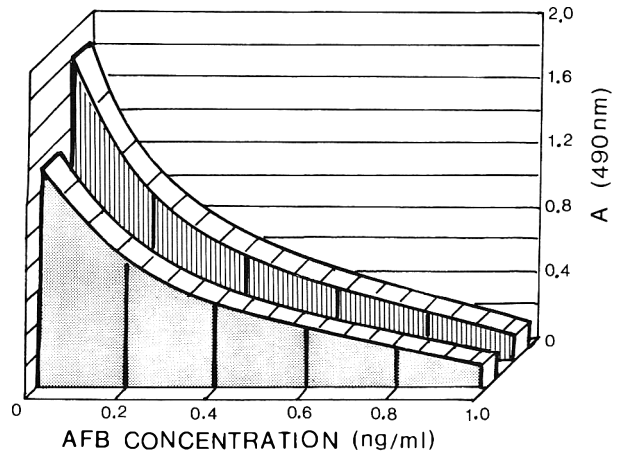

Figure 2. Effect of incubation time on ELISA of B_1. Top curve (back) represents incubation of standard solutions with aflatoxin B_1–horseradish peroxidase in wells for 30 min followed by 10 min of substrate incubation (30/10 system). Lower curve (front) represents 10 min incubation time for both steps (10/10 system). AFB = aflatoxin B_1.

boxymethyl-oxime (CMO) B_1–BSA conjugate (20). Determine optimal dilutions by titration against B_1–horseradish peroxidase (POD) conjugate (9). Coat each well of ELISA plate with 100 μL diluted antisera (1:5000 dilutions in carbonate buffer) using method described by Saunders (21). Use Oyster Bay dispenser in the coating. Coated plates are ready to use the day after coating and can be stored at 4°C for at least 6 months.

(m) *Aflatoxin B_1–POD conjugates.*—Prepare aflatoxin B_1 peroxidase by conjugating CMO–B_1 to POD enzyme by *N*-hydroxysuccinimide (Sigma Chemical Co., St. Louis, MO) method of Kitagawa et al. (22, 23). Activate CMO–B_1 with *N*-hydroxysuccinimide in presence of 1,3-dicyclohexylcarbodiimide (Aldrich Chemical Co., Milwaukee, WI) and then react directly with enzyme.

Sample Preparations

Extract aflatoxin from sample directly by homogenizing sample with extraction solvent. In recovery study, B_1 is added to samples one day before the experiment and then subjected to the above procedures.

Weigh 20 g ground sample into blender jar and add 100 mL SES. Blend 2–3 min at high speed, let settle for several minutes, and filter upper phase through glass wool-packed funnel or centrifuge for 5 min at 2000 rpm. Dilute small portions of filtered extracts (1:20 or higher dilution) with dilution buffer (SDB). Sample is now ready for ELISA.

ELISA Protocol

Add 50 μl aflatoxin B_1 standard or diluted sample and 50 μL B_1–POD conjugate to each well, incubate at room temperature (15–30°C) 30 min. (Assay can be performed with 10 min incubation time.) Wash 3–5 times by adding 300 μL portions of water to each well and then aspirating contents. Add 100 μL substrate to wells, incubate 10 min at room temperature then add 100 μL stopping reagent. Read color at 490 nm. Run samples in triplicate.

Calculations

Calculate mean *A* (490 nm) for each standard and sample. Calculate B/B_0 for each standard and sample:

$$B/B_0 = [\text{mean } A \text{ (490 nm) standard or sample}]/[\text{mean } A \text{ (490 nm) for blank (0 ppb) standard}]$$

Calculate logit B/B_0. Plot standard curve on semilogarithmic graph paper, placing values of standard on *x*-axis, and corresponding logit B/B_0 values on *y*-axis. Read B_1 concentration in samples directly from curve (in ppb, or ng/assay) found in original samples.

Results

Effect of Sample Matrix on the ELISA

The effect of sample matrix on the improved direct ELISA was studied by incubation of different dilutions of sample extracts (peanut butter, white cornmeal, and yellow cornmeal) with the aflatoxin B_1–horseradish peroxidase in the absence of B_1 and followed by regular ELISA protocols. Results of such studies are presented in Figure 1. It is apparent that at sample dilution of 1:20—i.e., 10 mg sample/mL assay buffer—absorbances were comparable to those obtained from the buffer alone.

Standard Curve for AFB

The effect of sample incubation time on ELISA standard curves is shown in Figure 2. Standard curves established with a shorter incubation time (10/10 system, i.e., 10 min sample incubation time and 10 min substrate incubation time) gave lower absorbance readings compared to ones obtained with the longer incubation (30/10 system). A typical standard curve for quantitative analysis of B_1 by ELISA as plotted according to the method described is presented in Figure 3. Under the experimental conditions, the value for the blank standard (B_0) was generally about 1.6–1.8 and 0.8–1.2 for the 30/10 and 10/10 systems, respectively. Since the blank readings were considered in the calculations, no difference between the standard curve is established by the 30/10 and the 10/10 systems. However, shorter incubation time generally gave a higher standard deviation. Thus, it can only be recommended for a screening test. In the 30/10 system, the interassay coefficient of variation (CV) for the standards in the range of 0.05 to 1.0 ng/mL was 3.5–13.5% (av. 9%).

Analytical Recovery of Aflatoxin B_1 Added to Cornmeal and Peanut Butter Samples

Recovery of B_1 added to cornmeal and peanut butter was between 5 and 100 ppb as shown in Table 1. For each sample, 5 to 9 trials were made at different concentrations. Overall

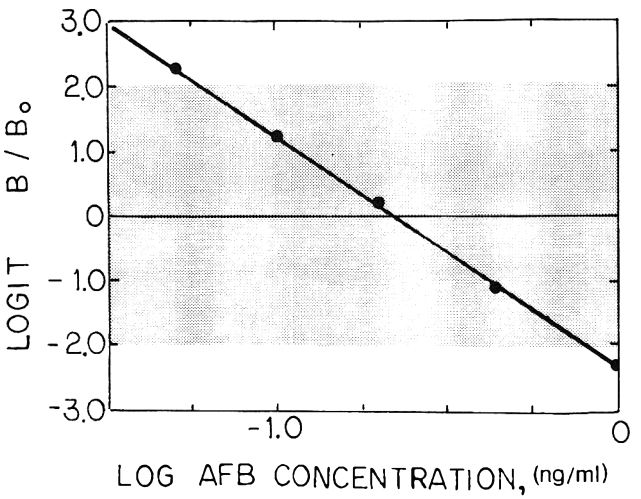

Figure 3. Standard curve for ELISA of B_1. Standard deviations were smaller than points indicated in curve; best range for assay is shown in shaded area. AFB = aflatoxin B_1.

recoveries for all concentrations tested for B_1 added to cornmeal and peanut butter were 91 and 95.4%, respectively. At concentrations of 20 ppb or above, the interassay CV was below 10%. Higher CV was observed at concentrations below 10 ppb. However, when samples were subjected to a Sep-Pak treatment, the CV at the 5 ppb level was about 10%.

Analysis of Naturally Contaminated Samples

To test the effectiveness of the present improved ELISA protocol, a large number of naturally contaminated samples that had been analyzed for aflatoxins by other laboratories were subjected to analysis. In the first experiment, about 30 corn samples, including both white and yellow corn which had been analyzed by a TLC method similar to the AOAC BF approach (24), were subjected to the ELISA analysis. Results of this study are shown in Figure 4. A correlation coefficient of 0.91 between TLC and ELISA data was obtained. In this study, the TLC data were about 11% higher than the ELISA data. Results for analysis of B_1 in a variety of naturally contaminated commodities are presented in Table 2. In those studies the variation between trials (interassay) over a period of 10 days and the variation between wells (interwell) in a single analysis for some samples were determined. In general, the interwell CV was 6–10%, whereas for interassay it was 2–19%. Data obtained from ELISA are generally slightly lower than those obtained by TLC. At concentrations below 20 ppb, a larger difference between the data obtained by TLC and ELISA methods was observed. In the 1985 WHO collaborative studies, the mean B_1 concentrations in yellow corn by CB, BF, European Economic Community (EEC), and liquid chromatographic (LC) methods were 66.54, 51.06, 82.18, and 73.10, respectively. The mean B_1 concentrations in peanut samples by CB, BF, EEC, and liquid chromatography were 72.49, 56.21, 93.38, and 85.42, respectively.

Discussion

Although previous investigations in our laboratory and others have led to a simple, specific, and sensitive ELISA method for B_1 in corn, peanuts, and other commodities as well as an ELISA method for M_1 in different dairy products (5–7), the assay is still prone to interference by the sample matrixes (5–7) and to a high CV, i.e., 20–30% (10, 14, 15). To overcome the interference problem, Ram et al. (14, 15) incorporated "toxin-free sample matrix extracts" or "high concentration of extraction solvents" in the preparation of the standard curve. However, such approaches often result in a loss of sensitivity and an increase in errors (14, 15). In the present study, another approach to resolve the problem was undertaken. We found that as long as the ELISA system provides good sensitivity, it is not necessary to incorporate the solvent extract of the sample matrix in the ELISA system. Consequently, we devoted our efforts to optimizing the ELISA protocol and to improving the overall sensitivity.

Table 1. Recovery of aflatoxin B. added to cornmeal and peanut butter, using ELISA

Added, ppb	Cornmeal		Peanut butter	
	ppb (%)	SD (CV)[a]	ppb (%)	SD (CV)
5	4.29 (85.8)	0.82 (19.1)	5.28 (105.6)	1.75 (33.1)
5[b]	4.75 (95.0)	0.42 (8.90)	5.09 (102.0)	0.54 (10.5)
10	9.43 (94.3)	1.91 (20.2)	9.28 (92.8)	1.90 (20.5)
20	17.92 (89.6)	0.86 (4.80)	18.33 (91.7)	0.30 (1.6)
50	46.97 (93.4)	4.33 (9.20)	47.95 (95.9)	3.47 (4.1)
100	91.92 (91.92)	3.75 (3.75)	91.80 (91.0)	7.73 (8.4)

[a] SD, standard deviation; CV, coefficient of variation.

[b] All samples in this row were subjected to Sep-Pak treatment before ELISA. In this study, 10 mL sample extract obtained from the second sample preparation step was mixed with 40 mL distilled water and passed through a prewashed C_{18} reverse-phase Sep-Pak cartridge (Waters Associates) according to the method described by Hu et al. (17). After cartridge was washed with 25 mL 25% aqueous methanol, B, was eluted with 10 mL 30% methanol in water which was then diluted for ELISA.

The improved ELISA protocol described herein is considerably more sensitive than the method previously reported by our laboratory (10) and others (14, 15); as little as 5 pg B_1 can readily be determined in each assay. Thus, after a simple extraction, samples can be used directly in ELISA. Practically no interference was observed at a sample concentration of 10 mg/mL in each assay (Figure 1). Because of the high sensitivity and possible matrix interference effect, a 1:20 dilution of sample was necessary in each assay. Consequently, less sample matrix was introduced into the assay system and, subsequently, less interference. This is one of

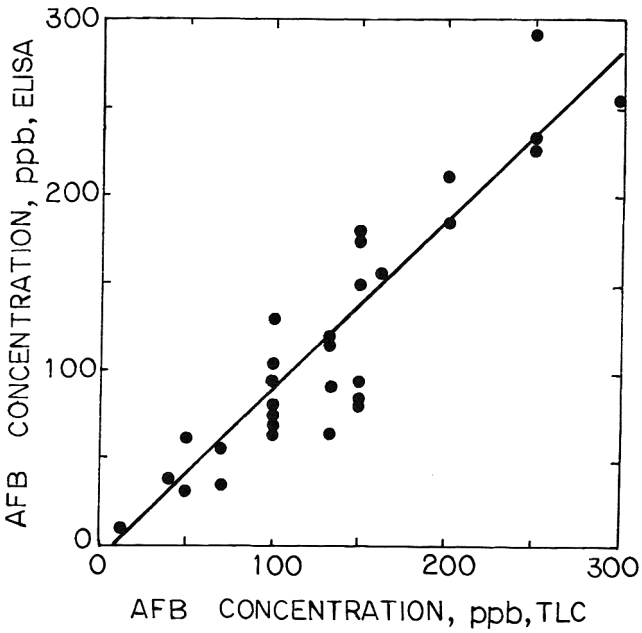

Figure 4. Correlation of ELISA with TLC data for analysis of B_1 in naturally contaminated corn samples. Linear regression equation of $Y = -9.8 + 0.99X$ with correlation coefficient of 0.91 was obtained. X and Y represent B_1 concentrations (μg/kg or ppb) as determined independently by TLC and ELISA in 2 laboratories. AFB = aflatoxin B_1.

Table 2. Comparison of recoveries of aflatoxin B_1 from naturally contaminated samples by ELISA and by other methods

Sample[a]	Recoveries by ELISA[b] ppb	SD	CV-A, %	CV-B, %	TLC or other methods, ppb
Corn 428	72.5	6.41	8.8 (6)[c]	7.2 (15)	83 (114.5)[d]
Corn 429	18.7	3.47	18.6 (5)	ND	13 (69.5)
Corn 430	11.5	0.57	4.9 (5)	ND	22 (191.3)
MF 325	44.0	4.86	11.0 (6)	8.3 (16)	52 (118.2)
MF 327	45.6	3.21	7.0 (5)	10.0 (20)	49 (107.5)
MF 328	146.0	2.83	1.9 (2)	5.5 (20)	158 (108.2)
MF 364	16.8	4.09	24.4 (4)	8.0 (11)	12 (71.4)
WHO yellow corn[e]	68.5	8.80	13.0 (2)	ND	64.5 (94.0)
WHO peanuts	62.2	11.0	17.0 (2)	ND	75.2 (114.0)

[a] Samples supplied and analyzed by FDA except as otherwise specified; MF = mixed feeds.

[b] SD, standard deviation; CV-A, coefficient of variation between separate trials on different days within a 10-day period (interassay); CV-B, coefficient of variation within a single plate (interwell); ND, not determined.

[c] Values in parentheses are number of determinations.

[d] Values in parentheses are percent of ELISA results.

[e] 1985 WHO collaborative study samples; analytical data for the WHO samples were the overall means of all the methods obtained from different laboratories.

the major advantages of the current method over the previous protocol which is still prone to considerable sample matrix interferences (10, 14, 15). The analytical time was also shortened to less than 1 h compared to the previously published methods which require about 2 h for the whole assay. The improved method can also be adapted for a screening test which requires less than 30 min for each assay (10/10 system).

The effectiveness of the improved ELISA protocol was tested both by analytical recovery studies and by analysis of naturally contaminated samples. Good analytical recovery was obtained in all the tested ranges for B_1 in corn and peanut butter. The CVs for the recovery experiments were low. Although a higher CV (20–30%) at levels below 10 ppb was found, a simple treatment of the extracted sample on a C-18 reverse-phase Sep-Pak cartridge improved the CV to about 10%. Therefore, if one needs very accurate data below 10 ppb, the Sep-Pak treatment step can be incorporated in the protocol. It is unnecessary to use the Sep-Pak treatment in the general assay. The ELISA data for the B_1 levels in naturally contaminated samples and the data obtained by other methods that were analyzed independently by 2 other laboratories were in good agreement. In the 1985 WHO collaborative studies, excellent agreement between ELISA and other methods was observed also. Because these analyses were carried out by different laboratories with different methods, present results indicate that the improved ELISA is a valid method for B_1 analysis. Data obtained from ELISA appear to be more accurate than those obtained by the TLC method. For example, it is apparent from Figure 4 that whereas TLC can only approximate the concentration range, ELISA can determine the B_1 level very accurately in a typical sample. Thus, it was common that B_1 levels in different samples were found to be identical by the TLC method, whereas ELISA provided a different concentration for each individual sample. Our results also show good reproducibility for ELISA as evident from the low interwell and interassay CVs for the standard and the naturally contaminated samples. This improvement was primarily due to the coating technique in which a mechanical device was used instead of the previously used manual method (9). In conclusion, the improved ELISA is a sensitive, simple, and rapid method and could be adopted for quantitative analysis of aflatoxin B_1 in a variety of commodities.

Acknowledgments

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Pesticide Confirmation by Triple Stage Quadrupole Mass Spectrometry: Etrimfos and Dimethoate

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The production of a protonated molecular ion. MH^+, for a pesticide under investigation is often preferred for primary identification purposes. However, the lack of fragment ions under such chemical ionization conditions can place the burden of proof on a single ion species for confirmation. Although a single ion representing the molecule at the correct retention time on a packed column or high-resolution capillary column might seem to have furnished sufficient evidence for unambiguous confirmation, in a number of cases additional specificity can improve the analytical result. With the introduction into commerce of triple stage quadrupole (TSQ) instruments, the possibility of improving the degree of specificity by chemical ionization has emerged for practical pesticide residue confirmation analysis. Use of collision-activated dissociation (CAD) experiments to form daughter ions from the protonated molecular ion of 2 representative organophosphorus pesticides has provided a second dimensional plane of characterization for confirmatory purposes at incurred residue levels.

During routine regulatory analysis for pesticides and industrial chemical residues, it is often necessary to confirm the presence of a particular contaminant by mass spectrometry before regulatory action can be initiated. The primary method of analysis is usually gas chromatography (GC) on various stationary phases (nonpolar, semipolar, and polar) and with various detectors (phosphorus, sulfur, nitrogen, halogen, etc.); added confirmation by mass spectrometry (MS) is normally required to provide unambiguous proof of the violation. In many of these cases, the concentration level is extremely low (< 1 ppm), and multiple ion detection (MID) techniques must be used to achieve the necessary level of sensitivity by mass spectrometry as well as to disregard selectively any endogenous materials in the sample matrix. The use of chemical ionization (CI) techniques to favor production of a molecular ion species for characterization purposes has also been a cornerstone for resolving such analytical problems.

To assist in identifying residues and "unknown analytical responses" (UARs), a comprehensive listing of pesticides and industrial chemicals has been organized according to molecular weight (1). Such a listing—containing all the possible compounds that might be encountered in regulatory analysis (both registered and unregistered) together with their respective spectra (both electron impact [EI] and chemical ionization), relative retention data on 3 columns, molecular formulas, and source reference data—has already proven to be a valuable asset (2). This database and its indexes were established to improve the success rate of identifying UARs, because cross-correlating GC retention data was time consuming and relied heavily on the exact recreation of the standard conditions under which the original data for comparison were formulated.

In essence, the present approach to structural elucidation of UARs, especially when low-resolution instruments are used, is to attempt to determine the molecular weight of the compound under investigation by using CI techniques (methane and ammonia). With knowledge of the molecular weight, the possibilities can be narrowed by searching the compiled database. If the compound is on the list, then absolute confirmation can be provided by recording the standard reference material available from the U.S. Environmental Protection Agency. This process is speedy and cost effective. If, however, the UAR does not correspond to an already-listed compound, a number of possibilities have been instantly eliminated, but nevertheless identification could require extended study via additional observed fragmentation pathways.

Supporting evidence from GC analysis using specific detectors to suggest that the compound contains phosphorus, nitrogen, sulfur, or halogen (i.e., potential for toxicity) should not be overlooked in the overall process of structural elucidation and priority attention. Indeed, GC detector data can be used to indicate potential toxicants as well as to supplement low-resolution measurements. With the combined knowledge of partial elemental composition, molecular weight determined by chemical ionization, and fragmentation pattern determined by electron impact or sometimes chemical ionization, UARs can quite often be identified without the use of more sophisticated techniques such as high-resolution measurements. In some cases, however, high-resolution measurements are mandatory to convert proposed fragmentation pathways into verified pathways.

In practical terms, the production of a protonated molecular ion, MH^+, for the pesticide under investigation is preferred for primary identification purposes because background ions from potential interferences are greatly reduced. The lack of fragment ions under such CI conditions, however, can place the entire burden of proof for confirmation on a single ion species. Although a single ion representing the molecule at the correct retention time on a packed column or high-resolution capillary column might seem to have furnished sufficient evidence for unambiguous confirmation, in a number of cases additional specificity is needed. Undoubtedly, the greater number of ions usually provided by EI detection can often be considered as definitive proof of the presence of a particular compound. However, with real residue extracts, whether cleaned up or not, the interpretation of the EI data obtained at the low-ppm level can become complicated. In many cases, the EI fragment ions represent several bond cleavages, thus specificity might be obscured, because generic ions would have to be used for confirmation (i.e., successive loss of CH_2 characteristic of hydrocarbons). The use of CI techniques has avoided some of these analytical difficulties.

With the introduction into commerce in 1981 of triple-stage quadrupole (TSQ) instruments, the possibility of improving the degree of specificity by chemical ionization emerged for practical pesticide residue confirmation analysis at low levels. Use of collision-activated dissociation (CAD)

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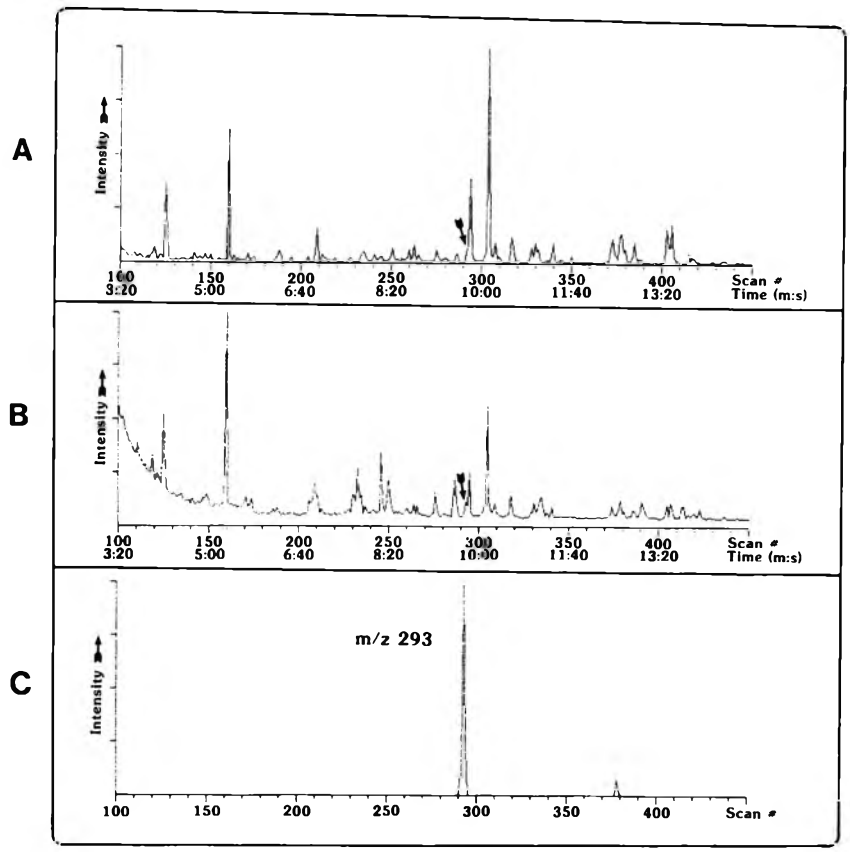

Figure 1. Mass spectral data acquired on bean extract: A, total ion current chromatogram under EI conditions; B, total ion current under methane CI conditions; C, mass chromatogram for m/z 293 performed on CI data.

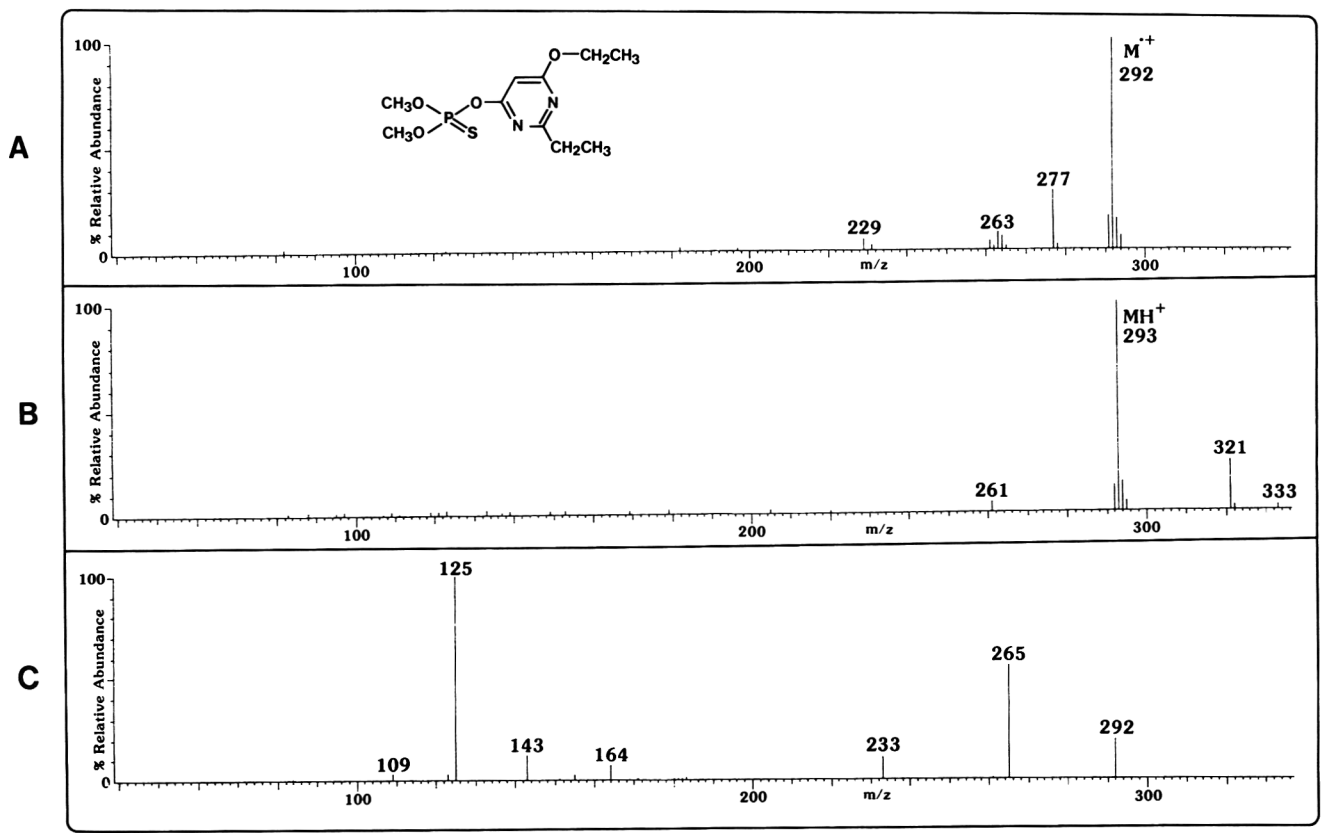

Figure 2. Mass spectral data for etrimfos: A, EI mass spectrum; B, CI mass spectrum; C, CAD daughter spectrum on m/z 293 obtained under CI conditions.

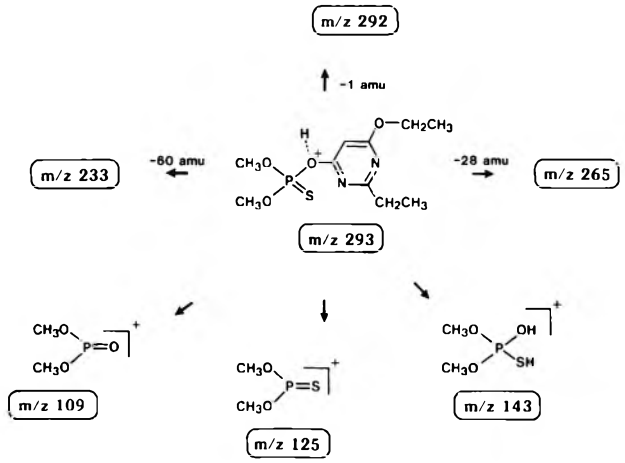

Figure 3. Proposed daughter fragmentation pathway of the protonated molecular ion at m/z 293 for etrimfos using CAD with argon as the collision gas.

experiments to enhance the formation of daughter ions from the protonated molecular ion has now provided a new dimensional plane of characterization.

This paper describes a recent incidence in an extract of beans of an organophosphorus pesticide UAR that was later identified as etrimfos (*O,O*-dimethyl-*O*-6-ethoxy-2-ethylpyrimidin-4-yl phosphorothioate) and was confirmed by CAD experiments. These experiments have now resulted in a detection protocol which uses generic daughter ions for identification of phosphorothionates and phosphorodithionates at low-ppm levels in produce extracts.

Experimental

Reagents

All chemicals were reagent grade except as indicated. Methane (99.9%) was purchased from Matheson Gas Products, Cucamonga, CA.

Mass Spectrometry

Mass spectra were recorded on a Finnigan Model 45A triple-stage quadrupole mass spectrometer equipped with a chemical ionization source and Incos data system. Operating conditions for residue samples were as follows: 30 m DB-5 capillary column programmed at 20°/min from 50° to 200°C and at 5/min from 200° to 250°C. Typical instrument parameters used were: extractor, −7 V; lens, −150 V; electron energy, 100 V; quad entrance, −27 V; source pressure, 300 mtorr; electron multiplier, −1050 V; source 170°C. Collision-activated dissociation studies were conducted using argon as the collision gas at a pressure of 1 mtorr with the collision energy voltage set between −14 and −29 V.

Results and Discussion

In the multiresidue procedure (3) adopted for routine use in this laboratory in the pesticide monitoring program on fruits and vegetables, a Hall electrolytic conductivity detector is used for organohalogen, organonitrogen, and organosulfur pesticides and a flame photometric detector (FPD-P) is used for organophosphorus pesticides. These detectors are now routinely sensitive in the sub-nanogram range. In the absence of a sample cleanup step, background peaks are kept to a minimum through the use of these element-sensitive detectors.

In the incident reported here, the unknown compound of interest had a retention time relative to chlorpyrifos of 0.56, 0.53, and 0.64 on OV-101, OV-225, and DEGS columns, respectively (4). With these relative retention data, a translation can be made to gas chromatography/mass spectrometry (GC/MS) from FPD-P analysis. The wealth of data usually gathered by a typical total ion current (TIC) chromatogram, as compared to that gathered by the highly specific GC detector, can give an entirely different elution profile. This dramatic change in elution profile (Figure 1) can cause some problems. First, the transition from a packed column used isothermally in gas chromatography to a cap-

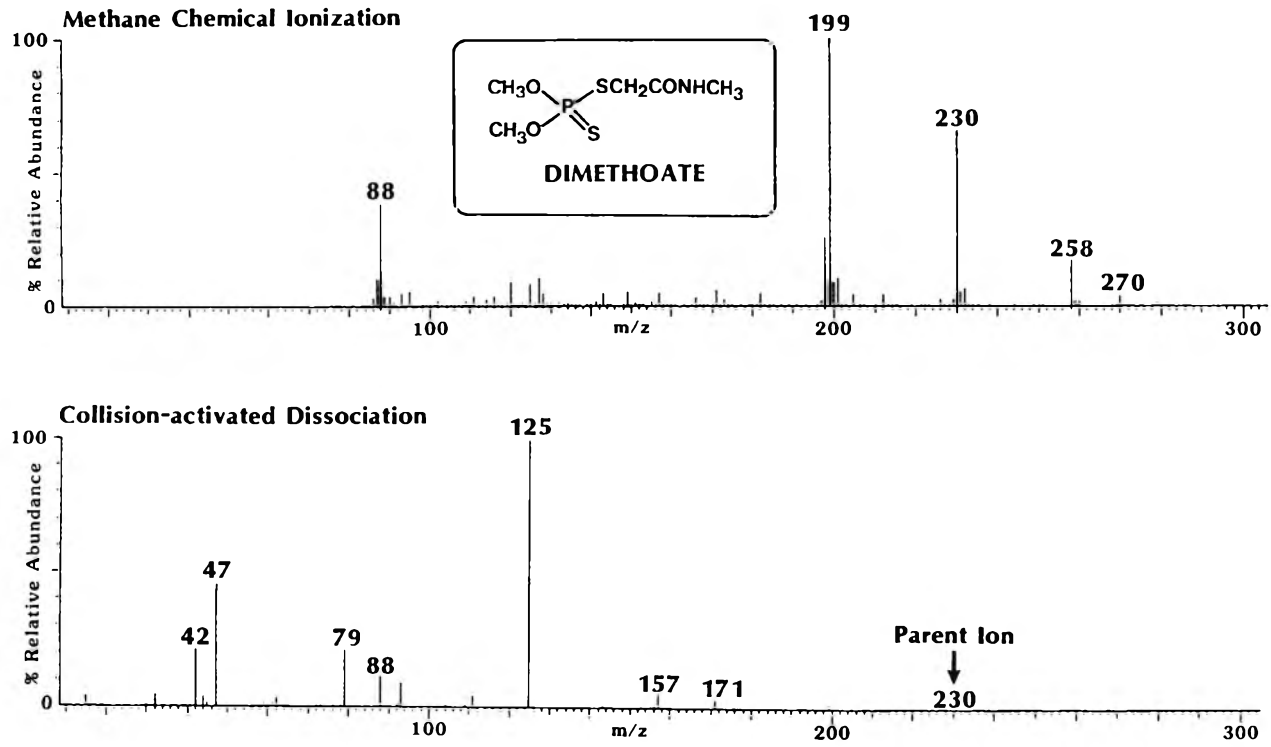

Figure 4. Mass spectral data for dimethoate: top, methane CI mass spectrum; bottom, CAD daughter spectrum on m/z 230 obtained under CI conditions.

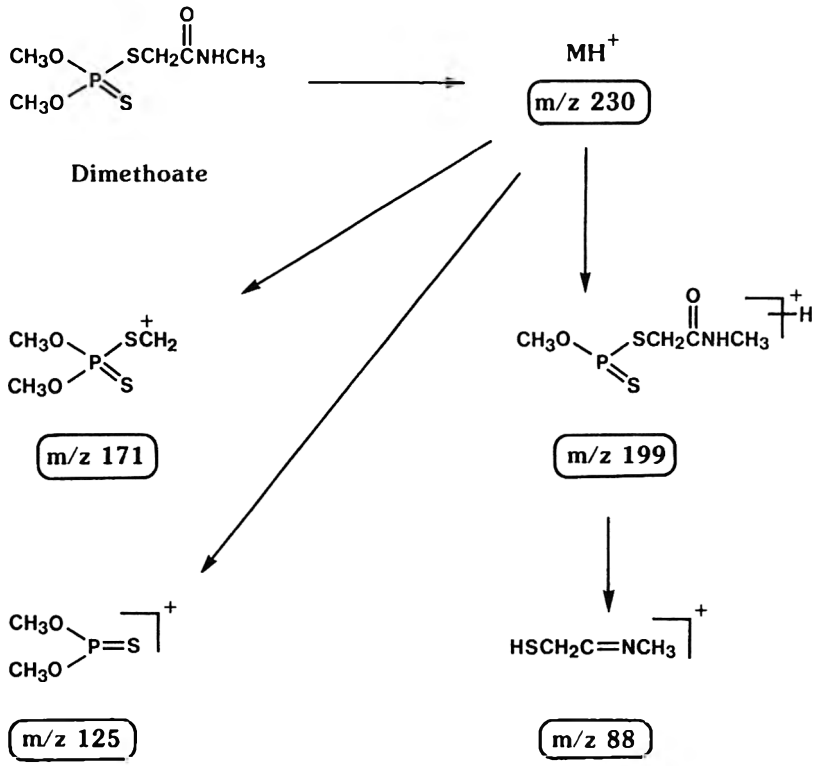

Figure 5. Proposed daughter fragmentation pathway of the protonated molecular ion at m/z 230 for dimethoate using CAD with argon as the collision gas.

illary column used with temperature programming in GC/MS analysis requires 2 additional reference standards with retention times that bracket the UAR. Such an approach has been useful in locating the precise retention window which contains the UAR of interest. Second, the ability of the GC/MS system to detect all eluting compounds rather than just those containing phosphorus requires detailed handling of the acquired data in the TIC mode to sort out the mass spectrum of the compound of interest. In many cases, the UAR is obscured by a coeluting compound in the extract.

In this particular bean extract, the 2 pesticide reference standards selected to locate the UAR were fonofos and pirimiphos-methyl. Examination of the TIC chromatogram produced under both EI and CI conditions (Figure 1) revealed gross interference by a coeluting hydrocarbon. In spite of this coeluting hydrocarbon, the UAR of interest was found (arrowed positions in Figure 1), by careful data profile analysis and subtraction techniques, to have a molecular weight of 292 (MH^+ = 293, Figure 1C). The complementary tandem use of EI and CI techniques to assist in the correct assignment of location and molecular weight is fundamental to the analytical approach adopted in such cases. Usually the only piece of data collected by this approach is a potential molecular weight since many fragment and adduct ions are completely obscured by coeluting compounds. However, this single piece of evidence, in conjunction with phosphorus being present from FPD-P, was sufficient to cause us to suspect, by consulting the U.S. Food and Drug Administration's molecular weight listing of pesticides, that the UAR could be phorate sulfone, diethyl carbethoxy dichloromethylphosphate, fensulfothion oxygen analog, etrimfos, or inezin. Whereas some of these possibilities could be instantly eliminated (e.g., no chlorine isotope ratios were observed), closer examination of the retention data in this listing revealed that etrimfos was a reasonable candidate. Repeating the experiment with a standard reference material for etrimfos confirmed the assumption. However, neither EI nor CI mass spectra (Figure 2) had sufficient fragmentation to extend the characterization beyond identification by the appropriate molecular weight. Although some fragment ions were present, the relative abundances in the residue were such as to preclude their use for absolute confirmation. All too often, fragment ions of low relative abundance either are lost in the background or cannot be assigned to the UAR because of strong coeluting compounds such as hydrocarbons. Therefore, in this particular case, the only ion of consequence was either at m/z 292 (EI, Figure 2A) or at m/z 293 (CI, Figure 2B). The presence of only one ion at the correct retention time cannot be viewed as unambiguous proof of the presence of a particular pesticide (5).

By selecting the protonated molecular ion at m/z 293 and by performing CAD experiments, daughter ions can be produced which are structurally related to the parent compound and hence provide a new dimensional plane of characterization for confirmation. In the case of etrimfos, the daughter ion spectrum derived from MH^+ at m/z 293 (Figure 2C) provided a number of significant ions suitable for confirmation purposes. In particular, the ion at m/z 125 was extremely useful because it provided proof of the presence of a dimethoxy phosphorothionate or phosphorodithionate (Figure 3). Furthermore, the copresence of 2 other daughter ions at m/z 109 and 143 provided additional evidence that the compound under investigation was a phosphorothionate. Had the potential identity of the UAR not been readily available, this CAD daughter spectrum would have been valuable in detecting the presence of a dimethoxy phosphorothionate. Other daughter ions produced from m/z 293 were the loss of 1, 28, and 60 daltons. The presence of the molecular ion at m/z 292 is revealing in that a suspected site for protonation of etrimfos is the ether linkage between the pyrimidinyl side chain and the phosphorus atom. Removal of a hydrogen atom by CAD using argon gas would seem to indicate that

this reagent gas hydrogen atom is weakly bound to that oxygen atom. Loss of 28 daltons from m/z 293 is probably loss of ethylene. Using the daughter ions derived from a study of the reference material, etrimfos in the bean extract at 0.1 ppm was confirmed using the protocol discussed above. With regard to sensitivity parameters, the lower detection level established for this confirmation procedure using 5 CAD daughter ions was 0.5 ng injected on-column.

To extrapolate the potential utility of the generic daughter ion spectrum observed for dimethoxy phosphorothionates, a dimethoxy phosphorodithionate (dimethoate) was then studied (Figure 4). It was apparent that the ion at m/z 125 was the dominant ion present representing the dimethoxy moiety discussed above (Figure 5). The lack of other daughter ions at m/z 109 and 143 was a strong indication that the compound was not a phosphorothionate. However, the weak presence of daughter ions at m/z 171 and 157 did confirm the dithionate as the basic structure with a methylene group adjacent to the second sulfur atom.

Conclusions

In defining the minimum criteria for confirmation of a pesticide residue, the ability to monitor at least 4 structurally related ions does not always present itself when such primary ionization techniques as EI or CI are used. The use of CI to produce a protonated molecular ion focuses attention on molecular weight of the compound but generally lacks fragment ions to provide unambiguous confirmation of presence. CAD experiments produce structurally important daughter ions not observable under EI or CI conditions, thereby providing an additional dimensional plane of characterization which, when used in tandem with the MH^+ from chemical ionization, can offer acceptable analytical protocols for confirmation as well as provide valuable structural clues to identify UARs. In the instance reported here, the presence of a daughter ion at m/z 125 from a pesticide known to contain phosphorus was strong evidence as to the presence of a phosphorothionate or phosphorodithionate.

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Assisted Distillation Cleanup of Pesticide Residues in Animal Fats: New Beadless Distillation Tube

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A new assisted distillation tube has been developed which contains no glass beads or glass wool. The sample is injected into the top of the tube, and a septum is not required. Large ground-glass joints (10/19) are used at the gas inlet and at the Florisil trap connection for maximum strength. Existing assisted distillation apparatus was easily modified to accept the new tubes. Recoveries of 9 organochlorine pesticides from butter and cattle fat ranged from 87 to 104% at a tube temperature of 230°C and a nitrogen flow rate of 250 mL/min. No significant change occurred in recoveries of 8 of the 9 pesticides after 85 uses of the beadless tube. Only the recovery of HCB from butter decreased, from 95 to 80%, after 51 runs. The new tube was compared with the Unitrex glass bead-packed concentric tube at flow rates of 230, 400, and 600 mL nitrogen/min. Recoveries from the Unitrex tube were similar to those of the beadless tube for all pesticides except DDT which was significantly lower, indicating incomplete distillation at 250 mL/min and possible partial breakdown at 400 and 600 mL/min. A procedure is introduced for pressurized solvent washing of distillation tubes. This procedure is more efficient than vacuum washing.

Assisted distillation as described by the authors (1, 2) is a refinement of the sweep codistillation technique of Storherr et al. (3) and is used for the cleanup of pesticide residues in animal fat and butter fat samples for pesticide residue analysis. This is achieved by introducing 1 g melted fat into a tube packed with glass beads and maintained at a high temperature so that a thin film of fat is formed over the beads. The fat is suspended in the vertical tube by a high-velocity flow of nitrogen. Over a period of approximately 30 min, the pesticides are distilled from the fat and collected in a Florisil trap (4); the fat remains in the tube.

Our major modification of the original technique (3) was the elimination of solvent injection into the distillation tube. We therefore renamed the modified technique "assisted distillation" because the sweeping codistilling solvent as Storherr et al. described it, was no longer used. Neidert and Saschenbrecker (5) investigated the effect of silanization on recoveries from sweep codistillation and assisted distillation tubes. A commercially constructed assisted distillation apparatus (Unitrex) was described by Luke et al. (6). Their compact design and the new top injection technique were significant improvements. The Unitrex distillation tube consists of 2 concentric tubes with the space between them packed with glass beads and glass wool.

Our aim was to develop a tube that did not contain glass wool or glass beads, thereby increasing the number of uses before cleaning was required. In addition, the tube should have top injection and be easily fitted to our existing apparatus. This paper reports the evaluation of the new tube over

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a limited range of temperatures and flow rates; its performance is also compared to that of a Unitrex tube.

Experimental

Optimization

All recoveries were obtained using butter fat and cattle fat fortified with HCB and lindane, 0.075 mg/kg; heptachlor, 0.17 mg/kg; aldrin, heptachlor epoxide, DDE, and dieldrin, 0.25 mg/kg; DDD, 0.50 mg/kg; and DDT, 0.75 mg/kg.

Depth of insertion of the distillation tube into the heating block was varied in the following ways to determine its effect on pesticide recovery from the beadless tube: bulb on top of block, 4 mm of bulb above block, and bulb wholly inserted into block. The following investigations were made with the bulb wholly inserted into the block: (*1*) nitrogen flow rates of 250, 400, and 600 mL/min at a block temperature of 230°C; (*2*) distillation temperatures through the range 220–250°C in 10° increments, using 250 mL nitrogen/min; (*3*) reproducibility of recoveries over the range of 230–250°C and 250 mL/min with butter fat only; and (*4*) recoveries from the Unitrex tube at nitrogen flow rates of 230, 400, and 600 mL/min at a temperature of 235°C, using cattle fat only.

Reagents and Apparatus

As described by Heath and Black (1, 2) with the following modifications:

(a) *New distillation tube.*[1]—See Figure 1.

(b) *Florisil trap.*—Figure 1. Similar to that of McDougall (4) but with the Florisil section widened and with a ground-glass cone instead of a socket. Removable tip of PTFE tubing containing small plug of glass wool.

(c) *Florisil.*—60–80 mesh, activated at 680°C for 3 h; partially deactivated by adding 5% w/w water when cool.

(d) *Assisted distillation apparatus (1).*—Modified to allow insertion of the bulb of new distillation tubes. This consisted of enlarging, to 25 mm diameter, the upper 25 mm of the eleven 16 mm diameter holes in the heating block. The side of the hole opposite the operator was also filed so that the bulb and outlet to the condenser could be closely fitted into the block.

(e) *Pressure washing reservoir.*—Stainless steel vessel of approximately 5 L capacity (fitted) with ¼ in. stainless steel inlet and outlet tubes. The outlet tube should reach to within approximately 6 mm of the bottom of the reservoir.

(f) *Petroleum ether.*—57–63°C bp.

(g) *10% diethyl ether in petroleum ether.*

(h) *Unitrex glass column tube.*—Packed with silanized glass beads, Part No. 093294.

Procedure

Set aluminum block temperature to 230°C and insert distillation tube (see Figure 1). Connect nitrogen supply to ground-glass socket so that gas flows down central tube. Add Florisil to trap by dispensing from separatory funnel; replace removable PTFE tip. Connect Florisil trap to other socket. Secure both ground-glass connections with slight twist. Measure nitrogen flow rate at outlet of trap with bubble meter and adjust to 250 mL/min. Disconnect nitrogen and inject 1.0 g melted fat into center tube, using 2 mL syringe with 50–100 mm, 16 gauge needle. Reconnect nitrogen.

After 30 min distillation, disconnect trap and place it in 20-30 mL test tube calibrated at 10 mL. Let trap cool and

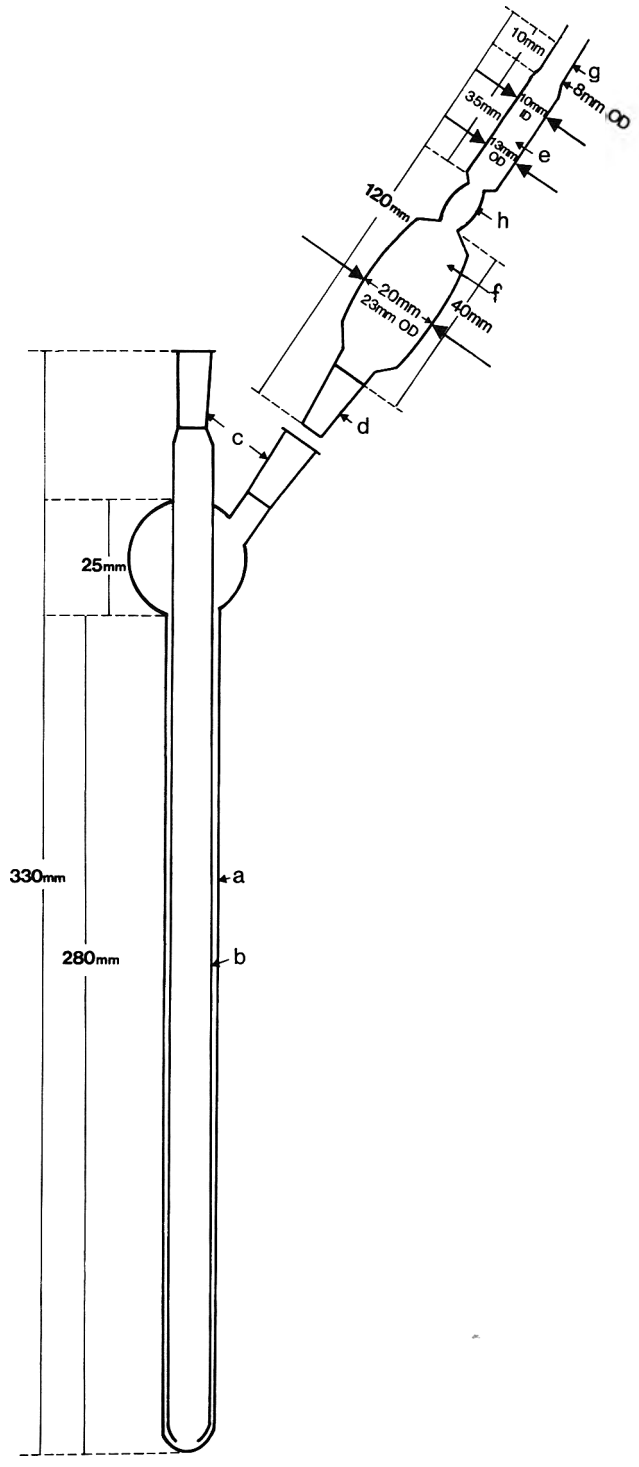

Figure 1. Distillation tube and condenser: a, outer tube wall thickness = 1.5 mm, id = 10.5 mm ± 0.02 mm (selected tube); b, inner tube wall thickness = 1.1 mm, od = 10.0 mm ± 0.02 mm (selected tube); c, ground-glass sockets 10/19; d, 10/19 ground-glass cone; e, Florisil; f, reservoir; g, removable PTFE tip packed with glass wool; h, glass wool.

rinse reservoir walls with 2 mL of 10% v/v diethyl ether in petroleum ether followed by additional 10 mL when the first wash has drained into Florisil. Adjust eluate to 10.0 mL for analysis. Remove distillation tube from heating block and support in fume cupboard. Connect pressure vessel to tube outlet and connect waste-collecting vessel to inlet with PTFE tubing and nylon or PTFE B10 stoppers. Wash hot tube by forcing approximately 35 mL petroleum ether at 12–15 psi through tube. Disconnect tube and drain, then dry by drawing

[1] Patent pending. Persons interested in a commercial model may contact the authors.

Table 1. Effect of depth of insertion of tube into heating block on condensation of pesticides in tube bulb

Pesticide	% Residue in bulb[a]		
	Wholly inserted	4 mm not inserted	Not inserted
DDT	<2	3.9 ± 2.4	15 ± 2.5
DDD	<2	2.2 ± 1.1	15 ± 3.8
Dieldrin	<2	<2	8 ± 5.9
DDE	<2	<2	12 ± 5.4

[a] Each recovery is the mean of 3 replicates ± SD.

stream of air through tube. It is important to conduct washing procedure in fume cupboard to control solvent vapors.

Glass wool plugs in removable tip and trap body may be reused.

Results and Discussion

The investigation of tube insertion depth into the heating block showed that no detectable levels of HCB, lindane, heptachlor, or heptachlor epoxide were recovered from the bulb when the tube was inserted at any of the 3 depths tested. Various levels of the other pesticides were found when the bulb was not fully inserted (Table 1). Less than 2% of any pesticide remained in the bulb when the tube was fully inserted. This was considered satisfactory.

Residues remaining in the bulb after 30 min distillation were determined by removing the tube from the heating block and letting it cool to approximately 20–30°C. The tube was then immersed in ice water to a point just below the bulb to solidify the fat. The tube was then removed, dried, and inverted over a 50 mm funnel on a small Florisil column. The inside surfaces of the bulb were then washed down with 3 mL of 10% diethyl ether/petroleum ether. This was followed by a further 10 mL to complete elution of the pesticides through the Florisil.

Table 2. Effect of nitrogen flow rate on percent recovery of pesticides from butter fat at 230°C

Pesticide	Recovery, %[a]		
	250 mL/min	400 mL/min	600 mL/min
HCB	95 ± 0.6	83 ± 4.4	85 ± 0.8
Lindane	100 ± 2.1	98 ± 7.5	91 ± 1.1
Heptachlor	89 ± 3.5	87 ± 2.9	47 ± 2.8
Heptachlor epoxide	104 ± 4.0	98 ± 6.1	99 ± 1.9
Aldrin	99 ± 1.0	100 ± 1.2	95 ± 1.3
Dieldrin	95 ± 2.1	104 ± 3.8	94 ± 5.7
DDE	98 ± 1.7	101 ± 1.2	93 ± 1.6
DDD	95 ± 2.6	99 ± 1.7	95 ± 2.7
DDT	88 ± 0.6	93 ± 0.6	94 ± 2.9

[a] Each recovery is the mean of 3 replicates ± SD.

Table 3. Effect of nitrogen flow rate on percent recovery of pesticides from cattle fat at 230°C

Pesticide	Recovery, %[a]		
	250 mL/min	400 mL/min	600 mL/min
HCB	95 ± 3.0	98 ± 3.1	96 ± 2.6
Lindane	99 ± 1.2	105 ± 2.5	101 ± 4.2
Heptachlor	91 ± 1.0	83 ± 2.5	67 ± 3.8
Heptachlor epoxide	98 ± 1.2	101 ± 1.7	105 ± 3.2
Aldrin	95 ± 3.5	93 ± 2.1	99 ± 0.6
Dieldrin	98 ± 1.2	100 ± 1.2	89 ± 1.5
DDE	100 ± 2.1	100 ± 3.1	98 ± 6.4
DDD	98 ± 5.5	101 ± 4.1	104 ± 1.2
DDT	87 ± 2.5	90 ± 2.1	94 ± 3.2

[a] Each recovery is the mean of 3 replicates ± SD.

Table 4. Effect of distillation tube temperature on percent recovery of pesticides from butter fat at 250 mL/min

Pesticide	Recovery, %[a]			
	220°C	230°C	240°C	250°C
HCB	95 ± 1.2	95 ± 0.6	84 ± 4.0	84 ± 1.5
Lindane	99 ± 4.0	100 ± 2.1	94 ± 3.6	97 ± 0
Heptachlor	99 ± 4.0	89 ± 3.5	86 ± 3.2	87 ± 11.1
Heptachlor epoxide	105 ± 3.7	104 ± 4.0	97 ± 1.5	99 ± 1.7
Aldrin	102 ± 7.0	99 ± 1.0	95 ± 5.0	101 ± 0.6
Dieldrin	91 ± 3.1	95 ± 2.1	106 ± 6.6	105 ± 2.1
DDE	99 ± 2.6	98 ± 1.7	99 ± 4.5	100 ± 2.1
DDD	78 ± 5.6	95 ± 2.6	90 ± 5.6	99 ± 0.6
DDT	68 ± 6.4	88 ± 0.6	88 ± 1.0	93 ± 2.7

[a] Each recovery is the mean of 3 replicates ± SD.

Pesticide recoveries from butter and cattle fat at nitrogen flow rates of 250, 400, and 600 mL/min at 230°C are shown in Tables 2 and 3. When the flow rate was increased from 250 to 400 mL/min, the recovery of DDT from butter fat increased by 5% but with no further increase at 600 mL/min. Recoveries of heptachlor decreased to 47 and 67% in butter and cattle fat, respectively, as the flow rate was increased to 600 mL/min. The higher flow rates also removed the water from the Florisil faster and hence reactivated it, causing lower dieldrin recoveries from the Florisil. Either more solvent or less Florisil is required to allow elution of dieldrin. No further heptachlor eluted, however. At this time, we are unable to explain the low recovery of heptachlor at this high flow rate.

Two types of distillation tubes were studied: (a) truly concentric, i.e., a uniform gap of 0.25 mm between tubes, and (b) nonconcentric, in which, although the average gap was 0.25 mm, the gap varied between 0.05 and 0.45 mm between tubes. Both types of tubes gave similar recoveries at 250 mL/min. When the truly concentric tube was used at flow rates above 270 mL/min, excess fat rose into the bulb and all recoveries were low (<20%). For this reason all the results reported were obtained with the nonconcentric (type b) tube which did not suffer from this problem.

For the pesticides studied, we recommend the use of 250 mL nitrogen/min to minimize the deleterious effects of the higher flow rates on the recovery of heptachlor and on the water content of the Florisil. This flow rate is also compatible with both concentric and nonconcentric distillation tubes.

Recoveries of 9 organochlorine pesticides from butter fat and cattle fat over a temperature range of 220–250°C at a nitrogen flow rate of 250 mL/min are shown in Tables 4 and 5. At 220°C, DDT recoveries from butter fat were on average

Table 5. Effect of distillation tube temperature on percent recovery of pesticides from cattle fat at 250 mL/min

Pesticide	Recovery, %[a]		
	230°C	240°C	250°C
HCB	95 ± 3.0	96 ± 2.0	97 ± 2.6
Lindane	99 ± 1.2	96 ± 7.8	104 ± 1.7
Heptachlor	91 ± 1.0	94 ± 7.0	81 ± 4.7
Heptachlor epoxide	99 ± 1.2	102 ± 0.6	103 ± 1.5
Aldrin	95 ± 3.5	103 ± 2.6	102 ± 1.0
Dieldrin	98 ± 1.2	102 ± 2.5	105 ± 4.6
DDE	100 ± 2.1	104 ± 3.5	107 ± 3.6
DDD	98 ± 5.5	101 ± 1.2	108 ± 1.7
DDT	87 ± 2.5	85 ± 0.6	85 ± 3.2

[a] Each recovery is the mean of 3 replicates ± SD.

Table 6. Reproducibility of percent recovery of pesticides from butter fat, using the beadless distillation tube at 230°C and 250 mL/min

Sample	HCB	Lindane	Heptachlor	Hept. epox.	Aldrin	Dieldrin	DDE	DDD	DDT
1	94	99	85	100	98	93	96	94	89
2	96	98	91	108	99	96	99	93	88
3	94	102	91	103	100	97	99	98	88
4	90	107	100	105	96	101	105	92	85
5	89	98	94	102	102	98	94	100	93
Mean	92.6	100.8	92.2	103.6	99.0	97.0	99.6	95.4	88.6
RSD	3.2	3.8	5.8	3.0	3.2	2.8	4.1	6.3	3.3

22% below those obtained at 230–250°C, indicating incomplete distillation. At 230, 240, and 250°C, there is no significant change in the recoveries of the other pesticides studied except for heptachlor in cattle fat which decreased significantly from 91% at 230°C to 81% ($P < 0.01$) at 250°C. In consideration of these data, we chose 230°C as our recommended temperature.

Table 6 shows the reproducibility of 5 recoveries from butter fat at 230°C and 250 mL nitrogen/min. These are comparable to those achieved in our previous investigation (1).

Table 7 shows recoveries obtained with the Unitrex tube silanized according to manufacturer's specifications and used at the recommended conditions (6) of 235°C and nitrogen flow rate of 230 mL/min. In addition, flow rates of 400 and 600 mL/min were studied for comparison with our tube. Because of the side arm on the Unitrex tube, we were unable to evaluate this tube in our heating block. We were successful, however, in evaluating the tube in a calibrated fan-forced gas chromatograph oven using an external Florisil trap; to ensure that results were not affected by the different heating medium, we carefully compared the results obtained from our beadless tube in the same oven. There was no significant difference in recoveries from our tube when heated in the air oven or the heating block, so we considered this to be an acceptable comparison of the 2 tubes. Generally, the results from the Unitrex tube at 230 mL nitrogen/min were comparable to results from our tube at 250 mL/min except for DDT which was recovered at 69% from the Unitrex tube (Table 7). (Our tube showed no significant difference in recoveries between 230 and 250 mL nitrogen/min.) When we continued the distillation with the Unitrex tube for another 30 min at this flow rate, a further 11% DDT and 15% DDD was collected, showing incomplete distillation. Distillation was still incomplete at 400 mL/min, when 4% DDT and 8% DDD were found in the second 30 min fraction. At 600 mL/min, less than 2% DDT and 2% DDD were found in the second fraction. The low 76% DDT recovery compared to the 87% reported by Luke et al. (6) may have occurred due to DDT breakdown on unpolished areas of glass beads. We also found that at the higher flow rates, droplets of fat entered the Florisil trap, increasing the probability of fat being eluted with the pesticides.

Table 7. Effect of nitrogen flow rate on percent recovery of pesticides from cattle fat at 235°C using the Unitrex tube

Pesticide	Recovery, %[a]		
	230 mL/min	400 mL/min	600 mL/min
HCB	98 ± 4.0	95 ± 1.2	94 ± 3.1
Lindane	101 ± 4.2	93 ± 9.7	106 ± 4.2
Heptachlor	95 ± 2.9	90 ± 6.1	71 ± 24.7
Heptachlor epoxide	99 ± 8.1	99 ± 3.1	98 ± 1.2
Aldrin	102 ± 7.4	99 ± 2.1	102 ± 2.1
Dieldrin	97 ± 6.9	92 ± 3.6	95 ± 7.8
DDE	99 ± 10.4	104 ± 4.0	95 ± 2.1
DDD	93 ± 6.4	100 ± 3.5	93 ± 9.2
DDT	69 ± 4.2	78 ± 4.2	76 ± 2.0

[a] Each recovery is the mean of 3 replicates ± SD.

Calculation shows that the beadless tube and the Unitrex tube have similar surface areas available for fat. We suggest that the higher DDT recoveries from cattle fat on the beadless tube may be due to the thinner film of fat formed by the higher gas velocity of 100 cm/s compared with 38 cm/s in the Unitrex tube we evaluated.

For 8 of 9 of the pesticides studied, the usable life of the beadless tube was in excess of 85 runs before nitric acid recleaning (2) was required.

After 51 runs, the recovery of HCB from butter fat decreased from 95 to 80%. No obvious charring or darkening of the tubes occurred over the 85 runs.

Conclusion

The new distillation tube presented shows that neither glass wool nor glass beads are necessary for the distillation of pesticides from fat. With no glass surfaces in contact, the need for acid cleaning is virtually eliminated, and the number of uses is greatly increased.

Pesticide recoveries obtained with this tube are comparable with those obtained with our previous glass bead-packed tubes and have been achieved at the lower flow rate of 250 mL/min.

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Total Arsenic in Foods after Sequential Wet Digestion, Dry Ashing, Coprecipitation with Ammonium Pyrrolidine Dithiocarbamate, and Graphite-Furnace Atomic Absorption Spectrometry

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A graphite-furnace atomic absorption (GFAAS) method is described for determining total arsenic (organic and inorganic compounds) in foods. Samples ranging from 1 to 40 g (depending on moisture content) were digested with HNO_3 and dry-ashed at 500°C overnight after addition of MgO. After dissolution in HCl, the arsenic was reduced with iodide and ascorbic acid and precipitated with ammonium pyrrolidine dithiocarbamate (APDC) in the presence of nickel carrier. Precipitates were collected on 0.3μm cellulose acetate filters and dissolved in 10% HNO_3 containing modifier. $Ba(NO_3)_2$ was added to remove a sulfate interference resulting from decomposition of APDC. Arsenic was determined using GFAAS. Accuracy of the method was good for 7 U.S. National Bureau of Standards (NBS) Standard Reference Materials and 3 National Research Council of Canada (NRCC) round-robin samples. Recovery of arsenic(V) from foods averaged 99.2% for peak heights and 97.1% for peak areas, with relative standard deviations (RSD) of 2.2% for peak heights and 3.3% for peak areas for all NBS and NRCC materials. Detection limit of the method was ca 10 ng arsenic.

A graphite-furnace atomic absorption spectrometric (GFAAS) coprecipitation method (1) recently developed by us for total arsenic in foods had several advantages over other methods: It could be automated at the instrumental step; only 1 flask and 1 funnel top per sample required washing, and disposable unwashed test tubes could be used for dissolution of precipitate and storage of analyte solutions prior to measurement; and synthetic standards could be used, thereby reducing analyst time. Nevertheless, the method also had some weaknesses: The detection limit was limited by sample size because only about 1 g organic matter could be dry-ashed. There were interferences for all liver samples, resulting in recoveries as low as 40–50%, and recoveries for other foods generally varied in the 80–100% range, depending on the food (1). Finally, application of the method by 3 analysts in our laboratory revealed that much technical skill was needed to achieve the correct pH of precipitation.

This paper presents the results of investigations to overcome the problems and limitations of the method described above. The improvements made to the original method include addition of a digestion step prior to dry-ashing to facilitate larger sample sizes and, hence, detection limits 3–10 times lower than previously obtained; changes to the matrix modifier to reduce sulfate and cationic interferences; a change in buffer so that use of a pH meter will be minimal; and simplification of the reduction procedure.

Experimental

Apparatus

(a) *Atomic absorption spectrometer.*—Varian Model 775-ABQ, equipped with simultaneous deuterium-continuum background corrector (Varian Associates of Canada, Ltd., Ottawa, Ontario).

(b) *Graphite furnace.*—Varian Model GTA-95 equipped with programmable autosampler, pyrolytically coated graphite tubes, and pyrolytic-graphite L'vov platform (Varian Associates).

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(c) *Vacuum filtering system.*—Consisting of water aspirator vacuum, filter base and clamp (Millipore Corp., Bedford, MA 01730, Cat. No. XX10 025 00) for holding 24 mm diam., 0.3μm cellulose acetate filters (Millipore Corp., Cat. No. PHWP 02400), and funnel top with 125 mL capacity and a base to match the Millipore filter base. The latter was custom-made by a glass blower.

(d) *Polystyrene centrifuge tubes with caps.*—15 mL capacity (Falcon Corp., Oxnard, CA 93030, Cat. No. 2095). Examine each tube against light and discard cracked tubes.

(e) *Repeater pipet.*—10 mL, inversion type (Fisher Scientific Co., Ottawa, Ontario, Cat. No. 13-683-10C).

(f) *Variable-volume hand pipet.*—Gilson-Pipetman® Model P5000, 5 mL (Mandel Scientific Co., 143 Dennis St., Rockwood, Ontario, Cat. No. GF23603).

Reagents

Unless otherwise specified, all reagents were reagent grade.

(a) *Deionized water.*—ASTM Type I was used throughout study.

(b) *Iron(III) solution.*—5000 μg/mL in 5% HNO_3. Dissolve 5 g high-purity (m4N8; t4N+) iron rod (Alpha Products, Danvers, MA 01923, Cat. No. 00164) by heating in solution containing 70 mL HNO_3 and 200 mL water. Cool and dilute to 1 L with water. Store indefinitely in tightly capped Pyrex bottle.

(c) *Copper(II) solution.*—5000 μg/mL in ca 5% HNO_3. Dissolve 5 g high-purity (m5N) copper metal (Alpha Products Cat. No. 400388) in 100 mL HNO_3 (1 + 1). Cool and dilute to 1 L with water. Store indefinitely in tightly capped Pyrex bottle.

(d) *Nickel(II) solution.*—1000 μg/mL in 10% HNO_3. Dissolve 4.95 g $Ni(NO_3)_2 \cdot 6\ H_2O$ in 10% HNO_3 and dilute to 1 L with 10% HNO_3.

(e) *Arsenic primary standard.*—500 μg/mL. Add 25 mL 1M NaOH to 0.660 g As_2O_3 and heat gently until dissolved. Cool, dilute to ca 150 mL with water, and neutralize with 1% HNO_3. Dilute to 1 L with water. Solution may be stored in Pyrex bottle several years.

(f) *Arsenic stock standard.*—10 μg/mL in 5% HNO_3. Pipet 2 mL arsenic primary standard **(e)** into 100 mL volumetric flask and dilute to volume with 5% HNO_3.

(g) *Arsenic stock standard.*—1 μg/mL in 0.5% HNO_3. Pipet 10 mL 10 μg/mL arsenic standard **(f)** into 100 mL volumetric flask and dilute to volume with water.

(h) *Light magnesium oxide.*—Cover several 250 mL beakers containing 100–150 g $Mg(NO_3)_2 \cdot 6\ H_2O$ (Baker Analyzed Reagent) with watch glasses and heat in muffle furnace at 200°C until samples liquefy. Heat at 350°C until evolution of nitric oxide (brown fumes) substantially subsides (ca 1.5 h). Heat at 500°C at least 4 h or overnight, cool, and grind to fine powder. Commercial high-purity, light MgO may be used after absence of arsenic blank is assured.

(i) *Ashing aid.*—Prepare fresh just before use. Add 250 mL water to 25 g MgO **(h)**, stir until homogeneous and ready for use, and transfer to repeater pipet **(e)**.

(j) *Hydrochloric acid.*—10M. Dilute 840 mL HCl to 1 L with water.

(k) *Reducing agent.*—Dissolve 25 g NaI and 2.5 g ascorbic acid in water and dilute to 100 mL with water. Prepare fresh once a month and store in refrigerator when not in use.

(l) *Ascorbic acid solution.*—2.5%. Dissolve 2.5 g ascorbic acid in water and dilute to 100 mL with water. Prepare fresh once a month and store in refrigerator when not in use.

(m) *Masking agent.*—Dissolve 2 g ethylenediaminetetraacetic acid disodium salt (EDTA) in water and dilute to 100 mL with water.

(n) *Buffer.*—Dissolve 136 g sodium acetate trihydrate in ca 450 mL water in 600 mL beaker. While stirring, cautiously add acetic acid until pH is 5.8. Transfer to 500 mL volumetric flask and dilute to volume with water. Final pH should be 5.6–5.7.

(o) *Carrier.*—100 μg/mL nickel(II) in 1% HNO_3. Into 100 mL volumetric flask, pipet 10 mL 1000 μg/mL nickel(II) solution (d), and dilute to volume with water.

(p) *Methyl violet indicator.*—0.1% (w/v) in water. Dissolve 0.2 g methyl violet in ca 200 mL water and filter through medium-porosity filter paper. Store in dark in refrigerator when not in use. Prepare fresh every 6 months.

(q) *APDC solution.*—1.5% (w/v) in water. Dissolve 3 g APDC in water and vacuum-filter through 0.3μm membrane filter. Dilute to 200 mL. Prepare fresh solution daily.

(r) *Dissolution solution.*—20 μg/mL nickel(II), 250 μg/mL iron(III), and 200 μg/mL copper(II) in 10% HNO_3. Into 2 L volumetric flask, transfer 40 mL 1000 μg/mL nickel(II) solution (d), 100 mL 5000 μg/mL iron(III) solution (b), 80 mL 5000 μg/mL copper(II) solution (c), and 200 mL HNO_3. Let cool and dilute to volume with water. Solution may be stored several years in original flask.

(s) *Barium solution.*—15 000 μg/mL barium. Dissolve 2.86 g $Ba(NO_3)_2$ in water and dilute to 100 mL with water.

Standard Preparation

Using pipet (f), transfer into each of six 100 mL volumetric flasks 5 mL of 5000 μg/mL iron(III) solution (b), 4 mL of 5000 μg/mL copper(II) solution (c), 3 mL of 1000 μg/mL nickel(II) solution (d), 5 mL of 15 000 μg/mL barium solution (s), and 9.25 mL HNO_3. Swirl, cool to room temperature, and pipet 0, 2, 4, 6, 8, and 10 mL of 1 μg/mL arsenic standard (g) into the respective flasks. Dilute to volume with water. Standards are stable at least 3 months.

Procedure

Washing.—Wash glassware with HNO_3 and rinse directly with water. Polystyrene test tubes from 10 lots did not require washing; however, the analyst should test each lot for the possibility of contamination.

Digestion and ashing.—Weigh food samples into 250 mL Erlenmeyer flasks, limiting weight to ca 5 g organic matter. Include 3 blanks. If necessary, heat foods in oven to remove bulk of moisture (120°C during day or 50°C overnight). Add 35 mL HNO_3 and let stand 4 h or overnight. Gently boil to reduce to 2–4 mL volume without charring. Repeat HNO_3 addition and digestion a second time and repeat a third time if digest is not homogeneous. Blanks containing the same total volume of HNO_3 may be boiled down more rapidly on a separate hot plate at medium-high temperature.

Add 10 mL ashing aid (i) to each flask and heat in muffle furnace at 120°C overnight. Increase temperature by ca 75°C/h to 300°C heat until evolution of nitrogen oxide gasses ceases (ca 1–2 h). Increase temperature to 500°C and ash at least 4 h or overnight. To cooled flasks add 5 mL water and 25 mL 10M HCl, washing down sides of flasks with acid. Discard solutions with an undissolved black residue, which indicates inadequate digestion with HNO_3. Inadequate digestion can be remedied by reducing sample size or increasing the number of digestions with HNO_3.

Table 1. Temperature program for the Varian Model GTA-95 graphite furnace using 15 μL total volume pipetted onto a L'vov platform

Step	Temp., °C	Time, s	Argon flow, L/min	Read
1	120	0.2	3	
2	120	10.0	3	
3	225	2.0	3	
4	225	35.0	3	
5	900	5.0	3	
6	900	5.0	3	
7	900	1.0	0	
8	2650	0.8	0	•
9	2650	2.0	0	•
10	2650	1.0	3	
11	40	13.0	3	
12	40	5.0	3	

Reduction of arsenic(V).—After dissolving the ash, add 1 mL reducing agent (k) and let stand 20 min. A yellow or yellow-brown color should appear. Dilute to ca 75 mL with water. The color should disappear. Heat solution near boiling until pale yellow color just appears, or for 30 min maximum if no color appears, then remove from heat. If pale yellow color continues to deepen, add 1 mL 2.5% ascorbic acid (l).

Precipitation.—To each flask, add 1 mL masking agent (m), 1 mL buffer (n), and 1 mL carrier (o).

To first flask, add 3 drops of methyl violet solution (p); then, dropwise, add ammonium hydroxide, swirling until color just changes from blue to violet. Add 5 mL buffer (n) and check pH. It should be 4.5, but 4.3 to 4.8 is acceptable. Repeat for other flasks. Add 10 mL 1.5% APDC solution (q) to each flask, swirl, and let stand 30 min. Filter solution (c). Rinse flask with ca 10 mL water and add rinse to filter funnel. Transfer filter to centrifuge tube (d).

Pipet 9.5 mL dissolution solution (r) into tubes, cap tubes, and let stand overnight or until dark precipitate dissolves. Pipet 0.5 mL barium solution (s) into each tube, cap tube, shake, and let stand at least 1 h.

Determinations by GFAAS

Set up and optimize spectrometer with simultaneous background correction and graphite furnace according to manufacturer's instructions for arsenic determinations (193.7 nm wavelength). Table 1 lists furnace temperature program used in this study. Peak height or area measurements may be used. Set autosampler to pipet 10 μL aliquot of sample or standard solution onto platform. If autosampler can be programmed to pick up blank or modifier simultaneously with each sample solution, use water in the modifier position and include 2 μL water in the autosampler program. This improves precision by washing out traces of standard or sample solution from the pipet.

Set up analytical sequence of solutions in autosampler in following order: standards in order of decreasing concentration, sample blanks, samples, and again standards in order of decreasing concentration.

Analyze solutions, making 3 replicate atomizations for each solution. Tabulate measurements and calculate mean for each solution. If absorbance for any sample solution exceeds that

Table 2. Recovery of organoarsenical spikes (400 ng as arsenic) from NBS Pine Needles under basic and acidic ashing conditions

Compound	Recovery, %			
	Acidic ash,[a]		Basic ash,[b]	
	Peak ht	Peak area	Peak ht	Peak area
Sodium methyl arsonate	97.3 ± 1.3	93.5 ± 4.0	101.5 ± 2.6	89.9 ± 4.3
Sodium methyl arsenate	101.1 ± 3.4	98.6 ± 0.9	106.6 ± 5.2	97.7 ± 7.1
Arsenobetaine	110.0 ± 4.2	99.7 ± 1.3	102.4 ± 13.6	98.6 ± 3.6
Dimethylarsenic acid	93.9 ± 9.5	94.6 ± 7.4	108.2 ± 2.9	99.1 ± 9.1

[a] Acidic ashing constituted digestion with HNO_3 followed by addition of an MgO-$Mg(NO_3)_2$ ashing aid (2) which contained insufficient oxide to neutralize the HNO_3.

[b] Basic ashing consisted of ashing with the same mixed ashing aid as above but without the HNO_3 digestion, so that the final mixture prior to drying and ashing was basic.

of the highest standard, dilute the solution by pipetting 0.9 mL sample blank and 0.1 mL sample into a sampling cup and reanalyze.

Calculations

Compare absorbances of the standards run before and after the samples. If 3 or more of the standards change by more than 10% in any one direction, reanalyze all solutions.

Calibrate using all the standards (run both before and after samples). This may be done either by drawing a calibration curve or by using a reliable calibration algorithm that can handle nonlinear curves. Calculate the solution concentrations of sample blanks and samples and subtract mean sample blank concentration from sample solution concentrations.

Calculate arsenic concentration in samples according to the formula:

$$\text{Arsenic, } \mu g/g = (C \times V)/(1000 \times \text{sample wt})$$

where C is the blank corrected concentration (ng/mL) of the sample solution, V is the final volume (mL) of solution, including dilutions, and sample weight is expressed in grams.

Results and Discussion

Destruction of Organic Matter

The original dry-ashing method (1) was developed using magnesium nitrate and magnesium oxide, which had been proved valid for inorganic as well as several organoarsenic species common to biological samples (2). In spite of its success, it was limited to sample sizes of ca 1 g organic matter. Periodically some samples would combust in the muffle furnace with a resultant arsenic loss, and some authors have reported an average of 5–6% arsenic losses (3, 4). Because there are still uncertainties about the relative merits of various wet digestion and dry-ashing techniques (2–4), the concept of dry-ashing was retained for this method, but ways to overcome some of the above problems were added.

Specifically, samples were digested with HNO_3 prior to being ashed, enabling a larger sample size to be used without fear of combustion losses during ashing (5). The amount of HNO_3 present after the digestion was only marginally neutralized with the original MgO–$Mg(NO_3)_2$ ashing aid, resulting in effects on the muffle furnace during drying. To overcome this, MgO was used in place of $Mg(NO_3)_2$ so that the total amount of magnesium present, 1 g as MgO per sample, would be the same as in the mixed ashing aid. This was sufficient to neutralize 3 mL HNO_3, reducing acid attack on the furnace. According to the method, however, 2–4 mL HNO_3 could remain after digestion, and the resulting ashing conditions could be either acidic or basic, depending on whether HNO_3 or MgO was in excess. The effect this might have on recovery of organoarsenic compounds was evaluated (Table 2) and found to be minimal. Use of a larger quantity of MgO to guarantee neutralization was judged impractical because larger quantities caused expansion and periodic loss of the ash (4), and because, for purity reasons, the MgO was prepared from the nitrate (6).

The sample size limitation of 5 g organic matter for this method was about 5 times that of the original method (1, 2).

Reduction of Arsenic(V)

The method of arsenic reduction was simplified over the previous method (1) by adding the reducing agent only once.

One of the problems encountered for isolated samples, using both the previous method and the present one, was that while the solution and the reducing agent were being heated, the pale yellow color continued to deepen after it was removed from the hot plate. If this occurred, arsenic was not quantitatively precipitated. Addition of 2.5% ascorbic acid solution to the hot sample solution when this occurred prevented arsenic loss.

Table 3. Levels of arsenic recovered from NBS Standard Reference Materials and NRCC round-robin samples

Sample	No. of analyses	Certified level,[a] μg/g	Found (±SD), μg/g	
			Peak ht	Peak area
Bovine liver	5	0.047 ± 0.006	0.053 ± 0.001	0.044 ± 0.001
Rice flour	7	0.41 ± 0.05	0.375 ± 0.007	0.375 ± 0.020
Orchard leaves	4	10.0 ± 2.0	10.64 ± 0.12	10.71 ± 0.44
Citrus leaves	4	3.1 ± 0.3	3.10 ± 0.04	3.15 ± 0.06
Tomato leaves	7	0.27 ± 0.05	0.311 ± 0.027	0.270 ± 0.018
Pine needles	6	0.21 ± 0.04	0.211 ± 0.006	0.198 ± 0.012
Oyster tissue	6	13.4 ± 1.9	13.78 ± 0.10	14.14 ± 0.26
Dogfish muscle[b]	3	30.8 ± 4.2	26.4 ± 0.5	28.2 ± 0.6
Scallops[b]	3	6.0 ± 0.9	5.45 ± 0.05	6.13 ± 0.02
Swordfish muscle[b]	3	4.1 ± 1.0	3.72 ± 0.03	4.16 ± 0.09

[a] Uncertainty expressed as 95% confidence level for NBS Standard Reference Materials.

[b] NRCC materials not certified and uncertainty expressed as standard deviation of round-robin results after rejection of outliers (8).

Table 4. Levels and recovery of arsenic(V) spikes from foods analyzed in a routine manner as part of a total-diet survey

Food	Peak ht		Peak area	
	Level,[a] ng/g	Rec.,[b] %	Level, ng/g	Rec., %
White bread	5.7 ± 0.5	102 ± 5	10.0 ± 0.3	102 ± 3
Donuts	5.0 ± 0.1	104 ± 2	4.9 ± 1.2	94 ± 0.2
Crackers	9.3 ± 1.8	95 ± 1	9.7 ± 3.7	86 ± 3
Wheat cereal	<3	103 ± 4	<3	104 ± 2
Whole milk	<0.4	92 ± 2	<0.8	88 ± 5
Peanut butter	12.4 ± 2.4	97 ± 4	16.8 ± 3.1	91 ± 5
Eggs	<0.4	96 ± 3	<2	98 ± 5
Luncheon meat	4.9 ± 0.4	107 ± 4	3.8 ± 0.8	98 ± 1
Raw beef muscle	14.7 ± 0.6	96 ± 7	16.2 ± 2.1	98 ± 6
Raw pork muscle	5.1 ± 0.8	98 ± 3	9.4 ± 0.6	97 ± 4
Apple juice	4.5 ± 0.1	96 ± 1	2.5 ± 1.3	100 ± 2
Apple sauce	<0.25	101 ± 0.1	<0.25	101 ± 1
Strawberries	3.8 ± 1.5	103 ± 0.2	3.6 ± 1.1	102 ± 1
Broccoli	2.4 ± 0.5	107 ± 0.6	3.1 ± 0.1	101 ± 0.6
Cauliflower	1.7 ± 0.3	92 ± 2	1.8 ± 0.2	98 ± 0.1
Baked potato	3.3 ± 1.0	94 ± 1	6.2 ± 0.8	90 ± 1
Boiled potato	3.9 ± 1.1	103 ± 2	2.0 ± 1.0	102 ± 2
Coffee (instant)	0.3 ± 0.07	101 ± 0.1	0.5 ± 0.07	98 ± 0.6
White sugar	<2.5	99 ± 7	<2.5	97 ± 7

[a] Uncertainties given as standard deviation. Two replicates were run for each of the spiked and unspiked samples.
[b] Spiking level, 400 ng arsenic(V).

A hypothesis of the mechanism for the effect described above was that the yellow color was due to interference from iodine. Addition of ascorbic acid reduced the excess iodine back to iodide.

Precipitation and Dissolution of Arsenic

The buffer solution of the original arsenic method (1) was modified so that a pH meter was not required on a routine basis.

Dissolution time for the precipitate was generally overnight. However, depending on sample type and weight and on pH of precipitation, 2 days were sometimes needed. The best indicators of complete dissolution were a clean filter and the absence of any dark brown residue on the bottom of the test tube.

Reduction of Sulfate and Cationic Interferences

Preliminary tests of the coprecipitation method revealed that, during dissolution of the precipitate, some APDC was being converted to sulfate, which interferes with arsenic in the furnace (7, 8). Since the amount of APDC in the precipitate depended on the concentration of other ions, such as iron and copper, in the food item, the interference was food related. This may explain why liver samples, which contain high concentrations of other cations, gave poor recoveries (1).

Addition of barium nitrate after dissolution of the sample eliminated the interference, and good recoveries ensued for all sample types. Two possible reasons for the reduction of the sulfate interference were removal of the sulfate by precipitating barium sulfate and interference reduction due to the presence of the barium ions themselves, simulating the sulfate interference reduction observed in the presence of magnesium ions (7). It was not necessary to filter the barium sulfate from the solution prior to analysis.

Preliminary investigations revealed that copper(II) and iron(III) caused, respectively, positive and negative interferences in the graphite furnace. The concentrations of these ions vary extensively in different foods and the elements are coprecipitated along with arsenic; thus, copper(II) and iron(III) were added as matrix modifiers to reduce interferences their presence in some foods might cause. A more complete discussion of choice of modifier will be presented in another publication.

Accuracy and Precision

Table 3 demonstrates that both peak heights and area were accurate for arsenic in NBS SRMs and NRCC round-robin samples (9). Recovery of arsenic(V) from foods analyzed in a routine manner as part of a dietary intake study ranged from 92 to 107% and averaged 99.2% for peak heights. Peak area recoveries averaged 97.1% (range 86–104%) (Table 4). Recovery from liver samples averaged 102%. In comparison, the earlier variation of the method gave recoveries averaging 61.3% for liver samples and 89.4% (range 77–102%) for other foods (1).

Precision averaged 2.2% RSD for peak heights and 3.3% RSD for peak areas for the NBS and NRCC materials (Table 3). For the foods in Table 4, precision for the spiked samples averaged 2.6% RSD for both peak heights and areas. Precision at the natural arsenic levels in Table 4 averaged 17% for peak heights and 21% for peak areas.

Blanks and Detection Limit

For routine analysis of foods, 3 blanks were included in each analytical series. The mean blank level over 20 analytical series was 1.7 ng arsenic absolute (range, −12.4 to 17.4 ng) for peak heights and 0.6 ng (range, −13.9 to 17.0 ng) for peak areas. The presence of negative blanks may have been due to a negative baseline during atomization because the instrument autozero was performed on a cold furnace before each atomization.

The mean standard deviation of replicate blanks over 20 analytical series was ±2.7 ng arsenic absolute (range ±4 to ±5.6) for peak heights and ±3.8 ng (range ±1 to ±8.2 ng) for peak areas. The detection limit of the method (3× the mean SD of blanks) averaged 8 ng for peak heights and 11 ng for peak areas.

Organoarsenic Compounds

Results for the NBS oyster tissue and 3 NRCC materials (Table 3) indicate applicability of the method to organoarsenic compounds, because Lawrence et al. (10) found that 71–91% of the arsenic in 25 fresh- or seawater fish and crustaceans, including scallops, was in the form of arsenobetaine, arsenocholine, or an unidentified methanol-extractable arsenic compound. Good recovery for sodium methyl arsenate, sodium methyl arsonate, arsenobetaine, and cacodylic acid can also be expected on the basis of results in Table 2.

GFAAS Conditions

Because the modifier elements, iron and copper, were present in large concentrations and because they atomize at high temperatures, the atomization temperature and times (Table 1) were chosen so that gradual buildup of the elements on the platform and within the tube would not occur. Thus, inclusion of a 1–2 s clean-out step immediately after atomization appeared to be the only critical factor in the temperature program.

Application of other models of graphite furnace may require modifications to the furnace parameters according to manufacturer's instructions. For instance, for larger-diameter graphite tubes used with a L'vov platform, a longer clean-out time may be required to remove the modifier elements

completely from the tube. The good agreement between peak height and area results indicates that the method may even work with atomization from the wall in the absence of a platform.

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Gas Chromatographic Determination of Mecarbam and Its Metabolites Mecarboxon, Diethoate, and Diethoxon in Cottonseeds

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A gas chromatographic method is described for determining residues of mecarbam and 3 of its metabolites, mecarboxon, diethoate, and diethoxon, in cottonseeds. For mecarbam analysis, following Soxhlet extraction with chloroform (after blending), the oily extract is partitioned with propylene carbonate and cleaned up on a silica gel column. Metabolites are extracted by the same method, followed by cleanup of mecarboxon on a silica gel column or diethoxon on an alumina column; cleanup of diethoate can be performed on either column. All 4 compounds are determined using a flame photometric detector equipped with a phosphorus filter. Average recoveries for cottonseed samples fortified with 0.03–1.0 ppm mecarbam ranged from 80 to 88%. Average recoveries were 81–88% for mecarboxon and 90–92% for diethoate (alumina column) and diethoxon from samples fortified with 0.05–1.0 ppm. Average recovery of diethoate from samples cleaned up on the silica gel column were 84–88% in the range of 0.05–0.2 ppm. Values obtained for mecarbam residues in field-treated samples are also presented.

Mecarbam, *S*-(*N*-ethoxycarbonyl-*N*-methylcarbamoylmethyl)-*O,O*-diethylphosphorodithioate (also known as Murfatox), is an organophosphorus compound having the structural formula $(EtO)_2P(S)\cdot SCH_2CO\cdot NMe\cdot CO\cdot OEt$. It is a slightly systemic insecticide and acaricide used to control scale insects, olive fly, and other fruit flies (1).

The 3 main metabolites of mecarbam, as described in studies of mecarbam degradation (2), are mecarboxon, structural formula $(EtO)_2P(O)S\cdot CH_2CO\cdot NMe\cdot CO\cdot OEt$, the oxygen analog of mecarbam; diethoate, structural formula $(EtO)_2P(S)SCH_2CO\cdot NHMe$; and diethoxon, structural formula $(EtO)_2P(O)SCH_2CONHMe$, the oxygen analog of diethoate.

A gas chromatographic (GC) method using a thermionic detector has been described for determining residues of the parent compound in several vegetables and in olive oil (3). In vegetables, the method involves extracting with hexane or hexane–acetone and partitioning with acetonitrile; in olive oil, acetonitrile is used for the extraction. In both cases the extract is cleaned up in 2 steps: charcoal and Florisil. As far as could be determined, there are no published methods for determining the metabolites.

The present work describes a procedure in which Soxhlet extraction with chloroform after blending is used to determine mecarbam and its 3 principal metabolites. For mecarbam determination, the extract is partitioned with propylene carbonate–petroleum ether (4, 5), cleaned up on a silica gel column, and quantitated. For determining the metabolites, the extraction procedure is followed only by column chromatographic cleanup of the oily extract and by quantitation. GC determination with a flame photometric detector (FPD) is used for all 4 compounds. Samples of cottonseed treated with mecarbam in an agricultural trial were also analyzed.

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METHOD

Apparatus

(a) *Gas chromatograph.*—Tracor Model 560 equipped with flame photometric detector set at 525 nm. GC columns: for mecarbam analysis, glass, 1.8 m × 3 mm id, packed with 5% OV-101 on 80–100 mesh Chromosorb W(HP); for metabolite analysis, glass, 1.25 m × 3 mm id, packed with 4% OV-17 on 80–100 mesh Gas Chrom Q. Operating conditions for all 4 compounds: gas flows (mL/min), nitrogen carrier 40, hydrogen 60, air 110; Westronics M/T recorder (Tracor), chart speed 0.4 cm/min; temperatures (°C), column oven 185, detector 200, injector 220.

(b) *Rotary evaporator.*—Heildolph (Kelheim, FRG), or equivalent.

(c) *Blender.*—Osterizer liquifier blender (Milwaukee, WI), or equivalent.

(d) *Chromatography columns.*—Small glass column, 30 × 0.75 cm id with capillary opening and funnel at top; large glass column, 40 × 1.70 cm id with 0.28 cm opening and funnel (ca 200 mL) at top.

(e) *Centrifuge tubes.*—100 and 200 mL with ground glass stoppers.

(f) *Filter paper.*—Whatman glass microfiber.

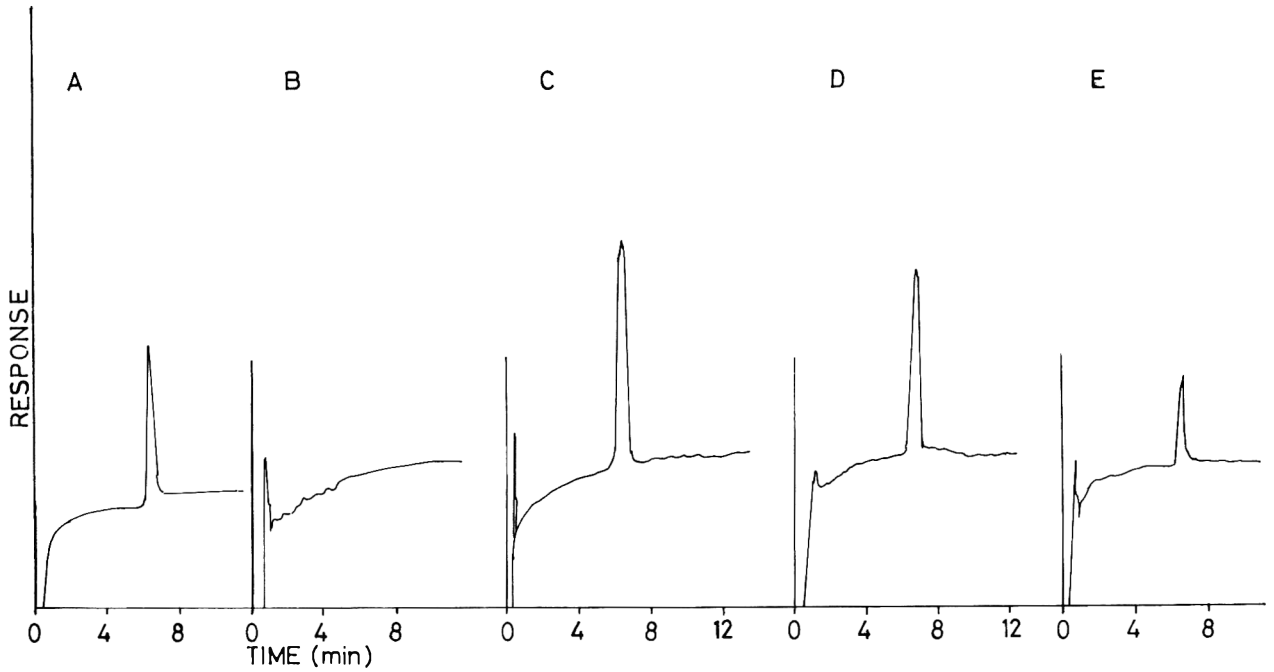

Figure 1. Chromatograms of mecarbam in cottonseeds, using 5% OV-101 GC column: A, 5 ng mecarbam standard (retention time relative to parathion, 1.36 min; RT for parathion, 4.1 min); B, extract equivalent to 70 mg cottonseeds; C, extract equivalent to 28 mg cottonseeds fortified with 0.3 ppm mecarbam; D, extract equivalent to 49 mg from field-treated sample (application 1.25 kg a.i./ha); E, extract equivalent to 70 mg cottonseeds fortified with 0.03 ppm mecarbam.

Reagents

(a) *Solvents.*—Petroleum ether (bp 40–60°C), analytical grade; chloroform; distilled-in-glass acetone (Frutarom Chemical Laboratory, Haifa, Israel); propylene carbonate, chemically pure ≥99% (Fluka, Buchs, SG, Switzerland) (d_4^{20}1.20).

(b) *Silica gel.*—70–230 mesh ASTM, Kieselgel 60 (Merck Chemical Co., Darmstadt, FRG).

(c) *Aluminum oxide-neutral.*—Fluka type 507C. Heat 4 h at 425°C, cool, add water (10%) with shaking, equilibrate 24 h.

(d) *Anhydrous granular sodium sulfate.*—(Merck). Heat 5–6 h at 130°C.

(e) *Mecarbam standard and metabolites mecarboxon, diethoxon, and diethoate.*—Supplied by Murphy Chemicals, Ltd, Wheathampstead, UK.

(f) *Stock solution.*—1 mg mecarbam/mL acetone and 0.5 mg mecarboxon, diethoate, or diethoxon/mL acetone.

Field-Treated Samples

In an agricultural trial conducted by the Luxembourg Chemical Co., cottonseed received 3 sprayings of 1.25 kg a.i./ha (formulation 50% a.i.) at 5-day intervals. Cottonseed samples were taken 8 days after last application. Three replicated samples of 500 g each from treated and untreated cottonseeds were supplied for analysis. To obtain information about the behavior of mecarbam in field-treated plants, we analyzed 2 samples of each of the 3 replicates. To determine metabolite residues, we analyzed one sample of each of the 3 field replicates. In the analysis for diethoate, one of the 3 samples was subjected, for purposes of comparison, to cleanup on both alumina and silica gel columns.

Extraction of Mecarbam and Its Metabolites from Cottonseed

The same extraction method can be used for all 4 compounds. The amount of cottonseed required depends on the number of compounds to be analyzed. The method described is for a 100 g sample of cottonseed, which is enough for the analysis of all 4 compounds.

Weigh 100 g (or 60 g with a corresponding 60% volume of solvent for determination of mecarbam residues only) of a well-mixed representative field sample of cottonseeds into 400 mL beaker. Add 150 mL chloroform and let stand 1–2 h. Because better blending is achieved with smaller amounts, transfer seeds in 2 portions to blender, add 140 mL solvent to each portion, and blend 30 s at low speed and 2½ min at high speed. Suction blended mixture through Whatman glass microfiber, using 2 filter papers. Rinse blender and extracted seeds with ca 100 mL chloroform. Quantitatively transfer extracted seeds to thimbles for continuous Soxhlet extraction with chloroform for 6 h. Dry chloroform extracts with anhydrous sodium sulfate and evaporate chloroform obtained from the 2 extraction steps in a weighed round-bottom flask on a rotary evaporator at ca 40°C. (Perform all evaporations this way.) Add a few mL of petroleum ether and evaporate again. Weigh flask again to calculate percentage of oil in cottonseed sample. Use chloroform-free extract for analysis. From 20–22 g oil extract, which is the amount usually obtained from 100 g cottonseed, use 10 g for mecarbam in the solvent partitioning step (4); transfer 4 g to silica gel column for mecarboxon and diethoate cleanup; and transfer 2 g to alumina column for diethoate and diethoxon cleanup.

Analysis of Mecarbam

Solvent partitioning.—Weigh 10 g cottonseed oil extract into small centrifuge tube, add 12 mL propylene carbonate, shake 2 min, and centrifuge 10 min at 1500 rpm. Pipet propylene carbonate phase (lower layer) into small separatory funnel. Again extract the oil in centrifuge tube with 8 mL propylene carbonate and add propylene carbonate layer obtained after centrifugation to first portion in separatory funnel. Allow any traces of oil to separate and drain off propylene carbonate into a beaker, leaving ca 1 mL (which contains remaining traces of oil). Transfer 10 mL aliquot (of total of

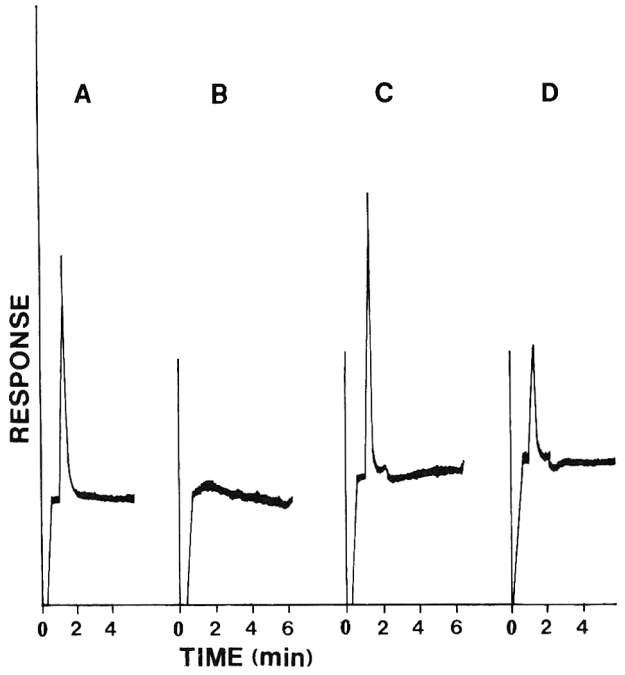

Figure 2. Chromatograms of mecarboxon in cottonseeds, using 4% OV-17 GC column: A, 24 ng mecarboxon standard (retention time relative to parathion, 0.28 min; RT for parathion, 4.6 min); B, extract equivalent to 156 mg cottonseeds; C, extract equivalent to 150 mg cottonseeds fortified with 0.2 ppm mecarboxon; D, extract equivalent to 160 mg cottonseeds fortified with 0.05 ppm mecarboxon.

ca 20 mL) of propylene carbonate extract to large (200 mL) centrifuge tube, add 50 mL each petroleum ether and water, and shake vigorously 2 min. Centrifuge and dry petroleum ether phase (46–48 mL volume, equivalent to 4.6–4.8 g oil) with anhydrous sodium sulfate held in a funnel. Concentrate petroleum ether extract to a few milliliters for silica gel cleanup step.

Silica gel chromatography.—Place small glass wool plug at bottom of small chromatographic column, add 4.5 g silica gel, and top with 1.5 cm layer of sodium sulfate. Tap column to settle adsorbent. Quantitatively transfer petroleum ether extract by rinsing flask with a total of 15 mL petroleum ether followed by 2 additional rinses of 3 mL 3% acetone in petroleum ether. Elute column with 100 mL 3% acetone in petroleum ether. Evaporate solvent almost to dryness and dissolve residue in acetone (minimum 3.0 mL) for GC/FPD determination.

Analysis of Metabolites

Silica gel chromatography.—Use for cleanup of mecarboxon and diethoate. Procedure consists of 2 successive cleanup steps on different-sized columns using silica gel as adsorbent. First, pack large column (described under *Apparatus*) by adding, in order, a glass wool plug, 12 g silica gel, and 1.5 cm anhydrous sodium sulfate. Transfer 4 g cottonseed oil extract dissolved in 40 mL petroleum ether to the dry column. Rinse flask 4–5 times with a total of 125 mL of 2% acetone in petroleum ether. Fraction 1 contains most of the oil and can be discarded. Maintain flow rate of 5–6 mL/min. Use 230 mL of 10% acetone in petroleum ether as second eluting solvent to elute mecarboxon residues (fraction 2). To recover diethoate from this column, change receiver and use third eluting solvent of 200 mL of 20% acetone in petroleum ether (fraction 3). Evaporate solvent of fraction 3 and add at least 2 mL acetone for GC determination.

Table 1. Recovery of mecarbam, mecarboxon, diethoate, and diethoxon from cottonseeds

	Rec., %[a]				
			Diethoate		
Added, ppm	Mecarbam	Mecarboxon	Alumina column	Silica gel column	Diethoxon
1.0	88 ± 5.1	88 ± 3.5	91 ± 1.9	—[b]	91 ± 1.9
0.5	86 ± 6.3	—	—	—	—
0.3	84 ± 1	—	—	—	—
0.2	—	86 ± 2.4	92 ± 0	88 ± 3.5	90 ± 0
0.1	84 ± 1	86 ± 3	90 ± 1.9	84 ± 0	92 ± 3.8
0.05	—	81 ± 0.9	90 ± 0	84 ± 3.8	90 ± 0
0.03	80 ± 1.1	—	—	—	—

[a] Values shown are average ± standard deviation of triplicate (mecarbam and mecarboxon) or duplicate (diethoate and diethoxon) analysis.
[b] Blanks indicate no recovery tests performed.

Some oil remains after evaporation of the solvent of fraction 2, which contains mecarboxon, and so, additional cleanup is performed using another silica gel column, this time a small one (described under *Apparatus*). Add 5 mL petroleum ether to oily residue (free of acetone) left after evaporation of the second eluate (fraction 2) and transfer to chromatographic column packed with silica gel as described for mecarbam cleanup. Rinse flask 3–4 times with 2% acetone in petroleum ether from a total of 90 mL eluting solvent used for first elution. Add 30 mL of 10% acetone in petroleum ether to column to remove last traces of oil. Change receiving flask and use 110 mL of 10% acetone in petroleum ether to elute mecarboxon residues. Evaporate solvent almost to dryness and dissolve in at least 1 mL acetone for GC determination.

Alumina column chromatography.—Use for cleanup of diethoate and diethoxon. Place a small glass wool plug at the bottom of small chromatographic column. Add 5 g alumina and cover with 2 cm anhydrous sodium sulfate. Transfer 2 g chloroform-free cottonseed oil extract dissolved in 20 mL petroleum ether to column. Rinse flask thoroughly 3–4 times with 3% acetone in petroleum ether and transfer to column. Use a total of 60 mL of 3% acetone in petroleum ether for rinsing and elution. Change receiving flask and add 100 mL of 20% acetone in petroleum ether to elute diethoxon and diethoate. Maintain flow rate of 2–3 mL/min for all elutions from the small column. (If the elution rate is too slow during washings, increase it by exerting slight pressure with glass stirring rod on sodium sulfate layer.) Evaporate solvent and dissolve residue in at least 1 mL acetone for GC determination.

GC analysis.—Use 2 columns for quantitation: 5% OV-101 column for mecarbam and 4% OV-17 column for the metabolites. Use peak height measurement for determination of all 4 compounds. Retention times for the metabolites on the 4% OV-17 column are different, thus enabling simultaneous determination. Note that since diethoate can be eluted from both alumina (together with diethoxon) and silica gel columns (fraction 3), quantitation of all 3 metabolites would still be possible even if there were no differences in retention times on a particular GC column.

Results and Discussion

For analysis of recovery, standard solution of mecarbam or of metabolites was added directly to the cottonseeds about 1 h before solvent was added. In most cases, cottonseeds were fortified with all 3 metabolites together. In a few cases, cottonseeds were fortified with diethoate alone or with die-

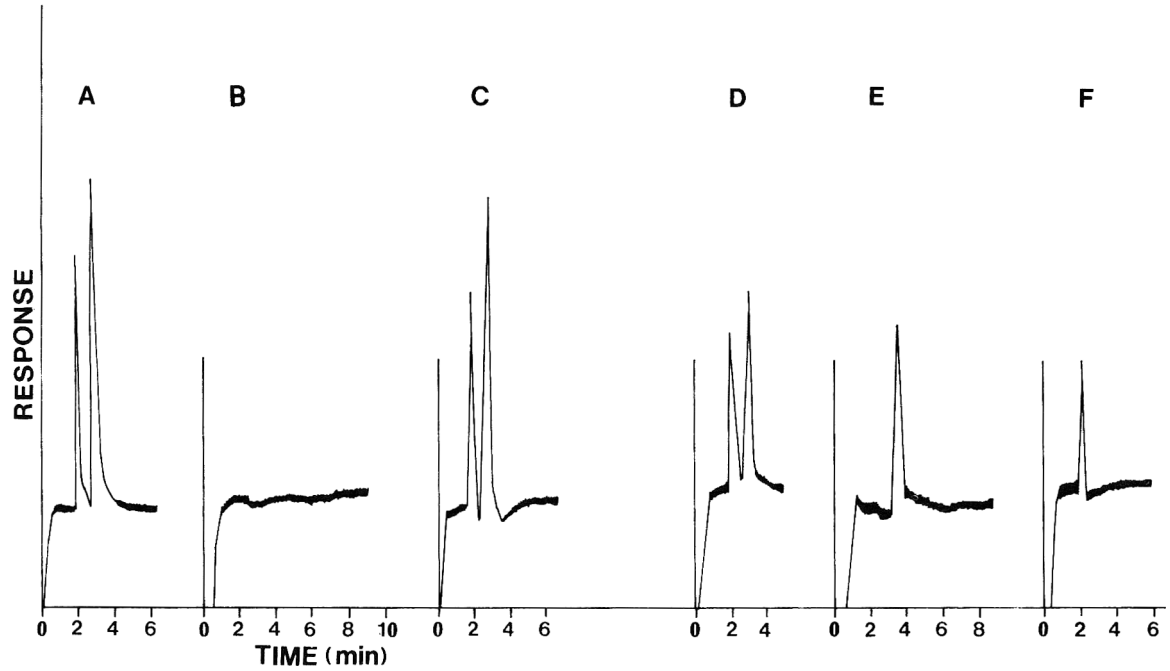

Figure 3. Chromatograms of diethoate and diethoxon in cottonseeds, cleaned up on an alumina column: A, 12 ng diethoate standard (retention time relative to parathion, 0.7 min) and 12 ng diethoxon standard (retention time relative to parathion, 0.5 min); B, extract equivalent to 83 mg cottonseeds; C, extract equivalent to 66 mg cottonseeds fortified with 0.2 ppm diethoate and diethoxon; D, 3.5 ng diethoate standard and 3.5 ng diethoxon standard; E, extract equivalent to 83 mg cottonseeds fortified with 0.05 ppm diethoate; F, extract equivalent to 87 mg cottonseeds fortified with 0.05 ppm diethoxon.

thoxon plus mecarboxon as an additional check for interfering peaks on the chromatogram of alumina column eluant.

The average recovery of mecarbam from cottonseeds fortified at levels of 0.03–1.0 ppm was 80–88% (Table 1). Typical chromatograms of a standard, untreated cottonseed sample and of samples fortified with 0.3 and 0.03 ppm are shown in Figure 1. Table 1 also shows recoveries of mecarboxon, diethoate, and diethoxon from cottonseeds fortified with different levels of metabolites. At spiking levels of 0.05–1.0 ppm, mecarboxon recovery was 81–88%, and recoveries of diethoate (alumina column) and diethoxon were 90–92%. For diethoate eluted from the large silica gel column, recovery was 84–88% at spiking levels of 0.05–0.2 ppm (Table 1). Typical chromatograms of standards, untreated products, and samples fortified with metabolites at 0.05–0.2 ppm are shown in Figures 2–4. Figures 3 and 4 show chromatograms of diethoate eluted from 2 different columns; in chromatograms C and E of Figure 3, diethoate is eluted from an alumina column, and in chromatogram B of Figure 4, it is eluted from the silica gel column.

The recommended maximum amounts (mg) injected for the 4 compounds are those indicated in each case on the chromatograms. Their volumes injected from the final extract solutions (see *Analysis of Mecarbam* and *Analysis of Metabolites*) were 8 μL for mecarbam, 9 μL for mecarboxon, and 10 μL for diethoate and diethoxon.

From the characteristics of the chromatograms of samples fortified at low levels and from the absence of interfering peaks in the untreated samples, the limit of detection based on an average signal-to-noise ratio of 5 (6) was about 0.015 ppm for mecarbam, diethoate, and diethoxon, and 0.020 for mecarboxon.

For all 3 metabolites, 3 columns are required in the cleanup step. If only diethoate and diethoxon are to be analyzed, an alumina column is sufficient, and if mecarboxon alone or mecarboxon and diethoate are to be analyzed, only silica gel columns are required. In the cleanup of mecarboxon, an attempt was made to use a single small silica gel column and only 1.5 g oil extract instead of using 2 columns for oil removal. However, it afforded lower recovery values and poorer reproducibility.

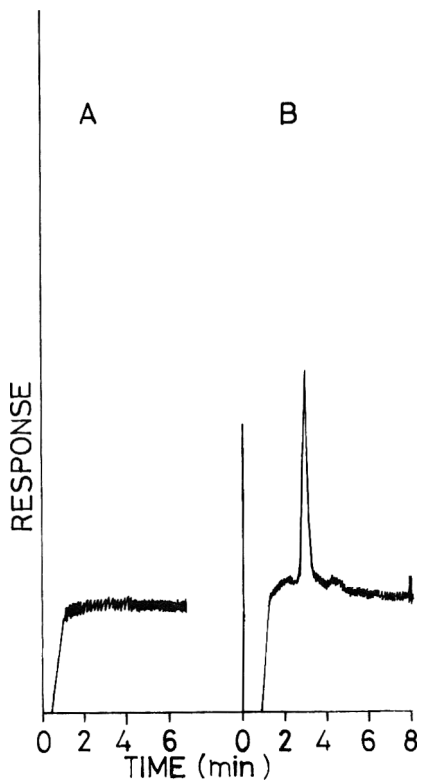

Figure 4. Chromatograms of diethoate in cottonseeds cleaned up on a silica gel column: A, extract equivalent to 83 mg cottonseeds; B, extract equivalent to 72 mg cottonseeds fortified with 0.05 ppm diethoate.

To determine the behavior of all 4 compounds on the GC column, the OV-17 column was tested also for mecarbam. In addition to mecarbam standard, final extracts of an untreated cottonseed sample and of a fortified sample were analyzed. About the same retention time relative to parathion, 1.40 on OV-17 vs 1.36 on OV-101, and response for mecarbam standard and similar chromatograms for the cottonseed samples were found on both GC columns.

In the analysis of field-treated samples (3 replicates, 2 samples each), the average values and standard deviations (ppm) for mecarbam residues were 0.11 ± 0.014, 0.148 ± 0.01, and 0.085 ± 0.035. The overall average and standard deviation for all 6 samples was 0.11 ± 0.04 ppm. A chromatogram of a field-treated sample containing 0.16 ppm mecarbam is shown in Figure 1D. None of the extracts of field-treated samples displayed peaks at the retention time of mecarboxon, diethoate, or diethoxon.

Of all the alternative methods used in working with cottonseeds in our laboratory, the extraction method described here, which combines advantages of 2 ways of extraction—namely, blending and Soxhlet—seems to be the most suitable. We have used it in recent years in the analysis of organophosphates, synthetic pyrethroids, and carbamates.

Acknowledgments

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Two Enzyme Immunoassays to Screen for 2,4-Dichlorophenoxyacetic Acid in Water

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Two solid-phase enzyme immunoassays were developed to measure 2,4-dichlorophenoxyacetic acid (2,4-D), using 2 sets of structurally distinct immunogens and enzyme ligands. The 2,4-D analog, 2-methyl-4-chlorophenoxyacetic acid (MCPA), gave a similar response with both methods, whereas other phenoxy herbicides cross-reacted differently. In method A, the aromatic moiety of 2,4-D was distal from the carrier protein and labeled enzyme, whereas in method B, the acetic acid portion of the herbicide was distal. The use of both methods to screen for this herbicide in ground water and municipal and river water reduced the number of false-positive responses. Water sources having a low background response could be monitored with either method alone. When a concentration step, with disposable C_{18} extraction columns, was used, the limit of sensitivity was 5 μg/L. Method A was the more sensitive of the 2 methods with a limit of detection of 10 μg/L without the concentration step.

The legally established limit for the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in potable water is 0.1 ppm (1). As an important pollutant, it is monitored in municipal water and ground water. It is most commonly determined by gas chromatography (GC) after derivatization to an ester (2). A radioimmunoassay (RIA) has been described for the determination of 2,4-D in ground water (3); however, many laboratories are not equipped for handling radioisotopes. Herein are described 2 enzyme immunoassay (EIA) methods that may be used together or alone to detect 2,4-D in water.

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Experimental

Apparatus

(a) *Extraction column.*—C_8 (Baker 7087-6). Before extraction of sample, wash column with 5 mL methanol followed by 5 mL water.

(b) *Gas chromatograph.*—Tracor Model 222 equipped with ^{63}Ni electron-capture detector and 2 mm × 1.6 m glass column packed with 10% OV-1 on 80–100 mesh Gas-Chrom Q. Operating conditions: column, 160°C, increase to 220°C at 15°/min, starting at time of injection; injector, 230°C; detector, 290°C; carrier gas, nitrogen at 40 mL/min; detector gas, nitrogen at 50 mL/min; detector sensitivity, 32 × 10^{-10} AFS.

(c) *Liquid scintillation counter.*

Reagents

(a) *Solvents.*—Reagent grade.

(b) *2,4-D[2-^{14}C].*—(Amersham Corp., Arlington Heights, IL 60005), 28 mCi/mmol. Check for radiochemical purity with silica gel thin layer plates.

(c) *Developing solvents.*—Diethyl ether–hexane–formic acid (70 + 30 + 2) and ethyl acetate–formic acid (98 + 2).

(d) *Analytical reference standards of chlorophenoxy acids.*—Obtained from EPA Standards Repository, Research Triangle Park, NC.

(e) *Internal standard.*—Standard toluene[^{14}C].

(f) *Solutions.—Pi buffer.*—0.1M sodium phosphate, 1mM

$MgCl_2$, 3mM NaN_3 (pH 7.4). *Assay buffer.*—Pi buffer supplemented with 0.1mM dithioerythritol. *Wash buffer.*—Pi buffer supplemented with 0.1% Tween 20. *Substrate solution.*—Assay buffer supplemented with 100 mg 4-methylumbelliferyl-β-galactoside/L. *Coating buffer.*—0.025M sodium carbonate (pH 9.0).

(g) *Immunogen A.*—To solution of 42 mg 2,4-D[^{14}C] (0.19 mmol, 2 μCi ^{14}C) and 22 mg *N*-hydroxysuccinimide (0.19 mmol) in 2 mL dioxane, add 39 mg (0.19 mmol) *N,N'*-dicyclohexylcarbodiimide in 0.5 mL dioxane. Let solution stand overnight at room temperature, then filter to remove precipitate. Remove solvent under reduced pressure at 35°C. To residue, add 3 mL 0.15M sodium borate (pH 9) containing 500 mg bovine serum albumin (BSA). After 45 min at room temperature, dialyze solution against Pi buffer, then with 0.1M ammonium formate, and finally against 2 changes of water. Freeze-dry dialyzed solution. To estimate amount of 2,4-D[^{14}C] bound to protein, dissolve 5 mg portion of preparation in 1 mL water containing 10 mg 2,4-D diethylamine salt. After 60 min, pass solution through Sephadex G-75 column. Measure ^{14}C found with void volume and use this value to estimate herbicide bound to protein. Typically, ca 15 moles of 2,4-D is bound per mole of protein.

(h) *Immunogen B.*—Prepare 2-chloro-4-nitrophenoxyacetic acid by the method of Brown and McCall (4). Dissolve 5 g of this compound in 60 mL ethyl acetate and add 0.8 g 5% Pd/C. With stirring, treat for 1 h with H_2 at atmospheric pressure to obtain 2-chloro-4-aminophenoxyacetic acid. Recrystallize from methanol–water (mp is greater than 250°C [5]). Stir suspension of phenoxyamino acid (160 mg, 0.9 mmol) in 4 mL of 1.0M HCl at 2–5°C and add 80 mg (1.17 mmol) sodium nitrite in 1 mL water over 3 min period. Continue stirring and cooling another 5 min. Add and dissolve 30 mg solid ammonium sulfamate. Add 2.0 mL of this solution dropwise to 500 mg porcine thyroglobulin in 40 mL of 1M sodium carbonate (pH 9.5), while stirring and cooling in ice-water bath. Continue stirring and cooling 2 h after addition is complete. Pass deep-red solution through Sephadex G-75 column equilibrated with 0.05M ammonium formate. Collect material eluting at void volume and freeze-dry. Repeat reaction using BSA in place of thyroglobulin.

(i) *Antisera.*—Dissolve immunogen in 0.9% saline (2–4 mg/mL) and homogenize with equal volume of Freund's complete adjuvant. Immunize rabbits every 10–14 days for 3 months with 0.5 mL portions of homogenized immunogen. Alternate use of BSA and thyroglobulin conjugates. Inject intramuscularly in flank, but on every third inoculation inject material subcutaneously at 6–8 sites on the back. Treat antisera with equal volume of 70% saturated ammonium sulfate. Centrifuge, then take up the pellet in 0.5 mL of 0.9% saline and dialyze against several changes of this solution. Add equal volume of glycerol to dialyzed solution and store globulin fraction at −20°C.

(j) *Labeled enzyme A.*—Prepare 2,4-D hydrazide by the method of Chao et al. (6). Reflux 1.7 g (7.8 mmol) 4-maleimidobenzoic acid in 20 mL toluene and 20 mL thionyl chloride for 60 min. Cool and remove solvents under reduced pressure at 35°C. Add 15 mL toluene and remove again under the same conditions. Take up acid chloride in 20 mL dry tetrahydrofuran (THF). With cooling, add acid chloride to stirred solution of 1.8 g 2,4-D hydrazide in 20 mL THF. Stir 60 min, then reflux 30 min. The product, 1-(4-maleimidobenzoyl)-2-(2,4-dichlorophenoxyacetyl)hydrazine (ligand A), separates on cooling. Recrystallize from dimethylformamide (DMF)–water (5 + 2) [mp = 240–242°C; ^{1}H NMR: δ(Me_4Si, DMSO-d_6–$CDCl_3$, 1 + 1) = 7.2–8.1 (*m*, 9H, aromatic), 4.8 (*s*, 2H, CH_2), 3.3 (*s*, 2H, NH—NH)].

To remove thiol reagents from commercial *E. coli* β-galactosidase (Sigma 5635), pass 2500 units through Sephadex G-75 column (1 × 15 cm) equilibrated with Pi buffer. Pool fractions containing enzyme (2.7 mL) and dilute with 50 μL DMF containing 50 μg ligand A. After 3 h, separate derivatized enzyme (labeled enzyme A) from unreacted ligand by chromatography on Sephadex G-100 equilibrated with assay buffer. Dilute labeled enzyme A with equal volume of glycerol and store at −20°C.

Estimate enzyme activity remaining after conjugation reaction and chromatography by the method of Dray et al. (7) using *ortho*-nitrophenyl-β-galactoside. (Sixty percent of the activity was recovered.)

(k) *Labeled enzyme B.*—Reflux 660 mg (3.2 mmol) 4-maleimidobenzoic acid in 10 mL toluene and 10 mL thionyl chloride 1 h and work up the preparation as described above. Dissolve acid chloride in 15 mL THF and add 600 mg (3 mmol) 2-chloro-4-aminophenoxyacetic acid. Stir suspension 60 min and reflux 30 min. Add 5 mL water while solution is warm. Let cool and obtain pale yellow crystals of 2-chloro-4-(4-maleimidobenzamido)phenoxyacetic acid (enzyme–ligand B). Recrystallize from ethanol [mp = 225–227°C; ^{1}H NMR: δ(Me_4Si, DMSO-d_6) = 7.2–8.3 (*m*, 9H, aromatic), 8.7 (*bs*, 1H, NH), 4.9 (*s*, 2H, CH_2)].

Remove thiol reagents from *E. coli* β-galactosidase (5000 units) using Sephadex chromatography as described above. Cool enzyme solution (2 mL) and dilute with 20 μL solution containing 10 mg enzyme–ligand B in 3 mL DMF. After 3 h, separate derivatized enzyme (labeled enzyme B) from unreacted ligand by chromatography on Sephadex G-100 equilibrated with assay buffer. Dilute with equal volume of glycerol and store at −20°C. (Fifty-six percent of the enzyme activity was recovered.)

Extraction of 2,4-D from Water Samples

Procedure is essentially that described by Yip (8). To water samples (1.0 L), add 5 mL 6M H_2SO_4 and store in covered glass containers in the dark at 4°C for up to 50 days (9). To extract 2,4-D, pass 50 mL aliquot of acidified water sample through prewashed C_8 column according to manufacturer's directions. Wash column with 5 mL of 0.01M HCl, then force gentle stream of air through column to remove excess water. Elute column with 5 mL methanol and collect eluate in small tube. Remove methanol by warming solution at 40°C while under stream of nitrogen gas. Take up residue in 1.0 mL Pi buffer. Use this solution directly in assay protocol.

Coating Titer Determination

Prepare serial dilutions (1:2 or 1:3) of antisera in coating buffer, starting at 1:1000 dilution. Add 1.0 mL portions of 95% ethanol to 12 × 75 mm polystyrene tubes. After 30 min, drain tubes and dry. Add 0.6 mL portions of immunoglobulin dilutions to duplicate tubes. Incubate 4 h at 37°C or 18 h at room temperature. Aspirate coating solution and rinse each tube once with 1.0 mL wash buffer and twice with 1.0 mL Pi buffer. To each tube add 0.009 unit of appropriate labeled enzyme in 0.5 mL assay buffer and incubate overnight (18 h) at room temperature. Aspirate solution and wash the tubes twice with 1.0 mL portions of assay buffer. Add 0.5 mL substrate solution and incubate 60 min at 37°C. Stop reaction with 1.0 mL 1M Na_2CO_3. Measure fluorescence with excitation at 360 nm and emission at 448 nm. Choose di-

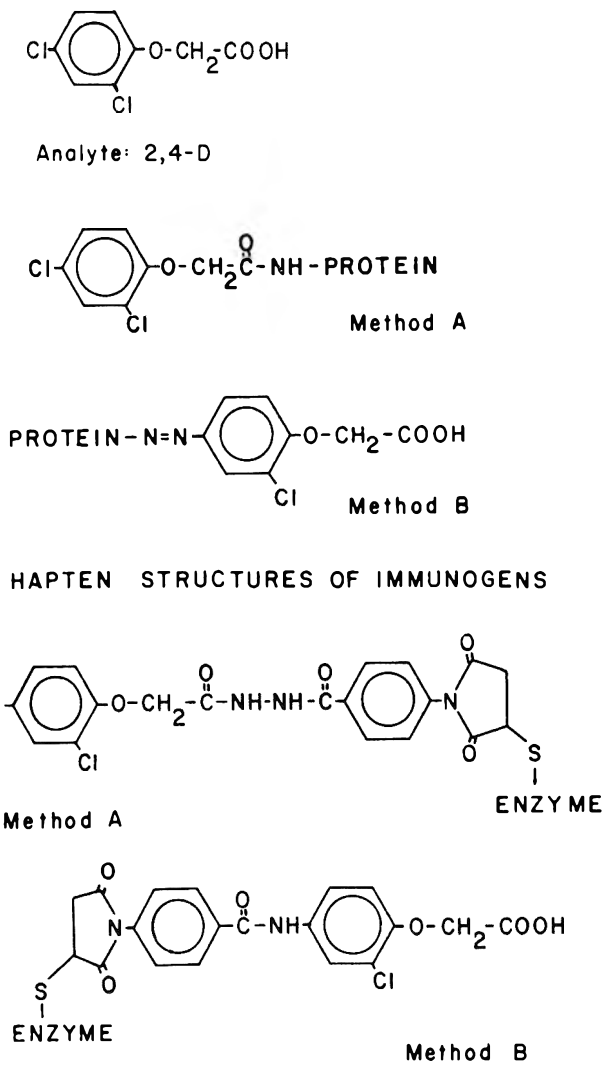

Figure 1. Structures of 2,4-D, the haptens of immunogens A and B, and the enzyme labels for methods A and B.

lution of antisera for use in immunoassay which binds ca 60% of maximum amount of labeled enzyme.

Assay Protocol

Coat and wash tubes as described for titer determination. To each tube add 0.1 mL sample, standard, or control solution in Pi buffer and 0.4 mL diluted labeled enzyme containing same total activity used in coating protocol. Incubate overnight (18 h) at room temperature. Aspirate solution and wash twice with 1.0 mL portions of assay buffer. Add 0.5 mL substrate solution and incubate 60 min at 37°C. Add 1.0 mL of 1M Na_2CO_3 and determine fluorescence. Prepare standard curves from tubes containing between 10 and 500 ng 2,4-D per tube. Determine each concentration in triplicate. Replicate blank for standard curve with a minimum of 12 tubes using Pi buffer. Concentration of 2,4-D that gives response 3 times the standard deviation of the mean blank response is limit of detection. Analyst must establish that distilled water carried through concentration protocol gives response similar to blanks.

Results and Discussion

Figure 1 shows the structures of the hapten moieties of the immunogens and the enzyme labels used in assay methods A and B. In method A, the distal portion of the hapten and enzyme label represent the aromatic moiety of the herbicide. In method B, the acetic acid moiety of 2,4-D is represented in the distal portion of the hapten and enzyme label. The antibodies produced to these haptens were expected to be most specific to the distal portion of the group.

Table 1. Cross-reactivity of antisera

	Cross-reactivity[a]	
	EIA method A	EIA method B
2,4-D	1.00	1.00
2,4-D amide	8.33	<0.003
2,4-D butoxypropyleneglycol ester	3.57	<0.003
4-(2,4-Dichlorophenoxy)butyric acid	0.50	<0.003
2,4,5-Trichlorophenoxyacetic acid	0.05	1.00
2-(2,4-Dichlorophenoxy)propionic acid	0.07	<0.003
3-(2,4-Dichlorophenoxy)propionic acid	0.35	<0.003
2-Methyl-4-chlorophenoxyacetic acid	0.60	0.72
2,4-Dichlorophenol	0.10	<0.005
Diclofop (acid)	<0.05	<0.003
Fluazifop (acid)	<0.003	<0.003

[a] Cross-reactivity is defined here as nmol 2,4-D causing 50% displacement of labeled enzyme per nmol of tested compound causing the same displacement.

Both methods detected 2-methyl-4-chlorophenoxyacetic acid (MCPA) nearly as well as 2,4-D (Table 1). Other phenoxy herbicides such as the esters of 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB) cross-reacted well with only one of the 2 methods. Esters of 2,4-D are rapidly hydrolyzed to 2,4-D in the environment (10), and the amide of 2,4-D is not used as an herbicide (11); therefore, these compounds are less likely to appear in ground or surface waters. When both assay methods are used, only 2,4-D and MCPA should give similar responses in either method.

The limit of quantitation for the study was the concentration of 2,4-D that gave a response 3 times the standard deviation of the mean of the blank response for the least sensitive method (method B). This was 5 ng/mL of 2,4-D in the solution to be extracted, or 50 ng/tube. The method A limit of quantitation under these conditions was 1 ng/mL; however, the recovery experiments were carried out with samples fortified at 5 ng/mL.

The recovery of 2,4-D[^{14}C] with the microcolumns was 94 ± 2% (n = 18) when ground water and river water were fortified at 5.0 ng/mL. The groundwater samples (n = 16) from this experiment were analyzed by the EIA methods. Method A gave an assay mean of 5.4 ± 1.9 ng/mL, and method B assay gave 5.2 ± 2.4 ng/mL.

The performance of an RIA method developed earlier in this laboratory (3) was not compared to the present EIA methods. The preparation of the iodinated ligand used in the RIA was necessary every 2 months, whereas the ligand-labeled enzymes have been stable over a 12 month period. The performance of the RIA varied with each preparation of the iodinated ligand.

The use of both EIA methods was helpful in evaluating some false-positive responses obtained when analyzing river water samples from sources in North Dakota and Minnesota. Method A indicated 7 of 11 samples contained >5 ng/mL 2,4-D, and method B indicated 4 of 11 samples as positive. Only 2 samples of the 11 were positive by both methods. Gas chromatography indicated all 11 samples contained <0.5 ng/mL of 2,4-D, MCPA, or 2,4,5-T. Sixty-nine groundwater samples were also analyzed by EIA, but no positive responses

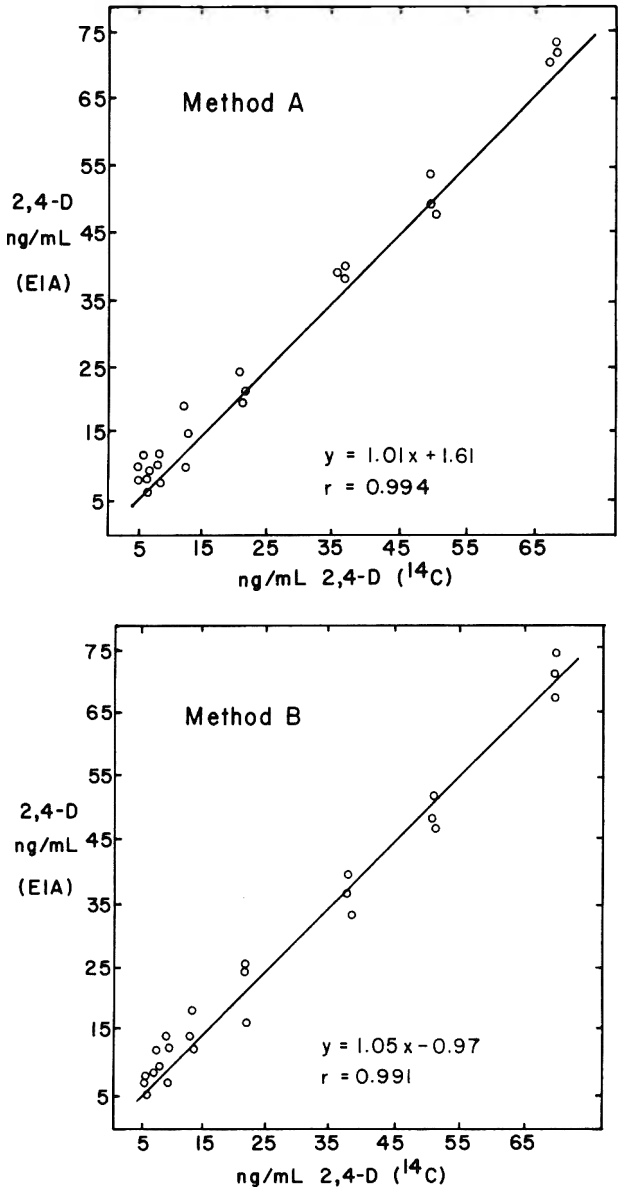

Figure 2. Comparison of the 2,4-D[^{14}C] levels measured by the immunoassay methods, to ^{14}C recovered. Water samples were fortified with ^{14}C-labeled herbicide and carried through a concentration step. Concentrates were assayed for ^{14}C and for 2,4-D by EIA methods A and B.

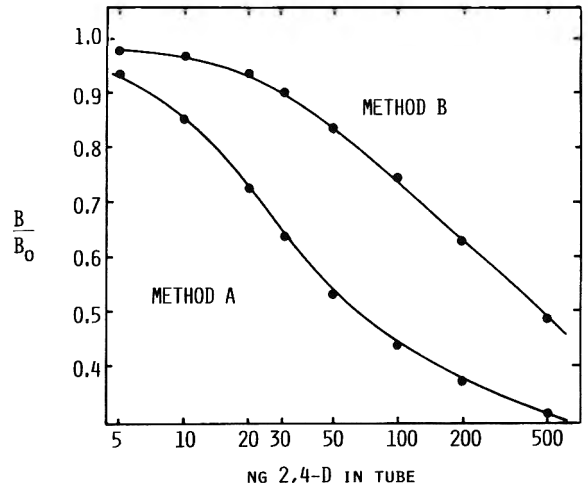

Figure 3. Standard curves for EIA methods A and B.

were obtained by EIA and no gas chromatography was carried out on these samples.

Substances present in ground water or surface water may affect the immunoassay technique. For instance, chaotropic agents such as detergents may be present in sufficient concentration to reduce the interaction between antibody and labeled enzyme and cause a false-positive response. Another source of false-positive responses (sample-matrix effects) is synthetic or natural substances that weakly interact with the antibody compared to the strong interaction by the analyte but are present at a sufficiently high concentration to give a false-positive value with the assay. A substance that interferes with one immunoassay system may interfere to a much different extent in a different immunoassay. The use of 2 immunoassay methods for 2,4-D, consisting of 2 different antibodies and 2 different enzyme-ligand structures for the same analyte, is an attempt to overcome partially the sample-matrix variation one may expect from ground water and surface water of diverse composition. When diverse water sources are analyzed, the matrix variation limits an immunoassay to screening purposes rather than quantitation. For a given water source, the compositional variation may be small, and then an immunoassay may be useful for quantitation.

Six groundwater samples that were known to contain <0.5 ng/mL of 2,4-D, MCPA, or 2,4,5-T were fortified with 2,4-D[^{14}C] at levels ranging from 5.0 to 70 ng/mL. The fortified samples were concentrated with the microcolumns and assayed by EIA methods A and B and by ^{14}C determination. The results are shown in Figure 2. Standard response curves are shown in Figure 3.

On a routine basis, the heterogeneous EIA technique described here can be used to analyze a large number of samples more rapidly than gas chromatography. A water source that chemically varies little from day to day, such as municipal water or ground water, could be analyzed by either of the 2 methods alone. In this case, method A could be used without a concentration step.

Immunoassay methods have been described for diflubenzuron (12), chlorsulfuron (13), diclorfop-methyl (14), atrazine (15), and paraquat (16, 17). Different labels were used in these methods, either fluorescent, enzyme, or radioisotopic, but all were solid-phase methods in which a polystyrene surface was coated with a protein-ligand conjugate or antibody. The solid-phase method, although it was used here, has a disadvantage in the time required for the coating and washing steps. An alternative technique, the homogeneous immunoassay, offers the advantage of eliminating these steps and should be examined for analysis of environmental samples.

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Gas Chromatographic Relative Retention Data for Pesticides on Nine Packed Columns: II. Organophosphorus and Organochlorine Pesticides, Using Electron-Capture Detection

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The retention time relative to parathion, absolute retention time, concentration range, peak asymmetry factor, and peak shape class are given for each of 42 organophosphorus pesticides and 28 organochlorine pesticides analyzed by gas chromatography (GC) on 9 different packed columns. The packing materials used were 3% SP-2100, 1% Dexsil-300, 3% OV-17, 1.5% OV-17 + 1.95% QF-1, 4% SE-30 + 6% QF-1, 3% OV-17 + 3% OV-210, 5% DC-200 + 7.5% QF-1, 3% Carbowax-20M, and 4% Reoplex-400. Retention data were determined at 200°C with a carrier gas flow at $\bar{u}_{opt}$, using a ^{63}Ni electron-capture detector. Results should be useful for preliminary identification of environmental samples and also for single or multiple pesticide residue analysis.

In continuation of the work done on relative retention data (1), it was thought necessary to generate data for the organochlorine as well as the organophosphorus pesticides on 9 packed columns by using a ^{63}Ni electron-capture detector (ECD). ECD is useful for single-component and multiresidue environmental samples containing organochlorine pesticides, but it can also be used to detect most organophosphorus pesticides.

A publication by Bot and Hollings (2) was used as a guide to choose the organophosphorus and organochlorine compounds. Furthermore, organochlorine metabolites as well as discontinued organochlorine pesticides such as dieldrin, endrin, and DDT, were included because they are still found in the environment (3, 4).

As previously stated, the trend in residue analysis is to use capillary columns (5), but packed columns are still widely used as illustrated in the most recent Transmittal Notices of the U.S. Environmental Protection Agency Pesticide Analytical Manual (6) where most analytical techniques for pesticides still specify packed columns.

The same criteria used to establish the previous retention index (1) are used again with some minor modifications.

Experimental

Apparatus

A Varian Model 6000 gas chromatograph, equipped with ^{63}Ni ECD, was coupled to a Varian Model 401 data system (Varian Instrument Group, Palo Alto, CA 94303). Operating conditions were as follows: temperatures (°C), injection port 225, column oven 200, and detector 280; nitrogen carrier gas flow was set at $\bar{u}_{opt}$ for each column, as determined with parathion.

Reagents

Reagents were the same as used in the first study (1), except that atmospheric air was used to determine gas holdup time for the calculation of average linear gas velocities.

Column Preparation

The same 9 columns evaluated previously (1) were used to obtain the results for the 2 pesticide groups with electron-capture detection.

Column Evaluation

An oven temperature of 200°C with an optimal carrier gas flow determined with parathion was used to evaluate all columns. The height equivalent to one theoretical plate (HETP) was determined as described previously (1). As mentioned earlier, atmospheric air instead of butane was used to determine the average linear gas velocity of each column by injecting ca 8 μL to produce an unretained peak. The number of theoretical plates (n) at $\bar{u}_{opt}$ for each column was the same as previously reported (1).

GC Procedure

The GC procedure was exactly as previously reported (1). In addition, special care was taken to use only pretested solvents and specially cleaned glassware to avoid contaminant peaks and abnormally wide solvent peaks that could interfere with the measurement of early-eluting peaks.

Table 1. Peak data of the 9 columns evaluated

Column	No. of compounds eluted	No. of compounds in each class[a]			
		1	2	3	4
3% SP-2100	48	21	19	6	2
1% Dexsil-300	48	8	35	4	1
3% OV-17	48	22	21	4	1
1.5% OV-17 + 1.95% QF-1	51	2	42	3	4
4% SE-30 + 6% QF-1	55	12	34	5	4
3% OV-17 + 3% OV-210	41	17	19	2	3
5% DC-200 + 7.5% QF-1	45	21	22	0	2
3% Carbowax-20M	43	18	19	5	1
4% Reoplex-400	48	10	34	3	4

[a] Class refers to peak symmetry.

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Table 2. Alphabetical list of all the organophosphorus and organochlorine pesticides tested on nine packed columns using electron capture detection

Pesticide	3% SP-2100			1% Dexsil-300			3% OV-17			1.5% OV-17 + 1.95% QF-1			4% SE-30 + 6% QF-1			3% OV-17 + 3% OV-210			5% DC-200 + 7.5% QF-1			3% Carbowax-20M			4% Reoplex-400		
	RRT[a]	As[b]	Dql[c]	RRT	As	Dql	RRT	As	Dql	RRT	As	Dql	RRT	As	Dql	RRT	As	Dql	RRT	As	Dql	RRT	As	Dql	RRT	As	Dql
Aldrin	1.00	1.0	A	0.70	1.5	A	0.75	1.0	A	0.57	1.3	A	0.52	1.3	A	0.61	1.3	A	0.46	1.0	A	0.33	1.4	A	0.29	1.1	A
Azinphos-et	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Azinphos-me	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alpha BHC	0.48	1.0	A	0.38	1.9	A	0.39	1.1	A	0.32	1.6	A	0.27	1.5	A	–	–	–	0.08	1.4	A	0.26	1.0	A	0.04	1.2	A
Beta BHC	0.53	1.0	B	0.46	1.1	A	0.55	1.2	A	0.44	1.5	A	0.34	1.4	A	0.47	1.3	A	0.34	1.3	A	0.75	1.1	A	0.22	1.1	A
Gamma BHC	0.79	1.1	A	0.46	1.8	A	0.49	1.2	A	0.38	1.4	A	0.33	1.3	A	0.42	1.5	A	0.32	1.0	A	0.10	3.2	A	0.12	1.3	A
Delta BHC	0.60	1.1	A	0.53	1.3	A	0.66	1.3	A	0.54	1.7	A	0.39	1.4	A	0.56	1.1	A	0.39	1.2	A	0.18	1.5	A	0.22	1.5	A
Bromchlorphos	–	–	–	–	–	–	–	–	–	0.33	2.4	C	0.29	1.8	C	–	–	–	–	–	–	–	–	–	–	–	–
Bromophos-et	1.40	1.0	A	1.20	1.4	B	1.35	1.2	A	1.04	1.3	B	0.91	1.5	B	1.11	1.0	C	0.80	1.0	B	0.76	1.0	C	0.55	1.3	C
Bromophos-me	1.09	1.2	A	0.95	1.6	B	1.09	1.4	B	0.85	1.6	C	0.73	1.0	C	0.90	1.3	C	0.65	1.1	C	–	–	–	1.54	1.6	C
Chlordane	1.22	1.4	B	1.32	1.6	A	1.23	1.5	A	1.02	1.5	A	0.88	1.4	A	1.04	1.2	A	0.78	1.1	A	0.75	1.4	A	0.76	1.1	A
Chlorfenvinphos	0.35	2.1	B	0.45	3.5	B	1.38	3.5	A	1.29	6.2	B	1.15	4.4	B	0.24	1.5	A	0.20	1.3	B	0.20	2.3	A	0.91	1.6	B
Chlormephos	0.28	2.0	A	0.26	2.0	C	0.16	1.1	B	0.14	1.2	B	0.12	1.3	C	0.13	1.6	B	0.13	1.7	C	0.10	1.1	B	0.08	1.3	B
Chlorobenzilate	–	–	–	–	–	–	–	–	–	1.83	4.8	C	1.47	3.8	C	0.89	4.2	C	0.70	4.3	C	–	–	–	–	–	–
Chlorpyrifos-et	0.97	1.5	B	0.83	1.7	B	0.94	1.2	B	0.73	1.5	B	0.61	1.0	B	0.77	1.1	B	0.56	1.0	B	0.50	1.0	B	0.44	1.3	B
Chlorpyrifos-me	0.76	1.2	B	0.66	1.7	B	0.68	1.2	C	0.52	1.5	B	0.42	1.3	C	0.57	1.1	B	0.41	1.0	B	0.44	1.3	C	0.35	1.3	B
op DDD	1.74	1.0	B	1.64	1.6	A	1.90	1.4	A	1.42	1.4	A	1.40	1.5	A	1.45	1.1	A	0.92	1.2	A	1.45	1.3	B	1.47	1.9	B
pp' DDD	2.12	1.1	B	2.22	1.2	A	2.40	1.2	B	1.87	1.5	B	1.06	1.4	A	1.87	1.2	A	1.24	1.4	B	1.95	1.2	B	2.51	1.8	A
op DDE	1.67	1.3	A	1.51	1.5	A	1.62	1.1	A	1.21	1.5	B	0.97	1.5	B	1.23	1.1	A	0.82	1.2	B	1.06	1.1	B	0.89	1.6	B
pp' DDE	1.66	1.0	A	1.51	1.5	A	1.62	1.2	A	1.21	1.7	B	0.97	1.5	B	1.23	1.1	A	0.81	1.2	B	1.05	1.2	B	0.89	1.8	B
op DDT	2.18	1.1	B	1.95	1.2	C	2.30	1.4	C	1.69	1.3	C	1.68	1.2	C	1.44	2.8	B	1.04	1.2	C	1.42	1.2	C	1.32	2.1	C
pp' DDT	1.67	1.5	B	2.45	1.4	C	1.63	1.3	B	2.22	1.1	C	1.21	1.2	C	1.86	1.6	C	1.40	1.3	B	1.03	3.3	B	0.97	2.7	B
Demeton S-me	0.46	8.0	C	–	–	–	0.32	3.0	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.26	1.8	D
Diazinon	0.57	1.3	B	0.43	2.0	C	0.45	1.0	C	0.35	1.3	C	0.31	1.1	C	0.36	1.0	B	0.30	1.0	B	0.20	1.2	C	0.16	1.2	C
Dichlorvos	0.24	1.8	C	–	–	–	0.11	3.5	B	0.12	2.5	B	0.10	3.3	B	–	–	–	–	–	–	0.70	1.6	C	0.06	1.5	C
Dicloran	0.56	1.6	B	0.56	1.3	A	0.49	1.5	A	0.44	3.5	A	0.41	2.5	A	0.46	1.6	A	0.40	1.3	B	0.72	1.3	B	0.74	2.0	A
Dicofol	1.06	2.8	B	0.99	2.5	A	1.02	2.3	A	0.83	1.9	B	0.71	2.3	A	0.86	2.1	A	0.71	1.4	A	0.82	1.5	A	0.81	1.6	A
Dicrotophos	–	–	–	–	–	–	–	–	–	–	–	–	1.14	3.0	D	–	–	–	–	–	–	–	–	–	–	–	–
Dieldrin	1.73	1.2	A	1.55	1.4	A	1.64	1.3	A	1.30	1.6	A	1.14	1.4	A	2.02	1.1	A	1.02	1.3	A	1.01	1.3	A	1.04	1.4	A
Dimethoate	0.34	1.7	C	–	–	–	0.56	1.8	C	0.60	5.8	D	–	–	–	–	–	–	–	–	–	–	–	–	1.12	1.5	D
Disulfoton	0.61	1.1	C	0.54	1.7	C	0.51	1.0	B	0.41	1.4	B	0.37	1.5	B	0.41	1.3	C	0.35	1.1	C	0.28	1.0	B	0.22	1.3	C
Endrin	–	–	–	2.61	1.1	A	–	–	–	1.59	2.0	B	1.30	1.4	B	–	–	–	–	–	–	1.11	1.1	B	1.17	1.6	B
Alpha Endosulfan	1.50	1.0	A	1.29	2.0	A	1.35	1.3	A	1.07	1.4	B	0.96	1.1	B	1.09	1.1	A	0.88	1.2	B	0.74	1.2	A	0.81	2.0	B
Beta Endosulfan	2.04	1.6	B	2.08	2.0	A	2.39	1.1	A	1.92	1.5	A	1.50	1.4	A	2.98	1.1	A	1.39	1.3	A	0.32	1.0	A	2.49	2.3	B
Endosulfan SO4	2.58	1.6	C	2.93	1.3	A	3.33	1.8	A	3.14	1.6	A	2.75	1.3	A	0.49	1.8	B	2.64	1.5	A	0.33	3.0	A	1.12	3.6	B
Fenchlorphos	1.34	1.0	C	0.71	6.4	C	–	–	–	0.59	1.3	C	0.52	1.7	B	0.60	1.2	C	0.48	1.2	C	0.50	1.4	C	0.44	1.2	C
Fenitrothion	0.88	2.5	B	0.86	1.8	B	0.96	1.5	D	0.91	1.8	B	0.88	1.6	B	0.93	1.6	C	0.85	1.2	C	–	–	–	1.12	1.5	C
Fenthion	0.94	2.0	B	–	–	–	1.03	1.0	B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Folpet	–	–	–	–	–	–	–	–	–	–	–	–	1.10	1.9	C	–	–	–	–	–	–	0.67	4.2	D	0.54	6.5	D
Formothion	–	–	–	–	–	–	0.80	1.5	C	–	–	–	0.76	4.0	C	1.32	2.8	C	–	–	–	–	–	–	–	–	–
Heptachlor	0.83	1.0	A	0.68	2.0	A	0.62	1.3	A	0.46	1.3	A	0.44	1.2	A	0.50	1.3	A	0.40	1.5	A	0.30	1.0	A	0.28	1.2	A
Heptenophos	0.19	1.6	B	–	–	–	0.13	1.0	B	0.10	1.6	B	0.08	1.8	B	0.09	3.7	C	0.09	1.1	A	0.07	2.0	B	0.07	2.4	B
Isofenphos	1.25	1.5	C	1.06	1.3	D	1.24	1.7	C	1.05	1.5	C	0.93	1.7	D	1.09	1.7	D	0.86	1.3	B	0.76	1.3	C	0.72	1.4	C
Jodfenphos	1.58	1.8	B	1.45	1.1	C	1.87	1.1	B	1.42	1.3	C	1.05	1.0	B	0.40	1.2	C	0.90	1.0	C	–	–	–	1.26	1.3	C
Leptophos	–	–	–	5.04	2.2	C	–	–	–	5.05	1.4	C	–	–	–	–	–	–	2.51	1.7	D	–	–	–	–	–	–
Malathion	0.93	2.1	C	0.81	2.6	C	0.99	1.5	C	–	–	–	0.78	1.4	B	–	–	–	0.76	1.4	B	0.75	1.8	C	0.73	1.3	B
Methamidophos	–	–	–	4.76	2.1	D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Methidathion	–	–	–	1.37	1.3	D	–	–	–	–	–	–	1.20	1.8	D	–	–	–	–	–	–	–	–	–	–	–	–
Mevinphos	0.56	2.5	C	–	–	–	0.18	2.8	C	0.21	3.6	C	0.22	7.0	D	–	–	–	–	–	–	0.14	1.5	C	0.13	1.9	C
Parathion-et(REF)	1.00	1.2	B	1.00	1.3	B	1.00	1.1	B	1.00	2.4	C	1.00	1.2	B	1.00	1.2	B	1.00	1.2	B	1.00	1.2	B	1.00	1.3	B
Parathion-me	0.79	2.6	B	0.71	1.8	B	0.79	1.2	C	0.79	1.6	C	0.77	1.4	C	0.78	1.6	B	0.77	1.6	C	–	–	–	1.07	1.7	C
Phenthoate	–	–	–	1.08	1.0	B	–	–	–	1.23	1.5	C	0.95	1.2	B	–	–	–	0.84	1.4	B	1.17	1.3	C	1.05	1.5	B
Phorate	0.46	1.1	B	0.40	1.4	B	0.34	1.0	B	0.29	1.3	B	0.25	1.5	C	0.28	1.5	B	0.25	1.3	C	0.19	1.0	C	0.16	1.1	B
Phosalone	–	–	–	5.98	1.2	D	–	–	–	6.77	1.5	D	5.00	1.7	C	–	–	–	–	–	–	–	–	–	–	–	–
Pirimiphos-me	0.85	1.6	C	0.73	1.6	D	0.82	1.3	D	–	–	–	0.52	1.4	D	0.67	1.2	C	–	–	–	0.48	1.9	D	–	–	–
Profenofos	–	–	–	1.01	1.3	D	–	–	–	1.63	2.1	C	1.39	3.0	C	–	–	–	–	–	–	–	–	–	1.09	1.1	D
Propetamphos	0.55	1.3	B	0.44	3.5	C	0.44	1.0	B	0.40	1.5	C	0.38	1.6	C	0.41	1.9	C	0.36	1.2	C	0.41	1.7	B	0.36	1.0	C
Prothiophos	1.62	1.2	B	–	–	–	1.60	1.3	D	1.25	1.5	C	1.03	1.0	D	1.39	1.3	D	0.89	1.5	C	0.89	1.2	D	0.36	1.5	C
Pyrazophos	–	–	–	7.48	2.0	D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tetrachlorvinphos	0.49	2.5	B	0.50	2.3	C	0.36	1.8	B	0.31	2.3	B	0.29	1.4	C	1.52	1.7	C	0.29	1.8	C	0.26	2.5	B	0.34	2.6	C
Tetradifon	4.64	2.7	B	5.40	1.3	C	6.47	1.3	D	5.89	1.5	D	4.54	1.4	C	–	–	–	4.10	1.9	D	–	–	–	–	–	–
Thiometon	0.49	1.1	B	0.44	1.0	C	0.42	1.0	C	0.34	1.5	B	0.29	1.2	C	–	–	–	0.28	1.5	C	0.28	2.5	C	0.26	1.3	B
Triadimefon	1.35	5.0	C	–	–	–	0.97	5.3	C	0.96	8.5	D	0.97	2.5	C	1.21	6.0	D	0.98	4.6	D	0.87	2.0	C	–	–	–

Peak Shape

The peak classification criteria described in the previous study (1) were used, where the following 4 classes of peak shapes were distinguished: 1, asymmetry factor < 1.2; 2, > 1.2 but ≤ 2.4; 3, > 2.4 but ≤ 3.6; and 4, > 3.6. Class 1 is essentially a symmetrical peak.

Detectable Quantity Levels for Pesticides

The same criteria described before (1) were applied to the electron-capture detector. The electron-capture detector was not affected by the low average linear gas velocities, and much lower concentration levels could be detected. Four new quantity levels were thus selected and given the following symbols: A = 1–10 pg; B = 10–100 pg; C = 100–1000 pg; D = 1000–10 000 pg.

Results and Discussion

The peak shape classes, order of elution, absolute retention times, relative retention times of all the pesticides, and the exact absolute retention times of chlorpyrifos-ethyl and parathion are presented in Figures 1–9. A summary of the number of compounds that were detected on each column as well as the number in each class is presented in Table 1. Retention times relative to parathion, peak asymmetry factors, and concentration ranges for all pesticides are presented in Table 2.

If the relative retention times of the organophosphorus pesticides in Table 2 are compared with the relative retention times in Table 2 of the previous paper (1), it seems that the majority of data for a given column agree, irrespective of the detector used. Certain pesticides such as tetrachlorvinphos and heptenophos showed large differences on almost all columns evaluated. Some pesticides showed differences only on specific columns, e.g., bromchlorphos (OV-17 + QF-1) and dicrotophos (SE-30 + QF-1). The possibility that the differences are due to error is ruled out because of precautionary measures described earlier (1).

These differences can be ascribed to the thermolabile nature of the relevant pesticides and the fact that thermal degradation may take place the moment the pesticide is injected into a heated GC column (7). It could, therefore, be that the part of the pesticide containing a phosphorus atom may separate from the part to which the ECD is sensitive. Detection of the pesticide by electron capture and by the flame photometric detector (FPD) at the same retention time does not necessarily indicate that the pesticide did not break down, but that both detectors are sensitive to the same thermal degradation product.

Recent literature (8) indicates that chlorpyrifos is preferred as a reference compound for relative retention data. Unfortunately, much of the data for this paper had already been obtained by using parathion when that study was published. The absolute retention times of both parathion and chlorpyrifos in Figures 1–9 are reported so that the data can easily be converted to those for chlorpyrifos as reference compound.

The same average linear gas velocities were used for each column as described previously (1). According to Kirkland et al. (9), serious errors in plate counts can occur if the equation $n = (t_R/W_h)^2$ is used on even moderately tailing peaks. Three columns gave asymmetry values of more than 1.2 with parathion, namely, the Dexsil-300, the OV-17 + QF-1, and the Reoplex-400. In the previous paper (1), 6 columns gave asymmetry values of more than 1.2. This anomaly cannot be explained, but it could be attributable to the detector because all other conditions and parameters were the same for both studies. The reasons for using parathion to determine $\bar{u}_{opt}$ in spite of the fact that it gives asymmetry values of more than 1.2 on some columns was previously discussed (1).

Acephate, chlordimeform, diflubenzuron, fenamiphos, monocrotophos, oxydemeton-me, omethoate, phoxim, temephos, triazophos, and trichlorfon did not elute within 2 h ± 10 min from any of the columns used, whereas other pesticides did not elute from certain columns (shown by dashes). The reason may be that the pesticides have very short retention times and eluted with the solvent front, or that they eluted after the 2 h time limit, or that the electron-capture detector was not sensitive to the compound or its thermal degradation products. Some compounds exhibited multiple peaks, which probably indicate decomposition or impurities, and in these cases the major peak was used to determine the given data.

On the SP-2100 column, 48 compounds were detected, of which 40 fell into classes 1 and 2, and 8 fell into classes 3 and 4. On the Dexsil-300 column, most compounds fell into class 2 with only a few in class 1 and classes 3 and 4. These 2 columns may be more suitable for single-component analysis since separation between peaks is very poor.

The distribution of peaks on the OV-17 column was better than on the previous 2 columns, but problems with separation might still be encountered in certain retention time slots, particularly if multiresidue analysis is undertaken. A reasonable separation was achieved among the organochlorines (shaded peaks, Figure 3) except near the 14 min slot. On this column, 48 compounds were detected, the vast majority of which fell into classes 1 and 2.

On the OV-17 + QF-1 column, 51 peaks were detected of which 42 occurred in class 2. Separation problems occurred at various retention times but reasonable separation among the organochlorine compounds (shaded peaks, Figure 4) was found. All of the organochlorine peaks fell into classes 1 and 2, with the last peak (endosulfan sulfate) eluting at ca 30 min, making the column most suitable for quantitative and qualitative analysis of organochlorine pesticides.

The SE-30 + QF-1 column produced the most peaks (55) with 46 in classes 1 and 2. Problems with separation occurred near the 5 min and 17 min slots. Separation of the organochlorines was reasonable.

The smallest number of peaks (41) was detected on the OV-17 + OV-210 column. Most of the peaks fell into classes 1 and 2 and were almost evenly distributed between the 2 classes; only 5 components fell into classes 3 and 4. The components were well distributed over the whole time range with separation problems occurring at ca 9, 19, and 26 min. As indicated by the shaded peaks in Figure 6, this column is not suitable for separation of the organochlorine compounds tested.

On the DC-200 + QF-1 column, 45 peaks were detected. Minor separation problems can occur with this column at the 7 and 9 min slots (Figure 7). The peaks were well distributed over the whole time range, making this column ideal for multiresidue analyses. For single component analyses it is also a valuable column because almost all of the peaks fell into classes 1 and 2; only 2 peaks fell into class 4. Separation of the organochlorine pesticides was good, but a slight problem occurred at 9–10 min and 33–34 min.

On the Carbowax-20M column, 43 pesticides were detected. Major separation problems occurred at ca 1–5, 11–12, and 14–17 min. This column is not suitable for multiresidue analyses.

The Reoplex-400 column produced 48 peaks with the majority occurring in class 2. The peaks were reasonably well distributed over the 82 min time period with some separation problems.

It is clear from the results that no one column was able to separate all the compounds that were detected. Some columns performed better than others, but the data reported should give the analyst a large choice of columns.

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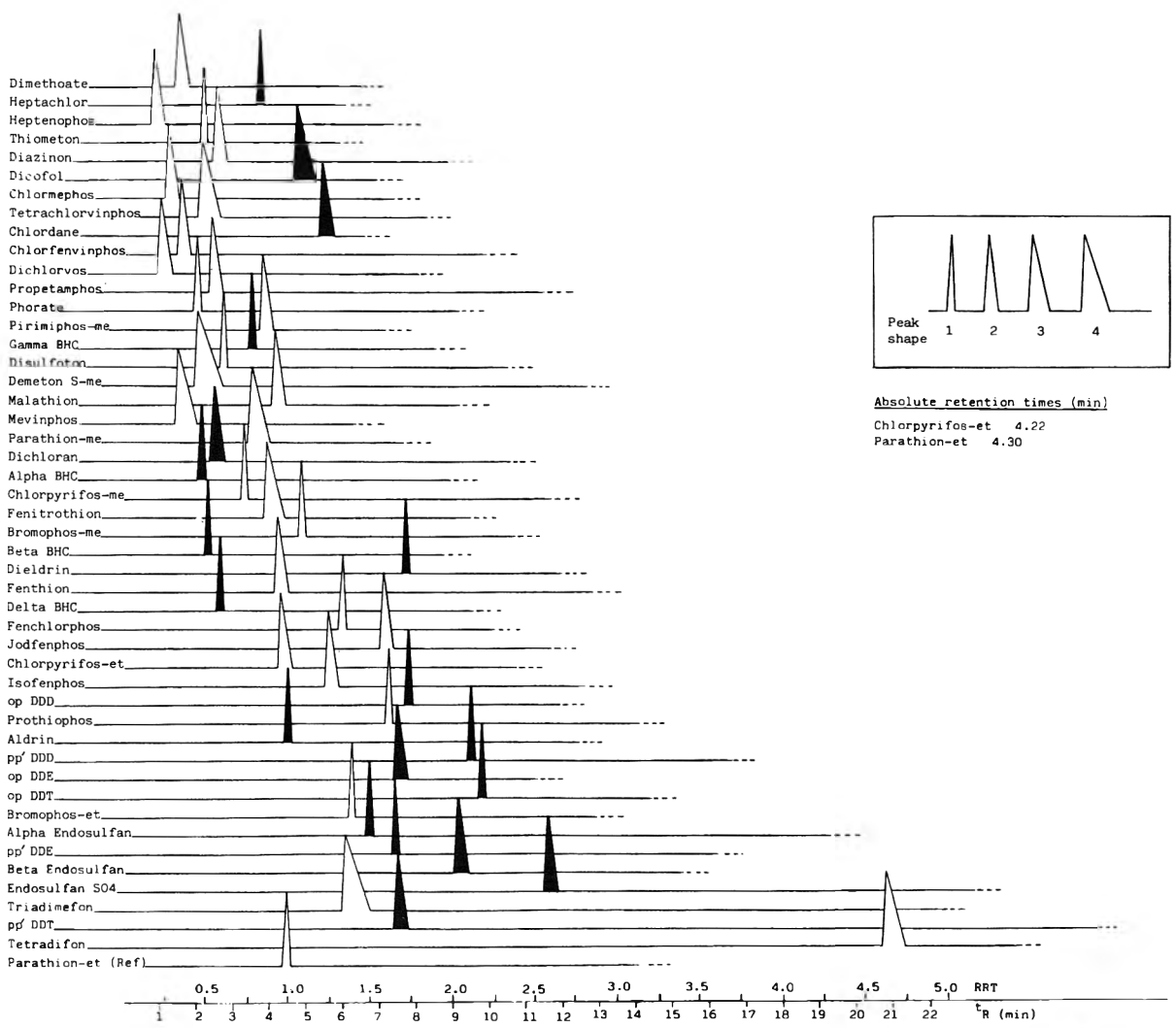
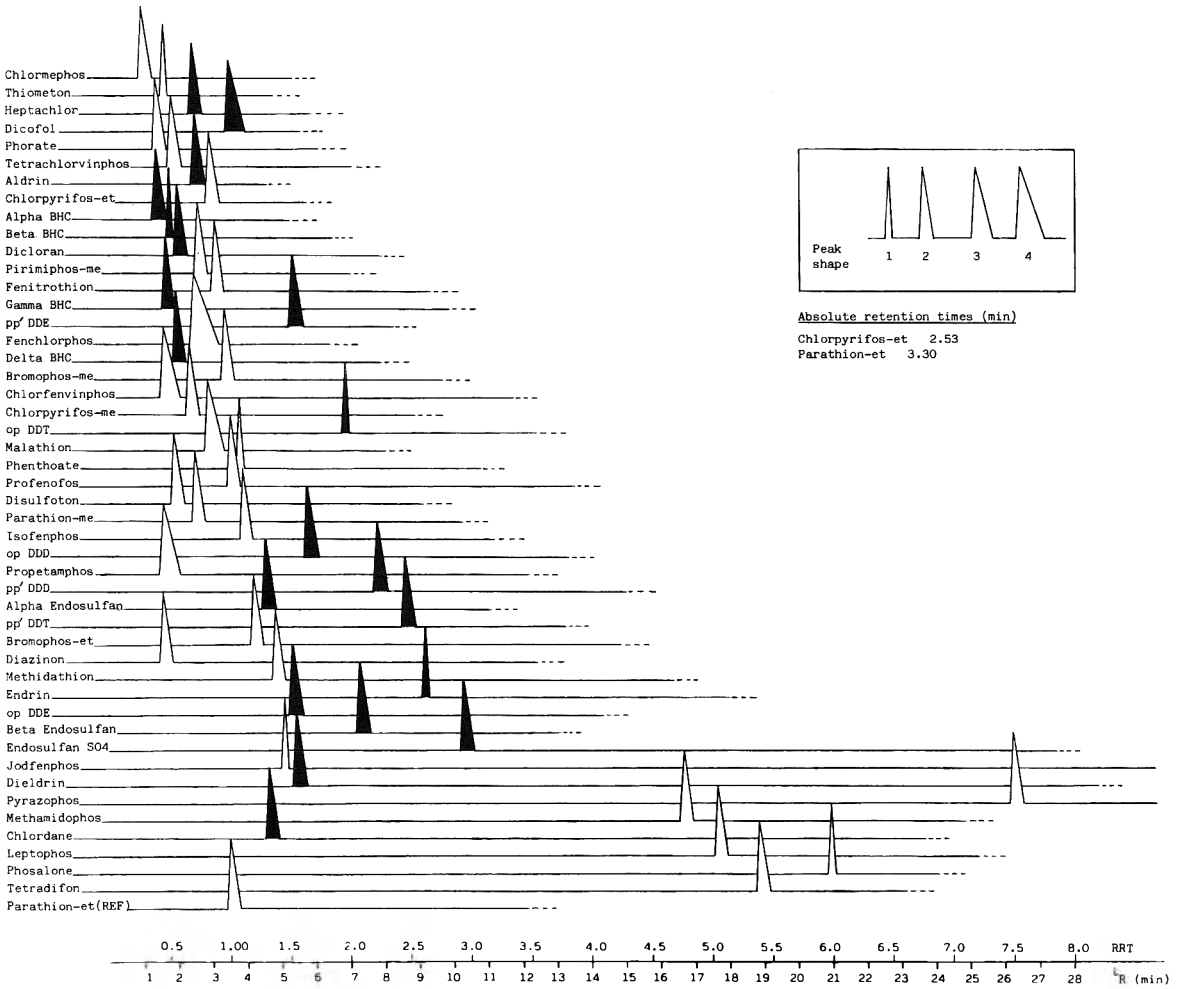
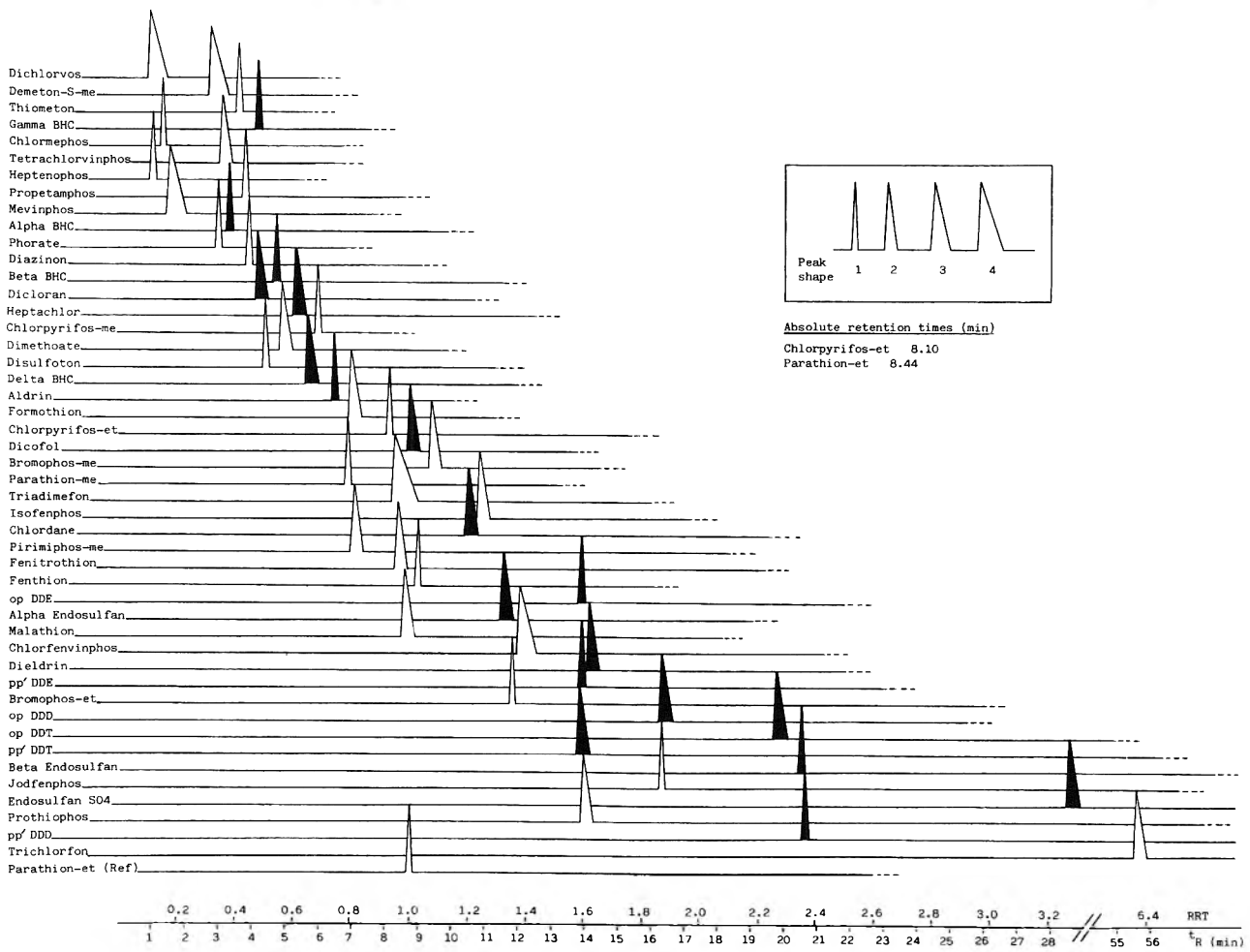


Figure 1. Retention data and peak shape classifications for pesticides analyzed by GC on 3% SP-2100 column at $\bar{u}_{opt}$ of 6.20 cm/s (25.5 mL/min).


Chlormephos
Thiometon
Heptachlor
Dicofol
Phorate
Tetrachlorvinphos
Aldrin
Chlorpyrifos-et
Alpha BHC
Beta BHC
Dicloran
Pirimiphos-me
Fenitrothion
Gamma BHC
pp' DDE
Fenchlorphos
Delta BHC
Bromophos-me
Chlorfenvinphos
Chlorpyrifos-me
op DDT
Malathion
Phenthoate
Profenofos
Disulfoton
Parathion-me
Isofenphos
op DDD
Propetamphos
pp' DDD
Alpha Endosulfan
pp' DDT
Bromophos-et
Diazinon
Methidathion
Endrin
op DDE
Beta Endosulfan
Endosulfan SO4
Jodfenphos
Dieldrin
Pyrazophos
Methamidophos
Chlordane
Leptophos
Phosalone
Tetradifon
Parathion-et(REF)
Peak shape
1 2 3 4
Absolute retention times (min)
Chlorpyrifos-et 2.53
Parathion-et 3.30
0.5 1.00 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 RRT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 tR (min)


Dichlorvos
Demeton-S-me
Thiometon
Gamma BHC
Chlormephos
Tetrachlorvinphos
Heptenophos
Propetamphos
Mevinphos
Alpha BHC
Phorate
Diazinon
Beta BHC
Dicloran
Heptachlor
Chlorpyrifos-me
Dimethoate
Disulfoton
Delta BHC
Aldrin
Formothion
Chlorpyrifos-et
Dicofol
Bromophos-me
Parathion-me
Triadimefon
Isofenphos
Chlordane
Pirimiphos-me
Fenitrothion
Fenthion
op DDE
Alpha Endosulfan
Malathion
Chlorfenvinphos
Dieldrin
pp' DDE
Bromophos-et
op DDD
op DDT
pp' DDT
Beta Endosulfan
Jodfenphos
Endosulfan SO4
Prothiophos
pp' DDD
Trichlorfon
Parathion-et (Ref)
Peak shape
1 2 3 4
Absolute retention times (min)
Chlorpyrifos-et 8.10
Parathion-et 8.44
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 6.4 RRT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 55 56 tR (min)

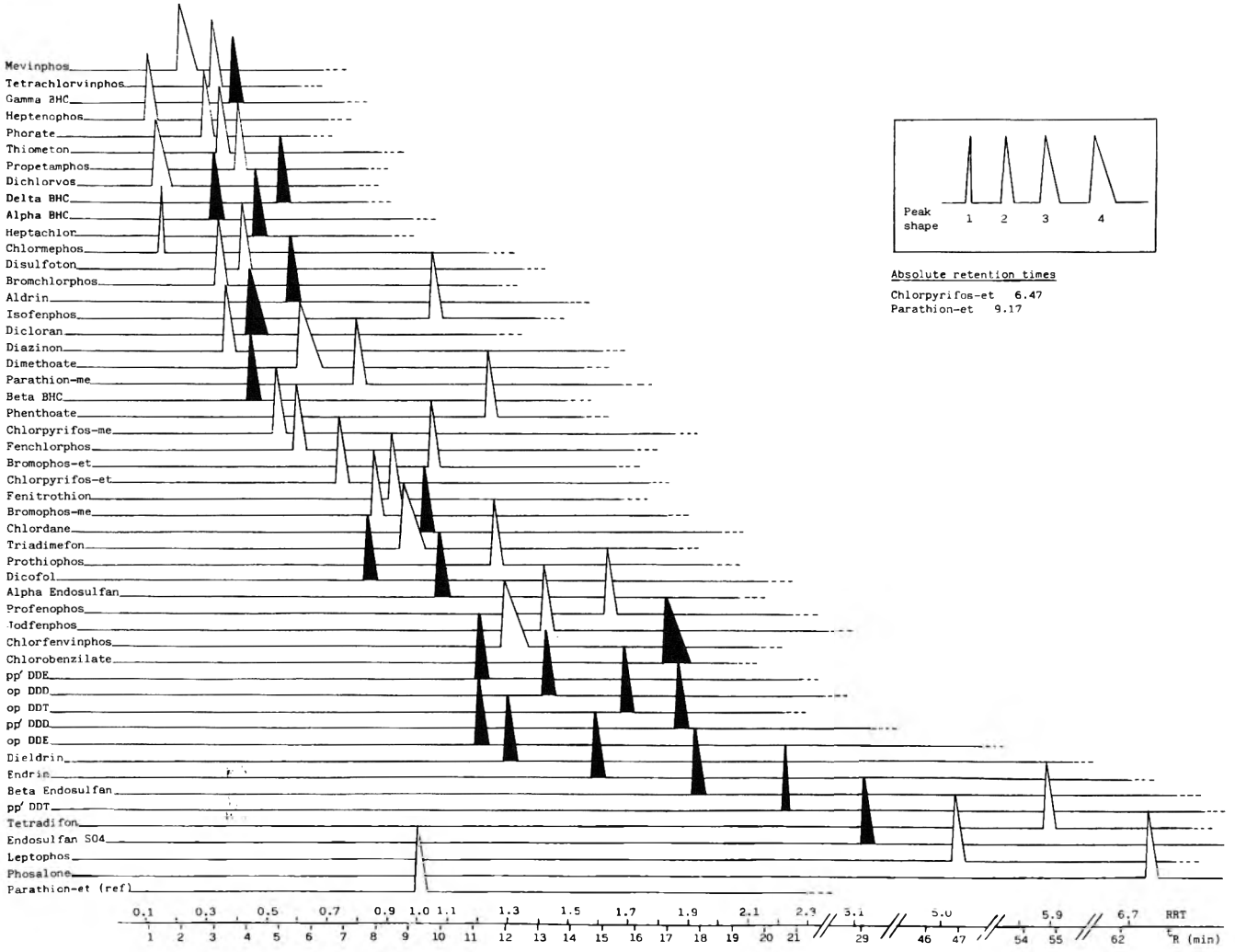


Figure 4. Retention data and peak shape classifications for pesticides analyzed by GC on 1.5% OV-17 + 1.95% QF-1 column at $\bar{u}_{opt}$ of 5.71 cm/s (23.0 mL/min).

←

Figures 2 (top) and 3 (bottom). Retention data and peak shape classifications for pesticides analyzed by GC on: Figure 2, 1% Dexsil-300 column at $\bar{u}_{opt}$ of 6.30 cm/s (25.8 mL/min), and Figure 3, 3% OV-17 column at $\bar{u}_{opt}$ of 7.10 cm/s (30.0 mL/min).

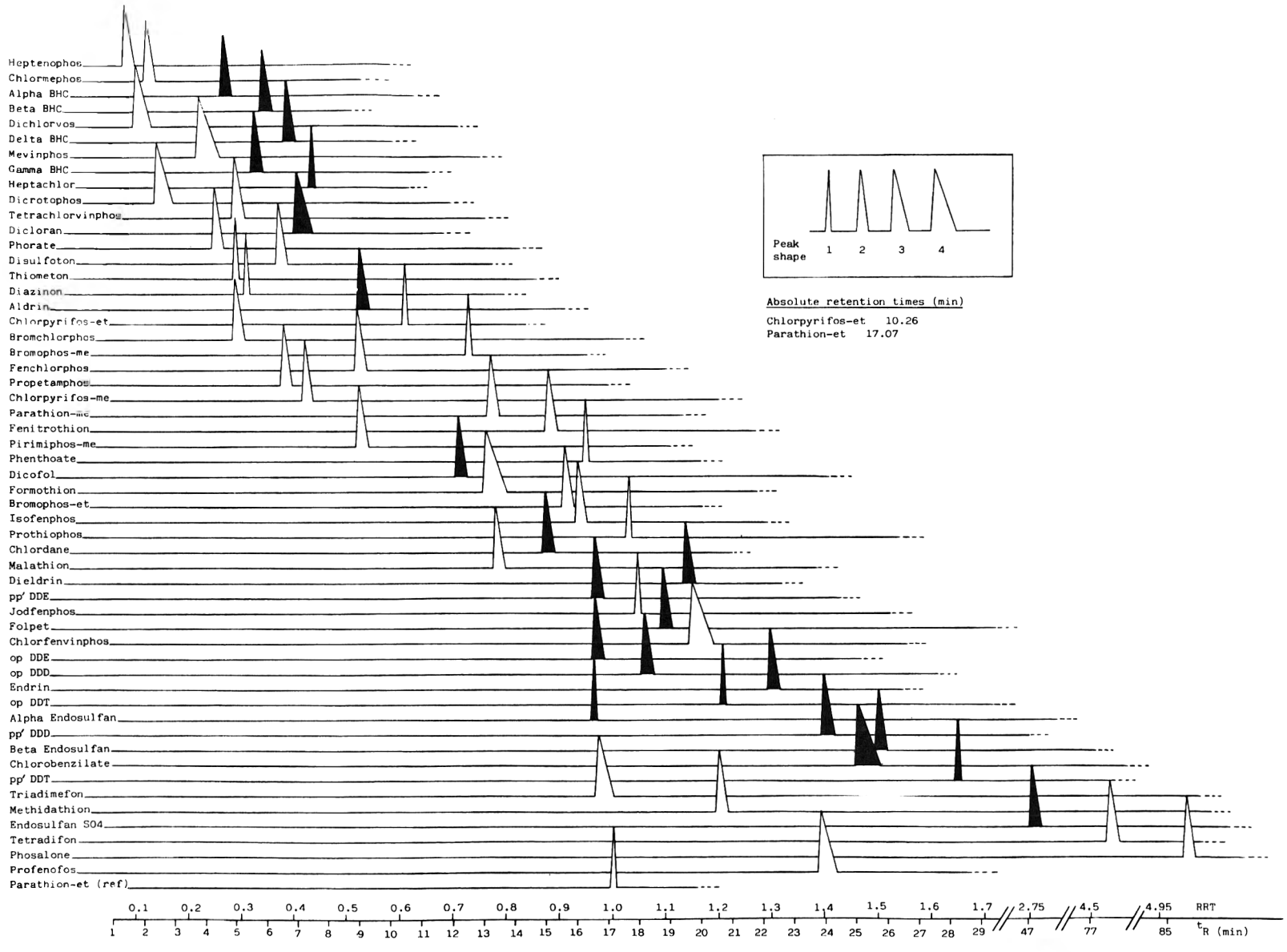


Figure 5. Retention data and peak shape classifications for pesticides analyzed by GC on 4% SE-30 + 6% QF-1 column at $\bar{u}_{opt}$ of 7.71 cm/s (33.5 mL/min).

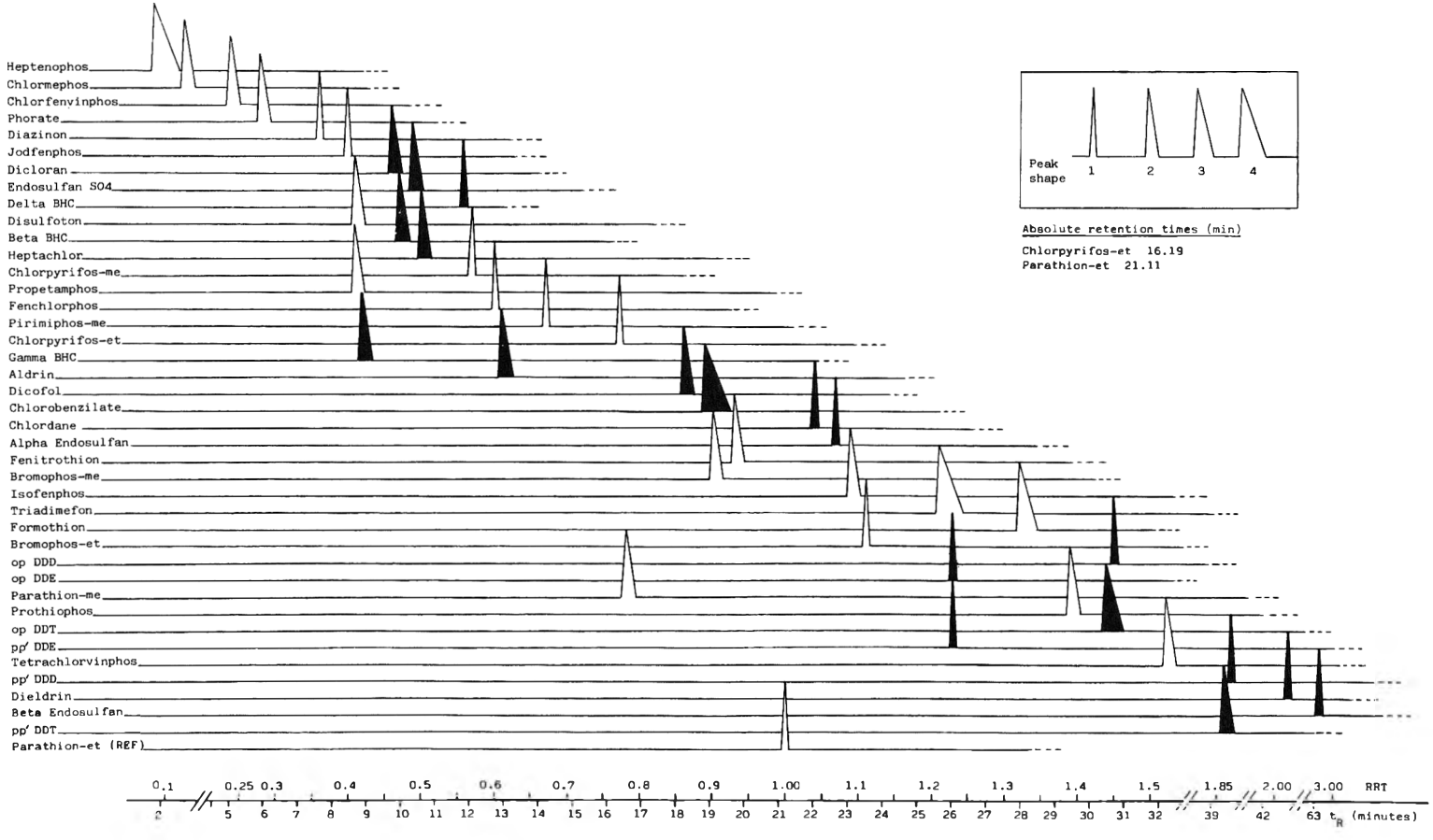


Figure 6. Retention data and peak shape classifications for pesticides analyzed by GC on 3% OV-17 + 3% OV-210 column at $\bar{u}_{opt}$ of 5.67 cm/s (22.9 mL/min).

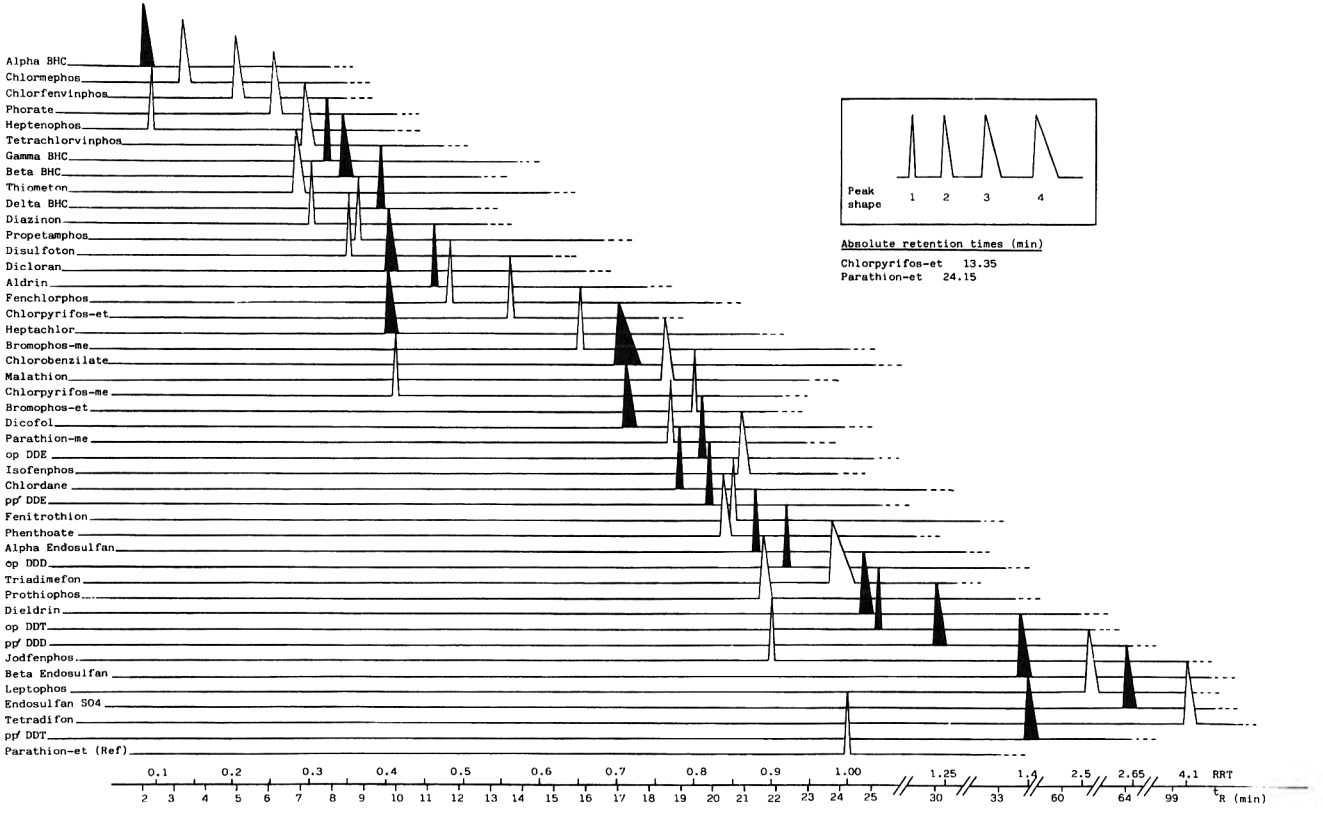


Figure 7. Retention data and peak shape classifications for pesticides analyzed by GC on 5% DC-200 + 7.5% QF-1 column at $\bar{u}_{opt}$ of 4.47 cm/s (17.2 mL/min).

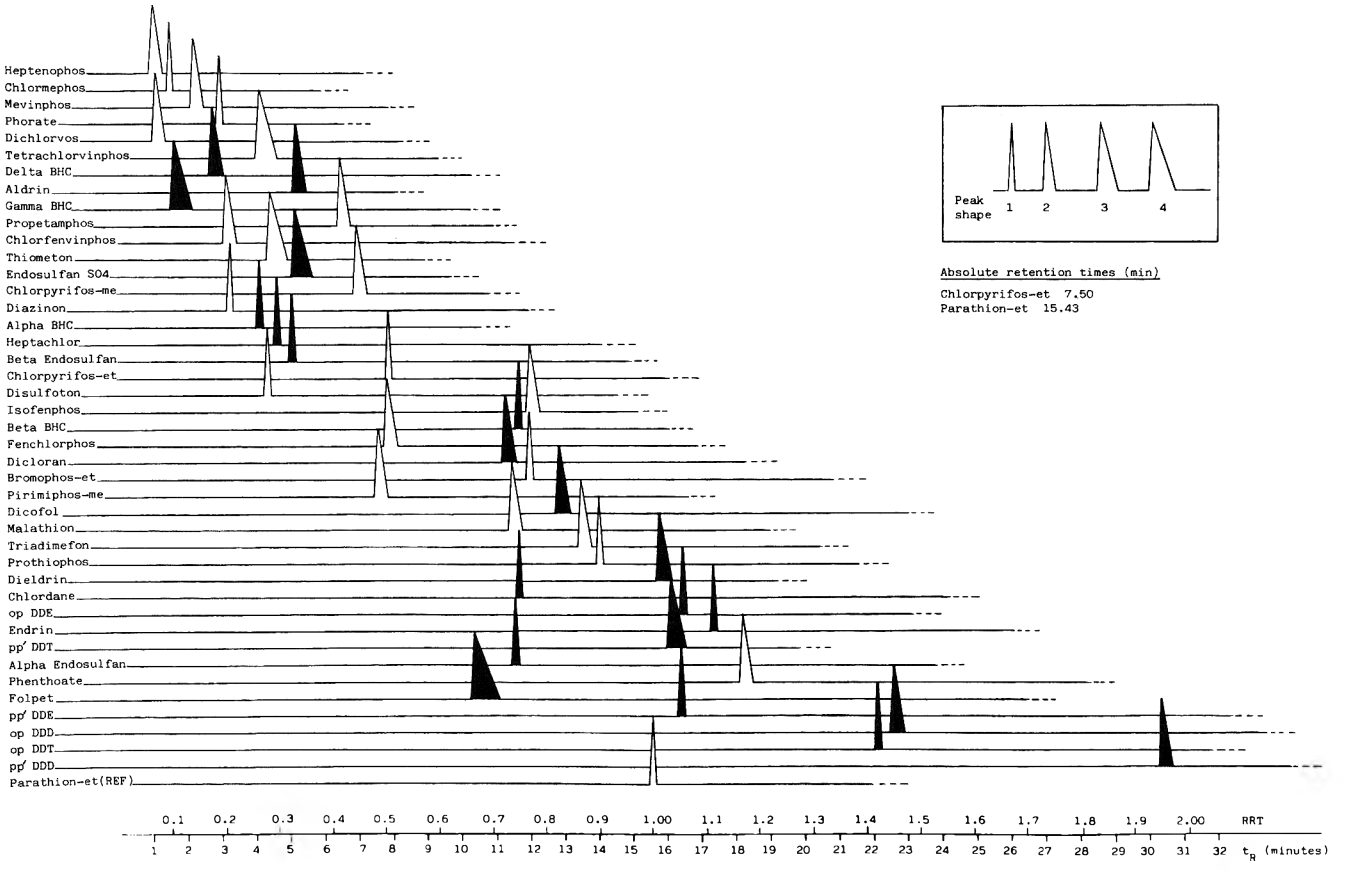


Figure 8. Retention data and peak shape classifications for pesticides analyzed by GC on 3% Carbowax-20M column at $\bar{u}_{opt}$ of 5.80 cm/s (23.2 mL/min).

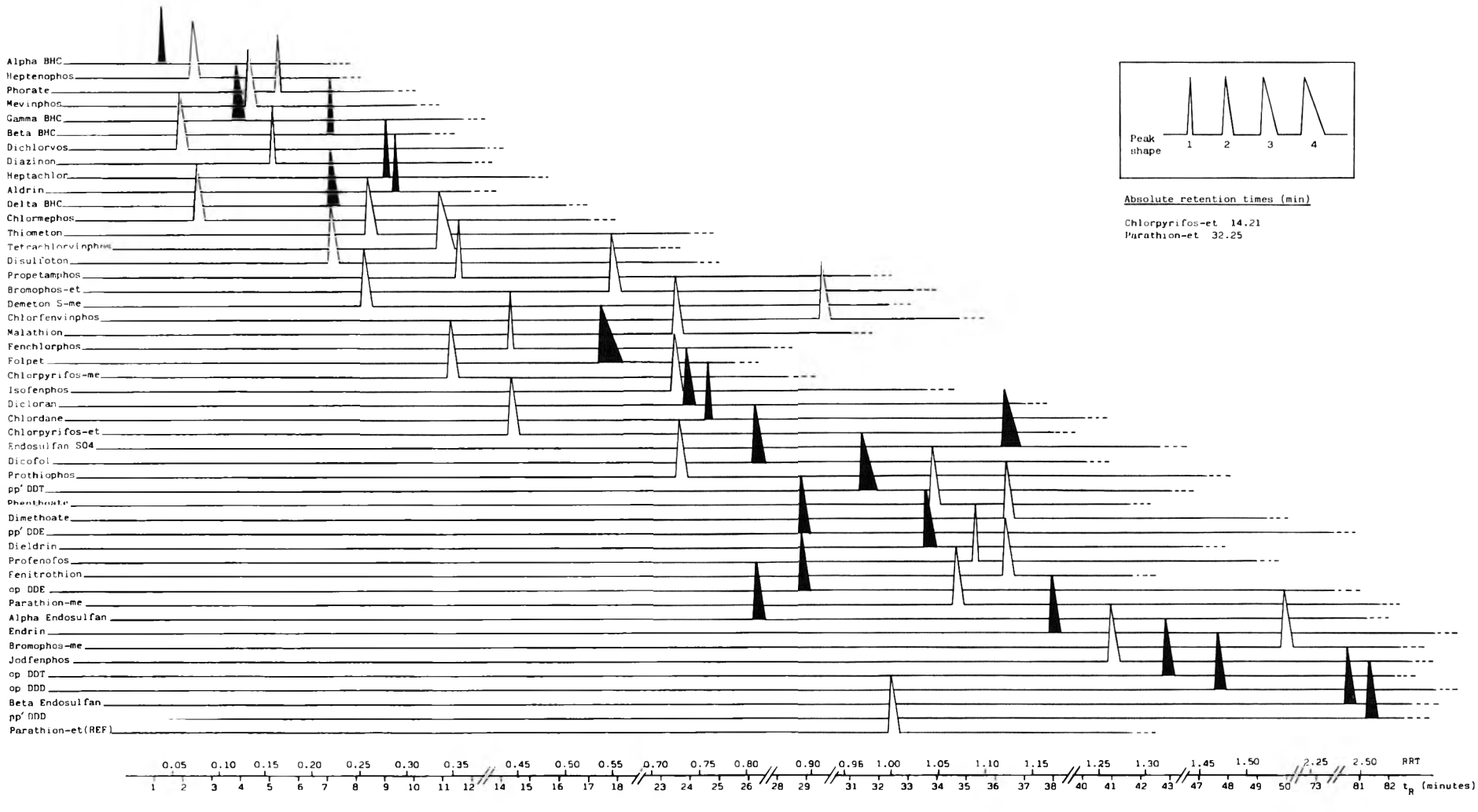


Figure 9. Retention data and peak shape classifications for pesticides analyzed by GC on 4% Reoplex-400 column at $\bar{u}_{opt}$ of 5.40 cm/s (21.5 mL/min).

Pentafluorobenzylation Derivatization for Determination of Chlorinated Herbicide Acids

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Pentafluorobenzyl and methyl esterification methods for several common herbicide acids have been evaluated and compared. Gas chromatography/electron capture instrumental detection limits for 2 of 7 pentafluorobenzyl esters were better than the corresponding methyl esters, and comparable for the other 5 herbicide esters. The linear dynamic concentration range was generally wider for the methyl esters. Gas chromatography of some pentafluorobenzyl derivatives was complicated by coelution of certain derivatives with each other and with byproducts of the derivatization reaction. The ruggedness-optimized pentafluorobenzylation procedure was subjected to linear dynamic range and detection limit studies. The choice between methyl or pentafluorobenzyl esters is contingent on the relative importance of detection limits and linear dynamic range.

Chlorophenoxy-type herbicides are widely used and are currently monitored in water by the U.S. Environmental Protection Agency (EPA). EPA method No. 615 (1) is a modification of an older American Society for Testing and Materials (ASTM) procedure for herbicides in water (2). Recently, a gas chromatography/electron capture (GC/EC) method for determining herbicide acids in solid wastes, as their methyl esters, was reported by Gurka et al. (3).

GC/EC determination of pentafluorobenzyl (PFB) esters has been proposed as an ultrasensitive derivatization technique by Nazareth et al. (4). This technique has been reported for a few herbicide acids by Waliszewski et al. (5), for phenols and organophosphorus compounds by Lee and Chau (6), and for amino acids by Netting and Duffield (7). A PFB phase-transfer derivatization has recently been published by Adams et al. (8). Of particular interest to this study is that successful PFB derivatization of 2-methyl-4-chlorophenoxy acetic acid (MCPA) has been achieved by 4 groups (9–13). Intuitively, it would be expected that the GC/EC detector would be more sensitive to the PFB derivatives than to the methyl esters.

Multiresidue analyses of PFB-esterified chlorinated herbicides in water and sediment were reported by Agemian and Chau (14) and Lee and Chau (6), and an optimization of the PFB method for a few compounds was reported by Chau and Terry (15). Later, a comparison of the GC behavior of PFB vs methyl esters of 10 chlorophenoxyalkyl acids was published by de Beer et al. (16). However, coelution of several PFB herbicide derivatives was observed on nonpolar GC columns by Lee and Chau (6) who recommended the use of 20 m Ultrabond columns.

De Beer and coworkers (16) noted that the mass spectral properties of PFB esters of 9 chlorophenoxy acids show this derivative gives useful spectral data for identification after chromatographic separation. The mass spectra show a characteristic m/z 181 ion with generally weak molecular ions (absent in the mass spectrum of 2,4,5-TB). This creates the possibility of gas chromatographic/mass spectrometric (GC/MS) confirmation of GC/EC data.

An earlier GC/EC study of the methyl esters of dicamba, silvex, 2,4-D, 2,4-DB, 2,4,5-T, dinoseb, 2-(2-methyl-4-chlorophenoxy)-propionic acid (MCPP), and MCPA by Shore et al. (17) indicated a range of instrumental detection limits exceeding 2 orders of magnitude. It was deemed advisable to attempt improved detection limits by optimizing a pentafluorobenzylation technique. After a preliminary evaluation, the PFB GC/EC procedure was subjected to ruggedness testing, linear dynamic range studies, and instrumental detection limit determinations for 8 of the 10 target herbicide acids.

Experimental

Apparatus

(a) *Gas chromatograph.*—Tracor Model 540 (Tracor Instruments, Austin, TX) equipped with Tracor Model 770 autosampler, ^{63}Ni electron-capture detector, and IBM CS9000 data system (IBM Instruments, Inc., Danbury, CT), with helium carrier gas at column head pressure of 10 psi, and Grob-type 30 s splitless injection.

(b) *GC columns.*—(*1*) DB-5 capillary column: 0.25 mm × 30 m, 0.25 μm film thickness (J & W Scientific, Inc., Rancho Cordova, CA); temperature program, 70°C for 1 min, increase 10°/min to 240°C, hold 17 min. (*2*) SP-2250 capillary column: 0.25 mm × 30 m, 0.25 μm film thickness (Supelco, Bellefonte, PA); temperature program, 70°C for 1 min, increase 10°/min to 240°C, hold 10 min. (*3*) DB-5 capillary column: 0.32 mm × 30 m, 1.0 μm film thickness (J & W Scientific); temperature program, 70°C for 1 min, increase 10°/min to 240°C, hold 10 min.

(c) *Vortex mixer.*—Vortex Genie (Scientific Industries, Inc., Springfield, MA).

(d) *Cleanup column.*—Bond-Elut silica column (Analytichem, Harbor City, CA).

Derivatization and Cleanup

Hydrolysis of herbicide esters and conversion of the free acids to methyl esters has been optimized by Shore et al. (17). Shore and coworkers ruggedness-tested the PFB pro-

Table 1. Retention times (min) of herbicide-PFB derivatives

Herbicide	Gas chromatography column		
	Thin-film DB-5	SP-2250	Thick-film DB-5
Dalapon	10.41	12.94	13.54
MCPP	18.22	22.30	22.98
Dicamba	18.73	23.57	23.94
MCPA	18.88	23.95	24.18
Dichlorprop	19.10	24.10	24.70
2,4-D	19.84	26.33	26.20
Silvex	21.00	27.90	29.02
2,4,5-T	22.03	31.45	31.36
Dinoseb	22.11	28.93	31.57
2,4-DB	23.85	35.61	35.97

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Mention of tradenames or commercial products does not constitute endorsement or recommendation for use.
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Table 2. Design for ruggedness test of experimental conditions

Experimental condition	Values of conditions in determination number[a]							
	1	2	3	4	5	6	7	8
1	A	A	A	A	a	a	a	a
2	B	B	b	b	B	B	b	b
3	C	c	C	c	C	c	C	c
4	D	D	d	d	d	d	D	D
5	E	e	E	e	e	E	e	E
6	F	f	f	F	F	f	f	F
7	G	g	g	G	g	G	G	g

[a] Two levels for variables listed in Table 3.

Table 3. Ruggedness test for PFB-herbicide method

Condition	Value for condition *X*	Value for condition *x*	Mean difference $(X - x)$
Reaction solvent for PFBBr plus herbicide	acetone	acetonitrile	1.1
Base catalysis for reaction	30% K_2CO_3	10% K_2CO_3	−4.2
Concentration of PFBBr for reaction	5%	3%	−2.1
Reaction time	4 h	3 h	−2.1
Addition of hexane to reaction residue and evaporation to dryness	+	−	1.9
Silica column for cleanup	Bond Elut®	packed Pasteur pipet 5% deactivated silica	0.7
Additional water in derivatization reaction	+30 μL	no water added	−0.5

cedure of Lee and Chau (6), using the method of Youden (18), for 2 concentration levels. The method used for linearity and detection limit studies is described below.

Derivatize acidic herbicides from extraction by dissolving residue in 4.0 mL acetone and adding 30 μL of 10% K_2CO_3 and 200 μL of 3% PFBBr in acetone. Close tube with glass stopper and mix on vortex mixer. Heat in tube heater at 60°C for 3 h. After reaction, evaporate solution to 0.5 mL with gentle stream of nitrogen. Add 2 mL hexane and repeat evaporation just to dryness at room temperature. Redissolve residue in 2 mL toluene–hexane (1 + 6) for column cleanup.

To clean up derivatives, top silica column with 0.5 cm hydrous Na_2SO_4. Prewet column with 5 mL hexane and let the solvent drain just to top of adsorbent. To column, quantitatively transfer reaction residue with several separate rinsings (total 2–3 mL) of toluene–hexane (1 + 6). Elute column with the same solvent to collect 8 mL. Discard this fraction which contains excess reagent. Elute same column with toluene–hexane (9 + 1) to collect 8.0 mL which contains PFB derivatives. Analyze this fraction by GC/EC.

Table 5. Linear range and instrumental detection limits[a] for methyl- and PFB-herbicide derivatives

Analyte[b]	Linear concn range, ng/g		Instrumental detection limits, ng/g	
	PFB	ME[c]	PFB	ME[c]
MCPP	4.0–160	3.1–309	0.74	333.0
Dicamba	3.2–130	2.6–520	0.49	0.60
MCPA	8.0–320	3.1–306	2.35	218.0
Dichlorprop	4.8–190	7.5–15 000	1.37	1.90
Silvex	8.0–320	2.1–4140	1.16	0.53
2,4,5-T	10.0–420	2.1–4110	1.90	0.78
2,4-DB	6.4–640	20.2–40 300	0.51	20.2

[a] Determined from standard solutions corrected back to 50 g samples with 9 mL final volume and 5 μL injection.
[b] Standard concentrations same as Table 4.
[c] Gurka et al. (3).

Measure instrumental detection limit (signal = 3 × noise) for each analyte. (Linearity was tested using 7 standard solutions varying in concentration from below the detection limit, to 10 times the linear range observed.)

Results and Discussion

The ability of DB-5 and SP-2250 capillary columns of various thicknesses to separate the 10 PFB esters was tested. These results are listed in Table 1 and show that a thick-film DB-5 column offers the best separation capability, although 2,4,5-T and dinoseb are close eluters. All further work was then carried out with a thick-film DB-5 capillary column.

Next, the PFB derivatization step was ruggedness-tested using the method of Youden. Seven experimental variables were tested at 2 levels, as summarized in Tables 2 and 3. The 7 variables were the reaction solvent, the base, water and reagent derivative concentrations, reaction time, choice of cleanup column, and the effect of adding hexane to the reaction residue. The small mean differences noted in Table 3 indicate that, at least for these variables, the method is rugged.

From the ruggedness results, a PFB derivatization procedure was prepared. Using this procedure the PFB derivative recoveries were determined for the 8 derivatives with 8 determinations. As shown in Table 4, mean recoveries range from 70% for 2,4-D to 96% for MCPP, with 7 of 8 recovery means exceeding 84%. The determination means ranged from 85.3 to 93.4%. These recoveries seem adequate for complex, multicomponent, environmental analyses. However, during this test, it was discovered that the PFB ester of 2,4-D chromatographically coeluted with a nonherbicide byproduct which carried through the cleanup procedure. Accordingly, work on the 2,4-D PFB ester was suspended. The linear

Table 4. Relative recoveries of PFB-herbicides using ruggedness-optimized test conditions

Analyte	Std concn, μg/mL	Relative recoveries, %								Mean
		1	2	3	4	5	6	7	8	
MCPP	5.1	95.6	88.8	97.1	100	95.5	97.2	98.1	98.2	96.3
Dicamba	3.9	91.4	99.2	100	92.7	84.0	93.0	91.1	90.1	92.7
MCPA	10.1	89.6	79.7	87.0	100	89.5	84.9	92.3	98.6	90.2
Dichlorprop	6.0	88.4	80.3	89.5	100	85.2	87.9	84.5	90.5	88.3
2,4-D	9.8	55.6	90.3	100	65.9	58.3	61.6	60.8	67.6	70.0
Silvex	10.4	95.3	85.8	91.5	100	91.3	95.0	91.1	96.0	93.3
2,4,5-T	12.8	78.7	65.6	69.2	100	81.6	90.1	84.3	98.5	83.5
2,4-DB	20.1	99.8	96.3	100	88.4	97.1	92.4	94.2	91.6	95.0
Mean		86.8	85.7	91.8	93.4	85.3	89.0	87.1	91.4	

dynamic concentration range and the GC/EC instrumental detection limits for the herbicide PFB and methyl derivatives are compared in Table 5. As expected, the PFB detection limits are at least 2 orders of magnitude better for the PFB derivatives of MCPP and MCPA and one order of magnitude better for 2,4-DB. The instrumental detection limits of the methyl derivatives of silvex and 2,4,5-T are about a factor of 2 better than those of the corresponding PFB compounds, whereas those of dicamba and dichlorprop are about the same.

Although the comparative detection limits for the 2 derivatives are varied, a comparison of the linear dynamic ranges is more straightforward. With the single exception of MCPA, the methyl derivatives provide a wider GC/EC quantitation range. Although the effect is not pronounced for MCPP, dicamba, and MCPA, it exceeds an order of magnitude for the other herbicides. The dynamic range plots level off at 10^7 GC/EC area counts indicating GC column overloading or detector saturation.

These results suggest that the herbicide methyl esters are a *generally* more useful approach to herbicide ester analysis than the PFB approach. However, if lower detection limits are the primary concern, and if the narrower linear dynamic ranges can be tolerated, it may be advantageous to use the PFB method in certain cases.

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FOOD ADDITIVES

Reverse-Phase Liquid Chromatographic Determination of Benzoic and Sorbic Acids in Foods

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An isocratic liquid chromatographic (LC) technique is described for the determination of benzoic acid and sorbic acid in foods such as beverages, fruits, seafood, vegetables, sauces, and dairy, bakery, and confectionery products. A C_{18} column is used with methanol–phosphate buffer (5 + 95) as mobile phase and 4-hydroxyacetanilide or 3,5-dinitrobenzoic acid as internal standard. Sample preparation is simple, rapid, and produces a sample extract that has a minimum effect on the column performance and life. Specificity of the method was checked against common food additives such as L-ascorbic acid, caffeine, artificial sweeteners (saccharin, cyclamate, aspartame), antioxidants (BHT, BHA) and artificial colors. Also described are 2 procedures for confirmation of the preservatives, using either redox reaction of sorbic acid with potassium permanganate or gas chromatography/mass spectrometry. Mean recoveries of 90–105% were obtained with a precision of 1–6% and a detection limit of 20 mg/kg for the 2 preservatives.

Antimicrobial agents are commonly used in foods to combat the proliferation of bacteria, yeasts, and molds. Benzoic acid and sorbic acid are the 2 preservatives most frequently used. Because the minimum permitted concentrations of benzoic acid and sorbic acid are controlled by legislation, their determination is important to analysts involved in the routine analysis of foods. Some analytical methods for their determination have been described in the literature. The nonspecificity and extensive sample extraction procedures of photometric methods have been discussed (1–3). Gas chromatographic methods (4, 5) are sensitive, specific, and accurate but may require lengthy sample preparation and derivatization of the 2 acid analytes. In contrast, liquid chromatography (LC) offers high specificity with minimal sample preparation and does not require derivatization. Several LC methods for the simultaneous determination of benzoic and sorbic acids have been reported (3, 6–12), but these methods are not applicable to a wide range of foodstuffs.

This paper reports on a reverse-phase LC system for the simultaneous determination of benzoic acid and sorbic acid. The method has been used for a variety of foods such as beverages, fruits, seafoods, vegetables, sauces, and dairy, bakery, and confectionery products.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Hewlett-Packard Model 1084B with variable-volume injector and variable-wavelength detector with built-in software integrator. Operating conditions: detection wavelength, 227 nm; mobile phase, phosphate buffer (0.03M, pH 6.5)–methanol (95 + 5), 2 mL/min; injection volume, ≤15 μL; temperature, ambient; chart speed, 0.4 cm/min.

(b) *Column.*—Spheri, RP18, 10 μm (Brownlee Labs, Inc., Santa Clara, CA 95050).

(c) *Precolumn.*—RP18 (Brownlee Labs).

(d) *Sample clarification kit.*—0.45 μm filters (Millex-HV, Millipore catalog No. SLHV025NS, Millipore Corp., Bedford, MA 01730), or equivalent.

(e) *Blender.*—Waring Model 32BL 79, or equivalent.

(f) *Food processor.*—Bamix Model M122, or equivalent.

(g) *High-speed homogenizer.*—Ultra-Turrax type TP/18/2 (IKA Werk, Stauffen im Breisgau, FRG), or equivalent.

(h) *Gas chromatograph/mass spectrometer.*—Hewlett-Packard Model 5985A. Operating conditions: carrier gas, helium, 1–2 mL/min; injection port, 250°C, splitless injection system; oven, 50°C isothermal.

(i) *Capillary column.*—Fused silica column, Ultra 1 (bonded OV-101), 12.5 m × 0.2 mm id × 0.33 μm film thickness.

Reagents

(a) Methanol, acetonitrile, benzoic acid, orthophosphoric acid, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, analytical reagent grade (Ajax Chemicals, Sydney, Australia); sorbic acid, 4-hydroxyacetanilide (HA), 3,5-dinitrobenzoic acid (DNBA), laboratory reagent grade (BDH, Sydney, Australia).

(b) *Potassium permanganate solution.*—Dissolve 14 g $KMnO_4$ in 100 mL water. Filter through glass wool.

(c) *Orthophosphoric acid solution (2%).*—Dilute 2 mL orthophosphoric acid (Univar grade, Ajax Chemicals) to 100 mL with water.

(d) *Stock internal standard solutions (1 mg/mL).*—Dissolve 100 mg HA (or DNBA) in 20 mL methanol and dilute to 100 mL with distilled water. Internal standard solutions can be refrigerated (4°C) for 1 month. DNBA internal standard is used only for analysis of cheese and yogurt.

(e) *Stock mixed standard solutions.*—Dissolve 50 mg benzoic acid, 50 mg sorbic acid, and 100 mg of appropriate internal standard (HA or DNBA) in 20 mL methanol and dilute to 100 mL with distilled water. This solution can be refrigerated (4°C) for 1 month.

(f) *Mobile phase.*—Phosphate buffer (0.03M, pH 6.5)–methanol (95 + 5). Prepare as follows: Dissolve 3.8 g K_2HPO_4 and 5 g KH_2PO_4 in 2 L water. Mix 1900 mL of this phosphate buffer with 100 mL methanol, then pass resulting solution through 0.45 μm filter (Millipore catalog No. HVLP 04700). Prepare and degas mobile phase daily.

Preliminary Sample Preparation

Samples must be pretreated to ensure their homogeneity.

(a) *Liquid foods.*—Shake carefully before sampling.

(b) *Powdered milk.*—Reconstitute powder to recommended strength with water and use 20 g of this mixture for analysis.

(c) *Sauces, mustard, yogurt, coconut cream.*—Mix with a spatula.

(d) *Fruit yogurt.*—Homogenize with a food processor.

(e) *Fruit products, canned seafoods, cakes.*—Mix in blender with equal weight of water.

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Table 1. Sample weights and solution volumes for extraction procedure 2, using acetonitrile

Samples	Sample weight, g	Internal standard solution, mL (V_1)	Diluent 2% orthophosphoric acid solution, mL (V_2)	Acetonitrile, mL (V_3)
Cheese, yogurt	10.0	5.0[a]	25	60
Bread, flour	5.0	5.0[b]	20[c]	20
Coconut milk or cream	10.0	5.0[b]	0	20
Milk (cow or goat), reconstituted milk	20.0	2.0[b]	10	50

[a] 3,5-Dinitrobenzoic acid.
[b] 4-Hydroxyacetanilide.
[c] Water.

(f) *Bread.*—Prepare as described in *Official Methods of Analysis* **sec. 14:086n** (13).

(g) *Cheese.*—Grate ca 100 g sample and mix carefully.

Sample Preparation

With methanol.—This procedure can be applied to beverages, mustard, sauces (oyster, tomato, shrimp, anchovy, satay), fruit products, canned seafoods (prawn, crab, caviar), and cakes. Mix 5.0 g prepared food with 2.0 mL internal standard (HA, 1 mg/mL) and dilute to 15 mL with methanol. Centrifuge (1500 × *g*) 10 min and filter small portion of supernate through 0.45 μm filter for chromatographic analysis. Remainder is used for confirmatory tests.

With acetonitrile.—This procedure is applicable to cheese, yogurt, bread, flour, coconut milk or cream, and milk. Weights and volumes (V_1, V_2, V_3) are shown in Table 1. Accurately weigh prepared sample into a quickfit conical flask, add V_1 of appropriate internal standard solution (1 mg/mL) and V_2 of diluent (water or 2% orthophosphoric acid solution). Blend mixture in high-speed homogenizer (for cheese, bread, and flour) until homogeneous suspension is obtained (ca 1 min). Add V_3 of acetonitrile and shake. For samples of yogurt, coconut milk or cream, and milk, omit homogenization stage. Filter mixture simultaneously through 2 filter papers—a Whatman No. 41 placed on top of a No. 42. Evaporate ca 7 mL of filtrate down to ca 3.5 mL under a stream of nitrogen in water bath at 30°C. Dilute to 7 mL with water. Filter 2 mL of this solution through 0.45 μm filter for chromatographic analysis. Remainder is used for confirmatory tests.

Preparation of Working Mixed Standard Solutions

Dilute appropriate stock mixed standard solution with water and methanol or with water and acetonitrile (with or without the addition of 2% orthophosphoric acid solution) to obtain working mixed standard solution similar in composition to that of prepared sample solution. For example, for analysis of fruit juice, dilute 2 mL stock mixed standard solution containing HA with ca 5 mL water and 8 mL methanol. For analysis of cheese, dilute 5 mL stock mixed standard solution containing DNBA with 25 mL 2% orthophosphoric acid solution, 15 mL acetonitrile, and 45 mL water.

Determinations

(a) *Relative response factors.*—Inject 10–15 μL working mixed standard solution several times until retention times and peak area ratios, benzoic acid/internal standard and sorbic acid/internal standard, have stabilized. Once reproducibility of these parameters is ensured, calculate mean relative response factors K_B and K_S for benzoic and sorbic acids, respectively, using 2 chromatographic runs, as follows:

K_B = (concn internal standard/concn benzoic acid) × (area benzoic acid/area internal standard)
= 2 × (area benzoic acid/area internal standard)

Similarly,

K_S = 2 × (area sorbic acid/area internal standard)

(b) *Preservative content.*—Inject 10–15 μL sample solution twice and calculate the mean area ratios, benzoic acid/internal standard and sorbic acid/internal standard. Calculate preservative content using the following formulas:

$$\text{Benzoic acid, mg/kg} = (m/K_B) \times a \times (1000/M)$$
$$\text{Sorbic acid, mg/kg} = (m/K_S) \times b \times (1000/M)$$

where, a = mean area ratio benzoic acid/internal standard; b = mean area ratio sorbic acid/internal standard; m = internal standard amount (mg), and M = sample amount (g). *Note*: Where a sample is blended with an equal weight of water, the result must be multiplied by 2.

After analysis of ca 10 samples, flush column 15 min with 50% methanol followed by equilibration with mobile phase before resuming analysis. After use, flush column 15–20 min with 5% methanol to remove phosphate buffer, then flush column 15 min with 100% methanol or acetonitrile.

Confirmation

The presence of the 2 preservatives can be confirmed by the following procedures:

(a) *Sorbic acid.*—Add 3–5 drops of potassium permanganate solution to 5 mL sample solution, shake and let mixture stand 15 min at room temperature. If violet color still persists, add dropwise sodium sulfite solution (5% w/v) to destroy excess oxidizing agent. Filter, using a Whatman No. 42 filter paper, and pass filtrate through 0.45 μm filter. Inject filtrate into chromatograph. If sorbic acid is present, no peak corresponding to sorbic acid will appear. Similarly, internal standard HA would also be oxidized, and the absence from the above chromatogram will be noted. DNBA peak will be unchanged.

(b) *Benzoic acid and sorbic acid by gas chromatography/mass spectrometry (GC/MS).*—Add 1 mL of 0.5N sulfuric acid to 5 mL sample solution and extract with 7 mL diethyl ether. Separate ether layer and extract it with 2 mL of 0.5N sodium hydroxide. Remove aqueous layer, acidify with 3 mL of 0.5N sulfuric acid, and extract with 5 mL diethyl ether. Dry ether extract with 1 g anhydrous sodium sulfate and remove ether by evaporation. Dissolve residue in 200 μL chloroform and add 0.5 mL freshly prepared diazomethane (in hexane). Let mixture stand 2 h at room temperature before GC/MS analysis. Prepare reference solution by treating 1 mL solution benzoic acid (100 mg/L) and sorbic acid (100 mg/L) in chloroform with 3 mL diazomethane (in hexane) and letting mixture stand 2 h before analysis.

(c) *GC/MS analysis.*—Inject 1 μL volumes. Approximate retention times are 2 min for sorbic acid and 3 min for benzoic acid. Use selective ion monitoring for low levels of preservatives: benzoic acid (m/z, % abundance)—136, 31; 105, 100; 77, 60; 51, 16; sorbic acid (m/z, % abundance)—126, 46; 111, 100; 95, 58; 67, 87.

Results and Discussion

Because the separation of ionic compounds in liquid chromatography is pH dependent (8, 14), the mobile phase consists of a pH 6.5 phosphate buffer, used with a C_{18} reverse-phase column. Methanol (5%) was added to the buffer to

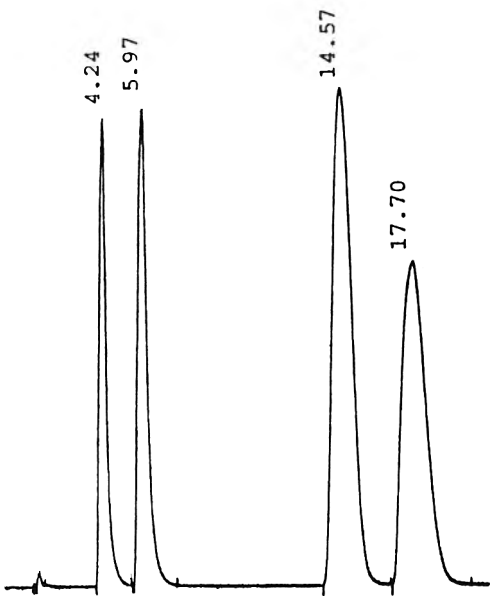

Figure 1. Separation of benzoic acid (4.24 min) and sorbic acid (5.97 min) on a new column; internal standards, HA (14.57 min), DNBA (17.70 min). Concentrations: benzoic acid and sorbic acid, 100 mg/L; HA and DNBA, 200 mg/ L. Injection volume: 10 μL.

reduce the analysis time. This pH value does not give an optimal separation of benzoic and sorbic acids, but for a method to be applicable to a wide range of food products, a compromise of optimum conditions is necessary for a satisfactory separation of the preservatives from background interference. Resolution of benzoic acid, sorbic acid, and internal standard (HA or DNBA) under the chosen conditions is illustrated in Figure 1.

The chance of false positives has been minimized by the chromatographic conditions which separate benzoic and sorbic acids from most interferences. The specificity of the method has been checked against common food additives such as L-ascorbic acid, caffeine, artificial sweeteners (saccharin, cyclamate, aspartame), antioxidants (BHT, BHA), and artificial colors. An LC result usually needs to be confirmed by an alternative method, and 2 methods have been described for this purpose. The first method is used to confirm the presence of sorbic acid on the basis of its oxidation with potassium permanganate (15), and the second method is a simultaneous confirmation of benzoic and sorbic acids using the inherent sensitivity and specificity of GC/MS.

This LC method does not require isolation of the preservatives from the food matrix, thus avoiding steam distillation or extraction, with either Soxhlet apparatus or separating funnels. The precipitation of proteins and fat by the addition of methanol or acetonitrile, followed by centrifugation and/or filtration, provided a clear solution suitable for chromatographic analysis. Various cleanup procedures using disposable columns or Carrez solutions were unnecessary. Using the sample preparation methods described above, no interference from background peaks with benzoic and sorbic acids was observed in the routine analysis of preservatives when working at levels above 20 mg/kg. The column life was not significantly affected by the sample extracts which underwent only partial cleanup prior to injection. Column blockages were reported in the determination of sorbic acid in yogurt by direct chromatography using phosphate buffer (0.1M, pH 5.5) with 40% methanol as mobile phase, and a 3-step extraction scheme was therefore proposed (12).

Under the present conditions, with a dilute phosphate buffer (0.03*M*, pH 6.5) containing a small quantity of methanol, the problem mentioned above was eliminated. For a 12 month period during which the system was in continuous use with an average of 40 injections per week, we used a new column for the determination of benzoic and sorbic acids in various food products including yogurt and cheese. Chromatograms of the standard solutions during the trial period were randomly chosen and the separation parameters (*S*) (16) for benzoic and sorbic acid were calculated to examine the performance of the column. The average values of *S* in successive 3 month terms during the 1 year period were 0.16, 0.15, 0.14, and 0.13, indicating a slow deterioration of the column performance. Separation of the 2 preservatives in the 10th month of the trial period, however, was still satisfactory.

Table 2. Results of 6 replicate analyses of the benzoic acid and sorbic acid content of 5 different orange juice samples

Sample	Preservatives	Mean, mg/kg	SD, mg/kg	CV, %
1	Benzoic acid	442	5	1
	Sorbic acid	—	—	—
2	Benzoic acid	170	1.5	1
	Sorbic acid	288	3	1
3	Benzoic acid	111.5	1	1
	Sorbic acid	52	2	4
4	Benzoic acid	87	1	1
	Sorbic acid	57	2	2
5	Benzoic acid	88.5	2	2
	Sorbic acid	58	1	1

Acetonitrile was suitable for the precipitation of protein and fat, but peak splitting occurred when the sample solution injected onto the column contained a significant amount of acetonitrile. Considerable adsorption of acetonitrile onto the solid phase has been measured (17), and an adsorbed layer of an organic liquid was shown to act as a separate stationary phase (18), which may account for the peak splitting observed. The problem may be eliminated by reducing the injection volume, diluting the sample solution with the mobile phase, or using methanol as an alternative denaturant solvent for less-fatty food products. If acetonitrile is used, it is preferable to remove the acetonitrile from the sample solution on a water bath at 30°C under a stream of nitrogen, particularly when working with low levels of preservatives.

The advantages of an internal standard method for precise and accurate quantitative analysis have been discussed (19). In the procedure described above, the use of an internal standard also facilitates the final steps of the sample preparation where the sample solutions are concentrated or diluted, because there is no need for an accurate measurement of volumes. 4-Hydroxyacetanilide (HA) was a suitable internal standard for most food products investigated in this work, but some cheeses and yogurts gave background peaks that interfered with the HA peak. 3,5-Dinitrobenzoic acid (DNBA), with a longer retention time, was chosen as an internal standard for the 2 types of dairy products examined. Mixed standard solutions were prepared with a concentration of 100 mg/L for HA and a range of concentrations (from 20 mg/L to 500 mg/L) for benzoic and sorbic acids. These solutions were kept in a refrigerator (4°C) and were examined weekly. Duplicate injections were made, and the mean peak area ratios benzoic acid/HA and sorbic acid/HA were calculated. The results obtained for 4 weeks differed by less than 4%, indicating no decomposition of the components in the mixed standard solutions. When the peak area ratios (y) were plotted against the weight ratios (x), a straight line passing

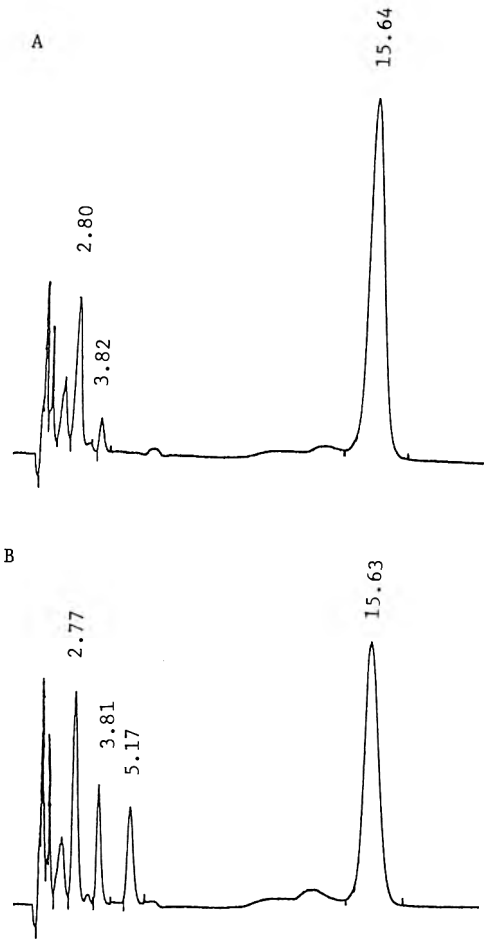

Figure 2. A, chromatogram of a blank sample of Gloucester milk cheese on an aged column, showing a trace of naturally occurring benzoic acid (3.82 min), internal standard, DNBA (15.6 min); B, chromatogram of a Gloucester milk cheese spiked with benzoic acid (3.81 min) and sorbic acid (5.17 min) at 40 mg/kg level, internal standard: DNBA (15.63 min).

through the origin was obtained for each preservative. The relative response factor for each preservative (slope of standard curve) was therefore constant over the range of concentration ratios examined. Similar results were obtained with DNBA as the alternative internal standard.

The detection limit of a method is governed by sample matrix interferences which vary from one class of sample to another. Although the method described can detect naturally occurring benzoic acid in dairy products at levels below 25 mg/kg, the quantitation is not accurate at such low levels. The detection limit (i.e., the level of preservative that will produce a signal equal to twice the noise level) of the method is estimated to be 20 mg/kg for each of the preservatives in complex foodstuffs such as dairy products; it is much lower (about 5 mg/kg) in samples with less-complex matrixes, such as soft drinks. The detection limit for sorbic acid can be lowered by using its wavelength of maximum absorption (255 nm in the present mobile phase) at the expense of decreased sensitivity for benzoic acid.

A very low detection limit is not necessary, however, since the minimum concentration of benzoic and sorbic acids for effective inhibitory action against bacteria, yeasts, and molds in foods is generally above 20 mg/kg (20). To examine the precision of the method, the standard deviation was determined for 2 types of foods. Five commercial orange juice samples (containing benzoic and sorbic acids), which constitute a typical simple matrix, were analyzed. Six separate

Table 3. Results of replicate analyses of benzoic acid and sorbic acid content of 6 types of food

Sample	Preservatives	Spiking level, mg/kg	Recovery, %
Cheese[a]	Benzoic acid	250	99–102
		50	102
	Sorbic acid	250	95–106
		50	110
Juices[b]	Benzoic acid	250	99–102
		50	96–104
	Sorbic acid	250	98–99
		50	90–100
Goat's milk[c]	Benzoic acid	250	98–104
		50	92–106
	Sorbic acid	250	99–101
		50	95–104
Cow's milk[c]	Benzoic acid	250	99–100
		50	94–106
	Sorbic acid	250	98–101
		50	94–104
Canned seafoods[d]	Benzoic acid	250	97–101
		50	92–102
	Sorbic acid	250	97–98
		50	93–101
Yogurt[e]	Benzoic acid	250	98–103
		50	93–108
	Sorbic acid	250	98–103
		50	90–100

[a] Six replicate analyses each on mozzarella, pasturella, matured cheddar, cream, elbostyle, and edam.
[b] Duplicate analyses on pineapple, grapefruit, vegetable fruit, tropical and apple, and tomato juice.
[c] Duplicate analyses.
[d] Duplicate analyses on seafood mix, clams, lumpfish roe, prawns, and crabs.
[e] Duplicate analyses on skim milk natural yogurt, natural low-fat yogurt, premium fruit salad low-fat yogurt, traditional natural yogurt, and strawberry yogurt.

analyses were run on each sample. The analytical results are shown in Table 2 together with the calculated standard deviations. The coefficients of variation (CV) range from 1 to 2% for benzoic acid and from 1 to 4% for sorbic acid.

Table 3 shows recovery data for cheese, fruit juices, goat's milk, cow's milk, canned seafood, and yogurt. Six replicate analyses of 6 samples of cheese, which represents a more complex matrix, were spiked with benzoic and sorbic acids at levels of 50 and 250 mg/kg. Means and standard deviations ranged from 51 ± 2 mg/kg to 296 ± 7 mg/kg. The CV was <4% for benzoic acid and <6% for sorbic acid; recoveries were 95–110%. Recovery experiments and blank determinations were performed on the other 5 categories of foods as follows: Five samples within each category, differing in formulation or origin, were spiked with benzoic and sorbic acids at 250 mg/kg and 50 mg/kg, and each sample was analyzed in duplicate. Blank determinations were performed to ensure that no peaks corresponding to the preservatives were present in the unspiked samples. Where a low level of benzoic acid was detected in the unspiked dairy products (Fig. 2A), GC/MS was used for confirmation. It has been shown (5) that benzoic acid does occur naturally at low levels in some milk products.

Figure 2B shows the separation of added benzoic and sorbic acids from the interfering background peaks usually found in cheese samples; other types of food examined showed less interference, with the exception of tuna in oil, herring in tomato sauce, and samples containing garlic. Benzoic and sorbic acids would not be determined in these products be-

cause of the presence of interfering peaks with similar retention times.

Recoveries of benzoic acid and sorbic acid from the food products investigated in this work (see *Sample Preparation*) were in the range of 90–105%, except for dairy products spiked with preservatives at 50 mg/kg in which higher recoveries (up to 110%) were found.

The method described is not suitable for the analysis of trace levels (less than 20 mg/kg) of benzoic and sorbic acids, but it can be successfully applied to their determination when they are added in a variety of foods.

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AIR

Simultaneous Ultrasonic Extraction and Silylation for Determination of Organic Acids, Alcohol, and Phenols from Airborne Particulate Matter

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Three methods for the extraction and derivatization of alcohols, carboxylic acids, and phenols from airborne particulates were compared and evaluated. Derivatization of the sample during ultrasonic agitation to form trimethylsilyl derivatives increased the recovery of "reactive oxygenated hydrocarbons" compared to the standard method of extraction followed by derivatization. A single solvent, methylene chloride, with a strong and volatile silylating reagent, *N,O*-bis(trimethylsilyl)trifluoroacetamide, extracted a large number of reactive components with a minimum of interference. When the extraction/derivatization procedure was repeated 4 or more times for a total extraction time of at least 30 h, greater than 90% of the major mono- and difunctional group-containing components was recovered.

Oxidation processes which occur with organic molecules in ambient air are complex and not well understood. While the majority of the oxygenated organic compounds contained in air are believed to be in the vapor state, many of these compounds exist on or as condensed particles. A number of potential sources exist for that fraction of oxygenated organic matter contained in the atmospheric particulate matter. Particles can be directly emitted into the atmosphere through natural or anthropogenic sources; nonpolar hydrocarbons can be oxidized in the gas phase by either photooxidation or chemical oxidation to produce polar compounds that in turn can either condense to form a particle or can adsorb onto particles already present; and, finally, oxidation may occur while the organic matter is in the particulate state. Needless to say, sorting out the various potential pathways in which organic oxidation can occur is a complicated matter.

The first step in determining any chemical mechanism is to identify the starting material, the intermediate species, and the final products. After identities of the principal species are known, then quantitative data must also be obtained. Niki et al. (1), in a discussion of air chemistry, made the following perceptive comment: "Clearly, to gain further insight into these complex reactions, it is imperative to identify and quantify the relevant products. The current knowledge of atmospheric reactions is, in general, limited largely by the lack of analytical methods suitable for monitoring the reactants and products under simulated atmospheric conditions."

The analytical technique that has provided the most qualitative information on organics in particles is high resolution thermal mass spectrometric analysis (HRTMSA) (2, 3). With this technique, samples are collected on glass fiber filters which are inserted directly into the electron impact (EI) ionization source with a temperature-controlled probe. As the components volatilize, high resolution mass spectra are obtained. This technique has inherent ambiguities in identification and quantitation of individual components that may have fragments with the same exact mass and overlapping volatilities. Moreover, oxygenated compounds that are adsorbed to particles may undergo thermal degradation at the higher probe temperatures.

Specific attempts to determine carboxylic acids on particulates have been made using procedures which require extraction of the particulate fraction followed by some form of derivatization (4–7). Not only is this 2-step process time consuming, the polar nature of the underivatized acid, alcohol, or phenol is expected to reduce the extraction efficiency with moderately polar to nonpolar solvents. The primary objective of the present study was to investigate the extraction efficiency of these compounds from airborne particulate matter when the extraction and derivatization processes were accomplished simultaneously.

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Experimental

Procedure

Air samples were taken from the roof of Dana Hall on the campus of Washington State University in Pullman, WA. The air particles were collected onto 8 × 10 in. glass fiber filters (Gelman Sciences, Ann Arbor, MI) with a HiVol sampler (General Metal Works, Cleves, OH). The glass fiber filters were pretreated in an annealing oven at 500°C to remove organic contaminants. The filters were weighed before and after sampling to determine the mass of particles collected. The glass fiber filters were found not to be hygroscopic, and stable mass measurements could be made without allowing for equilibration with room humidity.

Three extraction/derivatization procedures were compared: ultrasonic bath extraction followed by silylation (UEFS), simultaneous ultrasonic bath extraction silylation (SUES), and Soxhlet extraction followed by silylation (SEFS). In each case, methylene chloride (Burdick and Jackson Laboratories, Muskegon, MI) was used as the solvent and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Inc., Bellefonte, PA) was used as the silylating reagent. Methylene chloride was chosen because of its availability in highly pure form, its ability to dissolve the compounds of interest, and its hydrophobic character (minimizing the possibility of water contamination and interference with the silylation reaction). Also, the high volatility of methylene chloride allows concentration steps at room temperature without sample loss, and it elutes before sample components from the GC column with a sharp solvent peak. BSTFA was used as the silylating reagent because of its ability to react swiftly and completely with alcohols, phenols, and carboxylic acids, its availability in pure form, and its quick elution time which, like the solvent, did not interfere with sample peaks. Trimethylchlorosilane (TMCS) (Pierce Chemical Co., Rockford, IL) was added at a 1% level (compared with BSTFA) to catalyze the silylation reaction.

Ultrasonic Bath Extraction and Silylation

The glass fiber filter was cut into strips and covered with 50 mL glass-distilled methylene chloride in a round-bottom flask. This was fitted with a condenser, placed in an ultrasonic bath (Bransonic 220, Shelton, CN), and agitated for a predetermined time. The agitation maintained the bath temperature at 45°C, which refluxed the extracting solvent. The ground-glass joints were wrapped with Teflon tape to prevent

Table 1. Sampling and ultrasonic extraction conditions, sequential (seq.) and simultaneous (sim.) treatment

	Sample No.									
Condition	1210		1229		115		128		131	
Sampling time, h	167.0		168.8		50.1		71.67		98.33	
Amount sampled, cu. m	18 200		16 600		5650		8080		10 700	
Mass of particles, g	0.5277		0.5571		0.2828		0.1565		0.5515	
Procedure:	Seq.	Sim.	Seq.	Sim.	Seq.	Sim.	Seq.	Sim.	Seq.	Sim.
BSTFA added, mL	—	0.40	—	0.75	—	0.75	—	0.50	—	0.50
Extraction time, h	2	2	3	3	2	2	2	2	3	3

loss of solvent through the ungreased joints. After agitation, the solvent was filtered and concentrated to 2 mL at room temperature with a rotovaporator. The sample was concentrated to its final volume with a stream of prepurified nitrogen at room temperature. After this underivatized sample was analyzed by GC, 0.2 mL BSTFA with 1% TMCS was added to silylate the sample, and the sample was concentrated to the original volume with a flow of nitrogen and sealed in a reaction vial. The vial was heated to 45°C for 2 h. The silylated sample was then analyzed by gas chromatography.

Simultaneous Extraction/Silylation

The silylating reagent, BSTFA with 1% TMCS, was added with the methylene chloride to the filter strips in the round-bottom flask. The amount of BSTFA added varied with the sample. Ultrasonic extraction conditions were identical to those used above. Enough silylation reagent was added to ensure that a large excess was always available. The filtered extract was concentrated in the same manner as above and analyzed by gas chromatography.

Soxhlet Extraction and Silylation

The filter strips were placed into a glass Soxhlet extraction thimble with a coarse porous frit and extracted in a Soxhlet extractor for 8 h. After extraction, the extract was silylated and analyzed as described above.

Instrumentation

A Hewlett-Packard 5810 gas chromatograph containing an all-glass split-injection system with a minimum split ratio of 1:13 at an injection port temperature of 250°C was used for the analysis. Split ratios less than this resulted in a broad solvent peak. Hydrogen was used as the carrier gas at a linear velocity of 38 cm/s with an oven temperature of 250°C.

Two columns were used: (*1*) 30 m × 0.25 mm id, 0.25 μm film thickness, SE-30 fused-silica column; and (*2*) for all quantitative work, 30 m × 0.25 mm id, DB-1 0.25 μm film thickness, Durabond fused-silica column (J & W Scientific, Rancho Cordova, CA). For all samples, an initial oven temperature of 70°C with a 2 min hold was followed by a 4°/min increase to a final temperature of 250°C. A standard flame ionization detector with a make-up flow of 30 mL/min of nitrogen was used to help the column effluent into the flame and to enhance the FID response. The silicon-selective hydrogen atmosphere flame ionization detector [HAFID (Si)] was constructed and used as described by Osman et al. (8). The hydrogen flow was doped with ferrocene vapors by passing a controlled flow of nitrogen through a column of 80–100 mesh Chromosorb W (Manville, Denver, CO) saturated with ferrocene (Sigma Chemical Co., St. Louis, MO). Both the FID and the HAFID (Si) signals were amplified by the HP 5710A electrometer, recorded on a strip chart recorder, and integrated by interfacing with an auxiliary A/D board of an HP 5830 gas chromatograph.

Results and Discussion

Comparison of Ultrasonic Derivatization Procedures

After 5 high-volume air samples ranging from 5000 to 18 000 cu. m were collected over 1 week, the glass fiber filters were carefully cut into 2 equal parts. Extractions were begun within 24 h of each sample collection. Table 1 lists the sampling and extraction conditions.

One half of the glass fiber filter from each sample was extracted with pure methylene chloride. The extract was then split with one half remaining underivatized; the other was treated with BSTFA. Figure 1 illustrates typical chromatograms obtained from both the unsilylated and the silylated extracts. The particulate sample used in Figure 1 was number 1229, but similar results were obtained for all particulate samples. Chromatograms in Figure 1 clearly demonstrate the necessity of derivatization if organic analysis of particulate matter is to be attempted. A massive number of additional peaks was detectable when the extract was derivatized.

The object of this investigation was to compare simultaneous extraction and derivatization with sequential extraction and derivatization. Thus, the real purpose for comparisons of the type shown in Figure 1 was to identify important nonpolar components in the mixture, i.e., those compounds which do not require derivatization prior to chromatography. By comparing the silylated chromatogram from the sequential extraction/silylation procedure (Figure 1A) with the unsilylated chromatogram from the same extraction (Figure 1B), unsilylated peaks, representing hydrocarbons and other organic compounds nonreactive with BSTFA, could be distinguished from the reactive oxygenated compounds. The peaks that appear in chromatogram B but are not in chromatogram A are unsilylated reactive organic compounds.

Once a number of underivatized peaks were established for each of the 5 test samples, these peaks could be used to normalize the extraction efficiency between the simultaneous and the sequential extraction and derivatization procedures.

By identifying the underivatized peaks in both procedures, their areas could be compared, because it was assumed that they would be extracted with the same efficiency by both procedures. The differences found in the areas of these peaks would be due either to differences in the amount of particles on the 2 filter halves or to losses during preparation of the sample. Knowing the magnitude of these differences, a correction was made by finding the average area ratio between the 2 procedures for the underivatized peaks. These values are shown in Table 2. The areas of the silylated peaks were then adjusted by using these average ratios, allowing the efficiency of the 2 procedures in extracting and derivatizing the reactive organic compounds to be compared.

For each of the samples, the adjusted areas obtained by both procedures were compared for the major silylated peaks. Table 3 lists the assigned retention time and the adjusted area ratios for these peaks. In certain cases where no peak

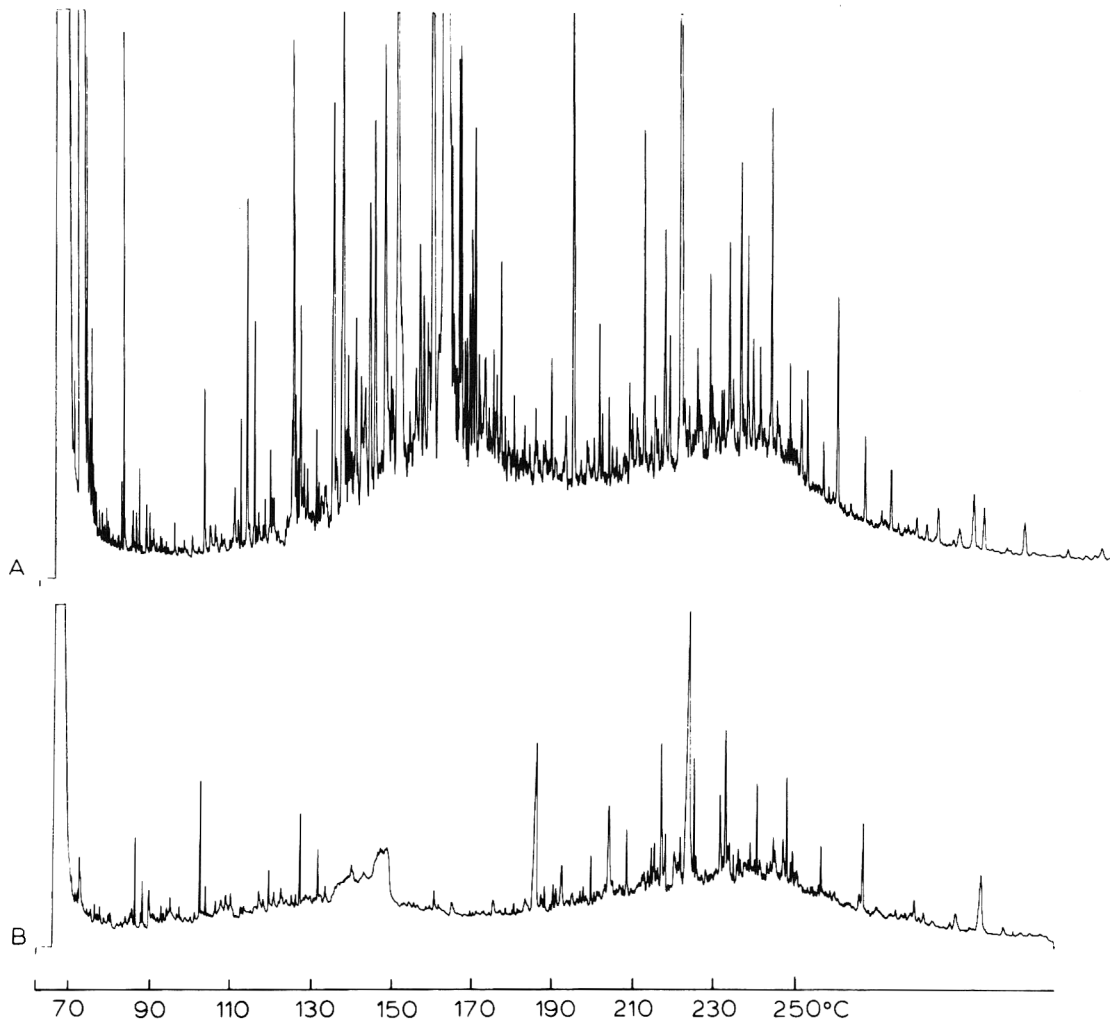

Figure 1. Chromatograms of silylated (A) vs unsilylated (B) ultrasonic extract. These 2 chromatograms from column 1 demonstrate typical results obtained when HiVol filters (Sample 1229) are extracted with methylene chloride by ultrasonication followed by silylation with BSTFA.

was detected in sequential treatment, the area reject value, which is set in the integrater to ignore smaller peaks, was used to determine a greater than (>) value for the ratio.

Figure 2 graphically shows the average of these values with the standard deviations marked. Below a relative retention time of 1.0, simultaneous treatment was substantially more effective than sequential treatment in extracting and derivatizing the reactive compounds from the particles.

The wide fluctuations in ratios between different compounds indicate that the compounds which react to form TMS derivatives vary widely in their attraction to the particles and/or their solubility in methylene chloride. A noticeable trend toward lower ratios occurs as the retention time increases. The longer retained peaks may be less polar due to increased proportion of nonpolar components compared to the number of polar functional groups as the compounds increase in size.

Table 2. Area ratios of underivatized peaks: simultaneous/sequential treatment

Sample No.	No. of peaks	Ratio of unreactive response ratio	RSD, %
1210	7	1.34	11.3
1229	14	0.695	26.7
115	13	0.775	32.4
128	22	0.803	42.5
131	20	1.13	25.7

Another observation is the large values for the standard deviation for some of the compounds with higher ratios. This is primarily due to the fact the that larger uncertainties in the smaller value, which were sometimes close to the detection limit, are amplified by the division process. This also could indicate that the more difficult-to-extract compounds, which give the higher ratios, are more susceptible to differences in sample matrixes and extraction conditions. Such factors as moisture content, inorganic composition, and physical parameters such as size and surface area could vary among samples.

Extraction Efficiency of Simultaneous Treatment Protocol

The quantitative recovery of alcohols, phenols, and carboxylic acids was investigated using repetitive extractions of the same sample filter. Full recovery was assumed when further repetition of the extraction produced no detectable sample peaks. For comparison, repetitive extractions using the sequential and Soxhlet methods were also obtained.

From high volume samples, collected as described in Table 4, each filter was split into 4 equal parts. One quarter was Soxhlet-extracted (fraction D), 2 quarters were simultaneously extracted and silylated (fractions A and C), and one quarter was sequentially extracted and silylated (fraction B). The extraction procedure was repeated for various intervals as shown in Table 5.

The percent recovery was determined for a variety of com-

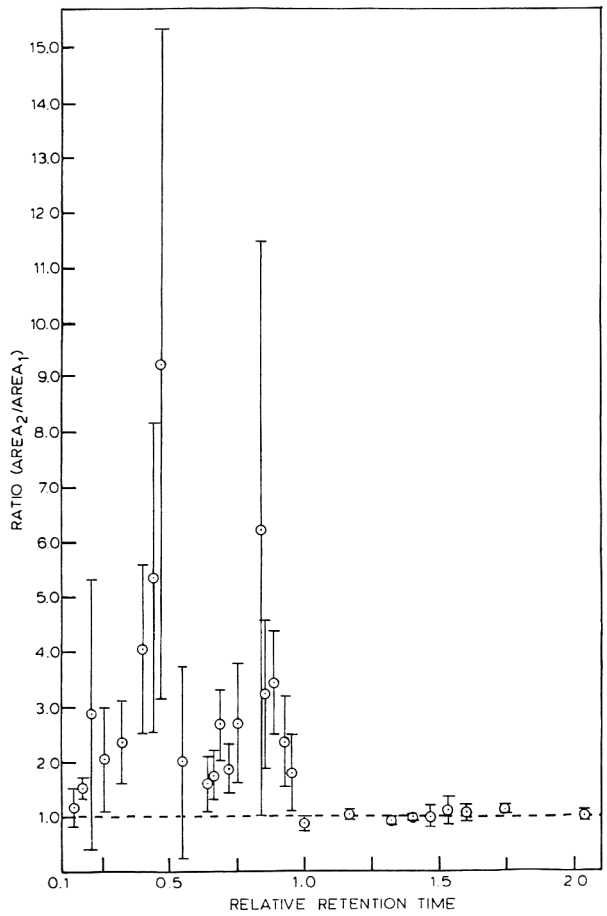

Figure 2. Adjusted area ratios of silylated compounds as a function of relative retention times. Plot of data presented in Table 3: point plotted is average of ratios for each sample at a given relative retention time. Average values are bracketed by standard deviation of 5 different samples.

pounds in order to represent a wide range of functional groups. Compounds chosen were glycolic acid (a hydroxyacid); glutaric acid (a diacid); phthalic acid (an aromatic diacid); 2-naphthol (a phenol); 1-naphthalene acetic acid (a polycyclic aromatic acid); dodecanol (a fatty alcohol), and palmitic acid (a fatty acid). Palmitic, phthalic, and glycolic acids were identified by mass spectrometry. The other compounds were identified by comparison of retention times with known standards.

Figures 3 and 4 illustrate the results of the extraction study for these test compounds. Figure 3 reports the percent recovery for the nonaromatic compounds; Figure 4 provides information on the aromatic compound. The information provided by these experiments not only corroborates what was indicated in Figure 2, but dramatically illustrates that the simultaneous method of extraction and derivatization is more efficient than either of the other 2 methods in which derivatization follows the extraction process.

The data from the sequential method, which are denoted by the diamond-shaped symbols in the graphs of Figures 3 and 4, indicate that the maximum efficiency of the extraction process ranged from about 10 to 20% for most of the test compounds. Palmitic acid was an exception to this trend although the maximum extraction efficiency for this compound was still only about 50%. Data from the Soxhlet extraction method, denoted by the cross marks, indicated that the maximum extraction efficiency was somewhat higher than for the former, ranging from 10 to 50%.

Since Soxhlet extraction is a continuously repetitive pro-

Table 3. Adjusted area ratios for silylated peaks: simultaneous/sequential treatment

Peak No.	Rel. time	Adjusted area ratios of samples				
		1210	1229	115	128	131
1	0.143	1.065	1.61	1.38	0.679	1.05
2	0.177	1.52	1.52	—	1.30	1.74
3	0.209	0.746	0.758	—	5.27	4.70
4	0.261	—	1.74	3.37	1.83	>1.2
5	0.332	>1.7	—	3.17	—	>2.2
6	0.399	>5.5	3.61	5.04	—	>2.1
7	0.436	6.17	2.01	4.59	9.61	4.37
8	0.468	3.97	2.65	17.5	9.78	12.3
9	0.548	1.77	0.848	0.682	1.63	>5.0
10	0.641	2.49	1.24	1.36	1.63	1.30
11	0.662	—	1.37	1.47	1.82	2.40
12	0.687	2.87	1.75	3.21	—	2.91
13	0.717	1.51	1.40	1.81	2.47	2.16
14	0.7521	2.78	1.40	4.39	2.58	2.40
15	0.837	1.53	13.9	2.19	4.40	9.11
16	0.854	2.12	2.76	2.40	5.47	3.41
17	0.888	3.83	3.46	1.91	4.40	3.62
18	0.928	1.12	2.93	3.28	2.41	2.10
19	0.953	1.59	1.28	2.13	2.86	1.14
20	1.000	1.02	—	0.923	0.826	0.688
21	1.167	1.02	1.19	1.03	0.912	1.06
22	1.322	1.02	0.989	0.932	0.884	0.851
23	1.401	1.03	0.952	—	0.963	0.996
24	1.464	1.00	0.865	—	1.29	0.867
25	1.531	1.53	1.02	1.03	1.03	0.956
26	1.601	1.05	1.05	0.999	1.32	0.985
27	1.745	1.27	1.05	1.12	1.12	1.13
28	2.040	1.03	0.902	—	—	1.11

Table 4. Sampling conditions used in extraction efficiency study

Sample No.	Sampling time, h	Total flow, cu. m	Sample mass, g	Total suspended particles, μg/cu. m
217	97.90	10 900	0.5606	32.2
33	163.8	18 100	0.3508	30.9

Table 5. Extraction conditions used in efficiency study

Extn method	Filter 1			Filter 2			
	A Sim.	B Seq.	C Sim.	A Sim.	B Sim.	C Sim.	D Sox.
1	0.3[a]	0	0.75	0.50	0.50	0.80	0
	2[b]	2	12	5	5	5	20
2	0.20	0	0.20	0.20	0.20	0.20	0
	2	2	2	11	11	11	20
3	0.20	0	0.20	0.20	0.20	0.20	—
	2	2	2	6	6	6	—
4	0.20	0	0.2	0.20	0.20	0.20	—
	2	2	2	12	12	12	—
5	0.20	0	0.20	0.20	0.20	0.20	—
	2	2	4	6	6	6	—
6	0.20	—	—	—	—	—	—
	2	2	—	—	—	—	—
7	0.20	—	—	—	—	—	—
	2	—	—	—	—	—	—
8	0.20	—	—	—	—	—	—
	2	—	—	—	—	—	—
9	0.20	—	—	—	—	—	—
	4	—	—	—	—	—	—
10	0.15	—	—	—	—	—	—
	5	—	—	—	—	—	—

[a] Throughout, first line is BSTFA, mL.
[b] Throughout, second line is duration, h.

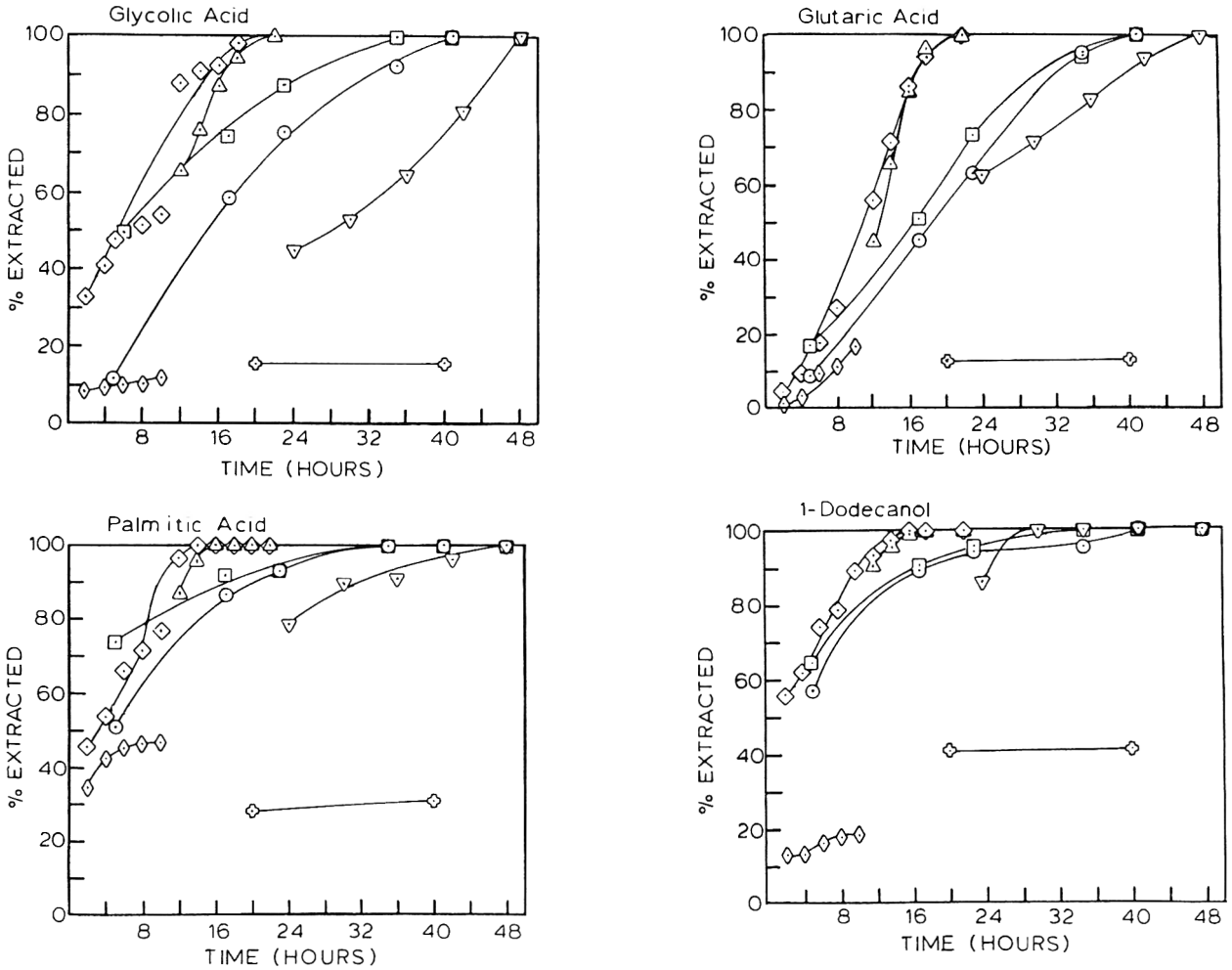

Figure 3. Extraction efficiencies of selected nonaromatic acids and alcohols. Extraction times and amounts of silylation reagents were varied for each extraction and are given in Table 5. Symbols: (◇) sim., filter section 1A; (◇) seq., 1B; (△) sim., 1C; (○) sim., 2A; (▽) sim., 2B; (□) sim., 2C; (⬭) sox., 2D.

cess, it is not surprising that it was more efficient than 5 cycles of the sequential method. Only for palmitic acid was the ultrasonic extraction procedure more efficient than the Soxhlet extraction method. The data set for this study was a very complicated one, similar to that shown in Figure 1. Thus, it is probable that with this one compound a contaminant coeluted with the silylated palmitic acid, producing a response greater than that obtained with the Soxhlet extraction method in which the contaminant did not exist or was sufficiently separated from the test compounds not to be integrated as the same peak. Also, it is important to realize that these experiments were not designed to compare specifically the Soxhlet and ultrasonic methods. Conditions for the 2 extraction processes were not matched. The ultrasonic extraction was performed for 10 h with 5 repetitions, while the Soxhlet extraction was continued for 40 h.

Table 6. Percent quantitative extraction efficiencies from synthetic samples

Compound	No. of extns	Total time extd, h	Extn recovery, %
Glycolic acid	5	36	92.3
Lactic acid	4	37	99.5
Catechol	5	36	92.8
Glutaric acid	5	36	88.3
1-Naphthol	5	36	99.1
1-Dodecanol	5	36	100
1-Naphthalene acetic acid	4	37	95.2
Fluoranthene	4	37	95.7
Palmitic acid	4	37	100
n-Docosane	4	37	93.0

The primary purpose of these experiments was to investigate the potential of simultaneous ultrasonic extraction and silylation. Using glass fiber filter sections numbered 1A, 1C, 2A, 2B, and 2C, the process was applied with different extraction times and silylation reagent concentrations as given in Table 5. The most efficient single extractions were obtained for section 1C, which was extracted for 12 h with 0.75 mL of BSTFA. Extraction efficiencies between 45 and 90% were achieved for these initial conditions. With subsequent extractions, 100% efficiency could be achieved for each of the test compounds within a 24 h extraction period. For all simultaneous extraction conditions investigated, 100% efficiencies were achievable within a 48 h extraction period.

Quantitative Extraction Studies

In the extraction efficiency studies described above, 100% efficiency was determined for each of the compounds when additional quantities of that compound could not be extracted with subsequent extractions. The advantage of this approach is that extraction efficiencies can be measured using real samples. The disadvantage is that absolute quantities cannot be measured, because it is not possible to know the true quantity of the compounds initially contained in the real samples.

An alternative procedure for investigating the effectiveness

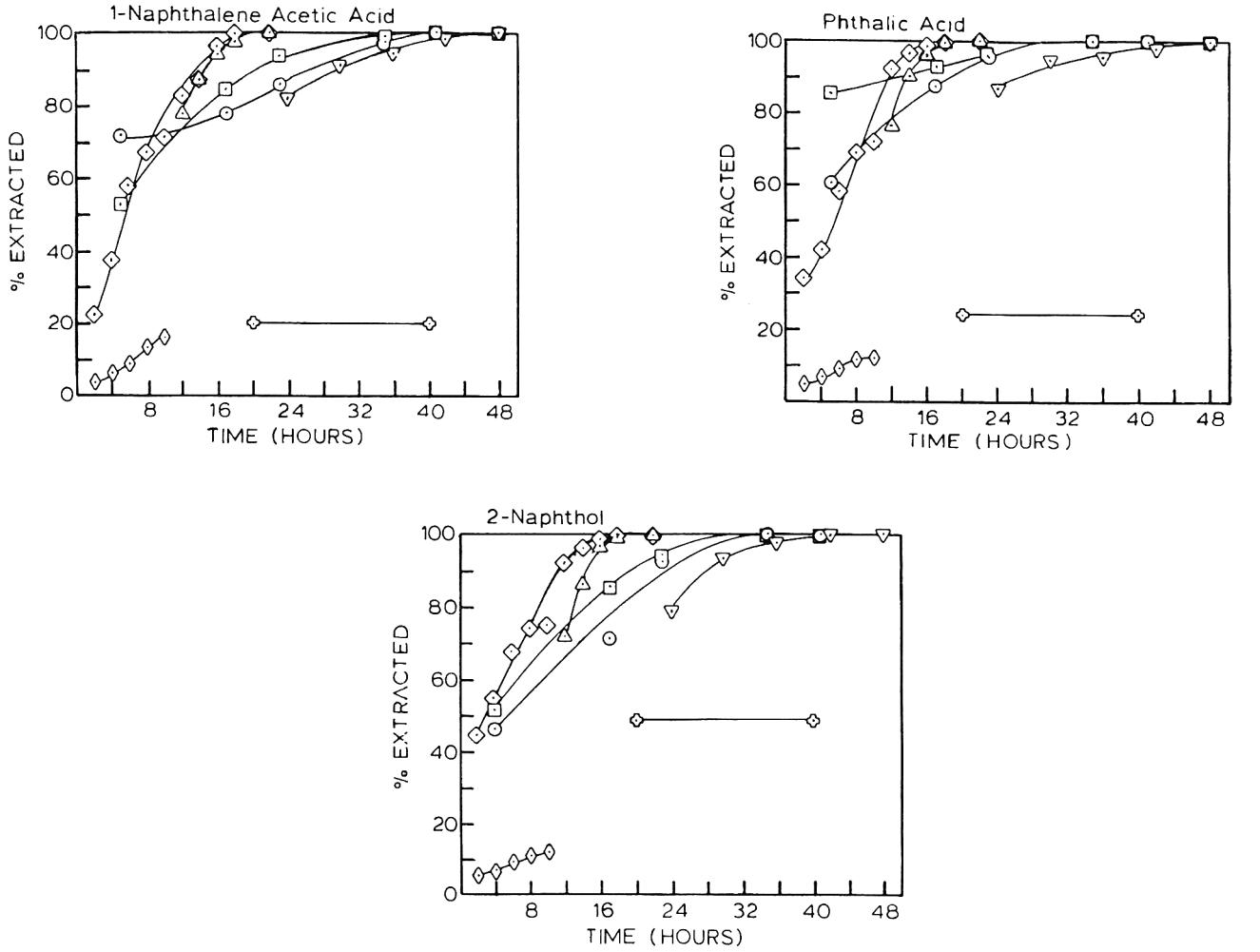

Figure 4. Extraction efficiencies of aromatic acids and phenols. Glass fiber filter sections, extraction times, extraction methods, and extraction conditions were the same as those given in Figure 3 and Table 5.

of an extraction method is to prepare a synthetic sample with known quantities of test compounds. In this study, a standard mixture of acids, alcohols, and phenols was prepared by dissolving underivatized standards in a compatible solvent. This solution was added to a glass fiber filter, and the solvent was allowed to evaporate at room temperature under nitrogen. Using the simultaneous method of extraction and silylation, this glass fiber filter was extracted in 5 steps over 36 h. An equal amount of the standard solution was added to a pure derivatizing agent and heated for 2 h at 60°C. These conditions were found to be sufficient for complete derivatization of the standard solution. The derivatized standard solution was adjusted to the same volume as that of the spiked filter extract, and the chromatograms of the 2 mixtures were compared to determine the percent recovery of the extraction process.

The results of this study are shown in Table 6. Better than 88% extraction recovery was obtained for all compounds in the standard mixture. Also, extraction profiles similar to those shown in Figures 3 and 4, using the simultaneous method, were obtained with the synthetic sample. Clearly, these studies have illustrated that many polar organic compounds are so tenaciously held to airborne particulate matter that the standard extraction techniques used to access the organic fraction of these particles are insufficient to recover completely many of these compounds. Although the studies reported here were not investigated in sufficient detail to recommend an optimal procedure, silylation of samples during ultrasonic agitation was shown to significantly increase the recovery of alcohols, phenols, and carboxylic acids compared to extraction followed by silylation.

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DISINFECTANTS

Evaluation of Penicylinders Used in Disinfectant Testing: Bacterial Attachment and Surface Texture

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Two possible deficiencies in the AOAC use-dilution method for registration of chemical disinfectants by the Environmental Protection Agency are examined: (1) the physical disparities among brands of penicylinders and (2) the variability of bacterial numbers on penicylinders depending upon test strain and penicylinder surface texture. Textural differences of 2 brands of stainless steel penicylinders, one brand of porcelain, and one brand of glass were assessed by scanning electron microscopy. A considerable variation in smoothness of both inner and outer surfaces of stainless steel and porcelain penicylinders was observed. Glass penicylinders were very smooth. Numbers of bacteria attached to a penicylinder were assessed by vortexing the penicylinders 30 s at No. 4 after using the AOAC method of bacterial inoculation and drying 40 min at 37°C. With this methodology, stainless steel carriers retained the 3 AOAC-recommended bacterial test strains differentially: ca 10^7 for *Pseudomonas aeruginosa*, 5×10^6 for *Staphylococcus aureus*, and 10^6 for *Salmonella choleraesuis*; glass retained 10^6-10^7 organisms of all 3 test strains; porcelain retained about that amount of *S. aureus* but 10^5-10^6 *P. aeruginosa* and 10^3-10^4 *S. choleraesuis*. These data suggest that disinfectants are not similarly challenged with the AOAC-recommended test bacteria and that an alternative method should be considered to ensure comparable numbers of bacteria on penicylinders.

The AOAC use-dilution method (1) is the official method for evaluating the bactericidal activity of hospital disinfectants. For many years, the Environmental Protection Agency (EPA) performed intramural pre- and postregistration efficacy testing of some chemical disinfectants. In 1982, the testing was curtailed, presumably for budgetary reasons and to reduce government regulation of business. Thus, manufacturers presently do not need verification of efficacy claims by EPA or by an independent testing laboratory when they apply to register a disinfectant. In the past 5 years, the test method has often been criticized, and several federal, commercial, state, and university laboratories performing the test have been unable, because of either intrinsic deficiencies of the disinfectants or problems with the method, to substantiate the bactericidal claims of some manufacturers (2).

The present investigation was undertaken to examine 2 deficiencies that have been proposed to account for the AOAC use-dilution test variability: physical disparities among brands of penicylinders and the relationship of such disparities to the adherence of bacteria to penicylinders.

Experimental

Media and Reagents

(a) *Nutrient broth.*—AOAC **4.001a.** Anatone (American Laboratories, Inc., Omaha, NE 68127) and beef extract (Difco Laboratories, Detroit, MI 48232) were used as specified and prepared according to directions with water. Prior to sterilization, pH was adjusted to 6.8.

(b) *Pour plate agar.*—Plate count agar (Difco).

(c) *Phosphate buffer dilution water.*—AOAC **4.020(f).**

(d) *Laboratory distilled water.*

(e) *Asparagine.*—Bacto asparagine (Difco) 0.1% (w/v) aqueous solution.

(f) *Ethanol.*—*(1)* 95% (v/v) for concentrations of 25, 50, 75, and 95%. *(2)* Absolute alcohol.

(g) *Freon 13.*—E. I. duPont de Nemours & Co., Wilmington, DE 19898).

(h) *Freon 113.*—(Polysciences Inc., Warrington, PA 18976).

(i) *Fixative.*—2% glutaraldehyde–2% paraformaldehyde. Glutaraldehyde 50% (Ted Pella, Inc., Tustin, CA 92680). Paraformaldehyde, reagent grade (Fisher Scientific, Pittsburgh, PA 15219).

(j) *Sorenson's phosphate buffer, 0.2M.*—To 363.5 mL water 10.325 g sodium phosphate, dibasic (Fisher Scientific) was added and dissolved; 136.5 mL water and 3.715 g potassium phosphate, monobasic, was added. pH was adjusted to 7.3.

(k) *Sorenson's phosphate buffer, 0.1M.*—Sorenson's 0.2M buffer was diluted 1:2 with water.

(l) *Osmium tetroxide, 1.0%.*—1.0 g osmium (Electron Microscopy Sciences, Ft. Washington, PA 19034) was dissolved in 50.0 mL water and diluted 1:2 with Sorenson's phosphate buffer 0.2M.

(m) *Dilution water.*—Phosphate buffer dilution water, AOAC **4.020(f)** (1).

Organisms

Staphylococcus aureus 6538, *Salmonella choleraesuis* 10708, *Pseudomonas aeruginosa* 15442 (American Type Culture Collection, Rockville, MD 20852).

Apparatus

(a) *Stainless steel penicylinders.*—8 ± 1 mm od, 6 ± 1 mm id, length 10 ± 1 mm (S&L Metal Products Corp., Maspeth, NY 11378; and Fisher Scientific).

(b) *Porcelain penicylinders.*—8 ± 1 mm od, 6 ± 1 mm id, length 10 ± 1 mm (Fisher Scientific).

(c) *Glass penicylinders.*—8 ± 1 mm od, 6 ± 1 mm id, length 10 ± 1 mm (University Research Glassware, Carrboro, NC 27510).

(d) *Colony counter.*—(New Brunswick Scientific, Edison, NJ 08818-4005.)

(e) *Vortex mixer.*—Vortex Genie (Fisher Scientific).

(f) *Screw-capped (SC) tubes.*—Flint glass, 20 × 150 mm (Fisher Scientific).

(g) *Petri dishes.*—Polystyrene, 100 × 15 mm (Fisher Scientific).

(h) *Scanning electron microscope.*—ETEC Autoscan (Perkin-Elmer Electron Beam Technology, Hayward, CA 94545).

(i) *Sonicator.*—Ultrasonic Cleaner Model 450 (RAI Research Corp., Hauppauge, NY 11787).

(j) *Filter paper.*—Whatman No. 2, 9 cm diameter (Whatman Ltd., Maidstone, UK).

Procedures

(a) *Broth medium.*—Nutrient broth was prepared as in AOAC **4.001a** (1), with 10 mL portions placed in 20 × 15

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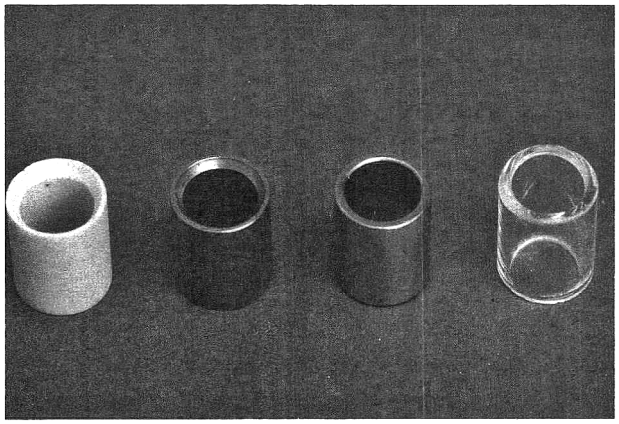

Figure 1. Types of available penicylinders for the use-dilution method. From left to right, porcelain (Fisher Scientific), stainless steel (Fisher Scientific), stainless steel (S&L Metal Products Corp.), and glass (University Research Glassware). All penicylinders are 8 ± 1 mm od, 6 ± 1 mm id, and 10 ± 1 mm long.

mm screw-capped, flint glass tubes, and steam sterilized at 121°C for 15 min. Broths were inoculated from stock culture slants and incubated overnight at 37°C. At least 3 consecutive 24 h broth transfers were made with 4 mm id platinum/rhodium loop (American Scientific Products, McGaw Park, IL 60085). Broths used for testing were inoculated and incubated 49 h. The 49 h broth cultures contain ca 1×10^9 *P. aeruginosa*/mL and 5×10^8 *S. aureus* and *S. choleraesuis*/mL.

(b) *Penicylinder preparation.*—New penicylinders were boiled in water for ≥10 min to remove oil residues. After water was poured off, penicylinders were sonicated in water for at least 5 min to remove additional debris. Used penicylinders were steam-sterilized for 15 min at 121°C prior to sonication. After sonic cleaning and rinsing in water, all penicylinders were placed in 1N NaOH overnight as specified in AOAC **4.009** (1). Penicylinders were then rinsed in tap water until neutral to phenolphthalein, rinsed twice in water, drained, placed 10 to a tube (20 × 150 mm, screwcap, flint glass), covered with fresh 0.1% asparagine solution, sterilized 15 min at 121°C (15 psi), and held at room temperature until ready for use. During preparation, any cylinders with noticeable imperfections (scratches, chips, etc.) were discarded.

(c) *Penicylinder inoculation.*—Test penicylinders were aseptically placed in swirled broth-cultures with volume of broth directly proportional to number of penicylinders (i.e., 1 mL broth/penicylinder). When we simultaneously inoculated more than one type of penicylinder, we alternated their placement into the tubes. If necessary, tubes were shaken to rearrange cylinders in broth so that all were covered by broth culture. Tubes remained undisturbed 15 min at room temperature.

(d) *Penicylinder drying.*—Following inoculation, penicylinders were removed from broth with a flamed wire hook and were placed on end in sterile glass petri dishes (no more than 10/dish) matted with 2 layers of 9 cm diameter Whatman No. 2 filter paper. The dishes were covered and placed in an incubator at 37°C and were dried 40 min at 25–35% relative humidity.

(e) *Evaluation of cell removal methods.*—Two methods of mechanically removing attached bacteria from penicylinders using a vortex mixer were compared. For each of the 3 bacteria tested, 9 penicylinders were inoculated as described above. Six were randomly selected, and each was placed into a 20 × 150 mm screw-capped, flint glass tube containing 10

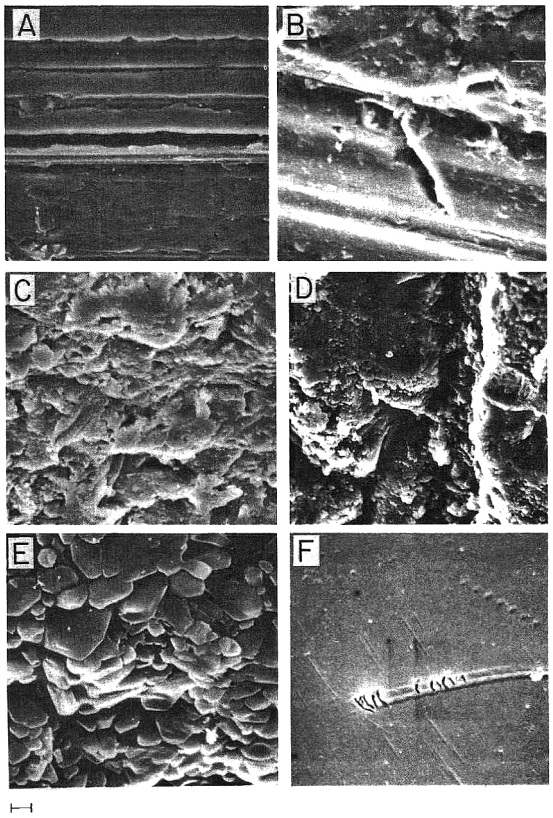

Figure 2. Scanning electron photomicrographs of penicylinder surface textures: S&L stainless steel, outside (A) and inside (B); Fisher stainless steel, outside (C) and inside (D); porcelain, outside (E); and glass, outside (F). Scale: bar = 1 μm.

mL sterile phosphate buffer dilution water (PBDW), AOAC **4.020(f)** (1). For method A, a vortex mixer set at No. 4 for 30 s was used; for method B, a vortex mixer set at No. 8 for 3 min was used. After each vortexing procedure, dilutions of 10^{-4} and 10^{-5} in PBDW were prepared for *S. aureus* and *P. aeruginosa,* and dilutions of 10^{-3} and 10^{-4} were prepared for *S. choleraesuis.* For each dilution, 1.0 mL was inoculated into each of two 15 × 100 mm sterile petri dishes to which 15–17 mL pour plate agar at 42–45°C was added. When all plates solidified, they were inverted and incubated 48 h at 37°C. Colonies on all plates were enumerated using a colony counter, and those with counts between 30 and 300 were used for calculations.

(f) *Bacterial removal from penicylinders.*—After they were dried, each of 5 penicylinders was aseptically placed into a 20 × 15 mm screw-capped, flint glass tube containing 10 mL sterile PBDW. Organisms were removed from dried penicylinders by placing tube on a vortex mixer set at No. 4 for 30 s. Dilutions of 10^{-3}, 10^{-4}, and 10^{-5} were prepared in PBDW using sample aliquots of 1.0 mL. At each of the 3 dilutions, 1.0 mL was plated in duplicate by the pour plate method, described above. When solidified, all plates were inverted and incubated 48 h at 37°C. Following incubation, colonies were enumerated using a colony counter, and those with counts between 30 and 300 were used for calculations.

(g) *Scanning electron microscopy (SEM).*—Sterile penicylinders for surface texture examination were prepared as described above. They were aseptically removed from sterile 0.1% asparagine solution and placed into sterile glass petri dishes matted with 2 layers of Whatman No. 2 filter paper and allowed to dry ≥30 min at 37°C. Penicylinders with adherent bacteria were inoculated and dried as described

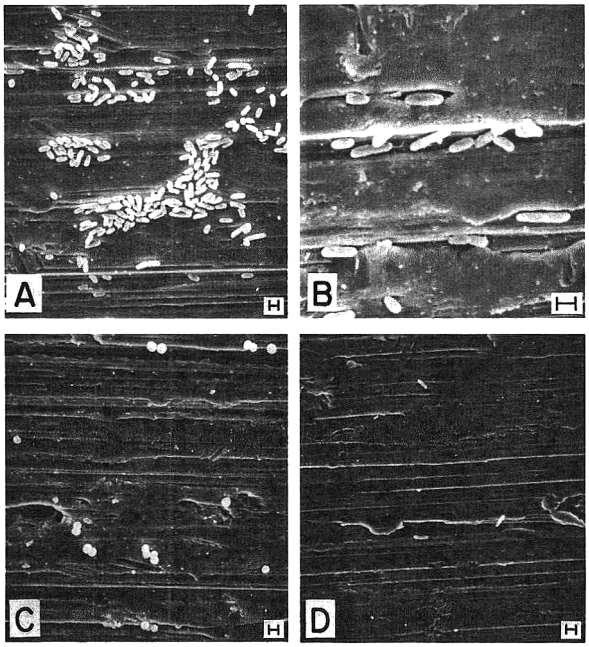

Figure 3. Comparison of numbers of use-dilution test bacteria seeded on recommended S&L stainless steel penicylinders: A, *P. aeruginosa*; B, *P. aeruginosa* residing in penicylinder (S&L) grooves; C, *S. aureus*; and D, *S. choleraesuis*. Scale: bars = 1 μm.

above. All penicylinders were immediately fixed in a mixture of 2% glutaraldehyde–2% paraformaldehyde (pH 7.2) and then rinsed in phosphate buffer. They were postfixed in a buffered 1% osmium tetroxide solution, dehydrated in a graded ethanol series, passed through graded solutions into a transition fluid of Freon 113, and dried by critical point technique using Freon 13. Penicylinders were mounted on SEM specimen stubs with silver paste and were spatter-coated with gold or gold–palladium. Specimens were examined in an ETEC Autoscan scanning electron microscope at an accelerating voltage of 20 kV. Prior to photographing a surface, at least 20 typical fields were examined.

Results

Surface Texture of Penicylinder Types

Examinations were performed to determine if physical differences exist among the 4 types of available penicylinders: stainless steel (both S&L and Fisher), porcelain, and glass (Figure 1). As can be seen in Figure 2, significant differences in surface texture were noted among the penicylinders when examined by SEM. The 2 brands of stainless steel penicylinders were markedly different, with the outer surfaces of the AOAC-recommended S&L brand principally smooth (Figure 2A) but exhibiting some grooves where bacteria could reside. The inner surface of the S&L penicylinder (Figure 2B) and the outer/inner surfaces of the occasionally used Fisher penicylinder (Figure 2C, D) showed deep grooves and significant pitting. Porcelain penicylinders were extremely irregular (Figure 2E) and the glass penicylinders were very smooth (Figure 2F).

Cell Removal from Penicylinders

For *S. aureus*, both methods A and B revealed identical mean values of 1.3×10^7 organisms ($P > 0.1$). SEM examination of a randomly selected penicylinder revealed no cells attached to the penicylinder following processing according to method A. Mean values for *S. choleraesuis* were 9.7×10^5 for method A, and 1.2×10^6 for method B ($P > 0.1$). *P. aeruginosa* showed a mean value of 6.3×10^6 cells using method A, and 1.5×10^7 cells using method B ($P > 0.1$). Because the number of cells per penicylinder did not differ by more than 0.5 log despite which removal method was used and because method B often resulted in tube breakage, method A was used.

Table 1. Mean numbers of organisms (±standard deviation) on penicylinders seeded with AOAC-recommended test bacteria

Penicylinder	No. of organisms,[a] $\times 10^6 \pm$ SD		
	S. aureus (ATCC 6538)	*P. aeruginosa* (ATCC 15442)	*S. choleraesuis* (ATCC 10708)
Stainless steel			
S&L	5.98 ± 1.61	13.70 ± 5.20	0.82 ± 0.11[b]
Fisher	5.38 ± 2.59	18.92 ± 12.48	0.99 ± 0.33[b]
Porcelain	7.04 ± 4.01	0.97 ± 0.94[b,c]	0.01 ± 0.009[b,c]
Glass	7.94 ± 8.92	13.22 ± 8.63	1.70 ± 0.34[b,c]

[a] All mean numbers of organisms represent 5 replicates except *P. aeruginosa* on porcelain penicylinders which has 4 replicates.
[b] Significant difference between test strains, $P < 0.05$.
[c] Significant difference between penicylinder types, $P < 0.05$.

Cell Attachment to Penicylinder Types

The mean numbers of *S. aureus* that adhered to S&L and Fisher stainless steel penicylinders were directly comparable (about 5×10^6), as they were on glass and porcelain (about 7×10^6) (Table 1); there were no significant differences among the 4 penicylinder types. Mean numbers of *P. aeruginosa* attached to S&L and Fisher stainless steel penicylinders also were directly comparable, whereas differences were observed between porcelain (low cell numbers) and the other penicylinders (Table 1). An SEM of a porcelain penicylinder inoculated with *P. aeruginosa*, however, showed cell attachment in large numbers, most likely indicating difficulty in removal of the cells from the rough surface. No significant difference was observed between glass and stainless steel. Mean numbers of *S. choleraesuis* on S&L and Fisher SS penicylinders again were directly comparable with about 10^6 organisms/penicylinder, while the porcelain and glass both differed significantly from the stainless steel. All 3 AOAC recommended test bacteria and all 4 brands of penicylinders varied considerably as demonstrated by the large standard deviations.

When numbers of the 3 AOAC test bacteria adhering to the recommended S&L penicylinder (Table 1) were assessed, *P. aeruginosa* appeared most adherent, with a mean number of organisms approaching 1.4×10^7; somewhat less *S. aureus* attached, at approximately 6.0×10^6; and *S. choleraesuis* attached the least, at 8.2×10^5. These findings were visually corroborated by SEM when inoculated penicylinders were examined (Figure 3).

Discussion

The surface texture of available penicylinders that might be used in the AOAC use-dilution method of disinfectant testing varies significantly. Bacteria that reside in penicylinder grooves may be protected from disinfectant exposure. The method recommends only the S&L brand of stainless steel penicylinder; however, in 1982, S&L Metal Products Corp. suspended the manufacture of their penicylinders for 14 months. At that time many laboratories were forced to purchase stainless steel penicylinders from the other major manufacturer, Fisher Scientific.

The Fisher penicylinders have the same measurements as those from S&L, but Fisher penicylinders have ends beveled to the inside. The degree of polish on the 2 brands also was visibly different. The Fisher penicylinder appeared less pol-

ished than the S&L, although the companies have manufacturing specifications that are nearly identical. S&L Metal Products Corp. manufactures their penicylinders using 304-grade stainless steel, with a maximum degree of polish of 32 μm on the outside and 36 μm on the inside. Fisher Scientific also uses 304-grade stainless steel but specifies a maximum degree of polish of 32 μm on the outside and the inside. A measurement of 32 μm indicates that the distance from the lowest to the highest point of a depression on the polished surface can be no greater than 32 μm. Scanning electron microscopy corroborated the marked textural difference between the 2 brands. The 3 bacterial test strains, however, exhibited similar adherence to both brands of penicylinders.

EPA requires that porcelain penicylinders be used as carriers in the use-dilution method if a germicidal claim is made for a porous surface. Fisher Scientific is the sole manufacturer and supplier of this penicylinder, which is identical to the Fisher stainless steel penicylinder in terms of size and design. The porcelain surface was found microscopically to be extremely rough. Bacterial adherence to the porcelain varied significantly among the test bacteria, although values for *P. aeruginosa* may be falsely low due to difficulty in removing cells from the matrix of the porcelain cylinder by the vortex method. In addition, the porcelain is susceptible to cracking and chipping. For these reasons it cannot be recommended as a substitute for stainless steel in use-dilution testing.

Glass penicylinders were included in this study because of their extremely smooth inner and outer surfaces. These were custom-made from Pyrex tubing (with fire polished ends) to the exact dimensions of the S&L stainless steel penicylinders. The most similar bacterial adherence (10^6–10^7) among the 3 test strains was found on glass penicylinders when compared with steel or porcelain penicylinders. However, glass penicylinders dried very poorly; therefore it is possible that the bacterial attachment is not entirely representative of organisms dried onto the glass surface, but rather represents organisms carried over in the remaining droplet of undried broth.

The most significant finding of the study was that the 3 test bacteria specified in the AOAC method attached to stainless steel penicylinders differently, with more than 10^7 for *P. aeruginosa*, approximately 5×10^6 for *S. aureus*, and 10^6 or less for *S. choleraesuis*. Thus, a 1.5 log difference or more among the test organisms is possible. Such a difference might not be unexpected considering differing numbers of cells in broth culture as well as other morphological and physiological differences among the test strains that may affect bacterial attachment to penicylinders.

This is the first demonstration that disinfectants claiming efficacy against various bacteria are not similarly challenged in the AOAC use-dilution method. When 18 laboratories tested 6 hospital disinfectants against the 3 AOAC-recommended test bacteria, the test disinfectants failed most frequently (62%) when they were challenged with *P. aeruginosa* (Rutala, Cole, unpublished results). This may be due to the larger number of *P. aeruginosa* per penicylinder, or to the intrinsic resistance of this organism, or both.

In addition to significant variability among the bacterial strains, variability is high among replicates using the same bacteria and penicylinders, as evidenced by large standard deviations. Test results could be affected by this intrinsic variability, because the time required for killing microorganisms is directly related to the initial number of cells (3). This may partially explain why in a test with 60 penicylinders, some of the penicylinders will pass and some will fail.

In conclusion, textural differences among the types of available penicylinders (porcelain, glass, and 2 brands of stainless steel) may affect the attachment of bacteria to them. Comparable numbers of each of the 3 AOAC-recommended bacterial test strains attached to both brands of stainless steel; however, the strains attached in different numbers for each organism, with more than 10^7 for *P. aeruginosa*, approximately 5×10^6 for *S. aureus*, and 10^6 or less for *S. choleraesuis*. In view of these findings, we recommend (*1*) that stainless steel be retained as the required carrier in future revision of the use-dilution method, (*2*) that S&L and Fisher brands of stainless steel penicylinders be comparatively studied, and (*3*) that alternative methodologies be considered to ensure comparable numbers of challenge bacteria.

Acknowledgment

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FEEDS

Comparison of HgO and $CuSO_4/TiO_2$ as Catalysts in Manual Kjeldahl Digestion for Determination of Crude Protein in Animal Feed: Collaborative Study

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Because of environmental concerns about HgO, and because of lengthy digestion requirements for HgO and $CuSO_4$, interest in alternative catalysts for the Kjeldahl determination of animal feeds remains high. A digestion system using a mixed $CuSO_4/TiO_2$ catalyst has been found to reduce digestion times to 40 min. A collaborative study was carried out to compare this system to the official AOAC HgO method, 7.015. Thirty-eight samples, consisting of blind duplicates of closely matched pairs and 2 standard materials, were analyzed once by each method. Results were received from 13 laboratories. Means and standard deviations of individual samples were comparable, with an overall difference of grand means of 0.005% protein. With only one exception, analyses of variance showed no significant method difference at the 95% confidence level. The $CuSO_4/TiO_2$ method has been approved interim official first action as an alternative method for determination of crude protein in animal feed.

The selection of catalyst in the Kjeldahl determination of crude protein continues to be a topic of great interest. The traditional catalyst of choice has been HgO, but this has raised much environmental concern. In addition, the official AOAC method using HgO, **7.015** (1), specifies a lengthy 2 h digestion whenever the sample contains organic material.

Because of these concerns, many laboratories have expressed the need for an alternative catalyst. A method using $CuSO_4$ as a catalyst (2) was successfully collaborated in 1984 (3), and is now an AOAC official first action method, **7.033-7.037** (1). The determination is mercury-free, and the digestion time is reduced from 120 to 90 min.

More recent work has shown a mixed catalyst of $CuSO_4$ and TiO_2 (4) to be more effective than $CuSO_4$ used alone. Using optimized amounts of $CuSO_4$ (0.01 g) and TiO_2 (0.6 g), it is possible to reduce the digestion time to 40 min, less than half the time required for $CuSO_4$ alone. This was considered a significant enough advantage to justify a collaborative study.

Collaborative Study

Thirty-eight samples were used in the collaborative study. They consisted of 18 blind duplicates as 9 closely matched pairs, plus 2 standard materials. The collaborators were instructed to perform boil tests to verify heat input, then to analyze each sample once by the official HgO method, **7.015** (1), and once by the $CuSO_4/TiO_2$ method.

Submitted for publication March 30, 1987.

This report of the Associate Referee was presented at the 100th AOAC Annual International Meeting, Sept. 15–18, 1986, at Scottsdale, AZ.

The recommendation of the Associate Referee was approved interim official first action by the General Referee, the Committee on Feeds, Fertilizers, and Related Materials, and the Chairman of the Official Methods Board. The method will be submitted for adoption official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) **71**, January/February issue.

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Protein (Crude) in Animal Feed

$CuSO_4/TiO_2$ Mixed Catalyst Kjeldahl Method

Interim First Action

(Caution: See 51.030, 51.037.)

Principle

Sample is digested in H_2SO_4, using $CuSO_4/TiO_2$ as catalysts, converting N to NH_3 which is distd and titrd.

Reagents

(a) *Sodium hydroxide soln.*—Dissolve ca 450 g NaOH pellets or flakes (low N) in H_2O, cool, and dil. to 1 L; or use soln with sp. gr. ≥ 1.36.

(b) *Boiling stones.*—Alundum, 8–14 mesh (No. 1590-D18; Thomas Scientific Co., 99 High Hill Rd at I-295, PO Box 99, Swedesboro, NJ 08085-0099).

(c) *Methyl red indicator.*—Dissolve 1 g Me red (Na salt) in 100 mL MeOH.

(d) *Hydrochloric or sulfuric acid std soln.*—0.5N. Prep. as in **50.011-50.017** or **50.039-50.040.**

(e) *Sodium hydroxide std soln.*—0.1N. Prep. as in **50.032-50.035.**

After stdzg both acid and base by methods suggested in **(d)** and **(e)**, also check one against the other. In addn, check entire method by analyzing NBS Std Ref. Material No. 194, $NH_4H_2PO_4$, certified 12.15% N, and a high purity lysine·HCl.

Table 1. Collaborative samples for Kjeldahl determination of crude protein

Closely matched pair No.[a]	Sample Nos of blind duplicates	Approx. % protein	Material
1	1,27	40	cattle concentrate
	17,32	40	cattle concentrate
2	2,34	17	alfalfa pellets
	15,23	17	dehydrated alfalfa meal
3	3,21	20	broiler finisher
	6,29	20	broiler finisher
4	4,33	88	soy protein concentrate
	20,25	88	soy protein concentrate
5	5,22	48	soybean meal, solv. extd
	11,31	48	soybean meal, solv. extd
6	7,37	80	blood meal
	9,30	80	blood meal
7	8,38	36	dry milk powder
	14,26	36	dry milk powder
8	10,35	80	feather meal
	19,28	80	feather meal
9	12	21.31	Fisher Scientific Co. primary std $(NH_4)_2SO_4$ lot 852132
10	13,36	50	meat meal
	16,24	50	meat meal
11	18	95.86	Aldrich Chemical Co. L-lysine·HCl (Gold Label), lot 0523AM

[a] Pairs are from different lots of same product from same manufacturer.

Table 2. Collaborative results for study of $CuSO_4/TiO_2$ and HgO catalysts for Kjeldahl determination of crude protein

	Coll. 1		Coll. 2		Coll. 3		Coll. 4		Coll. 5		Coll. 6	
Sample	Cu-Ti	HgO	Cu-Ti	HgO	Cu-Ti	HgO	Cu-Ti	HgO	Cu-Ti	HgO	Cu-Ti	HgO
1	40.98	40.06	40.15	39.64	41.9	41.0	40.18	40.35	41.24	40.63	41.38	41.03
2	16.98	17.16	16.40	17.27	15.9[b]	16.9	16.34	16.85	17.15	16.67	17.13	17.07
3	20.66	20.66	20.62	20.47	21.6	21.1	20.70	21.49	21.24	20.91	21.04	20.91
4	87.60	87.10	85.65	85.44	87.3	87.1	86.75	87.01	87.62	86.90	87.60	86.77
5	48.72	48.75	47.33	48.02	48.8	49.0	48.06	47.70	48.99	48.75	49.04	49.01
6	21.22	21.24	20.67	21.12	21.6	21.3	21.12	21.15	21.33	21.52	21.22	21.22
7	81.08	81.33	77.76	80.21	81.5	81.0	81.62	81.83	80.83	79.56	81.61	80.30
8	35.12	35.15	34.64	34.57	35.1	34.9	35.34	35.11	35.26	35.16	35.34	35.16
9	84.40	85.05	81.97[b]	82.98	84.0	84.3	84.38	84.80	83.72	83.23	85.15	84.96
10	83.96	84.15	82.13	83.08	84.6	84.6	84.25	84.29	84.51	84.18	84.64	84.02
11	48.82	48.20	45.30[b]	47.22	48.0	48.5	47.24	47.55	48.43	48.11	48.45	48.32
12	131.60	132.10	130.95	131.08	133.4	133.1	132.25	132.91	132.05	130.31	133.25	132.82
13	53.26	53.20	52.63	53.24	54.4	54.1	53.50	53.76	53.76	53.52	53.70	54.07
14	35.28	35.36	34.27[b]	34.77	35.2	35.1	35.11	35.31	35.16	35.40	35.21	35.01
15	17.22	17.50	16.92	17.24	17.4	17.3	17.04	17.04	17.58	17.82	17.17	17.24
16	57.40	57.46	56.00	56.49	56.0	57.3	57.56	57.53	57.16	56.94	57.24	57.07
17	40.78	40.54	40.49	40.22	42.3	41.7	41.30	40.86	40.29	40.71	42.72	41.74
18	94.64	96.08	92.67	95.32	95.3	95.5	94.96	94.55	95.25	95.66	95.22	96.60
19	78.50	79.68	77.55	77.55	77.9	79.2	79.64	79.30	78.87	78.87	78.97	78.78
20	88.73	89.13	88.24	88.40	88.2	87.2	85.95[b]	86.85[b]	89.03	88.66	89.00	88.96
21	21.04	20.98	20.35	20.72	21.4	21.3	21.00	20.75	21.19	21.00	20.99	20.85
22	47.58	47.74	46.67[b]	48.46	48.9	47.6	49.28	49.35	48.75	48.86	48.99	49.09
23	17.06	17.29	16.80	17.25	17.0	16.9	16.98	17.59	17.38	17.52	17.32	17.48
24	57.19	57.38	55.90	56.45	57.6	57.1	57.26	57.46	57.07	56.84	57.10	57.41
25	89.28	89.48	87.08	88.30	89.1	89.4	87.25	88.89	89.13	88.79	88.70	88.64
26	35.21	35.25	34.89	35.03	35.4	35.4	35.36	35.36	35.20	35.36	35.28	35.29
27	40.30	40.04	39.92	39.70	41.2	41.4	39.89	39.51	40.73	40.16	41.27	40.85
28	78.65	79.03	77.48	78.16	78.8	79.0	78.57	78.18	78.86	79.03	79.11	78.83
29	21.10	21.74	20.75	20.33	21.2	21.5	20.88	20.48	21.45	21.43	21.29	20.98
30	84.73	84.33	83.56	83.00	84.3	84.1	83.22	83.34	84.53	84.12	84.83	84.85
31	48.15	48.28	47.58	47.30	48.4	48.3	47.06	46.22[b]	48.30	48.10	48.37	48.55
32	40.33	41.20	40.75	38.65	40.5	40.1	40.16	39.30	40.51	40.31	41.85	42.00
33	87.60	87.98	86.39	86.60	86.4	87.4	87.38	89.25	86.96	87.70	87.55	88.09
34	17.08	17.08	17.07	16.99	17.2	17.3	16.85	17.11	17.20	17.25	17.04	17.18
35	84.50	84.50	83.19	83.09	80.8[b]	80.9[b]	84.38	82.52	84.28	84.32	84.02	84.15
36	53.90	53.90	52.88	53.74	54.1	54.1	53.54	53.52	53.71	54.00	54.06	54.32
37	79.45	80.60	77.88	79.12	78.9	80.7	79.43	79.25	80.97	79.46	81.08	80.97
38	35.11	35.26	34.55	35.03	35.7	35.2	34.82	34.44	35.15	35.23	35.28	35.16

[a] All data for collaborator 7 eliminated from further statistical consideration on basis of Steiner (6) and ranking tests (8).
[b] Outlier on basis of Steiner test (6).
[c] Outlier on basis of Dixon test (7).

Apparatus

(a) *Digestion.*—Kjeldahl flasks with capacity of 500–800 mL.

(b) *Distillation.*—Digestion flask (e.g., Corning No. 2020) connected to distn trap by rubber stopper. Distn trap is connected to condenser with low-S tubing. Outlet of condenser tube should be <4 mm diam.

Determination

Weigh 0.250–1.000 g sample into digestion flask. Add 16.7 g K_2SO_4, 0.01 g anhyd. $CuSO_4$, 0.6 g TiO_2, 0.3 g pumice, 0.5–1.0 g Alundum granules, and 20 mL H_2SO_4. (Add addnl 1.0 mL H_2SO_4 for each 0.1 g fat or 0.2 g other org. matter if sample wt is >1 g.)

Include at least 1 sample of high purity lysine·HCl in each day's run as check of correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.

To digest sample, first adjust heat to bring 250 mL H_2O at 25° to rolling boil in 5 min. Add a few boiling chips to prevent superheating. Then heat samples at this 5-min boil rate until dense white fumes clear bulb of flask (ca 10 min), swirl gently, and continue heating addnl 40 min. (Note: Reagent proportions, heat input, and digestion time are critical factors—do not change.) Cool, cautiously add ca 250 mL H_2O, and cool to room temp. (Note: Add H_2O as soon as possible to reduce amt of caking. If excessive bumping occurs during distn, increase diln H_2O from 250 mL to ca 300 mL.)

Prep. titrn beaker by adding appropriate vol. of acid std soln to amt of H_2O such that condenser tip will be sufficiently immersed to trap all NH_3 evolved. Add 3–4 drops of indicator soln (c).

Add addnl 0.5–1.0 g Alundum granules to cooled digestion flask. Optionally, 2–3 drops of tributyl citrate may also be added to reduce foaming. Slowly down side of flask, add sufficient NaOH soln (a) such that mixt. will be strongly alk. Immediately connect flask to distn app., mix completely, and distill at ca 7.5-min boil rate until ≥150 mL distillate is collected in titrn beaker.

Titr. excess std acid in distillate with NaOH std soln (e). Correct for blank detn on reagents. Calc. % nitrogen:

$$\% N = \{[(N_{acid})(mL_{acid}) - (mL_{bk})(N_{NaOH}) - (mL_{NaOH})(N_{NaOH})] \times 1400.67\}/mg\ sample$$

where mL_{NaOH} = mL std base needed to titr. sample; mL_{acid} = mL std acid used for that sample; mL_{bk} = mL std base needed to titr. 1 mL std acid minus mL std base needed to titr. reagent blank carried thru method and distd into 1 mL std acid; N_{acid} = normality of std acid; N_{NaOH} = normality of std base. Calc. % crude protein, defined as 6.25 × % nitrogen, or 5.7 × % nitrogen for wheat grains.

Results and Discussion

Table 1 identifies the sample types used in the collaborative study. In each case, closely matched pairs were obtained from the same manufacturing source and represent different lots of the same product. Results were received from 13 laboratories (Table 2). Pair No. 7, dry milk powder, was included in the study at the request of the American Dry Milk Institute because that group is also interested in the elimination of HgO catalyst. The instructions to collaborators directed that the 6.25 calculating factor be applied to all

Table 2. Continued

Coll. 7[a]		Coll. 8		Coll. 9		Coll. 10		Coll. 11		Coll. 12		Coll. 13	
Cu-Ti	HgO	Cu-Ti	HgO	Cu-Ti	HgO	Cu-Ti	HgO	Cu-Ti	HgO	Cu-Ti	HgO	Cu-Ti	HgO
39.50	39.56	41.31	41.13	40.71	40.74	40.95	40.95	40.67	40.96	40.10	40.53	40.25	39.31
16.19	16.94	17.31	17.13	17.14	17.38	17.26	17.21	16.72	17.06	16.98	16.68	17.23	16.71
20.19	20.88	21.13	21.25	21.32	21.18	21.13	21.25	20.79	21.10	20.94	19.94[b]	20.45	20.82
84.00	84.44	88.00	87.88	87.96	87.73	87.26	87.54	87.43	87.54	86.80	87.12	86.24	86.55
46.44	47.38	48.88	48.94	48.69	48.92	49.15	48.38	48.31	48.89	48.83	48.42	48.83	48.88
20.38	20.88	21.44	21.44	21.57	21.54	21.00	21.10	21.22	21.31	21.24	20.89	20.71	21.09
75.63	75.69	80.88	80.56	80.48	80.42	78.01	77.16[b]	80.12	81.31	79.85	80.57	80.47	78.81
33.00	33.06	35.56	35.63	35.22	35.43	34.65	35.32	34.75	35.06	34.61	35.24	34.14	35.19
77.56	79.88	85.25	85.25	84.86	84.91	84.22	84.29	84.09	83.74	84.62	82.90	84.15	84.76
81.50	80.63	84.88	84.88	84.55	84.85	83.88	83.60	84.26	84.55	84.49	83.87	83.61	84.18
45.75	47.19	49.06	48.56	48.27	48.54	47.92	47.80	47.85	48.25	48.01	47.90	47.68	47.78
129.13	129.31	132.38	133.31	132.32	132.48	130.50	131.54	131.90	132.06	132.92	130.42	129.44	131.62
51.44	52.19	54.31	54.13	53.97	53.91	53.69	53.90	53.82	53.84	53.70	53.76	57.04[b]	53.12
34.25	34.75	35.56	35.69	35.24	35.29	34.73	35.16	34.75	35.08	35.16	34.84	35.05	34.12[b]
16.44	17.13	17.44	17.31	17.51	17.57	17.20	17.30	17.00	17.27	17.22	17.40	17.78	17.40
55.25	56.06	58.00	57.56	57.32	57.50	56.88	57.43	57.07	56.61	57.05	56.04	57.70	56.68
40.31	39.19	41.50	41.06	42.52	42.53	41.67	40.87	40.78	40.78	41.27	41.58	41.05	40.33
75.94	93.75	96.38	96.31	95.62	96.12	94.93	95.97	95.52	95.53	92.28[c]	95.86	95.62	94.73
76.06	76.81	79.50	79.31	79.19	79.46	78.55	79.62	78.37	78.71	78.69	77.89	77.93	77.83
85.50	86.00	89.06	89.19	88.61	88.81	88.03	88.24	88.91	88.56	88.57	88.58	88.58	88.59
20.13	20.69	20.88	21.13	21.20	21.47	21.07	20.94	20.95	21.04	21.24	20.68	21.04	20.87
46.75	48.00	49.25	48.94	48.40	48.75	48.50	49.05	48.81	48.65	48.57	48.64	49.31	48.78
16.69	17.31	17.25	17.19	17.27	17.38	17.57	17.22	16.99	17.21	17.55	17.36	17.76	17.21
54.66	56.31	58.00	57.75	57.35	57.18	57.77	57.22	57.46	57.29	57.09	56.51	57.13	55.81
84.97	85.19	89.44	89.44	89.04	89.12	88.33	88.72	88.69	89.10	88.73	89.06	88.98	88.35
33.40	34.69	35.19	35.50	35.25	35.53	34.98	35.10	35.16	35.36	34.80	35.07	35.44	35.02
38.63	38.63	41.13	40.63	40.65	40.87	40.33	40.56	40.52	40.67	40.19	40.35	40.30	40.24
75.12	76.88	78.38	79.25	79.38	79.21	78.57	78.83	78.38	78.55	78.82	78.43	78.29	76.90
20.20	19.44	21.50	21.25	21.61	21.48	21.06	21.17	20.92	21.15	21.49	21.18	21.21	21.15
77.74	81.94	85.25	85.19	84.76	84.46	83.46	83.99	84.83	83.95	83.29	84.27	83.63	82.60
45.75	47.63	48.38	48.44	48.35	48.63	47.81	47.98	48.27	47.79	47.48	47.30	48.15	47.77
38.55	39.81	40.94	41.50	41.45	41.73	41.13	41.06	41.79	41.03	41.94	41.84	40.14	40.87
84.18	84.06	88.13	87.69	87.86	87.70	86.62	87.61	87.16	87.15	86.31	86.89	86.63	85.80
16.54	17.31	17.31	17.06	17.04	17.11	17.16	17.18	17.04	16.83	17.26	17.27	17.21	17.06
81.44	81.75	85.13	85.06	84.63	84.69	83.67	84.76	84.12	84.27	84.26	83.71	83.73	83.48
52.28	52.63	54.69	54.50	54.34	54.20	53.40	53.51	53.07	53.84	53.90	53.90	53.45	53.54
74.46	78.38	81.25	81.00	80.50	80.28	78.26	78.70	80.05	79.88	80.13	80.51	79.32	79.08
34.46	34.81	34.69	35.31	35.28	35.34	34.96	34.83	35.04	35.17	35.10	34.93	35.00	34.85

samples, the milk powder as well, to help preserve the blind duplicate nature of the study. However, the correct factor for the milk samples would be % N × 6.38.

There were a number of comments from collaborators. Several noted more foaming during the initial digestion stage for the $CuSO_4/TiO_2$ method. This required more analyst attention. A greater tendency to cake was also noted. Several collaborators suggested that dilution water be added as soon as possible after the digestion step was completed. If steps were not taken to control caking, bumping was observed during subsequent distillation, and there was the possibility of broken flasks. Extra dilution water can minimize this problem.

In addition, collaborator 12 noted lumps in samples 1, 17, 27, and 32 as received. Collaborators 5 and 11 thought the sample container of sample 33 was not tightly sealed. Collaborator 11 thought that some of the samples could have been more finely ground.

Collaborator 2 ran 2 repeat analyses of sample 18 (lysine) by the $CuSO_4/TiO_2$ method, with results similar to the low 92.67% value that he reported in Table 2. Finally, collaborator 5 analyzed a number of nicotinic acid samples by both methods, and found that the recoveries averaged about 60% using the $CuSO_4/TiO_2$ catalyst, and about 99% using the HgO catalyst. While non-HgO systems can be used for animal feed materials, it must be remembered that for some samples HgO performs better.

Two-sample X-Y charts were prepared according to Youden (5); blind duplicates were plotted together on the same chart. Examination of the charts suggested that one laboratory, collaborator 7, had an excessive number of low results. In general, the chart patterns were the typical elliptical shape, not excessively elongated, but with a few results in each chart spreading farther into the lower left quadrant. In other words, a low bias is much more frequent than is one in the positive direction.

The data were processed for outliers according to Steiner (6). In the case of samples 12 and 18, the Dixon test (7) was used instead because these samples did not have closely matched pairs, and therefore the number of data points was only 13. Of a total of 22 outlying values identified, 17 were from collaborator 7. Further examination of the data from collaborator 7 revealed that every result reported except one, by both the $CuSO_4/TiO_2$ and HgO methods, was lower than the appropriate mean of the results for all laboratories. A ranking test (8) confirmed that data from collaborator 7 should be excluded from all further statistical consideration. The most probable cause of this bias was improper acid and/or base standardization.

When the data were again processed for outliers as before, the following numbers of outliers were found by the Steiner and Dixon tests: Lab. 2, 4 outliers; Lab. 3, 3; Lab. 4, 3; Lab. 10, 1; Lab. 12, 2; Lab. 13, 2. These outliers are identified individually in Table 2. There were 9 outliers for the $CuSO_4/$

TiO_2 method and 6 outliers for the official HgO method, representing 1.6% of the total number of results.

The remaining data, with outliers removed, were processed for information on precision by using Youden's scheme for closely matched pairs (9). Blind duplicates of closely matched pairs were treated as if they were additional matched pair data points from additional laboratories, giving a total of 36 pairs. Table 3 contains the means of closely matched pairs (X and Y) and corresponding standard deviations for both methods: S_o, the estimate of random error, S_L, the estimate of interlaboratory bias, and S_x, the estimate of overall precision.

In general, standard deviations for the $CuSO_4/TiO_2$ method and the official HgO method are in close agreement. The $CuSO_4/TiO_2$ method had smaller standard deviations 12 times and the HgO method 15 times; none of the differences were cause for concern.

The same is true for the means. Means for the $CuSO_4/TiO_2$ method were larger 7 times and for the HgO method, 11 times. The largest difference was 0.235%; most differences were smaller. The overall average difference, $CuSO_4/TiO_2$ minus HgO grand means, was +0.005%.

Comparison of these data with corresponding data from the 1984 $CuSO_4$ catalyst collaborative study (3) indicates similar standard deviations. The $CuSO_4/TiO_2$ deviations are slightly smaller than the deviations for the 1984 $CuSO_4$ study, but the HgO deviations are also slightly smaller than the 1984 HgO deviations.

Sample 12, Fisher Scientific Co. primary standard $(NH_4)_2SO_4$, 133.19% protein equivalent, was included to obtain some estimate of the accuracy of the 2 methods. The mean of the $CuSO_4/TiO_2$ results was 131.79% with a standard deviation of 1.13. The mean of the HgO results was 131.98% with a standard deviation of 1.01. The slightly low recoveries by both methods on this material (99.0%) closely match the low recoveries on the standard material in the 1984 collaborative study (99.5%) (3). Careful standardization of acid and base and quality assurance procedures using standard materials are advised with any Kjeldahl methodology. Errors will tend to be most apparent on the high level N samples, of course.

Sample 18, Aldrich Chemical Co. lysine·HCl, 95.86% protein theoretical, 99+% purity, was included among the collaborative samples because it is a hard-to-digest material and therefore a good test of the ruggedness of the digestion parameters. The mean of the $CuSO_4/TiO_2$ results was 95.101% with a standard deviation of 0.93. The mean of the HgO results was 95.686% with a standard deviation of 0.61. While these values indicate low recoveries, they represent improvements over the corresponding values from the 1984 collaborative study of 94.02% mean, 1.34 standard deviation for $CuSO_4$, and 94.54% mean, 1.50 standard deviation for HgO.

Most collaborators in this study had digestion heaters which were tested as approximately in the 5-min boil range. An attempt to correlate low lysine recoveries with excessively high heater boil rates was not productive. Two analyses performed with heaters in the 8–9 min boil range resulted in lower lysine recoveries, but on the other hand, 2 analyses performed with heaters in the 5-min boil range also resulted in lower lysine recoveries. Heat input is not the only parameter that needs careful quality control. As was the conclusion in the 1984 study, proper digestion parameters need attention whatever Kjeldahl method is used, and this is more important with non-HgO catalyst systems if quality results are to be expected.

An analysis of variance using method, laboratory, and sample, was run on the data. There was no significant method effect at the 95% confidence level.

Conclusions and Recommendations

On a wide range of animal feed types there is no significant difference between the proposed method and the official AOAC HgO method at the 95% confidence level, with one exception. In this one case, the $CuSO_4/TiO_2$ mean is slightly higher than the HgO mean. Standard deviations of the 2 methods were in the same range, and were generally smaller than in the 1984 collaborative study. The $CuSO_4/TiO_2$ catalyst eliminates the environmental hazard associated with HgO, and the digestion is considerably more efficient than when $CuSO_4$ catalyst is used by itself. As with the $CuSO_4$ catalyst method, more attention to quality control in the digestion phase is advised compared with the official HgO catalyst method.

It is recommended that the $CuSO_4/TiO_2$ catalyst method be adopted official first action; that it be stipulated as a part of the method that lysine·HCl be analyzed during routine sample determinations as an ongoing check of analytical performance; that the digest be diluted as soon as possible to avoid excess caking; that if bumping during distillation is a problem the amount of dilution water may be increased from about 250 to about 300 mL.

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S. Allison, State of Missouri Agricultural Dept, Jefferson City, MO

P. Eggerman and R. Sweeney, University of Missouri, Columbia, MO

C. Gehrig, Indiana State Chemist's Laboratory, Purdue University, West Lafayette, IN

Table 3. Means and standard deviations for collaborative results: $CuSO_4/TiO_2$ vs HgO catalysts

Sample X-Y	$CuSO_4/TiO_2$					HgO				
	Mean X	Mean Y	S_o	S_x	S_L	Mean X	Mean Y	S_o	S_x	S_L
1,27-17,32	40.677	41.173	0.508	0.651	0.407	40.471	40.938	0.511	0.726	0.516
2,34-15,23	17.048	17.261	0.178	0.264	0.195	17.063	17.333	0.206	0.194	0.000[a]
3,21-6,29	20.999	21.200	0.152	0.266	0.218	20.994	21.212	0.216	0.288	0.191
4,33-20,25	87.150	88.640	0.501	0.624	0.373	87.262	88.766	0.542	0.655	0.368
5,22-11,31	48.756	48.111	0.406	0.441	0.174	48.618	48.051	0.342	0.439	0.275
7,37-9,30	80.060	84.314	0.685	0.905	0.592	80.237	84.134	0.606	0.834	0.573
8,38-14,26	35.034	35.157	0.209	0.261	0.156	35.108	35.230	0.168	0.243	0.176
10,35-19,28	84.160	78.615	0.366	0.596	0.470	84.122	78.722	0.424	0.672	0.521
13,36-16,24	53.730	57.135	0.378	0.516	0.351	53.818	57.042	0.350	0.437	0.262

[a] Note that for this sample S_L is zero because a negative S_L is not real. In this case it is assumed that S_o is greater than S_x due to sampling error.

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FOOD ADULTERATION

Determination of Phytosterols in Butter Samples by Using Capillary Column Gas Chromatography

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A positive bias in the gas chromatographic (GC) analysis of butter for β-sitosterol was discovered when attempting to confirm values by gas chromatography/mass spectrometry (GC/MS). The source of the problem was traced to an interfering material that was not effectively separated by packed column GC. Because capillary columns are known to provide superior separation, they were substituted for packed columns in the assay, and instrument parameters were modified accordingly. A compound with a similar retention time, identified by GC/MS as lanosterol, was separated from β-sitosterol by the capillary column. The capillary column technique was applied to over 300 butter samples. The results indicate that the method can accurately quantitate β-sitosterol in butter with no known interferences. The limit of detection for this method is 1 mg/100 g. Recoveries at a level of 3 mg/100 g averaged 98% with a coefficient of variation of 3.45%.

The adulteration of butter oil with vegetable oils has been and continues to be a major concern in the dairy industry. Methods for detection of adulteration include determination of the phytosterols, particularly β-sitosterol. In 1958, AOAC approved a method for sterol analysis that used a digitonin column to remove the sterols, followed by a melting point test for qualitative determination (1). Modifications of the method incorporating gas chromatography (GC) were developed in subsequent years (2–4). In 1980, Slover et al. (5) published a method for determining tocopherols and sterols in fats and oils by using capillary GC. The current AOAC official method for determining β-sitosterol in butter oils is applicable to oils containing ≥4 mg of this phytosterol (6).

The present investigation was conducted to detect adulteration of butter oils by determining the presence of β-sitosterol. When a direct saponification/extraction technique (7) and packed column GC (8) were used to analyze butter samples, an interfering compound, which was later confirmed as lanosterol, coeluted with β-sitosterol. We then investigated the applicability of capillary GC techniques, outlined by Slover et al. (5), to detect adulteration of butter oils by the determination of β-sitosterol.

METHOD

Apparatus

(a) *Centrifuge tubes.*—Pyrex® No. 13, 15 mL. Silanize tubes as follows: rinse clean tubes with anhydrous methanol and dry 30 min at 110°C. Transfer to desiccator and let cool to room temperature. Fill tubes with 10% solution of dimethyldichlorosilane in toluene, stopper, and let stand 1 h. Drain tubes and rinse thoroughly with anhydrous methanol. Dry in oven before use.

(b) *Gas chromatograph.*—Hewlett-Packard 5710A or HP5720A equipped with flame ionization detector and on-column injection system.

(c) *Packed column GC system.*—6 ft × 2 mm silanized glass column packed with 5% SP2401 on 100–200 Supelcoport®. Column temperature, 214°C; carrier gas, nitrogen at 30 mL/min; detector gases, hydrogen at 40 mL/min and air at 240 mL/min; injector port temperature, 250°C; detector temperature, 250°C.

(d) *Capillary column GC system.*—Hewlett-Packard, 25 m × 0.32 mm id fused silica capillary column with 5% phenyl methyl silicone, cross-linked. Column temperature, 190°C for 9.5 min, programmed at 20°/min to 260°C for 30 min; carrier gas, helium at 2 mL/min; make-up gas, nitrogen at 30 mL/min; detector gases, hydrogen at 40 mL/min and air at 240 mL/min; injector port temperature, 250°C; detector temperature, 300°C.

(e) *Integration.*—Hewlett-Packard 3357 Laboratory Automation System.

Reagents

(a) *Sterol standard solutions.*—Prepare stock solution containing >99% pure cholesterol (NuChek Prep, Inc., Elysian, MN 56028), >97% pure β-sitosterol (Alltech Associates, Deerfield, IL 60015), >99% pure campesterol (Research Plus Laboratories, Bayonne, NJ 16801), >99% pure lanosterol (Research Plus Laboratories) and >99% pure stigmasterol (Applied Science Laboratories, Deerfield, IL 60015), each at 1.0 mg/mL dimethylformamide (DMF). Prepare working standard solution of 0.3 mg/mL of each sterol by diluting 2.0 mg/mL stock solution in DMF. Store at room temperature and prepare monthly.

(b) *5α-Cholestane.*—>99% pure (Sigma Chemical Co., St. Louis, MO 63178). Prepare 0.2 mg/mL working solution in heptane. Store at room temperature and prepare monthly.

(c) *Reagent alcohol.*—Use one of the 2 following solutions: 95 + 5 (v/v) Formula No. 3A–isopropyl alcohol (American Scientific Products, McGaw Park, IL 60085, Catalog No. C4305) or 95 + 5 (v/v) ethanol–methanol (USI).

(d) *50% Potassium hydroxide (KOH) (w/v).*—Dissolve 115 g KOH pellets in deionized water and dilute to 100 mL.

(e) *1N KOH.*—Dissolve 65.2 g 85% KOH pellets in deionized water and dilute to 1 L.

(f) *0.5N KOH.*—Dilute 500 mL 1N KOH with 500 mL deionized water.

Direct Saponification

Accurately weigh 800 mg butter sample and 5 g water into 250 mL Erlenmeyer flask. Add 8 mL of 50% KOH (w/v) and 40 mL reagent alcohol. Attach Erlenmeyer flask to condenser and place on magnetic stirrer/hot plate. Reflux sample solution 1 h with gentle stirring, and then add 60 mL reagent alcohol through condenser into saponified solution. Remove from heat and, after sample solution has cooled to room temperature, remove flask from condenser and then stopper.

Unsaponifiable Extraction

Using volumetric pipet, add 100 mL toluene to flask and then stopper. Vigorously stir mixture at 200–300 rpm 1 min. Transfer solution to 500 mL separatory funnel that contains 100 mL 1N KOH and shake vigorously 30 s. Let resulting solutions separate into 2 distinct layers and discard lower

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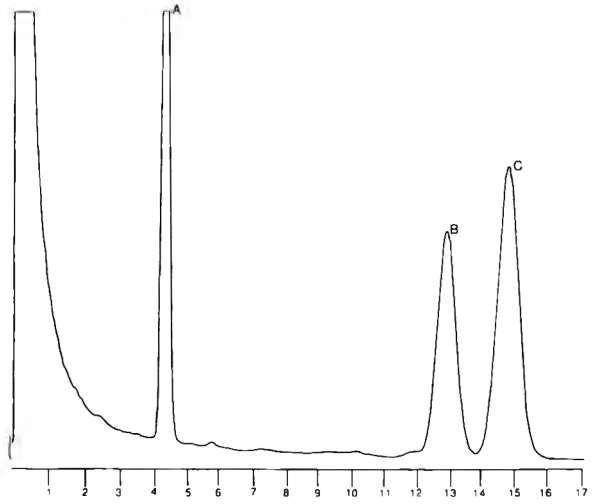

Figure 1A. Packed column gas chromatogram of sterol standards: A, cholestane (internal standard); B, stigmasterol; and C, β-sitosterol.

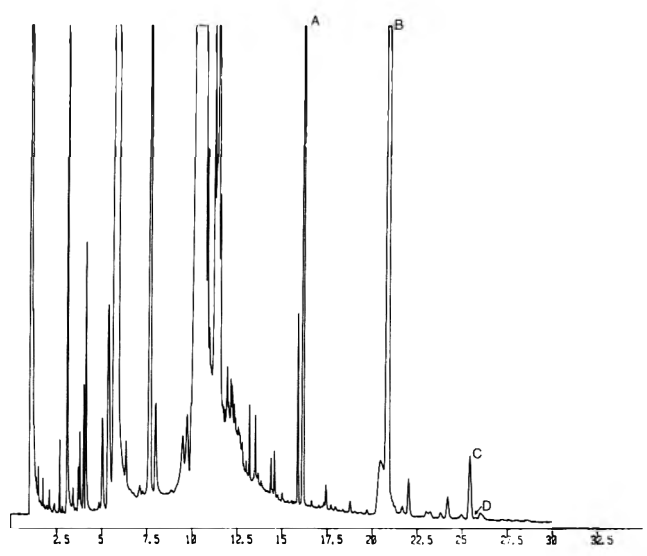

Figure 2A. Capillary gas chromatogram of unsaponifiable fraction of butter sample: A, cholestane (internal standard); B, cholesterol; C, lanosterol; and D, area in which β-sitosterol elutes.

aqueous phase. Add 40 mL 0.5N KOH to toluene layer and rotate separatory funnel gently for 30 s. Let the 2 layers separate completely and discard lower aqueous phase. Wash toluene phase with 5 portions of 100 mL deionized water. To avoid emulsion formation, conduct washes as follows: rinse sides of separatory funnel with first water wash without agitating, and then drain lower aqueous phase; gently rotate separatory funnel, end over end, with second water wash; moderately shake separatory funnel with third water wash; and vigorously shake separatory funnel with fourth and fifth water washes. Transfer toluene extract through sodium sulfate (Na_2SO_4) column into 125 mL glass-stopper Erlenmeyer flask that contains ca 20 g anhydrous Na_2SO_4, and then stopper. Let stand 15 min.

Pipet 50 mL aliquot of toluene extract into 125 mL round-bottom, glass-stopper flask and evaporate to dryness on rotary evaporator at 40°C. Rinse sides of round-bottom flask with 5 mL acetone, evaporate rinse to dryness, and stopper. Dissolve residue in 3.0 mL dimethylformamide.

Derivatization

Transfer duplicate 1.0 mL aliquots of 0.3 mg/mL standard sterol solution to separate 15 mL silanized centrifuge tubes. Transfer 1.0 mL aliquots of sample extract solutions to be analyzed to separate 15 mL silanized centrifuge tubes. Accurately add 0.2 mL hexamethyldisilazane, followed by 0.1 mL trimethylchlorosilane to each tube, stopper, and mix resulting solution on vortex mixer for 30 s. Let solution stand for 30 min; solution should clarify, with resulting ammonium chloride precipitate settling to bottom of tube. Accurately add 1.0 mL 0.2 mg/mL 5α-cholestane solution to each tube, gently add 10 mL distilled water to each tube, and vigorously shake 1 min. Centrifuge at 2000 rpm for 5 min and transfer portion of upper heptane layer, excluding lower aqueous layer, to injection vial.

Calibration of GC Apparatus and Sample Calculation

Inject 4 μL derivatized 0.3 mg/mL standard solutions into GC column and obtain 5α-cholestane and individual sterol peak areas for each injection. Obtain standard response factor for each individual sterol by the following calculation:

$$\text{Standard response ratio} = \text{sterol peak area/cholestane peak area}$$

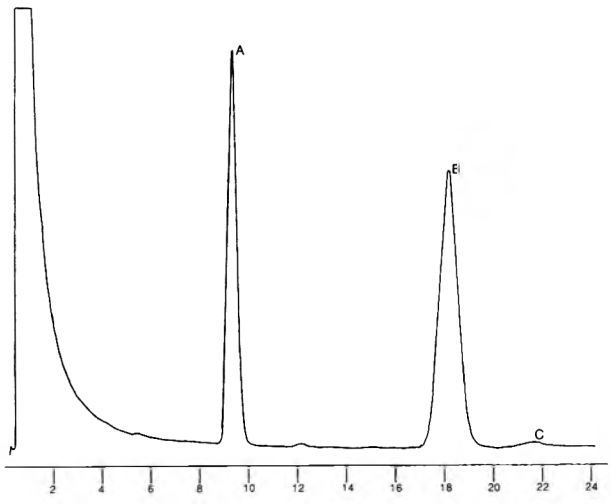

Figure 1B. Packed column gas chromatogram of unsaponifiable fraction of butter sample: A, cholestane; B, cholesterol; and C, β-sitosterol (suspected).

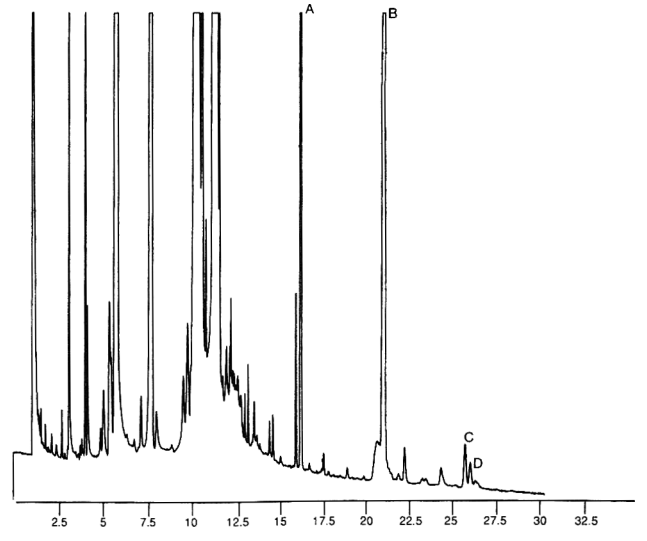

Figure 2B. Capillary gas chromatogram of unsaponifiable fraction of butter sample spiked with β-sitosterol: A, cholestane; B, cholesterol; C, lanosterol; and D, β-sitosterol.

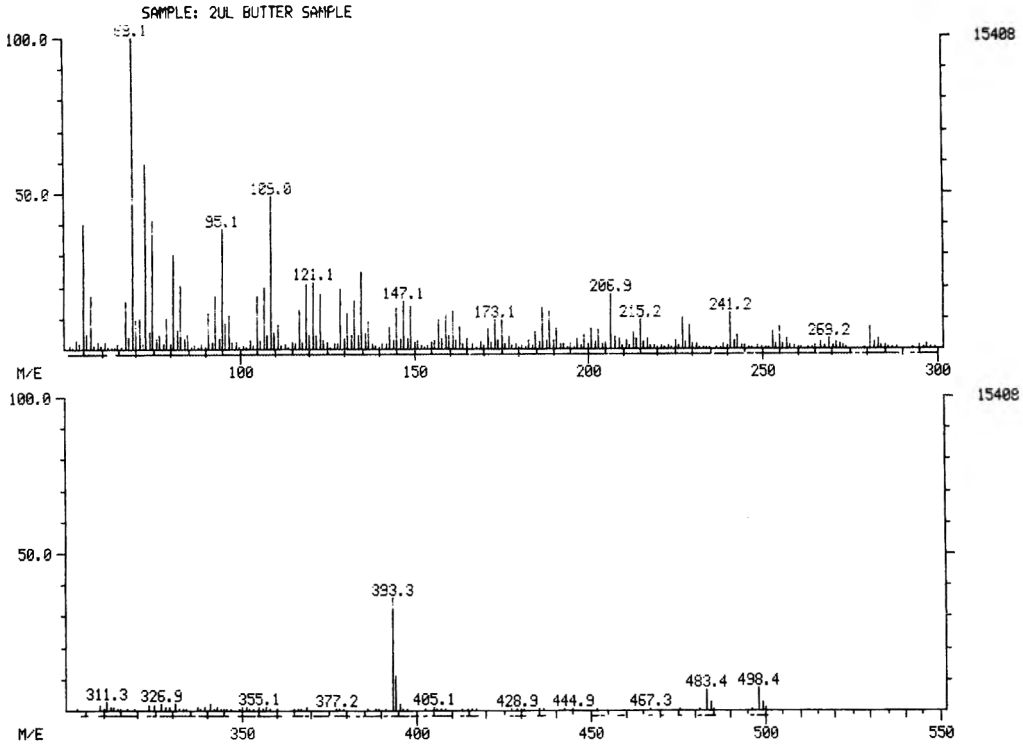

Figure 3A. Mass spectrum of unknown peak eluting at 25:57 (Figure 2).

Standard response factor = std response ratio/std soln concn (0.3 mg/mL)

Standard response factors obtained for the two 0.3 mg/mL standards should not vary >2%. If standard response factors are within 2% variance, inject 4 μL of each sample solution. Inject 4 μL standard solution after every 10 sample injections. Obtain individual sterol and cholestane peak areas for each sample injection. Calculate each sterol concentration in mg/mL for each sample as follows:

Cholesterol, mg/mL = sterol peak area/cholestane peak area/av. std response factor

Calculate sample sterol level in mg/100 g as follows:

Sterol, mg/100 g = [sterol (mg/mL) × DMF (mL) × 100 mL/50 mL × 100 g]/ sample wt (g)

Detection limit of this method has been determined to be 1.0 mg for each sterol/100 g sample material. This peak

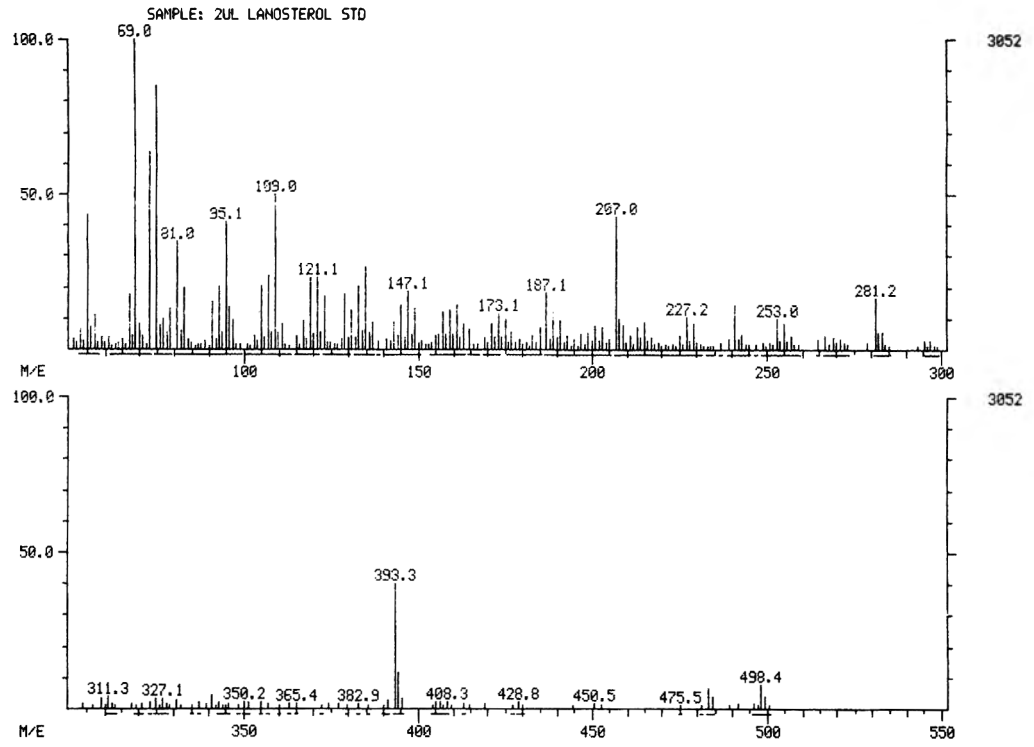

Figure 3B. Mass spectrum of 2 μg/mL lanosterol standard.

represents a 6-fold increase over normal background response.

Results and Discussion

Analyzing butter samples for the presence of phytosterols by using direct saponification followed by packed column GC analysis resulted in apparent β-sitosterol values of 4–7 mg/100 g. To confirm the accuracy of this method, a control butter was obtained from the University of Wisconsin Creamery. When analyzed by the above-mentioned method, this butter, known to contain no vegetable oil contamination, also appeared to contain β-sitosterol. Figures 1A and 1B show a chromatogram of a butter sample and a chromatogram of the appropriate standards obtained by the packed column procedure. A GC/MS analysis of the apparent β-sitosterol peak in the control butter sample was conducted. The mass spectra indicated the presence of an interfering compound that eluted at the approximate retention time of β-sitosterol.

The control butter sample was analyzed using the same techniques as above, except that the chromatography was conducted using a capillary system. The interfering compound (suspected to be lanosterol) was eluted just before the retention time of β-sitosterol (Figure 2A). The calculated β-sitosterol, using this technique, gave a value of <1.0 mg/100 g. This control butter was then spiked with corn oil and assayed for phytosterols, using the capillary column technique. β-Sitosterol was fully resolved from the suspected lanosterol (Figure 2B). A GC/MS analysis of the interfering compound showed it to be the animal sterol, lanosterol. The mass patterns of the ionized fragments of the suspected lanosterol peak match those of the lanosterol standard (Figure 3A and 3B). The GC/MS instrument parameters used for this analysis were as follows: The gas chromatograph was a Finnigan MAT 4021-T; the capillary column was a J & W Scientific DB-5 (30 m × 0.38 mm id). Operating conditions: carrier gas, helium; flow rate, 2 mL/min; injection temperature, 280°C; oven temperature, 75°C for 4 min, then increase 20°/min to 300°C; injection volume, 2 μL. Operating conditions for the mass spectrometer were: interface, direct; interface temperature, 300°C; source temperature, 280°C; mass range, 50–500 AMU; scan rate, 450 AMU/s; mode, electron impact; source pressure, 5×10^{-7} torr.

When the previous butter samples, which had showed β-sitosterol levels when analyzed via the packed column procedure, were reanalyzed, β-sitosterol values of <1.0 mg/100 g were obtained using the capillary column technique. This procedure was used to monitor 300 butter samples for the presence of β-sitosterol.

The capillary chromatography procedure was validated using the control butter obtained from the University of Wisconsin Creamery and subsequently spiked with 0.5% corn oil. The precision data obtained for β-sitosterol are as follows: $N = 16$; mean = 3.05 mg/100 g; standard deviation = 0.105 mg/100 g; coefficient of variation = 3.45%.

Spiked additions of β-sitosterol (at 1.0, 2.0, and 4.0 mg/100 g) in the control butter gave an average recovery of 98%.

In addition to β-sitosterol, this procedure can be used to monitor other sterols of interest. These include other phytosterols such as campesterol and stigmasterol and the animal sterol, cholesterol.

Recommendations

The use of a direct saponification/capillary GC procedure provides a rapid method for the determination of phytosterols in butter oils. The new procedure offers both increased sensitivity and selectivity over the current AOAC method (1.0 mg/100 g compared to 4.0 mg/100 g, respectively) for determining β-sitosterol in butter oils. The method is precise, gives excellent recoveries of added sterols, and is free of lanosterol interference.

On the basis of this study, it is recommended that all future phytosterol analyses of dairy products be conducted using capillary column gas chromatography.

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CHEMICAL CONTAMINANTS MONITORING

Correlations between Selected Trace Elements and Organic Matter and Texture in Sediments of Northern Prairie Wetlands

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Total concentrations of arsenic, cadmium, lead, mercury, and selenium previously determined in wetland sediments were grouped according to habitat type and geologic location and related to sediment organic matter and particle size by stepwise multiple regression analysis. Cadmium and Se were significantly correlated with the measured sediment properties in every geologic and hydrologic category. Arsenic and Pb were consistently correlated with sediment properties in riverine habitats but not in potholes. In contrast, good correlations were found with Hg in potholes but not riverine wetlands. Organic matter concentration was the most consistent overall predictor of trace element concentrations, but texture was also a good indicator in some instances. The lack of correlation observed for some of the elements within given geologic categories suggests that other factors, such as external loading or deposition rates, were responsible for the observed variation.

In a previous paper (1), we reported total concentrations of arsenic, cadmium, lead, mercury, and selenium in surface sediments of various wetlands in the north central United States. We noted that, with the exception of Hg, sediments from pothole wetlands contained significantly higher concentrations of these elements than did sediments from riverine locations. Except for this natural differentiation into 2 general habitat types, we had no information that would account for the observed variation in elemental concentrations. Several investigators (2–4) have discussed various factors that should be considered in interpreting data on elements in sediment. Among these are sediment properties such as particle size, organic matter content, and sulfide ion concentration, and external factors such as bedrock lithology, sedimentation rate, and anthropogenic loading.

On the samples that had been analyzed in the previous study (1), we determined organic matter content and particle size distribution. Because the original elemental data had suggested differences between pothole and riverine locations, we retained this hydrologic division and further grouped the sample sites according to major geologic divisions. A stepwise multiple regression analysis was then used to identify significant relations between trace element concentrations and sediment properties within each hydrologic and geologic category. This information may assist resource managers in this geographic region in distinguishing between natural levels of trace elements in sediments and levels indicative of anomalous enrichment.

Study Areas

Sample locations were previously identified geographically and described hydrologically (1). Location of each site with respect to major physiographic classification is shown in Table 1. All study areas lie within the physiographic region of the United States known as the Interior Plains. This is a vast, diverse lowland bounded on the west by the Rocky Mountains, on the east by the Appalachians, and on the south by the Interior Highlands and Atlantic Coastal Plain. The entire western portion of the Interior Plains, from Canada to Mexico, is the physiographic province known as the Great Plains. Adjacent to the Great Plains, along its entire eastern border, is the other major physiographic province within the Interior Plains—the Central Lowland. The Great Plains and the Central Lowland are, in themselves, rather diverse and are further subdivided into various physiographic divisions.

Three study areas were located in the Great Plains: (1) the Coteau du Missouri potholes, (2) the Upper Missouri Basin riverine, which is also in the Coteau du Missouri region, and (3) the High Plains potholes. The Coteau du Missouri is a large, glacial drift complex that is 90% dead-ice moraine, with small amounts of end moraine and outwash deposits. The thickness of this drift complex may reach 200 m; it consists of rock, sand, and clayey, silty till. The fine-grained material is believed to have been derived from local bedrock, although the larger rocks are known to have been transported from as far away as Canada by the glaciers. Cretaceous formations such as Pierre Shale, Fox Hills Sandstone, and Hell Creek make up the bedrock.

In contrast to the Coteau du Missouri, the surface of which was formed by glacial action less than 13 000 years ago, the surface of the High Plains has remained essentially unchanged for about 5 million years. This huge, nearly flat plateau was formed by Tertiary Age deposition of stream sediment over a period of more than 60 million years. From the end of the last deposition (Ogallala) to the present, the High Plains have been essentially untouched and unaffected by streams. Windblown silt deposits (loess) and sand dunes cover much of the northern High Plains, including the region surrounding our study area.

Table 1. Physiographic location of sediment sampling sites in the north central United States[a]

Physiographic region	Physiographic province	Physiographic division	Study area
Interior Plains	Great Plains	Coteau du Missouri	potholes (Jamestown, ND, and Coal Creek, ND)
			riverine—Upper Missouri Basin (Audubon, ND)
		High Plains	potholes (Hastings, NE)
	Central Lowland	Drift Prairie	riverine—Souris Basin (Des Lacs, ND, and J. Clark Salyer, ND)
			riverine—James Basin (Arrowwood, ND, and Sand Lake, SD)
		Coteau des Prairies	potholes (Madison, SD, and Wike, SD)
		Des Moines Drift Lobe	riverine—Blue Earth–Des Moines Basin (Union Slough, IA)

[a] Geographic names used by Martin and Hartman (1) are shown in parentheses.

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Table 2. Results of duplicate sample analyses

Constituent, unit	Number of duplicates	Concn			Mean difference (%)	Range of difference
		Min.	Max.	Mean		
As, mg/kg	9	1.0	7.2	3.3	0.2 (6)	0–0.5
Cd, mg/kg	10	0.06	1.2	0.42	0.03 (7)	0–0.10
Pb, mg/kg	10	3.4	16.8	9.0	0.6 (7)	0–2.6
Hg, mg/kg	10	0.01	0.07	0.04	<0.01 (12)	0–0.01
Se, mg/kg	10	0.02	1.7	0.63	0.05 (8)	0–0.10
Organic matter, % dry wt	18	0.5	14.0	5.2	0.9 (17)	0–5.8
Clay, % dry wt	12	1	43	28	3 (11)	0–6
Silt, % dry wt	12	1	78	41	3 (7)	0–11
Sand, % dry wt	12	1	98	32	3 (9)	0–13

Four study areas were located in the Central Lowland: (1) the Souris Basin riverine, (2) the James Basin riverine, (3) the Coteau des Prairies potholes, and (4) the Blue Earth–Des Moines Basin riverine. The Drift Prairie, which contains both the Souris River and James River basins, borders the Coteau du Missouri on the east. The dividing line between the Drift Prairie and the Coteau du Missouri also forms the border between the Great Plains and the Central Lowland in North Dakota. The Drift Prairie is characterized by low relief and integrated drainage. Extensive glacial lake deposits of silt and clay occur in the Souris and lower James (Lake Dakota) basins. The rest of the Drift Prairie is covered mostly by ground moraine, deposited beneath the glacier by active ice. In contrast with the high-relief, dead-ice moraine on the Coteau du Missouri, the ground moraine of the Drift Prairie forms an undulating plain of low relief. Bedrock in the vicinity of the James Basin study area consists primarily of Pierre Shale, whereas the Souris Basin is underlain by Fox Hills Sandstone and a mixture of several other Tertiary Age formations.

The Coteau des Prairies is a plateau in eastern South Dakota, continuously covered with glacial drift of irregular thickness, but often in excess of 100 m. Most of the drift consists of tills; it is made up of fragments of local rocks. The till is very rich in clay and silt, reflecting the predominance of shale in the bedrock strata of the region. Glacial erosion of the weak Cretaceous bedrock, primarily Pierre Shale, is believed to have been great.

The Des Moines Drift Lobe is yet another glacial feature of the Central Lowland, extending out from Minnesota and down into north-central Iowa. The age has been established at about 14 000 years. The lobe consists of Cary glacial drift, the surface of which has been modified by 2 major erosion cycles. End moraine predominates in the vicinity of the study area, although ground moraine is found at various places within the watershed. Bedrock to the west of the study area consists of Upper Cretaceous Dakota Sandstone, whereas older Paleozoic limestones and dolomites subcrop the glacial sediments along the eastern edge.

Geologically, some differences and similarities exist among the 7 study areas. Six are situated in regions recently formed by various stages of late Wisconsin Age glaciation, whereas one is located in an unglaciated region. Some are underlain by different bedrock formations, the influence of which may be reflected in the physical and chemical properties of surface soils and sediments.

The preceding discussion is intended only to provide a broad overview of the physiographic setting of the study areas. More detailed descriptions can be found elsewhere (5–10).

Experimental

Methods of sample collection, preservation, preparation, and elemental analysis were described previously (1). In the time elapsed between elemental analysis (1980–1981) and analysis for texture and organic matter (1983), the dried samples were stored at −20°C. Organic matter content was determined colorimetrically following wet oxidation with potassium dichromate and sulfuric acid (11). This method was preferable to loss-on-ignition methods because of error involved with low-temperature dehydration of clays (12). Soil separates (clay <0.002 mm, silt 0.002–0.062 mm, sand >0.062 mm) were determined by the pipet method following peroxide treatment (13). Texture and organic matter analyses were performed on appropriately sized samples that had been oven-dried at 100°C to a constant weight; results are reported as percent of dry weight.

Analytical precision and accuracy for elemental analyses were estimated by recovery of known additions, duplicate analyses, and analyses of certified standard reference materials (1). Analytical quality control for texture and organic matter consisted of duplicate analyses and cross-check analyses of samples by the Soil Testing Laboratory of the South Dakota State University, Agricultural Experiment Station. Statistical methods were from Steel and Torrie (14) and LeClerg et al. (15).

Table 3. Mean dry weight concentrations and coefficients of variation of trace elements, organic matter, and particle size distribution in sediments from northern Prairie Wetlands

Wetland type and physiographic region (n)	Trace element, mg/kg (CV, %)					Other constituents, % dry wt (CV, %)			
	As	Cd	Pb	Hg	Se	Organic matter	Clay	Silt	Sand
Potholes									
Coteau du Missouri (10)	5.1 (41)	0.46 (37)	11.0 (27)	0.03 (55)	0.87 (44)	14.0 (45)	23 (17)	52 (23)	24 (62)
High Plains (12)	3.2 (38)	0.44 (41)	14.1 (26)	0.03 (19)	0.50 (84)	2.9 (28)	40 (18)	46 (11)	14 (50)
Coteau des Prairies (18)	4.8 (21)	0.61 (15)	12.4 (27)	0.03 (34)	1.16 (37)	13.0 (42)	33 (39)	52 (17)	14 (79)
Combined (40)[a]	4.4a (36)	0.52a (31)	12.6a (28)	0.03a (38)	0.89a (55)	10.2a (67)	33a (33)	51a (18)	17a (71)
Riverine									
Souris Basin (24)	2.3 (52)	0.31 (45)	7.6 (33)	0.04 (38)	0.44 (59)	6.3 (52)	30 (57)	32 (38)	38 (61)
Upper Missouri Basin (9)	2.4 (54)	0.19 (32)	4.4 (41)	0.03 (48)	0.23 (57)	2.9 (41)	19 (63)	29 (45)	52 (31)
James Basin (18)	2.1 (38)	0.17 (53)	5.0 (62)	0.03 (38)	0.24 (58)	3.5 (74)	29 (31)	14 (57)	57 (28)
Blue Earth–Des Moines (9)	3.0 (43)	0.40 (25)	9.1 (29)	0.03 (22)	1.58 (84)	11.4 (38)	33 (18)	57 (23)	10 (110)
Combined (60)[a]	2.4b (50)	0.26b (54)	6.6b (47)	0.03a (38)	0.52b (135)	5.7b (72)	28a (46)	30b (60)	42b (57)

[a] Combined values designated with b are significantly different ($P < 0.05$) from combined values designated with a.

Results and Discussion

Results of the duplicate sample analyses (Table 2) suggest that elemental analyses might be more reproducible than analyses for the other sediment constituents. Mean differences of duplicate sets ranged from 6 to 12% of the mean concentration for each of the elements, whereas the corresponding values for the other sediment properties ranged from 7 to 17%. Organic matter determinations were the most variable, possibly due to the relatively small subsamples of sediment (0.1 g) taken for analysis. Also, homogeneity of organic matter in the overall sample is probably difficult to attain.

The same 12 samples that were used for duplicate analyses were also submitted to the independent laboratory for cross-check analysis of organic matter and particle size. Results for soil separates were comparable to our analyses, ranging within ±14% of our values. Results for organic matter were more variable. Two of the samples varied by −26 and +43% of our values, whereas the remainder were within ±10%. Again, we suspect that the major difficulties in organic matter analysis lie in the nonhomogeneous distribution of the organic matter in the parent sample and in the relatively small size of the subsamples used for analysis.

Arsenic concentrations in combined pothole sediments were somewhat less variable than those in combined riverine sediments (Table 3). Breakdown of the data into geologic divisions did not consistently reduce this variability, as evidenced by the coefficients of variation (CVs) in each category. The best equations for predicting As in both the combined pothole and combined riverine environments were based only on organic matter (Table 4). Arsenic was not significantly correlated with any of the sediment properties measured at any of the pothole locations or at one of the riverine sites. This lack of correlation suggests that factors other than those considered in this study were primarily responsible for the observed variation in As.

Cadmium concentrations in combined pothole sediments were less variable than those in combined riverine sediments. In riverine habitats, the breakdown into geologic subdivisions slightly reduced variability, whereas this was not the case in pothole locations. Cadmium concentrations in both types of habitat were best predicted by a combination of sand and organic matter. Correlations between Cd and the other sediment properties were significant in each of the geologic categories except one. This relatively high degree of correlation suggests that the observed variation in Cd was largely accounted for by the sediment properties measured.

Lead concentrations in combined pothole sediments were less variable than those in combined riverine sediments. The breakdown into geologic categories did not reduce the relative variability in pothole habitats but did reduce variation in 3 of the 4 riverine locations. Lead was not correlated with any of the measured sediment properties in the combined pothole category or in 2 of the 3 pothole geologic divisions. Correlations between Pb and other sediment properties were fairly good at all riverine locations studied. Apparently, Pb concentrations are dependent on factors other than those measured in the pothole sediments, whereas organic matter and texture provide a relatively good level of predictability in riverine sediments.

Relative variation in Hg was about equal in both combined pothole and combined riverine habitats. Geologic breakdown provided no consistent reduction of variation in either habitat type. Correlations between Hg and sediment properties were quite good in all pothole categories. Correlations were either not significant or fairly weak at the riverine locations. This suggests that Hg was strongly related to organic matter and texture in potholes, but at riverine locations other factors predominate.

Selenium was the most variable of all the elements measured in both pothole and riverine locations. The breakdown into geologic categories reduced the variation somewhat, but

Table 4. Correlation/regression analysis of trace element concentrations (mg/kg) vs other sediment properties

Element	Location[a]	Equation[b]	r or R[c]
Arsenic	CM	NS	—
	HP	NS	—
	CP	NS	—
	Combined potholes	0.106 (OM) + 3.3	0.46
	SB	0.258 (OM) + 0.7	0.69
	UMB	NS	—
	JB	0.051 (silt) + 1.4	0.52
	BE-DM	0.404 (OM) + 0.142 (clay) − 6.2	0.85
	Combined riverine	0.164 (OM) + 1.4	0.58
Cadmium	CM	−0.009 (sand) + 0.68	−0.77
	HP	0.016 (clay) − 0.20	0.62
	CP	−0.005 (sand) + 0.68	−0.56
	Combined potholes	−0.008 (sand) + 0.010 (OM) + 0.56	0.68
	SB	−0.003 (sand) + 0.021 (OM) + 0.29	0.92
	UMB	0.040 (OM) + 0.08	0.81
	JB	0.008 (silt) + 0.012 (OM) + 0.02	0.94
	BE-DM	NS	—
	Combined riverine	−0.003 (sand) + 0.016 (OM) + 0.28	0.90
Lead	CM	NS	—
	HP	NS	—
	CP	0.20 (silt) + 1.9	0.54
	Combined potholes	NS	—
	SB	0.50 (OM) − 0.03 (sand) + 5.6	0.84
	UMB	1.16 (OM) + 1.0	0.78
	JB	0.24 (silt) + 1.6	0.65
	BE-DM	0.81 (OM) + 0.38 (clay) − 12.6	0.80
	Combined riverine	0.42 (OM) − 0.03 (sand) + 5.6	0.78
Mercury	CM	0.0035 (OM) − 0.001 (silt) + 0.0347	0.96
	HP	0.0040 (OM) − 0.0005 (silt) + 0.0359	0.89
	CP	0.0005 (clay) + 0.019	0.52
	Combined potholes	0.0016 (OM) + 0.0010 (clay) + 0.0004 (sand) − 0.026	0.65
	SB	0.002 (OM) + 0.025	0.51
	UMB	NS	—
	JB	NS	—
	BE-DM	NS	—
	Combined riverine	−0.0002 (sand) + 0.041	−0.32
Selenium	CM	0.020 (silt) − 0.20	0.68
	HP	0.311 (OM) − 0.40	0.58
	CP	0.029 (silt) − 0.013 (clay) + 0.06	0.89
	Combined potholes	0.050 (OM) + 0.38	0.68
	SB	0.067 (OM) + 0.02	0.86
	UMB	0.099 (OM) + 0.06	0.95
	JB	0.021 (OM) + 0.008 (silt) + 0.05	0.83
	BE-DM	0.235 (OM) − 1.11	0.75
	Combined riverine	0.131 (OM) − 0.23	0.77

[a] CM, Coteau du Missouri; HP, High Plains; CP, Coteau des Prairies; SB, Souris Basin; UMB, Upper Missouri Basin; JB, James Basin; BE-DM, Blue Earth–Des Moines Basin.

[b] NS, no significant correlations; OM, organic matter.

[c] r = coefficient of linear correlation (applies to equations with one dependent variable); R = multiple correlation coefficient (applies to equations with 2 or more dependent variables). Only statistically significant correlations ($P < 0.05$) are shown for each location.

Se remained the most variable. Organic matter was important to the prediction of Se concentrations at most locations, and overall, correlation was good between Se and the sediment properties measured.

Considerable variation existed in the relationship between a given element and the various sediment properties that were measured. The correlations range from several that were not statistically significant to some with correlation coefficients greater than 0.90. Similar findings have been reported in the literature. Iskandar and Keeney (16) found little or no correlation between elemental concentration and organic matter or textural data, whereas Kemp et al. (17) reported a high degree of correlation. At times, a lack of correlation is interpreted as evidence for anthropogenic loading or contamination (e.g., 4). In our study, the poorest correlations were perhaps those for As and Pb in potholes and Hg in riverine environments. This lack of correlation may be due to variation in external loading at specific sites within these categories. On the other hand, the best correlations were found with Cd and Se, suggesting little or no outside contamination for these elements.

The relationships shown in Table 4 provide a starting point (or backdrop) for further examination of trace element concentrations in sediment in the Interior Plains region. Factors governing the concentrations of As and Pb in potholes and Hg in riverine environments need to be elucidated. Values of Cd or Se that vary significantly in their relationship to organic matter or texture from those relationships shown in Table 4 should be examined to determine probable cause. In addition, resource managers in this area who question whether a particular elemental sediment concentration is indicative of contamination will now have some baseline data (Table 3) for comparison.

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TECHNICAL COMMUNICATIONS

Liquid Chromatographic Determination of Amprolium in Poultry Feed and Premixes Using Postcolumn Chemistry with Fluorometric Detection

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Two extraction and liquid chromatographic procedures are presented which separate amprolium from compounds in poultry feed or premixes that could interfere with its fluorometric determination. The procedures are based on earlier work on the determination of thiamine in food samples. Amprolium is extracted from feed with a hexane-aqueous sulfosalicylic acid mix, separated on a C_{18} column, and detected fluorometrically after postcolumn derivatization. For premixes, water extraction is used. Values for the amprolium content of poultry feed obtained with these procedures are in good agreement with those obtained with AOAC official methods. It is suggested that these methods with suitable modifications may be of use for routine analysis of amprolium in feeds. The overall methods are rapid and appear to give reasonable results.

In recent work undertaken to develop a liquid chromatographic method for the determination of the levels of thiamine and its phosphate esters in foods, it was found that the anticoccidial amprolium could serve as an internal standard in the procedure (1). Fluorescence detection was used, and, because amprolium oxidizes to form the same type of fluorescent derivative as does thiamine and its phosphate esters (i.e., the thiochrome reaction) (2), amprolium satisfies all the normal criteria for an internal standard (3). Besides undergoing the same chemical reaction during the analysis, it also behaves like the thiamine vitamers during the entire extraction and analytical procedures. The developed methods gave reasonable results for a variety of food products and could detect the vitamers at the picomole level.

The results described above suggest that the roles of the vitamers and amprolium could be reversed if one were interested in determining the levels of the anticoccidial in poultry feed. For example, one might hope the levels of one of the thiamine vitamers would be low in feed, and this compound then could be used as the internal standard in the analysis for amprolium. To check this, a preliminary study was made on the levels of amprolium and the thiamine vitamers in a typical chicken feed mixture prepared at the Beltsville Human Nutrition Center.

In addition, the original extractions and analytical procedures were simplified to shorten the analysis when thiamine is not present in a sample, i.e., a premix. This procedure was checked on a commercially supplied premix containing amprolium but no thiamine.

The proposed chromatographic procedures offer the possibility of easy separation of amprolium from interfering compounds, and they offer short analysis times. In contrast to manual methods (4), the entire procedure including extraction and analysis can be automated using a laboratory robot if necessary (5). The use of fluorescence detection leads to much cleaner baselines than are observed with ultraviolet absorption (6).

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Experimental

Feed Mixtures

One ton of feed was mixed in a vertical mill at the Poultry Laboratory in Beltsville. The feed was principally ground corn and soybean meal (82% by weight) but did contain chlortetracycline HCl, thiamine, and amprolium. The latter was nominally 0.025% by weight or 250 μg/g. No further mixing was done before analysis because the poultry department was interested in the variation of amprolium from sample to sample.

The premix, Amprol 25, was commercially prepared (Merck Sharp & Dohme) and was nominally 25% amprolium by weight. This was analyzed without any further mixing.

Reagent and Standards

Thiamine, thiamine monophosphate, thiamine diphosphate, and pyrithiamine were obtained from Sigma Chemical Co. Amprolium was a gift from Merck Sharp & Dohme Research Laboratory. Sequanol grade sulfosalicylic acid was obtained from Pierce Chemical Co. All other chemicals were obtained from Fisher Scientific Co.

Method of Analysis

Two extraction and chromatographic procedures are described below. The one used for feed mixtures containing thiamine involves extraction of amprolium and an internal standard with sulfosalicyclic acid followed by a cleanup step and subsequent analysis by chromatography. The chromatographic analysis involves a step gradient elution of the compounds of interest with 2 separate buffers. This procedure separates the thiamine vitamers and amprolium from one another and from any interfering impurity peaks.

The second extraction and chromatographic procedure is appropriate for a premix containing no thiamine. Extraction of amprolium and the internal standard is accomplished with water with subsequent analysis by an isocratic chromatographic procedure. While simpler and faster than the previous method, thiamine diphosphate and thiamine monophosphate are not separated from one another nor from an interfering impurity peak. However, the thiamine and amprolium peaks are separated and are free from interference. For the premix tested with this procedure, a clean chromatographic baseline was obtained. However, if a water extraction instead of sulfosalicyclic acid is used in the analysis of a feed mixture, unidentified peaks which interfere with the analysis are observed.

Extraction Procedure

(a) *Feed mixture.*—To 1 g feed mixture are added 2 mL of a 5 μg/mL solution of thiamine monophosphate as the internal standard, 10 mL of a 5% (w/v) sulfosalicylic acid solution, and 10 mL hexane. This is vortex-mixed for 1 min, and centrifuged at 2400 *g* for 10 min. The water layer is removed and filtered through an ACRO LC 13 0.45 μm

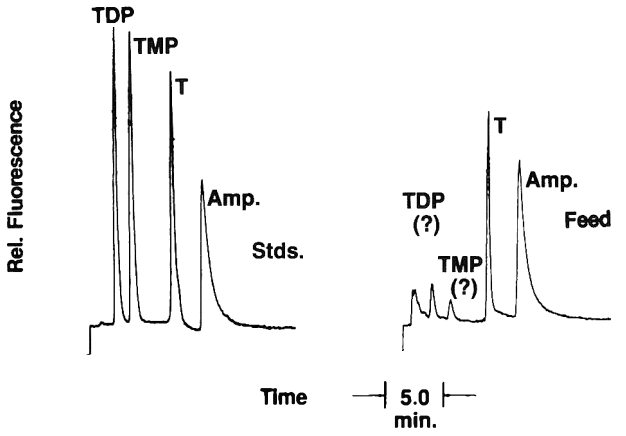

Figure 1. Chromatogram of a mixture of standards and an extract of chicken feed mixture. Notation is as follows: TDP, thiamine diphosphate; TMP, thiamine monophosphate; T, thiamine; Amp, amprolium.

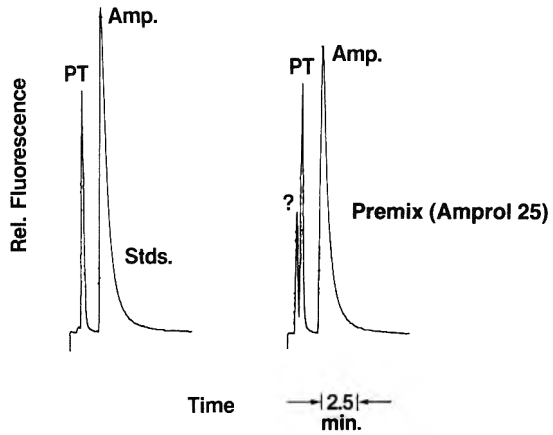

Figure 2. Chromatogram of a mixture of standards and an extract of premix (Amprol 25). Notation is as follows: PT, pyrithiamine; Amp, amprolium.

disposable filter (Gelman). The filtered water layer is then carried through the rest of the cleanup and analytical procedures described in detail previously (1, 7).

(b) *Premix.*—For a premix containing no thiamine, a simpler extraction procedure is used. A known amount (ca 0.1 mg) of internal standard, pyrithiamine, is added to a known amount (ca 0.8 mg) of sample in 10 mL water. This mixture is ground in an Omni-Mixer (Dupont Instruments) for 10 min, 10 mL hexane is added, and the mixture is ground for another 5 min. This is centrifuged at 2400 *g* for 10 min at 4°C (Beckman Model TJ-6); the water layer is removed and then filtered through a 0.45 μm Millipore filter. The filtrate is diluted 20 times in preparation for injection into the liquid chromatographic analytical system.

Liquid Chromatography

(a) *Hardware.*—The liquid chromatographic system is described in detail elsewhere (1). The system as set up is capable of performing a postcolumn chemical reaction. Separation is achieved on a 3 mm × 3 cm C_{18} column (Perkin-Elmer) packed with 3 μm particles. The eluting buffer (or buffers) is described below. Thiamine, its phosphate esters, and amprolium are oxidized postcolumn to their thiochrome derivatives with 0.01% (w/v) potassium ferricyanide in 15% (w/v) sodium hydroxide solution (pH ≤13). Tubing prior to the column is stainless steel (0.025 cm id); postcolumn tubing can be AWG 26 gauge (0.040 cm id) Teflon tubing. The flow rates of both the eluting buffer and the oxidizing stream are set at 1 mL/min. The streams are mixed in a "tee" behind the column. Before it enters the fluorescence detector, the mixed stream is passed through 7 m of 0.04 cm id Teflon tubing kept at 32°C. The output from the detector is fed to a previously calibrated recording integrator which automatically calibrates the amount of unknown on the basis of the amount of internal standard added to the original sample.

Table 1. Liquid chromatographic determination of amprolium in chicken feed mixture

Sample	Amprolium, μg/g	Recovery,[a] %
1	226	99
2	220	105
3	226	101
4	206	118
5	264	100
Av.	228 ± 22	105 ± 8

[a] (Amp added − 228) × 100/Amp added.

Peak areas are used in the calculation. A typical apparatus consists of a Perkin-Elmer Series 4 liquid chromatograph, a Haake water bath, a Shimadzu C-R1A recording integrator, and a Perkin-Elmer 650-40 fluorescence spectrophotometer with excitation and emission wavelengths set at 339 and 432 nm, respectively. The excitation and emission slits are normally set at 10 and 15 nm, respectively. A Milton Roy constant-volume pump is used for the oxidizing stream. The analytical column is protected with an RP-18, 3 cm × 4.6 mm, 5 μm particle size guard column (Rainin).

(b) *Eluting buffers.*—For the feed mixture, 2 separate buffers are used to elute the compounds of interest from the column. The column is first equilibrated for 15 min with a 0.1M sodium phosphate buffer at pH 5.5. After the sample is injected, the pH is kept at 5.5 for an additional 6 min. This is then followed with 0.1M sodium phosphate buffer at pH 2.6 for an additional 19 min to complete the elution of the sample contents from the column.

For the premix containing no thiamine, the elution is isocratic with only the one buffer at pH 2.6 being used.

Results

A standard solution of thiamine diphosphate, thiamine monophosphate, thiamine, and amprolium chromatographed under the conditions mentioned for the chicken feed mixture is shown in Figure 1. The elution times for thiamine diphosphate, thiamine monophosphate, thiamine, and amprolium in the standard solution are 3.28, 5.26, 10.21, and 13.98 min, respectively. Lower limits of detection are at the picomole level. Also shown is a chromatogram of extracted chicken feed mixture to which no thiamine or its phosphate esters have been added. The elution times are the same as for the solution of standards. Small peaks indicating perhaps the presence of thiamine diphosphate and thiamine monophosphate are observed as are those for thiamine and amprolium. Thiamine monophosphate was selected as the internal standard and, under the procedures described for an actual analysis for amprolium, enough thiamine monophosphate was added to ensure that its resulting peak was 12 times the area of that for an unspiked sample. Five analyses were performed, and the results are shown in Table 1 together with recovery studies. The average value of 228 μg/g obtained is in contrast with the nominal value of 250 μg/g for the prepared mixture. Values obtained by 2 commercial laboratories using the official spectroscopic method of analysis (4) were 230 and 236 μg/g, which are in reasonable

agreement with the results obtained here. The standard deviation shown in Table 1 leads to a coefficient of variation of 10%, which is double the value found in the earlier work in food samples. It is suspected that some of this variation is due to a slight inhomogeneity in the chicken feed mixture because no effort was made to homogenize the mixture further after mixing in the vertical mill. With the exception of one high value, the recoveries from spiked samples were close to 100% even though chlortetracycline HCl was in the mixture. This is in agreement with the earlier work of Kanora and Szalkowski (2) who found no enhanced recovery of amprolium when this drug was present, although some other drugs enhanced the recovery. One caveat is in order, however. The small peak of thiamine monophosphate present in the original sample will tend to lower the values of amprolium and its recovery when using the internal standard method because of the manner in which the integrator calculates concentration. It is estimated that the values are approximately 8% lower than they should be. Finally, the amounts of thiamine in the mixture can be determined along with amprolium. None are reported here, however, because, in our original work, it was not ascertained that the extraction procedure used effectively removed thiamine from ground corn or soy bean meal, which compose a large percentage of the mixture.

Shown in Figure 2 are chromatograms obtained when the extraction and analytical system described for premixes containing no thiamine is used on Amprol 25. Traces are shown for a standard solution containing pyrithiamine (as the internal standard) and amprolium and for an extracted sample of premix spiked with pyrithiamine. The elution times for pyrithiamine and amprolium are 1.62 and 4.03 min, respectively. Thiamine could also be used as the internal standard as it elutes at the same time as does pyrithiamine. The phosphate esters of thiamine elute earlier with unknown compounds.

Eight determinations of the amount of amprolium in the premix were made which yielded the percentage values of 32.3, 23.5, 31.7, 26.0, 34.2, 22.7, 26.0, and 28.1. The average value was 28.1 ± 4.3%. This large variation in amprolium content is likely due to the fact that very small sample amounts of premix (0.8 mg) had to be used and the prepared premix had not been further homogenized as was the intention. The method is so sensitive that even with these small samples, the extracted solution still had to be diluted 20-fold to stay within the range of the instument sensitivity. Under these conditions, it was considered impossible to do recovery studies since adequate representative samples were not obtained.

Conclusions

The 2 extraction liquid chromatographic separation procedures outlined here effectively separate amprolium from other compounds such as the thiamine vitamers in poultry feed which could interfere with fluorescence methods for the determination of amprolium in feed mixtures and premixes. As such, they allow for the analysis of this compound by either an internal or external standard method. The internal standard method as used here for feed mixtures may lead to slightly lower values of amprolium content due to the presence of a small amount of thiamine monophosphate in the feed. This same criticism does not apply to the analysis of premixes where no thiamine is present. At the present time, the methods are too sensitive for the determination of amprolium in premixes in that samples too small to be representative ones must be taken to stay within instrument sensitivity. A large reduction in instrument response would eliminate this problem.

It is suggested that the procedures outlined here may be useful to those who routinely analyze for amprolium in feeds. Although minor modification of the methods may be necessary, the analysis is straightforward and reasonably rapid.

Acknowledgments

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Simple Method for Distinguishing Maneb, Zineb, Mancozeb, and Selected Mixtures

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Maneb, zineb, mancozeb, and arbitrarily selected mixtures of those can be differentiated by a simple method. Compounds are differentiated on the basis of colors produced after treatment of saturated solutions of the fungicides in *n*-propanol-acetone mixture (1 + 1 v/v), first with dithizone and then with monosodium dihydrogen phosphate solution in the same solvent. The color depends on the type and concentration of metal present in the sample.

Various metallic ethylenebisdithiocarbamates and their mixtures are used widely as plant protectants; their identification requires simple methods. Much research on this subject has been done, and nearly all methods are based on the determination of carbon disulfide and the metal contained in the fungicides (1). Most of the metallic ethylenebisdithiocarbamates have similar color and appearance, and some of them give identical elemental analysis results. For example, mancozeb cannot be differentiated by this technique from a mixture of maneb-zineb or from a mixture of maneb and zinc salts. Although X-ray diffraction or other instrumental methods can be used for this purpose, these instruments are rarely available in conventional laboratories (2–4).

In 1970, Walker (5) reported a simple color spot test using dithizone to distinguish fungicidal mixtures, but it was not applicable to the differentiation of mancozeb from a mixture of maneb and zinc sulfate.

Stevenson (6) proposed a spot test which is an extension of Walker's method. This technique, as Stevenson stated in his report, needs practice, because determinations are based on color differences that are difficult to describe accurately, particularly when the colors are very similar. In addition, this technique does not definitely distinguish mancozeb and maneb–zinc sulfate mixtures.

In the present work, a method has been developed to distinguish colors of the complexes instrumentally and/or by the naked eye. Thus, subjective mistakes have been reduced to a minimum. This technique can be applied easily, and accurate results can be obtained with a simple colorimeter.

Experimental

Principles

Saturated solutions of samples in *n*-propanol–acetone are prepared and treated with a solution of dithizone in the same solvent. Colors of complexes vary with type and concentration of the metal present, thus mixtures can be distinguished by means of color differences. To obtain more accurate results, colors are observed after the addition of dithizone to the sample solutions, and then a saturated solution of monosodium dihydrogen phosphate in *n*-propanol–acetone is added. The colors following the addition of phosphate can be measured spectometrically at 510 nm. A reagent blank is used in all cases.

Reagents

(a) *Solvent mixture.*—Analytical grade *n*-propanol and acetone mixed in equal volumes.

(b) *Dithizone solution.*—4×10^{-4} molar dithizone solution prepared in solvent mixture.

(c) *Phosphate sclution.*—Saturated solution of $NaH_2PO_4 \cdot 2H_2O$ in solvent mixture.

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(d) *Reference solution I.*—10 mL of dithizone solution diluted to 50 mL with solvent mixture.

(e) *Reference solution II.*—10 mL dithizone solution and 10 mL phosphate solution, mixed and diluted to 50 mL with solvent mixture.

Procedure

A 1.00 g sample of fungicide (maneb, zineb, mancozeb, maneb + zinc sulfate mixture or maneb + zineb mixture) is weighed into glass-stopper conical flask, and 20 mL *n*-propanol–acetone mixture is added. The solution is stirred ca 1 h and then filtered through ashless Whatman No. 42 paper. Two 1 mL aliquots of the filtrate are transferred to separate test tubes; to the first is added 1 mL dithizone solution and 1 mL phosphate solution; to the second is added 1 mL dithizone solution. The contents of both tubes are diluted to 5 mL with solvent mixture. The subsequent colors are visually compared, and their absorbances are recorded against reagent blank at 510 nm. The same procedure is applied to samples of maneb, zineb, mancozeb, the mixture of maneb + zineb, and the mixture of maneb + zinc sulfate.

Results and Discussion

The basic aim of this investigation was the differentiation of mancozeb from a mixture of maneb and zinc salts or from a mixture of maneb and zineb.

Zineb is the zinc complex of ethylenebisdithiocarbamic acid, and, due to its greater stability, zinc dithizonate is not formed on the addition of dithizone to a zineb organic solution. Mancozeb is the zinc complex of manganese ethylenebisdithiocarbamate, and the strength of the bond between zinc ions and the ligand is weaker than in zineb. Thus, zinc(II) can dissociate to a greater extent in the organic solution of mancozeb, and the ligand displacement can occur with dithizone. Consequently, as shown in Table 1, zineb does not alter the dithizone green, whereas mancozeb gives a pink-violet zinc complex. With maneb, the Mn(II) ion concentration in the organic solution is sufficient to form a complex with dithizone. Even though the color of the zinc dithizonate and manganese dithizonate complexes are different (the former is pink-violet while the latter is orange), the Mn(II) ions are masked with phosphate to obtain a further differentiation; as a result, Mn(II) ions in the case of mancozeb can no longer complex with dithizone, and the only observable color in solution is the pink-violet zinc dithizonate complex. Either in a mixture of maneb–zineb or in a mixture of maneb–zinc sulfate, zinc ions are not at a sufficient concentration in the organic solution to form the dithizonate complex, while Mn(II)

Table 1. Colors and absorbances of sample solutions

	Sample + dithizone		Sample + dithizone + phosphate	
Compound	Color	Abs.	Color	Abs.
Zineb	green	0.01	green	0.00
Maneb	orange	0.98	green	0.01
Mancozeb	pink-violet	1.08	pink-violet	0.49
Maneb–zineb (7 + 1 w/w)	orange	1.04	green	0.01
Maneb–$ZnSO_4$ (7 + 1 w/w)	orange	0.99	green	0.01

ions can easily form these complexes, which fade on the addition of phosphate.

As can be seen from Table 1, maneb, a mixture of maneb + zineb, and a mixture of maneb + zinc sulfate show identical behavior on the sequential addition of dithizone and phosphate. When differentiation of these substances is desired, the presence of Zn(II) is tested (by dithizone or other standard procedures). For this purpose, the test sample is first leached with water and then with diluted H_2SO_4. The maneb + zineb mixture can only give a positive zinc test in the H_2SO_4 extract, while the maneb + zinc sulfate mixture can give positive zinc results in both water and H_2SO_4 extracts. On the other hand, maneb gives negative results for both extracts.

The differentiation of mancozeb from the maneb + zineb mixture is not affected by a water content of 8% (by volume) in the organic solution. The same tolerance, valid for the differentiation of mancozeb from the mixture of maneb and zinc sulfate, is limited to a water content of 2%. It is apparent that absolute drying of the solvents is not strictly necessary. Therefore, the method is simple and convenient (for most laboratories lacking instrumental devices) for the analysis of the indicated fungicides, which is of benefit to both users and sellers.

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Chloride Determination in Foods with Ion-Selective Electrode After Isolation as Hydrogen Chloride

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This report describes a sample preparation method in which chloride is isolated as hydrogen chloride from food samples prior to analysis with the chloride ion-selective electrode. Chloride analyses of selected foods with this method agreed with chloride values reported in food composition tables. Chloride analysis with the present procedure also agreed with the certified value for the chloride content of the National Bureau of Standards (NBS) Standard Reference Material, Nonfat Milk Powder. Reliability of the chloride isolation procedure was evident by the complete recovery of chloride added to food samples and a narrow range of 95% confidence limits calculated for each set of analyses. The usefulness of the chloride ion-selective electrode to determine chloride in foods is greatly enhanced by this procedure because matrix interference by other sample components is removed prior to analysis.

Chloride in foods is routinely determined by titration of solubilized chloride with a standard solution of silver nitrate (1). The titration end point can be visualized either by using a color indicator or by using the chloride electrode as an indicator electrode. Neither of these methods is entirely satisfactory because of serious interference by other sample components, for example, the determination of chloride in meat and meat products (2). Direct determination of chloride with the chloride ion-selective electrode has also met with limited success because the electrode is subject to serious interference by other sample components, including fouling of the membrane surface with protein, and detection of other halides also present (3). Direct determination of chloride in milk, for example, required pretreatment of the sample to remove protein (4); otherwise, an erroneously high chloride determination was obtained. Although it has been reported that much of the electrode interference can be removed by pretreatment of the sample with an oxidative reagent (5), the ideal situation for the determination of chloride in foods would be to isolate chloride from interfering substances prior to its measurement. To accomplish this, methods of varying degree of complexity and expense have been suggested including ion-exchange chromatography, gas chromatography, and distillation methods (6).

In the present study, we describe the use of a simple, inexpensive microdiffusion procedure using Conway cells (7) to isolate chloride as hydrogen chloride from foods prior to analysis with the chloride ion-selective electrode.

Experimental

Reagents, Apparatus, Procedure

A detailed description of the reagents, apparatus, and procedure required for the determination of chloride with the ion-selective electrode following isolation as hydrogen chloride has already been published (8). In brief, the sample ring which surrounds the center well of a Conway plate contains the sample and sufficient water to make a final volume of 1 mL. The center well contains 0.2 mL 1.25M NaOH and 2 drops of absolute ethanol to help spread the NaOH solution over the well surface. Diffusion of hydrogen chloride begins by the addition of 3 mL cold (5°C) 60% H_2SO_4 to the sample compartment. A petri dish cover is immediately applied to each cell and the contents are swirled to mix. Diffusion is allowed to proceed for 22 h at 37°C. Then, Petri dish covers are removed and 2.3 mL water and 0.2 mL 1.25M HNO_3 are added to the center well. Center well contents are mixed and transferred to a 10 mL disposable beaker cup that contains 2.4 mL water and 0.1 mL 5M $NaNO_3$. Chloride is then determined with the chloride ion-selective electrode. Under

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Table 1. Chloride content of food samples[a]

Food	Cl^-, mg/100 g: Present method (95% conf. interval)	Lit. range (Ref.)
Milk, cow, lowfat (2%)	86–92	78–120 (11)
Milk, evaporated	202–214	200–220 (11)
Infant formula (Similac)	94–100	95–105[b]
Egg (whole)	171–177	170–180 (9, 10)
Ham (smoked)	2124–2195	1400–2100 (9)
Tomato juice, canned	482–497	380 (10)
Carrot juice, canned	32–34	29–53 (9)
Peas, drained solids, canned	298–309	267–410 (9)
Flour, whole wheat	55–57	—
Flour, white, all purpose	66–70	71 (10)
NBS SRM 1549 (Nonfat Milk Powder)	1080–1120	1070–1100[c]

[a] n = 5, except for NBS Reference Standard, where n = 12.
[b] Personal communication, Ross Laboratories, Columbus, OH.
[c] 95% confidence interval, National Bureau of Standards (NBS), Gaithersburg, MD.

the conditions described, a linear analysis will be obtained when the sample introduced into the sample ring contains 50–1000 μg chloride. In a 5 mL analysis volume, the lower end of the stated range corresponds to 10 ppm Cl^-, which is well above the electrode minimum detection limit of 1.8 ppm.

Sample Preparation

(a) For liquid samples such as milk and juices, no sample preparation is required. Pipet a sufficiently large sample into the sample ring of the Conway cell that will yield 200–500 μg chloride. Add redistilled water to the sample ring to give a final volume of 1.0 mL.

(b) For solid samples such as ham, vegetables, and egg, it is necessary first to solubilize chlorides in the food. Add 5.0 g of a representative sample to a blender. Add 45 mL 0.1N reagent grade HNO_3 and blend 30 s at low speed to extract chlorides. Centrifuge 10 min at 400 × g. For chloride analysis, remove an aliquot that will yield 200–500 μg chloride.

(c) For some solid food samples such as flour, it is possible to diffuse chloride directly from the sample, because the amount of chloride diffused will be in the desired 200–500 μg range.

Calculations

Diffuse standard amount of chloride (100, 200, 500 μg) with each set of samples. Calculate chloride content of the sample from the regression equation generated from the standards. Express final results as mg Cl^-/100 g, to compare results directly with available food composition references (9, 10), according to the following equation:

$$Cl^-, \text{mg}/100\text{ g} = (C \times V \times D/W) \times 100$$

where C = concentration (mg/L) of chloride in analysis volume corrected for percent recovery, V = sample volume (5 mL), D = dilution factor, and W = sample weight (g).

Results and Discussion

The original microdiffusion method on which the present paper was based was designed to determine the chloride content of urine and serum by using the chloride ion-selective electrode (8). To determine the possible application of this method to the determination of chloride in food, an effort was made to select representative food items from each of the major food groups where comparable chloride content of foods was available from food composition tables (9, 10) or literature references (11). In addition, the present method was also applied to chloride determination in a randomly selected infant formula reflecting recent concerns (12) as well as to the National Bureau of Standards Standard Reference Material 1549, Nonfat Milk Powder, which has been certified for chloride content.

Table 2. Recovery of chloride added to food samples[a]

Food	Chloride, μg: Added	Found	Recd	Rec., %
Milk, lowfat (2%)	0	178.8 ± 6.9	—	—
	200	377.6 ± 8.2	198.8	99.4
Milk, evaporated	0	208.2 ± 7.1	—	—
	200	413.1 ± 6.3	204.9	102.5
Peas, drained solids	0	151.7 ± 3.1	—	—
	200	347.6 ± 5.9	195.9	98.0
Egg, whole	0	139.0 ± 3.0	—	—
	200	344.5 ± 4.9	205.5	102.8

[a] Each value represents the mean ± SD of 5 determinations.

Results for the determination of chloride content in food by using the present method are shown in Table 1. Determinations have been expressed as 95% confidence intervals to facilitate comparisons with ranges of chloride content of foods reported in food composition tables. Expression of results in this way also facilitates comparison of the present method with other methods, because the certified value for chloride in the NBS standard is expressed as a 95% confidence interval. Methods used by the National Bureau of Standards to certify the chloride content of nonfat milk powder were ion-exchange chromatography and instrumental neutron activation analysis. Chloride determinations in foods with the present method were in close agreement with the available reference values as shown in Table 1. The narrow range of 95% confidence intervals attests to the fact that the present method for the determination of chloride in food is highly reproducible. Furthermore, the determination of chloride in the NBS standard with the present method was in excellent agreement with the certified value.

As shown in Table 2, the present method allows for excellent, reproducible recovery of chloride added to foods as sodium chloride. These results indicated little or no matrix interference on the diffusion of chloride as hydrogen chloride from these samples.

In summary, the sample preparation method used in this study to determine chloride content of foods greatly enhances the usefulness of the chloride ion-selective electrode because electrode interference by other sample components is removed. The procedure does not require ashing or deproteinization of food samples. The method requires inexpensive equipment and yet yields reliable, reproducible results equivalent to those obtained with much more sophisticated instrumentation.

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Gas Chromatographic Determination of Capsaicinoids in Green *Capsicum* Fruits

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A new gas chromatographic (GC) method is described that uses a simple on-column methylation for the quantitative determination of the major naturally occurring capsaicinoids. Capsaicinoids are extracted from the dry sample with acetone in a Soxhlet apparatus, purified by extraction with petroleum ether and water, mixed in a microsyringe with trimethylanilinium hydroxide solution, and injected onto the GC column. The methylated capsaicinoids thus formed require 16 min for elution. The gas chromatographic response is linear with increased concentration of capsaicinoids.

Capsaicin and its analogs, called capsaicinoids, are the pungent compounds of the *Capsicum* fruit. Five naturally occurring capsaicinoids have been reported (1): capsaicin (C), dihydrocapsaicin (DC), nordihydrocapsaicin (NDC), homocapsaicin (HC), and homodihydrocapsaicin (HDC). C, DC, and NDC are the major components of most *Capsicum* species, constituting about 95% or more of the total capsaicinoid content.

Gas chromatography (GC) has been widely applied for separation, identification, and quantitation of individual capsaicinoids of natural and synthetic origin in fresh peppers, oleoresins, spices, and medicinal preparations. When capsaicin and its analogs are analyzed by GC in the free form, strong adsorption of the compounds to most columns results in severe tailing of peaks and hampers quantitation. Therefore, derivatization is necessary for efficient GC separation of capsaicinoids. Two derivatization procedures for capsaicinoids have been reported: trimethylsilylation of capsaicinoids (2–7) and hydrolysis of capsaicinoids to yield fatty acids, and subsequent esterification (8, 9).

The GC method described here is based on a methylation reaction of the phenolic group of capsaicinoid with trimethylanilinium hydroxide (TMAH). This derivatization method yields symmetrical chromatographic peaks without evidence of adsorption on the column. TMAH has been proven to be a powerful methylating agent for GC quantitation and identification of high molecular weight compounds of biological interest such as barbiturates, phenolic alkaloids, xanthine bases, and others (10, 11). A simple on-column technique is used for methylation with TMAH (12). When it is mixed with an extract and heated in the injector port, TMAH reacts with the active hydroxyl, amino, or carboxyl group to yield methylated nonpolar derivatives which can be easily chromatographed.

The GC method proposed here has been successfully applied to quantitative determinations of capsaicinoids in natural products.

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METHOD

Apparatus

(a) *Gas chromatograph.*—Varian Model 3700 (Varian Associates, Palo Alto, CA 94303) equipped with flame ionization detector; column, 2.1 m × 2 mm id glass, packed with 5% SP2100 on 100–120 mesh Chromosorb WAW DMCS; column temperature program: initial temperature 200°C, initial time 0 min, program rate 4°/min, final temperature 245°C, final time 7 min; detector temperature, 250°C; injector temperature, 250°C; nitrogen flow rate, 30 mL/min; air flow rate, 300 mL/min; hydrogen flow rate, 30 mL/min; sensitivity, 8×10^{-10} AFS; sample size, 2 μL.

(b) *Freeze-drier.*—The VirTis Co., Gardiner, NY 12525.

(c) *Grinder.*—Wiley mill (Arthur H. Thomas Co., Philadelphia, PA 19105).

(d) *Rotavapor.*—Brinkman Instruments, Westbury, NY 11590.

Reagents

(a) *Natural capsaicin.*—Mixture of naturally occurring capsaicinoids (Pfaltz and Bauer, Inc., Waterbury, CT 06708).

(b) *Pure NDC, C, and DC standards.*—Obtained by low pressure liquid chromatographic separation of natural capsaicin (13).

(c) *Trimethylanilinium hydroxide (TMAH).*—0.1M solution in methanol (Fisher Scientific, Pittsburgh, PA 15219).

(d) *Internal standard (IS).*—Tetracosane (Fisher Scientific). Prepare IS solution by dissolving 250 mg tetracosane in 1000 mL tetrahydrofuran.

(e) *Sodium sulfate anhydrous.*—J. T. Baker, Phillipsburg, NJ 08865.

(f) *Solvents.*—Tetrahydrofuran, methanol, acetone, diethyl ether anhydrous, petroleum ether (50–110°C) (all "Baker Analyzed" Reagents).

Standard Curve

Prepare stock solution of capsaicinoids containing 0.4, 1.4, and 0.8 mg/mL of NDC, C, and DC, respectively in IS solution. Transfer aliquots of stock solution to 5 mL volumetric flasks and dilute to volume with IS solution to obtain a series of standard mixtures in the range 0.4–0.1, 1.4–0.35, and 0.8–0.2 mg/mL for NDC, C, and DC, respectively. Draw 1 μL TMAH solution and 2 μL standard solution into microsyringe and inject into gas chromatograph. (Figure 1 illustrates chromatogram of standard mixture of capsaicinoids with IS.) Measure peak heights of capsaicinoids and IS. Calculate ratios of each peak to IS and plot, separately for each compound, obtained values vs concentration.

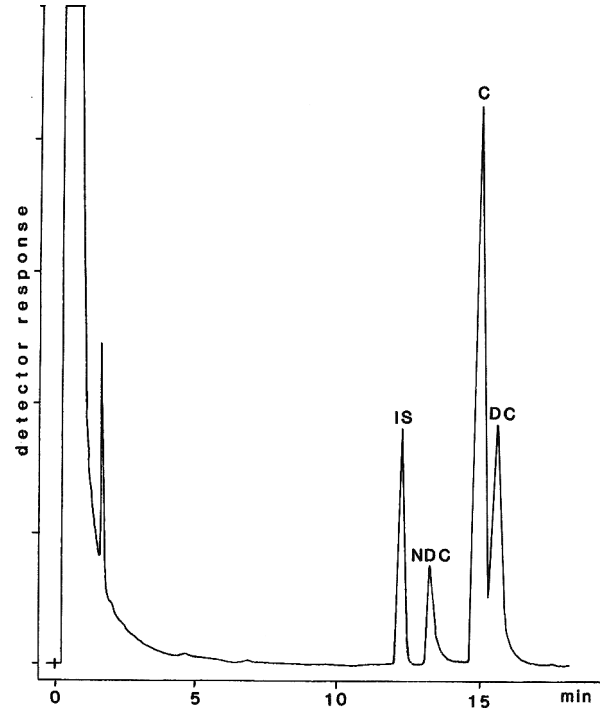

Figure 1. Gas chromatogram of methylated standard mixture of capsaicinoids; IS = internal standard (tetracosane); NDC = nordihydrocapsaicin; C = capsaicin; DC = dihydrocapsaicin; 2 μL injected representing 0.6, 2.1, and 1.2 μg of NDC, C, and DC, respectively.

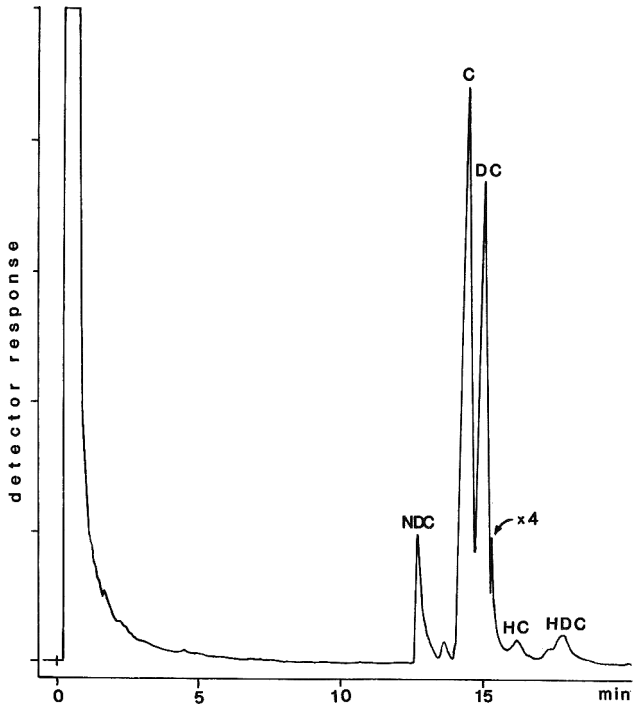

Figure 2. Gas chromatogram of methylated natural capsaicin; codes same as in Figure 1 plus HC = homocapsaicin, HDC = homodihydrocapsaicin; sensitivity changed to 4 × 10^{-10} AFS after 15.4 min; 1 μL injected representing 5.85 μg natural capsaicin.

Sample Preparation

Cut fresh peppers into 1 cm strips. Discard stems. Freeze in blast freezer and dry in freeze-drier to constant weight (ca 48 h). Determine water content of fresh peppers. Grind dry peppers in Wiley mill. Extract 3 g powdered sample in Soxhlet apparatus 3 h with 150 mL acetone. Evaporate acetone in Rotavapor. Dissolve residue in ca 50 mL methanol and ca 50 mL petroleum ether (add several drops of water to avoid mixing of petroleum ether in methanol). Transfer to 250 mL separatory funnel. Shake and let layers separate. Discard petroleum ether layer. Evaporate methanol in Rotavapor. Dissolve residue in diethyl ether and water. Transfer to 250 mL separatory funnel. Swirl gently to avoid emulsion formation. Let layers separate. Wash water layer with 2 more portions of diethyl ether. Dry combined ether fractions with anhydrous Na_2SO_4, filter and evaporate to dryness in Rotavapor. Dissolve residue in 5 mL IS solution. Draw 1 μL TMAH solution and 2 μL extract into a microsyringe and inject into gas chromatograph.

Results and Discussion

GC separation of methylated capsaicinoids was very good using the chromatographic conditions described (Figure 2). Peaks are well resolved and therefore easy to measure. As can be seen in Figure 2, HC and HDC can be detected in mixture with other capsaicinoids only if sensitivity of the instrument is increased. Because of the very low content of HC and HDC in peppers, their determination was not included in this project.

On-column methylation with TMAH was 100% effective, without premixing reagent and extract. No difference in peak response was observed when TMAH was added to the extract at 0, 5, 10, and 30 min prior to injection. Therefore, the method of mixing TMAH solution with the extract in the syringe was used throughout this experiment. Amount of TMAH used was 5- to 10-fold in excess and peak response was the same in this range.

The purification procedure yielded a light green extract and effectively removed interfering substances. Figure 3 illustrates a typical chromatogram of a green pepper extract. The petroleum ether extraction eliminated most of the pigments and lipids and was essential for extended column life and performance. The diethyl ether and water partition removed the substances interfering with capsaicinoid peaks.

By this procedure, bell peppers contained no capsaicinoids and therefore bell pepper samples were spiked and used for recovery studies. The results (Table 1) indicate a satisfactory level of recovery and good reproducibility. The minimum

Table 1. Recovery of capsaicinoids from spiked bell pepper samples

Capsaicinoid	Added, mg	Recovery,[a] % Mean ± SD	CV, %
NDC	0.82	102.0 ± 5.8	5.7
C	5.20	87.5 ± 4.7	5.4
DC	2.66	80.5 ± 4.3	5.3

[a] Average of 6 determinations.

Table 2. Determination of capsaicinoid content in green *Capsicum* fruits

Capsaicinoid	Jalapeno peppers Content[a] ± SD	CV, %	Finger hot peppers Content[a] ± SD	CV, %
NDC	4.80 ± 0.17	3.5	7.49 ± 0.27	3.6
C	18.70 ± 1.20	6.4	19.30 ± 1.34	6.9
DC	16.83 ± 1.03	6.1	18.18 ± 0.97	5.3

[a] mg/100 g fresh pepper; average of 6 determinations.

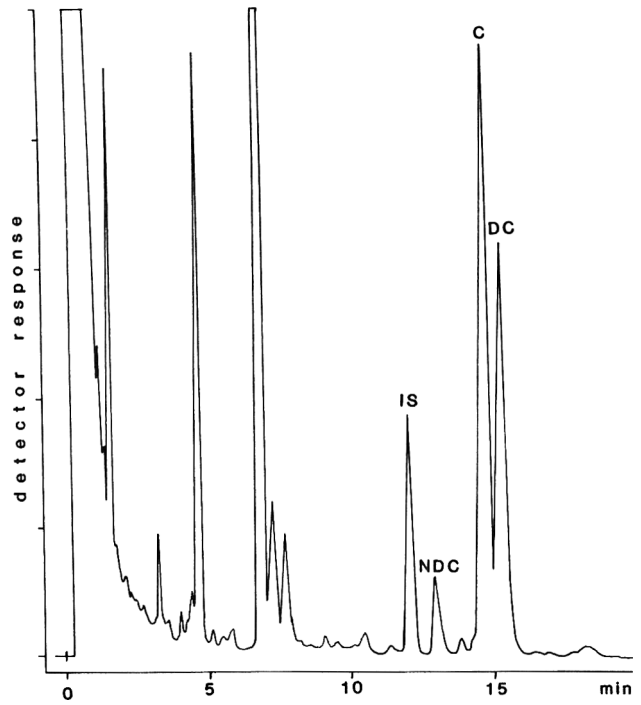

Figure 3. Gas chromatogram of methylated jalapeno pepper extract; codes same as in Figures 1 and 2.

detectable levels by this method are 12.0, 12.5, and 14.0 ng for NDC, C, and DC, respectively.

Relationships between the ratio of peak heights of individual capsaicinoids to internal standard were linear with the concentration of capsaicinoids (r = 0.99) in the ranges 0.4–0.1, 1.4–0.35, and 0.8–0.2 mg/mL for NDC, C, and DC, respectively. The relative concentrations of each capsaicinoid in the standard mixtures were such that they reflected the relative content of capsaicinoids in *Capsicum* fruits.

The method was successfully used to determine capsaicinoid levels in 2 kinds of hot green pepper available on the local market, i.e., jalapeno pepper and finger hot pepper (see Table 2). The results indicate that the described GC method offers a useful alternative for determinations of capsaicinoids in *Capsicum* fruits.

This method can also be applied to detect adulterants such as vanillylamide of *n*-nonanoic acid, which, under the chromatographic conditions described here, has a peak with a retention time of 13.7 min and is clearly resolved from NDC and C peaks.

Other chromatographic methods such as liquid chromatography (LC) (14–16) and high performance thin layer chromatography (HPTLC) (17) have been successfully used for determination of individual capsaicinoids. However, the method proposed here is less costly than LC which requires large amounts of high purity solvents and HPTLC which requires expensive, nonreusable plates. Also, many more laboratories have gas chromatographs than have LC equipment. As compared with other gas chromatographic procedures, the main advantage of the method described here is the ease of the derivation reaction. No premixing of the reagents, heating, or waiting before injection is required. TMAH solution and an extract are simply drawn into the syringe and injected. Some other methods—sensory, colorimetric, spectrophotometric, or thin layer chromatographic procedures—are not effective for the determination of individual compounds and can be used only for the analysis of total capsaicinoids.

Although sample preparation described here is somewhat time consuming, it results in extracts suitable for chromatography. If time is limited, sample preparation may be shortened by omitting the petroleum ether purification of the extracts. This will have little effect on the appearance of the final chromatogram because none of the substances removed by petroleum ether overlap with capsaicinoid peaks. However, neglecting to perform this purification will cause contamination of the GC column, and therefore loss of resolution, due to the deposition of fats and lipids present in the extracts.

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Solvent Effects on Response Factors for Polynuclear Aromatic Hydrocarbons Determined by Capillary Gas Chromatography Using Splitless Injections

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When standard solutions of polynuclear aromatic hydrocarbons (PAHs) were analyzed by capillary column gas chromatography using splitless injections, response factors were observed to be dependent on the solvent used to prepare the standard. This report presents the response factors for 16 individual PAHs in 5 commonly used solvents: acetonitrile, methanol, toluene, isooctane, and cyclohexane. To minimize quantitation errors due to differences in transfer efficiency, samples and standards of PAHs should be prepared in the same solvent.

Due to the ubiquitous nature and the persistence, carcinogenicity, and mutagenicity of many polynuclear aromatic hydrocarbons (PAHs), such compounds are frequently monitored in water, sediment, biota, and other matrixes. A variety of analytical methodologies such as gas chromatography (GC) (1), gas chromatography/mass spectrometry (GC/MS) (2), and liquid chromatography (LC) (3) have so far been developed for the determination of PAHs. Because of different requirements in the detection system, standard solutions are frequently prepared in different solvents. For example, for reverse phase LC analysis, PAH standard solutions are often prepared in polar solvents such as acetonitrile and methanol. On the other hand, for GC or GC/MS analysis of PAHs, standards are usually prepared in aliphatic or aromatic hydrocarbons or other nonpolar solvents. Different cleanup procedures can also produce sample extracts in solvents different from the calibration standards.

Recently, we observed unexpected results when our in-house PAH standard solution prepared in toluene was calibrated against a PAH reference sample (NBS SRM 1647) prepared in acetonitrile. Although solvent effects on peak shape (4) and response factors (5) in capillary gas chromatography have been reported, very few quantitative results on the response factors caused by solvents of different polarity appeared in the literature. In this paper, we report the solvent effects on the retention times and response factors for the 16 U.S. Environmental Protection Agency PAH priority pollutants (EPA Method 612) determined by splitless injections onto a fused silica capillary column.

Experimental

Gas Chromatography

We used a Hewlett-Packard 5880A gas chromatograph equipped with split/splitless capillary injection port, flame ionization detector, 7671A autosampler, and level IV terminals. For all PAH analyses, we used a 30 m × 0.25 mm id DB-5 fused silica capillary column of 0.25 μm film thickness available from J & W Scientific Co. Operating temperatures: injection port and detector, 275°C; oven, initial 70°C, hold 0.75 min at 70°C, programming rate 1, 30°/min (from 70 to 140°C), programming rate 2, 3°/min (from 140 to 260°C), hold 15 min at 260°C. Flow rates: hydrogen, 35 mL/min; air, 240 mL/min; detector makeup gas (helium), 25 mL/min. Carrier gas (helium) head pressure, 15 psi. Linear velocity, 23.3 cm/s at 260°C. Splitless valve on for 0.75 min. Approximately 3 μL of each sample was injected to the column, using the autosampler. An electronic integrator was used to measure peak areas for the subsequent calculation of response factors.

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PAH Standard Solutions

Stock solutions of individual PAHs were prepared with concentrations between 500 and 1000 μg/mL toluene. Also a concentrated mixture of the 16 PAHs in toluene was prepared by combining 1.0 mL of each stock solution. Each of five 500 μL aliquots of this mixture was diluted to 10.0 mL with the following 5 solvents: *(1)* toluene, *(2)* acetonitrile, *(3)* methanol, *(4)* isooctane, and *(5)* cyclohexane. *Caution*: Extreme care should be exercised in handling concentrated PAH stock solutions because some of them are known carcinogens.

Results and Discussion

In our laboratory, all sediment extracts and standard solutions for PAH analysis are prepared in toluene. This solvent was chosen because it is suitable for flame ionization detection and mass selective detection without further solvent exchange. Toluene is also the solvent used in the final column cleanup step for PAH analysis, and its lower volatility reduces evaporation of solvent in sample extracts and standard solutions during storage. Although PAH samples in toluene are not suitable for direct injection into a reverse phase LC system, toluene is miscible with methanol or acetonitrile in ratios greater than 1:10. Thus, after dilution with the LC solvent, the sample can be analyzed without solvent replacement which usually causes losses of the more volatile PAHs. In this work, standard solutions of PAHs were also prepared in acetonitrile, methanol, isooctane, and cyclohexane for the following comparison studies. These solvents were chosen because they were frequently used in PAH analysis.

Table 1 summarizes the average retention times (based on 7 replicate injections) of 16 PAHs in the 5 solvents. The injections were made by an autosampler onto a 30 m capillary column in the splitless mode, and the chromatographic data were recorded by an electronic integrator. For the same solvent, the between-run variation in retention times for each PAH seldom exceeded 0.01 min. For the less volatile PAHs,

Table 1. Retention times (min) of PAHs in 5 different solvents (see *Experimental* for GC conditions)

PAH	Acetonitrile	Methanol	Toluene	Isooctane	Cyclohexane
Naphthalene	4.21	4.34	4.21	4.21	4.20
Acenaphthylene	6.55	6.47	6.52	6.56	6.52
Acenaphthene	6.97	6.89	6.94	6.98	6.95
Florene	8.37	8.28	8.34	8.37	8.35
Phenanthrene	12.11	12.04	12.08	12.11	12.09
Anthracene	12.32	12.26	12.29	12.33	12.30
Fluoranthene	18.97	18.96	18.96	18.98	18.97
Pyrene	20.32	20.30	20.30	20.32	20.31
Benzo[a]anthracene	29.27	29.28	29.25	29.28	29.27
Chrysene	29.55	29.55	29.52	29.56	29.54
Benzo[b]fluoranthene	37.06	37.07	37.05	37.07	37.06
Benzo[k]fluoranthene	37.25	37.25	37.23	37.24	37.24
Benzo[a]pyrene	39.07	39.09	39.06	39.08	39.08
Indeno[123-cd]pyrene	46.40	46.40	46.38	46.40	46.39
Dibenz[ah]anthracene	46.94	46.94	46.92	46.93	46.94
Benzo[ghi]perylene	48.13	48.14	48.11	48.12	48.14

Table 2. Response factors of PAHs in acetonitrile, methanol, isooctane, and cyclohexane relative to those in toluene

PAH	Acetonitrile	Methanol	Toluene	Isooctane	Cyclohexane
Naphthalene	0.26 (8.7)−[a]	0.39 (5.2)−	1.00 (4.3)	0.95 (3.7)+	0.86 (2.8)−
Acenaphthylene	0.43 (9.6)−	0.45 (5.6)−	1.00 (4.0)	1.03 (3.6)+	0.93 (2.7)+
Acenaphthene	0.40 (9.4)−	0.45 (5.3)−	1.00 (3.9)	1.04 (3.6)+	0.93 (2.6)+
Florene	0.45 (6.4)−	0.47 (6.6)−	1.00 (4.2)	1.04 (3.6)+	0.95 (2.6)+
Phenanthrene	0.51 (5.7)−	0.52 (8.7)−	1.00 (5.0)	1.04 (4.8)+	0.95 (3.0)+
Anthracene	0.51 (6.0)−	0.51 (9.3)−	1.00 (5.4)	1.04 (5.0)+	0.95 (3.2)+
Fluoranthene	0.55 (7.0)−	0.57 (10.0)−	1.00 (6.9)	1.03 (6.5)+	0.88 (4.8)+
Pyrene	0.55 (7.6)−	0.62 (8.8)−	1.00 (5.7)	1.00 (7.8)+	0.85 (4.7)−
Benzo[a]anthracene	0.59 (5.5)−	0.70 (7.4)−	1.00 (9.0)	1.01 (6.0)+	0.75 (5.8)−
Chrysene	0.61 (5.5)−	0.72 (7.0)−	1.00 (6.6)	1.04 (6.3)+	0.77 (5.8)−
Benzo[b]fluoranthene	0.68 (3.6)−	0.80 (6.3)−	1.00 (7.7)	1.20 (5.9)−	0.79 (5.2)−
Benzo[k]fluoranthene	0.67 (3.8)−	0.78 (7.0)−	1.00 (6.2)	1.18 (6.0)−	0.77 (3.8)−
Benzo[a]pyrene	0.71 (3.6)−	0.79 (5.5)−	1.00 (6.6)	1.25 (5.1)−	0.80 (5.6)−
Indeno[123-cd]pyrene	0.94 (6.0)+	0.80 (6.7)−	1.00 (11.0)	1.49 (7.7)−	0.87 (4.9)−
Dibenz[ah]anthracene	0.97 (4.6)+	0.84 (7.0)−	1.00 (8.9)	1.56 (7.9)−	0.91 (8.5)+
Benzo[ghi]perylene	0.95 (6.1)+	0.83 (5.1)−	1.00 (11.1)	1.48 (8.4)−	0.88 (7.9)+

[a] + and − signs denote the absence or presence, respectively, of significant difference in response factors (toluene vs other solvents) concluded by the *t*-test at 5% significance level. Precision of injections in % RSD is given in parentheses.

i.e., fluoranthene and lower in Table 1, the retention times varied only slightly between different solvents. The maximum between-solvent differences in retention times were all between 0.02 and 0.04 min for the same PAH. However, larger between-solvent differences in retention times were observed for the more volatile PAHs. For example, the retention times for naphthalene in methanol and cyclohexane were 4.34 and 4.20 min, respectively. Therefore, in cases where standard solution and sample extract were prepared in solvents that produced a large difference in retention time, misidentification of peaks could occur.

Table 2 lists the mean response factors for each PAH in different solvents relative to toluene. In all cases, the response factors were obtained by averaging the results of 6 or 7 injections. To compensate for any long-term drifting in detector sensitivity, samples in different solvents were analyzed in sets of fives. Huge differences in relative response factors were observed for many PAHs between toluene and the polar solvents, i.e., acetonitrile and methanol. In the most extreme case, the detector responses of naphthalene as determined in acetonitrile and methanol were only 26 and 39%, respectively, of that determined in toluene. For the other PAHs, detector responses were also lower in methanol and acetonitrile, and they were mostly between 40 and 80% of those determined in toluene. At 5% level of significance, the Student's *t*-test indicated that response factors of all 16 PAHs determined in methanol were significantly different from those obtained in toluene. Except for indeno[123-cd]pyrene, dibenz[ah]anthracene, and benzo[ghi]perylene, response factors for PAHs determined in acetonitrile were also concluded as significantly different by the same test.

With the exception of naphthalene, PAHs had higher detector responses in isooctane than toluene. On the other hand, detector responses for PAHs in cyclohexane were 5–25% lower than the corresponding values in toluene. A *t*-test indicated that response factors for benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[123-cd]pyrene, dibenz[ah]anthracene, and benzo[ghi]perylene were significantly different for toluene and isooctane. For cyclohexane and toluene, significant differences in response factors were found for naphthalene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, and indeno[123-cd]pyrene.

Comparisons of response factors for PAHs were also made between solvents of similar polarity, i.e., acetonitrile vs methanol, as well as isooctane vs cyclohexane. Between the 2 polar solvents, *t*-tests indicated significant difference ($P < 0.05$) in response factors for all PAHs except acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene. Similar tests also suggested that response factors for all PAHs determined in isooctane were significantly different from those determined in cyclohexane.

The results in Table 2 clearly indicate that, among the 5 solvents tested, isooctane was the most efficient in transferring PAHs from the injection port to the column when splitless injections were made. On the other hand, acetonitrile and methanol were much less efficient in transferring PAHs. Therefore, when PAHs are analyzed by splitless injections onto a capillary column, the solvent used to make up the samples must be identical to that in the calibration standard. Otherwise, difference in transfer efficiency of different solvents may cause significant (25 to over 100%) errors in the quantitation of PAHs. Because the reference standard solution for PAHs from NBS (SRM 1647) is prepared in acetonitrile, and the quality assurance samples from EPA are mostly prepared in methanol, direct application of these materials using splitless injection for quantitative purposes must be avoided unless the samples are also in the same solvent as the standard.

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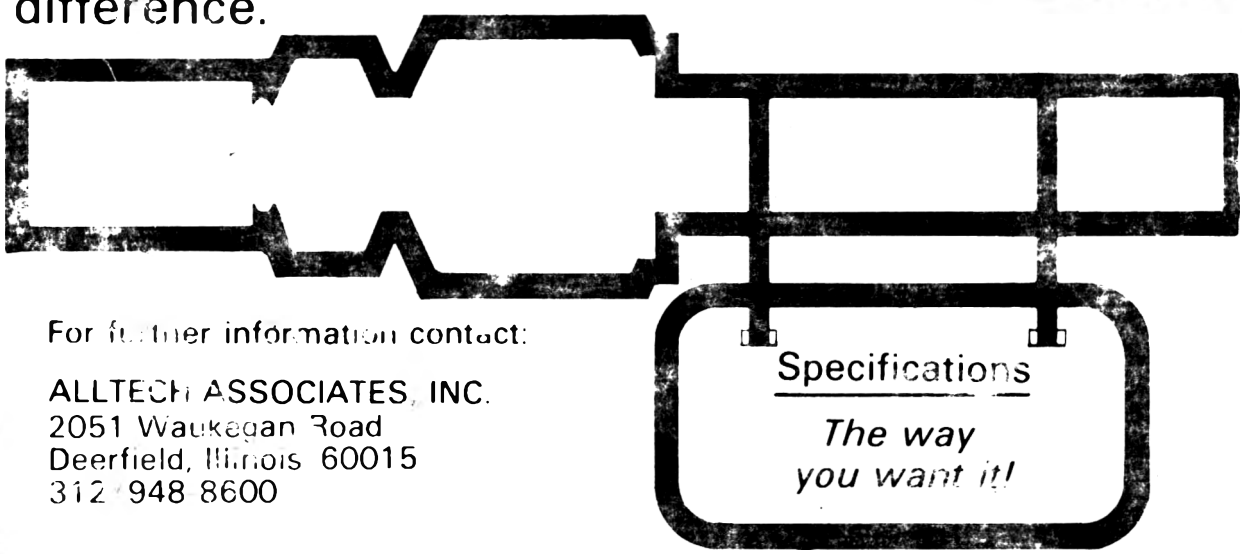

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