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ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

JOURNAL

NOVEMBER/DECEMBER 1989
VOL. 72, NO. 6

JANCA 72(6) 873–1038 (1989)
ISSN 0004-5756

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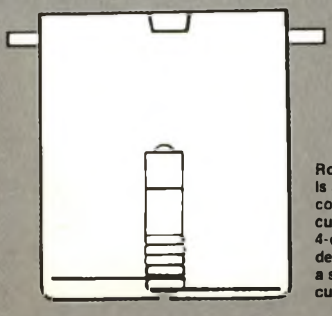

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AOAC Analytical Technology Week
April 2-6, 1990
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Valley Forge, Pennsylvania

You can sign up for one or more of the following program items.

TOPICAL CONFERENCE ON FOOD IRRADIATION

April 2-4* - *Chairman: Leon Lakritz, U.S. Department of Agriculture*

- Basic Irradiation Chemistry
- The Effects of Ionizing Radiation on Foods
- The Effects of Irradiation on the Microflora of Foods
- Analytical Methods Applicable to the Detection of Irradiated Foods
- Regulatory Roundtable

WORKSHOP ON IMMUNOASSAYS

April 4-5 - *Chairman: Jim Pestka, Michigan State University*

- Fundamentals of Immunoassay
- Immunoassay of Foodborne Pathogens
- Immunoassay of Residues in Foods
- Tutorials, Workshops

FOUR TWO-DAY SHORTCOURSES

April 2-3 - Quality Assurance for Analytical Laboratories — *Updated*
April 3-4 - Statistics for Methodology — ***New***!
April 3-5* - Improving Sampling for Analysis of Foods, Drugs, and Agricultural Materials — *Updated*
April 5-6 - Quality Assurance for Microbiology Laboratories

*Starts at 1:00 PM on beginning date and concludes at noon on ending date.

To receive additional information on **Analytical Technology Week** as it becomes available, fill out and mail this coupon to AOAC, Suite 400, 2200 Wilson Blvd., Arlington, VA 22201-3301 USA; or FAX to (703) 522-5468.

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I am especially interested in:

☐ The Food Irradiation Conference
☐ The Immunoassays Workshop
☐ The Shortcourse(s) on ☐ Statistics, ☐ Sampling, ☐ QA for Analytical Labs, ☐ QA for Microbiological Labs.

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

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

NOVEMBER/DECEMBER 1989
VOL. 72, NO. 6
JANCA 72(6)873–1038 (1989

 The *Journal of the Association of Official Analytical Chemists* (ISSN 0004-5756) is published bimonthly by the Association of Official Analytical Chemists, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. **Subscriptions** for Vol. 72 (1989) are: Members, $84.50 in U.S., $94.50 outside U.S.; Nonmembers, $125.00 in U.S., $145.00 outside U.S. Second class postage paid at Arlington, VA, and additional mailing offices. Abstracted or indexed in AGRICOLA, Analytical Abstracts, Biological Abstracts, Cambridge Scientific Abstracts, Chemical Abstracts, Current Advances in Ecological Sciences, Current Contents, and others. Printed in USA.
POSTMASTER: Send address changes to AOAC, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA.

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

Quality Assurance Principles for Analytical Laboratories

by Frederick M. Garfield

former Assistant Administrator
U.S. Drug Enforcement Administration

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

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

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

CONTENTS:

Chapters—

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

Appendices—

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

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

Price—Members: $43.25 in U.S., $48.25 outside U.S.
Nonmembers: $47.50 in U.S., $52.50 outside U.S.
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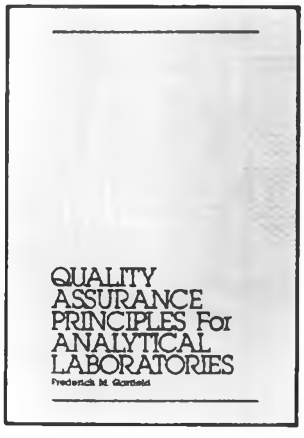

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Chromatography Data System Using HP Integrators

Chrom Perfect software turns the HP 3396 or HP 3393 integrators into complete PC-based chromatography data systems. The software acquires data simultaneously from up to two integrators while leaving the integrator free for instrument control and the PC free for running other programs such as Lotus, Rbase, etc. The software plots chromatographs on-screen at over 1000 points/s with baselines drawn and peaks labeled. Justice Innovations, Inc.

Circle No. 372 on reader service card.

Biocompatible LC Autosampler

The Alcott/Micromeritics biocompatible LC autosampler is a modified version of the former standard model. Designated as the 728B, it contains biocompatible materials in key sample transfer areas. The needle block is fashioned from titanium, connecting tubing is of PEEK, and the user can select an injection valve from a number of different biocompatible materials, such as titanium, PEEK, or Hastelloy C. Alcott Chromatography.

Circle No. 373 on reader service card.

Ductless Fume Enclosure

The Mystaire Model FE-500 ductless fume enclosure is designed for use in industrial, clinical, academic, and biomedical laboratories. The FE-500 eliminates hazardous fumes, gases, and noxious odors by means of adsorption technology. The multistage filtration system removes contaminants by adsorption onto activated carbon or specially manufactured chemisorptive filters. Applications include organic solvents, acids, formaldehyde, and many other commonly used chemicals. Heat Systems.

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Calcium Ion-Selective Electrode

The Calcium ISE offers rapid, low-cost analysis with little sample preparation. It provides easy, accurate measurement of calcium in virtually all aqueous sample types, including colored and turbid samples. The calcium electrode features excellent linearity and a wide measurement range (0.04-40,000 mg/L). It employs an extremely low-solubility solvent for improved electrode life and performance. A replaceable calcium membrane sensor module and refillable internal filling solution allow for convenient, economical operation. Hach Company.

Circle No. 375 on reader service card.

Scrubber Apparatus To Remove Fumes During Digestions

The FumeScrubber safely removes acid fumes during Kjeldahl nitrogen and trace metal digestions. The FumeScrubber works in conjunction with the fume removal system of Labconco's rapid digestor. A two-step pre-separator neutralizes and condenses up to 97% of the vapors while the remaining fumes are drawn off by a centrifugal washer. To accommodate individual requirements, the vacuum applied to the fume removal system is adjustable. Labconco Corporation.

Circle No. 376 on reader service card.

Basic LS Systemic Simplifies Decision-Making

The LS 6000SE liquid scintillation counter incorporates easy-to-use, built-in help screens, preset counting regions, and quench curve set up parameters that help reduce the number of decisions to be made while using the LS system with either an integral liquid crystal display or an optional, external cathode ray tube. The system generally can be used for radioimmunoassay studies. Beckman Instruments, Inc.

Circle No. 377 on reader service card.

Combination Package Leak Detector and Seal Strength Test System

The 1520 S package test system gives an accurate and totally reproducible measurement of package integrity and seal strength. This fully automatic test system identifies within approximately 30 s the stress areas and weaknesses of the package. A pre-programmed hand-held calculator computes leakage area and theoretical hole size based on pressure fall back. Mocon Modern Controls, Inc.

Circle No. 378 on reader service card.

Quick IR Software Helps Provide Fast Analysis of IR Data

A powerful program for timesaving analysis of infrared data, Quick IR gives the infrared spectroscopist or

microscopist a complete set of data manipulation and plotting functions including quantitative analysis, library search, Kramers-Kronig transformation, and Fourier self-deconvolution. Now available are special optional spectral libraries: "Polymers," "Environmental Toxins," and "Forensics." Spectra-Tech Inc.
Circle No. 379 on reader service card.

High-Productivity Manifold

With a precise, highly controlled flow rate, a see-through vacuum manifold handles simultaneous extraction of up to 12 solid phase extraction cartridges while also accommodating a wide variety of cartridge sizes and configurations from any manufacturer. A two-position lid design increases the versatility of the manifold. One position allows for discarding undesired sample eluates while the second position may be used for collection of eluates. J & W Scientific.
Circle No. 380 on reader service card.

Affinichrom Immunoaffinity Chromatography Columns

A new line of immunoaffinity chromatography columns, called Affinichrom, maximize antibody utilization through proper orientation on the column packing. The columns are packed with protein-coated, non-porous glass beads. Four types of beads are available; avidin-, streptavidin-, protein A-, and Con A-coated beads. Kontes Biotechnology.
Circle No. 381 on reader service card.

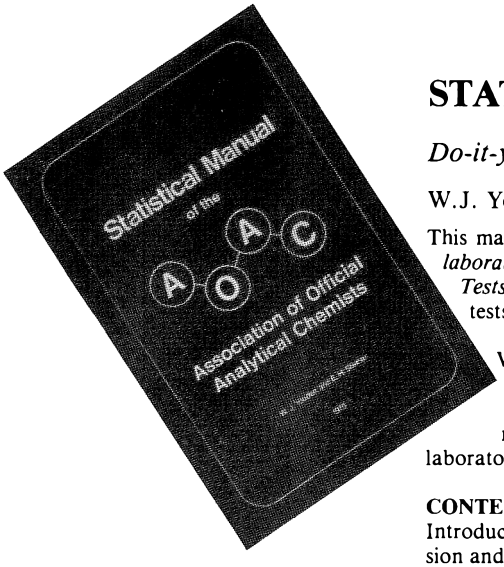
High-Resolution Oscilloscope for Millimeter OTDR Applications

The OOS-1 optical oscilloscope for optical time domain reflectometry applications is used in combination with the Hamamatsu's PLP-01 light pulser to deliver finer resolutions via a more flexible system than alternative methods. The OOS-1 is the only oscilloscope that accepts direct optical input enabling users to measure pulses without ringing, cable mismatches, or instrument distortion. This particular approach to OTDR offers extra flexibility because the oscilloscope can be used for other light pulse measurements as well. Hamamatsu Photonic Systems Corporation.
Circle No. 382 on reader service card.

Ozone in Air Monitor

The TG-800-KA portable battery powered ozone detector continuously monitors levels below 0.1 ppm. Featuring an AC adaptor and recorder output, it is possible to monitor for extended periods with recorded results. On battery power, the detector, weighing less than a pound, can operate for 30 h. Using a dedicated electrochemical sensor, unaffected by normal interfering gases, the sensor responds in seconds with digital readout in ppm. It has audio and visual alarms that are user adjustable. CEA Instruments, Inc.
Circle No. 383 on reader service card.

INDISPENSABLE STATISTICAL REFERENCE BOOKS for EVERY ANALYTICAL SCIENTIST



STATISTICAL MANUAL OF THE AOAC

Do-it-yourself statistical techniques for interlaboratory collaborative tests

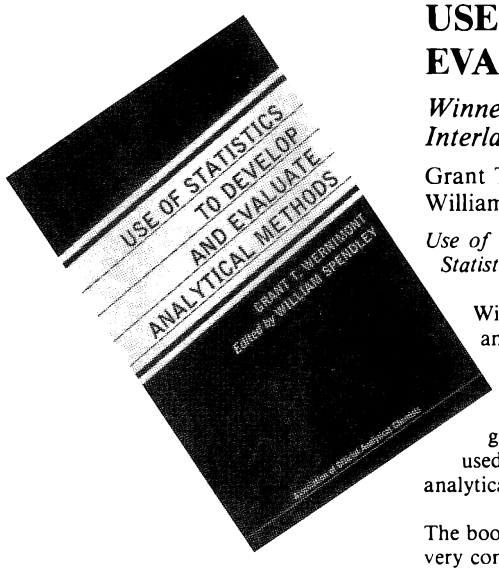
W.J. Youden and E.H. Steiner

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

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

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

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



USE OF STATISTICS TO DEVELOP AND EVALUATE ANALYTICAL METHODS

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

Grant T. Wernimont, Author
William Spendley, Editor

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

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

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

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

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

1985. 2nd printing, 1987. xvi + 183 pages. 11 figures. 54 tables. Index. Glossary. Softbound. ISBN 0-935584-31-5.
Price—Members: $49.55 in U.S., $54.55 outside U.S. Nonmembers: $54.50 in U.S., $59.50 outside U.S.
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Principles and Practice of Chromatography. By B. Ravindranath. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1989. 502 pp. Price: $79.95. ISBN 0-470-21328-0.

This book is an overview of the whole range of chromatographic techniques, incorporating theories and principles, methods and instrumentation, practice and applications, ranging from the oldest known method (paper chromatography) to the most modern (supercritical fluid chromatography-mass spectrometry). Emphasizing modern methods, the book covers all the latest developments, bringing together all the related aspects to facilitate an understanding of the principles and mechanisms of separation. Unlike many existing texts, it places importance on the chemical principles governing the separation mechanism and the choice of method. A bibliography of recent and thorough reviews provides sources of additional relevant information.

Symmetry in Molecules and Crystals. By M. F. C. Ladd. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1989. 274 pp. Price: $51.95. ISBN 0-470-21376-0.

This book is a presentation of molecular and crystal symmetry, from a geometrical point of view, based on courses in crystallography, chemical physics, and chemistry given over the past 30 years. Copiously illustrated, with numerous stereoviews of molecules and lattices, it does not require mathematics much beyond A-level (advanced high school) standard. This text will clarify a subject with which students often find some difficulty. The author uses both the Hermann-Mauguin and the Schoenflies symmetry notations in his treatment of crystal and molecular symmetry and elementary group theory. He also discusses applications of interests in crystallographic, spectroscopic, and theoretical studies.

The Sprouse Collection of Infrared Spectra, Book IV: Common Solvents—Condensed Phase, Vapor Phase, and Mass Spectra. Edited by Diana L. Hansen. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam. The Netherlands, 1988. 776 pp. Price: U.S. $240.00/Dfl. 520.00. ISBN 0-942595-04-1.

The Sprouse Collection is a unique series of reference spectra collections produced by Sprouse Scientific Systems of Paoli, PA. Its objective is to provide reliable, high quality reference spectra that can be used for structural elucidation, qualitative analysis, quantitative analytical methods development, computerized library searching, and software and algorithm development. Each volume in the series will be devoted to a special class of compounds. Book I is devoted to polymers, Book II to solvents by cylindrical internal reflectance, and Book III to surface active agents.

Agricultural Chemicals Book Series, Agricultural Chemicals Book I—Insecticides, Acaricides, and Ovicides; Agricultural Chemicals Book II—Herbicides; Agricultural Chemicals Book III—Fumigants, Growth Regulators, Repellants, and Rodenticides. By W. T. Thomson. Published by Thomson Publications, PO Box 9335, Fresno, CA 93791, 1989. 250; 260; 190 pp. Price: $16.50 each.

Newly revised, these books describe in detail each major category of pesticides. The different chemicals are listed separately by trade names, chemical structure, chemical formula, toxicity, formulations available, registered usage, application directions, pests controlled, precautions, and other pertinent information. Many new experimental materials are listed to prolong the effectiveness of these books. Used as major reference books by many people for over 20 years, anyone in the agricultural business from researcher to chemical fieldman should not be without these handy guides. Written in layman terms, they are readily adapted to field usage. Pesticides developed outside the United States are included to make this series useful on a worldwide basis.

Nomenclature of Inorganic Chemistry. Edited by G. J. Leigh. Published by Blackwell Scientific Publications Ltd, Osney Mead, Oxford OX2 OEL, U.K., 1989. 304 pp. Price: £39.50/$70.00. ISBN 0-632-02319-8.

The International Union of Pure and Applied Chemistry has the responsibility to propose rules for nomenclature to the chemistry community. In 1978, the IUPAC Commission for Nomenclature in Inorganic Chemistry decided to revise thoroughly the 1970 edition of Nomenclature in Inorganic Chemistry ("The Red Book"). Due to the fact that many of the new fields in chemistry are very highly specialized and need complex types of names, this edition will be published in two parts. This present book, representing general inorganic chemistry is Part 1, and in it can be found rules to name compounds ranging from the simplest molecules to oxoacids and their derivatives, coordinate compounds, and simple boron compounds.

Chemical Structure Information Systems, Interfaces, Communication, and Standards, ACS Symposium Series No. 400. Edited by Wendy A. Warr. Published by the American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1989. 146 pp. Price: U.S. & Canada $34.95/Export $41.95. ISBN 0-8412-1664-9.

With the advent of computer graphics, the proliferation of PCs, and the development of user-friendly software, the average chemist can now build chemical databases, search structures and substructures, and generate chemical reports. However, these many systems and databases cannot be linked in a "seamless" manner. This book addresses this concern in 12 chapters written by people from diverse backgrounds, from software developers to information specialists. Some of the specific topics covered include: the need for flexibility in field formats to enable free exchange of chemical data, the interrelated forces that restrict compatability, the attributes of a standard interface, chemical structure browsing, the use of a host language interface PC-to-mainframe communication programs, and the standard molecular data format as an integration tool.

Enzyme Assays for Food Scientists. By Clyade E. Stauffer. Published by Van Nostrand Reinhold/AVI, 115 5th Ave, New York, NY 10003, 1989. 320 pp. Price: $62.95. ISBN 0-442-20765-4.

In this single volume, food scientists will find accurate, reliable enzyme as-

says for all the major categories of foods and food-related systems. Incorporated here are specific assay descriptions, explanations for making assay choices, step-by-step techniques for preparing substrates and assay reagents, a critical discussion of good assay design, as well as ready-to-use computer programs for the PC.

Production Wine Analysis. By Bruce W. Zoecklein. Published by Van Nostrand Reinhold/AVI, 115 5th Ave, New York, NY 10003, 1989. 550 pp. Price: $54.95. ISBN 0-442-23463-5.

Here is a detailed, state-of-the-art guide to wine analysis techniques as they are performed in wine industry production laboratories. Each chapter unveils the theories, techniques, and precautions for each analysis, with an emphasis on the role they play in overall winery operations. Remedial techniques to correct problems revealed by analysis are also presented. Appendices on chromatographic techniques, reagent preparations, and laboratory media and strains are included.

Surfactant-Based Separation Processes. Edited by John F. Scamehorn and Jeffrey H. Harwell. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1989. 360 pp. Price: U.S. & Canada $115.00/Export $138.00. ISBN 0-8247-7929-0.

The first book dedicated solely to separation processes utilizing surfactants, "Surfactant-Based Separation Processes" focuses on novel techniques and also views established methods—tying together the numerous ways in which surfactants can be used to effect separations as well as showing the commonality of these technologies. Presenting original research on many surfactant-based separation processes—a number of which have the potential for wide industrial applications—this useful resource describes new extraction techniques, introduces micellar-enhanced ultrafiltration and admicellar chromatography, discusses protein extraction using reverse micelles, covers surfactant-enhanced carbon regeneration, demonstrates new methods of turning waste stream containing dilute concentrations of valuable materials into product streams, and examines such traditional surfactant-based methods as froth flotations and foam fractionation.

Analytical Artifacts. By B. S. Middleditch. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1989. 1028 pp. Price: U.S. $260.50/Dfl. 495.00. ISBN 0-444-87158-6.

This book is an easy-to-use compendium of problems encountered when using various common analytical techniques. Emphasis is on impurities, by-products, contaminants and other artifacts. A separate entry is provided for each artifact. For specific chemicals, this entry provides the common name, mass spectrum, gas chromatographic data, CAS name and registry number, synonyms, and a narrative discussion. More than 1100 entries are included. Mass spectral data are indexed in a six-peak index (molecular ion, base peak, second peak, third peak) and there are also formula, author, and subject indexes.

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Meetings

February 4-6, 1990: Southwest AOAC Regional Section Meeting. Contact: William Y. Cobb, Texas A & M University, Office of the Texas State Chemist, PO Box 3160, College Station, TX 77841-3160, telephone 409/845-1121.

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

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

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

Layloff Is Incoming AOAC President

Thomas P. Layloff was named 1989-1990 President of AOAC at the 103rd Annual International Meeting and Exposition held in St. Louis, MO, September 25-28.

Layloff has a long history of service to AOAC. He has served on the Validation Council, Governance Council, and Interlaboratory Studies Committee, and as a member and Chairman of the Committee on Drugs and Related Topics (Committee B) for 7 years. As Chairman of Committee B, he has also been a member of the Official Methods Board and has headed the OMB Subcommittee on Candidate Methods. He was elected to the Board of Directors in 1985 and became President-Elect in 1988.

A graduate of Washington University in St. Louis, MO, and the University of Kansas, Layloff has been associated with the U.S. Food and Drug Administration since 1967 when he became a consultant to the National Center for Drug Analysis (currently the Division of Drug Analysis) in St. Louis. He continued in this position until 1976 when he joined this organization as director. Responsibilities of the Division include analyzing bulk drug materials submitted for approval to the Agency and validating methods submitted by manufacturers for the control of their products.

The Division also serves as the federal government's collaborating laboratory on the acceptance of reference materials for the USP. The Division has been specifically cited in the Preface of the 1990 *The United States Pharmacopeia and The National Formularly* as being "the primary governmental participant in the ongoing evaluation of established and proposed new USP Reference Standards."

Prior to assuming this position at FDA in 1976, Layloff served as a professor of analytical chemistry at St. Louis University for 7 years. In addition to outside awards, Layloff has received the FDA Commendable Service Award (1980) and the Commissioner's Special Citation (1985).

1989-90 AOAC Board of Directors

The following individuals will serve on the 1989-90 Board of Directors with newly named AOAC President Thomas P. Layloff: President-Elect H. Michael Wehr, Secretary/Treasurer Edgar R. Elkins, Directors Harry B. S. Conacher, Nicole Hardin, Arvid Munson, Albert W. Tiedemann, Alex Williams, and Past President Odette L. Shotwell.

1989 Fellows of the AOAC

In recognition of at least 10 years of meritorious service, the following individuals received honors as 1989 Fellows of the AOAC at the 103rd Annual International Meeting and Exposition in St. Louis, MO.

Aram Beloian, U.S. Environmental Protection Agency. Associate Referee: 1963-1966, Disinfectants: Antimicrobial Agents Used on Fabrics and Materials and in Laundry Processes. General Referee: 1970-1978 and 1983-present, Disinfectants. Member, 1979-1982, Methods Committee.

Raffaele Bernetti, CPC International, Inc. Associate Referee (4 methods adopted): 1979-present, Corn Syrup and Corn Sugars; 1983-1987, Water Determination in Feeds; 1980-1984, Karl Fischer Method in Fats and Oils. Member, 1987-present, Validation Council. Member, 1984-1986, and Chairman, 1987-present, Editorial Board. Member, 1988-present, Governance Council. Member, 1986-present, Joint Mycotoxin Committee.

Thomas L. Jensen, Nebraska State Dept. of Agriculture. Associate Referee: 1979-1983, Disulfoton. General Referee (16 methods approved): 1980-1986, Fungicides and Disinfectants; 1980-1986, Herbicides III. Member, 1986-present, Official Methods Board. Member, 1986, Methods Committee. Member, 1979-1988, Committee on State and Provincial Participation. Member, 1987-present, Committee on Meetings, Symposia, and Educational Programs.

Peter F. Kane, Office of the Indiana State Chemist. Associate Referee (4 methods adopted): 1979-present, K_2O in Fertilizer (Potash); 1982-present, Crude Protein in Animal Feeds. Member, 1983-1987, Committee on Instrumental Methods and Data Handling. Associate Referee of the Year, 1982.

Frederick J. King, National Marine Fisheries Service. Associate Referee (2 methods adopted): 1977-1983, Drip Fluid in Fish Fillets and Fish Fillet Blocks; 1975-1978, Quantitation of Seafood in Crabmeat, Shrimp, or Seafood Cocktail; 1980-1985, Drained Weight of Block Frozen Raw, Peeled Shrimp; 1985-present, Minced Fish in Fillet Blocks. Co-Associate Referee: 1982-present, Determination of Fish Content in Coated Products.

James B. Kotteman, retired, U.S. Food and Drug Administration. Member, 1981-present, Committee on the Constitution. Member, 1980-1984, Board of Directors. Member, 1977-1983, Methods Committee. Member, 1978-1982, Committee on Collaborative Studies.

Thomas L. Layloff, U.S. Food and Drug Administration. Member, 1985-present, Board of Directors. Member, 1988-present, Validation Council. Member, 1982-1983, and Chairman, 1984-1986, Methods Committee. Member, 1984-1986, Official Methods Board. Member, 1987, and Chairman, 1988, Governance Council. Member, 1985-1987, Interlaboratory Studies Committee.

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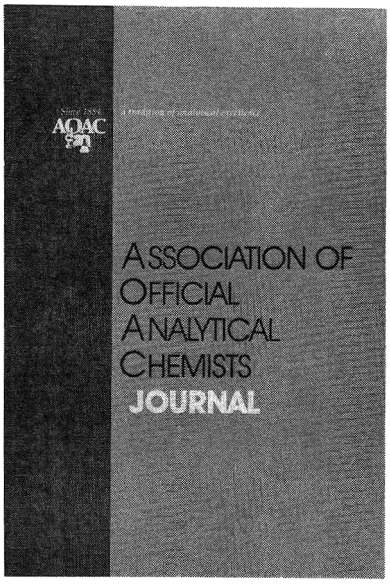

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Hussein S. Ragheb, Office of the Indiana State Chemist. Associate Referee (1 method adopted): 1975–1981, Chlortracycline (Feeds); 1974–1981, Bacitracin in Feeds; 1986–present, Virginiamycin by Turbidimetric Assay. General Referee: 1987–present, Antibiotic Analysis in Feeds. Member, 1982–present, Interlaboratory Study Committee.

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.

General Referee Award to Lopez-Avila and Scott

The 1989 General Referee Award, granted by the Official Methods Board in recognition of outstanding leadership and substantial contribution to method development, was shared this year by Viorica Lopez-Avila, General Referee for Organics in Surface and Waste Water, and Peter M. Scott, General Referee for Mycotoxins. In these capacities, Lopez-Avila and Scott oversee method development in 12 and 14 topic areas, respectively. Under their leadership, a total of four methods have been adopted.

Lopez-Avila is employed at the Acurex Corp., Mountain View, CA. Her work has focused on the development of sensitive analytical methods for various environmental matrixes, characterization of organic compounds in effluents from chemical specialty plants and hazardous waste samples from disposal lagoons and other sources, and study of chemical fate and transport. She has been serving as Associate Referee for Chlorinated pesticides in Groundwater—GC/EC Method since 1988.

Scott who is also this year's Harvey W. Wiley Award winner works for the Health Protection Branch, Health and Welfare Canada, Ottawa. He has been actively involved with AOAC for many years, serving as Associate Referee for Patulin from 1972 to 1982, as Associate Referee for Mycotoxins in Cocoa and Tea/Mycotoxins in Cocoa from 1968 to 1979, as a member and Chairman of the Joint Mycotoxin Committee, as a member of the Validation Council, and as organizer of the mycotoxins session at the 1981 AOAC Spring Workshop in Ottawa. He was named a Fellow in 1983.

Methods Committee Associate Referee Awards for 1989

Created in 1986, the Methods Committee Associate Referee Awards recognize the best Associate Referee in a committee for a given year. Those named for 1989 are: Eugene C. Cole, Committee on Pesticide Formulations and Disinfectants; Eileen Bargo, Committee on Drugs and Related Topics; Jonathan DeVries, Committee on Foods II; Gayle A. Lancette, Committee on Microbiology and Extraneous Materials; and Karen S. Harlin, Committee on Feeds, Fertilizers, and Related Agricultural Products.

Collaborative Study of the Year Award—1989

The Official Methods Board named Rose A. Sweeney the winner of the Collaborative Study of the Year Award for 1989 for "Crude Protein Determination by a Combustion Method Based on Current Leco Analyzers." Sweeney works for the Analytical Biochemistry Laboratories, Inc., in Columbia, MO.

The award recognizes the collaborative study judged to be best for the year as determined by scientific innovation and soundness of design, implementation, and reporting.

New Sustaining Members

AOAC welcomes the following new private sustaining members: Advanced Instruments, Inc., Needham Heights, MA, and A.E. Staley Manufacturing Co., Decatur, IL.

AOAC Announces Analytical Technology Week for April 1990

AOAC will hold its first Analytical Technology Week in Valley Forge, PA, April 2–6, 1990.

Analytical Technology Week will consist of a conference on food irradiation, a workshop on immunoassay techniques, and four short courses: Quality Assurance for Analytical Laboratories; Quality Assurance for Microbiology Laboratories; Improving Sampling for Analysis of Food, Drugs, and Agricultural Materials; and Statistics for Methodology.

The conference and workshop will run consecutively. The short courses will be staggered throughout the week.

The conference on food irradiation will be co-chaired by Leon Lakritz, U.S. Dept of Agriculture, Philadelphia, PA, and Roger Wood, Ministry of Agriculture, Fisheries, and Food, London, U.K. It will cover basic irradiation chemistry, the effects of ionizing radiation on foods, the effects of irradiation on the microflora of foods, food irradiation as viewed by the food industry and by the food processors, and analytical methods, applicable to the detection of irradiated foods.

The conference will also feature a regulatory roundtable of international speakers who will discuss the current rules, regulations, and policies on irradiation of foods in their respective countries.

The workshop on immunoassay techniques will cover fundamentals of various immunoassays, including ELISA, immunoaffinity chromatography, and new technologies. It will also cover immunoassays of foodborne pathogens, including *Salmonella*, *Listeria*, and *Escherichia coli*, as well as immunoassays of contaminants and residues in foods, such as mycotoxins, pesticides, and drugs. Finally, statistical approaches, collaborative studies, and regulatory concerns will be discussed, and hands-on experience will be offered with the immunoassays covered in the discussion sessions.

Jeffery L. Cawley, Northwest Analytical, Inc., Portland, OR, will teach AOAC's first statistics short course, Statistics for Methodology, during Analytical Technology Week.

His text will be *Use of Statistics to Develop and Evaluate Analytical Methods* by Grant T. Wernimont, published by AOAC in 1985.

The course will address AOAC collaborative studies and method certification; the process of developing an analytical method; and using statistics and related quantitative methods to evaluate methods.

The three other short courses offered have been reviewed and significantly updated and restructured to reflect advancing technology and expanding regulatory requirements. These are Quality Assurance for Analytical Laboratories; Quality Assurance for Microbiology Laboratories; and Improving Sampling for Analysis of Food, Drugs, and Agricultural Materials.

Analytical Technology Week is being planned and organized by the Committee on Meetings, Symposia, and Educational Programs.

New Methods

The following methods have been approved interim official first action by the appropriate General Referee and Methods Committee, and by the Chairman of the Official Methods Board: by the Methods Committee on Foods II—(*1*) Determination of Total Sulfite in Shrimp, Potatoes, Dried Pineapple, and White Wine by Flow Injection Analysis, submitted by J. J. Sullivan, T. A. Hollingworth, M. M. Wekell, V. A. Meo, H. H. Saba, A. Etemad-Moghadam, and C. Eklund (Food and Drug Administration, Seafood Products Research Center, Bothell, WA); J. G. Phillips (U.S. Department of Agriculture, Agricultural Research Service, Philadelphia, PA); and B. H. Gump (California State University, Department of Chemistry, Fresno); (*2*) Determination of Free Sulfite in Wine by Flow Injection Anaylsis, submitted by J. J. Sullivan, T. A. Hollingworth, M. M. Wekell, V. A. Meo, and A. Etemad-Moghadam (Food and Drug Administration, Seafood Products Research Center, Bothell, WA); J. G. Phillips (U.S. Department of Agriculture, Agricultural Research Service, Philadelphia, PA); and B. H. Gump (California State University, Department of Chemistry, Fresno); (*3*) Determination of Total Sulfite in Foods and Beverages by Ion Exclusion Chromatography with Electrochemical Detection, submitted H-J. Kim (U.S. Army, Natick Research, Development, and Engineering Center, Natick, MA); by the Methods Committee on Residues—Determination of Copper, Iron, and Nickel in Edible Oils and Copper and Iron in Edible Fats by Direct Graphite Furnace Atomic Absorption Spectrometry, IUPAC-AOAC Method, submitted by S. G. Capar (Food and Drug Administration, Division of Contaminants Chemistry, Washington, DC); by the Methods Committee on Microbiology and Extraneous Materials—(*1*) Colorimetric Deoxyribonucleic Acid Hybridization Assay for Rapid Screening of *Salmonella* in Foods, submitted by M. S. Curiale and M. J. Klatt (Silliker Laboratories, Chicago Heights, IL) and M. A. Mozola (Gene-Trak Systems, Framingham, MA); (*2*) Dry Rehydratable Film Method for Enumeration of Total Aerobic Bacteria in Foods, submitted by M. S. Curiale and T. Sons (Silliker Laboratories, Chicago Heights, IL) and J. S. McAllister, B. Halsey, and T. L. Fox (3M Center, St. Paul, MN); and by the Methods Committee on Environmental Quality—(*1*) Digestion and Inductively Coupled Plasma Atomic Emission Spectroscopic Method for Elemental Analysis of Solid Waste Materials, submitted by E. E. Hargesheimer (City of Calgary, Glenmore Waterworks Laboratory, Calgary, Alberta, Canada); (*2*) Gas Chromatographic-Electron Capture Detection Method for Determination of 29 Chlorinated Pesticides in Finished Drinking Water, submitted by V. Lopez-Avila (Acurex Corp., Mountain View, CA), R. Wesselman (U.S. Environmental Protection Agency, Cincinnati, OH), and K. Edgell (Bionetics Corp., Cincinnati, OH).

The methods were adopted official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Copies of the methods are available from AOAC Scientific Publications.

Official Methods to be Published in Two Volumes

AOAC is taking a first step toward creating a multivolume collection of official methods by publishing the 15th edition of *Official Methods of Analysis* as a two-volume set. The two volumes will only be sold as a set.

Due to be published in January 1990, the book will be divided roughly between food composition analyses and analyses dealing with agricultural chemicals, contaminants, and drugs.

A 1986 user survey showed an overwhelming preference for the bound printed book, compared with loose-leaf or electronic versions. However, users also expressed a desire for more easily handled units that were targeted to specific types of analyses.

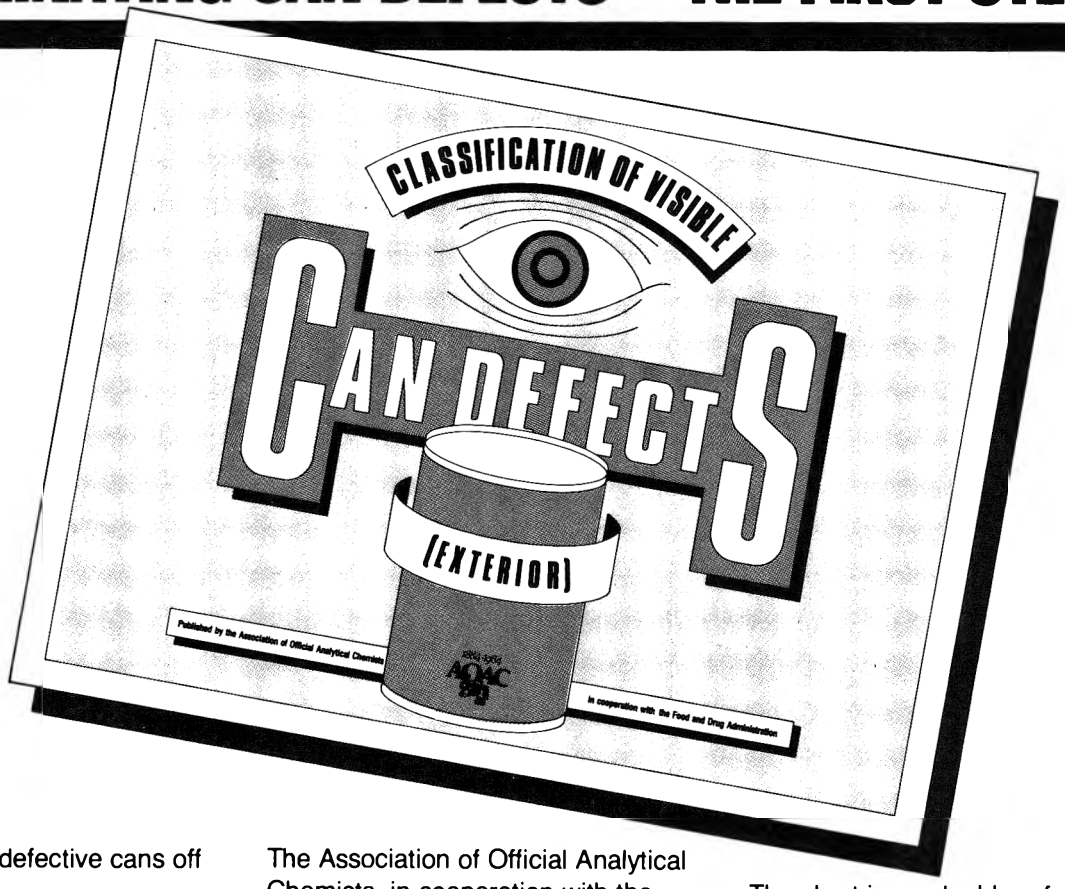
The Editorial Board, considering these preferences, recommended a division of chapters somewhat along AOAC Methods Committee groupings, but strongly supported ensuring the integrity of the publication as a whole.

The Board feels that presentation of AOAC official methods in two volumes enhances the book's usefulness to research and quality control laboratories and institutions involved in a wide range of analyses.

Another step taken by the Board in response to the survey is that, beginning with methods adopted at the 1989 annual international meeting and published in the first supplement to the 15th edition, methods will no longer be written in condensed form.

Standard abbreviations for units of measure will be the only abbreviations used.

ELIMINATING CAN DEFECTS—THE FIRST STEP



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

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

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

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

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   JOURNAL ARTICLE REFERENCE

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

   BOOK CHAPTER REFERENCE

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

   BOOK REFERENCE

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

   OFFICIAL METHODS REFERENCE

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

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CUMULATIVE AUTHOR INDEX 1989

determination of available phosphorus. With the extraction cell, the possibility of automation becomes apparent. Results compare favorably with those for the AOAC method. By using the extraction cell, precision should be improved and analysis time should be reduced.

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(1) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA
(2) Baker, A. M., Trimm, J. R., & Sikora, F. J. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 867–869
(3) Sikora, F. J., Dilland, E. F., Copeland, J. P., & Mullins, G. L. (1989) *J. Assoc. Off. Anal. Chem.* **72,** 852–856

CHEMICAL CONTAMINANTS MONITORING

Ethyl Carbamate Levels in Selected Fermented Foods and Beverages

BENJAMIN J. CANAS, DONALD C. HAVERY, LOUIS R. ROBINSON, MICHAEL P. SULLIVAN, FRANK L. JOE, JR, and GREGORY W. DIACHENKO
Food and Drug Administration, Division of Food Chemistry and Technology, Washington, DC 20204

Ethyl carbamate (EC), also known as urethane, is an animal carcinogen and a by-product of fermentation. Because EC has been found in distilled spirits and wines, a variety of fermented foods and beverages were analyzed to assess its occurrence in other products. Previously described methods using a gas chromatograph-thermal energy analyzer with a nitrogen converter were modified for each matrix and gave recoveries of >80%, with a limit of detection in the 1–2 μg/kg (ppb) range. A total of 152 test samples were analyzed; EC levels ranged from none found to 3 ppb in 15 cheeses, 6 teas, 12 yogurts, and 8 ciders; from none found to 13 ppb in 30 breads and 69 malt beverages; and from none found to 84 ppb in 12 soy sauces. Gas chromatography/mass spectrometry/mass spectrometry was used to confirm EC identity and to quantitate EC in selected food extracts.

Ethyl carbamate (EC), also known as urethane, has exhibited carcinogenic activity in laboratory animals (1, 2). The presence of EC in alcoholic beverages first received attention in 1971 when Gejvall and Lofroth (3) reported that diethyl pyrocarbonate (DEPC), an antimicrobial food additive, reacted with ammonia at neutral or alkaline pH to produce EC in wines. Consequently, in 1972, under the Federal Food, Drug, and Cosmetic Act of 1958, the regulation permitting the use of DEPC in the United States was rescinded (4). In 1976, Ough (5, 6) questioned the amounts of EC that could actually result from added DEPC and reported that a nominal amount of EC (<5 μg/kg (ppb)) occurred naturally in fermented foods and beverages. The reaction of naturally occurring carbamyl phosphate with ethanol was suggested as the source of EC.

In late 1985, the Health Protection Branch of Health and Welfare Canada reported the presence of EC in distilled spirits and wines and established regulatory limits on the content of EC in these products (7). The Bureau of Alcohol, Tobacco, and Firearms and the U.S. Food and Drug Administration (FDA) also conducted surveys to assess the frequency and levels of EC in alcoholic beverages (8). The EC content in alcoholic beverages was found to be highly variable, ranging from <5 ppb to ppm levels in some cases. In 1986, Ough (9) reported that naturally produced urea, from yeast metabolism of the amino acid arginine, and the amino acid citrulline were also possible sources of EC. Ethanol solutions of urea or citrulline produced EC when heated. Ingledew et al. (10) conducted experiments indicating that yeast fermentation itself did not produce detectable EC (20 ppb EC, detection limit), but when heat was subsequently applied to fermented beverages in which urea was present, EC was formed in significant quantities.

In the present study, the EC levels found in fermented food products such as malt beverages, bread, soy sauce, yogurt, cheese, and cider are reported. These products were chosen because of their high consumption volume and were purchased from the Washington, DC, retail market. Results from this study will permit an estimate of the contribution of these fermented foods and beverages to the total average daily intake of EC in the American diet. The basic methodology used in this study was described previously (11). A technique using a gas chromatograph-thermal energy analyzer with nitrogen converter (GC-N/TEA) was used for detection and quantitation of EC. In the case of cheese, a vacuum distillation procedure for nitrosamines (12) was applied to overcome the high fat content in this matrix. In some instances, chromatographic interferences were overcome with modifications to the temperature program of the GC oven and/or the use of disposable chromatography columns of silica gel and Florisil. Confirmation and quantitation by gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS) were obtained for selected extracts by using a previously described technique (13).

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METHOD

Apparatus and Reagents

Apparatus and reagents were previously described (11, 12). In addition, the following are required.

(a) *Omni-Mixer.*—Ivan Sorvall Inc., Norwalk, CT.

(b) *Silica gel disposable column.*—J.T. Baker Chemical Co., Phillipsburg, NJ 08865.

(c) *Sep-Pak Florisil cartridge.*—Waters Associates, Milford, MA 01757.

(d) *Gas chromatograph-thermal energy analyzer with nitrogen converter.*—Hewlett-Packard Model 5710A gas chromatograph interfaced to thermal energy analyzer Model 502L (Thermedics Inc., Woburn, MA 01888) with nitrogen converter Model 610R. Operating conditions: DB-wax fused silica capillary column, 30 m × 0.53 mm id, with 1 μm film coating (J & W Scientific, Inc., Folsom, CA 95630); helium carrier gas at 8 mL/min; injector 200°C; GC interface 225°C, pyrolyzer furnace 825°C, oven (isothermal) at 110°C, or program as follows: hold at 90°C for 8 min, from 90 to 110°C at 2°C/min, hold at 110°C for 2 min; TEA operating pressure 0.3–0.4 torr.

Extraction

Basic procedure for all matrixes except cheese is similar to that for alcoholic beverages described by Canas et al. (11). Brief description of basic procedure follows: Thoroughly mix 15 g test portion with 15 g Celite and pack in chromatographic column containing 10 g deactivated alumina capped with 40 g layer of sodium sulfate. Add 100 mL methylene chloride and collect eluate in 300 mL conical flask. Evaporate eluate to 1–2 mL, using rotary evaporator with 30°C water bath. Transfer concentrate to graduated 4 mL concentrator tube. Rinse flask with 1 mL methylene chloride and add rinsings to tube. Concentrate to 1 mL final volume under gentle stream of nitrogen. For increased sensitivity in malt beverage analyses, use 25 g test portion and 25 g Celite; elute with 150 mL methylene chloride.

For bread analyses, tear bread slice or test sample into very small pieces (5 sq mm) and weigh 10.0 g into Sorvall cup. Add 15 mL distilled water. Homogenize 10 s at medium speed. Add 25 g Celite and homogenize 10 s again. Complete mixing with spatula until mixture is homogeneous. Pack in

column, following procedure described above; elute with 150 mL methylene chloride. Concentrate eluate to 1–2 mL.

To confirm identity of EC (from bread analysis) by GC/MS/MS, remove fat by performing Sep-Pak Florisil cartridge cleanup described by Dennis et al. (14). Brief description of Sep-Pak cartridge cleanup follows: Wash Florisil cartridge with 10 mL methylene chloride and add 1–2 mL concentrate to cartridge. Rinse flask with 5 mL methylene chloride and add rinsings to cartridge. Collect methylene chloride washings and discard. Elute EC from cartridge with 5 mL 7% methanol in methylene chloride into concentrator tube. Concentrate under nitrogen to 1 mL final volume.

Prepare tea test samples by following label instructions, or place 1 tea bag in 250 mL hot water for 5 min, mix, and let cool to room temperature. Analyze 15 g test portion by procedure described above.

Analyze cheese by mineral oil vacuum distillation technique described by Havery et al. (12). Brief description of technique follows: Homogenize 25 g test portion with water and distill under reduced pressure with mineral oil. Extract distillate with three 50 mL portions of methylene chloride, and concentrate to 1–2 mL by using rotary evaporator. Clean extract by using silica gel disposable column as follows: Wash column with 2 mL acetone followed by 2 mL methylene chloride. Load extract into column and drain. Rinse flask with 2 mL methylene chloride, add wash to column, and drain. Elute EC with 2 mL acetone, and evaporate under nitrogen to 1 mL final volume. Mix concentrate and save for GC-N/TEA analysis.

GC-N/TEA Analysis

Inject 2 μL concentrated extract into gas chromatograph-thermal energy analyzer. Use isothermal oven conditions at 110°C for cheese, yogurt, apple cider, and tea extracts. Retention time of EC at 110°C is about 8.5 min. For malt beverage, bread, and soy sauce extracts, use temperature program specified in *Apparatus and Reagents*, **(d)**. EC retention time obtained by using this program is about 17 min. Injection of 0.2 ng EC at attenuation 16 gave 25% full scale deflection. Quantitate EC by comparing peak height at EC retention time to that from 2 μL injection of EC working standard prepared as previously described (11). Upon completion of each GC analysis, heat oven 15 min to 200°C to eliminate interferences that might appear in next chromatogram.

GC/MS/MS Analysis

This technique was previously described by Brumley et al. (13). Brief description of technique follows: Quantitate EC and confirm identity by GC/MS/MS by adding known amount of ^{13}C, ^{15}N-labeled ethyl carbamate (LEC) to final extract or to test portion before extraction. Amount of LEC added should be similar to EC level expected or to that previously found by GC-N/TEA. Using isobutane chemical ionization to produce $(M+H)^+$ ion for EC and for LEC internal standard, monitor collision-activated decomposition of m/z 90 and 92 by multiple ion detection of m/z 90, 62, and 44 for EC and m/z 92, 64, and 46 for LEC. Quantitate EC by using ratio of daughter ions m/z 62 for EC and 64 for LEC. Quantitate without confirming identity when intensity of daughter ion m/z 62 for EC is large enough to permit quantitation, but intensity of m/z 44 is not measurable (i.e., EC identity is not considered confirmed unless all 3 ions are present and in correct ratio).

Results and Discussion

Detection and quantitation limits of the GC-N/TEA methods, based on a 15 g test portion and 1 mL final volume of extract, were 1.5 and 3.0 ppb, respectively. These values were calculated by following the guidelines for data acquisition by the American Chemical Society Committee on Environmental Improvement (15). Test portions of 25 g were used for malt beverage and cheese analyses to lower the detection and quantitation limits to 1 and 2 ppb, respectively. In the analyses of bread, in which a 10 g test portion was mixed with 15 g water, the detection limit was approximately 2 ppb. Typical chromatograms of soy sauce, bread, and malt beverage extracts containing 11, 8.0, and 4.5 ppb EC, respectively, are shown in Figure 1. Typical chromatograms of soy sauce, bread, and malt beverage extracts containing nondetectable levels of EC are shown in Figure 2.

Recovery studies were conducted with each food type. The results are shown in Table 1. Foods previously found to contain no detectable levels of EC were fortified at levels ranging from 4 to 24 ppb EC. Mean recoveries from each matrix ranged from 89.6 to 100%, with an overall average of 94.0 ± 4.1%. GC-N/TEA data reported in this study were not corrected for recovery.

Table 2 shows results from analyses of 33 domestic (United States) and 36 imported malt beverages, representing 29 domestic and 22 imported brands; analyses of cheese, yogurt, apple cider, and tea; and 30 analyses of bread, representing 21 brands. For malt beverages, 7 of the 29 domestic brands contained detectable levels of EC that were below the quantitation limit; 4 of these were domestic ales. No domestic brand contained quantifiable levels of EC. The mean EC level in all domestic malt beverages analyzed was 0.24 ppb. Most imported malt beverages contained <5 ppb EC; 2 test samples

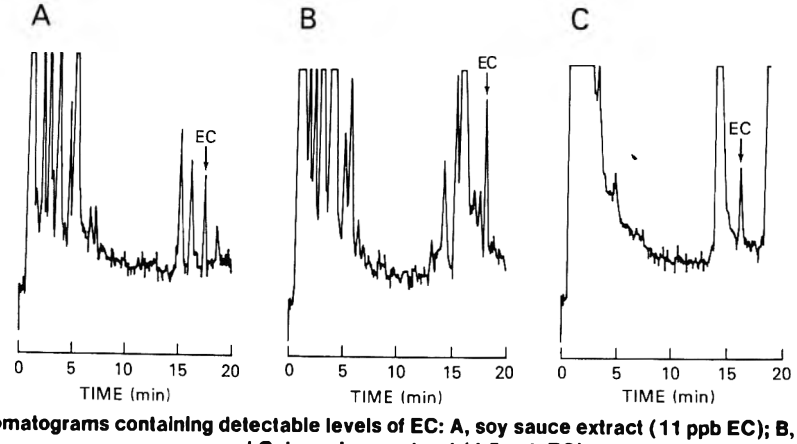

Figure 1. GC-N/TEA chromatograms containing detectable levels of EC: A, soy sauce extract (11 ppb EC); B, bread extract (8.0 ppb EC); and C, lager beer extract (4.5 ppb EC).

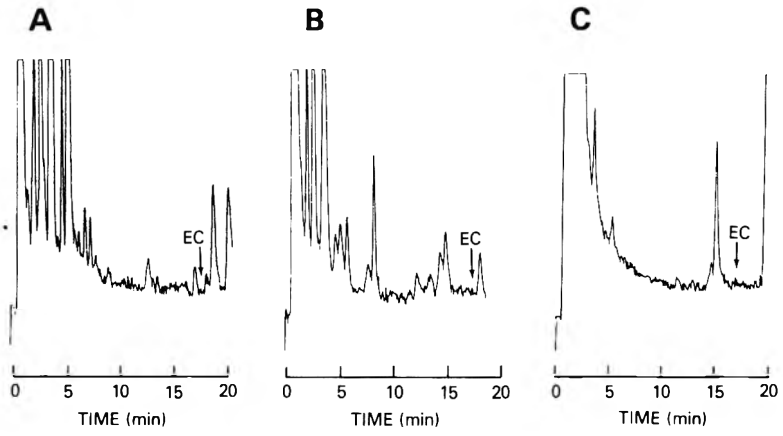

Figure 2. GC-N/TEA chromatograms containing nondetectable levels of EC: A, soy sauce extract; B, bread extract; and C, lager beer extract.

contained 13 ppb. The mean EC level in all imported malt beverages analyzed was 2.8 ppb. For a limited number of malt beverages, different test samples of the same brand were analyzed to obtain some measure of lot variability. Although lot numbers could not be readily identified for some brands, purchases of the same brand were made at least several weeks apart to increase the likelihood of obtaining different lots. The results from this limited number of analyses showed little or no variability. GC/MS/MS was used to quantitate EC in 18 selected extracts in which EC had been found by GC-N/TEA. The results obtained by the 2 techniques usually differed by ≤1 ppb.

In the cheese analyses, no quantifiable levels of EC were found in 16 test samples of 5 different cheese types. In analyses of yogurt, EC was found in only 2 of 12 test samples, at levels between the detection and quantitation limits. No EC was found in analyses of 3 non-alcoholic apple ciders; however, 3 ppb EC was found in 1 of 5 alcoholic ciders analyzed. No EC was found in 6 tea test samples.

In the bread analyses, 8 of 21 initial test samples contained EC levels above the 2 ppb limit of detection. A subsequent purchase of these brands was made 4 weeks later, and additional test samples were analyzed to obtain variability data. Results of the initial and additional analyses differed by only 1–2 ppb. Quantitative results obtained by GC/MS/MS for selected bread extracts were in agreement with GC-N/TEA results. The mean EC levels in all white and wheat breads analyzed were 3.0 and 1.2 ppb, respectively. Other breads analyzed included bagel, pita, Italian, French, rye, pumpernickel, English muffin, and sour dough. The mean EC level found in these products was 0.9 ppb.

Because of reports that linked heating to increased levels of EC in alcoholic beverages (9, 10), the effect of toasting on the EC levels in bread was also studied. In Table 3, EC levels are reported in ng/slice instead of ng/g (ppb) to avoid confusion due to the loss of weight associated with toasting. Two bread slices of similar weight were chosen for analysis. The weight of the whole slice to be analyzed fresh was recorded. The other slice was toasted to a light brown color in a common toaster and allowed to cool to room temperature, and its weight was recorded. Test portions of 10 g from each slice were analyzed, and the results in ng/g were multiplied by the weight of the respective slice. The EC levels in ppb in fresh bread are shown in Table 2. The results in Table 3 from the analysis of 9 test samples show the effect of toasting on the EC levels in sliced bread. EC levels in toasted bread were as much as 6 times the levels in their fresh counterparts. Toasting increased the mean level of EC by a factor of 2.6 from 45 to 117 ng/slice.

Table 1. Recovery of EC from selected fermented foods

Food type	n	Test portion, g	EC added, ng	Mean rec. ± SD, %
Malt beverage	10	25	100	94.3 ± 4.0
Bread	5	10	200	89.6 ± 3.5
Cheese	6	25	600	89.7 ± 11.0
Yogurt	3	15	200	97.6 ± 2.2
Apple cider	1	15	100	100
Tea	3	15	200	91.0 ± 4.4
Soy sauce	1	15	100	96

Table 2. EC found in selected fermented foods and beverages

Food type	No. of test samples	No. of brands	No. of test samples containing EC, ppb				EC mean, ppb[b]
			ND[a]	1–4	5–8	9–13	
Malt beverage							
Domestic							
Non-alcoholic	2	2	2				—
Malt liquor	4	4	3	1			0.3
Lager beer	16	13	14	2			0.1
Low calorie	3	3	3				—
Ale	6	6	1	5			0.8
Dark beer	2	1	2				—
Imported							
Lager beer	18	14	5	7	4	2	3.5
Ale	5	2	1	4			2.4
Dark beer	13	6	2	11			2.1
Cheese							
Cheddar	4	3	4				—
Cottage	3	3	3				—
Cream	2	2	2				—
Blue	2	2	2				—
Processed	5	5	5				—
Yogurt	12	12	10	2			0.4
Apple cider							
Non-alcoholic	3	3	3				—
Alcoholic	5	5	4	1			0.6
Tea	6	6	6				—
Bread							
White	10	6	3	5	2		3.0
Wheat	6	5	4	2			1.2
Other	14	10	10	4			0.9

[a] ND = none detected.

[b] In calculating the mean, a value of zero was used for a test sample that contained no detectable EC; an estimated value was used for a test sample that contained an EC level between the detection and quantitation limits.

Table 3. EC found in fresh and toasted bread

Test sample	Bread type	EC, ng/slice[a]	
		Fresh	Toasted
1	white	42	90
2	white	33	57
3	white	62	63
4	white	42	97
5	wheat	52	312
6	wheat	82	226
7	pumpernickel	18	81
8	rye	54	85
9	English muffin	19	45
Mean		45	117

[a] Average weight of 1 slice (fresh) = 27.1 g; average weight of 1 slice (toasted) = 22.5 g.

Table 4 shows results for 12 soy sauce test samples analyzed by the previously described GC-N/TEA (11) and GC/MS/MS (13) procedures. EC levels in soy sauce showed wide variability and ranged from none detected to 84 ppb found by GC-N/TEA and from 0.3 to 76 ppb found by GC/MS/MS. Although the 2 sets of values are in good agreement, the slightly higher GC-N/TEA values for some extracts could indicate the presence of a small amount of co-eluting interference. The mean and median levels of EC in soy sauce were 18 and 7 ppb, respectively.

Table 4. EC found in soy sauce by GC-N/TEA and GC/MS/MS procedures

Test sample	Country of origin	EC, ppb	
		GC-N/TEA	GC/MS/MS
1	United States	ND[a]	NA[b]
2	United States	ND	NA
3	United States	35	30[c]
4	United States	46	40[c]
5	China	ND	NA
6	China	6	7[d]
7	China	7	7[d]
8	China	24	24[c]
9	Japan	7	5[d]
10	Japan	11	10[c]
11	Japan	84	76[c]
12	Philippines	ND	0.3[d]
Mean		18	
Median		7	

[a] ND = none detected; a value of zero was used in calculating the mean.
[b] NA = not analyzed.
[c] EC identity was confirmed.
[d] EC identity was not confirmed because the proper ratio of m/z 90, 62, and 44 was not maintained.

Conclusions

By making a few modifications to the basic Celite extraction procedure, selected fermented foods and beverages were analyzed for EC. With the exception of some soy sauce test samples, most foods analyzed contained EC levels of <5 ppb, as previously reported by Ough (5, 6). GC/MS/MS quantitation and confirmation of identity of EC in selected food extracts demonstrated the accuracy of the GC-N/TEA results. Analyses of other types of fermented foods are under way.

Acknowledgment

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DAIRY PRODUCTS

Spectrophotometric Method for Measurement of Lactose in Milk and Milk Products by Using Dialysis

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A simple method for measuring lactose in milk and milk products by using dialysis is described. A sample of milk, diluted milk powder, cultured buttermilk, or Cheddar cheese whey is dialyzed against water in a test tube at 45°C for 1.5 h. Lactose from the product is extracted into the water and Teles reagent is added. Absorbance was converted to percent lactose using a standard curve. Lactose obtained by the dialysis method was comparable to results obtained by the ultraviolet enzymatic and infrared methods. The method was not suitable for yogurt, which contains galactose that reacts with the Teles reagent. The new method for lactose measurement is simple and does not require precipitation of proteins, filtration, or centrifugation.

Lactose is an important constituent of milk and milk products, and many methods are presently available for its quantitation (1-19). Most of these methods require many steps including extraction of lactose from the sample prior to quantitation. Lactose is generally extracted by precipitation of proteins, followed by filtration and/or centrifugation. Exceptions are the cryoscopic (1), liquid chromatographic (11), and infrared spectroscopic (17) methods, which require expensive, specialized instrumentation.

A simple technique for measuring lactose makes use of Teles reagent (16). Lactose is extracted from milk by precipitation of proteins, filtration, and centrifugation; the filtrate containing lactose is then reacted with Teles reagent, in which picric acid is the primary reactant. Color intensity is then measured in a spectrophotometer and converted to percent lactose from a standard curve.

The extraction of lactose may be considerably simplified by applying dialysis. Beloff and Anfinsen (20), Harmsen and Kolff (21), and Hestrin et al. (22) used dialysis to separate and measure soluble end products of microbial and enzymatic activity. The same principle has also been used to develop sensitive methods for measuring alkaline phosphatase and tyrosine in milk (23, 24). The objective of the present study was to develop a simple method for measuring lactose in milk and milk products by using dialysis to extract lactose from the product and adding Teles reagent for color development.

Experimental

Apparatus and Reagents

(a) *Dialysis tubes.*—Spectra/Por 4 dialysis tubing of molecular weight cut-off of 12 000-14 000 Daltons (Fisher Scientific, Minneapolis, MN) was used. Approximately 15 cm sections of dialysis tubes were cut and washed thoroughly with deionized distilled water. Enough sections were prepared for use over 2 days. Dialysis tube closures (Spectra closures) were used to seal tubes during incubation.

(b) *Test tubes.*—32 mm (od) × 200 mm (100 mL water capacity to rim) (Corning Products, Corning, NY).

(c) *Teles reagent.*—1% phenol, 5% NaOH, 1% picric acid, and 1% sodium bisulfite, prepared fresh daily as described by Teles et al. (16). (*Caution*: Picric acid is a hazardous chemical and must be handled with care. It is toxic; hence gloves should be used when handling. Picric acid can also be explosive under dry conditions; therefore, it should not be allowed to dry out.)

(d) *Milk and milk products.*—Commercial fluid 2% low fat, and homogenized whole milk samples were obtained from local stores and were analyzed for lactose on the same day by all methods. Commercial Grade A nonfat dry milk (NFDM) was obtained from the SDSU dairy plant. Fresh Cheddar cheese whey was obtained from a local cheese plant; commercial cultured buttermilk and plain yogurt samples were obtained from local stores.

(e) *Lactose/galactose UV kit.*—Catalog No. 176303, Boehringer-Mannheim Biochemicals, Indianapolis, IN 46280.

(f) *Lactose stock solution.*—10 g lactose monohydrate (Sigma Chemicals, St. Louis, MO) was diluted to 100 mL with deionized distilled water. This stock solution was used to prepare lactose standards of various concentrations.

(g) *Spectrophotometer.*—Beckman DU50 (Irvine, CA).

(h) *Water bath.*—Thermostatically controlled at 45°C.

Procedure

(a) *Standard curve.*—Lactose stock solution was diluted with water to concentrations ranging from 1% (1 mg/mL) to 7% (7 mg/mL) lactose monohydrate (Table 1). Three mL of each concentration was pipetted in duplicate into preknotted, washed dialysis tubes. Care was taken not to stretch the dialysis tubing. The open end of the tube was then sealed with a closure, leaving no air space or air bubbles in the tube. The sealed dialysis tube was placed in a test tube containing 90 mL deionized distilled water, taking care that the closure was directly beneath the water level in the test tube and that all dialysis tubes were at the same depth. To prevent the tube from sinking during incubation, the portion of the tube above the closure was folded over the rim of the test tube and held with a rubber band.

The test tube containing dialysis tube was immediately placed in a water bath maintained at 45°C. After 1.5 h, the test tube was removed from the water bath and the dialysis tube was discarded. The contents of the test tube were then mixed, and 1 mL was pipetted into a 20 mL capacity test tube. Fresh Teles reagent (2.5 mL) was added and the mixture was placed in a boiling water bath for exactly 6 min for color development and then in a cold water bath for 2 min to stop color development. Nine mL deionized distilled water was then added and, using color developed with water as a blank, absorbance was measured at 520 nm.

In the absence of lactose, picric acid produced a clear yellow color. In the presence of lactose, the color was red-brown. If the blank showed any traces of brown coloration, a fresh batch of Teles reagent was prepared. The standard curve was replicated 3 times, hence 6 absorbance values were obtained for each lactose concentration. Absorbance was

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Table 1. Lactose standards for preparing standard curve for the dialysis test

Lactose stock soln[a]	Distd water, %	Lactose monohydrate in mixt., %	Lactose anhydrous in mixt., %
1	9	1	0.95
2	8	2	1.90
3	7	3	2.85
4	6	4	3.80
5	5	5	4.75
6	4	6	5.70
7	3	7	6.65

[a] Lactose stock solution = 100 mg lactose monohydrate/mL.

plotted against percent anhydrous lactose, and an equation was obtained by linear regression using Statistical Analysis Systems (SAS, Cary, NC).

(b) *Lactose in milk.*—Three mL of each milk sample described above was pipetted into a preknotted, washed dialysis tube. Incubation and color development were as described previously for standard curve. The standard curve was used to convert absorbance to percent anhydrous lactose.

(c) *Lactose in NFDM.*—Ten g NFDM was diluted with 90 mL deionized distilled water and mixed well, giving a 10× dilution factor. Lactose was measured in the diluted sample as for fluid milk, except that the dilution factor was used to obtain percent anhydrous lactose in the powder.

(d) *Lactose in whey.*—Cheddar cheese whey (held for ca 15 h at < 4°C) was pasteurized in a water bath at approximately 72°C for 15–17 s to kill all lactic acid bacteria. It was then cooled to 20°C, and lactose was determined by the dialysis method as for fluid milk.

(e) *Lactose in cultured buttermilk.*—To prevent growth of lactic acid bacteria and subsequent lactose metabolism, buttermilk was pasteurized at 72°C for 15–17 s and cooled to 20°C. Pasteurization of a cultured milk product normally leads to separation of milk protein and whey due to the low pH. Therefore, the buttermilk was neutralized with 1N NaOH to pH 7.0 prior to pasteurization. Four mL 1N NaOH was required per 50 g buttermilk having a pH of 4.6. After pasteurization, lactose was measured as in fluid milk. Dilution due to the addition of NaOH was accounted for when absorbance was converted to percent lactose.

(f) *Lactose in yogurt.*—Yogurt was also neutralized and pasteurized. Due to the high viscosity of yogurt, it also had to be diluted with water to improve handling. Fifty g plain yogurt with a pH ca 4.3 was diluted with 44 mL deionized distilled water and 6 mL 1N NaOH, giving a 2-fold dilution. The diluted, neutralized yogurt was pasteurized at 72°C for 15–17 s and cooled to 20°C. Lactose was determined as for fluid milk.

(g) *Ultraviolet and infrared methods for lactose.*—For comparison, lactose in all of the products was determined by the lactose/galactose UV enzymatic method according to AOAC (25). Lactose in milk and Cheddar cheese whey was also determined at 2 local cheese plants with a Multispec M (Multispec, Inc., Newhall, CA) infrared milk analyzer. This instrument at both the cheese plants was calibrated for lactose by samples provided by the Dairy Quality Control Institute, St Paul, MN.

Lactose measurement for each sample was replicated 5 times, and color development with the dialysis method was performed in duplicate for each replicate, giving a total of 10 measurements for each sample.

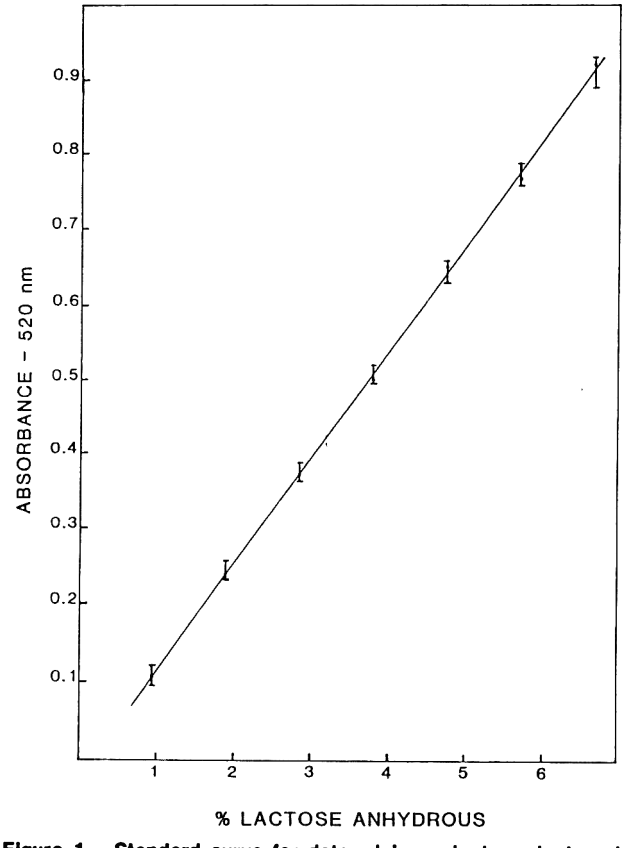

Figure 1. Standard curve for determining anhydrous lactose by dialysis method.

Results

The linear standard curve (r = 0.996) for anhydrous lactose concentrations, ranging from 0.95 to 6.65%, is shown in Figure 1. The upper and lower 95% confidence limits indicate that the variability between replicates was small for all concentrations. This standard curve was used to convert absorbance values for samples to percent anhydrous lactose. Table 2 shows percent lactose in 5 commercial fluid milk samples by the new dialysis method and the 2 reference methods. The overall means of the 3 methods were comparable. The overall mean percent lactose by the dialysis method was not significantly different from the mean by the infrared method ($P < 0.05$). The standard deviation for the milk samples was < 0.10% by the dialysis method, indicating good repeatability. Means with the infrared method had the lowest standard deviation.

Determination of lactose in other milk products is shown in Table 3. Of these products, only Cheddar cheese whey was analyzed with all 3 methods. Results with the dialysis and enzyme methods were comparable. The correlation coeffi-

Table 2. Determination of lactose[a] in commercial fluid milk samples by dialysis, ultraviolet, and infrared methods

		Dialysis		UV		IR	
Sample	Trials	Mean	SD	Mean	SD	Mean	SD
A, 2% fat	5	4.89	0.10	4.69	0.09	4.77	0.01
B, 2% fat	5	4.98	0.09	4.57	0.14	4.83	0.02
C, Whole	5	4.74	0.08	4.71	0.02	4.82	0.01
D, Whole	5	4.64	0.08	4.74	0.04	4.83	0.01
E, 2% fat	5	4.68	0.10	4.82	0.03	4.82	0.01
Overall mean[b]		4.79a		4.71b		4.81a	

[a] For all methods lactose is expressed as percent anhydrous lactose.

[b] Means with same letter are not significantly different from each other at $P < 0.05$.

Table 3. Determination of lactose[a] in milk products by dialysis, ultraviolet, and/or infrared methods

		Dialysis		UV		IR	
Product	Trials	Mean[b]	SD	Mean[b]	SD	Mean[b,c]	SD
Nonfat dry milk	5	48.50a	1.36	48.7a	0.90	———	
Cheddar cheese whey	5	4.71a	0.14	4.75a	0.07	4.62a	0
Cultured buttermilk	5	3.20a	0.13	3.68b	0.08	———	

[a] Lactose is expressed as % anhydrous lactose.

[b] Means with same letter for a product are not significantly different from each other at $P < 0.05$.

[c] Infrared analysis was not conducted for nonfat dry milk and buttermilk. Mean for whey with infrared method represents 2 trials.

cient for all products (milk, NFDM, whey, and buttermilk) between the dialysis method and the UV method was 0.999.

The new method was also used to determine the lactose content of commercial plain yogurt (Table 4). Unlike other products tested, the dialysis method consistently gave significantly higher results than the UV method ($P < 0.05$). The standard deviation with both methods was low, indicating good repeatability. "Lactose" in this product was also measured by difference, i.e., by subtracting fat, protein, and ash from total solids. These results include lactose, other carbohydrates, as well as lactic acid (1%). The UV method, which measures true lactose, produced the lowest results for yogurt (Table 4).

Discussion

When milk and milk products are dialyzed against water, water-soluble components, including lactose, minerals, and vitamins, will permeate the dialysis tubing and mix with water in the test tube. The resulting mixture can then be reacted with a coloring agent specific for lactose, such as picric acid. The color intensity will be directly proportional to the quantity of lactose in the mixture, which in turn, will be directly proportional to the amount of lactose in the original sample that was placed in the dialysis tube. This principle provided a simple method for extracting lactose from milk and milk products for quantitation, and did not require precipitation, filtration, and centrifugation, which is required by most of the other methods.

The quantity of water outside the dialysis tube, incubation time and temperature, and sample size were important factors which determined the quantity of lactose dialyzed. Preliminary experiments showed that with only 1 h of dialysis at 45°C, the variability of the results were high. Variability reduced considerably when the incubation time was increased to 1.5 h, hence 1.5 h was chosen as the incubation time.

Fluid milk samples could be tested with relative ease. Samples required no pretreatment or dilution. Fermented products such as cheese whey and cultured buttermilk were pasteurized prior to dialysis to prevent lactose metabolism during incubation by lactic acid bacteria. For the same reasons, raw milk must also be pasteurized prior to dialysis. Preservatives (such as hydrogen peroxide, potassium dichromate, formalin, and others) are not recommended because they are strong oxidizing agents and will interfere with the color reaction. Antibiotics are also not recommended since most common antibiotics are water-soluble and dialyze out during incubation. They would therefore not be available to suppress bacterial growth.

When cultured products such as cultured buttermilk are pasteurized, protein precipitation may occur. Thus, it would be difficult to obtain a representative sample for lactose analysis. Neutralization of the acidity in cultured products prior to pasteurization prevented protein precipitation. Neutralization was not necessary for whey, as no precipitation occurred during pasteurization.

Table 4. Determination of lactose[a] in commercial plain yogurt by dialysis and ultraviolet methods, and calculated by difference

		Lactose, %	
Method	Trials	Mean	SD
Dialysis	5	5.85	0.08
UV	5	4.23	0.02
Diff.[b]	2	6.7	0.001

[a] Lactose is expressed as percent anhydrous lactose.

[b] Lactose by difference is calculated by subtracting percent protein, fat, and ash from total solids.

Measurement of lactose in yogurt does not appear feasible with the new method. Yogurt is prepared with thermophilic lactic acid bacteria, which are unable to completely utilize galactose (26). Thus, yogurt normally contains 1.2–1.4% galactose (18). Galactose, being a reducing sugar, also reacts with picric acid and hence overestimates lactose concentration. The UV method distinguishes lactose and galactose and measures only lactose. Work is currently under way to adapt the dialysis method for measuring lactose in yogurt.

Conclusions

A simple method for measurement of lactose in milk and some milk products has been developed. Lactose is extracted from liquid milk by dialysis and without any pretreatment. The extracted lactose is then reacted with picric acid for color development. Absorbance is measured in a spectrophotometer and then converted to lactose by using a standard curve. This method does not require expensive, specialized instruments, and it would therefore be suitable for any quality control laboratory.

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DETERGENTS

Rapid Colorimetric Determination of Activity of Subtilisin Enzymes in Cleaning Products

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A new colorimetric method is described for the determination of enzymatic activity of subtilisin in cleaning products. The procedure is more rapid and precise than the casein digestion methods commonly used to assay protease activity. The principle of the colorimetric method depends on the determination rate of *p*-nitrophenol released on hydrolysis of *N*-CBZ-L-leucine-*p*-nitrophenyl ester at pH 8.0 by subtilisin, with correction for any nonenzymatic (spontaneous) hydrolysis of the substrate. Because of the broad range of hydrolytic activity of this enzyme, and the difficulties in predicting its proteolytic activity, this hydrolytic rate was chosen as a general indicator of subtilisin enzyme behavior. The slope for 7 replicate standard curves generated over a 6 week period exhibited a relative standard deviation of 7.5%, and 8.0% for 20 replicates with an enzyme cleaning product. Papain does not interfere with this assay.

Subtilisin, a mixed alkaline protease derived from bacteria (1), has been applied successfully as cleaning and food processing agents. They are typically used in detergents and contact lens cleaning tablets but may also be used in the commercial preparation of starch syrups, beer, and other food products employing protein hydrolysates (2). Subtilisin shows a broad range of hydrolytic activity, including both protease and esterase activities. If the type of subtilisin (i.e., the strain of *Bacillus* from which it has been obtained) is unknown, a determination of the relative first-order rate constants, using 2 different ester substrates, has been recommended to qualify the subtilisin (3). The protease activity of subtilisin is influenced by the amino acid residues on either side of the peptide bond in the substrate protein being hydrolyzed. Consequently, the specificity and proteolytic activity of the enzyme is especially dependent on the stucture of the particular protein substrate (4).

Published assays for subtilisin include determination of the catalytic rate of hydrolysis by using either ester or peptide substrates. Casein as a peptide substrate has been described (5), using a turbidity method similar to those specified in the USP for other proteases (6). Nonetheless, the utility of casein as a substrate for subtilisin is dependent on its homogeneity and molecular consistency from lot to lot due to the specificity of subtilisin for particular amino acid residues. In addition, this assay is slow, insensitive, and relatively inaccurate. *N*-4-Toluenesulphonyl-L-arginine methyl ester (TAME) has been employed as a substrate using pH-stat procedure for activity, and *N*-benzoyltyrosine ethyl ester has been used with a spectrophotometric procedure (7). In addition, a spectrophotometric active-site titration using *N-trans*-cinnamoylimidazole has been employed for kinetic studies (8). These methods are limited by a lengthy analysis time, inability to quantify the enzyme at low concentrations, or the relatively poor precision of the results.

The purpose of the present study was to develop a simple and rapid analytical procedure that would provide a reproducible indication of the hydrolytic activity of subtilisin with good precision.

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METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Shimadzu Model UV-160 double beam (Shimadzu Scientific Instruments, Inc., Columbia, MD 21046) equipped with microprocessor-driven internal timer for repetitive scanning and thermostated cell holder. Cell is thermostatically controlled to 25 ± 0.5°C using circulating water. Disposable 1 cm plastic cuvets (Baxter Healthcare Corp., McGaw Park, IL 60085) were used.

(b) *Hand-held mixer.*—Bio-Vortexer (Cole-Parmer Instrument Co., Chicago, IL 60648).

(c) *Buffer solution.*—Dissolve 14.2 g sodium phosphate dibasic, 22.4 g potassium chloride, and 0.036 g EDTA disodium salt (all ACS reagent grade, J.T. Baker Inc., Phillipsburg, NJ 08865, in 900 mL water, adjust to pH 8.0 with hydrochloric acid, and dilute to 1 L.

(d) *Substrate solution.*—Dissolve 0.25 g *N*-carbobenzoxy-L-leucine-*p*-nitrophenyl ester (Sigma Chemical Co., St. Louis, MO 63178) in 25 mL dimethylsulfoxide (ACS reagent grade, Fisher Scientific, Pittsburgh, PA 15219).

(e) *Subtilisin standard.*—Standard was established from commercial lot of subtilisin (Enzyme Development Corp., New York, NY 10121) by comparing its activity using proposed method to independently established activity of reference subtilisin (Boehringer Mannheim Biochemicals, Indianapolis, IN 46250). Sample (1 mg) of standard material had enzyme activity equivalent to 25 (μg) (4%) of reference subtilisin (on basis of 5 replicate determinations).

Preparation of Standard Curve

Dilute weighed sample of subtilisin standard with water to prepare 2.5 mg/mL stock solution. Prepare working standards of 0.25, 0.50, 0.75, and 1.0 mg/mL subtilisin standard in water by serial dilution of stock solution. Prepare stock standard solution and dilute working standard solutions fresh daily. Assay working standard as described below. Plot corrected absorbance differences against concentration to obtain linear standard curve with no significant y-intercept value.

Preparation of Sample

Dilute weighed sample containing equivalent of approximately 25 mg subtilisin material with 50 mL water.

Assay

Pipet 2.8 mL buffer solution into each of 2 cuvets, one for blank measurement and other for test measurement. Add 0.10 mL substrate solution to each cuvet and mix thoroughly using the hand-held mixer. Adjust absorbance reading of spectrophotometer to zero with cuvets in cell holders. Pipet 0.10 mL water into blank cuvet and 0.10 mL aliquot of either working standard or sample into test cuvet. Mix thoroughly. Two and 3 min after addition of working standard or sample, measure absorbance of *p*-nitrophenol in test solution at 400 nm against blank. Calculate corrected absorbance difference by subtracting absorbance at 2 min from that at 3 min.

Table 1. Comparative assay results for activity units of different subtilisin materials by proposed method and by USP casein digestion method

Subtilisin sample	Proposed method, AU/mg	Casein digestion, USP units/mg
Crystalline[a]	22	16900
A	1.0	920
B	1.1	1390
C	3.8	5450
D	5.0	5070

[a] Subtilisin from Boehringer Mannheim Biochemicals, with no inert additives. Other samples were white or tan powders.

Determine enzyme concentration by interpolation from standard curve. Enzyme concentration may be expressed in activity units (AU): 1 mg subtilisin standard = 1 AU.

Results and Discussion

The application of *p*-nitrophenyl amino acid esters as substrates for hydrolytic enzymes has been reported, and the relative rate of nonenzymatic hydrolysis of various amino acid substrate analogs has been calculated (9). The CBZ-L-leucine analog was chosen as the substrate for the proposed method based on its reportedly low rate of nonenzymatic hydrolysis. Other amino acid analogs were evaluated but either gave significant absorbance at 400 nm due to autolysis or had limited solubility in the buffer system. These compounds included *N*-CBZ-L-phenylalanine-*p*-nitroanilide and Ala-*p*-nitroanilide.

The rate of formation for *p*-nitrophenol from the substrate catalyzed by the subtilisin standard was linear with respect to the enzyme concentration up to 1 mg/mL over the reaction period from 1 to 4 min following substrate addition. The most consistent results were obtained for absorbance measurements taken at the 2 and 3 min intervals following substrate addition.

A comparison of results obtained for a variety of subtilisins using the proposed method as well as a casein digestion method for proteolytic activity (6) is given in Table 1. For the 5 subtilisin materials, the correlation coefficient is 0.989, demonstrating a correspondence in results between the 2 assays. This result could indicate that the rate limiting reaction is similar for subtilisin using the 2 different substrates. In contrast, the *p*-nitrophenol method gives no measurable result when applied to a papain reference standard with known proteolytic activity. The specificity of the proposed method toward other proteases has not been determined.

The presence of buffers, salts, or enzyme activators or inhibitors in subtilisin formulations can affect the slope of the curve for the proposed assay. If excipient content is known, its effect can be compensated for by preparing the standards in the presence of these additional components rather than in water. This assay is routinely performed in the presence of borate, phosphate, carbonate, chloride, and EDTA.

Table 2. Standard curves of subtilisin generated using proposed method

Curve No.	Slope	Intercept
1	0.262	−0.001
2	0.247	0.002
3	0.251	0.004
4	0.288	0.003
5	0.246	0.013
6	0.224	0.013
7	0.246	0.006
Mean	0.252[a]	0.006
RSD, %	7.54	

[a] The slope (0.25) represents the ratio of 1 absorbance unit to 100 μg subtilisin/mL.

Standard curves were generated over a 6 week period using the proposed method to demonstrate reproducibility. The results for 7 standard curves are given in Table 2. The precision of the method was determined by the analysis of 20 replicates of a cleaning tablet. Results ranged from 17.5 to 22.3 AU/sample (mean, 19.9; RSD, 8.0%). The data indicate that the method is precise, reproducible, and representative of the total hydrolytic activity of subtilisin in the cleaning formulations.

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DRUG FORMULATIONS

Liquid Chromatographic Determination of Penicillin V Potassium in Tablets: Collaborative Study

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A liquid chromatographic method for the determination of penicillin V potassium in tablets was collaboratively studied by 7 laboratories. The method uses an octadecyl silane reverse-phase column, a mobile phase of acetonitrile–methanol–0.01M potassium phosphate monobasic (21 + 4 + 75 v/v/v), photometric detection at 225 nm, and sulfadimethoxine as an internal standard. Each collaborator received 6 samples: powdered composites of 2 commercial tablet preparations and 1 synthetic tablet powder mixture, each with blind duplicates. The mean repeatability and reproducibility relative standard deviations for commercial samples were, respectively, 1.10 and 1.46% (250 mg dosage), and 0.84 and 2.82% (500 mg dosage). The average standard recovery from the synthetic formulation was 99.1%, with repeatability and reproducibility relative standard deviations of 1.30 and 3.66%, respectively. The method has been adopted official first action.

A simple and selective reverse-phase liquid chromatographic (LC) method for the determination of penicillin V potassium in tablets was previously described by this laboratory (1). The LC method represents a significant improvement over the time-consuming microbiological and nonselective iodometric titration methods for penicillin V in oral dosage forms, which are specified in the *Code of Federal Regulations* (2). The *U.S. Pharmacopeia* (USP) has proposed a similar LC assay procedure for penicillin V, which uses a mobile phase of water, acetonitrile, and glacial acetic acid (650 + 350 + 5.75), a penicillin G resolution compound, and detection at 254 nm (3). The present report describes a collaborative study of the LC method.

Collaborative Study

Seven collaborators received a total of 6 samples as blind duplicates of 2 composited commercial tablet formulations, and 1 synthetic tablet mixture. The collaborators were also provided with vials of the standard drug substance and detailed instructions. Each collaborator was requested to submit all relevant data, chromatograms, summary of results, and comments to the Associate Referee.

Penicillin V Potassium in Tablets

Liquid Chromatographic Method

First Action

Method Performance:

250 mg/tablet

s_r = 1.17; s_R = 1.55; RSD_r = 1.10%; RSD_R = 1.46%

500 mg/tablet

s_r = 0.90; s_R = 3.01; RSD_r = 0.84%; RSD_R = 2.82%

Recovery

s_r = 1.29; s_R = 3.63; RSD_r = 1.30%; RSD_R = 3.66%

A. Principle

Penicillin V potassium is determined by isocratic liquid chromatography on reverse-phase C_{18} column, using mobile phase of CH_3CN–CH_3OH–0.01M KH_2PO_4 (21 + 4 + 75 v/v/v), photometric detection at 225 nm, and sulfadimethoxine as internal standard.

B. Apparatus

(a) *Liquid chromatograph.*—Model 510 solvent pump, Model 481 variable wavelength detector, Model U6K injector valve, and Model 730 integrating recorder (Waters Chromatography Div.), or equivalent LC system.

(b) *Column.*—Stainless steel, 30 cm × 3.9 mm id prepacked with 10 μm μBondapak C_{18} (Waters Chromatography Div.) preceded by 40 mm LC guard column, 30–50 μm, Co:Pell ODS (Whatman Inc.), or equivalent column that meets system suitability requirement, *E.*

C. Reagents

(a) *Solvents.*—LC grade acetonitrile and methanol.

(b) *Potassium dihydrogen phosphate solution.*—0.01M KH_2PO_4, 1.361 g/L. Pass solution through suitable filter of 0.5 μm or finer porosity.

(c) *Mobile phase.*—CH_3CN–CH_3OH–0.01M KH_2PO_4 (21 + 4 + 75 v/v/v). Deaerate by suitable means (e.g., vacuum, sonication).

(d) *Penicillin V potassium standard solution.*—Accurately weigh ca 12 mg USP Penicillin V Potassium Reference Standard into 100 mL volumetric flask. Add 15.0 mL internal standard solution, dilute to volume with water, and mix well.

(e) *Internal standard solution.*—Dissolve USP Sulfadimethoxine Reference Standard in 50% CH_3CN–H_2O to obtain concentration ca 0.4 mg/mL.

D. Sample Preparation

Weigh and powder ≥20 tablets. Transfer portion of powder to volumetric flask of size suitable to obtain final concentration of ca 0.6 mg penicillin V potassium/mL. Dilute to volume with water, mix well, and filter. Pipet 20.0 mL filtrate into 100 mL volumetric flask, add 15.0 mL of internal standard solution, dilute to volume with water, and mix well.

E. Suitability Test and Determination

Equilibrate column with mobile phase flowing at 0.5 mL/min for ≥4 h prior to injection. Inject 20 μL each of standard solution and sample solution into liquid chromatograph by means of suitable sampling valve or microsyringe. Pump mobile phase at 1.0–1.5 mL/min and monitor absorption at 225 nm. Adjust sensitivity so that peak response for penicillin V is between 40 and 75% full scale deflection. In suitable system, coefficient of variation of peak response ratios of 5

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The recommendation has been 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 was adopted official first action at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73**, January/February issue.

Table 1. Collaborative results for determination of penicillin V potassium in commercial and synthetic tablets

Coll.	Found, % of declared 250 mg/tab.	500 mg/tab.	Rec., %[a]
1	105.8	105.8	100.6
	106.5	106.5	99.9
2	107.3	105.0	99.2
	106.4	106.0	98.2
3	109.1	106.1	100.4
	108.3	104.2	100.3
4	107.1	106.2	102.0
	102.9	105.3	101.3
5	106.4	112.4	92.9
	106.6	112.2	92.2
6[b]	98.8	100.5	80.6
	103.0	89.5	98.8
7	107.6	105.1	101.0
	107.4	104.9	101.0
Mean	106.8	106.6	99.1
SD	1.52	2.72	3.21
s_r	1.17	0.90	1.29
s_R	1.55	3.01	3.63
RSD, %	1.43	2.55	3.24
RSD_r, %	1.10	0.84	1.30
RSD_R, %	1.46	2.82	3.66

[a] Recovery from synthetic tablet formulation prepared to contain 54.3 mg penicillin V potassium per 100 mg formulation.

[b] Outlier by Cochran test. Not included in statistical evaluation.

replicate injections of standard preparation is ≤1.5; resolution factor, R, is ≥3; and tailing factor, T, for penicillin V peak is ≤2.

Calculate resolution factor, R, as follows:

$$R = [2(t_2 - t_1)]/(W_2 + W_1)$$

where t_2 and t_1 = retention times of the 2 components, and W_2 and W_1 = corresponding widths of bases of peaks, obtained by extrapolating relatively straight sides of peaks to baseline.

Calculate tailing factor, T, as follows:

$$T = W_{0.05}/2f$$

where $W_{0.05}$ = distance from leading edge to training edge of peak; and f = distance from peak maximum to leading edge of peak, both measured at point 5% of peak height from baseline.

F. Calculations

Calculate quantity of penicillin V in portion of composite taken as follows:

$$\text{Penicillin V, mg/tab} = (R/R') \times C \times D \times (T/W) \times (1/F)$$

where R and R' = average response ratios (peak area or height) relative to internal standard for sample and standard solutions, respectively; C = concentration of standard solution, U/mL; D = sample dilution factor; T = average tablet weight, g; W = sample composite taken for analysis, g; and F = equivalency factor of penicillin V, 1600 U/mg.

Ref.: JAOAC **72,** November/December issue (1989).
CAS-132-98-9 (penicillin V potassium)
CAS-87-08-1 (penicillin V)

Results and Discussion

The analytical results obtained by the 7 collaborators are summarized in Table 1. Statistical evaluation of the method was performed with data from 6 collaborators (36 determinations). The data from Collaborator 6 were eliminated as outliers because duplicate results failed Cochran's test for homogeneous values; the wide variation in results between duplicate samples was attributed to failure to properly equilibrate the chromatographic system. The results from Collaborator 5 were not rejected even though they failed Dixon's test, because these results indicated good reproducibility and the source of random errors could not be established. For the 6 sets of data evaluated, repeatability and reproducibility relative standard deviations (RSD_r and RSD_R) for commercial tablet samples were, respectively, 1.10 and 1.46% for the 250 mg potency and 0.84 and 2.82% for the 500 mg potency. The recovery of known spikes of drug substance from synthetic tablet mixtures showed RSD_r and RSD_R values of 1.30 and 3.66%, respectively.

Collaborators' Comments

The 6 collaborators whose data were included in the statistical evaluation reported no major problems with the method. However, Collaborators 3 and 4 observed minor chromatographic fronting and small peaks associated with the penicillin peak. This anomalous behavior, apparently caused by the amphoteric nature of penicillin and by interaction of the solvent with the column, was resolved by increasing the time of column equilibration with the mobile phase or by changing columns to meet system suitability requirements.

Of the 7 collaborators, four used Waters μBondapak C_{18} columns, Collaborator 2 used an EM Science Hibar LiChrosorb RP-18 column, and Collaborator 6 used a Varian Micropak C_{18} IP-4 column.

Recommendation

The proposed LC method is suitable for the determination of penicillin V in tablets and represents a significant improvement over the CFR microbiological method (2). Advantages of the LC method include greater precision and selectivity, comparable sensitivity, and the ability to detect degradation products (1).

It is recommended that the liquid chromatographic method for determination of penicillin V potassium in tablets be adopted official first action.

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Spectrophotometric Determination of Some Benzene Sulfonamides with 7,7,8,8-Tetracyanoquinodimethane

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A new spectrophotometric method for the determination of some unsubstituted benzene sulfonamides is presented. The method is based on the interaction of these derivatives with 7,7,8,8-tetracyanoquinodimethane at pH 9.0–9.5 to produce intense blue products. The quantitation of the products was carried out at 578 nm. Beer's law was obeyed over a wide range of concentrations for all sulfonamide compounds studied. Optimum analytical conditions were determined, and the color produced was stable for at least 90 min at 25°C. Analytical data for determination of sulfonamide compounds in pure form are presented together with application of the proposed method for analysis of some commercially available pharmaceutical preparations. The results are in good agreement with those obtained by official procedures.

Several compendial and noncompendial methods have been reported for the quantitative determination of the studied sulfonamides in pure form, in commercial pharmaceutical preparations, and in biological fluids. These methods include titrimetric (1–5), chromatographic (6–14), densitometric (15), ultraviolet and visible spectrophotometric (1, 2, 15–22), and fluorometric (23) procedures.

7,7,8,8-Tetracyanoquinodimethane (TCNQ) is a strong electron-acceptor and has been used for the quantitation of numerous electron-donors such as vitamin A (24), alkaloids (25), procaine and related local anesthetics (26), some nitrogen bases (27), some drugs containing imidazoline ring (28), antihistamines (29), pentazocaine (30), and some sulfa drugs (31).

TCNQ, in the presence of sodium acetate, was used successfully in the present study for determination of 8 unsubstituted sulfonamides in pure form and in commercial pharmaceutical preparations. The reaction conditions were standardized. The proposed method is simple, sensitive, and accurate.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Zeiss Model No. PM2DL (Carl Zeiss West Germany, Oberkochen, GFR).

(b) *Chemicals.*—Pharmaceutical grade chlorthalidon (Swiss-Pharma), clopamide (Sandoz), frusemide (Hoechst), mefruside (Bayer), quinethazone (Lederle), sulfanilamide (Memphis), sulthiame (Bayer), and *p*-toluene sulfonamide were obtained as gifts and used as working standards without further treatment. Use analytical grade solvents.

(c) *TCNQ solution.*—0.15% w/v TCNQ in acetonitrile. Prepare fresh daily.

(d) *Sodium acetate solution.*—0.5M sodium acetate in water.

(e) *Dosage forms.*—Various commercial preparations purchased from local sources.

(f) *Thin-layer plates.*—Precoated (0.25 mm) aluminum silica gel sheets, 20 × 20 cm (fluorescent indicator, E. Merck, Darmstadt, GFR).

Preparation of Standards

Accurately weigh 40 mg each sulfonamide compound and dissolve as free base in 100 mL acetonitrile. Dilute 5 mL aliquot to 100 mL with same solvent to obtain working standard solution of 20 μg/mL for each drug.

Preparation of Samples

Tablets.—Weigh and powder 20 tablets. Accurately weigh portion of powder, equivalent to 40 mg each sulfonamide compound, and transfer to 100 mL volumetric flask. Add 70 mL acetonitrile and shake 10 min; then dilute to volume with acetonitrile. Filter and discard first portion of filtrate. Use clear solution as stock solution. Dilute 5 mL aliquot to 100 mL with acetonitrile to obtain 20 μg/mL for each drug. Use these solutions as final sample dilutions.

Injections.—Mix well contents of 10 ampoules. Accurately measure volume of injections, equivalent to 20 mg frusemide and dilute quantitatively with acetonitrile to obtain 20 μg frusemide/mL. This is final sample dilution.

Recovery study.—Accurately weigh quantity of declared drug for each preparation and transfer to 100 mL volumetric flask containing accurately weighed portion of powdered tablets or accurately measured volume of injections. Either dissolve contents of flask in acetonitrile and treat as described for tablets or dilute quantitatively with acetonitrile to obtain required concentration as described for injections.

Determination

Pipet 5 mL standard or sample sulfonamide solution into 50 mL volumetric flask. Add 5 mL TCNQ solution followed by 5 mL sodium acetate solution, then let stand about 5 min. Dilute to volume with acetonitrile and let stand 25 min at 25°C. Measure absorbances of solutions at 578 nm in 1 cm cells against reagent blank. Prepare reagent blank as described above, except use 1 mL acetonitrile instead of standard or sample sulfonamide solution.

Thin-Layer Chromatography Study

Apply about 20 μL each of acetonitrile solution of standard *p*-toluene sulfonamide, *p*-toluene sulfonamide–TCNQ reaction mixture, reagent blank, TCNQ, and TCNQ radical anion obtained by iodide reduction method (26) on same TLC plate. Place prepared plate in saturated tank containing developing solvent system (chloroform–methanol, 80 + 20 v/v). After development to 14–15 cm, air-dry plates and examine in daylight and under UV lamp.

Results and Discussion

The proposed method was successfully used for the analysis of some unsubstituted sulfonamides in pure form and in commercial pharmaceutical preparations. The method involves interaction of sulfonamides with TCNQ at pH 9.0–9.5 to produce intense blue products. Figure 1 shows the absorption spectra of the colored products from *p*-toluene sulfonamide and clopamide as representative examples of the sulfonamides studied. Absorbances were measured at 578 nm, which is the most intense peak. In addition, the reagent blank has negligible absorbance readings in the range of 540–620 nm against pure solvent. Therefore, the very low reagent background obviously recommends this method for routine analysis with increased sensitivity and minimum interferences.

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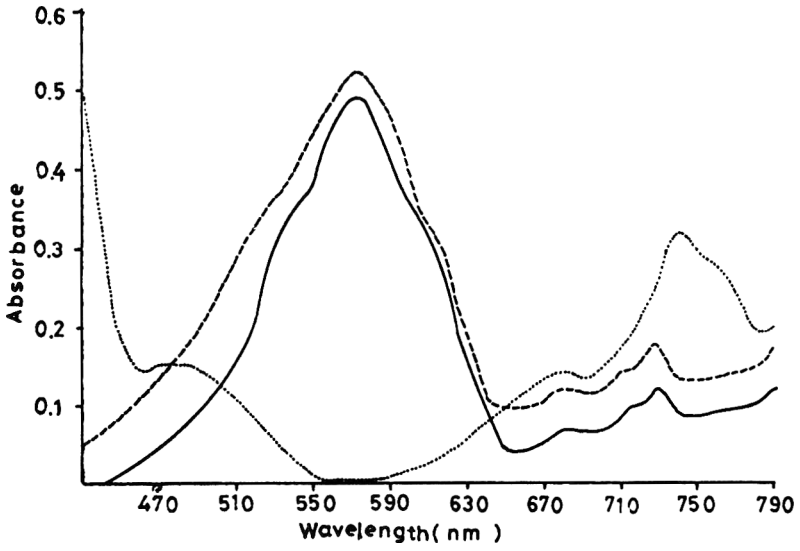


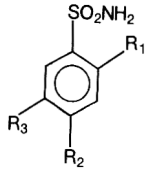
Figure 1. Absorption spectra of colored products of *p*-toluene sulfonamide (- - - - - -) and clopamide (———), as well as reagent blank (• • • • • • • • • • •) measured against pure solvent.

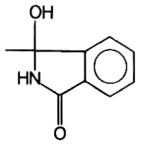
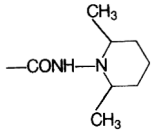
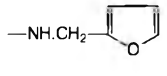
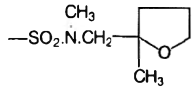
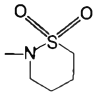
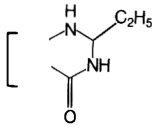
Beer's law was obeyed for all sulfonamide compounds studied in the general concentration range of 0.5–8.0 μg/mL. Table 1 shows typical linear regression correlations for each drug studied. Coefficients of variation were ≤2% for separate determinations at different concentration levels for each drug.

Effect of TCNQ Concentration

Figure 2 shows the effect of TCNQ concentration on color intensity of reaction products of *p*-toluene sulfonamide and clopamide. Highest color intensity and most reproducible results were obtained by using TCNQ concentrations from 0.12 to 0.27%. Therefore, 0.15% TCNQ solution is recommended.

Table 1. Linear regression correlations for sulfonamide drugs in study



Drug	R_1	R_2	R_3	εmax. at 578 nm	Slope[a] (b)	Intercept[a] (a)	Corr. coeff.
p-Toluene sulfonamide	H	CH_3	H	2.36×10^4	0.135	0.0160	1.0000
Sulfanilamide	H	NH_2	H	2.40×10^4	0.137	0.0170	0.9999
Chlorthalidone	Cl	H		4.23×10^4	0.124	0.0037	0.9998
Clopamide	Cl	H		5.09×10^4	0.146	0.0070	0.9999
Frusemide	Cl		—COOH	4.56×10^4	0.134	0.0193	0.9998
Mefruside	Cl	H		4.75×10^4	0.134	0.0144	0.9998
Sulthiam	H		H	5.26×10^4	0.180	0.0077	0.9999
Quinethazone	Cl	4, 5		5.93×10^4	0.202	0.0136	0.9999

[a] In all cases, n = 12

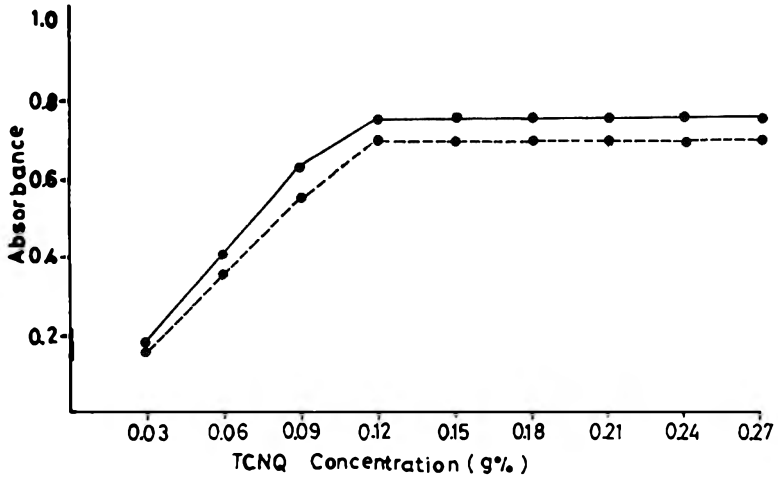


Figure 2. Effect of TCNQ concentration on absorption intensity of colored products of 5 μg *p*-toluene sulfonamide/mL (- - - ● - - -), and 5 μg clopamide/mL (—●—).

Table 2. Effect of pH on absorbance (at 578 nm) of reaction product of clopamide (5 μg/mL) with TCNQ in water–acetonitrile solution (1 + 9)

	Buffers			
pH	A	B	C	D
7.0	—	—	0.000	—
7.5	—	—	0.040	—
7.8	0.080	—	0.070	—
7.9	—	—	0.080	0.050 (0.001)
8.0	0.102	—	0.090	—
8.4	0.437	—	0.420	0.514 (0.01)
8.8	0.612	—	0.600	—
8.9	—	—	0.610	0.745 (0.1)
9.0	0.660	—	0.623	0.753 (0.2)
9.1	—	—	0.628	0.755 (0.3)
9.2	0.662	0.653	0.622	0.751 (0.45)
9.3	—	—	0.624	0.756 (0.7)
9.4	0.664	0.655	0.620	0.752 (1.1)
9.5	—	—	0.622	0.750 (1.7)
9.6	0.648	0.640	0.600	—
9.7	—	—	0.590	—
9.8	0.610	0.609	0.570	—
9.9	—	—	0.554	—
10.0	0.570	0.582	0.533	—

A = Clark and Lubs buffer, pH 7.8–10.0 (32).
B = Kolthoff and Vleeschhouwer buffer, pH 9.2–11.0 (32).
C = Theorell and Stenhagen buffer, pH 2.0–12.0 (32).
D = Sodium acetate solution (molarity).

Effect of pH

Studies on the effect of pH showed that the reaction is strongly pH-dependent. No blue products were produced at pH ≤7. Highest color intensity was obtained in the pH range 9.0–9.5 (Table 2). Significant differences in the intensity and stability of colors were observed when the constituents of buffers were different. Maximum color intensity was obtained by using sodium acetate solution (0.2–2.0M).

Effect of Dilution by Different Solvents

Acetonitrile, dimethylformamide, dimethylsulfoxide, isobutyl methyl ketone, methylene chloride, 1,2-dichloroethane, chloroform, dioxane, methanol, and ethanol were examined as solvents. Maximum color stability and intensity were achieved when acetonitrile was used as a diluent. In addition, the solvent power of acetonitrile for TCNQ (up to 0.3%) and for all drugs studied is good. No other solvent studied was as satisfactory.

Effect of Temperature and Time

The color was produced at room temperature; no heating was required. Optimum reaction time was determined by following the color development at 25°C in a thermostatically controlled water bath. Figure 3 illustrates the results of this study with clopamide as a representative example of the sulfonamides studied. The color develops completely in 20 min after dilution with the solvent and remains stable for at

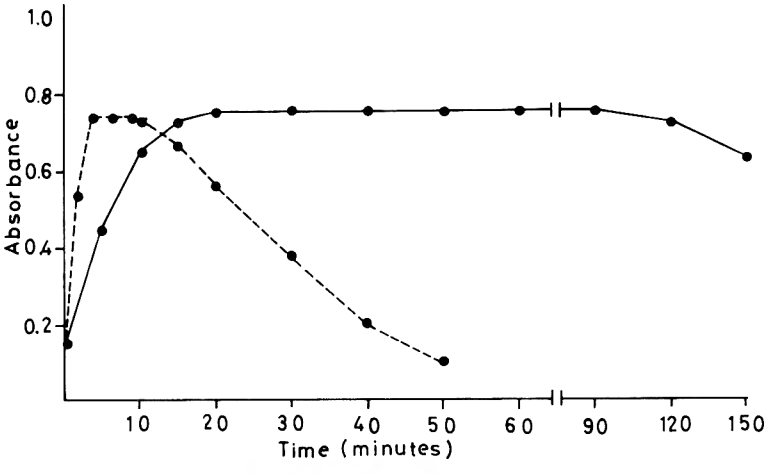


Figure 3. Effect of temperature and reaction time on absorption intensity of colored products of 5 μg clopamide/mL; (—●—) 25°C, (- - - ● - - -) 60°C.

Table 3. Analysis of some unsubstituted sulfonamides in commercial pharmaceutical preparations by proposed and official methods[a]

Product	Source	Content	Label claim, mg	Proposed method			BP method (1980)
				Found, %	Added	Recovery, %	Found, %
Hygroton tablets	Swiss-Pharma	chlorthalidone	50/tab.	96.45 ± 0.84 t = 0.333 F = 1.16	50	99.74 ± 0.70	96.28 ± 0.78
Brinaldix tablets	Sandoz	clopamide	20/tab.	97.35 ± 0.98	20	99.83 ± 0.59	—[b]
Lasix tablets	Hoechst	frusemide	40/tab.	98.21 ± 0.89 t = 0.143 F = 1.99	40	99.76 ± 0.65	98.14 ± 0.63
Lasix ampoules	Hoechst	frusemide	10/mL	96.58 ± 0.59 t = 0.764 F = 0.82	10	99.97 ± 0.50	96.28 ± 0.65
Baycaron tablets	Bayer	mefruside	50/tab.	98.21 ± 0.79	50	99.05 ± 0.50	—[b]
Aquamox tablets	Lederle	quinethazone	50/tab.	96.49 ± 0.71 t = 0.815 F = 0.790	50	99.04 ± 0.69	96.10 ± 0.80[c]
Ospolot tablets	Bayer	sulthiame	50/tab.	96.99 ± 0.80 t = 1.60 F = 0.91	50	100.07 ± 0.52	97.82 ± 0.84

[a] Average of 5 determinations ± SD.
[b] Not an official preparation.
[c] Analyzed according to USP 1980. Theoretical values at 95% confidence limit, t = 2.78 and F = 6.39.

least 90 min. From the other point of view, heating increases the reaction rate but decreases the color stability. A marked loss of color results at temperatures above 50°C.

Specificity of Reaction

To assess the specificity of the method for analysis of unsubstituted sulfonamides, several drugs and related compounds were tested under the recommended reaction conditions. Substituted sulfonamides (R-Ar-SO_2NHR'), sulfonacetamides (R-Ar-$SO_2NHCOCH_3$), and sulfonylureas (R-Ar-$SO_2NHCONH$-R') exhibited zero absorbance at 578 nm. Primary aromatic amines produced yellow-to-orange products with absorption maxima at 330 and 475 nm. They exhibited negligible absorbance at 578 nm. The reaction products formed with different unsubstituted sulfonamides possess similar spectral characteristics. Thus, the method is not suitable for analysis of their mixtures without prior separation. However, most drugs studied are commercially available in plain dosage forms, and the problem of interference does not arise.

Analysis of Dosage Forms

The proposed method was applied for the determination of chlorthalidone, clopamide, frusemide, mefruside, quinethazone, and sulthiam as the drug entities in various pharmaceutical formulations. The results were compared statistically with those obtained by applying the official methods (1, 2). In the t- and F-tests, no significant differences were found between the calculated and theoretical values (95% confidence) of the proposed and official methods (Table 3). This indicates similar precision and accuracy. In addition, recovery experiments were carried out for each drug in its respective pharmaceutical formulations. As shown in Table 3, the recoveries (99.04–100.07%) indicate the absence of interference from frequently encountered excipients, additives, or coloring matter.

The molar ratio determined according to Job's method of continuous variation (33) indicated a ratio of 1:1 for sulfonamides and TCNQ in the presence of 0.5M sodium acetate (Figure 4).

The possible mechanism of the reaction of TCNQ with

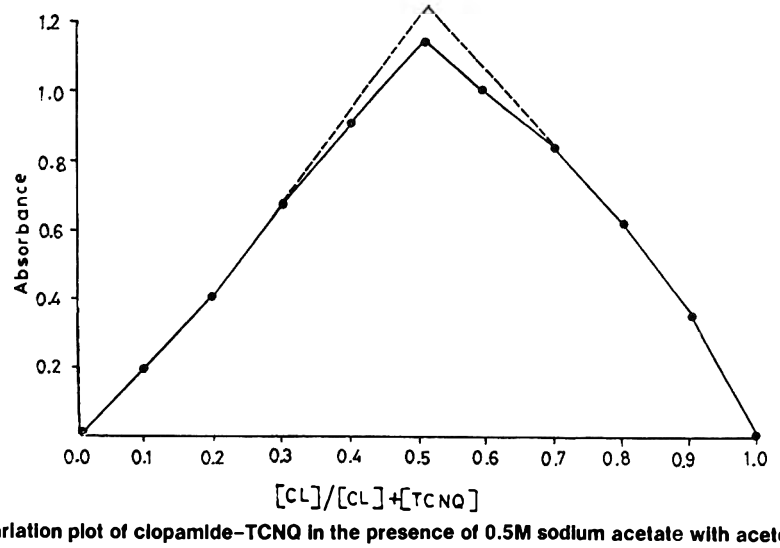


Figure 4. Continuous variation plot of clopamide–TCNQ in the presence of 0.5M sodium acetate with acetonitrile as dilution solvent.

Table 4. Spot colors (TLC), R_f values, and wavelength maxima of eluted spots of *p*-toluene sulfonamide–TCNQ reaction mixture, reagent blank, TCNQ, and TCNQ radical anion

Color	R_f	Wavelength maxima	Reaction mixture	Reagent blank	TCNQ	TCNQ radical anion
Yellow	0.96	(395)[c], 840	++	++	++++	+
Yellowish green	0.85	420, 680, 745, (845)	+	++	+	++++
Orange	0.65	(325)	+	+	–	–
Violet[a]	0.58	(578), 603	++++	–	–	–
Colorless[b]	0.25	–	–	–	–	+++

[a] Changed to blue on 5 min exposure to air or UV radiation.

[b] Changed to faint yellow on exposure to air.

[c] Numbers in parentheses represent peaks of the highest absorption intensity.

unsubstituted sulfonamides is not a simple one. First, the products absorbed appreciably at 325, 420, 680, 735, 805 and 845 nm, in addition to the major peak at 578 nm. Second, the multiplicity of spots on the TLC plates (Table 4) indicates that the reaction is more than the formation of TCNQ-radical anion. Third, TCNQ is a reactive compound of a multifunctional nature. Polycyano-1,4-benzoquinone methide is a strong electron acceptor and has enhanced reactivity toward the neucleophilic addition of nitrogenous compounds (34–36). In the end, the major violet product could be isolated by several techniques, including column chromatography and extraction procedures using different solvents with different polarities. Unfortunately the isolated products could not be successfully characterized because they were unstable.

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ENVIRONMENTAL ANALYSIS

Liquid Chromatographic Method for Determination of Extractable Nitroaromatic and Nitramine Residues in Soil

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An analytical method was developed to determine the concentration of nitroaromatic and nitramine residues in soil. Air-dried soil samples are ground with a mortar and pestle and extracted with acetonitrile in an ultrasonic bath. A portion of extract is diluted with aqueous $CaCl_2$ to flocculate suspended particles, filtered, and analyzed by liquid chromatography. The method provides linear calibration curves over a wide range of concentration. Detection limits ranged from 0.03 to 1.27 μg/g. Recovery of spiked analyte was better than 80% for all analytes tested. Each step in the analytical procedure was optimized using spiked and field-contaminated soils. This optimization included tests to (*1*) assess the effectiveness and kinetics associated with various extraction methods, solvents, and soil-to-solvent ratios; (*2*) compare separations achievable using various combinations of reverse-phase columns and eluants; (*3*) assess analyte recovery and ease of use for various procedures to remove particles from extracts; and (*4*) document stability of soil extracts and analytical stock and working standards. A ruggedness test and a preliminary assessment at 2 other laboratories indicated that the method was sufficiently rugged to justify a full-scale collaborative test. A comparison of extraction kinetics for spiked soil vs field-contaminated soil revealed very different kinetic behavior, indicating it is unwise to develop extraction procedures based solely on spiked soils.

A common environmental problem at many U.S. Army installations is the presence of soil contaminated with munitions residues (1). This contamination has occurred over the last 50 years due to waste discharges from munitions manufacturing and the destruction of out-of-date or off-specification material. The most common residues contain 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and associated nitroaromatic and nitramine impurities and degradation products (Figure 1). Unlike many other organic contaminants, TNT and RDX have little affinity for surface soils and can rapidly migrate to produce groundwater pollution (1, 2). To locate contamination sources, a method was needed to enable the rapid, precise, and sensitive determination of these analytes in a wide range of soil and sediment matrixes.

Because a number of functionally similar analytes are often present coincidentally, the method of choice must include a separation step. Existing methods for one or more of these analytes in environmental matrixes and forensic analysis have used thin-layer chromatography (3–5); gas chromatography with a variety of detectors including the electron-capture detector (6–13), thermal energy analyzer (TEA) (14–16), nitrogen-phosphorus detector (17), flame ionization detector (18), and mass spectrometry (19, 20); and liquid chromatography (LC) using ultraviolet (UV) detection (13, 17, 21–27), TEA (28–30), electrochemical detection (31–33), electron capture (34), and LC/MS (35–36). None of these methods, however, has been optimized for this specific suite of contaminants in a soil matrix. Because some of these explosives are both high melting and thermally unstable (13), LC was selected as the most promising technique.

All of the analytes of interest are polar (octanol/water partition coefficients ranging from 1.3 to 263) and a polar extractant was found to be most efficient. To allow direct analysis of this polar extract, reverse-phase LC (RPLC) methods were investigated. Use of normal-phase LC would require a solvent exchange step to transfer analytes into a nonpolar solvent. A method for several of these analytes in water, based on RPLC, has been previously developed and successfully validated in a full-scale collaborative test (24).

Since few commercial laboratories are equipped to use either the electrochemical detector or TEA with LC, and the intent is to develop a method for general use, we concentrated on UV detection. A wavelength of 254 nm was chosen because a variety of manufacturers offer a fixed-wavelength detector based on the mercury lamp at 254 nm and all the analytes of interest absorb strongly at this wavelength, while potential interferences such as chlorinated organic compounds do not.

An initial study indicated that ultrasonic extraction was particularly suitable for these analytes in soil (37). The objective of the study reported here was to carefully develop a step-by-step method based on ultrasonic extraction and RPLC determination which could subsequently be validated via a full-scale collaborative test.

Experimental

Analytical standards for the following were obtained from the U.S. Army Toxic and Hazardous Materials Agency, Aberdeen Proving Ground, MD: octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 1,3,5-trinitrobenzene (TNB), 1,3-dinitrobenzene (DNB), nitrobenzene (NB), methyl-2,4,6-trinitrophenylnitramine (tetryl), 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT). Standard material for 2-amino-4,6-dinitrotoluene (2-AmDNT) and 4-amino-2,6-dinitrotoluene (4-AmDNT) was obtained from David Kaplan, Natick Laboratories, Natick, MA. Individual stock standards were prepared in acetonitrile (ChromAR HPLC grade, Mallinckrodt). Methanol used in the preparation of the eluant was Baker Analyzed Reagent Grade. Water used in the eluant was obtained from a Milli-Q reagent water system (Millipore Corp.).

Field-contaminated soils used for method development were obtained from a variety of military sites in 5 states. Uncontaminated soils used in spiking experiments were obtained locally. All soils were air-dried to constant weight and ground with a mortar and pestle to pass a 30 mesh sieve (22).

LC separations were obtained on a modular system composed of a Spectra Physics SP8810 isocratic pump, a Dynatech Precision Sampling LC-241 autosampler equipped with a Rheodyne Model 7010A sample loop injector, a Spectra Physics SP8490 variable wavelength UV detector set at 254

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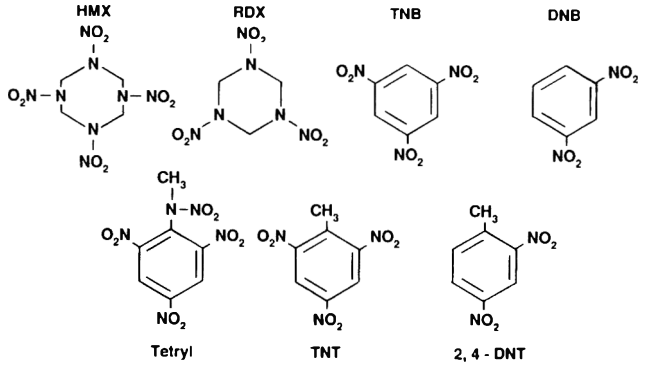

Figure 1. Structures of primary analytes.

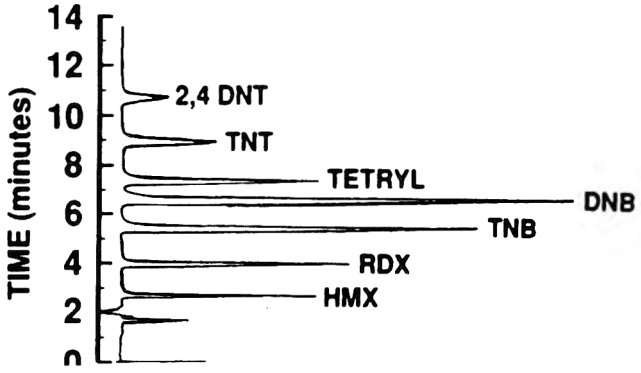

Figure 2. Chromatogram of standards for 7 primary analytes on LC-18 column eluted with 1.5 mL/min of methanol–water (1 + 1).

nm, and a Hewlett-Packard HP3393A digital integrator. Samples were introduced by overfilling a 100 μL loop. Results were generally obtained in the peak height mode, which was found to give much better reproducibility for low concentration samples than automated peak area measurements.

Soils were extracted with acetonitrile in an ultrasonic bath (Cole Parmer 8855-10 Ultrasonic Cleaner). Extracts were filtered through Millex SR 0.5 μm membrane filters (Millipore Corp.). The optimization of the various steps in this method is the subject of this investigation.

Results and Discussion

Analytical Separations

Several reverse-phase columns (Supelco) were tested including LC-1, LC-8, LC-18, LC-CN, LC-DP, LC-NH_2, and LC-diol under isocratic conditions using a variety of binary and tertiary eluants composed of water, methanol, and acetonitrile. The initial objective was to find a column/eluant combination that could provide baseline separation of HMX, RDX, TNB, DNB, tetryl, TNT, and 2,4-DNT under isocratic conditions within 15 min. A 25 cm × 4.6 mm LC-18 column (5 μm) eluted with methanol–water (1 + 1, v/v) at 1.5 mL/min (Figure 2) satisfied this objective. Retention times and capacity factors for the primary analytes, potential interferences, and degradation products are presented in Table 1. Separation on a 25 cm × 4.6 mm LC-CN column (5 μm), using the same eluant and flow rate, was chosen for analyte confirmation because the order of elution (Table 1) was very different. Details on separations achievable using other column/eluant combinations are presented elsewhere (38).

We have used these 2 columns to analyze extracts from over 100 soil samples from 14 states. In no case were major interferences observed which precluded the use of the LC-18 column for quantitation. When high concentrations of the analytes were found, however, unidentified shoulders on the major peaks were observed (Figure 3). Use of the LC-CN column for analyte confirmation proved to be very useful. The analytes most often observed in field contaminated soils were TNT, RDX, TNB, 2,4-DNT, HMX, 4-Am-DNT, and 2-Am-DNT. Nitrobenzene and the isomers of nitrotoluene have never been observed in any of our analyses. Several examples of LC-18 chromatograms for extracts from field-contaminated soils are shown in Figure 3.

Linearity Testing

To determine whether detector response (integrator peak height) was a linear function of analyte concentration, 2 sets of calibration standards containing 7 analytes were independently prepared and analyzed in random order. A linear model was fit to each set of absorbance vs concentration data using standard regression analysis. The residual sums of squares were tested for significance using a lack-of-fit test at the 95% confidence level. Generally, the F-ratios indicated that the data were adequately described by a linear model. Next, a linear model with zero intercept was fitted to each data set. The residuals for the 2 models were compared. The F-ratios at the 95% confidence level indicated that a linear model through the origin adequately described the data. Thus, for daily calibration, a replicated single-point standard is sufficient. The sensitivities and demonstrated ranges of linear response are given in Table 2.

Soil Extraction

The following 4 extraction methods were compared by using field-contaminated soils for both total analyte recovery and process kinetics using methanol and acetonitrile: Soxhlet, wrist-action shaker, ultrasonic bath, and homogenizer-sonicator (37, 39). Acetonitrile was superior to methanol for extraction of RDX and HMX, in part due to their much greater solubility in acetonitrile (D.C. Leggett, U.S. Army Cold Regions Research and Engineering Laboratory), and equivalent to methanol for extraction of the nitroaromatics. Soxhlet and ultrasonic bath methods were better than methods using the wrist-action shaker or homogenizer-sonicator

Table 1. Retention times and capacity factors for major analytes and potential interferences on LC-18 and LC-CN columns[a]

Substance	Retention time, min		Capacity factor, k	
	LC-18	LC-CN	LC-18	LC-CN
HMX	2.44	8.35	0.49	2.52
RDX	3.73	6.15	1.27	1.59
TNBA	4.60	5.01	1.80	1.12
TNB	5.11	4.05	2.12	0.71
DNB	6.16	4.18	2.76	0.76
Tetryl	6.93	7.36	3.23	2.11
Nitrobenzene	7.23	3.81	3.41	0.61
TNT	8.42	5.00	4.13	1.11
4-Am-DNT	8.88	5.10	4.41	1.15
2-Am-DNT	9.12	5.65	4.56	1.38
2,6-DNT	9.82	4.61	4.99	0.95
2,4-DNT	10.05	4.87	5.13	1.05
Benzene	11.22	3.48	5.84	0.47
o-Nitrotoluene	12.26	4.37	6.48	0.84
p-Nitrotoluene	13.26	4.41	7.09	0.86
m-Nitrotoluene	14.23	4.45	7.68	0.88
Toluene	23.0	3.93	13.0	0.66

[a] Eluants were water–methanol (1 + 1) for both LC-18 and LC-CN columns. Flow rate was 1.5 mL/min for both columns. Void volumes were obtained using nitrate ion.

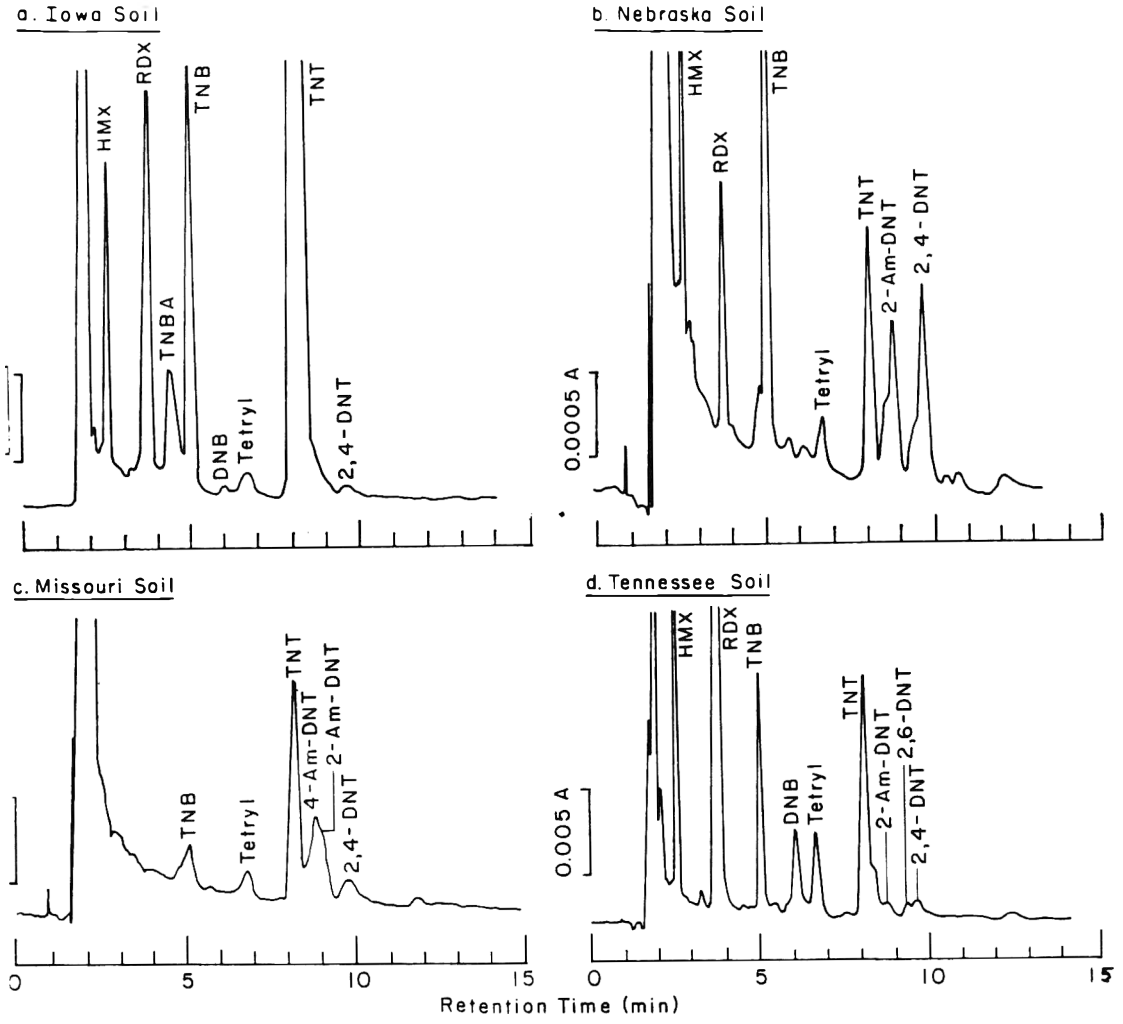

Figure 3. Chromatograms of typical soil extracts from field-contaminated soils on LC-18.

both in total amount recovered and kinetics. The sonic bath method is preferred for several reasons. The extracted residue in the Soxhlet method is held at the boiling point of the extraction solvent for long periods of time and can produce degradation of thermally labile compounds. In addition, a large number of samples can be processed simultaneously in an ultrasonic bath while an individual Soxhlet device must be used for each sample.

Additional kinetic studies were performed to determine optimum extraction time for the ultrasonic bath method. A variety of field-contaminated soils from a number of different states were tested. These soils contained HMX, RDX, TNB, TNT, 2-Am-DNT, and 2,4-DNT at concentrations ranging from <1 to 15000 μg/g. Extraction rates for spiked soils were also studied.

Table 2. Detector sensitivities and proven range of linear response

Analyte	Sensitivity,[a] absorbance/μg	Proven linear range, μg/L
HMX	2.96×10^{-2}	10–10072
RDX	3.55×10^{-2}	10–10037
TNB	6.67×10^{-2}	10–20052
DNB	8.77×10^{-2}	10–10013
Tetryl	4.90×10^{-2}	10–20130
TNT	5.22×10^{-2}	10–10132
2,4-DNT	6.24×10^{-2}	10–5007

[a] Using 100 μL loop injected with standard–water (1 + 1); UV detector 254 nm.

The extraction kinetics for field-contaminated soils varied with analyte and soil. In general, extraction periods of less than 4 h often recovered less than 80% of the total extractable

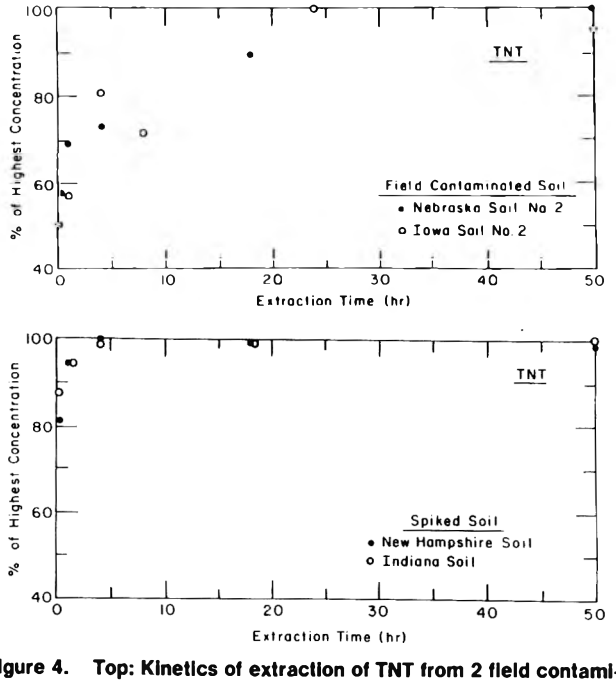

Figure 4. Top: kinetics of extraction of TNT from 2 field contaminated soils; bottom: kinetics of extraction of TNT from 2 spiked soils.

Table 3. Summary of results for soil-to-solvent ratio test

Analyte	Mean concn, μg/g[a] 2 g/50 mL	2 g/25 mL	2 g/10 mL	% Diff.[b] $\frac{\text{highest-lowest}}{\text{lowest}} \times 100$
		Iowa soil		
HMX	1990 (a)	2000 (a)	1970 (a)	1.6 NS
RDX	13600 (b)	13300 (b)	12700 (c)	7.1 ••
TNB	484 (d)	479 (d)	474 (d)	2.1 NS
DNB	38.4 (e)	38.3 (e)	39.6 (e)	3.4 NS
Tetryl	390 (f)	420 (f)	398 (f)	7.7 NS
TNT	14900 (g)	14800 (g, h)	14500 (h)	3.0 ••
		Louisiana soil		
HMX	224 (i)	228 (i)	264 (j)	17.8 ••
RDX	878 (k)	871 (k, l)	828 (l)	6.0 ••
TNB	1.8 (m)	1.7 (m)	1.7 (m)	5.9 NS
DNB	<d	<d	0.15	—
TNT	12.2 (n)	12.0 (n)	11.6 (n)	5.2 NS

[a] Numbers identified with the same letter are not significantly different at the 95% confidence level by ANOVA and least significant difference test.
[b] % Difference = [(highest-lowest)/lowest] × 100. NS indicates that difference among 3 treatments was not significant at the 95% confidence level using ANOVA.
•• Differences were significant at the 95% confidence level using ANOVA.

analyte. An extraction of 18 h, on the other hand, recovered greater than 90% of all the extractable analytes in a variety of field-contaminated soils (Figure 4). An 18 hr period is also convenient since the extraction can be started in the late afternoon and the extracts processed the following morning.

The rate of analyte extraction from spiked soils was quite different than from field-contaminated soils. Despite the fact that the spiked soils were aged for 2 days to allow solvent evaporation and analyte interaction with the soils, only 15 min was required for greater than 90% analyte recovery (Figure 4). Clearly it is unrealistic to develop extraction methodology based solely on spiked material.

Soil-to-Solvent Ratio

Initially, the method utilized 2 g soil and 50 mL acetonitrile. To determine if this ratio could be increased without affecting method performance, we selected 2 field-contaminated soils from Iowa and Louisiana which had very different concentrations of analytes. A set of 18 replicate 2.00 g subsamples of each soil was randomly divided into 3 groups of 6 subsamples. One group of 6 subsamples for each soil type was extracted with 50 mL solvent. The other 2 groups were extracted with 25 and 10 mL solvent, respectively. Results are summarized in Table 3. An analysis of variance of analyte concentrations showed significant differences among the 3 treatments in 4 of the 10 cases where comparisons were possible: RDX for both soils, HMX for the Louisiana soil, and TNT for the Iowa soil. In the Iowa soil, the concentrations of TNT and RDX both exceeded 1% of the dry weight of soil, and poorer recovery was found when the 2 g subsample was extracted with only 10 mL acetonitrile. These differences, however, amounted to only 7.1% for RDX and only 3.0% for TNT on a μg/g basis.

For the Louisiana soil, a similar result was observed for RDX where the result was 6.0% lower for the 10 mL extracts than for the 50 mL extracts. For HMX, analyte concentrations were 17.7% higher in the 10 mL extracts than in the 50 mL extracts on a μg/g basis. This anomalous result for HMX was due to some interference from unretained salts and polar compounds which elute just before HMX. In the 10 mL extracts, the concentration of these compounds can be as much as 5 times higher than in the 50 mL extracts and may overload the column, thereby causing greater interference with the early eluting HMX.

The higher solution concentrations achieved for the extracts with 2 g in 10 mL did permit quantitation of DNB for the Louisiana soil when it was not possible to do so for the 25 mL and 50 mL extracts. This was expected since the concentration in the extract was approaching the lower limit of detection.

Overall, the method is quite rugged with respect to soil-to-solvent ratio. A 2 g-to-10 mL ratio was selected since it gave the best low level detection capability. Higher soil-to-solvent ratios were not tested because we needed sufficient volume of processed extract to allow both primary analysis and secondary analyte confirmation.

Particulate Removal

Prior to analysis by LC, the soil extracts had to be filtered to protect expensive reverse-phase columns. Because of the long period of sonication required for maximum analyte recovery, the soil aggregates were dispersed into very fine particles with long settling times. These fine particles made filtration of the extracts extremely difficult, even after the extracts were centrifuged at 5000 rpm for extended periods of time. Rupturing of the membranes sometimes occurred due to the excessive pressure required.

Introduction of aqueous $CaCl_2$ produced flocculation of the fine particles and rapid settling. After a 15 min settling time, the clarified solutions were readily filtered through 0.5 μm filters. Since dilution with water was required to match the solvent strength of the sample to that of the eluant, we decided to determine the conditions under which flocculation with aqueous $CaCl_2$ was effective and whether the flocculation process modified analyte concentrations. Concentrations greater than 60 g/L resulted in salting out of the acetonitrile and formation of 2 layers at room temperature. Salting-out also occurred at concentrations greater than 10 g/L if the samples were refrigerated. Aqueous concentrations ranging from 0.1 to 10 g/L were effective in causing flocculation, although the rate varied. We chose 5 g/L, which has worked well for most soils we have studied.

To test whether flocculation affected analyte concentrations, a screening experiment was conducted with a series of 8

Table 4. Means and standard deviations for aliquots from centrifugation (c) and flocculation (F) procedures with determinations conducted in quadruplicate

	Concentration, μg/g											
	HMX		RDX		TNB		DNB		Tetryl		TNT	
Statistic	F	C	F	C	F	C	F	C	F	C	F	C
Tennessee soil No. 1												
$\bar{X}$	71.2	71.1	439	436	1.9	2.1	0.86	0.77	34.7	34.2	28.0	27.9
S	0.98	0.70	6.3	0.96	0.32	0.26	0.23	0.15	0.96	0.36	0.97	0.59
t[a]	0.17		0.79		1.09		0.65		0.97		0.26	
% Diff.[b]	0.1		0.7		10.5		11.7		1.4		0.3	
Tennessee soil No. 2												
$\bar{X}$	24.2	23.1	171	173	4.7	4.7	1.5	1.2	—	—	10.3	10.6
S	2.7	0.51	0.96	0.58	0.54	0.91	0.17	0.17	—	—	0.29	0.69
t	0.81		3.13[c]		0.05		2.48[c]				0.80	
% Diff.[b]	4.8		1.1		0.0		25.0				2.9	
Iowa soil												
$\bar{X}$	116	118	80.6	79.7	66.7	82.7	—	—	—	—	754	757
S	0.96	1.26	2.0	0.99	1.21	1.63	—	—	—	—	4.1	1.0
t	2.53[c]		0.88		15.8[c]						1.19	
% Diff.[b]	1.7		1.1		24.0						0.4	

[a] Critical value for $t_{0.95}$ (df = 6) = 2.447, where df is degrees of freedom.
[b] % Difference = [(highest-lowest)/lowest] × 100.
[c] Significant difference detected at 95% confidence level.

field-contaminated soils from Iowa, Louisiana, and Tennessee. A 2.00 g portion of each soil was extracted in the sonic bath with 50.0 mL acetonitrile. From each extract, one 10.0 mL aliquot was mixed with 10.0 mL water and centrifuged at 2000 rpm for 15 min. Another 10.0 mL aliquot was mixed with aqueous $CaCl_2$ and allowed to stand 30 min. Then these solutions were filtered and analyzed.

The mean concentration ratios between centrifuged and flocculated aliquots for HMX, RDX, TNB, and TNT ranged from 0.98 to 1.00, indicating very little difference between the 2 processing techniques (40). In the absence of analytical replication, it was impossible to determine if the small differences found for individual samples were statistically significant. Consequently, 3 soils from Tennessee and Iowa, including 2 that showed the largest difference between the 2 types of processing in the screening experiment, were further analyzed. Each soil was extracted and processed as above. Centrifugation was conducted at 5000 rpm for 20 min. The solutions resulting from the 2 treatments for each soil were analyzed in quadruplicate. Means and standard deviations are presented in Table 4.

Of the 15 analyte/soil comparisons that could be made, based on the analytes present in these field-contaminated soils, only one (TNB for the Iowa soil) was of practical significance, although 3 others were statistically significant as well (Table 4). The mean values for TNB in the Iowa soil were 66.7 and 82.7 μg/g for flocculated and centrifuged aliquots, respectively, a difference of 24%. Analytical replication was excellent in both cases, so the difference appears both real and important. Chromatograms for these extracts indicated that a small, broad peak eluted just ahead of the TNB peak in the flocculated subsample (Figure 3a). Additional research indicated that this peak was due to the presence of 2,4,6-trinitrobenzaldehyde (TNBA) in the Iowa soil. Like TNB, TNBA is a photodecomposition product of TNT (40). TNBA is converted to TNB by decarbonylation (41). In the centrifuged samples, this reaction has proceeded to completion so the TNB observed is a sum of the TNB and TNBA initially present. In the flocculated samples, the TNBA is more stable, perhaps due to some complexation from the high concentration of $CaCl_2$. However, if samples are allowed to stand at room temperature overnight, the TNBA slowly converts to TNB.

Overall, the flocculation step is a major improvement in extract processing and does not alter analyte concentrations.

Power Dissipation in Sonic Bath

Processing a large number of samples simultaneously might lessen the efficiency of sonic dispersion (B. Tomkins, Oak Ridge Naval Laboratory). To investigate this, 4 replicate 2.00 g portions of Iowa soil were extracted for 18 h as usual with no other tubes in the bath, and another 4 portions were processed in an identical manner along with 32 additional samples in the bath. Iowa soil was chosen since it contains measurable levels of 5 of the 7 analytes of interest. No significant differences were found between the 2 treatments at the 95% confidence level for any of the analytes (40).

Photodegradation Study

TNT is known to degrade in aqueous solutions exposed to sunlight. However, the susceptibility to photodegradation of TNT and other munitions while associated with soil is unknown. The method we developed requires air-drying of soils for at least 24 h prior to extraction. Thus it was necessary to determine whether special precautions are required to minimize exposure of the soils to sunlight during drying.

Two soils from Louisiana and Iowa were selected for study on the basis of their previously determined concentrations of TNT. A bulk sample of each was air-dried, ground, and sieved under low light conditions, homogenized, and divided into 2 portions. One portion of each soil was spread in a thin layer in aluminum pans and exposed on the sill of a south-facing window, ensuring maximum exposure to whatever sunlight was available for 10 days (2 days were sunny and the other 8 days were mostly overcast). Fluorescent lights in the room were left on continuously. The pans were shaken several times per day to refresh the soil surface exposed to light. The

Table 5. Results of photodegradation experiment

Statistic	Concentration, μg/g									
	HMX		RDX		TNB		DNB		TNT	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light
Louisiana soil										
$\bar{X}$	54.8	54.7	161	169	2.4	2.4	—	—	11.6	10.6
S	3.7	7.4	2.1	5.4	0.14	0.21	—	—	0.59	0.28
t^a	0.04		3.23[b]		0.48				3.36[b]	
Iowa soil										
$\bar{X}$	67.8	66.1	73.6	87.3	65.2	69.7	0.60	0.56	734	655
S	22.3	18.5	8.9	21.1	3.1	3.3	0.20	0.12	16.6	7.5
t	0.15		0.77		2.46[b]		0.43		10.7[b]	

[a] Table value for $t_{0.95}$ (10 df) = 2.23.

[b] Significant difference detected at 95% confidence level.

second portion of each soil was also spread evenly in aluminum pans, which were kept in the dark in the same room as the exposed samples. Residual moisture contents of the soils maintained in the dark and those exposed to room light were found to be equivalent.

After 10 days of exposure, six 2.00 g subsamples of each soil treatment were extracted and analyzed (Table 5). Statistically significant differences in analyte concentrations for the 2 treatments at the 95% confidence level were observed for RDX and TNT in the Louisiana soil and for TNB and TNT in the Iowa soil. Losses of 8.6 and 10.8% for TNT were observed for the light-exposed subsamples of the Louisiana and Iowa soils, respectively. A coincident increase of 6.9% for TNB in Iowa soil confirms that it is a product of TNT photodegradation (41). A 5.0% increase in RDX concentration was also observed in the light-exposed subsamples for the Louisiana soil, although the reason is unclear. Thus soil samples should be air-dried out of direct sunlight to minimize concentration changes due to photodegradation.

Ruggedness Testing

To complete the ruggedness testing of this method, we identified 4 factors that could potentially affect performance and might be varied by individual analysts. These factors were varied systematically by way of a full factorial experiment to assess the sensitivity of the method to each variable or their interactions (42). A full factorial experiment was preferred to eliminate confounding of main effects from high order interactions. A full 2^4 factorial experiment in duplicate requires 32 trials, which is about the maximum number of analyses that can be completed in one 8 h day. Conducting all analyses in one day eliminates variability resulting from differences in daily calibration curves.

One important factor was aggregate size. The initial method specified grinding the soil to pass a 30 mesh sieve. Further grinding to pass a 60 mesh sieve might alter analyte recovery.

The second factor was vortex mixing to initially disperse soil in the extracting solvent. Some analysts might choose to eliminate this step in favor of manual shaking. The levels tested were the normal 1 min vortex mixing vs 15 s of manual shaking.

The third factor was the concentration of the aqueous $CaCl_2$ solution added to the acetonitrile extracts. We chose 20 and 4 g/L as the 2 levels to be tested. The high level is near the maximum concentration of $CaCl_2$ that can be used without causing the acetonitrile to "salt out" at room temperature, and the low level is slightly below our recommended level but still adequate for efficient flocculation.

The final factor was idle time, the settling time allowed for flocculation after aqueous $CaCl_2$ is added. The 2 levels chosen were 15 min, the minimum time necessary to allow the floc to settle, and 4 h, both at room temperature.

Two separate factorial experiments were conducted: one used field-contaminated soil from Iowa and the other used field-contaminated soil from Nebraska. These 2 soils represented extremes in analyte concentrations, thereby addressing concentration as a possible determining influence in whether these factors significantly affected overall method performance.

Experimentally, the combinations specified by the design matrix were obtained as follows: Soil previously ground to pass a 30 mesh sieve was mixed thoroughly and split into two 40 g portions. One portion was further ground to pass a 60 mesh sieve. Sixteen 2.00 g subsamples of each portion were then weighed into individual 25 × 200 mm test tubes equipped with Teflon-lined screw caps.

A 50 mL aliquot of acetonitrile was added to each tube. Half of the tubes were vortex-mixed for 1 min and placed into the sonic bath. The second half of the tubes were manually shaken for 15 s and added to the sonic bath along with the vortex-mixed tubes.

After 18 h in the sonic bath, the tubes were allowed to cool, and 5.00 mL portions of each were removed with a glass volumetric pipet and placed in glass scintillation vials containing 5.00 mL aliquots of one of the 2 aqueous $CaCl_2$ solutions. The vials were shaken briefly.

Half of the vials were allowed to stand for 15 min, the rest for 4 h, during which the suspended particles flocculated and settled. Approximately 6 mL portions of the clear supernatant liquid were filtered through 0.5 μm Millex SR disposable filters into clean scintillation vials. The first 3 mL portion of each filtrate was discarded, and the second 3 mL portion was retained for analysis. Details of the experimental design and individual results are presented elsewhere (40).

An analysis of variance (ANOVA) was performed separately on each analyte to discover whether any of the 4 factors had significant effects on analyte concentration. Table 6 summarizes the main effects expressed as the percentage change relative to the grand average. An effect is defined as the response at (+) setting minus response at (−) setting.

Particle size.—Smaller particle size enhanced recovery for HMX and DNB in Iowa soil but not in Nebraska soil, where the concentrations were much lower. When concentration is high HMX may be heterogeneously distributed, perhaps as localized deposits or discrete crystals, which may be less efficiently solubilized than a more evenly distributed analyte.

Table 6. Ruggedness test main effects[a] for Iowa and Nebraska soils expressed as percent of grand average

Iowa soil					
Variable	HMX	RDX	TNB	DNB	TNT
Size	15.3*	11.4	0.8	23.4*	0.5
Agitation	−4.8	1.0	−0.6	−15.5	−0.4
$CaCl_2$	−23.5*	8.0	−0.9	9.3	0.3
Idle time	7.0	−4.2	7.3*	−5.8	−0.1
Nebraska soil					
Variable	HMX	RDX	TNB	TNT	DNT
Size	−4.0	−7.7	−0.9	−1.6	−13.8*
Agitation	3.7	−11.0*	−1.2	−7.7	7.0
$CaCl_2$	−1.3	−14.0*	−3.8	−1.2	−3.7
Idle time	−3.1	−0.5	4.2	4.3	6.8

[a] Negative effect favors 30 mesh, vortex mixing, 20 g/L $CaCl_2$, or 15 min idle time.
* Effects are significant at 95% probability level.

Following the original contamination event, water may have evaporated, leading to precipitation of HMX instead of adsorption because of its inherently low water solubility and high concentration in this soil. This interpretation is supported by an earlier study of extraction methods (37), in which increasing variance with decreasing sample size was found for HMX and RDX in Iowa soil. This trend is consistent with heterogeneous analyte distribution. An additional confirmation of analyte heterogeneity was found in the ruggedness test where the variance of results of 30 mesh particles was significantly greater than that for 60 mesh particles. Although this difference contravenes an assumption in ANOVA of homogeneous variance, and tends to desensitize the significance tests, the effects were still significant for HMX.

The reasons for lower recovery of DNT in Nebraska soil and higher recovery of DNB in Iowa soil at smaller particle size are uncertain, but there is no evidence that heterogeneous distribution is responsible.

Agitation method.—For the most part, manual mixing is just as good as vortex mixing, but the latter gave somewhat better recovery for RDX in Nebraska soil, although the size of the effect was small. Since agitation is strictly a mechanical phenomenon, it is not clear why the main effect would be significant for one but not all analytes.

Although manual shaking is nearly equivalent to vortex mixing and requires no special equipment, it is not generally recommended because manual shaking styles are likely to be very different among laboratories. Uniform use of a vortex mixer at a given speed and duration would eliminate this potential source of interlaboratory variance. If a vortex mixer were unavailable, however, manual shaking would be acceptable.

Concentration of $CaCl_2$ used for flocculation.—HMX in Iowa soil and RDX in Nebraska soil were recovered less effectively at the 4 g/L level of $CaCl_2$ (Table 6). This behavior was not observed for these analytes in the other soil. While the effect for HMX in Iowa soil (23.5%) is the largest main effect observed, we are unable to suggest a reasonable physical explanation since the addition of $CaCl_2$ occurs after extraction is complete. It could be due to accidental inclusion of subsamples from a "hot spot" within the total soil sample. Recall that HMX variability in Iowa soil was greater than that for the other analytes. Inspection of the interaction between agitation method and $CaCl_2$ concentration shows that the concentration effect for RDX in Nebraska soil is dominated by the contribution from manual agitation. Thus vortex mixing would minimize this effect.

Idle time.—The recovery of TNB in Iowa soil was improved at longer idle time (Table 6). This effect is consistent with that discussed earlier for this soil where TNBA is slowly converted to TNB when diluted extracts are allowed to stand at room temperature. This effect was not significant for Nebraska soil and has not been found in the majority of other soils subsequently tested.

Summary of Ruggedness Test Results

The method is quite rugged overall. A few effects were found to be significant at the 95% probability level, but none was dominant for all analytes and soil types (40). The recommended parameter levels for the method are grinding of soil to pass a 30 mesh sieve prior to extraction, vortex mixing, and 15 min of idle time after addition of 5 g/L of $CaCl_2$.

Stability of Stock Standards

A major question for all analytical procedures requiring calibration is how often stock standards must be replaced. To answer this question, we took advantage of the availability of stock standards of explosives prepared over a period of 19 months. In all cases these stock standards were prepared by weighing out SARM-grade material, transferring it to volumetric flasks, and diluting it to volume with either methanol or acetonitrile. The stock standards were stored in a refrigerator at 4°C in the dark, and the stoppers were wrapped with Parafilm to retard solvent evaporation.

Three sets of individual stock standards were tested. The first set was prepared in methanol in August 1985; the 1985 HMX and RDX stocks contained 40% acetonitrile to assist in initial dissolution. The second and third sets of standards used only acetonitrile and were prepared in June 1986 and March 1987.

In July 1987, three replicate composite standards were prepared from each set of stock standards. Detailed results of the analysis of the diluted combined standards are presented elsewhere (40). None of the analytes showed a consistent trend toward decreasing concentrations with increasing storage time, and the variation in standards prepared and stored over 23 months is minimal (generally $< 5\%$). We conclude that stock standards of these explosives stored in glass at 4°C in the dark, with precautions taken to minimize solvent evaporation, can be safely used for periods up to 1 year.

Stability of Dilute Working Standard

Another question we investigated was how often diluted working standards need to be replaced. To test the stability of the dilute working standards, duplicate combined stock standards and duplicate dilute working standards were prepared about every 5 days over a 28-day period. These dilute working standards were stored over this period at 4°C in the dark with stoppered joints wrapped with Parafilm to retard evaporation. Another set of duplicates was prepared on the first day of the experiment, but they were warmed to room temperature and a small portion was removed every 5 days to simulate a working standard that was being used over this 28-day period. The 16 individual working standards were analyzed as a group in random order on the day following the last preparation. Response factors were obtained from the mean responses of the most recent working standard. Detailed results are presented elsewhere (40).

An analysis of variance was conducted for each of the 7 analytes. For all the analytes except tetryl, variations were not statistically significant at the 95% confidence level. This is in spite of excellent agreement between duplicates, with relative standard deviations ranging from 0.52 to 1.15%. For

Table 7. Results of method testing in 2 collaborating laboratories using field-contaminated soils

Analyte	Laboratory 1				Laboratory 2			
	Soil 1 concn, μg/g		Soil 2 concn, μg/g		Soil 3 concn, μg/g		Soil 4 concn, μg/g	
	Known	Detd	Known	Detd	Knowr	Detd	Known	Detd
HMX	4.2	2.1	124	117	79	98	30	25
RDX	<d[a]	<d	1162	1120	68	93	135	149
TNB	2.0	2.6	159	170	75	62	5	5
DNB	<d	<d	<d	0.5	<d	1.3	<d	1.6
Tetryl	<d	<d	<d	<d	<d	<d	<d	<d
TNT	<d	1.0	380	375	740	718	5	8
2,4-DNT	<d	<d	4.2	3.3	<d	<d	<d	<d

[a] Concentrations less than CRL values given in Table 8.

tetryl, a statistically significant difference was observed. A least-significant-difference computation indicated that the standard stored for 24 days was significantly different from the most recent standard, but those stored 28 days were not significantly different. This inconsistency suggests that the 24-day result was anomalous. We conclude that working standards can be prepared and used over a 28-day period if they are refrigerated and kept in the dark when not in use.

Stability of Soil Extracts

Another unresolved question is the stability of soil extracts. To investigate this question a series of 5 field-contaminated soils were extracted and processed. The extracts were allowed to stand at room temperature for 24 h and were then analyzed immediately. The extracts were also analyzed after being stored at 4°C in the dark for 3, 6, 18, 27, and 71 days. The results are presented elsewhere (40). HMX, RDX, DNB, and TNT were found to be stable over the entire 71-day period in these extracts.

It appears that the concentration of TNB in the extracts from the Iowa and Nebraska soil No. 2 slowly increased over the time the extracts were held. The increase amounted to about 18% for the Iowa soil (78.6 to 92.6 μg/g) and 32% for Nebraska soil No. 2 (360 to 475 μg/g). The increase in TNB was not accompanied by a measurable change in the concentration of other analytes, but the small chromatographic peak attributed to TNBA, discussed earlier, declined over storage. Thus the increase in TNB concentration was probably a result of TNBA decomposing and forming TNB during the extended cold storage period. In general, extracts can be held for up to 2 months without large adverse effect.

Initial Method Testing in Other Laboratories

All results discussed thus far were obtained in our laboratory. To assess the utility of the method for more general application, the method and several test samples were supplied to 2 other laboratories that had no previous experience with the determination of explosive residues in soil but were acquainted with the use of LC. Four different previously characterized field-contaminated soils were provided, 2 to each laboratory.

The results of these analyses are presented in Table 7 along with values we obtained for the same soil (known values). For both laboratories, the results compared favorably with those obtained at CRREL, particularly considering that the laboratories analyzed different subsamples of field-contaminated soil that had some inherent heterogeneity.

Estimation of Detection Capability and Overall Analyte Recovery

Estimates of detection capability of the method were delayed until it was tested in other laboratories. The results justified experiments to estimate method detection limits (MDLs) (43) and certified reporting limits (CRLs) (44, 45). The test concentrations used in conducting the MDL study were obtained by estimating the expected values from results of a similar method developed for analysis of water for these analytes (27). Ten replicate 2 g subsamples of uncontaminated soils were spiked at the appropriate levels and the analytes were extracted and analyzed. MDLs were obtained by calculating the standard deviation for the set of 10 replicates for each analyte at the 0.5 μg/g level and multiplying the result by the appropriate t-statistic at the 99% confidence level (45). MDLs ranged from 0.03 μg/g for 2,4-DNT to 1.27 μg/g for HMX (Table 8).

CRL estimates were obtained from experiments conducted on 4 separate days. Duplicate spiked samples at 0.5, 1, 2, 5, and 10 times the levels used for the MDL test were processed on each day (46). The mean and variance for the 8 values at each level were calculated and Bartlett's test used to identify the range of homogeneous variance for each analyte. Least-squares analysis was used to obtain the best linear relationship between found and spiked concentration over the ranges of homogeneous variance. Confidence intervals about this line were established at the 90% level (5% each side). The CRL is defined as the point on the "target concentration" axis which corresponds to the point on the lower confidence band where the value on the upper confidence band intersects the "found concentration" axis.

CRL results are shown in Table 8. Except for TNB, MDL values are lower than CRLs. This is principally due to the inclusion of day-to-day calibration variability in the CRL estimates while MDL values are obtained from data collected on a single day. The α- and β-risks are also very different (46). Using either detection capability estimate, the method provides a measurement capability of less than 1 μg/g except for HMX. Overall method accuracy is also presented in Table 8. These values were obtained from the slope of the

Table 8. Detection capability estimates and overall analyte recovery

Analyte	MDL (43), μg/g	CRL (45), μg/g	Rec., (%)
HMX	1.27	2.15	80
RDX	0.74	1.03	84
TNB	0.29	0.24	119
DNB	0.11	0.12	105
Tetryl	0.12	0.65	83
NB	0.08	0.11	100
TNT	0.08	0.24	102
2-Am-DNT	0.03	0.11	91
2,6-DNT	0.07	0.16	107
2,4-DNT	0.03	0.07	102
o-NT	0.07	0.24	92
p-NT	0.07	0.22	103
m-NT	0.07	0.25	101

zero intercept linear regression equations for spiked versus found concentrations from the CRL tests. Measured percent recoveries of individual analytes averaged about 98%, ranging from 80% for HMX to 119% for TNB.

Overview of Method

The results of this study have been used to establish the step-by-step method described below. This method has been evaluated by means of a full-scale collaborative test, the results of which are presented in the report of the collaborative study (47).

Soil samples are visually inspected to ensure no chunks of solid explosive are present and air-dried out of direct sunlight to constant weight. Soil is then ground with a mortar and pestle to a fine powder (approximately 30 mesh) and homogenized by placing in a closed container and shaking thoroughly. A 2.00 g subsample is then placed in a 6 dram glass vial equipped with a Teflon-lined cap.

A 10.0 mL aliquot of acetonitrile is added, and the soil is dispersed by vortex mixing for 1 min and extracted in an ultrasonic bath for 18 h. The bath is maintained at ambient temperature during extraction to minimize loss of tetryl which is thermally degradable (47).

Vials are removed from the bath and allowed to settle for 30 min. A 5.00 mL aliquot is removed, placed in a glass scintillation vial and combined with 5.00 mL aqueous $CaCl_2$ (5 g/L). The vials are shaken and allowed to stand for 15 min to permit fine particles to flocculate. A 5 mL aliquot is filtered through a 0.5 μm Millex SR filter into a clean scintillation vial. Extracts are stored at 4°C in the dark until analyzed.

On the day of analysis, the vials are warmed to room temperature and shaken thoroughly in case high salt concentrations have caused the acetonitrile to salt out during storage at 4°C. Each extract is analyzed by RPLC on an LC-18 column (Supelco) eluted with 1.5 mL/min of water–methanol (1 + 1). If peaks with retention times corresponding to analytes of interest are observed, a confirmation determination is made on an LC-CN column (Supelco) using an identical eluant and flow rate.

Because analyte concentrations may vary from <1 μg/g to 10% of the sample mass, precautions must be taken to prevent cross contamination of samples. For example, the mortar and pestle must be thoroughly cleaned between samples by washing in hot soapy water and rinsing with acetonitrile. We do not recommend sieving the soil prior to subsampling because the adequate cleaning of sieves is inconvenient and time-consuming. While variability will be increased to some extent by including larger aggregates typically low in analyte concentration, the potential for cross contamination is an overriding concern. Disposable glassware is used wherever possible (soil extraction, flocculation, and storage of filtrates), and disposable syringes and filter units are used only once. These steps will improve the quality of the data by preventing false positive results, and the cost increase is minor compared to the overall analytical cost.

Conclusions

The method described in this paper is used to determine nitroaromatics and nitramines in soil. Concentrations as low as 0.1 μg/g may be measured quantitatively. The method has been used successfully to analyze soils from several present and former Army sites, and has been subjected to a full-scale collaborative test (47).

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Formaldehyde Quantitation in Air Samples by Thiazolidine Derivatization: Factors Affecting Analysis

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A new method for the determination of trace levels of formaldehyde in air was developed and validated. The method is based on the reaction of formaldehyde with cysteamine to form thiazolidine. Air samples containing trace levels of formaldehyde were prepared from paraformaldehyde. The percent yield of formaldehyde from paraformaldehyde was 85.1 ± 1.14%. Air samples were bubbled into an aqueous cysteamine trap. Thiazolidine formed from formaldehyde and cysteamine in the trap was determined by gas chromatography with a fused silica capillary column and a nitrogen-phosphorus detector (NPD). The lowest detection level for thiazolidine was 17.2 pg, equivalent to 5.80 pg formaldehyde. The recovery efficiency of trace gas phase formaldehyde in air was greater than 90%. Formaldehyde levels in ambient laboratory air were 48.9–56.2 ppb (v/v).

The determination of formaldehyde in air is of great importance because it is a major pollutant in a variety of industrial and domestic environments (1). The most common method for measuring formaldehyde is to pass an air sample through a trapping solution containing a derivatizing reagent such as pararosaniline (2), 3-methyl-2-benzothiazolone hydrazone hydrochloride (3), or 2,4-dinitrophenylhydrazine (4–7). Formaldehyde derivatives may be determined by colorimetry, gas chromatography (GC), or liquid chromatography (LC). A major drawback of these methods is that the analysis requires adverse reaction conditions, such as low pH, which may alter the original sample.

Recently, we developed a simple and accurate analytical method for determining trace levels of formaldehyde in foods and beverages (8). Formaldehyde is reacted with cysteamine at room temperature and neutral pH to form thiazolidine, which is then determined by gas chromatography using a fused silica capillary column and a nitrogen-phosphorus detector (NPD). Cysteamine reacted with all aldehydes except α,β-unsaturated aldehydes, but a high resolution fused silica capillary column was able to separate all resulting derivatives.

In the present study, the application of the cysteamine method to the determination of formaldehyde in air was investigated.

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METHOD

Apparatus

(a) *GC column.*—30 m × 0.25 mm id DB-WAX (J & W Scientific, Folsom, CA 95630) fused silica column.

(b) *Gas chromatograph (GC).*—H-P (Hewlett-Packard, Avondale, PA 19311) Model 5890 equipped with nitrogen-phosphorus detector. Operating conditions: initial column temperature 100°C, program at 4°/min to 190°C; injector 250°C; detector 300°C; helium carrier gas flow 31.8 cm/s; injector split ratio 1:30.

(c) *Integrator.*—Spectra Physics Model 4290.

(d) *Gas chromatograph/mass spectrometer (GC/MS).*—H-P Model 5890 GC interfaced to VG TRio II mass spectrometer with VG 11-250 computer data system. Operating conditions: ionization voltage 70 eV; ion source temperature 150°C.

Reagents

(a) *Cysteamine hydrochloride.*—Aldrich Chemical Co., Milwaukee, WI.

(b) *Formaldehyde.*—37% in water (Aldrich Chemical Co.)

(c) *Paraformaldehyde.*—mp, 163–165°C (Aldrich Chemical Co.)

(d) *Anhydrous sodium sulfate.*—Fisher Scientific, Pittsburgh, PA.

(e) *Hydrochloric acid.*—Fisher Scientific.

(f) *Internal standard for GC analysis.*—Add 10 μg *N*-methylacetamide to 1 mL chloroform and store at 5°C.

Table 1. Effect of pH on percent yield of thiazolidine from reaction of cysteamine and formaldehyde from sodium bisulfite adduct or from aqueous solution

Source of formaldehyde	pH 5	6	7	8	9	10
Sodium bisulfite adduct	3.1	28.6	55.2	59.1	77.8	—[a]
Aqueous formaldehyde solution	a	37.4	95.2	93.6	99.9	99.2

[a] Not tested.

(g) *Extraction solvent.*—Chloroform (J. T. Baker Inc., Phillipsburg, NJ.).

(h) *Aqueous standard stock solution of formaldehyde.*—Add 2.7 mL 37% formaldehyde solution to 0.5 L deionized water and adjust volume of solution to 1 L with deionized water. Determine actual formaldehyde concentration in stock solution by using procedure of National Institute for Occupational Safety and Health (9).

Quantitative Analysis of Formaldehyde by GC

Prepare calibration curve for thiazolidine with *N*-methylacetamide as internal standard. Add 50 mL stock internal standard solution to 10 mL sample. Inject 1 μL sample solution on GC apparatus equipped with NP detector. Obtain thiazolidine concentration from calibration curve by using GC peak area ratio of thiazolidine, derived from formaldehyde, and internal standard.

Experimental

Transfer Efficiency of Formaldehyde from Its Sodium Bisulfite Adduct to Thiazolidine

Add 1 mL formaldehyde solution (100 μg as formaldehyde) to 40 mL 1% sodium bisulfite solution, stir solution, and then add 0.5 g cysteamine hydrochloride. Release formaldehyde from its bisulfite adduct with 5 mL 2.4N HCl. Adjust the solution to pH 8 with 2N NaOH solution and leave for 2 h. Extract with chloroform and dry over anhydrous sodium sulfate. Adjust volume of solution to 50 mL with chloroform. Add 50 μL of GC internal standard to 10 mL sample solution and determine formaldehyde as thiazolidine by GC analysis with NP detector.

Effect of pH on Derivatization of Formaldehyde to Thiazolidine

Use sodium bisulfite adduct or aqueous formaldehyde solution (37%) as source of formaldehyde to test effect of pH on thiazolidine formation. Add 1.5 g cysteamine hydrochloride to 20 mL deionized water. Adjust pH of cysteamine solution with 2N NaOH and add either 1 mL of adduct solution (4.47 mg/mL water), equal to 1 mg formaldehyde, or 1 mL aqueous formaldehyde solution, equal to 100 μg formaldehyde. Stir well, and then leave reaction solution for 4 h at room temperature. Extract with three 10 mL portions of chloroform and dry combined extracts over anhydrous sodium sulfate. Filter off sodium sulfate and adjust volume of extract to 50 mL with chloroform. Add 10 μL internal standard to 5 mL of this extract and measure thiazolidine by GC analysis.

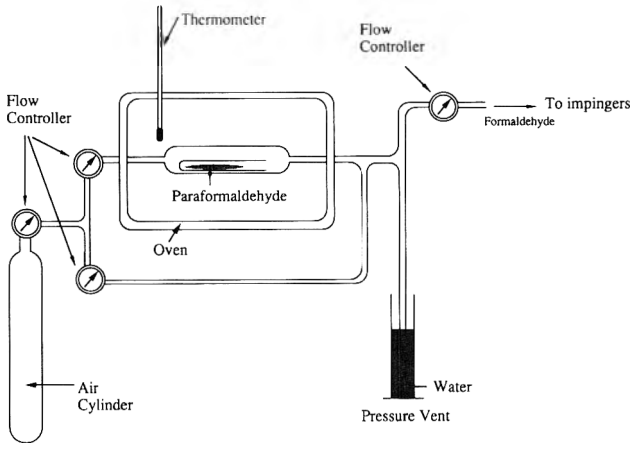

Figure 1. Apparatus used for producing air samples with trace formaldehyde levels.

Effect of Sodium Bisulfite Adduct Concentration on Thiazolidine Formation

Dissolve 0.5 g cysteamine hydrochloride in 40 mL sodium bicarbonate solution. Add adduct (0.89–4.47 mg) and leave for 4 h at room temperature. Extract and determine formaldehyde as thiazolidine as described above.

Effect of Cysteamine Concentration on Derivatization of Formaldehyde to Thiazolidine

Dissolve cysteamine hydrochloride (0.1–1.5 g) in 40 mL sodium bicarbonate solution (pH = 7.0–7.1). Add 1 mL adduct solution (4.47 mg/mL) and leave for 4 h at room temperature. Extract with chloroform and determine formaldehyde as thiazolidine as described above.

Effect of Reaction Time on Thiazolidine Formation from Cysteamine and Formaldehyde

Add 0.5 g cysteamine to 40 mL sodium bicarbonate solution. Add 1 mL stock solution of formaldehyde (100 μg/mL) to cysteamine solution and leave for 1–4 h. Extract with chloroform and determine formaldehyde as thiazolidine as described above.

Determination of Trace Formaldehyde in Air

Obtain vapor-phase formaldehyde according to Andrawes (10). Figure 1 shows diagram of apparatus used for producing air samples with trace formaldehyde levels. Fill 2 impingers connected in series with 40 mL sodium bicarbonate solution containing either 0.5 g cysteamine hydrochloride or 0.5 g sodium bisulfite. Bubble air samples into impingers with air flow rate of 50 mL/min for 24 h and then extract resulting thiazolidine with chloroform. Determine formaldehyde as described above.

Percent Yield of Formaldehyde from Paraformaldehyde

Add 11.3 mg paraformaldehyde to 5 mL sodium bicarbonate solution. Add 1 drop of 2N NaOH and adjust volume to 10 mL with deionized water. Heat solution and determine formaldehyde as thiazolidine as described above.

Table 2. Effect of cysteamine and sodium bisulfite adduct quantities on thiazolidine formation

Parameter	1	2	3	4	5	6	7	8	9	10
Adduct, mg	0.89	1.79	2.68	3.58	4.47	4.47	4.47	4.47	4.47	4.47
Cysteamine, g	0.5	0.5	0.5	0.5	0.5	0.1	0.2	0.3	0.4	1.0
Recovery, %	85	80	80	77	75	74	76	77	76	77

Table 3. Recovery of trace formaldehyde in air

Number of replications	Amount of paraformaldehyde sublimed, mg	Amount of formaldehyde recovered from impingers, μg	Recovery, %
1	3.21	294	9.0
2	0.76	348	45.8
3	1.24	736	59.4
4	1.13	715	63.1

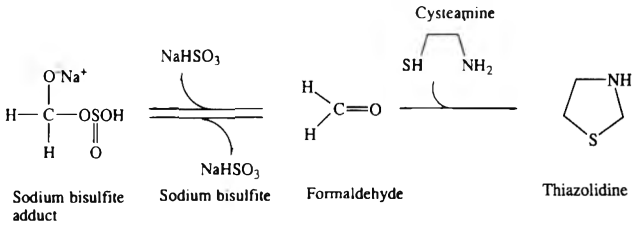

Figure 2. Reaction mechanisms of formaldehyde with sodium bisulfite and cysteamine.

Comparison Between Cysteamine and Sodium Bisulfite Methods

To test recovery efficiencies of cysteamine and sodium bisulfite traps, fill 2 impingers, connected in series, with either 40 mL sodium bicarbonate solution (5.9 g/L) containing 0.5 g cysteamine or 40 mL 1% sodium bisulfite solution. For sodium bisulfite trap, recover formaldehyde as thiazolidine as described above.

Yield of Adduct from Formaldehyde and Sodium Bisulfite

Add 1 mL aqueous formaldehyde solution (containing 100 μg formaldehyde) to 40 mL 1% sodium bisulfite solution. Stir solution and add 0.5 g cysteamine hydrochloride. Remove formaldehyde from its adduct by adding 5 mL 2N HCl solution dropwise and then adjust solution to pH 8.0 with 6N NaOH solution. Extract with chloroform and measure formaldehyde as thiazolidine by GC analysis as described above.

Determination of Trace Formaldehyde in Ambient Air

Fill 2 impingers, connected in series, with 40 mL sodium bicarbonate solution (5.9 g/L) containing 1 g cysteamine hydrochloride. Purge ambient laboratory air through impingers either by vacuum suction for 88 h at flow rate of 12 mL/min or by peristaltic pump for 72 h at flow rate of 15 mL/min.

Results and Discussion

To trap trace formaldehyde in air, an aqueous sodium bisulfite solution (1%) was used along with cysteamine solution to evaluate the NIOSH analytical method (9). Formaldehyde was released from its sodium bisulfite adduct either with acid or base and then determined by GC analysis as thiazolidine, instead of by colorimetry as described in the NIOSH method. The results are shown in Table 1. The recovery efficiency increased as pH increased. However, a blank value became significantly high when the pH exceeded 8. Therefore, a pH below 8 is recommended for this method.

It is quite tedious to adjust the pH of many trapping solutions for routine analysis. To eliminate this need, further experiments were conducted using 40 mL sodium bicarbonate solution (5.9 g/L), which gave a solution of pH 7.0–7.1 unless specific pH adjustment was required. Relative peak area ratio of thiazolidine (10 ng) and *N*-methylacetamide (50 ng) was 1.57 ± 0.03 (SD), based on 6 replications. The lowest detectable level for thiazolidine was 17.2 pg, equivalent to 5.80 pg formaldehyde.

Formaldehyde was recovered more efficiently from an aqueous solution than from a sodium bisulfite adduct solution (Table 1). This may be due to a low transfer efficiency for formaldehyde from its sodium bisulfite adduct to thiazolidine. The reaction mechanisms of formaldehyde with sodium bisulfite and cysteamine are shown in Figure 2. The results of experiments to examine effect of cysteamine and sodium bisulfite adduct quantities on thiazolidine formation are shown in Table 2. This result also indicates that the concentration of cysteamine did not influence the transfer efficiencies of formaldehyde from sodium bisulfite to thiazolidine. Use of 0.5 g cysteamine is recommended as most economical. A linear relationship between adduct concentration and thiazolidine yield was obtained, even though recovery efficiencies varied from 74 to 85%.

When aqueous formaldehyde was reacted with cysteamine for periods of 1, 2, 3, or 4 h, the percent yields of thiazolidine were 90.5, 91.5, 92.5, and 94.2%, respectively. These results indicate that the reaction proceeds quickly and that a 1 h reaction time is sufficient.

Determination of Trace Gas-Phase Formaldehyde in Air

It is extremely difficult to obtain accurate concentrations of trace levels of formaldehyde in air. Paraformaldehyde was reportedly used to obtain known or specific formaldehyde concentrations in air (10). The percent yield of formaldehyde from paraformaldehyde was 85.1 ± 1.14 for 3 replications. The recovery of gas-phase formaldehyde was considerably low in the first experiment (Table 3); this may be due to glass surface activity of the apparatus (Figure 1). After the surface was saturated with formaldehyde, recovery increased. The recovery of gas-phase formaldehyde was approximately 60%.

The recovery efficiencies of trace gas-phase formaldehyde in air were approximately 90% with a cysteamine trap and 95% with 1% sodium bisulfite trap, calculated using the factor 0.85 for conversion from paraformaldehyde to formaldehyde and 0.60 for recovery efficiency factor of gas-phase formaldehyde. The sodium bisulfite method appears to give better results, but it requires an additional step of transferring formaldehyde from adduct to thiazolidine. If experimental errors are considered, the 2 methods should not significantly differ in efficiency.

The results of the analysis of formaldehyde in the ambient air of our laboratory were 48.9 ppb (v/v) when a vacuum pump was used, and 56.2 ppb (v/v) when a peristaltic pump was used.

Conclusions

The working range of the NIOSH method for formaldehyde in air is 20 to 400 ppb. The cysteamine method can be used in the range of 1 ppb to 10 000 ppm. Some contaminants such as phenols, ethanol, and higher MW alcohols interfere in the NIOSH method. In the present method, such contaminants do not interfere with the analysis because a high-resolution, fused silica capillary column and a highly selective and sensitive detector are used. Moreover, because cysteamine reacts with all aldehydes except α,β-unsaturated aldehydes, it is possible to perform a multiple determination of aldehydes in a sample.

Acknowledgments

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FOOD ADDITIVES

Liquid Chromatographic Determination of Sulfite in Grapes and Selected Grape Products

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A liquid chromatographic (LC) method is described for the determination of sulfite in grapes and certain grape products. Sulfite is extracted from grapes with aqueous formaldehyde solution buffered at pH 5; free sulfite is converted to hydroxymethylsulfonate (HMS), which is extremely stable at pH 3–7. Subsequent heating to 80°C for 30 min converts reversibly bound forms of sulfite to HMS. The extract is then analyzed by reverse-phase ion-pairing liquid chromatography, using a C_{18} column and a mobile phase of aqueous 0.005M tetrabutylammonium ion in 0.05M acetate, pH 4.7, and a flow rate of 1 mL/min. Aqueous KOH is added to the eluate to convert HMS to free sulfite, which is then treated with 5,5′-dithiobis[2-nitrobenzoic acid]. This reaction produces the 3-carboxy-4-nitrothiophenolate anion, which is determined by measurement of electronic absorption at 450 nm. For grapes spiked with HMS at 5–20 ppm (as SO_2), recoveries ranged from 92 to 112%, with a coefficient of variation of 4.6%. The method was also used to determine sulfite in various grape products. Results were comparable to those obtained by the AOAC official Monier-Williams method.

Many analytical procedures have recently been developed to enable the rapid and specific determination of sulfites in foods. Several ion chromatographic methods have been proposed (1–4). Recently Warner et al. (5) published a reverse-phase ion-pairing liquid chromatographic (LC) method for determining total sulfite in food. Sulfite is stabilized by addition of formaldehyde, which causes the formation of hydroxymethylsulfonate (HMS). This species is extremely stable in the pH range 3–7 (6). HMS is determined by reverse-phase ion-pairing liquid chromatography and a 2-step post-column reaction. In the first step, KOH solution is added to convert HMS to free sulfite; in the second step, the free sulfite is combined with 5,5′-dithiobis[2-nitrobenzoic acid] to produce the 3-carboxy-4-nitrothiophenolate anion, which is then determined by measurement of electronic absorption at 450 nm. The present study describes modification of the Warner method (5) to improve chromatography and permit more accurate and precise determination of reversibly bound sulfite in grapes and grape products.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Model 8700 solvent delivery system (Spectra-Physics, Piscataway, NJ 08854), equipped with Model 7125 injection valve with 100 μL loop (Rheodyne, Inc., Berkeley, CA 94710), and 4.6 × 250 mm stainless steel column packed with Zorbax ODS (E. I. du Pont de Nemours & Co., Wilmington, DE 19898). Spectroflow 773 absorption monitor (Kratos, Westwood, NJ 07675) fitted with tungsten lamp set at 450 nm was used for detection. Chromatograms were recorded and peaks were integrated with Spectra-Physics Model 4270 integrator.

(b) *Auxiliary pumps.*—Two Spectra-Physics Model 3500B pumps were used to deliver post-column reagents. Eluate from column was mixed with reagent A in stainless steel tee. This mixture was then passed through short length of tubing into second tee, where it was mixed with reagent B. The second mixture was then passed through Kratos 1 mL reaction tube immersed in 50°C water bath, and finally into detector.

(c) *Heating module.*—Pierce Reacti-Therm set at 80°C (Pierce Chemical Co., Rockford, IL 61105).

Reagents and Experimental Materials

(a) *Water.*—Obtained from Milli-Q water purification system (Millipore Corp., Bedford, MA 01730).

(b) *Buffered formaldehyde solutions.*—Prepare 2% solution buffered to pH 5 by dissolving 1.02 g potassium hydrogen phthalate and 0.1 g KOH in water, adding 54 mL 37% formaldehyde solution, and diluting to 1 L with water. Prepare 0.5% formaldehyde solution by diluting this solution with water (1 + 3).

(c) *5,5′-Dithiobis[2-nitrobenzoic acid]* (*DTNB*).—(Eastman Kodak Co., Rochester, NY 14650).

(d) *Tetrabutylammonium hydroxide* (TBA^+OH^-).—40% aqueous solution (Aldrich Chemical Co., Inc., Milwaukee, WI 53233).

(e) *Membrane filters.*—Nylon-66, 0.45 μm pore size (Rainin Instrument Co., Inc., Woburn, MA 01801). Use 47 mm diameter filter for mobile phase and post-column reagents.

(f) *Solid phase extraction* (*SPE*) *columns.*—Baker-10 octadecyl, 3 mL columns. Condition with 2 mL methanol followed by 2 mL 0.5% formaldehyde solution (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(g) *Mobile phase.*—Prepare 0.05M TBA^+ in 0.5M acetate buffer, pH 4.7, by dissolving 17 mL glacial acetic acid, 27.2 g sodium acetate, and 32.5 mL TBA^+OH^- solution in water, and diluting to 1 L. Filter through 0.45 μm membrane filter. Remove trace organics by passing solution through any high-capacity reverse-phase column. (We used an old reverse-phase column that no longer exhibited peak efficiency. This cleanup column should be pre-cleaned with acetonitrile before use.) Dilute eluate with water (1 + 9) to obtain final, purified mobile phase: 0.005M TBA^+ in 0.05M acetate, pH 4.7.

(h) *Post-column reagent A.*—0.064N aqueous KOH solution. Filter through Nylon-66 filter.

(i) *Post-column reagent B.*—Dissolve 27.2 g KH_2PO_4 in water. Dissolve 360 mg DTNB in 5 mL ethanol, add it to KH_2PO_4 solution, mix well, and dilute to 1 L with water. Filter through Nylon-66 filter.

(j) *Standard sulfite solutions.*—Prepare 1 mg/mL solution of Na_2SO_3 (equivalent to ca 0.5 mg/mL as SO_2) in 2% buffered formaldehyde solution. Dilute with water (1 + 3) to obtain 250 μg/mL solution (equivalent to ca 125 μg/mL as SO_2). Dilute further with 0.5% buffered formaldehyde solution to prepare working standards of desired concentrations, typically in 0.2–10 μg/mL range (equivalent to ca 0.1–5 μg/mL as SO_2).

Preparation

Accurately weigh ca 200 g grapes into blender jar, add

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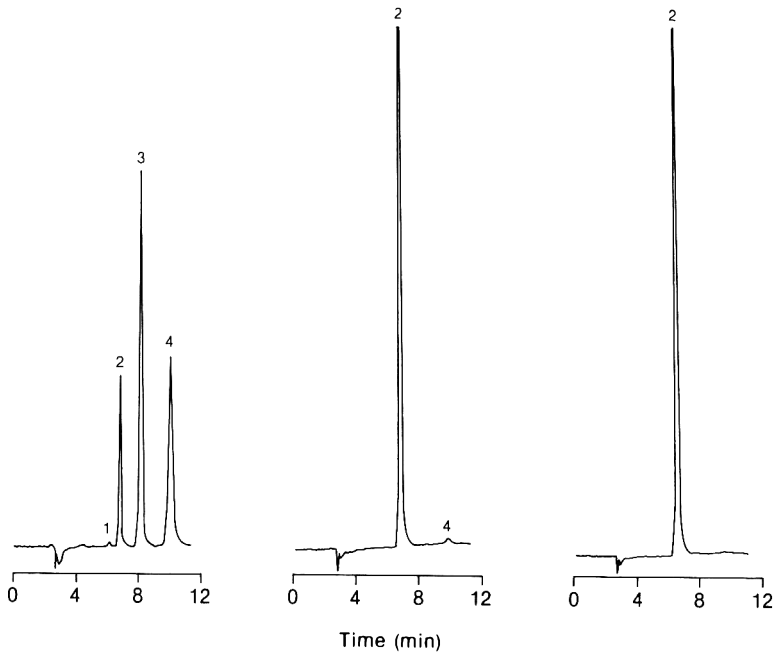

Figure 1. Chromatograms of a grape extract. Left, injected immediately after preparation; center, injected 3 days after preparation; right, subjected to heat treatment, cooled, and injected. Peaks 1 and 4, unknowns; peak 2, HMS; peak 3, HES. Conditions given in *Experimental* section.

known weight (ca 25 mL) of water, and blend 1 min. Accurately weigh ca 12 g homogenate into 50 mL centrifuge tube. Calculate actual weight of grapes in centrifuge tube, w_a, as follows:

$$w_a = w_s \times w_g/(w_g + w_h)$$

where w_s is weight of homogenate in centrifuge tube, w_g is weight of grapes placed in blender, and w_h is weight of water added. Extract homogenate with three 25 mL portions of 0.5% buffered formaldehyde solution, and centrifuge 5 min at 2000 rpm after each extraction. Combine extracts in 100 mL stoppered graduated cylinder and dilute to 100 mL with 0.5% buffered formaldehyde solution. Pass ca 4 mL of this solution through preconditioned C_{18} SPE column, and discard first 2 mL. Collect remainder in 2 mL screw-cap vial, and heat to 80°C for 30 min. Let cool to room temperature before injection.

Chromatographic Conditions

Inject 100 μL aliquots of grape extract and 3 standards (see reagent (j)), using flow rate of 1 mL/min for main pump and both auxiliary pumps. Set detector to 450 nm. Determine peak heights of grape extract and standards. Perform linear regression analysis on standard data to determine slope (m) and intercept (b). Calculate SO_2 content of grape extract from following equation:

$$SO_2, \text{ ppm} = [(H - b)/m] \times (100/w_a)$$

where H is peak height of grape extract.

Results and Discussion

Anionic substances such as HMS are normally not retained on a reverse-phase column, but may often be retained by adding an ion-pairing reagent to the mobile phase. In the present study, the mobile phase contains tetrabutylammonium ion (TBA^+) as the ion-pairing reagent, along with acetate buffer to maintain suitable pH. Retention is influenced primarily by the concentration of TBA^+ and by the total concentration of acetate. The retention of HMS increases with increasing [TBA^+], but not to a great extent. A 10-fold increase in [TBA^+] was found to cause only a 20% increase in retention time. Total acetate concentration, however, has a very pronounced effect on retention; a doubling of the total acetate concentration causes a 50% decrease in the retention time. Thus, acetate concentration may be manipulated to adjust the retention time of HMS as necessary. A pH of 4.7 was chosen because it is close to the pK_a of acetate and, thus, results in a high buffering capacity, and because HMS is very stable at this pH.

The procedure developed for the determination of sulfite in grapes is straightforward, consisting of multiple extractions, centrifugation, SPE column cleanup, and heating. Experiments with fortified grapes showed that multiple extractions of the grape solids yielded higher recoveries. The SPE column cleanup is used mainly to prolong column life; the grape pigments are irreversibly retained on reverse-phase packing. Thus, the pigments are strongly retained on the C_{18} SPE column, while HMS passes through unretained.

Early in the study, peaks due to compounds other than HMS were observed in the chromatograms of some grape extracts. These peaks were found to decrease with time, usually disappearing completely for extracts injected 24 h after preparation. At the same time, the HMS peak became substantially larger. For example, Figure 1 shows chromatograms for a single grape extract. In the chromatogram at the left, the extract was injected immediately after preparation. Four peaks are observed. Peak 2 is HMS, and peak 3 has been identified as the sulfite adduct of acetaldehyde, hydroxyethylsulfonate (HES). Peaks 1 and 4 have not yet been identified, but they are presumed to be sulfite adducts of other carbonyl compounds. The central chromatogram represents the same grape extract injected 3 days after preparation. Conversion of HMS is almost complete; a small amount of the peak 4 compound still persists. In the chromatogram at the right, a portion of the extract has been heated (immediately after preparation) to 80°C for 30 min, cooled to room

Table 1. Recoveries and coefficients of variation of HMS from grapes

Level, ppm (as SO_2)	Av. rec., %[a]	CV, %
5	104	6.2
10	103	2.1
20	100	3.7

[a] Average of 20 replicate analyses.

temperature, and injected. Conversion to HMS is complete. For this reason, a heating step was added to the procedure to speed up conversion of all sulfite adducts to HMS.

A recovery study of sulfite in grapes was carried out. Grapes cannot be spiked with sulfite itself because some portion of the sulfite reacts irreversibly with the matrix. Instead, the grapes were spiked with sulfite in the form of HMS, as follows: The grapes were blended with water, and portions of the homogenate were weighed into individual centrifuge tubes. The appropriate amount of HMS standard solution was then added to each tube. Spiking levels of 5, 10, and 20 ppm (as SO_2) were used; 20 replicates were analyzed at each level, and both red and white seedless grapes were used. The data are summarized in Table 1. Recovery of sulfite was essentially quantitative at all 3 spiking levels, and no differences in recovery were observed for red and white grapes. For all grapes, recoveries ranged from 92 to 112%, with an average recovery of 103% and a coefficient of variation of 4.6%. These results are similar to those obtained by Warner et al. (5), who added free sulfite to foods previously blended with the formaldehyde extractant.

Because the Monier-Williams method (7) is the most widely used of the AOAC official methods for sulfite in foods, we were interested in comparing our method with it. Grapes purchased at local supermarkets were analyzed by the LC method and by a modification of the Monier-Williams method (8), recently adopted as official first action, which permits SO_2 determination at the 10 ppm level. The grapes were homogenized with water and portions were weighed immediately for both analyses. As shown in Table 2, numbers 5–8, the grapes contained very low levels of sulfite. However, even at low levels, agreement between the 2 methods is generally good. For number 5, the LC analysis was carried out immediately after blending, whereas there was a delay of several hours before the modified Monier-Williams method was run. The delay probably caused the lower value obtained by the Monier-Williams method. Number 6 was the same bunch of grapes analyzed 4 days later; in this case, the 2 methods agree well. The lower value is consistent with the general observation that sulfite is lost from grapes on standing. Numbers 1–4 were grapes fumigated in-house. (Grapes were fumigated by filling a tube with SO_2 gas, cooling the tube with liquid nitrogen, placing the tube in a desiccator containing the grapes, and allowing the grapes to sit for several hours.) Agreement between the 2 methods for numbers 1–4 is very good. Unfortunately, we were not successful in preparing grapes containing low levels of SO_2 (e.g., 10 ppm).

Table 2. Comparison of LC and Monier-Williams methods for determining sulfite in grapes[a]

	Sulfite, ppm (as SO_2)	
Extract	LC	Monier-Williams
1	71.8	70.0
2	66.2	68.5
3	58.4	59.3
4	31.3	34.2
5	5.7	2.4
6	1.6	1.6
7	0.8	0.8
8	0.7	0.8

[a] Grapes containing >30 ppm SO_2 were prepared by fumigation with SO_2 in the laboratory. All other grapes were purchased at local supermarkets.

Table 3. Comparison of LC and Monier-Williams methods for determining sulfite in grape products

	Sulfite, ppm (as SO_2)	
Product	LC	Monier-Williams
White wine		
A	125	122
B	100	103
C	101	105
Red wine		
A	85	100
B	58	72
C	84	122
White grape juice	79	85
Red wine vinegar	79	101
Red grape juice		
A	1.6	20
B	0.3	5.8
C	0.4	10

Some grape products, including grape juice, wine, and red wine vinegar, were also analyzed by the 2 methods (Table 3). For these liquid products, LC preparation consisted of diluting the sample (1 + 49) with 0.5% buffered formaldehyde solution, filtering through an SPE column, and heating to 80°C for 30 min. Agreement between the 2 methods is good for white grape juice and white wine. However, for the red wines, red grape juices, and red wine vinegar, results by the modified Monier-Williams method are considerably higher than those by the LC method. The fact that the acid-base titration in the Monier-Williams method is nonspecific could be one reason for these results; any volatile acid in the sample may produce a positive interference. The high result for vinegar by the Monier-Williams method, which has also been observed by Kim et al. (4), could be attributed to the presence of acetic acid. To determine if this was indeed the case, a second grape extract was analyzed by a variation of the Monier-Williams method, in which SO_2 in the distillate was converted to HMS by trapping in formaldehyde solution and determined by LC analysis. The result, 86 ppm as SO_2, is close to the value of 79 ppm determined by LC alone, indicating that the higher SO_2 level measured by the Monier-Williams method may be due to volatile acids such as acetic acid rather than SO_2 from sulfites. This same argument cannot be used to explain the differences observed for the red wines and red grape juices. The results obtained by the modified Monier-Williams method were in agreement with the results obtained by the method described above for vinegar, which confirms that the method was measuring only SO_2, rather than interfering volatile acids. Some other factor appears to be at work here. This phenomenon is currently being investigated.

In summary, a simple, rapid LC method has been developed for determining total sulfite in grapes. The method is specific and permits quantitation at low parts-per-million levels. Recoveries at 5, 10, and 20 ppm were quantitative. The results of the method were comparable to those obtained by the modified Monier-Williams method. Results of the 2

methods for selected grape products revealed some anomalies, particularly for red grape products, which are currently under investigation. With suitable modification, the LC method could be used for analyses of other food matrixes.

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FOOD ADULTERATION

Honey Protein as Internal Standard for Stable Carbon Isotope Ratio Detection of Adulteration of Honey

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Using the difference in stable carbon isotope ratio between a honey and its protein fraction permits objective evaluation of possible adulteration of honey with small amounts (7–20%) as well as larger amounts of corn or cane sugar. The present uncertainty in interpretation of results from pure honeys with $\delta^{13}C$ values outside the generally accepted limits for pure honey of −27.5‰ to −23.5‰ is eliminated; likewise TLC testing to resolve questionable samples with $\delta^{13}C$ values between −23.5 and −21.5‰ is not needed. Fifty certified samples of pure honey were used to establish criteria for purity, and 38 other samples with $\delta^{13}C$ values in the "questionable" or "adulterated" range for the AOAC official method were tested. A difference of 1.0‰ or more between honey and protein fractions is proposed to indicate adulteration.

A stable carbon isotope ratio procedure was developed, validated, and adopted in 1977 (1) as an AOAC official method for detecting corn or cane sugar adulteration of honey. The wide range of values found for pure honey (mean −25.4‰ range −22.5 to −27.4‰), produces a considerable area of uncertainty within which no positive judgment can be made about purity without other testing. For regulatory purposes, the $\delta^{13}C$ limit for unequivocal adulteration was set at 4s beyond the mean, or −21.5‰. A TLC test (2–4) was developed for this "gray area," the range between 2s and 4s. It has been useful even though it does not respond to cane sugars and has other shortcomings: it requires subjective judgment of frequently faint traces on the TLC plate and may be vulnerable to the more highly purified high-fructose corn syrups (HFCS) with much reduced oligosaccharide content.

After validation of the isotope ratio test, it was established that values for citrus honey are significantly less negative than others, requiring special limits for this floral type (5). Furthermore, it is likely that the isotope ratio values for catsclaw (*Acacia* spp.) honey are even less negative. It has been speculated that honey from so-called CAM plants should be significantly less negative in $\delta^{13}C$ (6), since $\delta^{13}C$ values for several such nectars are in the range −13.1 to −14.4‰.

The need for improved testing became apparent during the U.S. government (U.S. Department of Agriculture, Agricultural Stabilization and Conservation Service) honey loan program. To avoid acquiring adulterated honey, the government has required isotope ratio testing of honey submitted for participation. With hundreds of millions of pounds of honey in the program, problems inevitably arose; TLC testing was applied during one year to a limited number of samples with $\delta^{13}C$ values less negative than −23.5‰, as required by AOAC, but it soon became evident that the test is too time-consuming and too subjective in interpretation; it is used only sparingly in the USDA program.

Further, it has always been apparent that the wide range of values found for authentic honeys could be exploited by adding as much as 12% corn syrup to a honey with $\delta^{13}C$ about −25.7‰, which would change the value to about −23.7‰, still within the "normal" range. It is not known if this is actually practiced, but it shows that basing decisions on average values for authentic honey may invite adulteration. The AOAC official method has been criticized (7) for setting the limit for unequivocally adulterated honey at −21.5‰, which was done to avoid unjust discrimination, even though as much as 25% of corn or cane sugars could be present. A means to narrow this range is urgently needed.

To eliminate these difficulties, we sought a procedure by which each sample could provide its own natural internal standard. After examining several possibilities, we developed a procedure using the isotope ratio value of the protein fraction of a honey as the internal standard; the purity is then judged by the difference between that value and that of the whole honey. This principle was proposed by Parker (8) using the pulp fraction of orange juice as the internal standard and was further examined by Bricout and Koziet (9) with 39 samples of orange juice. Mean value for pulp was −25.6‰ (s = 0.8); mean value for solubles was −25.1‰ (s = 0.9).

A candidate component for this approach should be a normal constituent, present in sufficient quantity and easily isolated. The most abundant constituent in honey after the sugars and oligosaccharides is gluconic acid. Furthermore, gluconic acid is known to originate from glucose during ripening from nectar in the hive (10); therefore, it should represent the isotopic composition of the original sugars. We examined an ion-exchange procedure for its isolation.

The constituent next in abundance is the protein, which averages 169 mg/100 g and ranges from 58 to 786 mg/100 g (11). It originates largely from the honeybee. We examined 3 procedures for isolation and purification of the protein complex and chose tungstic acid precipitation with extensive washing of the precipitate to remove sugars.

After an encouraging preliminary study using 19 samples of unknown purity, we analyzed 50 samples of known provenance and producer-certified purity to establish variability of the differences in stable carbon isotope ratio between the original honeys and their protein fraction. Finally, we analyzed 31 samples of unknown origin and purity (submitted by ASCS for purity determination) and 7 samples offered commercially for import to demonstrate the utility of the procedure.

Preliminary Studies

Ion-exchange.—A procedure previously used (12) to isolate gluconic acid from honey was evaluated.

Dialysis.—A protein fraction was isolated from 2 commercial honey samples by 24-h dialysis of 25 g portions against tap water followed by evaporation on a hot plate to about 2 mL.

Alumina cream precipitation.—The protein material was obtained from the same samples by precipitation with alumina cream, commonly used for clarifying solutions for polarimetry. Aluminum hydroxide (which interferes with recovery of CO_2 during combustion) was removed by adding con-

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Table 1. Isotope ratio values (‰) of honey fractions

Sample	Honey	Dialyzate	Precipitate	
			Alumina cream	Tungstic acid
A	−24.6	−25.2	−25.5	−25.2
B	−25.9	−25.7	−25.6	−25.4

centrated HCl, heating to boiling, and washing with water 4 times by decantation.

Tungstic acid precipitation.—The procedure finally used was adapted from Bianchi's (13) modification for honey of the Folin-Wu blood protein procedure.

METHOD

Reagents

(a) *Tungstic acid, sodium salt.*—10% aqueous solution (Sigma Chemical Co., St. Louis, MO, Cat. T2629).

(b) *Sulfuric acid.*—2/3N. Add 1.88 mL H_2SO_4 to water and dilute to 100 mL.

Equipment

(a) *Centrifuge.*—Accepting 50 mL tubes and capable of RCF = 1500 × *g* (IEC HN S-II is suitable).

(b) *Isotope ratio mass spectrometer.*

Procedure

(a) *Honey.*—Determine $\delta^{13}C$ of honey sample as directed in *Official Methods of Analysis*, **31.158** (14).

(b) *Protein.*—If appreciable amounts of solid matter are present, strain honey through 100–150 mesh (nylon stocking material is excellent) because any insoluble material heavier than water will contaminate the protein precipitate. Add 4 mL water to 10–12 g honey in clear 50 mL centrifuge tube and mix well. To small test tube, add 2.0 mL 10% sodium tungstate solution and 2.0 mL 2/3N H_2SO_4, mix, immediately add to honey sample, and mix well. Swirl tube in ca 80°C water bath until visible floc forms, with clear supernate. If no visible floc forms, or if supernate remains turbid, add dilute acid in 2 mL increments and repeat heating between additions.

Fill tube with water, mix, and centrifuge 5 min at 1500 × *g*; decant supernate and wash pellet 5 times with ca 50 mL portions of water, thoroughly dispersing pellet each time. Transfer washed pellet with minimum of water (Pasteur pipet) to small vial, cap, and place in boiling water for 2 min to hold for isotope ratio analysis, unless this is to be done immediately.

Place 15–25 mg (wet weight) protein in ceramic combustion boat similar to that used for honey samples. Dry protein in oven at ca 75°C ≥ 3 h. Combust protein samples by same method used for honey (14) and determine $\delta^{13}C$ with the same instrument used for honey.

Calculate apparent corn/cane sugar content as follows:

$$\text{Adulteration, \%} = 100 \times [\delta^{13}C(\text{protein}) - \delta^{13}C(\text{honey})]/[\delta^{13}C(\text{protein}) - (-9.7)]$$

Report negative values for % adulteration as 0%.

Results and Discussion

Ion-exchange isolation of gluconic acid yielded a product with $\delta^{13}C$ of −20.6‰ from a honey with $\delta^{13}C$ of −26.1‰. This rather large difference and the cumbersome isolation and purification procedure discouraged its use.

Results of the preliminary isolations of the protein fractions are shown in Table 1. No significant differences are apparent among the 3 procedures, so the simplest was chosen, described above. It was applied to 19 samples of honey of unknown purity and provenance to evaluate it generally. Results are shown in Table 2. Samples 6–19 in the table were selected from ASCS samples with known $\delta^{13}C$ values including those common to pure honey and also some degree of possible adulteration. The others were commercial samples. Three of the samples (Nos. 6, 15, 17) seemed sufficiently different from the others to be considered adulterated and were excluded from the calculation of means and standard deviations. The close agreement of the means for honey and protein is gratifying; the difference is within the error of the isotope ratio measurement (0.2‰). Furthermore, the standard deviation for the protein value is appreciably less than that for the whole honeys.

Sample 13 in Table 2 was sample 14 with 20% HFCS added. The value of 21.6% corn syrup indicates satisfactory agreement with the amount added. The linear relationship of $\delta^{13}C$ values of mixtures of corn syrup and honey was established earlier (1). Whether the apparent elevated corn/cane contents of samples 6, 15, and 17 are results of its deliberate addition by the producers after harvest or of exposing corn/cane syrup in the apiary during the normal honey flow is not addressed here. It is not likely that it resulted from feeding sugar in the absence of a nectar flow.

These data encouraged us to examine a representative group of samples of known purity and provenance. Forty-six of these samples were taken from the 1974–75 crops originally collected for the research which led to the establishment of the official isotope ratio method (1). These had been certified by their producers as pure and had been stored at or below 10°F since. Most of them had also been used in a study of their bound galactose content in an effort to detect added beet sugar (15).[1] The other four (Nos. 57, 64, 65, 66), from the 1988 crop, were also certified pure by their producers.

During the analysis of these samples, 6 replicates of a

Table 2. Feasibility study of use of honey protein as internal standard in isotope ratio testing of honey

Number	Stable carbon isotope ratio ($\delta^{13}C$), ‰			Apparent corn/cane content, %
	Honey	Protein	Diff.[a]	
1	−24.6	−25.2	−0.6	3.9
2	−25.9	−25.4	0.5	
3	−25.9	−25.4	0.5	
4	−23.6	−23.7	−0.1	0.7
5	−22.2	−22.6	−0.4	3.1
6	−21.7	−24.1	−2.4	16.7[b]
7	−25.4	−24.8	0.6	
8	−26.0	−25.5	0.5	
9	−26.6	−25.6	1.0	
10	−27.0	−26.0	1.0	
11	−24.5	−24.4	0.1	
12	−24.0	−24.0	0.0	
13	−21.7	−25.0	−3.3	21.6[b,c]
14	−25.0	−25.1	−0.1	0.6
15	−20.4	−23.3	−2.9	21.3[b]
16	−22.5	−21.8	0.7	
17	−21.1	−26.1	−5.0	35.5[b]
18	−23.2	−23.8	−0.6	4.2
19	−19.7	−20.4	−0.7	6.5
Mean	−24.40	−24.24		
s	1.94	1.58		

[a] Protein value minus honey value.

[b] Excluded from calculation of mean (see text).

[c] Sample 14 with 20% added HFCS. Also excluded from mean.

Table 3. Reproducibility of procedure (‰ $\delta^{13}C$)

Run	Honey	Protein	Diff.[a]
A	−22.9	−23.6	−0.7
B	−22.8	−23.0	−0.2
C	−22.7	−23.4	−0.7
D	−22.8	−23.5	−0.7
E	−22.8	−23.4	−0.6
F	−22.8	−22.8	0.0
Mean	−22.8	−23.28	−0.48
s	0.06	0.31	0.31

[a] Protein value minus honey value.

commercial citrus honey were analyzed, 2 on each of 3 days, to evaluate reproducibility of the entire procedure. Results of the replication are shown in Table 3. The larger standard deviation of the protein values (0.31‰ vs 0.08‰ for the honey) must be due to the procedure of isolating the proteins and possibly to the much smaller sample available for the combustion and mass spectrometry.

[1] The bound galactose content of these honeys averaged only one-tenth that of the average of a variety of beet sugar products, but values for about 10% of the honeys fell within and beyond the beet sugar range, so that additional testing is required to demonstrate significant beet sugar addition.

Table 4. Stable carbon isotope ratios of certified honeys and their protein fractions

No.	Floral type	State of origin	Stable carbon isotope ratio ($\delta^{13}C$), ‰			Apparent corn/cane content, %
			Honey	Protein	Diff.[a]	
20	Pepper tree	FL	−24.6	−24.3	0.3	
21	Alfalfa	SD	−24.5	−24.8	−0.3	2.0
22	Basswood	WI	−25.5	−25.0	0.5	
23	Goldenrod	MN	−25.4	−25.3	0.1	
24	Clover	NE	−24.1	−23.5	0.6	
25	Alfalfa-sunflower	ID	−24.0	−23.8	0.2	
26	Citrus	FL	−23.0	−23.6	−0.6	4.3
27	Tupelo	FL	−25.3	−24.8	0.5	
28	Palmetto	FL	−23.4	−23.7	−0.3	2.1
29	Clover	AR	−25.1	−25.2	−0.1	0.6
30	Locust	TN	−23.4	−23.9	−0.5	3.5
31	Ti-ti	GA	−23.1	−23.7	−0.6	4.3
32	Clover	MI	−25.2	−24.4	0.8	
33	Clover	OH	−24.0	−24.4	−0.4	4.1
34	Huajillo	TX	−23.6	−23.8	−0.2	1.4
35	Clover-alfalfa	MT	−23.1	−23.8	−0.7	5.0
36	Chinese tallow	TX	−25.2	−25.8	−0.6	3.7
37	Clover	SD	−24.9	−24.3	0.6	
38	Clover	WI	−24.8	−24.4	0.4	
39	Spearmint	WA	−24.2	−23.7	0.5	
40	Wild buckwheat	CA	−23.4	−23.2	0.2	
41	Mesquite	TX	−25.8	−25.5	0.3	
42	Safflower	CA	−24.4	−24.8	−0.4	2.6
43	Trefoil	IA	−24.9	−25.2	−0.3	1.9
44	Alfalfa	AZ	−25.3	−24.0	0.3	
45	Soybean	MN	−24.8	−24.7	0.1	
46	Blackberry-thistle	WA	−25.5	−24.7	0.8	
47	Clover	IL	−25.1	−24.7	0.4	
48	Clover	IN	−24.4	−24.3	0.1	
49	Clover	NY	−24.0	−24.3	−0.3	2.0
50	Tulip poplar	NC	−24.4	−24.3	0.1	
51	Alfalfa	NE	−24.6	−23.1	1.5	
52	Cotton	OK	−23.7	−21.8	1.9	
53	Sunflower	ND	−24.5	−24.6	−0.1	0.7
54	Season blend	MI	−25.1	−24.9	0.2	
55	Alfalfa blend	IA	−25.2	−24.0	1.2	
56	Season blend	MN	−23.8	−24.4	−0.6	4.1
57	Citrus	FL	−23.1	−23.2	−0.1	0.7
58	Alfalfa blend	UT	−23.6	−23.6	0.0	
59	Thistle-vetch	OR	−25.2	−24.5	0.7	
60	Alfalfa-sweetclover	WY	−24.5	−23.4	1.1	
61	Season blend	LA	−26.7	−25.4	1.3	
62	Lima bean	CA	−23.7	−20.6	3.1	
63	Aster	OH	−24.9	−24.6	0.3	
64	Catsclaw	AZ	−22.3	−22.8	−0.5	3.8
65	Catsclaw	AZ	−22.9	−22.9	0.0	
66	Catsclaw	AZ	−22.4	−22.0	0.4	
67	Citrus	FL	−22.8	−23.2	−0.4	3.0
68	Sweetclover	ND	−25.7	−25.1	0.6	
69	Catsclaw	AZ	−22.0	−21.9	0.1	
Mean			−24.30	−24.04		
s			1.02	1.03		

[a] Protein value minus honey value.

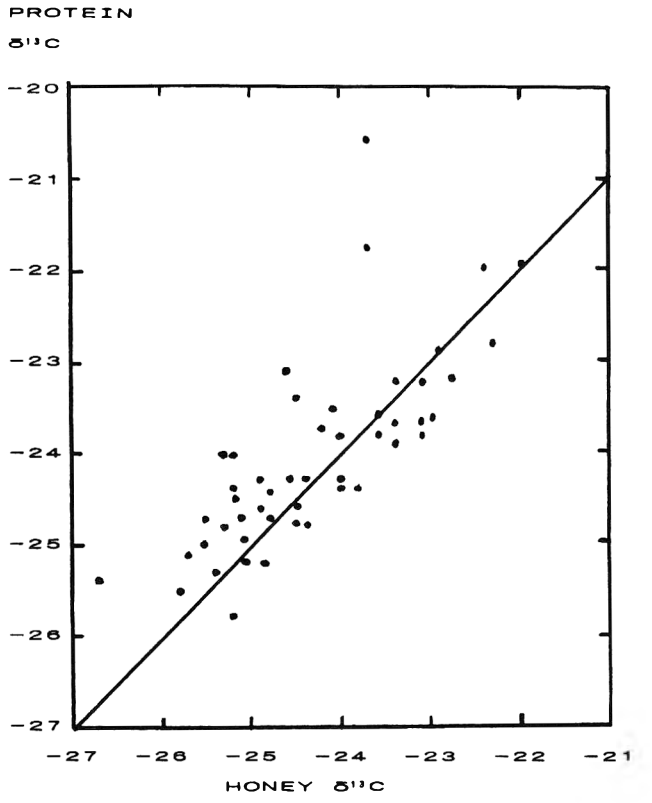

Figure 1. Stable carbon isotope ratios of 50 pure honeys and their protein fractions. Line indicates equivalence.

Results of the analysis of the authentic samples are shown in Table 4. Figure 1 shows the values for each sample. The $\delta^{13}C$ values previously published for 46 of these samples when analyzed earlier (1) by another laboratory averaged −25.2‰. For these same samples after 14 years of frozen storage (which should have no effect on isotope ratios), values in this work averaged −24.4‰. This difference, although significant, is not surprising when compared with interlaboratory differences in $\delta^{13}C$ shown for 6 laboratories in a recent collaborative study (16). This difference emphasizes the importance of measuring the isotope ratio values of both honey and protein on the same instrument, as specified in the method.

The relationship between the $\delta^{13}C$ values for protein and honey is expressed by the least squares line $y = -5.67 + 0.756\,x$; $r = 0.75$. The difference between the mean $\delta^{13}C$ for honeys and for the protein isolates for the certified samples in Table 4 is 0.26‰ and the standard deviations, 1.02‰ and 1.03‰, are similar. The t-test with $t = 1.26$ ($t_{0.005} = 2.68$) confirms that the difference is not significant, as does an analysis of variance (Table 5). We consider this conclusive evidence that the principle examined is valid and that, after appropriate limits are imposed, the occurrence and extent of honey adulteration by corn or cane syrups can be established.

When the extent of addition of these syrups is estimated, only the differences between protein and honey values in the less negative direction are of significance. Using the protein value as reference for a sample, the amount of adulteration can be calculated for each sample with far greater accuracy than the approximations used in the official method (1), in which the average value for honey (−25.4‰) and that of HFCS (−9.7‰) are used as a basis for the extent of adulteration.

Table 5. Analysis of variance of data in Table 4

Variance assoc. with	DF	SS	MS	F
Materials	1	1.74	1.74	1.66 (ns)[a]
Error	98	102.89	1.05	
Total	99	104.63		

[a] ($F_{0.05}$ = ca 4.04).

Table 6. Rejection criteria for internal standard isotope ratio testing of honey for adulteration

Probability of error	Limit	Diff.,[a] ‰	Corn/cane, %
1 in 6	Mean[b] + s	−0.35	2.4
1 in 44	Mean + 2s	−0.57	4.0
1 in 770	Mean + 3s	−0.79	5.5
1 in 25000	Mean + 4s	−1.01	7.1

[a] Protein value minus honey value.
[b] Mean negative difference (Table 4).

Since the differences when positive may be taken to indicate the absence of adulteration, we have considered them as zero for the purpose of calculation. Thus Table 4 shows 18 certified honeys with small negative differences, 2 exactly equal, and 30 with positive differences. Considering the latter as zero, the mean negative difference is −0.13‰, s = 0.22‰. Table 6 provides guidelines for estimating corn or cane syrups at various levels of probability of error. It is proposed that a negative difference of 1.0‰, (about 7% corn or cane addition) be adopted as the limit for considering a sample adulterated. This corresponds to the mean negative difference for authentic honeys plus 4s.

To test further the performance of this procedure, 31 samples of honey were selected from those analyzed for ASCS. All had $\delta^{13}C$ less negative than −23.5‰, the value above which honey has been rejected by ASCS as adulterated unless proven otherwise by a TLC test. Results are shown in Table 7. Of these, 23 are in the −23.5 to −21.7‰ range, which requires a TLC test under existing AOAC procedures. Using the new procedure, 11 of these were found acceptable, and 12 were adulterated. The other 8 samples would be rejected under the present AOAC procedures without further testing, since their values were less negative than −21.7‰. Our testing found 2 (Nos. 70, 75) to be pure, and 6 to be adulterated, ranging from 8.7% to 75%.

Table 8 shows 7 commercial samples of honey offered for import. Two (Nos. 101, 102) represented so-called "Mexican mesquite" honey, which has consistently been rejected over the years on the basis of its $\delta^{13}C$ value. TLC tests on this honey type have been negative; these were not so tested. It is apparent that the somewhat less negative values are characteristic of this honey and do not represent adulteration. The reason for this difference from U.S. mesquite honey is not clear. The remaining 5 samples in Table 8 were offered as "Russian" honey. All are heavily adulterated.

The larger dispersion of the differences (Table 4) when compared with the results of the replication (Table 3) implies that other factors contribute to the spread of values. It is well established (17–19) that the $\delta^{13}C$ values of animals and their products are dependent on that of their diet, although some small differences are seen in various categories of product. Although a small part of the differences in the negative direction seen here may be due to isotope effects in the synthesis of the protein material from dietary constituents in the honeybee, the rather wide range implies other influences.

Honey as marketed by the producer is a blend from a number of colonies, from one or more locations, and is fre-

Table 7. Internal standard isotope ratio testing of selected honeys[a] offered for loan program

Number	Stable carbon isotope ratio ($\delta^{13}C$), ‰ Honey	Protein	Diff.	Corn/cane content, %
70	−21.6	−21.8	−0.2	1.6
71	−22.6	−23.7	−1.1	7.8
72	−21.3	−22.4	−1.1	8.7
73	−22.3	−23.5	−1.2	8.7
74	−22.0	−23.0	−1.0	7.5
75	−21.1	−21.8	−0.7	5.8
76	−21.9	−22.3	−0.4	3.1
77	−22.2	−23.8	−1.6	11.3
78	−22.8	−23.6	−0.8	5.7
79	−22.1	−23.3	−1.2	8.8
80	−22.9	−23.3	−0.4	2.9
81	−22.4	−23.2	−0.8	5.9
82	−22.5	−24.6	−2.1	13.9
83	−21.5	−22.9	−1.4	10.6
84	−21.4	−23.5	−2.1	15.0
85	−22.4	−23.2	−0.8	5.9
86	−22.5	−23.3	−0.8	5.9
87	−22.8	−22.9	−0.1	0.7
88	−21.1	−23.3	−2.2	16.2
89	−22.2	−23.1	−0.9	6.7
90	−19.2	−23.9	−4.7	33.0
91	−22.2	−23.4	−1.2	8.7
92	−22.8	−23.0	−0.2	1.5
93	−12.3	−20.1	−7.8	75
94	−22.6	−24.3	−1.7	11.6
95	−22.3	−23.1	−0.8	6.0
96	−22.3	−23.6	−1.3	9.4
97	−22.2	−23.2	−1.0	7.4
98	−22.2	−24.1	−1.9	13.2
99	−22.6	−22.7	−0.1	0.8
100	−22.0	−22.7	−0.7	5.4

[a] Selected for $\delta^{13}C$ between −23 and −21‰, except Nos. 90, 93.

quently collected over a considerable number of weeks by the bees; each colony may have a foraging range of 70–80 square miles. This all provides an extremely large opportunity for diversity. During a honey flow, honeybees have a life-span of about 3–4 weeks; the population is constantly renewed and the new population is fed with honey collected by the same colony previously. Before a honey flow, particularly in early spring, it is common practice to built up colony strength by feeding sugar syrup; HFCS as well as sucrose or invert sugar is used. The protein (enzymes) added by the bees to stores during this period would reflect the isotopic composition of that feed. When sufficient nectar becomes available, the feeding is discontinued and the short lifespan of the bees ensures that the contribution of corn/cane to the $\delta^{13}C$ of the protein component of the extracted honey is rapidly diluted.

Another potential cause for the wider range in the more negative direction could be differences in pollen content from various sources. Pollen, heavier than water, will be present in the protein isolate. It can be calculated that as much as 1.3 mg pollen could be included in the isolate, up to 10% of the total. Since pollen is collected concurrently with nectar, its presence in naturally varying amounts will not vitiate the procedure. Nectar sources vary in the abundance of pollen available (20); a good nectar source may be a poor pollen source so that the relative proportions of pollen and nectar from different plants may contribute to the width of the range.

Table 8. Internal standard isotope ratio testing of selected honeys offered for import

Number	Stable carbon isotope ratio ($\delta^{13}C$), ‰ Honey	Protein	Diff.	Corn/cane content, %
101	−22.0	−21.9	0.1	0.0
102	−21.9	−21.8	0.1	0.0
103	−13.3	−18.3	−5.0	58
104	−15.0	−18.7	−3.7	41
105	−15.3	−21.0	−5.7	50
106	−16.5	−20.4	−3.9	36
107	−14.9	−20.0	−5.1	49

In addition to resolving uncertainties for samples within the "gray area," this procedure settles authenticity questions for honeys falling outside the so-called normal range, such as citrus, catsclaw, and others not yet identified. Honey from CAM plants, or mixtures containing appreciable amounts of such honey, can be also be judged for purity.

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FOOD COMPOSITION

Precolumn Phenylisothiocyanate Derivatization and Liquid Chromatography of Amino Acids in Food

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A precolumn phenylisothiocyanate derivatization method is described for the determination of amino acids in protein hydrolysates from a wide variety of complex food matrixes, with and without performic acid oxidation pretreatment. Analysis of samples that were not pretreated with performic acid was necessary since this pretreatment destroyed an average of 25% of the histidine and 87% of the tyrosine present in the food samples. This method is rapid and reproducible; coefficients of variation between duplicate analyses of the same food item were less than 5% for a majority of the amino acids. Occasionally, variation between duplicate analyses for histidine and tyrosine was greater than 10%. Recoveries of amino acids added to samples were in the 100% range.

The use of phenylisothiocyanate (PITC, or Edman's reagent) for amino acid analysis was first described by Heinrickson and Meredith (1) and developed commercially by Waters (Milford, MA) as the Pico-Tag method (2, 3). In the Pico-Tag method, PITC is used for precolumn derivatization of both primary and secondary amino acids after protein hydrolysis. Reverse-phase gradient elution liquid chromatography (LC) is used to separate the phenylthiocarbamyl (PTC) derivatives (typically in less than 20 min), which are then detected by their UV absorbance at a single wavelength. This technique has been used extensively for the analysis of amino acids in pure proteins and peptides because it is sensitive, reproducible, and rapid, especially when compared to traditional amino acid analysis techniques (4).

With some minor modifications in sample preparation and chromatography, the Pico-Tag method has been applied to the determination of amino acids in food matrixes (5–9). To date, however, only relatively simple food matrixes have been analyzed.

The present paper outlines the determination of amino acids, including the sulfur-containing amino acids in complex recipe-prepared foods, using a modification of the original Water's Pico-Tag method.

Experimental

The Pico-Tag method for sample preparation and analysis was used (Liquid chromatographic analysis of amino acids in feeds and foods using a modification of the Pico-Tag method, revision 1987, Millipore Corp.), with some modifications as described below.

Reagents and Apparatus

Reagents.—Reagent grade concentrated hydrochloric acid, formic acid, hydrogen bromide, hydrogen peroxide, and LC grade glacial acetic acid, acetonitrile, methanol, and sodium acetate trihydrate were obtained from J. T. Baker (Phillipsburg, NJ). Pierce H amino acid standard and phenylisothiocyanate (PITC) were obtained from Pierce Chemical Co. (Rockford, IL). Triethylamine (TEA) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Alpha-amino-*n*-butyric acid (AABA), cysteic acid (CYA), methionine sulfone (MET-O_2) cystine (CYS), methionine (MET), and 1-octanol were obtained from Sigma Chemical Co. (St. Louis, MO).

Apparatus.—High purity water was supplied by a Milli-Q purification system (Millipore, Bedford, MA). Hydrolysis (25 × 50 mm) and derivatization (6 × 50 mm) tubes were Pyrex brand (Corning Glass Co., Corning, NY). Vacuum vials and resealable enclosures were obtained from Waters (Milford, MA). HV-type membrane filters (0.45 μm pore size, Millipore) were used for sample and solvent filtration. Samples were heated for hydrolysis in an electric oven. Samples were derivatized and dried in a Waters Pico-Tag vacuum station. Samples subjected to performic acid oxidation were dried with an Evapo-Mix (Buchler Instruments, Fort Lee, NJ).

Sample Handling Prior to Hydrolysis or Oxidation

Food samples with high water content (potatoes and complete recipe-prepared foods) were homogenized in a food processor, frozen with liquid nitrogen, freeze-dried, and ground to pass a 20 or 40 mesh sieve. Dry food samples (wheat, legumes) were ground directly to pass a 40 mesh sieve. Protein samples (casein, U.S. Biochemical) were analyzed directly.

All glassware used in sample preparation and analysis was washed with 6N HCl, rinsed with Milli-Q water, and dried. The protein content of each sample was determined by the micro-Kjeldahl method (10).

Acid Hydrolysis

Enough sample to contain 40 mg protein (wet weight basis, WB) was weighed into a hydrolysis tube, and 15 mL 6N HCl was added. The tube was then flushed for 30 s with nitrogen gas and immediately sealed with a Teflon-lined cap. The tube was placed in an electric oven for 24 h at 110°C for sample hydrolysis. The tube was then cooled to room temperature, and 10 mL internal standard solution (AABA, 10 μmol/mL) was added. The contents of the tube were quantitatively transferred to a 50 mL volumetric flask with washings of Milli-Q water. After thorough mixing, ca 1 mL dilute sample was filtered, and 10 μL filtrate was placed in a derivatization tube. (If derivatization cannot immediately follow hydrolysis, the filtrate can be stored at −20°C for at least 3 months.)

Performic Acid Oxidation for Cystine and Methionine

A sample to contain 40 mg protein (WB) was weighed into a hydrolysis tube and chilled in an ice bath for 30 min. Ten mL of a mixture of 30% hydrogen peroxide–88% formic acid

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Table 1. Amino acid content of casein, beef pepper steak, and potatoes, and published data for casein and potatoes[a]

Amino acid	Casein g/100 DB (CV)	PV casein[b] g/100 DB	Beef g/100 WB (CV)	Potatoes g/100 DB (CV)	PV potatoes[c] g/100 DB
CYA	0.566 (0.21)	0.632	0.173 (7.27)	0.113 (0.42)	0.106
ASP	6.889 (0.03)	7.301	0.813 (3.65)	2.241 (0.92)	1.963
GLU	23.271 (1.26)	22.728	1.735 (0.94)	1.630 (0.65)	1.290
SER	5.171 (3.49)	5.777	0.354 (7.92)	0.282 (8.82)	0.271
GLY	1.821 (0.87)	1.894	0.356 (2.73)	0.211 (4.79)	0.220
HIS	2.641 (5.60)	2.813	0.364 (9.21)	0.160 (4.79)	0.150
ARG	4.002 (1.10)	3.513	0.572 (1.56)	0.357 (1.12)	0.411
THR	3.874 (0.29)	4.394	0.367 (8.09)	0.249 (2.18)	0.255
ALA	3.124 (0.49)	3.125	0.501 (1.07)	0.320 (0.52)	0.216
PRO	10.556 (0.99)	11.100	0.403 (1.25)	0.254 (1.57)	0.190
MET-O_2	2.982 (2.27)	2.355	0.231 (1.51)	0.100 (4.08)	0.143
TYR	5.949 (1.94)	5.720	0.301 (9.27)	0.183 (9.40)	0.326
VAL	6.979 (1.18)	6.487	0.459 (3.98)	0.498 (0.43)	0.450
ILE	5.402 (2.18)	5.076	0.412 (4.88)	0.305 (0.46)	0.298
LEU	9.250 (0.16)	9.622	0.724 (1.46)	0.428 (0.22)	0.417
PHE	4.952 (1.58)	5.180	0.391 (8.74)	0.334 (2.32)	0.317
LYS	7.453 (0.10)	7.993	0.697 (6.71)	0.479 (7.30)	0.389
Protein WB, % (N X 6.25)	92.98	94.70	9.31	1.95	

[a] Abbreviations: PV = published value; DB = dry basis; CV = percent coefficient of variation; WB = wet basis.
[b] Ref. 11.
[c] Ref. 12.

(1 + 9, v/v) (which had been mixed gently at room temperature for 1 h and chilled in an ice bath for 30 min) was added to the hydrolysis tube. The tube contents were swirled gently and the tube was sealed with a Teflon-lined cap. The tube was stored in an ice bath at 5°C in a refrigerator for 16 h. With the tube still in an ice bath and under a fume hood, 3 drops of 1-octanol were added, and 3 mL chilled 48% hydrogen bromide was added, slowly, while tube contents were swirled. The tube was kept in an ice bath for an additional 30 min. The tube contents were then dried in an evaporator-mixer. At this point, sample preparation was continued with the addition of 6N HCl, as described in the acid hydrolysis section.

Derivatization of Amino Acids with PITC

A derivatization tube containing 10 μL filtrate from the acid hydrolysis procedure was placed in a vacuum vial and sealed with a Teflon-lined closure. The tube contents were dried under vacuum (to less than 75 millitorr pressure) in the Pico-Tag vacuum station and 30 μL of a redry solution was added (mixture of LC grade methanol–0.2N LC grade sodium acetate trihydrate in Milli-Q water–TEA (2 + 2 + 1)). After contents were again dried to less than 75 millitorr pressure, 30 μL derivatization reagent was added (mixture of LC grade methanol–Milli-Q water–TEA–PITC (7 + 1 + 1 + 1)) and the tube was held at room temperature and atmospheric pressure for 20 min. The tube was dried under vacuum for 15 min; then 30 μL LC grade methanol was added. Drying was continued to a pressure of less than 75 millitorr.

After derivatization, the sample was either stored dry at −20°C (samples are stable under these conditions for at least 3 weeks) or 100 μL diluent was added (5mM sodium phosphate monobasic, titrated to pH 7.5 with LC grade glacial acetic acid, and 5% (v/v) LC grade acetonitrile). The sample was then transferred to a limited volume (300 μL) insert contained in a Waters Intelligent Sample Processor (WISP, Waters) injection vial. This vial was sealed with a Teflon-lined closure and contents were analyzed within 12 h. For chromatographic analysis, a 20 μL aliquot of sample (containing 1 nmol of the internal standard, AABA) was injected.

Standard Preparation

The working standard consisted of a 1:1 (v/v) mixture of Pierce H amino acid standard (which contains 2.5 μmol/mL of each amino acid, except for 1.25 μmol/mL of cystine), and a solution containing 2.5 μmol/mL of AABA, CYA, MET-O_2. Ten μL working standard was placed in a derivatization tube and carried through the same derivatization procedure as the samples, except that 250 μL diluent was added to the tube. For chromatographic standardization, a 20 μL aliquot (containing 1 nmol of each amino acid, except for 0.5 nmol cystine) was injected.

Chromatography and Quantification

Chromatography.—A Model ALC 204 liquid chromatograph (Waters) which consisted of 2 M6000A solvent delivery systems (Waters) controlled by an M660 gradient programmer and one M440 fixed-wavelength spectrophotometer (254 nm/0.2 AUFS) was used. Samples were injected using an M712 WISP automatic injector (Waters).

The column was an application-specific Pico-Tag analysis column (Waters) (3.9 X 150 mm, stainless steel). The temperature was controlled at 40°C with a column heater (Waters). The solvent system consisted of 2 eluants: (*1*) 940 mL of an aqueous buffer of 0.14M LC grade sodium acetate trihydrate containing 0.75 mL/L of TEA titrated to pH 6.2 with LC grade glacial acetic acid, and 60 mL LC grade acetonitrile, and (*2*) 60% LC grade acetonitrile and 40% Milli-Q water. Eluant A was pumped isocratically at 1.0 mL/min for 3 min; then a gradient was run from 0% B to 68% B in 17 min using a linear curve at 1.0 mL/min. An additional step to 100% B for 1 min was used to flush highly retained components from the column. The program was then returned to 100% A at 1.5 mL/min for 8 min to regenerate the column, at which time the flow rate was reduced to 1.0 mL/min. The total time between injections was 29 min. These gradient conditions were optimized for use with a manual pump control system. If an automated pump controller is available, total run time (including column regeneration) can be reduced to 20 min (6).

Quantification.—Two instruments were used for quantifi-

Table 2. Amino acid content of spring and winter wheat varieties, and published data for soft red winter wheat variety[a]

Amino acid	Daws spring, g/100 WB (CV)	Daws winter, g/100 WB (CV)	Tres winter, g/100 WB (CV)	Soft red winter,[b] g/100 WB
CYA	0.496 (1.60)	0.462 (1.38)	0.457 (1.90)	0.423
ASP	0.607 (1.07)	0.569 (1.41)	0.577 (0.55)	0.557
GLU	3.561 (0.38)	3.370 (2.21)	3.529 (1.15)	3.663
SER	0.518 (1.94)	0.484 (6.01)	0.500 (0.58)	0.540
GLY	0.492 (0.81)	0.460 (4.19)	0.431 (2.42)	0.455
HIS	0.255 (10.04)	0.233 (11.11)	0.237 (11.34)	0.256
ARG	0.559 (6.97)	0.545 (3.46)	0.525 (4.26)	0.522
THR	0.303 (2.47)	0.289 (6.01)	0.281 (3.22)	0.342
ALA	0.596 (1.43)	0.556 (2.30)	0.583 (1.07)	0.412
PRO	1.280 (0.01)	1.179 (2.79)	1.237 (0.74)	1.166
MET-O_2	0.293 (0.79)	0.287 (5.54)	0.274 (0.22)	0.211
TYR	0.220 (14.50)	0.226 (14.31)	0.193 (14.42)	0.327
VAL	0.551 (2.97)	0.507 (1.55)	0.500 (1.66)	0.498
ILE	0.423 (1.86)	0.386 (1.14)	0.393 (1.99)	0.396
LEU	0.841 (0.44)	0.780 (2.97)	0.766 (1.02)	0.763
PHE	0.541 (1.09)	0.505 (0.42)	0.536 (6.75)	0.508
LYS	0.331 (1.93)	0.384 (8.58)	0.326 (4.62)	0.315
Protein WB, %	11.31	9.71	10.33	10.35

[a] Abbreviations: see Table 1.
[b] Ref. 13.

cation. One was an M730 data module (Waters) operated in the internal standard mode. In addition, data were collected and processed with Maxima 820 software (Dynamic Solutions, Ventura, CA) via a System Interface Module (SIM) (Waters) between the detector and an IBM (Armonk, NY) PC/XT computer. Both instruments gave comparable results.

Standard Addition and Standard Recovery Studies

To test for loss of amino acids during sample preparation and hydrolysis, 1 mL Pierce H standard was added to duplicate samples of a freeze-dried food recipe (LaChoy beef pepper steak oriental), and 1 mL Milli-Q water was added to 2 additional duplicate samples of this same food recipe. These 4 samples were hydrolyzed, derivatized, and analyzed as described above. Two "samples" containing 2 mL Pierce H standard were "hydrolyzed," derivatized, and analyzed.

To test for loss of amino acids during performic acid oxidation and subsequent hydrolysis, 2 standard addition recovery studies were performed. In one case, 1 mg additions of CYA and MET-O_2 (in aqueous solutions) were added to duplicate samples of the food recipe, while equivalent volumes of Milli-Q water were added to 2 additional samples. Oxidation, hydrolysis, derivatization, and analysis followed. In another experiment, 1 mg additions of histidine, tyrosine, and phenylalanine (in aqueous solutions) were added to replicate samples of the food recipe, while the equivalent quantity of water was added to 2 additional samples. These samples were then oxidized, hydrolyzed, derivatized, and analyzed.

To test for conversion of CYS and MET to CYA and MET-O_2, respectively, during performic acid oxidation, an additional test was conducted. One mg spikes of CYS and MET were added as aqueous solutions to 2 samples of the food recipe, while an equal volume of Milli-Q water was added to additional replicate samples. Oxidation, hydrolysis, derivatization, and analysis of these samples followed.

Results and Discussion

A total of 41 recipe-prepared foods were analyzed with this method. This was a wide variety of food matrixes, with protein, carbohydrate, and fat contents ranging from (on a wet weight basis) 0.83 to 19.4%, from 2.63 to 35.48%, and from 1.93 to 27.0%, respectively. The recipes included salads, sauces, casseroles, and desserts. These foods were analyzed for the USDA Human Nutrition Information Service as a part of Contract No. 53-3198-6-51. We chose a mixed beef dish (LaChoy canned beef pepper steak oriental) as a representative recipe-prepared food because the amino acid profile and analytical variability experienced with this sample was typical of the other recipes analyzed.

Some individual food items were also analyzed to allow for comparison of this procedure with published methods. These included 3 varieties of wheat, 2 varieties of dry peas, a legume sample, Russett Burbank potato, and casein.

Duplicate samples of each food item were analyzed. The coefficients of variation (CV) between duplicate analyses were less than 5% for a majority of the amino acids and were never greater than 15%. CV between duplicate injections were always less than 5%. The amino acid contents of the representative recipe and the individual food items, along with the CV between duplicates, are shown in Tables 1–3. The amino acids are listed in their elution order (the internal standard AABA elutes just prior to tyrosine). Chromatograms of the representative recipe and the working standard are shown in Figure 1. The internal standard (AABA) peak in both chromatograms, and the peaks for the amino acids in the working standard, represent 1 nmol injected.

Variability was highest for histidine (HIS) and tyrosine (TYR). HIS peak shape was irregular in some cases due to degradation of eluant A. This problem was overcome by using freshly prepared eluant A and/or a slightly elevated TEA content in eluant A. HIS peak shape can also be improved by adding $(Na)_2$EDTA at 200 ppb to eluants 1 and 2. TYR may have been oxidized in some duplicates during sample preparation, leading to low recoveries (15). Occasionally, the so-called small reagent peak co-elutes with phenylalanine (PHE). Adequate drying after derivatization (to less than 75 millitorr pressure) reduces the size of this reagent peak, and a slight increase in the pH of eluant A (to 6.3) can be used to completely resolve this peak from PHE. In some cases, there was relatively high variability for PHE and lysine (although never greater than 10% CV). These are the last

Table 3. Amino acid content of peas and lentils, and published data for split peas[a]

Amino acid	Alaska peas, g/100 WB (CV)	Melrose peas, g/100 WB (CV)	Brewer lentils, g/100 WB (CV)	Split peas,[b] g/100 WB
CYA	0.455 (3.17)	0.527 (4.59)	0.479 (6.13)	0.526
ASP	2.589 (1.72)	2.237 (0.22)	2.588 (0.24)	2.896
GLU	4.225 (1.04)	3.416 (0.33)	3.864 (0.36)	4.196
SER	1.117 (0.76)	1.032 (0.28)	1.154 (1.91)	1.080
GLY	0.987 (0.81)	0.880 (1.20)	1.059 (0.38)	1.092
HIS	0.535 (5.59)	0.494 (1.14)	0.515 (0.12)	0.597
ARG	2.146 (0.47)	1.795 (2.06)	1.896 (0.84)	2.188
THR	0.716 (0.16)	0.592 (0.08)	0.711 (2.67)	0.872
ALA	0.952 (1.20)	0.812 (1.39)	0.930 (0.18)	1.080
PRO	0.942 (1.59)	0.849 (0.09)	0.957 (0.71)	1.014
MET-O_2	0.383 (4.62)	0.353 (4.95)	0.348 (2.05)	0.305
TYR	0.724 (0.57)	0.613 (6.65)	0.707 (5.51)	0.711
VAL	1.071 (0.17)	0.854 (3.09)	1.122 (1.93)	1.159
ILE	0.942 (2.43)	0.742 (5.91)	0.939 (6.40)	1.014
LEU	1.682 (2.10)	1.382 (3.96)	1.686 (1.51)	1.760
PHE	1.043 (2.20)	0.825 (8.43)	1.236 (5.84)	1.132
LYS	1.465 (1.20)	1.251 (0.90)	1.436 (3.51)	1.772
Protein WB, % (N X 6.25)	22.48	19.10	23.62	24.55

[a] Abbreviations: see Table 1.
[b] Ref. 14.

amino acids to elute from the column; variation in response for these peaks can be reduced by strict timing of the gradient conditions.

While all of the recipes were analyzed after freeze-drying (which was done to ensure adequate homogenization and preservation), 2 recipes (chocolate pudding and sweet and sour pork) were also analyzed before freeze-drying. There were no significant differences in amino acid content between the wet and dry samples.

The amino acid contents as determined by the Pico-Tag method agree well with published values when normalized for differences in protein content (see Tables 1–3).

Recovery of amino acids from the Pierce H standard added to a recipe that was subjected to acid hydrolysis (no performic acid oxidation) averaged 106% (excluding cystine); no cystine was recovered. When the Pierce H standard was "hydrolyzed" and analyzed, recovery averaged 96% (excluding CYS); once again, no CYS was recovered. Recovery of MET-O_2 and CYA added to samples that were oxidized with performic acid, hydrolyzed, and analyzed averaged 105%. Recovery of MET and CYS added to samples that were oxidized, hydrolyzed, and analyzed averaged 96 and 108%, respectively. The results of the standard addition and standard recovery studies are summarized in Table 4.

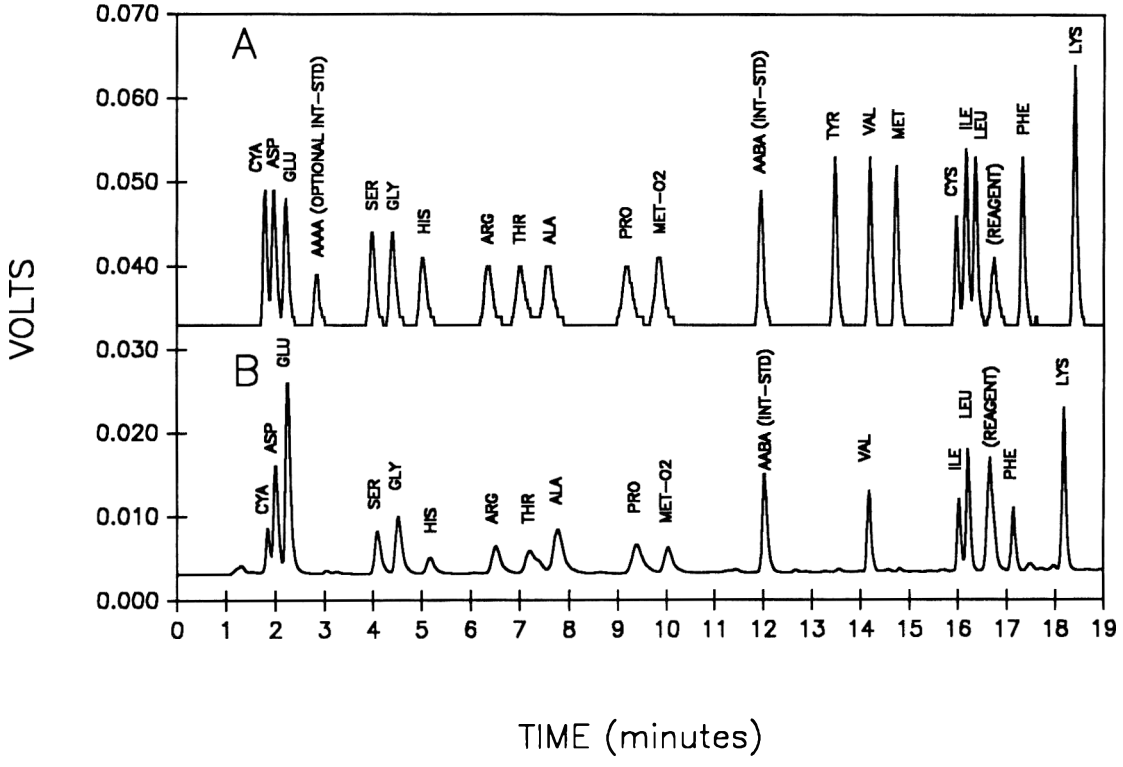

Figure 1. Chromatograms of working standard (A), and representative recipe, canned beef (B), which was subjected to performic acid oxidation.

Table 4. Standard addition and standard recovery data

Amino acid	Canned Beef, Av. % rec. (CV)	Pierce H std, % found (CV)
ASP	102.9 (1.32)	91.1 (1.53)
GLU	104.9 (0.12)	100.6 (3.85)
SER	103.1 (5.49)	91.1 (2.17)
GLY	105.5 (1.49)	94.4 (3.53)
HIS	106.1 (3.42)	93.7 (1.72)
ARG	106.8 (2.60)	91.5 (1.57)
THR	104.8 (4.04)	85.1 (2.47)
ALA	104.8 (2.41)	107.1 (2.62)
PRO	105.2 (4.23)	95.4 (2.28)
TYR	105.7 (7.20)	99.4 (1.83)
VAL	108.9 (1.47)	101.6 (0.48)
MET	109.6 (5.87)	96.4 (5.42)
CYS	0.0 (0.00)	0.0 (0.00)
ILE	106.9 (4.28)	98.1 (2.33)
LEU	108.0 (1.21)	95.2 (1.74)
PHE	108.6 (4.13)	93.4 (1.10)
LYS	107.3 (4.60)	97.7 (9.67)
Performic acid-oxidized samples:		
CYA	106.4 (1.73)	
MET-O_2	104.6 (0.03)	
CYS	108.3 (0.92)	
MET	95.6 (1.03)	

It is possible to quantify most of the amino acids besides CYS and MET after performic acid oxidation, except for HIS, TYR, and PHE. In a further standard addition recovery study, we found a 25% loss of HIS, an 87% loss of TYR, and a 33% increase in PHE (possibly due to the presence of co-eluting compounds) in samples that were analyzed after performic acid oxidation. Similar results were obtained by Elkin and Griffith (16). While CYS was totally destroyed in samples that were not oxidized, recovery of MET was often complete. Thus, we found that it was possible to quantify all of the nutritionally important amino acids except for CYS and tryptophan without performic acid oxidation.

Conclusion

The results demonstrate that it is possible to use PTC-based amino acid analysis with a variety of complex food matrixes. By analyzing samples with and without performic acid oxidation, we were able to quantify all of the nutritionally important amino acids except tryptophan. The method described is rapid and generates reproducible results which correlate well with conventional amino acid analysis.

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Determination of Allicin in Garlic and Commercial Garlic Products by Gas Chromatography with Flame Photometric Detection

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A simple and rapid method has been developed for the determination of allicin in garlic and commercial garlic products by gas chromatography (GC) with flame photometric detection. Samples containing allicin are homogenized with distilled water and centrifuged. An aliquot of the supernate is cleaned up on an Extrelut column using diethyl ether as an eluant. The GC separation is carried out on a 1% OV-1 or 2% Advance DS+ H_3PO_4 column. The calibration curve was rectilinear in the range from 0.2 to 2.0 μg/mL. The overall recovery of allicin added to garlic and garlic products at 0.4 mg/g was more than 84% with a detection limit of 0.02 mg/g. Allicin was found in spices; whereas, no allicin was found in garlic products marketed as health foods.

Garlic (*Allium sativum L.*) has been used as a folk medicine since ancient times. Consisting of various physiologically active components, garlic shows antibacterial activity (1) and a platelet aggregation (2, 3). Recently, garlic products have been marketed as health foods with purported physiological effects to humans. The quality of these garlic products is questionable. Therefore, the development of a method for controlling the quality and evaluating the safety of commercial garlic products is required.

Because allicin (diallyl thiosulfinate; $C_6H_{10}OS_2$) is a main component of garlic flavor, the determination of allicin is expected to be essential for evaluating the quality of garlic products. Paper chromatography (4) and gas chromatography (GC) (5, 6) have been reported for qualitative analysis of allicin while colorimetry (7) and oxygen flask combustion (8), which measure total sulfur, have been reported for quantitative assay. These methods have been used mostly for the detection of allicin in fresh garlic bulbs. Miething (9), on the other hand, used a liquid chromatographic (LC) method to detect allicin in garlic products.

Gas chromatography with flame photometric detection (FPD) is preferable to other methods on the basis of sensitivity and specificity. Allicin is a volatile organic compound containing sulfur. During gas chromatography (5), allicin undergoes dehydration and forms 2 isomeric disulfides (3-vinyl-1,2-dithi-5-ene, 3-vinyl-1,2-dithi-4-ene, $C_6H_8S_2$). The formation of these disulfides is proportional to the allicin content. Therefore, allicin can be determined using disulfide as an indicator.

The present study describes the development of a GC method for the determination of allicin in garlic products. Alliin (*S*-allyl-L-cysteine sulfoxide, $C_6H_{11}NO_3S$), a precursor of allicin, is also determined by the method of Mochizuki et al. (10) for evaluating the quality of garlic products.

METHOD

Materials and Reagents

(a) *Samples.*—Locally purchased garlic bulbs and garlic products (health food and spice).

(b) *Allicin and alliin.*—Supplied by Wakunaga Pharmaceutical Co.

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(c) *Allicin standard solutions.*—Prepare 10 mg/mL diethyl ether solution. Dilute with diethyl ether to appropriate concentrations. Store these standard solutions in freezer (−20°C) until use.

(d) *Alliin standard solutions.*—Prepare 10 mg/mL aqueous solution. Dilute with water to appropriate concentrations.

(e) *Allinase solution.*—Prepare 10 mL crude enzyme solution from 10 g garlic bulbs according to procedure of Mochizuki et al. (10).

(f) *Extraction and cleanup.*—Plug syringe (5 cm × 1.2 cm id) with cotton. Place 1 g anhydrous sodium sulfate and 0.5 g Extrelut (E. Merck) successively into syringe and fit needle (32 mm × 0.7 mm id) as shown in Figure 1.

(g) *Solvents.*—Analytical grade diethyl ether (Wako Pure Chemical Industries Ltd); water, distilled and deionized.

Apparatus

(a) *Gas chromatograph.*—Shimadzu GC-7A system (Shimadzu Corp.) with flame photometric detector. Electric signal of flame photometric detector was proportional to square of allicin concentration. Record and calculate output data on Shimadzu C-R3A data processor. Operating conditions: glass column 2.1 m × 3.2 mm id coated with 1% OV-1 on 80–100 mesh Chromosorb W AW-DMCS and 1.5 m × 3 mm id coated with 2% Advance DS (identical to DEGS) + 0.5% H_3PO_4 on 80–100 mesh Chromosorb W AW-DMCS; column temperature 100°C, injection port temperature 140°C, nitrogen carrier gas 40 mL/min, injection volume 5 μL.

(b) *Gas chromatograph-mass spectrometer (GC-MS).*—Shimadzu QP-1000A. Operating conditions: glass column 2.1 m × 3.2 mm id coated with 1% OV-1 on 80–100 mesh Chromosorb W AW-DMCS, helium carrier gas 40 mL/min, column temperature 100°C, injection port temperature 140°C, ion source and separator temperature 250°C, ionization energy 70 eV (EI), 200 eV (CI), reaction gas isobutane.

(c) *High speed homogenizer.*—Hiscotolon (Nichion Irika Kikai).

Extraction and Cleanup

Weigh 0.5–1 g sample into 10 mL test tube and add 8 mL water. Homogenize 1 min and dilute to 10 mL with water. Centrifuge 5 min at 3000 rpm. Filtrate upper layer through paper (Toyo Roshi No. 5A). Place 1 mL filtrate on Extrelut column and let soak 10 min. Elute allicin from column with 6 mL diethyl ether. Collect first 5 mL portion of eluate for analysis.

Preparation of Standard Curve

Inject 5 μL allicin solution (0.2–2.0 μg/mL) into FPD-GC system. Prepare analytical calibration curve by plotting different concentrations of allicin against observed peak height on logarithmic (log-log) graph paper. Inject 5 μL sample solution into FPD-GC system and calculate allicin content in sample from calibration curve.

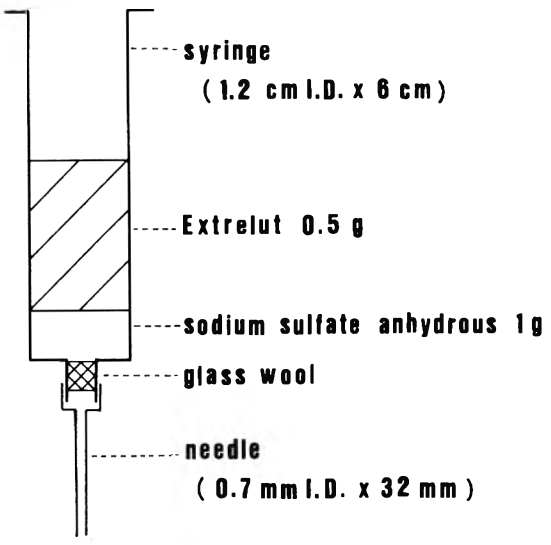

Figure 1. Schematic illustration of column for extraction and cleanup.

Enzymatic Treatment of Alliin

Mix 1 mL alliin solution (100 μg/mL) with 1 mL allinase solution and incubate mixture 15 min at 37°C. Load mixture on Extrelut column and follow the procedure previously described. Use 1 mL water as control instead of allinase solution.

Results and Discussion

Extraction of Allicin

Garlic products contain several vitamins, sugar, organic acid, and vegetable oil. Allicin is first extracted with water, and then re-extracted with diethyl ether, according to the procedure of Nakata et al. (7) who used repeated partitions with diethyl ether from water extract of garlic. This step resulted in the formation of emulsions especially during the analysis of garlic products. Therefore, a liquid-liquid partitioning step with an Extrelut column was investigated instead of diethyl ether extraction.

The elution pattern with diethyl ether was studied on the Extrelut column with aqueous allicin solution (20 μg/mL). All allicin was found in the 4 mL eluate, and, thus, 5 mL diethyl ether was chosen as the eluant.

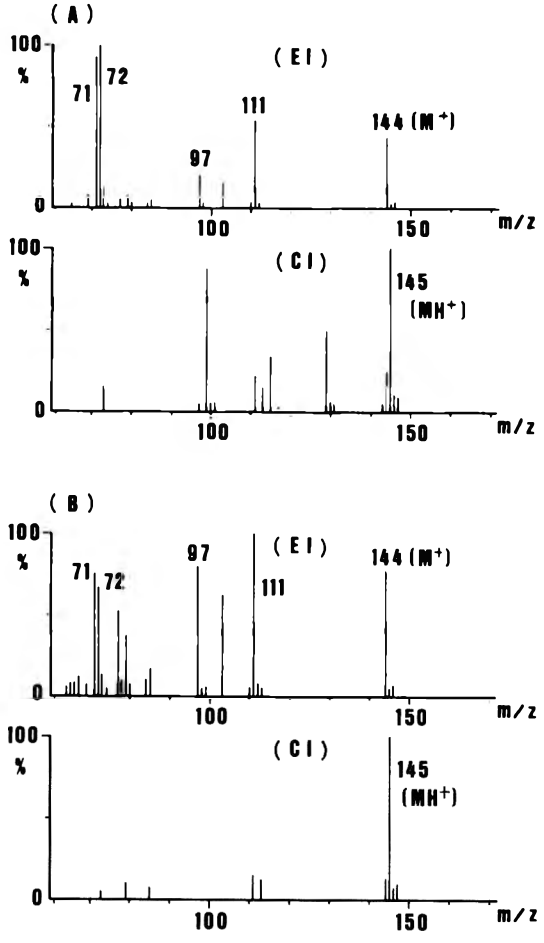

Figure 3. GC-MS spectra for 2 eluting components (A and B) measured by EI and CI methods. Peaks A and B: isomeric disulfides derived from allicin.

Separation of Allicin

Brodnitz et al. (5) used a PEG-20M packed column and a Goley column to separate several volatile compounds including allicin from garlic oil, with different coating. The separation of allicin was also examined on GC columns. They included: OV-1, SE-30, and DC-200 as nonpolar coatings; OV-17, XE-60, and QF-1 as slightly polar coatings; SP-1000 and OV-330 as moderately polar coatings; and Advance DS+H_3PO_4, PEG-20M, Thermon 1000, Thermon 3000, and

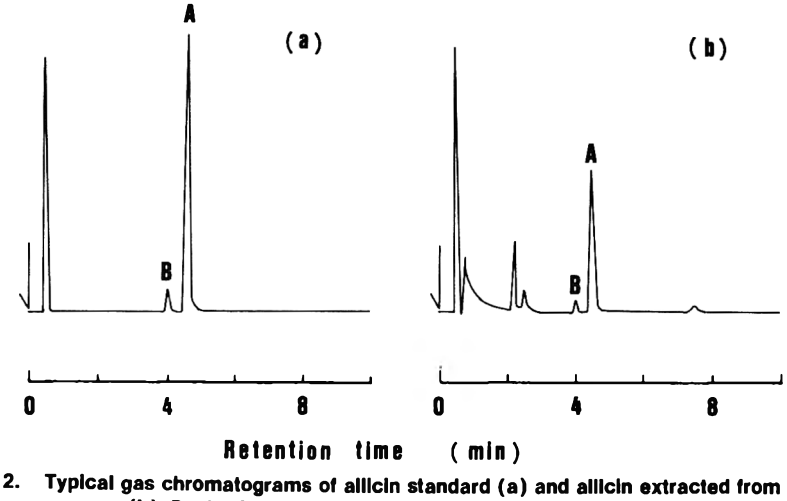

Figure 2. Typical gas chromatograms of allicin standard (a) and allicin extracted from garlic bulb (b). Peaks A and B: isomeric disulfides derived from allicin.

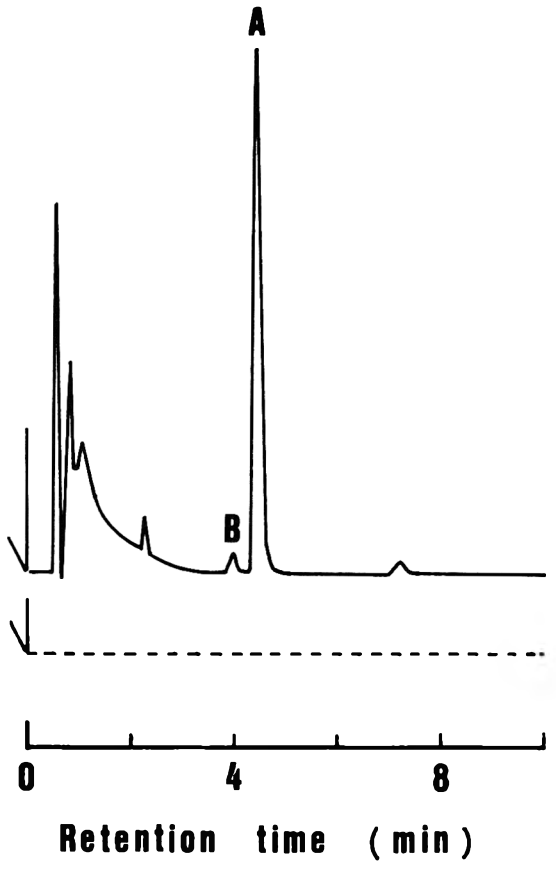

Figure 4. Gas chromatograms of alliin with treatment (—) or without treatment (---) of allinase. Peaks A and B: isomeric disulfides derived from allicin.

1,2,3-TCEP as polar coatings. The maximum sensitivity and reproducibility of the columns was assessed. Similar peak patterns of allicin were obtained by using columns with either nonpolar or slightly polar coatings. The separation of allicin was sufficient only on OV-330 and Advance DS+H_3PO_4 coated columns among those columns with moderately polar and polar coatings. The columns coated with OV-1 and Advance DS+H_3PO_4 showed excellent resolution of allicin from other volatile sulfur compounds, as well as excellent sensitivity and peak shape. The OV-1 coated column gave the best sensitivity. Based on the present study, the OV-1 coated column was chosen for a quantitative determination, and the Advance DS+H_3PO_4 coated column was chosen for cross-checking. A typical GC chromatogram for allicin is shown in Figure 2.

Mass Spectroscopy of Allicin

Gas chromatography–mass spectrometry was carried out to identify allicin peaks (A) and (B) (Figure 2). The molecular weight of allicin is m/z 162. Figure 3 shows that each peak had a molecular ion of m/z 144 (M^+) by EI mode and a protonated molecular ion of m/z 145 (MH^+) by CI mode. The splitting patterns of fragment ions by EI mode between both peaks were almost the same while the intensity of each fragment ion was different. Based on the results of these experiments, peaks (A) and (B) indicate the dehydration of allicin molecules and subsequent formation of 2 isomeric disulfides at the GC injection port. This observation was similar to that reported by Brodnitz et al. (5).

The intensity of peak (B) shown in Figure 2 is about 1/20 that of peak (A). The logarithmic plot of allicin concentrations against the height of peak (A) is linear from 0.2 to 2.0 μg/mL. Thus, the height of peak (A) was adopted as an indicator for the quantitative determination of allicin.

Table 1. Recoveries of allicin from commercial garlic products

Samples[a]	Added, mg/g	Recovery, %[b]	CV, %[c]
Garlic bulbs	0.4	84.0	7.4
Powder formulation	0.4	86.2	4.5
Tablet formulation	0.4	84.8	4.9
Liquid formulation	0.4	98.0	2.9

[a] No allicin-containing products were used. Garlic bulbs were used after inactivation of allicin and allinase by heating.
[b] Average of 5 determination.
[c] Coefficient of variation.

Enzymatic Treatment of Alliin

Alliin, a precursor of allicin, undergoes an enzymatic reaction by allinase to produce allicin (11, 12). To confirm allicin, which is detected by gas chromatography with flame photometric detection, the enzymatic treatment of alliin with allinase was studied. The treatment resulted in the appearance of the peak established as allicin on a GC chromatogram (Figure 4). The peaks provided the proof that the peaks were derived from allicin.

Recovery Study and Analysis of Commercial Samples

Fresh garlic bulbs and garlic products such as powders, tablets, capsules, and liquid formulas were fortified with 0.4 mg/g allicin for the recovery study. Recoveries were greater than 84%, and coefficients of variation were satisfactory (Table 1).

The GC method was applied to the analysis of commercial garlic products. Determination of alliin, the precursor of allicin, was also carried out by the LC method of Mochizuki et al. (10) to assess the quality of garlic products. Table 2 shows the content of allicin and alliin in garlic products. The spice samples contain allicin and not alliin; whereas, health foods contain alliin and not allicin. Commercial health foods are often advertised as odorless products. Allicin and other flavor components may be purposely eliminated from the products, but flavor components such as allicin, which are

Table 2. Contents of allicin and alliin in commercial garlic products

Sample (formulation)	Allicin, mg/g	Alliin, mg/g
Spice A (powder)	4.3	—[a]
B (powder)	3.5	—
C (powder)	1.9	—
D (powder)	2.7	—
E (granule)	3.1	—
F (grated)	0.7	—
G (grated)	<0.02	—
Health food H (powder)	—	8.5
I (powder)	—	5.8
J (powder)	—	—
K (tablet)	—	2.7
L (tablet)	—	0.2
M (tablet)	—	—
N (tablet)	—	—
O (capsule)	—	4.1
P (capsule)	—	—
Q (capsule)	—	—
R (capsule)	—	—
S (paste)	—	—

[a] Not detected.

essential to spices, are unavailable in odorless form. Samples J, M, N, P, Q, R, S, and G that contain neither allicin nor alliin are less qualified to be considered garlic products.

From these results, the quantitative analyses of allicin by a GC method and alliin by a LC method can be evaluated in terms of quality assessment of garlic products.

Acknowledgment

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MEATS AND MEAT PRODUCTS

Detection of Pork and Lard as Adulterants in Processed Meat: Liquid Chromatographic Analysis of Derivatized Triglycerides

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A new method is described for detection of pork and lard as adulterants in processed beef and mutton mixtures. The unsaturated triglycerides in the fat are ozonized and then derivatized. The mixture of derivatized and saturated triglycerides is analyzed by liquid chromatography using a reverse-phase column and a UV detector. Pork fat has larger amounts of triglyceride containing saturated fatty acid at the C-2 position than does the fat of other meat. The ratio of triglyceride containing saturated fatty acid vs triglyceride containing unsaturated fatty acid at the same (C-2) position (SSU/SUS) in a sample is compared with those of pure meats. The presence of pork in the sample causes the ratio to increase compared with ratios for pure beef or mutton. The increase in the SSU/SUS ratio is significant for the addition of 1% pork in beef. In the case of mutton, the addition of 3% pork causes a noticeable change. The method is reliable and is also applicable to samples containing only fat. Processing (heating or cooking) does not affect the ratios.

Detection of pork and lard (pig fat) as adulterants in many food and consumable products is of considerable importance and interest in many parts of the world. The Islamic religion prohibits the consumption of these products in any form. Many Muslim countries have banned the import or production of pork- and lard-containing products. In the absence of a reliable and dependable detection procedure, the law that bans these products is difficult to implement. The detection of pork as an adulterant has, therefore, been the subject of many studies (1-7). The analysis of fat has formed the basis of many of the studies because of the unique composition of pork fat and the stability of the fat during processing. Saeed et al. reported that 11,14-eicosadienoic acid (C20:2) is present in pork fat and absent in other commonly used meats and fats (6). Therefore, the presence of C20:2 was suggested as an indicator for the presence of pork or lard in the sample. C20:2 acid was, however, claimed to be present in some beef and mutton samples (8).

The unique composition of pork triglycerides has also been used for detection purposes. In contrast with other animal fats, pork triglycerides are mostly esterified by saturated fatty acid (especially palmitic acid) at the C-2 position (9). In the case of pork, a high proportion (80%) of the total palmitic acid content is esterified at the C-2 position (10). In triglycerides of other animals, such as beef, lamb, rabbit, and deer, only 15-27% of the total palmitic acid content is esterified at the C-2 position (10). Chacko and Perkins (11) reported that pork fat contains 38% of SSU (S = saturated fatty acid, U = unsaturated fatty acid), 41% of USU, and very low percentages of SUS (1%) and UUS (7%). In other animal fats, the triglyceride composition is distinctly different (SSU, 9-14%; USU, 7-14%; SUS, 13-38%; and UUS, 26-38%) (9). This characteristic composition of pork triglycerides has been used in many studies for the detection of pork and lard as adulterants in other meats and fats. El Sayed and El Dashlauty (1) used pancreatic lipase hydrolysis to prepare 2-monoglycerides. The ratio of unsaturated acid in monoglyceride and triglyceride has been used for the detection of pork. Rugraff and Karleskind (5) separated saturated triglycerides and obtained 2-monoglycerides by pancreatic lipase. Gas chromatographic analysis was used to determine the amount of palmitic acid in monoglycerides as well as triglycerides, the ratio of which was used to detect pork in the sample. The activity of the pancreatic lipase enzyme has been problematic: different batches have shown different activities (12). The range of these differences has been quite large; some show no activity at all (probably because of improper shipping or storage). To avoid the use of pancreatic lipase, a liquid chromatographic (LC) method was developed for the separation of triglyceride isomers after derivatization. The method was designed to give the ratio of SSU/SUS. The present paper describes the results obtained by using this method for the detection of pork in processed meat.

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METHODS

Apparatus

(a) *Pasteur pipets.*—Disposable.

(b) *Homogenizer.*—High speed with rheostat, Ultra-Turrax Model No. T-25 (Janke & Kunkel GmbH & Co. KG Staufen, West Germany), or equivalent.

(c) *Rotary evaporator.*—With 40°C water bath.

(d) *Micro-ozonizer.*—Supelco Model No. 6-0615 (Supelco, Bellefonte, PA), or equivalent.

(e) *Boiling flask.*—100 mL, short-neck.

(f) *Conical flask.*—25 mL, short-neck.

(g) *Autoclave.*—Hirayama Model No. HL42AE (Hirayama Mfg. Corp., Tokyo, Japan), or equivalent.

(h) *Chromatographic column.*—Glass, 1.5 × 20 cm, with Teflon stop cock.

(i) *Vials.*—40 mL, with Teflon-faced screw caps.

(j) *Thermometer.*—Range −20 to 50°C.

(k) *Magnetic stirrer with hot plate.*

(l) *Food chopper.*—With glass jar, stainless steel blades, and multispeed settings, Osterizer (Oster Corp., Milwaukee, WI), or equivalent.

(m) *Liquid chromatograph.*—Shimadzu Model No. LC-4A with universal injector (Model No. SIL-1A), or equivalent.

(n) *Spectrophotometric detector.*—Shimadzu Model No. SPD-2AS, variable UV, or equivalent.

(o) *Recorder/integrator.*—Shimadzu Chromatopac Model No. C-R3A, or equivalent.

(p) *Column.*—5 μm; reverse-phase (octadecyl); 25 cm × 0.5 cm (Alltech Associates, Deerfield, IL).

Reagents

(a) *Solvents.*—Methanol, chloroform, diethyl ether, hexane, acetone; all analytical grade (BDH Ltd, Poole, England).

(b) *LC solvents.*—Acetonitrile, methylene chloride; LC grade (E. Merck, Darmstadt, West Germany).

(c) *Water.*—Distilled and deionized.

(d) *Chemicals.*—Sulfuric acid, hydrochloric acid, potassium iodide, starch, pyridine; all analytical grade (BDH Ltd).

(e) *Lindlar catalyst.*—Cat. No. 62145 (Fluka AG Chemische Fabrik, Buchs, West Germany).

(f) *O-(4-Nitrobenzyl)hydroxylamine hydrochloride (PNBA).*—Cat. No. 73200 (Fluka AG Chemische Fabrik).

(g) *Florisil.*—60–100 mesh (J. T. Baker Inc., Phillipsburg, NJ).

(h) *Triglyceride standards.*—Trilinolein (Cat. No. 4-4303); 1,3-palmitate-2-oleate (SUS, CE-41, Cat. No. 4-4312); 2,3-palmitate-1-oleate (SSU, CE-41, Cat. No. 4-4353); 1-palmitate-2-stearate-3-oleate (SSU, CE-43, Cat. No. 4-4381); 1-palmitate-2-oleate-3-stearate (SUS, CE-43, Cat. No. 4-4382); 1,3-stearate-2-oleate (SUS, CE-45, Cat. No. 4-4327); 1,3-oleate-2-palmitate (USU, Cat. No. 4-4360); 1-palmitate-2,3-oleate (SUU, Cat. No. 4-4384) (Supelco).

Preparation of Florisil

Mix 300 g Florisil with 900 mL concentrated hydrochloric acid and heat 3 L on steam bath. Decant hot supernatant liquid and wash adsorbent with small amount of acid and heat overnight with further 900 mL acid. Filter and wash with water until neutral. Wash neutral residue with ca 400 mL each of methanol, chloroform–methanol (1 + 1 v/v), chloroform, and diethyl ether. Air-dry and then activate product by heating overnight at 110–120°C. Deactivate with 7% water. (*Caution:* Hydrochloric acid, methanol, chloroform, and diethyl ether are hazardous chemicals and should be used only under hood or in well-ventilated area.)

Samples

Authentic samples (40) of pork from animals grown under controlled conditions were obtained from the Danish Meat Research Institute (Roskilde, Denmark). Samples (15 each) of beef and mutton from various countries were obtained from the local market. For pure meats, samples were taken that had some visible fat. Mixtures were made with either pure fat or lean meat (without any visible fat). Samples of pure meats were minced, weighed, and mixed thoroughly before processing. Meat processing was simulated by autoclaving sample 30 min at 120°C.

Sample Preparation

To extract fat, add 15 mL chloroform–methanol (2 + 1), to 1–2 g sample, homogenize 2 min, and then filter. Repeat extraction with fresh 15 mL solvent. Combine filtrates and evaporate solvent until dry. Store extracted fat in chloroform. (*Caution:* Chloroform is suspected carcinogen. Use under hood.)

Separation of Triglycerides

Pure triglycerides are separated from fat by column chromatography on acid-treated Florisil (13).

Fill chromatographic column (1.5 × 20 cm) with 30 g acid-treated Florisil as slurry in hexane. Let Florisil settle with gentle tapping. Adjust flow to 1 mL/min. Introduce sample (100 mg) as hexane solution at top of column. Elute column with 20 mL hexane followed by 20 mL 5% diethyl ether in hexane. Discard both fractions. Elute column with 20 mL 15% ether in hexane. Collect this fraction, which contains triglycerides.

Ozonolysis of Triglycerides

Disperse 10 mg triglycerides in 5 mL hexane in centrifuge tube. Cool tube in mixture of dry ice and acetone (−18°C). Let ozone from micro-ozonizer flow through solution for about 15 min at 200 mL/min (until iodine/starch indicator solution changes color). Flush tube with nitrogen to purge unreacted ozone.

Hydrogenation of Ozonide

Transfer contents of tube to 50 mL conical flask. Cleave formed ozonide to aldehyde by adding 2–3 mg Lindlar catalyst and by stirring solution about 30 min under hydrogen.

Derivative Formation

Transfer aldehyde solution to reaction vial. Evaporate solvent under gentle stream of nitrogen and add 10 mg *O*-(4-nitrobenzyl)hydroxylamine hydrochloride (PNBA) and 100 μL pyridine. Cap vial and heat 1 h at 50°C. After completion of reaction, evaporate pyridine under stream of nitrogen and dissolve residue in methylene chloride. Wash residue twice with 3 mL portions of water.

Liquid Chromatography of Derivatized Triglycerides

Liquid chromatographic analysis of derivatized triglycerides was set up to optimize resolution for isomers and for homologs. Different samples of pork were analyzed under isocratic conditions. Acetonitrile–methylene chloride (90 + 10) was used as mobile phase at flow rate of 1.5 mL/min. All other samples were run using following gradient program: 5% methylene chloride in acetonitrile for first 10 min, programmed to 25% methylene chloride in 5 min, and held for 25 min. Flow rate was adjusted to 1.5 mL/min. UV detector was operated at 254 nm. 25 μL sample, in mobile phase, was injected.

Identification of Triglycerides

Triglycerides in samples were identified by comparison of their retention times with those of standard triglycerides treated and analyzed in same manner. Additional confirmation was obtained by comparing results with those reported by Tinsley et al. (12).

Results and Discussions

Liquid chromatographic analysis of (intact) underivatized triglycerides requires the use of a refractive index (RI) detector. Because of low sensitivity of the RI detector, relatively large amounts of sample need to be injected, which adversely affects column performance. In recent years, some studies have reported (14, 15) the UV detection of underivatized triglycerides at wavelengths shorter than 230 nm. Such an approach had limited use; the available LC-grade solvents were not pure enough at wavelengths significantly shorter than 254 nm. Derivatization of triglycerides provided a solution to these problems.

Liquid chromatographic analysis of the triglycerides obtained from meat samples (containing saturated and derivatized triglycerides) on a reverse-phase silica column showed that the retention time of the S_2U fraction was higher than that of the SU_2 fraction. The triglycerides in different samples were identified by analyzing standard derivatized triglyceride samples and also by comparing the results to those reported by Tinsley et al. (12).

Liquid chromatographic analysis of derivatized triglycerides in processed pure meats showed that the pork triglyceride profile was distinctly different from that of beef or mutton. Such a difference was not unexpected and was in agreement with reported observations (11). The chromatographic profile also indicated the presence of S_2U and SU_2 triglycerides in pure meat samples as confirmed by analyzing standard derivatized triglyceride samples. For the resolution of

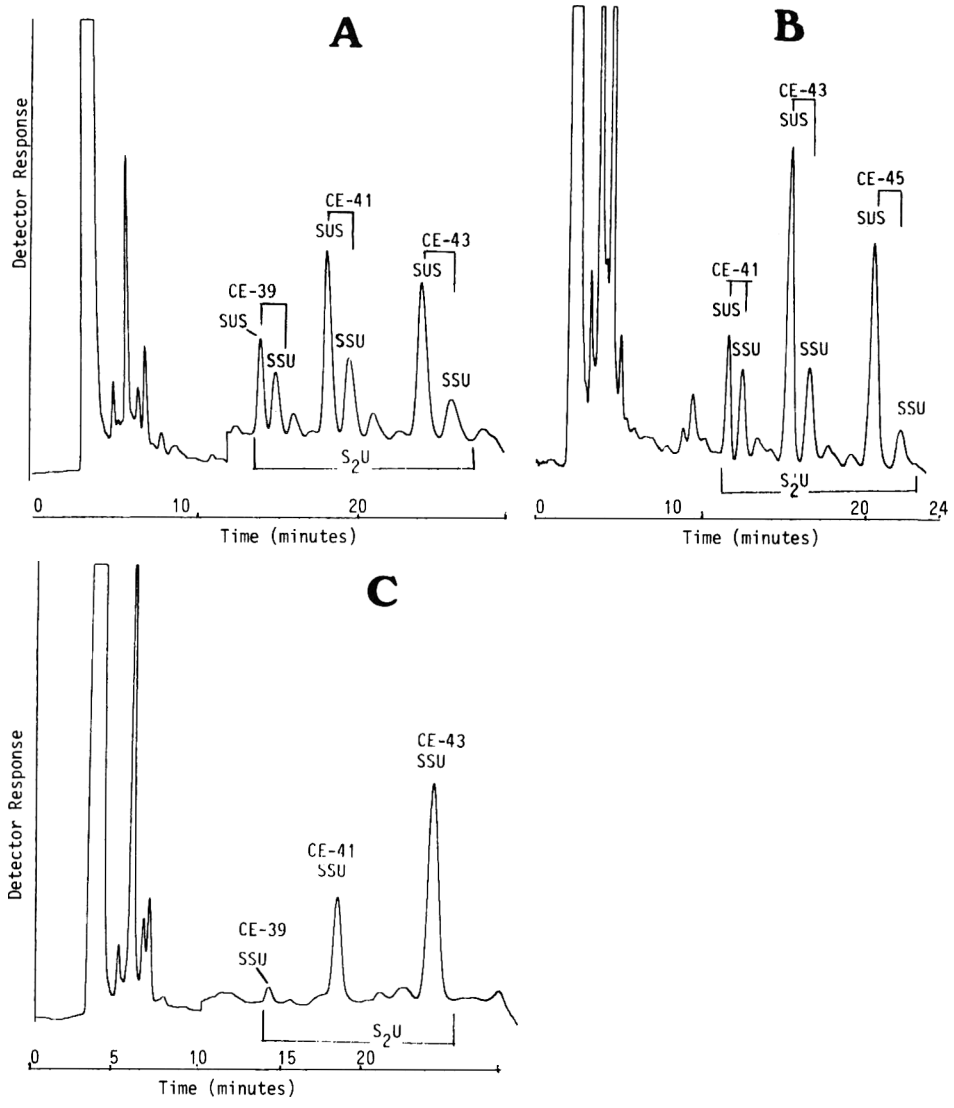

Figure 1. LC chromatograms of derivatized triglycerides of (A) mutton, (B) beef, and (C) pork.

isomers in both fractions, different analytical conditions were tried, including such parameters as mobile phase composition, isocratic as well as gradient elution, columns, $-NH_2$, —CN, and silica. Nevertheless, the SU_2 isomers (SUU and USU) could not be separated under these conditions. However, isomers for the S_2U fraction (SUS and SSU) were well separated. Figure 1 shows LC chromatograms of the 3 pure meats. The S_2U isomer profile for pork is clearly different from that of beef and mutton. The major difference is the absence of SUS isomers of the 3 homologs of triglycerides. Only SSU isomers were detected. This is in agreement with the literature (11) where only 1% SUS levels were reported in pork fat.

Triglycerides of beef and mutton, on the other hand, showed the presence of both SUS and SSU isomers for all homologs. The amount of SUS was higher than SSU. The addition of pork to beef or mutton should, therefore, increase the percentage of the SSU isomers in the sample in preference to SUS, which will, thus, be reflected in a higher ratio of SSU to SUS than that of pure beef. The change in the SSU/SUS ratio for different triglyceride homologs, however, will not be the same because the amounts of SSU of these homologs in pork are not present in the same proportion. The SSU/SUS ratio for CE-43 (carbon equivalent (CE) is the total number of carbon atoms in the fatty acid chains of derivatized triglycerides) will, thus, change more significantly than that for CE-41. On the other hand, the ratio for CE-45 will not change much because the percentage of SSU of this carbon is present in pork in very small amounts.

Pork–Beef Mixtures

To study the effect on SSU/SUS ratios of adding pork to beef, mixtures of beef and pork ranging from 0 to 50% pork were prepared and processed, and their triglyceride composition was determined. Results obtained (Table 1) showed that the ratio for CE-41, CE-43, and the sum of CE-41 and CE-43 increased with increasing amounts of pork in the mixture.

Table 1. Derivatized triglyceride SSU/SUS ratios[a] of beef–pork mixtures (processed)

Pork concn, %	CE-41	CE-43	Total[b]
0	0.68 ± 0.02	0.38 ± 0.023	0.43 ± 0.02
1	0.70 ± 0.015	0.41 ± 0.015	0.47 ± 0.015
3	0.74 ± 0.02	0.44 ± 0.015	0.51 ± 0.016
5	0.78 ± 0.012	0.47 ± 0.015	0.56 ± 0.014
10	0.90 ± 0.01	0.64 ± 0.01	0.72 ± 0.01
30	1.27 ± 0.013	1.12 ± 0.007	1.14 ± 0.01
50	1.86 ± 0.008	1.87 ± 0.01	1.85 ± 0.01

[a] Average of 5 replicate analyses ± standard deviation.
[b] SSU (CE-41 + CE-43)/SUS (CE-41 + CE-43).

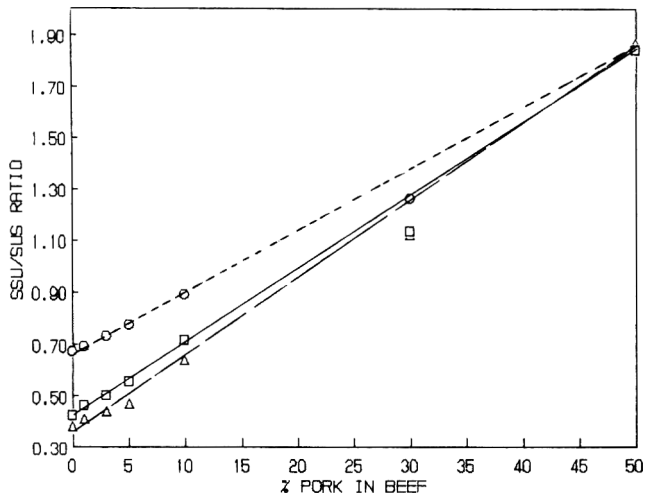

Figure 2a. Plot of pork concentrations (0–50%) in beef vs derivatized SSU/SUS ratios: O CE-41; △ CE-43; □ Total.

The addition of a small amount of pork (3%) caused a significant increase in CE-41 and CE-43 as well as in the sum of both. The increase of the SSU/SUS ratio with the increasing amounts of pork appears to be linear as illustrated in Figure 2a, but the value for 30% pork was slightly out of trend probably because of experimental error. No significant differences in the ratios of SSU to SUS were observed between fresh and processed beef, mutton, or pork.

When the ratios are plotted against low percentages of pork in beef, the linearity of these curves can be seen more clearly (Figure 2b). In this graph, the curve for CE-41 was very linear and showed a steady increase in the SSU/SUS ratio. The curve for CE-43 was not as linear, and the value for 5% pork was slightly out of trend. The curve for the sum of CE-41 and CE-43 followed the same pattern as CE-43. These figures show that the addition of pork to beef affected the ratios and, hence, the ratios can be used for detection purposes. The reproducibility of these ratios at low percentages was not very high, and correct integration of the peak area in the chromatogram becomes critical. Even under the best conditions, some variations were observed. In the case of beef, these variations were not very high, and the values remained close for the same sample run many times. Analysis of duplicate sample preparations also gave quite close results.

Table 2. Derivatized triglyceride SSU/SUS ratios[a] of mutton–pork mixtures (processed)

Pork concn, %	CE-41	CE-43	Total[b]
0	0.85 ± 0.018	0.65 ± 0.022	0.70 ± 0.02
1	0.87 ± 0.016	0.70 ± 0.015	0.74 ± 0.015
3	0.91 ± 0.02	0.88 ± 0.015	0.85 ± 0.017
5	0.95 ± 0.014	0.96 ± 0.013	0.95 ± 0.013
10	1.12 ± 0.015	1.30 ± 0.01	1.20 ± 0.012
30	1.40 ± 0.011	2.40 ± 0.013	2.10 ± 0.012
50	1.70 ± 0.01	3.10 ± 0.01	2.55 ± 0.01

[a] Average of 5 replicate analyses ± standard deviation.
[b] SSU (CE-41 + CE-43)/SUS (CE-41 + CE-43).

Pork–Mutton Mixtures

Different mixtures of mutton and pork (from 0 to 50% pork) were prepared (as was done for beef). Table 2 lists the results obtained for these mixtures. Mutton fat did not contain any appreciable amount of CE-45. As with beef, the increasing amount of pork in mutton is reflected in the SSU/SUS ratios for CE-41 and CE-43. As the concentration of pork increased in the mixture, the SSU/SUS ratio also increased. The relationship of the pork concentration in mutton mixtures with the SSU/SUS ratio is shown graphically in Figure 3a. The effect of pork concentration on the SSU/SUS ratio for CE-41 TG was quite linear except at high pork concentrations where it seemed to level off. This is apparently because of the value obtained for the sample containing 30% pork that was slightly less than expected. The same observation was made for CE-43 and the sum of CE-41 and CE-43. The graph, however, is reasonably linear below the 30% level. Figure 3b shows the graph for the SSU/SUS ratio plotted against the lower concentrations of pork in mutton. The CE-41 plot, as for beef, is reasonably linear, whereas for CE-43 it is slightly off. The plot for the total, however, is acceptably linear.

The reproducibility of the values for 5% or less pork was not as good as that for beef, but the difference in values for pure mutton and mutton containing 1% pork are substantial. The presence of 3% pork caused such a marked difference that the variation in the SSU/SUS ratio because of experimental or other errors is easily offset.

A preliminary study of the triglyceride composition variability of pork showed that sex, breed, diet, and muscle type

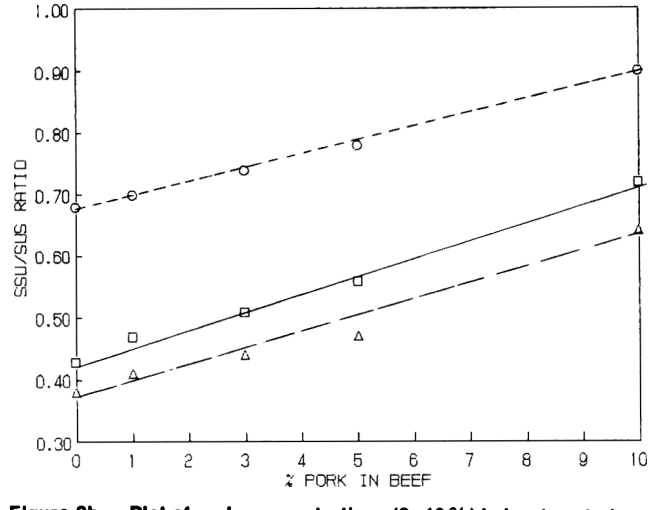

Figure 2b. Plot of pork concentrations (0–10%) in beef vs derivatized SSU/SUS ratios: O CE-41; △ CE-43; □ Total.

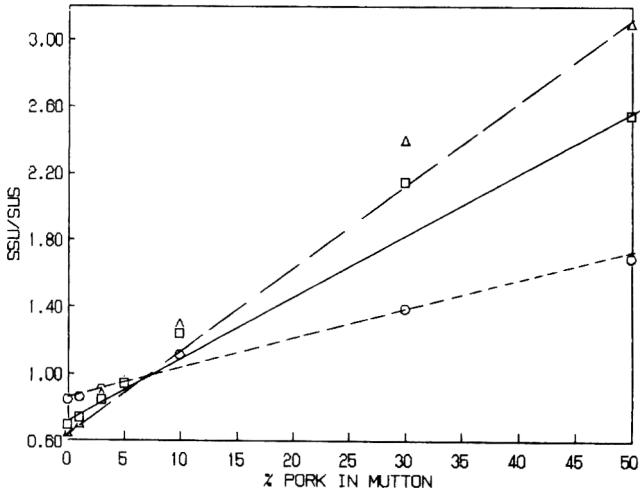

Figure 3a. Plot of pork concentrations (0–50%) in mutton vs derivatized SSU/SUS ratios: O CE-41; △ CE-43; □ Total.

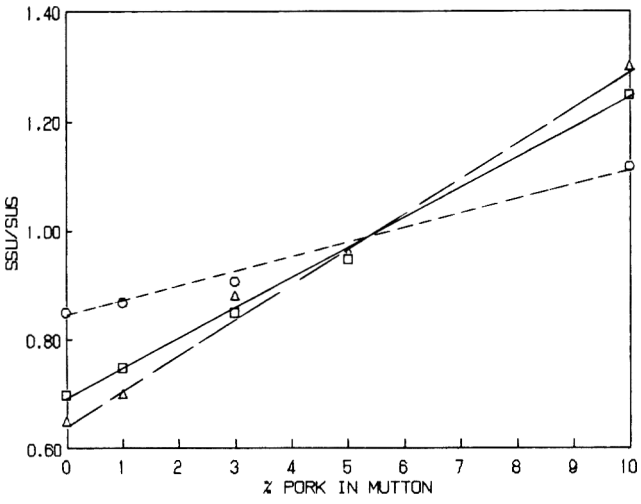

Figure 3b. Plot of pork concentrations (0–10%) in mutton vs derivatized SSU/SUS ratios: ○ CE-41; △ CE-43; □ Total.

did not affect the triglyceride profile significantly. The relative amounts of different triglyceride homologs (CE-39, CE-41, and CE-43) were quite consistent.

Based on these results, liquid chromatographic analysis can detect pork or lard as adulterants in processed meats. The detection limit of pork or lard was 1% in beef and 3% in mutton. The method can be applied to samples that do not contain meat, but contain fat (e.g., biscuits and other bakery products). Unlike protein, fat is not affected greatly by heating or cooking. This method can, therefore, be applied to both fresh and processed products.

Acknowledgments

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METALS

Liquid Chromatographic–Atomic Absorption Spectrophotometric Method for Determination of Methyl Mercury in Seafood: Collaborative Study

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A previously developed method that uses a simplified sample preparation procedure and atomic absorption detection of liquid chromatographic eluates for the determination of methyl mercury in seafood has been collaboratively studied. The unique feature of the method involves the use of a specially designed interface for the generation of mercury vapor. Methyl mercury is isolated from the blended sample by chloroform elution from a diatomaceous earth–hydrochloric acid column. The organomercury compound is then extracted into a small volume of 0.01M sodium thiosulfate solution. An aliquot of this solution is injected onto a Zorbax ODS column and eluted with methanol–ammonium acetate solution (3 + 2), pH 5.7, containing 0.01% mercaptoethanol. Mercury is detected by flameless atomic absorption spectrophotometry using the interface. The samples analyzed in the study were unspiked swordfish, unspiked and spiked lobster, and unspiked and spiked tuna. The spiked samples contained methyl mercury both above and below the U.S. Food and Drug Administration guideline level of 1 μg Hg/g. Reproducibility relative standard deviations ranged from 10.5% at 1 μg Hg/g to 18.2% at about 0.1 μg Hg/g. Accuracy, measured by comparison to reference values obtained by the Associate Referee, ranged from 94.4 to 99.6%. The method has been adopted official first action.

Two AOAC official methods are currently used for the determination of mercury in foods: the flameless atomic absorption spectrophotometric (AAS) method for total mercury (1) and 2 variations of a gas chromatographic method for methyl mercury (2, 3). Since the U.S. Food and Drug Administration (FDA) guideline for mercury in seafood is 1 ppm derived from methyl mercury, the method capable of speciation is the method of choice. Several years ago, a liquid chromatographic (LC) method using flameless AAS for detection was published (4). This potentially attractive approach combined 2 popular techniques: LC for separating mercury compounds, and flameless AAS for sensitive and specific detection. An interface for generation of mercury vapor was constructed (5), which is suitable for routine use. The method has been used in this laboratory for approximately 1 year to screen seafood samples for methyl mercury. In addition, an interlaboratory trial of the method was successfully conducted. As a result, a number of improvements were incorporated, and the method was subjected to a collaborative study. The present paper describes the results of the study.

Collaborative Study

Two samples of swordfish, one tuna and one tuna spike, and one lobster and one lobster spike were used in the study. The edible portion of each sample was blended in a food processor to obtain a homogeneous mass. Ten g (10.00 g) portions of each blended sample were weighed into 4 oz wide-mouth polyethylene bottles. One-half of the bottles containing 10 g portions of lobster and tuna samples were spiked with aliquots of aqueous solutions of methyl mercuric chloride standard measured by micropipet. Lobster and tuna were spiked at 0.40 and 2.30 μg/g, respectively. The swordfish samples already contained the desired levels of methyl mercury, so they were used unspiked.

Before the interlaboratory study was initiated, the samples were repetitively analyzed in this laboratory to obtain a reference value, as well as to check for homogeneity and stability. Only the tuna spike exhibited some loss of mercury during storage. Initial recoveries were quantitative, but after approximately 2 weeks the value stabilized at 1.87 μg/g, corresponding to a recovery of 74.8%. To confirm the above result, this sample was analyzed by the AOAC flameless AAS method (1). A value of 1.86 μg/g was obtained, confirming the accuracy of the earlier findings. The following summarizes the Associate Referee's data, mean, ± standard deviation (n = 6), μg/g, and range, respectively: swordfish (1), 1.08 ± 0.080 (0.99–1.119); swordfish (2), 1.68 ± 0.0109 (1.57–1.87); tuna, 0.15 ± 0.015 (0.14–0.18); tuna spike, 1.87 ± 0.092 (1.74–2.00); lobster spike, 0.42 ± 0.021 (0.39–0.44).

Duplicate portions of each sample were sent to each collaborator. The samples were randomly numbered so that the presence of duplicates was not identified. The samples were stored in a freezer and shipped frozen under dry ice. They were allowed to thaw to room temperature prior to analysis. Each collaborator also received an LC/AAS interface, a copy of the method, instructions, a practice sample, and a practice sample spiked with 1 μg Hg/g in the same manner as the unknowns. The collaborators were instructed to analyze the practice sample and the spike and then to proceed with the unknowns if recoveries were 100 ± 10%.

The collaborators were instructed to add 15.00 g of 1.8N HCl to each sample in the bottle and to mix the contents with a Polytron homogenizer. Ten-g (10.00 ± 0.30 g) portions of the fine suspension thus obtained were to be weighed for analysis.

Mercury (Methyl) in Seafood

Liquid Chromatographic–Atomic Absorption Spectrophotometric Method

First Action

Method Performance:

s_r = 0.02–0.12; s_R = 0.03–0.25; RSD_r = 4.5–18.2%; RSD_R = 10.5–18.2%

A. Principle

LC effluent is heated to produce Hg vapor from organomercury compounds. Hg vapor, together with vaporized mobile phase, is directed into water-cooled condenser where mobile phase is liquefied. Hg vapor is swept with nitrogen

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This report was presented at the 102nd AOAC Annual International Meeting, August 29–September 1, 1988, at Palm Beach, FL.
The recommendation has been approved interim official first action by the General Referee, the Committee on Residues, and the Chairman of the Official Methods Board. The method will be submitted for adoption at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* **73**, January/February issue.

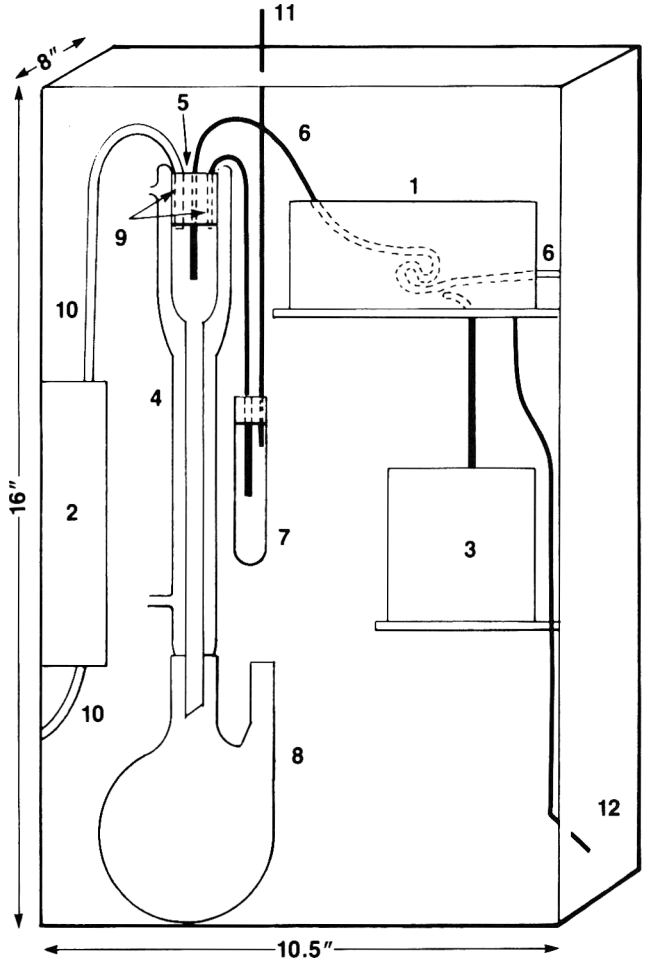

Fig. A—LC/AAS Interface. Assemble as described in D. (1) Disk heater; (2) flow meter; (3) pyrometer; (4) short condenser; (5) rubber stopper; (6) stainless steel tubing; (7) trap; (8) boiling flask; (9) stainless steel tubing; (10) and (11) plastic tubing; (12) electrical connection to variable voltage transformer.

into absorption cell in light path of atomic absorption spectrophotometer.

B. Apparatus

Wash all glassware with detergent (Micro Laboratory Cleaner, International Products, Trenton, NJ 08601; or equivalent) and rinse thoroughly with hot tap water followed by distilled or deionized water. Rinse with acetone and let dry.

(a) *LC/AAS interface.*—See Fig. A. Assemble as described in **D.** (*1*) Heater (HSP disk heater, 120 V, 200 W, 3.25 in. od × 11/32 in. thick, Leo C. Pilkus, 170 Worcester St, Wellesley, MA 02181) with magnesia and alumina insulation, 1 in. thick. (*2*) Flow meter (No. 7908, Alltech Associates, Inc., 2051 Waukegan Rd, Deerfield, IL 60015). (*3*) Temperature-indicating device (pyrometer, series 7000, 0–1370°C, 3.5 in. case, part No. 7035K2500; thermocouple, TJ 18-CAIN-116G-6-LUG, Omega Engineering, One Omega Dr, Stamford, CT 06907). (*4*) Short condenser, 175 mm jacket length, T 24/40 (Aldrich No. Z11, 545-4). (*5*) Rubber stopper, No. 5, solid neoprene (No. 14-141F, Fisher Scientific Co.). (*6*) Stainless steel tubing, 1/16 in. od, 0.04 in. id (No. 38.3067, Rainin Instrument Co., Mack Rd, Woburn, MA 01801). (*7*) Trap, test tube, 125 × 15 mm. (*8*) Boiling flask, 2-neck, 500 mL (Kontes No. K605000). (*9*) Stainless steel tubing, two 6 in. lengths (see item *6*). (*10*) Plastic tubing, spaghetti type, 0.057–0.067 in. id (No. 13-9045-12, Ace Scientific Supply Co., Inc., 40-A Cutter Ln, East Brunswick, NJ 08816). (*11*) Plastic tubing, spaghetti type, as connector to AAS system (see item *10*). (*12*) Electrical connection, standard 120 V plug, to variable voltage transformer.

(b) *LC pump.*—Tracor 950, or equivalent, with 100 μL fixed loop injector (Rheodyne, Berkeley, CA).

(c) *LC column.*—Zorbax ODS, 5 μm, 4.6 mm × 25 cm (DuPont).

(d) *Guard column.*—7 cm × 2.1 mm id, packed with 25–37 μm CO:Pell ODS (Whatman, Inc., Clifton, NJ; or equivalent).

(e) *Atomic absorption spectrophotometer.*—Equipped with Hg hollow cathode lamp, deuterium background corrector, and gas flow-thru cell with open ends or quartz closed ends (10–25 mm id × 100–115 mm). Follow manufacturer's operating instructions for Hg determination using wavelength of 253.7 nm and deuterium background correction. Typical response for injection of 0.100 μg Hg/100 μL standard is ca 0.20 *A*, using cell 25 mm id × 115 mm. Use recording device set to obtain ca 30–50% full scale for injection of 0.100 μg Hg/100 μL standard. Working range is between ca 0.01 and 0.25 μg Hg/100 μL injected.

(f) *Glass chromatographic columns.*—25 mm od, 22 mm id, × 250 mm.

(g) *Homogenizer.*—Polytron with PT-10-ST head (Brinkmann Instruments, Westbury, NY), or equivalent.

(h) *Variable transformer.*—0–140 V.

C. Reagents

(Use water double-distilled in glass.)

(a) *Sodium thiosulfate solution.*—0.01N. Dissolve 2.5 g $Na_2S_2O_3 \cdot 5H_2O$ and dilute to 1 L with water.

(b) *Hydrochloric acid solution.*—1.8M. Dilute 148 mL concentrated HCl to 1 L.

(c) *Celite 545.*—Acid-washed (Johns-Manville Products Corp., Denver, CO).

(d) *Methyl mercuric chloride stock standard solution.*—100 μg Hg/mL. Weigh 0.125 g CH_3HgCl (Alfa Inorganics, Inc., Danvers, MA) into 1 L volumetric flask. Add 20 mL methanol, shake to dissolve CH_3HgCl, dilute solution to volume with water, and mix. (*Caution:* See **51.079.**)

(e) *Ammonium acetate solution.*—0.05M. Dissolve 3.9 g ammonium acetate and dilute to 1 L with water.

(f) *Mobile phase.*—Methanol–0.05M ammonium acetate (3 + 2). Mix 3 volumes of methanol with 2 volumes of ammonium acetate solution and adjust pH to 5.7 ± 0.2 with glacial acetic acid. Degas with He. Add 0.1 mL 2-mercaptoethanol/L immediately before use. Shake very gently to thoroughly mix, taking care to incorporate as little air as possible into solution.

D. Instrument Set-Up

Fig. A is a diagram of the LC/AAS interface. Components are placed inside shop-made box of dimensions shown. Box has Plexiglas door at front, and back and top are removable. Items 1, 2, and 3 are bolted to sides of box.

Set up remaining items as follows: Bend 30 in. stainless steel tubing (item 6) as shown to provide additional heating surface. Place bent portion, together with thermocouple element, between 2 disks of heater, which are held tightly together by screw at center of upper disk. Enclose heater assembly in 1 in. thick magnesia–alumina insulation, and secure to aluminum plate support by means of aluminum cover and screws.

Push stainless steel tubing from heater outlet through center of rubber stopper (item 5) so that end of tubing is near constricted portion of condenser when stopper is inserted

tightly into top of condenser. Push 2 additional 6 in. lengths of stainless steel tubing through rubber stopper to serve as nitrogen inlet and Hg vapor outlet, respectively. Connect these lengths of tubing to flow meter and to test tube trap, respectively, by means of spaghetti-type tubing. Connect nitrogen tank to flow meter by means of spaghetti-type tubing and standard Swagelok fittings and unions.

Connect outlet from LC column to 0.01 in. id stainless steel tube, which is connected to inlet of heating tube by standard 1/16 in. Swagelok fittings and zero dead volume union.

Connect outlet of test-tube trap (spaghetti tubing, item 11) to AAS cell by small rubber stopper inserted into side arm of cell.

E. Operating Conditions for LC/AAS Interface

(a) *To turn system ON.*—(*1*) Adjust mobile phase flow rate to 0.7 mL/min. (*2*) Introduce water into condenser. (*3*) Adjust nitrogen sweep to 0.1 L/min (tank pressure 15 psi and 10.0 setting on flow meter). (*4*) Gradually adjust temperature of interface heater to 550° (transformer setting ca 65). (*5*) After temperature reaches 550°, check system stability by injecting several aliquots of methyl mercury standard solutions. Retention time of methyl mercury is 5–6 min. Precision between methyl mercury peak heights should be ≤5%. Inject all standard solutions to check *linearity*. If these parameters cannot be achieved, check for leaks or, after long use, replace effluent tubing.

Note: To conserve analytical standard solutions, another set of standards of same concentration may be prepared by direct dilution of stock standard solution with 0.01N $Na_2S_2O_3$. Use these standards only for instrument checking. To prepare 0.05, 0.100, 0.150, 0.200, and 0.250 μg Hg/100 μL, dilute 100 μg Hg/mL standard solution with 0.01N $Na_2S_2O_3$ solution as follows: 1, 1, 3, 2, and 5 mL to 200, 100, 200, 100, and 200 mL, respectively.

(b) *To turn system OFF.*—(*1*) Turn off interface heater and let cool to near room temperature. (*2*) Shut off other components, but DO NOT shut off mobile phase flow while heater is hot. If this is done, carbon may deposit and clog effluent tube. For same reason do not pump neat organic solvents, such as methanol, to clean column while heater is hot. (*3*) After heater has cooled to room temperature, pump methanol to rinse column.

F. Preparation of Samples

Use only edible portion of seafood. Homogenize sample in blender or food processor. Water (double-distilled in glass) may be added to aid homogenization, but added water must be accounted for in final calculation. Weigh 10.00 ± 0.30 g (wet) portion of homogenized sample into 100 mL beaker. Prepare analytical solution by adding HCl solution so that mass of analytical portion plus HCl solution totals 25.00 ± 0.30 g. Blend analytical solution in homogenizer (ca 1 min) to obtain fine suspension. Immediately weigh 10.00 g homogenized solution into beaker containing 10 g Celite 545, and mix well. Quantitatively transfer mixture, with aid of Scupula™, to chromatographic column, **B(f)**, containing pledget of glass wool at bottom. Compact mixture moderately with tamping rod to height of ca 8 cm, and place pledget of glass wool on top.

Elute column by adding 20 mL followed by four 5 mL aliquots of $CHCl_3$. Collect first 20 mL eluate in tall 25 mL glass-stopper graduated cylinder. Add 4.0 mL $Na_2S_2O_3$ solution by pipet, shake mixture gently 1 min, and let stand 5 min. Using disposable pipet, transfer upper aqueous layer containing methyl mercury–thiosulfate complex together with any emulsion into 25 mL erlenmeyer. Blow moderately strong stream of nitrogen into flask for 1–2 min to break up emulsion and expel droplets of $CHCl_3$. (*Note:* To aid in breaking emulsion, hold and rotate the flask at 45° angle with one hand, and direct nitrogen stream at thin layer of emulsion that adheres to bottom of flask as it rotates.)

Note: Some species of seafood may produce cloudy extracts. If this occurs, extract may be filtered through membrane filter.

G. Preparation of Reagent Blank Solution

Prepare reagent blank analytical solution by weighing 25.00 g HCl solution into 100 mL beaker. Proceed as in *Preparation of Samples*, **F**, beginning "Immediately weigh 10.00 g. . .".

H. Preparation of Standard Solutions

Prepare 0.050, 0.100, 0.150, 0.200, and 0.250 μg Hg/100 μL standard solutions by adding, respectively, 20, 40, 60, 80, and 100 μL aliquots of CH_3HgCl stock standard solution, **(d)**, to 20 mL $CHCl_3$ in separate 25 mL glass-stopper graduated cylinders. Proceed as in *Preparation of Samples*, **F**, beginning "Add 4.0 mL $Na_2S_2O_3$ solution. . .".

I. Determination

Inject 100 μL aliquot of sample solution (0.100 g injected for 10.00 g analytical portion) into LC/AAS system. After methyl mercury peak appears, inject 100 μL aliquot of standard solution that produces peak height equal to or slightly higher than sample peak height. Repeat by injecting sample again followed by selected standard. If sample peak height is higher than peak height for highest standard, dilute appropriate aliquot of sample (not less than 1.00 mL, using positive displacement pipets) with $Na_2S_2O_3$ solution. Account for dilution in final calculation.

J. Calculation

Additional dilutions must be accounted for in final calculation. Do not vary injection volume.

Measure peak heights above baseline and calculate methyl-bound mercury concentration in sample, μg Hg/g, by comparing average peak heights of sample solution to average peak heights of standard solution as follows:

$$\text{Hg, } \mu g/g = (R/R') \times (W'/W)$$

where R = average peak height of sample solution (A); R' = average peak height of standard solution (A); W' = amount standard injected (μg Hg); W = amount sample injected (g), and

$$W = (D/E) \times [F \times (0.100 \text{ mL}/4.0 \text{ mL})]$$

where D = mass of analytical portion (g); E = mass of analytical solution (g); and F = mass of analytical solution added to Celite (g).

If necessary, correct A for sample solution for A for correspondingly diluted blank solution. Quantitation limit, defined as 10× standard deviation of reagent blank, is 0.006 μg Hg/100 mL injected. This corresponds to quantitation limit of 0.06 μg Hg/g for 10 g sample treated according to method.

Ref.: JAOAC **72**, November/December issue (1989).

CAS-7439-97-6 (mercury)

Results and Discussion

Six collaborators submitted their results on the forms pro-

Table 1. Collaborative results for determination of methyl mercury (μg Hg/g) in seafood by LC/AAS method

Coll.	Swordfish (1)	Swordfish (2)	Tuna	Tuna spike	Lobster	Lobster spike
Reference[a]	1.08	1.68	0.15	1.87	0.03	0.42
1	1.15	1.52	0.15	1.90	0.0	0.44
	1.20	1.61	0.15	1.97	0.0	0.41
2	1.00	1.49	0.16	1.78	0.0	0.42
	1.10	1.66	0.14	1.80	0.0	0.38
3	1.30	1.82	0.18	1.97	0.05	0.47
	1.00	1.94	0.11	2.19	0.06	0.48
4	1.23[b]	1.80	0.20[b]	2.01	0.0	0.41
	1.55[b]	1.69	0.20[b]	1.70	0.05	0.42
5	0.93	1.38	0.13	2.04	0.03	0.37
	1.10	1.39	0.15	2.09	0.02	0.32
6	1.12	1.50	0.09[c]	1.46	0.05	0.36
	1.12	1.50	0.10[c]	1.44	0.02	0.38
Av.	1.02	1.608	0.146	1.863	0.023[d]	0.405
Accuracy, %	94.4	95.7	93.3	99.6	—	96.4
s_r	0.115	0.073	0.027	0.113	—	0.020
RSD_r, %	10.5	4.5	18.2	6.1	—	4.9
s_R	0.115	0.185	0.027	0.245	—	0.048
RSD_R, %	10.5	11.5	18.2	13.2	—	11.8

[a] Average of results from Associate Referee's laboratory, n = 6.
[b] Data not used (see text).
[c] Data determined to be outliers by Dixon test on blind duplicate average value; not used in statistics.
[d] Below quantitation limit.

vided to report data in a uniform format. For each laboratory, there were 12 individual concentration values or 72 values for the entire study (Table 1). One collaborator used a 50 μL injection loop instead of the specified 100 μL loop. This proved to be adequate for all samples except the tuna sample, which contained only 0.146 μg Hg/g. (The lobster sample contained Hg below the limit of quantitation and will not be considered.) The 50 μL injection of tuna sample extract produced a very low absorbance value on the recorder so that the signal could not be measured accurately. The original data from the same collaborator show that the responses of the standards in obtaining the results for the swordfish 1 sample differed greatly, while the responses of the sample duplicates were similar. This could indicate a possible problem with these sets of standards. Consequently, the results for tuna and swordfish 1 samples from Collaborator 4 were not included in calculating the statistical parameters.

The Dixon test (6) was applied to identify any stray data points. This test, which is sensitive to values that lie outside the range that is expected for randomly distributed results, was applied to the sum of replicates for each material and for each spike. Finding any value that does not pass the test results in the rejection of both replicate partners. Only one set of values, the results for tuna from Collaborator 6, was rejected by the Dixon test.

The rest of the data was subjected to an analysis of variance to estimate the precision between laboratories and between replicates. The deviation of replication (the within-laboratory standard deviation, or repeatability (s_r)), together with the corresponding relative standard deviation (RSD_r) as well as the deviation between laboratories (the among-laboratories standard deviation, or reproducibility (s_R)), together with its relative standard deviation (RSD_R), are listed in Table 1. Repeatability values represent the standard deviation to be expected for a single determination when compared with other results within 1 laboratory.

The reproducibility values represent the standard deviation to be expected for a single determination when compared with other results from many laboratories. The RSD_R values in this study ranged from 10.5% at the 1 μg Hg/g level to 18.2% at about 0.1 μg Hg/g. Historically, RSD_R values for AOAC studies have been 16 and 23% at 1 and 0.1 μg Hg/g, respectively (7). The accuracy may be evaluated by comparing the average collaborator values for each sample to the reference values listed in Table 1, i.e., the results obtained by the Associate Referee. As can be seen, the agreement is quite good. In addition, the recovery of Hg from the lobster spike sample was 96.4%.

Collaborators' Comments

Comments concerning this proposed method were solicited from each collaborator and most responded positively as well as with some suggestions, which were considered carefully by the Associate Referee.

One collaborator inquired about the possibility of doubling the sample size so that it could be extracted into a larger volume, making more sample extract available in case of a chromatographic problem. This is certainly a possibility, but probably not necessary in most situations; the 4 mL extract is sufficient for multiple injections. Furthermore, the system can be tested with the standards provided for that purpose in the method. The same collaborator suggested some modifications to the LC/AAS interface in order to facilitate maintenance. The Associate Referee agrees and the future models will have access from the top for easy replacement of the tubing.

One collaborator experienced a phenomenon resembling the memory effect, which was attributed to the interface. Low standards gave higher responses when injected after high standards. One explanation suggested the possibility of active sites within the interface in contact with the mercury vapor, probably as a result of accumulation of various substances during long use. It is recommended that the tubing between the interface and the absorption cell be periodically changed. The same collaborator observed a small variation in peak height, depending on the level of mobile phase that had collected in the round-bottom flask. This has not been observed by the Associate Referee. This variation should not affect the results because bracketing of samples by standards is used for quantitation.

Recommendation

The LC/AAS method provides an alternative technique for determination of methyl mercury in seafood in support of the FDA guideline level of 1 μg Hg/g. The proposed method is an extension of the AOAC flameless AAS method, **25.131–25.133**, with the added capability of speciating mercury compounds.

It is recommended that the liquid chromatographic–atomic absorption spectrophotometric method be adopted official first action for determination of methyl mercury in seafood.

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D. Edge, Atlanta Regional Laboratory, Atlanta, GA

R. W. Marts, Kansas City District Laboratory, Kansas City, MO

B. Patel, New York Regional Laboratory, Brooklyn, NY

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METHOD PERFORMANCE

Reliability of Repeatability and Reproducibility Measures in Collaborative Trials

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The acceptability of a new analytical method is generally assessed in terms of repeatability and reproducibility estimates derived from a collaborative study. Procedures are presented for calculating confidence intervals and operating characteristic curves for acceptance criteria based on the repeatability and reproducibility estimates. Comparisons of the reliability of estimates are provided for various numbers of collaborators. With a small number of collaborators, the estimates of reproducibility are not reliable and decisions regarding acceptability of a method will be heavily based on the method's repeatability rather than the property of most interest, namely, the reproducibility of the method.

The main purpose of carrying out collaborative tests on an analytical method is to determine the precision of the method under actual working conditions. Precision has 2 components which are generally referred to as repeatability and reproducibility. The repeatability measure is derived from the within-laboratory variability. This is a measure of the variability among repeated measurements taken under presumably identical conditions. The reproducibility measure is a composite of within-laboratory variability and variations in results among laboratories which arise from different conditions specific to the individual laboratories.

The present minimum requirement for a collaborative study conducted under the auspices of the Association of Official Analytical Chemists (AOAC) is 6 laboratories and 5 samples (1). Each laboratory generally runs 2 repeat determinations on each of the samples and this provides a substantial amount of data for a pooled estimate of repeatability. Even with as few as 3 laboratories and 5 samples (as proposed for a new "candidate method" classification (2)), the information provided on repeatability may be adequate. However, the determining factor in evaluating a method is its reproducibility, and a small number of collaborators may not provide an adequate basis to properly assess how laboratories vary among themselves with respect to their systematic errors.

This article explores some of the consequences of using a reduced number of laboratories in a collaborative study. Detailed assessments of statistical properties of repeatability and reproducibility estimates are provided for collaboratives with: 3 laboratories, as in the minimum requirement for the proposed candidate method classification of the AOAC; 6 laboratories, as in the current minimum requirement for AOAC collaborative studies; and 12 laboratories, a number more consistent with the numbers suggested by Youden and Steiner (3).

Confidence Intervals

Detailed instructions for comprehensive analysis of collaborative studies are provided in the *Statistical Manual of the AOAC* (3). After preliminary checks for outliers and tests for homogeneity of variances, the data are subjected to an analysis of variance. For r replicates, s samples, and k laboratories, an analysis of variance table can be constructed as in Table 1.

Estimates of repeatability and reproducibility are provided, respectively, by

$$\sqrt{S_3^2} \text{ and } \sqrt{\frac{1}{rs}S_1^2 + \left(\frac{1}{r} - \frac{1}{rs}\right)S_2^2 + \left(1 - \frac{1}{r}\right)S_3^2}$$

Interpretation of the above quantities depends on the scale used for statistical analysis. If no transformation is applied to the data, the quantities are interpreted as standard deviations. If analysis has been carried out on the natural log scale, the quantities are interpreted as "coefficients of variation/100."

In analytical studies, there is a strong tendency to express precision in terms of the coefficient of variation (CV). This appears to stem from the fact that analytical errors often appear to be multiplicative rather than additive and, consequently, the variance frequently increases as the concentration increases in a manner such that the CV is relatively constant. Thus the CV is a convenient dimensionless summary measure of precision in this situation, and analysis of the data should be conducted on the log scale. Although results in this report could be applied equally well to standard deviations derived from an analysis of untransformed data, the remaining discussion will be restricted to CVs derived from a log scale analysis.

A 95% confidence interval for repeatability (CV_w) is provided by

$$\left(100\sqrt{n_3S_3^2/\chi^2_{n_3(.975)}},\quad 100\sqrt{n_3S_3^2/\chi^2_{n_3(.025)}}\right)$$

where $\chi^2_{n_3(\alpha)}$ denotes the 100α percentile of the chi-square distribution with n_3 degrees of freedom. For reproducibility (CV_b), no exact interval exists, but a good approximate 95% confidence interval is provided by

$$\left(100\left[\frac{1}{sr}S_1^2 + \frac{s-1}{sr}S_2^2 + \frac{r-1}{r}S_3^2 - \left(\frac{L_1^2}{s^2r^2}S_1^4 + \frac{(s-1)^2L_2^2}{s^2r^2}S_2^4 + \frac{(r-1)^2L_3^2}{r^2}S_3^4\right)^{1/2}\right]^{1/2},\right.$$

$$\left.100\left[\frac{1}{sr}S_1^2 + \frac{s-1}{sr}S_2^2 + \frac{r-1}{r}S_3^2 + \left(\frac{H_1^2}{s^2r^2}S_1^4 + \frac{(s-1)^2H_2^2}{s^2r^2}S_2^4 + \frac{(r-1)^2H_3^2}{r^2}S_3^4\right)^{1/2}\right]^{1/2}\right)$$

This formula is based on the intervals given by Graybill and Wang (4) for linear combinations of chi-square variables; values of L_1 and H_1 must be calculated from tables provided in John (5). For the 3 cases considered in this report, L_1 and H_1 values are listed in Table 2. Mathematical details relating to the above intervals are provided in the Appendix.

Results from a collaborative study by Sen et al. (6) were used to illustrate the relative magnitudes of the confidence intervals. Sen et al. (6) had conducted an analysis on the log scale and presented the following estimates of the variance components:

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Table 1. General analysis of variance table

Source of variation	Degrees of freedom	Mean square	Expected mean square
Between laboratories	$n_1 = k - 1$	S_1^2	$\theta_1 = \sigma^2 + r\,\sigma_{LS}^2 + rs\sigma_L^2$
Between samples	$s - 1$	—	
Laboratory–sample interaction	$n_2 = (k - 1)(s - 1)$	S_2^2	$\theta_2 = \sigma^2 + r\,\sigma_{LS}^2$
Between replicates	$n_3 = ks(r - 1)$	S_3^2	$\theta_3 = \sigma^2$

Table 2. Coefficients for reproducibility confidence intervals

Case	L_1	L_2	L_3	H_1	H_2	H_3
k = 3, s = 5, r = 2	0.7901	0.5758	0.4758	22.5849	2.3146	1.2758
k = 6, s = 5, r = 2	0.6520	0.4322	0.3745	4.0556	1.0884	0.7437
k = 12, s = 5, r = 2	0.5245	0.3243	0.2872	1.6831	0.5699	0.4648

$$\hat{\sigma} = 0.17 \qquad \hat{\sigma}_{LS} = 0.096 \qquad \hat{\sigma}_L = 0.18$$

Using these values, CV_w = 17% and CV_b = 27%. Degrees of freedom for the various mean squares were calculated as though these estimates had arisen from the 3 collaboratives considered in this report. The resulting confidence limits are presented in Table 3.

Operating Characteristic Curves

While there do not appear to be any strict guidelines for assessing a method, a decision rule framework can be postulated as follows:

(i) accept repeatability if the observed $CV_W < 24\%$, reject repeatability if the observed $CV_w > 24\%$;

(ii) accept reproducibility if the observed $CV_b < 40\%$, reject reproducibility if the observed $CV_b > 40\%$.

The 40% limit for the observed CV_b is consistent with the range considered to be acceptable in the survey of collaboratives studied by Horwitz et al. (7). Horwitz et al. (7) also reported that 'observed CV_w/observed CV_b' was generally close to 0.6 and hence 24% (= 0.6 × 40%) was taken as a reasonable limit for the observed CV_w.

Operating characteristic (OC) curves for rule (i) are provided in Figure 1 where the probability of rejecting a method is plotted against the true CV_w. For reproducibility, no single set of OC curves can be constructed. This is due to the fact that information on both between- and within-laboratory components of variation is necessary in the calculation of probabilities. OC curves (Figure 2) were therefore constructed for the special case where CV_w is 24% and laboratory-sample interaction is negligible. This case corresponds to the situation where the within-laboratory variance on the log scale is a fixed constant and the increases in the true CV_b are entirely due to increased between-laboratory variance. In practice, CV_w is known to some degree since extensive testing will have been conducted in a laboratory during the development stages and a ruggedness test will have been completed. OC curves could therefore be drawn as in Figure 2, using this estimated CV_w to obtain OC curves which may be more relevant to the particular method being tested. OC curves for other cases can be determined using the mathematics outlined in the Appendix.

Table 3. Example 95% confidence intervals for log scale analysis with $\hat{\sigma}$ = 0.17, $\hat{\sigma}_{LS}$ = 0.096, and $\hat{\sigma}_L$ = 0.18

CV	k = 3, s = 5, r = 2	k = 6, s = 5, r = 2	k = 12, s = 5, r = 2
CV_w	(12.6%, 26.3%)	(13.6%, 22.7%)	(14.4%, 20.7%)
CV_b	(19.6%, 95.4%)	(21.1%, 47.2%)	(22.3%, 36.6%)

The probabilities of actually accepting or rejecting a method will naturally be a composite of the probabilities in Figure 1 and probabilities similar to those presented for the special case in Figure 2. Assuming that a method must satisfy both the CV_w and CV_b criteria to be accepted, series of probability curves have been calculated for collaborative studies with 3 laboratories (Figure 3), collaborative studies wth 6 laboratories (Figure 4), and collaborative studies with 12 laboratories (Figure 5). As in Figure 2, the laboratory–sample interaction was assumed to be negligible.

Probabilities of rejecting a method with a specific combination of true CV_w and true CV_b can be easily derived from these graphs. Within each graph, the true CV_w determines the appropriate probability curve. This curve is used to determine the rejection probability corresponding to the specified true CV_b. Illustrations of probability determinations for 2 example (CV_w, CV_b) combinations are provided in Figures 3, 4, and 5; the rejection probabilities are summarized in Table 4.

A noteworthy point regarding these graphs is the importance of CV_w relative to CV_b for designs with different numbers of laboratories. From Figure 5, it is evident that, with 12 laboratories, increases in either the repeatability or reproducibility measure result in a rapid increase in the probability of rejecting a method. Figure 3 illustrates that, with 3 laboratories, the probability of rejecting a method also increases fairly rapidly as its repeatability properties deteriorate. However, for any specific CV_w, the probability profiles are quite flat. Therefore, with 3 laboratories, the decision regarding the method's acceptability will be based largely on the method's repeatability and not on the method's reproducibility.

Conclusions

Assessment of repeatability may be adequately carried out with a collaborative study involving as few as 3 laboratories. With duplicate determinations and a sufficient number of samples, a substantial amount of data will be available for a pooled estimate of repeatability. With an increased number of participants, estimates and decisions regarding repeatability will be more reliable and chances are better of detecting

Table 4. Examples of rejection probabilities derived from Figures 3, 4, and 5

True CV_w, %	True CV_b, %	3 Labs (Fig. 3)	6 Labs (Fig. 4)	12 Labs (Fig. 5)
20	30	0.21	0.10	0.02
20	50	0.61	0.73	0.85

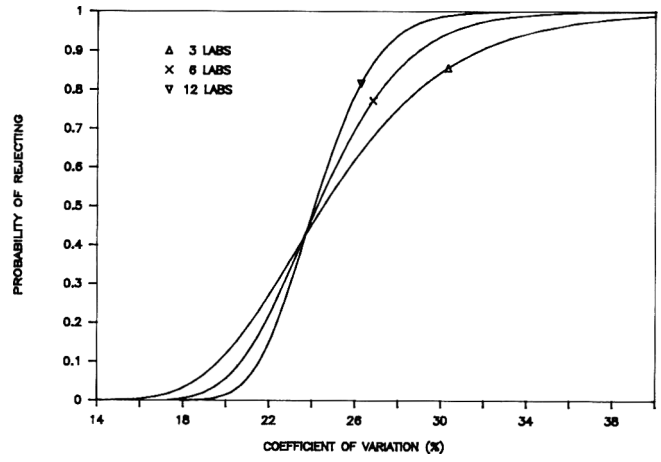

Figure 1. Repeatability OC curves for collaborative studies with 5 samples, 2 replicates, and rejection limit of 24% for CV_w.

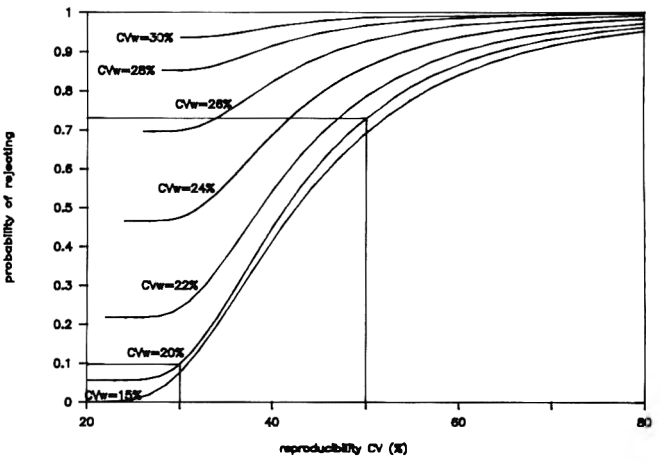

Figure 4. Probabilities of rejecting method in 6-laboratory collaborative studies with rejection limits of 24% and 40% for CV_w and CV_b, respectively. Lines illustrate probability determinations for (true CV_w, true CV_b) combinations of (20%, 30%) and (20%, 50%).

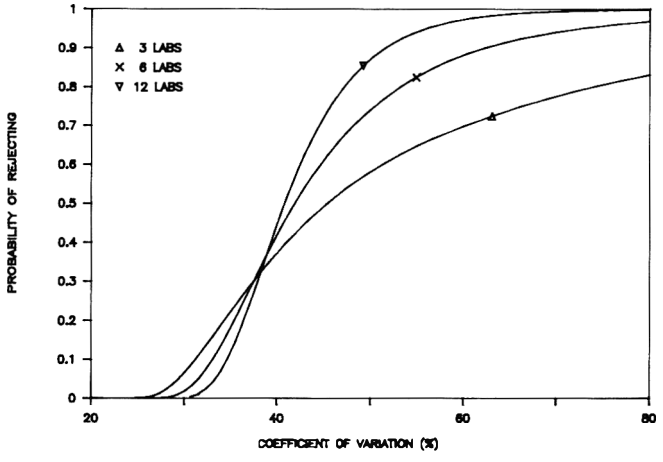

Figure 2. Reproducibility OC curves for collaborative studies with 5 samples, 2 replicates, $CV_w = 24\%$, $\sigma_{LS}^2 = 0$, and rejection limit of 40% for CV_b.

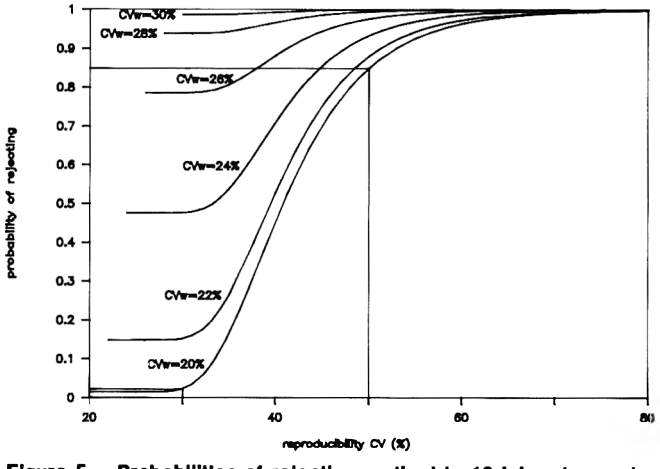

Figure 5. Probabilities of rejecting method in 12-laboratory collaborative studies with rejection limits of 24% and 40% for CV_w and CV_b, respectively. Lines illustrate probability determinations for (true CV_w, true CV_b) combinations of (20%, 30%) and (20%, 50%).

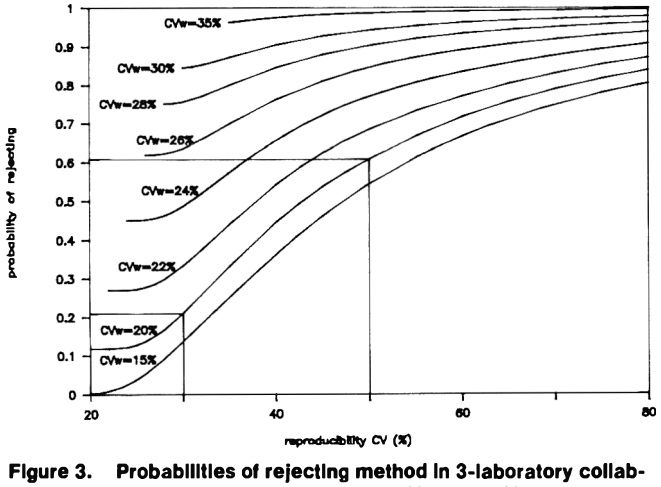

Figure 3. Probabilities of rejecting method in 3-laboratory collaborative studies with rejection limits of 24% and 40% for CV_w and CV_b, respectively. Lines illustrate probability determinations for (true CV_w, true CV_b) combinations of (20%, 30%) and (20%, 50%).

outliers which might artificially inflate the observed coefficients of variation. However, the marginal gains in information on repeatability may not justify the increased cost of a larger collaborative study.

Reduction in the number of participating laboratories has a much greater impact on estimates and tests of a method's reproducibility. Reliable estimates of reproducibility cannot be obtained from a collaborative study involving only 3 laboratories. Decisions regarding the acceptability of a method would be heavily based on the method's repeatability rather than the property of most interest in a collaborative study, namely, the reproducibility of the method.

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Appendix

The quantities of interest will be taken to be the standard deviations or CV/100, depending on whether the analysis is based on the original scale or on the log scale, respectively. In either case, with notation as in Table 1, the 2 quantities of interest are

$$\sigma \text{ and } \sqrt{\sigma_L^2 + \sigma_{LS}^2 + \sigma^2}$$

and are estimated by

$$S_3 \text{ and } \sqrt{\frac{1}{rs}S_1^2 + \left(\frac{1}{r} - \frac{1}{rs}\right)S_2^2 + \left(1 - \frac{1}{r}\right)S_3^2}$$

Confidence intervals and probability statements regarding repeatability follow directly from the fact that

$$\frac{n_3 S_3^2}{\sigma^2} \sim \chi^2_{n_3}$$

For reproducibility, the quantity

$$\frac{1}{s(r-1)}S_1^2 + \frac{s-1}{s(r-1)}S_2^2 + S_3^2$$

has the form $\sum_i C_i S_i^2$ with

$$C_i \geq 0 \text{ and } C_3 = 1$$

$$n_i S_i^2/\theta_i$$

independently distributed as chi-square variables with n_i degrees of freedom, and

$$\sum_i C_i S_i^2$$

as an unbiased estimator of $\theta = \sum_i C_i \theta_i = \frac{r}{r-1}(\sigma_L^2 + \sigma_{LS}^2 + \sigma^2)$.

Therefore, as in Graybill and Wang (4), the shortest unbiased approximate confidence intervals for reproducibility are provided by

$$100\left[\frac{r-1}{r}\left(\sum_i C_i S_i^2 - \left(\sum_i L_i^2 C_i^2 S_i^4\right)^{1/2}\right)\right]^{1/2}, \qquad 100\left[\frac{r-1}{r}\left(\sum C_i S_i^2 + \left(\sum H_i^2 C_i^2 S_i^4\right)^{1/2}\right)\right]^{1/2}$$

where $L_i = 1 - 1/F_{1i}$ and $H_i = -1 + 1/F_{2i}$, with values of F_{1i} and F_{2i} given in John (5).

Evaluation of the operating characteristic (OC) curves for reproducibility is based on a Corollary provided in Fleiss (8) concerning the distribution of linear combinations of chi-square variables. The required probability statements can be expressed as

$$\Pr\left\{\frac{1}{rs}\frac{S_1^2}{\theta_3} + \frac{s-1}{rs}\frac{S_2^2}{\theta_3} + \frac{r-1}{r}\frac{S_3^2}{\theta_3} \leq \frac{K^2}{\theta_3}\right\}$$

which, after setting $\sigma_{LS}^2 = R_2\sigma^2$ and $\sigma_L^2 = R_1\sigma^2$ and rearranging terms,

$$= \Pr\left\{\frac{k(1 + rR_2 + rsR_1)}{k-1}U_1 + \frac{k(1+rR_2)}{k-1}U_2 + U_3 \leq \frac{ksr(1 + R_1 + R_2)K^2}{\sigma^2}\right\}$$

where $U_i \sim \chi^2_{n_i}$, i = 1, 2, 3. For given values of σ^2, R_1, and R_2, the conditions of the Fleiss (8) Corollary are satisfied and the required probability is given by

$$\frac{\Gamma\left(\frac{n}{2}\right)}{\prod_i \Gamma\left(\frac{n_i}{2}\right)} \int_0^1 \int_0^1 w_2^{\frac{n_2}{2}-1} w_3^{\frac{n_3}{2}-1} (1 - w_2 - w_3)^{\frac{n_1}{2}-1} \chi^2_n \left\{\frac{z}{\sum_i a_i w_i}\right\} dw_2 dw_3$$

$$w_2 + w_3 \leq 1$$

$$w_1 = 1 - w_2 - w_3$$

where $n = n_1 + n_2 + n_3$,

$$a_1 = \frac{k(1 + r R_2 + rs R_1)}{k - 1}, \quad a_2 = \frac{k(1 + r R_2)}{k - 1}, \quad a_3 = 1, \quad z = \frac{ksr(1 + R_1 + R_2)K^2}{\sigma^2}$$

For the particular application in this report $\theta_3 = \sigma^2$ was taken as a fixed known quantity and $R_2 = 0$. Thus, for any given CV_b, R_1 is determined by

$$R_1 = \frac{(CV_b/100)^2}{\theta_3} - 1$$

A numerical integration routine using Simpson's rule was used to evaluate the integral.

MICROBIOLOGICAL METHODS

Hydrophobic Grid Membrane Filter/MUG Method for Total Coliform and *Escherichia coli* Enumeration in Foods: Collaborative Study

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Twenty-four laboratories participated in a collaborative study to validate a hydrophobic grid membrane filter (HGMF) method incorporating the use of 4-methylumbelliferyl-β-D-glucuronide (MUG) for enumeration of total coliform and *Escherichia coli* bacteria in foods by comparing its performance against the AOAC 3-tube MPN method (46.013–46.016). Raw milk, raw ground poultry, whole egg powder, cheese powder, and ground black pepper were included in the study. The total coliform methods did not differ significantly, except that the 3-tube method detected a significantly higher level of total coliforms than did the HGMF method in the ground black pepper. Conversely, the HGMF/MUG *E. coli* method detected significantly higher numbers of *E. coli* present in the egg powder, cheese powder, and ground black pepper samples, while not differing significantly from the 3-tube method for the raw milk and raw ground poultry samples. The overall confirmation rate of MUG-positive colonies isolated using the HGMF method was 99.5%. The hydrophobic grid membrane filter/MUG method has been adopted official first action as an additional method to AOAC official final action method 46.030–46.034.

A hydrophobic grid membrane filter (HGMF) enumeration method using M-FC agar for total coliforms and tryptone bile agar for *Escherichia coli* was adopted by AOAC in 1984 **(46.030–46.034)** (1). The method has performed well; however, the 4-h resuscitation step and 44.5°C incubation temperature specified for *E. coli* are somewhat inconvenient. Since those methods were adopted, incorporation of 4-methylumbelliferyl-β-D-glucuronide (MUG) into coliform media has been introduced for differential enumeration of *E. coli* and has gradually gained acceptance. We redesigned the HGMF total coliform/*E. coli* method to take advantage of the properties of MUG to (*1*) eliminate the elevated temperature incubation, (*2*) eliminate the 4-h resuscitation, and (*3*) combine the total coliform and *E. coli* enumeration on a single HGMF. The new method was subjected to detailed in-house validation over 25 food product categories (manuscript in preparation), and was at least equivalent in overall performance to the AOAC 3-tube most probable number (MPN) method **(46.013–46.016)** (1). The present report describes the results of a collaborative study of the new HGMF total coliform/*E. coli* enumeration method.

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

Collaborative Study

Twenty-four laboratories took part in the study. Seven laboratories participated in the raw milk and raw ground poultry segments of the study. Thirteen to 18 laboratories took part in the analysis of ground black pepper, whole egg powder, and cheese powder. The laboratories involved in the analysis of each food product are indicated in Tables 1–11, inclusive. Each collaborator received a complete set of instructions, data sheets, and a set of 6 or 8 samples (see Table 12) for each portion of the study in which the laboratory was participating. The Associate Referee furnished HGMFs, filtration units (where needed), and the ingredients for lactose monensin glucuronate agar (LMG) and buffered MUG agar (BMA), the 2 media required for the HGMF method; all other materials were furnished by the collaborating laboratories.

The milk and poultry meat were naturally contaminated with coliforms and *E. coli*. Production lots of raw milk and raw ground poultry were screened in advance and chosen to provide low-, moderate-, and high-count samples of these foods for the collaborative study. The pepper, egg powder, and cheese powder were inoculated with a lyophilized mixture of *E. coli* (5 strains), *Enterobacter aerogenes*, *Enterobacter agglomerans*, *Klebsiella pneumoniae*, and *Citrobacter freundii*, targeted to produce low (20/g), moderate (200/g), and high (500/g) levels of total coliforms in the samples. The targeted ratio of *E. coli* to other coliforms in the inocula was approximately 1:1. The inocula were prepared and mixed into the samples as described previously (2).

The collaborative study was carried out over a 5-week period, with each product analyzed during a separate week. Collaborators were asked to analyze all samples both by the HGMF method and by the AOAC 3-tube MPN method, **46.013–46.016** (1). Procedures for the methods were followed throughout, except the sample dilutions(s) to be tested by both methods were specified by the Associate Referee. Collaborators calculated and reported 3-tube method MPN/g or mL and HGMF MPN/g or mL for all samples, and also reported all raw data. To validate the specificity of the HGMF MUG reaction to *E. coli*, collaborators were instructed to subculture 2 MUG-positive colonies from each HGMF and determine Gram reaction, IMViC pattern, and production of acid/gas in lauryl sulfate tryptose broth in 48 h

Table 1. Collaborative study results for total coliforms in raw milk (MPN/mL) by HGMF/MUG and AOAC 3-tube methods

Coll.	3-Tube method						HGMF method					
	1	2	3	4	5	6	1	2	3	4	5	6
2	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	3.8×10^4	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$
3	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	9.2×10^3	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	5.4×10^3
6	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$
20	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	4.6×10^4
22	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$
23	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	8.9×10^4	$>9.2 \times 10^4$	2.8×10^3	$>9.2 \times 10^4$	9.6×10^2	8.5×10^4
26	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$	$>9.2 \times 10^4$

Table 2. Collaborative study results for total coliforms in raw milk (MPN/mL) by HGMF/MUG and AOAC 3-tube methods - reset

Coll.	3-Tube method						HGMF method					
	1	2	3	4	5	6	1	2	3	4	5	6
3	2.4×10^5	$>1.1 \times 10^6$	$>1.2 \times 10^5$	$>1.1 \times 10^6$	1.1×10^6	4.6×10^5	1.3×10^6	1.9×10^6	7.2×10^5	1.8×10^6	4.5×10^5	1.8×10^5
6	1.1×10^6	$>1.1 \times 10^6$	1.1×10^6	$>1.1 \times 10^6$	4.6×10^5	$>1.1 \times 10^6$	1.6×10^6	9.7×10^6	1.1×10^7	1.0×10^7	7.4×10^6	1.2×10^6
22	2.4×10^5	$>1.1 \times 10^6$	$>1.1 \times 10^6$	$>1.1 \times 10^6$	$>1.1 \times 10^6$	1.5×10^5	6.9×10^5	1.5×10^6	7.7×10^5	2.1×10^6	1.4×10^6	7.9×10^5
26	$>1.1 \times 10^6$	$>1.1 \times 10^6$	$>1.1 \times 10^6$	$>1.1 \times 10^6$	$>1.1 \times 10^6$	$>1.1 \times 10^6$	1.2×10^6	8.8×10^6	2.6×10^6	5.9×10^6	1.0×10^7	7.5×10^5

Table 3. Collaborative study results for total coliforms in raw ground poultry (MPN/g) by HGMF/MUG and AOAC 3-tube methods

Coll.	3-Tube method						HGMF method					
	1	2	3	4	5	6	1	2	3	4	5	6
2[a]	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	$>1.1 \times 10^3$	1.0×10^1	2.0×10^1	3.0×10^1	2.2×10^3	2.0×10^1	2.0×10^1
3	9.1×10^0	4.3×10^1	9.3×10^1	2.3×10^1	2.3×10^1	4.3×10^1	2.0×10^1	7.0×10^1	1.1×10^2	2.0×10^1	2.0×10^1	4.0×10^1
6	1.4×10^1	4.6×10^2	9.3×10^1	2.3×10^1	2.3×10^1	1.5×10^1	3.0×10^1	3.1×10^2	4.0×10^1	$<1.0 \times 10^1$	3.0×10^1	6.0×10^1
20	7.5×10^1	9.3×10^1	2.4×10^2	4.3×10^1	9.3×10^1	1.1×10^3	5.0×10^1	2.1×10^2	1.0×10^2	$<1.0 \times 10^1$	4.0×10^1	2.0×10^2
22	2.3×10^1	4.6×10^2	2.3×10^1	2.3×10^1	9.3×10^1	4.6×10^2	5.0×10^1	2.3×10^2	5.0×10^1	6.0×10^1	1.6×10^2	2.4×10^2
23	9.1×10^0	9.3×10^1	4.3×10^1	9.1×10^0	9.1×10^0	4.3×10^1	$<1.0 \times 10^1$	1.5×10^2	3.0×10^1	1.0×10^1	2.0×10^1	6.0×10^1
26	4.3×10^1	2.4×10^2	9.3×10^1	2.3×10^1	4.3×10^1	9.3×10^1	1.0×10^1	2.5×10^2	3.0×10^1	1.0×10^1	2.0×10^1	6.0×10^1

[a]Samples improperly stored due to freezer failure. All data excluded from statistical evaluation.

at 35°C. Any non-*E. coli* isolates were to be retested to verify their MUG-positive reaction on BMA.

Total Coliform and *Escherichia coli* Counts in Foods

Hydrophobic Grid Membrane Filter/MUG Method

First Action

(Applicable to enumeration of total coliform and *Escherichia coli* bacteria in all foods)

Method Performance:

Total coliform count

$s_r = 0.11–0.48$; $s_R = 0.32–0.52$; $RSD_r = 1.2–32.8\%$; $RSD_R = 5.0–38.2\%$

E. coli count

$s_r = 0.08–0.45$; $s_R = 0.33–0.67$; $RSD_r = 0.0–48.9\%$; $RSD_R = 0.0–46.3\%$

A. Principle

Hydrophobic grid membrane filter (HGMF) method uses membrane filter imprinted with hydrophobic material in grid pattern. Hydrophobic lines act as barriers to spread of colonies, thereby dividing membrane filter surface into separate compartments of equal and known size. Number of squares occupied by colonies is enumerated and converted to most probable number (MPN) value of organisms by using formu-

Table 4a. Collaborative study results for total coliforms in ground black pepper (MPN/g) by AOAC 3-tube method

Coll.	Total coliforms, MPN/g							
	1	2	3	4	5	6	7	8
1	2.3×10^1	9.3×10^1	4.6×10^3	2.1×10^3	4.3×10^1	3.6×10^0	$<3.0 \times 10^0$	2.3×10^1
2	$<3.0 \times 10^0$	1.1×10^3	1.1×10^4	1.1×10^4	9.3×10^1	2.4×10^2	$<3.0 \times 10^0$	1.5×10^1
3	$<3.0 \times 10^0$	1.1×10^3	4.6×10^3	4.6×10^3	9.3×10^1	4.3×10^1	$<3.0 \times 10^0$	4.3×10^1
5	$<3.0 \times 10^0$	2.1×10^1	1.1×10^4	1.1×10^4	1.1×10^3	2.3×10^1	$<3.0 \times 10^0$	2.3×10^1
7	1.1×10^3	$<3.0 \times 10^0$	1.1×10^4	1.1×10^4	1.1×10^3	1.1×10^3	1.1×10^3	1.1×10^3
8	2.4×10^2	1.1×10^3	1.1×10^4	1.1×10^4	1.1×10^3	4.3×10^1	$<3.0 \times 10^0$	1.5×10^2
10	$<3.0 \times 10^0$	1.5×10^2	4.6×10^3	1.1×10^4	1.1×10^3	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
14	$<3.0 \times 10^0$	7.2×10^0	1.1×10^4	1.1×10^4	2.3×10^1	9.1×10^0	$<3.0 \times 10^0$	3.6×10^0
15	2.3×10^1	2.1×10^2	1.1×10^4	1.1×10^4	7.5×10^1	2.3×10^1	$<3.0 \times 10^0$	3.6×10^0
16	$<3.0 \times 10^0$	2.4×10^2	1.1×10^4	1.1×10^4	1.1×10^3	1.5×10^1	$<3.0 \times 10^0$	2.3×10^1
17	$<3.0 \times 10^0$	2.3×10^1	4.6×10^3	4.6×10^3	4.6×10^2	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$
19	$<3.0 \times 10^0$	1.1×10^3	1.1×10^4	1.1×10^4	2.4×10^2	3.6×10^0	$<3.0 \times 10^0$	3.6×10^0
21	9.3×10^1	1.1×10^3	1.1×10^4	1.1×10^4	4.6×10^2	2.8×10^1	$<3.0 \times 10^0$	1.1×10^3
24[a]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.1×10^4	1.1×10^4	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
26	$<3.0 \times 10^0$	1.1×10^3	4.6×10^3	1.1×10^4	9.3×10^1	2.4×10^2	$<3.0 \times 10^0$	9.1×10^0

[a]Method deviation due late initiation of analyses. Data for HGMF and 3-tube methods excluded from statistical evaluation.

Table 4b. Collaborative study results for total coliforms in ground black pepper (MPN/g) by HGMF/MUG method

Coll.	Total coliforms, MPN/g							
	1	2	3	4	5	6	7	8
1	2.0×10^2	9.0×10^2	6.0×10^2	1.2×10^3	4.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	8.0×10^1
2	$<1.0 \times 10^1$	1.0×10^1	2.3×10^3	2.9×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
3	$<1.0 \times 10^1$	4.0×10^1	1.0×10^3	2.8×10^3	6.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1
5	$<1.0 \times 10^1$	7.0×10^1	3.7×10^3	6.4×10^3	2.3×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1
7	$<1.0 \times 10^1$	2.6×10^2	6.1×10^3	$<1.0 \times 10^2$	4.0×10^1	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$
8	6.0×10^1	1.0×10^1	4.0×10^3	2.3×10^3	2.3×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1
10	$<1.0 \times 10^1$	1.2×10^2	3.8×10^3	1.3×10^4	2.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1
14	$<1.0 \times 10^1$	9.5×10^3	6.5×10^3	1.1×10^4	3.4×10^2	1.0×10^1	$<1.0 \times 10^1$	1.0×10^1
15	3.3×10^2	6.0×10^1	1.5×10^3	9.4×10^3	5.1×10^2	8.0×10^1	2.0×10^1	$<1.0 \times 10^1$
16	$<1.0 \times 10^1$	8.0×10^1	1.3×10^3	6.9×10^3	1.8×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
17	$<1.0 \times 10^1$	4.0×10^1	3.1×10^3	1.0×10^4	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$	1.0×10^1
19	$<1.0 \times 10^1$	5.0×10^1	4.2×10^3	3.3×10^3	3.0×10^1	8.0×10^1	$<1.0 \times 10^1$	1.0×10^1
21	8.0×10^1	4.0×10^1	3.2×10^3	2.6×10^3	1.0×10^1	1.0×10^1	$<1.0 \times 10^1$	1.0×10^1
24[a]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^3	1.9×10^3	$.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.5×10^2
26	$<1.0 \times 10^1$	8.0×10^1	2.0×10^3	1.3×10^3	2.8×10^2	2.0×10^1	$<1.0 \times 10^1$	1.0×10^1

[a]See corresponding footnote to Table 4a.

Table 5a. Collaborative study results for total coliforms in whole egg powder (MPN/g) by AOAC 3-tube method

Coll.	Total coliforms, MPN/g							
	1	2	3	4	5	6	7	8
1	4.6×10^2	2.3×10^1	4.3×10^1	2.3×10^1	4.3×10^1	1.1×10^3	4.6×10^2	$<3.0 \times 10^0$
2	2.3×10^1	$<3.0 \times 10^0$	9.3×10^1	2.3×10^1	4.3×10^1	2.4×10^2	4.3×10^1	$<3.0 \times 10^0$
3	2.4×10^2	$<3.0 \times 10^0$	9.3×10^1	9.1×10^0	4.3×10^1	2.4×10^2	4.3×10^1	$<3.0 \times 10^0$
5	4.3×10^1	$<3.0 \times 10^0$	4.3×10^1	3.6×10^0	2.3×10^1	2.4×10^2	2.4×10^2	$<3.0 \times 10^0$
7	9.3×10^1	$<3.0 \times 10^0$	9.3×10^1	7.3×10^0	9.3×10^1	9.3×10^1	9.3×10^1	$<3.0 \times 10^0$
8	2.4×10^2	$<3.0 \times 10^0$	9.3×10^1	1.5×10^1	9.1×10^0	2.4×10^2	4.3×10^1	$<3.0 \times 10^0$
9	9.3×10^1	$<3.0 \times 10^0$	2.0×10^1	9.1×10^0	3.6×10^0	9.3×10^1	4.6×10^2	$<3.0 \times 10^0$
10	1.5×10^2	$<3.0 \times 10^0$	2.1×10^1	4.3×10^1	4.3×10^1	4.3×10^1	9.3×10^1	$<3.0 \times 10^0$
11	4.3×10^1	$<3.0 \times 10^0$	4.3×10^1	9.1×10^0	3.6×10^0	2.4×10^2	4.3×10^1	$<3.0 \times 10^0$
16[a]	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^0	3.6×10^0	$<3.0 \times 10^0$
19	9.3×10^1	$<3.0 \times 10^0$	4.3×10^1	3.6×10^0	2.3×10^1	4.3×10^1	1.5×10^1	$<3.0 \times 10^0$
21	2.4×10^2	$<3.0 \times 10^0$	2.4×10^2	2.1×10^1	2.3×10^1	2.4×10^2	9.3×10^1	$<3.0 \times 10^0$
26	4.3×10^1	$<3.0 \times 10^0$	2.3×10^1	9.1×10^0	2.3×10^1	2.4×10^2	4.3×10^1	$<3.0 \times 10^0$

[a]Statistical outlier for 3-tube method. 3-Tube method data excluded from statistical evaluation.

Table 5b. Collaborative study results for total coliforms in whole egg powder (MPN/g) by HGMF/MUG method

Coll.	Total coliforms, MPN/g							
	1	2	3	4	5	6	7	8
1	4.9×10^2	$<1.0 \times 10^1$	3.0×10^1	1.0×10^1	$<1.0 \times 10^1$	3.0×10^2	2.0×10^1	$<1.0 \times 10^1$
2	4.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1	7.0×10^1	$<1.0 \times 10^1$
3	3.0×10^1	$<1.0 \times 10^1$	3.0×10^1	1.0×10^1	2.0×10^1	1.0×10^1	3.0×10^1	$<1.0 \times 10^1$
5	1.1×10^2	$<1.0 \times 10^1$	4.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	3.0×10^1	$<1.0 \times 10^1$
7	3.0×10^1	$<1.0 \times 10^1$	1.0×10^1	2.0×10^1	1.0×10^1	8.0×10^1	6.3×10^3	$<1.0 \times 10^1$
8	9.0×10^1	$<1.0 \times 10^1$	8.0×10^1	1.0×10^1	1.0×10^1	5.0×10^1	8.0×10^1	$<1.0 \times 10^1$
9	3.0×10^1	$<1.0 \times 10^1$	3.0×10^1	1.0×10^1	1.0×10^1	9.0×10^1	3.0×10^2	$<1.0 \times 10^1$
10	3.0×10^1	$<1.0 \times 10^1$	3.0×10^1	$<1.0 \times 10^1$	1.0×10^1	4.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$
11	4.0×10^1	$<1.0 \times 10^1$	1.0×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.5×10^2	2.7×10^2	$<1.0 \times 10^1$
16[a]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.6×10^2	$<1.0 \times 10^1$
19	1.4×10^2	$<1.0 \times 10^1$	1.0×10^1	1.0×10^1	1.0×10^1	1.1×10^2	6.0×10^1	$<1.0 \times 10^1$
21	5.0×10^1	$<1.0 \times 10^1$	2.0×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.3×10^2	7.0×10^1	$<1.0 \times 10^1$
26	1.6×10^2	$<1.0 \times 10^1$	3.0×10^1	1.0×10^1	3.0×10^1	1.2×10^2	2.0×10^1	$<1.0 \times 10^1$

[a]See corresponding footnote to Table 5a.

la. *E. coli* produce β-glucuronidase, which degrades 4-methylumbelliferyl-β-D-glucuronide (MUG) fluorogenic substrate and releases fluorescent 4-methylumbelliferone. MUG-positive colonies fluoresce blue-white under longwave (366 nm) UV light.

B. Apparatus, Culture Media, and Reagents

See **46.B02(a)–(g)** and **(i)–(r)** and in addition:

(a) *Longwave (366 nm) UV light source.*

(b) *Lactose monensin glucuronate agar (LMG).*—10.0 g tryptose, 5.0 g proteose peptone No. 3, 3.0 g yeast extract, 12.5 g lactose, 0.038 g monensin (dissolved in 10 mL 95% ethanol), 0.25 mL Tergitol anionic 7, 0.1 g aniline blue (Colour Index No. 42755. Fisher Scientific Cat. No. A-967, or equivalent), 0.5 g glucuronic acid Na salt, and 15.0 g agar diluted to 1 L with water (LMG agar, QA Laboratories, Cat.

Table 6a. Collaborative study results for total coliforms in cheese powder (MPN/g) by AOAC 3-tube method

Coll.	Total coliforms, MPN/g							
	1	2	3	4	5	6	7	8
1	1.5×10^2	4.6×10^2	$<3.0 \times 10^0$	2.4×10^2	$<3.0 \times 10^0$	2.1×10^1	9.3×10^1	2.3×10^1
2	9.3×10^1	2.4×10^2	$<3.0 \times 10^0$	2.4×10^2	$<3.0 \times 10^0$	4.3×10^1	9.3×10^1	3.6×10^0
3	1.5×10^2	4.6×10^2	$<3.0 \times 10^0$	4.3×10^1	$<3.0 \times 10^0$	4.3×10^1	1.5×10^2	2.3×10^1
4	4.3×10^1	2.4×10^2	$<3.0 \times 10^0$	3.9×10^1	$<3.0 \times 10^0$	9.1×10^0	2.4×10^2	1.5×10^1
5	9.3×10^1	9.3×10^1	$<3.0 \times 10^0$	4.3×10^1	$<3.0 \times 10^0$	9.1×10^0	2.4×10^2	1.5×10^1
7	2.4×10^2	1.5×10^2	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^0$	2.3×10^1	9.3×10^1	4.3×10^1
8	9.3×10^1	2.4×10^2	$<3.0 \times 10^0$	1.5×10^2	$<3.0 \times 10^0$	2.3×10^1	9.3×10^1	2.3×10^1
10	2.3×10^1	2.4×10^2	$<3.0 \times 10^0$	9.3×10^1	$<3.0 \times 10^0$	9.3×10^1	9.3×10^1	2.3×10^1
11	9.3×10^1	2.4×10^2	$<3.0 \times 10^0$	3.9×10^1	$<3.0 \times 10^0$	3.6×10^0	4.6×10^2	9.1×10^0
12	7.5×10^1	9.3×10^1	$<3.0 \times 10^0$	1.2×10^2	$<3.0 \times 10^0$	4.3×10^1	2.4×10^2	3.9×10^1
13[a]	9.3×10^1	9.3×10^1	$<3.0 \times 10^0$	4.3×10^1	$<3.0 \times 10^0$	2.3×10^1	4.3×10^1	9.1×10^0
14	2.3×10^1	2.0×10^1	$<3.0 \times 10^0$	4.3×10^1	$<3.0 \times 10^0$	2.3×10^1	4.3×10^1	2.3×10^1
15	4.3×10^1	9.3×10^1	$<3.0 \times 10^0$	4.6×10^2	$<3.0 \times 10^0$	4.3×10^1	2.4×10^2	2.3×10^1
16[b]	7.3×10^0	9.1×10^0	$<3.0 \times 10^0$	1.1×10^1	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.5×10^1	3.0×10^0
17	9.3×10^1	4.3×10^1	$<3.0 \times 10^0$	2.3×10^1	$<3.0 \times 10^0$	3.6×10^0	2.4×10^2	2.3×10^1
20	4.3×10^1	4.6×10^2	$<3.0 \times 10^0$	2.4×10^2	$<3.0 \times 10^0$	7.5×10^1	2.4×10^2	9.1×10^0
21	1.2×10^2	4.6×10^2	$<3.0 \times 10^0$	9.3×10^1	$<3.0 \times 10^0$	1.5×10^2	4.6×10^2	2.3×10^1
26	9.3×10^1	4.6×10^2	$<3.0 \times 10^0$	2.4×10^2	$<3.0 \times 10^0$	4.3×10^1	4.6×10^2	9.3×10^1

[a]Method deviation due to incorrect dilution filtered. HGMF method data excluded from statistical evaluation.

[b]Statistical outlier for HGMF and 3-tube MPN methods. Data from both methods excluded from statistical evaluation.

No. A-003, is satisfactory). Heat to boiling. DO NOT AUTOCLAVE. Temper to 45–50°. Aseptically adjust pH to give final pH of 7.2 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use by inverting partly open for 15–20 min in 35° incubator.

(c) *Buffered MUG agar (BMA).*—8.23 g dibasic sodium phosphate, 1.20 g monobasic sodium phosphate, 5.0 g NaCl, 0.1 g 4-methylumbelliferyl-β-D-glucuronide, and 15.0 g agar diluted to 1.0 L with water (BMA agar, QA Laboratories, Cat. No. A-004, is satisfactory). Heat to boiling. Autoclave 15 min at 121°. Temper to 45–50°. Do not adjust pH. Final pH should be 7.2–7.6. If pH < 7.2 or > 7.6, discard mixture. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use by inverting partly open for 15–20 min in 35° incubator.

C. Sample Preparation

(a) *Liquid egg.*—Use sterile spoon or spatula to thoroughly mix sample. Prepare 1:10 dilution by aseptically weighing 11 g egg material into sterile wide-mouth, screw-top bottle; add 99 mL PT diluent, **(g),** and 1 tablespoonful of sterile glass shot. Thoroughly agitate 1:10 dilution to ensure complete solution or distribution of egg material in diluent by shaking each bottle rapidly 25 times, each shake an up-and-down movement of ca 30 cm, total time interval ≤ 7 s. Let bubbles escape. If enzyme treatment is needed (see Table **46:B1**), combine 5 mL of 1:10 dilution with 1 mL of appropriate enzyme stock solution. Incubate solution 20–30 min in 35–37° water bath. Correct for additional dilution by filtering 1.2 mL of enzyme-treated sample.

(b) *Other liquid samples.*—Thoroughly mix contents of sample container. To prepare 1:10 dilution, aseptically transfer 10 mL sample into 90 mL PT diluent, **(g),** in sterile wide-mouth, screw-top bottle. Mix by shaking bottle 25 times through 30 cm arc in ≤ 7 s. If enzyme treatment is needed (see Table **46:B1**), combine 5 mL of 1:10 dilution with 1 mL of appropriate enzyme stock solution. Incubate solution 20–30 min in 35–37° water bath. Correct for additional dilution by filtering 1.2 mL of enzyme-treated sample.

(c) *Whole egg powder.*—Use sterile spoon or spatula to thoroughly mix sample. Prepare 1:10 dilution by aseptically weighing 11 g egg material into sterile wide-mouth, screw-top bottle; add 99 mL PT diluent, **(g),** and 1 tablespoonful of sterile glass shot. Thoroughly agitate 1:10 dilution to ensure complete solution or distribution of egg material in diluent by shaking each bottle rapidly 25 times, each shake an up-and-down movement of ca 30 cm, time interval ≤ 7 s. Let bubbles escape. Prepare 1:100 dilution by aseptically transferring 10 mL of 1:10 dilution into 90 mL PT diluent, **(g),** and combining 10 mL of 1:100 dilution with 1 mL trypsin stock solution,

Table 6b. Collaborative study results for total coliforms in cheese powder (MPN/g) by HGMF/MUG method

Coll.	Total coliforms, MPN/g							
	1	2	3	4	5	6	7	8
1	$<1.0 \times 10^1$	5.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	6.1×10^2	6.0×10^1
2	9.0×10^1	9.0×10^1	$<1.0 \times 10^1$	8.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.7×10^2	1.0×10^1
3	2.1×10^2	4.3×10^2	$<1.0 \times 10^1$	1.9×10^2	$<1.0 \times 10^1$	9.0×10^1	4.5×10^2	5.0×10^1
4	6.0×10^1	7.0×10^1	$<1.0 \times 10^1$	4.0×10^1	$<1.0 \times 10^1$	3.0×10^1	4.0×10^1	$<1.0 \times 10^1$
5	9.0×10^1	1.6×10^2	$<1.0 \times 10^1$	1.2×10^2	$<1.0 \times 10^1$	1.0×10^1	1.8×10^2	1.0×10^1
7	5.0×10^1	1.3×10^2	$<1.0 \times 10^1$	2.0×10^1	$<1.0 \times 10^1$	1.0×10^1	1.2×10^2	3.0×10^1
8	1.3×10^2	2.4×10^2	$<1.0 \times 10^1$	1.7×10^2	$<1.0 \times 10^1$	1.0×10^1	2.4×10^2	1.0×10^1
10	1.4×10^2	3.4×10^2	$<1.0 \times 10^1$	1.1×10^2	$<1.0 \times 10^1$	4.0×10^1	2.9×10^2	7.0×10^1
11	1.0×10^2	5.0×10^2	$<1.0 \times 10^1$	1.6×10^2	$<1.0 \times 10^1$	4.0×10^1	2.8×10^2	1.0×10^1
12	6.0×10^1	1.8×10^2	$<1.0 \times 10^1$	1.7×10^2	$<1.0 \times 10^1$	7.0×10^1	2.8×10^2	2.0×10^1
13[a]	$<1.0 \times 10^2$	2.0×10^2	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.0×10^2	$<1.0 \times 10^2$
14	1.0×10^2	3.0×10^2	$<1.0 \times 10^1$	1.0×10^2	$<1.0 \times 10^1$	5.0×10^1	4.4×10^2	8.0×10^1
15	1.9×10^2	3.9×10^2	$<1.0 \times 10^1$	9.0×10^1	$<1.0 \times 10^1$	4.0×10^1	2.1×10^2	3.0×10^1
16[b]	2.0×10^1	5.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^1	1.0×10^2	$<1.0 \times 10^1$
17	3.0×10^1	3.1×10^2	$<1.0 \times 10^1$	2.0×10^1	$<1.0 \times 10^1$	8.0×10^1	2.9×10^2	5.0×10^1
20	2.2×10^2	3.9×10^2	$<1.0 \times 10^1$	1.2×10^2	$<1.0 \times 10^1$	5.0×10^1	4.9×10^2	4.0×10^1
21	1.3×10^2	4.9×10^2	$<1.0 \times 10^1$	2.2×10^2	$<1.0 \times 10^1$	9.0×10^1	5.7×10^2	7.0×10^1
26	1.0×10^2	2.8×10^2	$<1.0 \times 10^1$	2.0×10^2	$<1.0 \times 10^1$	4.0×10^1	5.3×10^2	8.0×10^1

[a,b]See corresponding footnotes to Table 6a.

Table 7. Collaborative study results for E. coli in raw milk (MPN/mL) by HGMF/MUG and AOAC 3-tube methods

Coll.	3-Tube method						HGMF method					
	1	2	3	4	5	6	1	2	3	4	5	6
2[a]	9.1×10^0	9.1×10^0	4.3×10^1	1.5×10^2	$>1.1 \times 10^3$	1.5×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
3	4.6×10^2	4.6×10^2	4.6×10^2	2.9×10^2	$>1.1 \times 10^3$	2.8×10^1	4.4×10^2	3.8×10^2	5.5×10^2	4.4×10^2	6.3×10^2	6.0×10^2
6[b]	3.0×10^0	3.0×10^0	3.0×10^0	3.0×10^0	1.5×10^1	7.2×10^0	5.2×10^2	3.2×10^2	6.0×10^2	2.7×10^2	6.0×10^2	5.0×10^2
20	3.6×10^1	1.1×10^3	$>1.1 \times 10^3$	4.6×10^2	3.6×10^1	1.1×10^3	8.4×10^2	3.3×10^2	1.0×10^3	2.9×10^2	1.3×10^3	4.4×10^2
22	$>1.1 \times 10^3$	4.6×10^2	$>1.1 \times 10^3$	4.4×10^1	$>1.1 \times 10^3$	4.6×10^2	9.3×10^2	2.7×10^2	1.0×10^3	2.5×10^2	6.3×10^2	7.4×10^2
23	2.7×10^1	2.8×10^1	1.1×10^1	1.1×10^1	7.3×10^0	4.6×10^2	4.7×10^2	4.3×10^2	1.4×10^3	4.4×10^2	1.3×10^3	7.5×10^2
26	2.4×10^2	1.1×10^3	$>1.1 \times 10^3$	1.1×10^3	$>1.1 \times 10^3$	$>1.1 \times 10^3$	5.3×10^2	2.7×10^2	6.8×10^2	3.4×10^2	8.7×10^2	5.2×10^2

[a]Method deviation due to faulty ultraviolet lamp. HGMF method data excluded from statistical evaluation.

[b]E. coli confirmation tests incomplete. 3-Tube method data excluded from statistical evaluation.

(p). Incubate solution 20–30 min in 35–37° water bath. Filter entire 11 mL volume to test 1:10 dilution.

(d) *Other foods.*—To prepare 1:10 dilution aseptically weigh 10 g sample into sterile blender jar. Add 90 mL PT diluent, **(g),** and blend 2 min at low speed (10 000–12 000 rpm). If enzyme treatment is needed (see Table **46:B1**), combine 5 mL of 1:10 dilution with 1 mL of appropriate enzyme stock solution. Incubate solution 20–30 min in 35–37° water bath. Correct for additional dilution by filtering 1.2 mL of enzyme-treated sample.

D. Analysis

(See Figs. **46:B1** and **46:B2.**) Turn on vacuum source. Place sterile filtration unit on manifold or vacuum flask. Open clamp A. Rotate back funnel portion C. Aseptically place sterile HGMF on surface of base D. Rotate funnel

Table 8. Collaborative study results for E. coli in raw ground poultry (MPN/g) by HGMF/MUG and AOAC 3-tube methods

Coll.	3-Tube method						HGMF method					
	1	2	3	4	5	6	1	2	3	4	5	6
2[a]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
3	3.6×10^0	9.1×10^0	4.3×10^1	9.1×10^0	9.1×10^0	4.3×10^1	1.0×10^1	7.0×10^1	5.0×10^1	2.0×10^1	1.0×10^1	2.0×10^1
6[b]	$<3.0 \times 10^0$	1.4×10^1	4.3×10^1	$<3.0 \times 10^0$	2.3×10^1	2.3×10^1	1.0×10^1	3.0×10^1	4.0×10^1	$<1.0 \times 10^1$	2.0×10^1	4.0×10^1
20	2.3×10^1	4.3×10^1	4.3×10^1	3.6×10^0	4.3×10^1	4.6×10^2	4.0×10^1	9.0×10^1	1.1×10^2	$<1.0 \times 10^1$	5.0×10^1	2.3×10^2
22	9.1×10^0	9.3×10^1	2.3×10^1	2.3×10^1	2.3×10^1	2.4×10^2	3.0×10^1	1.2×10^2	1.0×10^1	6.0×10^1	1.2×10^2	2.0×10^2
23	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<1.0 \times 10^1$	6.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	5.0×10^1
26	1.5×10^1	4.3×10^1	4.3×10^1	2.3×10^1	7.3×10^0	2.3×10^1	1.0×10^1	5.0×10^1	1.0×10^1	1.0×10^1	1.0×10^1	6.0×10^1

[a]Samples improperly stored due to freezer failure. All data excluded from statistical evaluation.

[b]E. coli confirmation tests incomplete. 3-Tube method data excluded from statistical evaluation.

Table 9a. Collaborative study results for E. coli in ground black pepper (MPN/g) by AOAC 3-tube method

Coll.	E. coli, MPN/g							
	1	2	3	4	5	6	7	8
1	$<3.0 \times 10^0$	4.3×10^1	9.1×10^1	9.1×10^1	3.0×10^0	3.6×10^0	$<3.0 \times 10^0$	3.6×10^0
2[a]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^1$	$<3.0 \times 10^1$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
3	$<3.0 \times 10^0$	9.1×10^0	9.1×10^1	3.6×10^1	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
5	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^1$	$<3.0 \times 10^1$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
7	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.1×10^1	$<3.0 \times 10^1$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
8	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^1$	$<3.0 \times 10^1$	2.3×10^1	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
10	$<3.0 \times 10^0$	3.6×10^0	$<3.0 \times 10^1$	$<3.0 \times 10^1$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
14[b]	$<3.0 \times 10^0$	3.6×10^0	N/A	N/A	N/A	$<3.0 \times 10^0$	$<3.0 \times 10^0$	N/A
15	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^1$	7.3×10^1	3.6×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
16	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^1$	$<3.0 \times 10^1$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
17	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^1$	$<3.0 \times 10^1$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
19	$<3.0 \times 10^0$	3.6×10^0	$<3.0 \times 10^0$	3.0×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
21	$<3.0 \times 10^0$	3.9×10^1	9.1×10^1	3.6×10^1	9.1×10^0	3.6×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$
24[c]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
26	$<3.0 \times 10^0$	3.6×10^1	3.6×10^1	3.6×10^1	3.6×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$

[a]Method deviation due to failure of 45.5°C water bath. 3-Tube method data excluded from statistical evaluation.

[b]Method deviation in samples 3, 4, 5, and 8; E. coli confirmations incomplete. 3-Tube method data for these samples excluded from statistical evaluation.

[c]Method deviation due late initiation of analyses. Data for HGMF and 3-tube MPN methods excluded from statistical evaluation.

forward. Clamp shut by sliding jaws L of stainless steel clamp over entire length of flanges B that extend from both sides of funnel C and base D, and rotating moving arm K into horizontal (locked) position.

Aseptically add ca 15–20 mL sterile H_2O to funnel. Pipet 1.0 mL of 1:10 dilution (or appropriate volume of enzyme-treated sample) into funnel. Apply free end of vacuum tubing E to suction hole F to draw liquid through prefilter mesh G. Aseptically add additional 10–15 mL sterile H_2O to funnel and draw through mesh as before. Close clamp A to direct vacuum to base of filtration unit and draw liquid through HGMF.

Open clamp A. Rotate moving arm K of stainless steel clamp into unlocked (ca 45° angle) position and slide jaws L off flanges B. Rotate back funnel C.

(a) *Total coliform count.*—Place HGMF on surface of

Table 9b. Collaborative study results for E. coli in ground black pepper (MPN/g) by HGMF/MUG method

Coll.	E. coli, MPN/g							
	1	2	3	4	5	6	7	8
1	$<1.0 \times 10^1$	1.4×10^2	5.0×10^2	5.0×10^2	4.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1
2[a]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
3	$<1.0 \times 10^1$	1.0×10^1	2.0×10^2	$<1.0 \times 10^2$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
5	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	2.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
7	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^2	$<1.0 \times 10^2$	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$
8	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^2	$<1.0 \times 10^2$	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1
10	$<1.0 \times 10^1$	2.0×10^1	$<1.0 \times 10^2$	$<1.0 \times 10^2$	2.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1
14[b]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^2	$<1.0 \times 10^2$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
15	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
16	$<1.0 \times 10^1$	2.0×10^1	6.0×10^2	1.9×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
17	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
19	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^2$	1.0×10^2	3.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
21	$<1.0 \times 10^1$	2.0×10^1	2.0×10^2	5.0×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
24[c]	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
26	$<1.0 \times 10^1$	2.0×10^1	1.0×10^2	1.2×10^3	4.0×10^1	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$

[a,b,c]See corresponding footnotes to Table 9a.

Table 10a. Collaborative study results for E. coli in whole egg powder (MPN/g) by AOAC 3-tube method

Coll.	E. coli, MPN/g							
	1	2	3	4	5	6	7	8
1	1.5×10^1	$<3.0 \times 10^0$	1.5×10^1	3.6×10^0	2.3×10^1	1.1×10^3	7.5×10^1	$<3.0 \times 10^0$
2[a]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
3	2.4×10^2	$<3.0 \times 10^0$	3.6×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.3×10^1	$<3.0 \times 10^0$	$<3.0 \times 10^0$
5	4.3×10^1	$<3.0 \times 10^0$	4.3×10^1	3.6×10^0	$<3.0 \times 10^0$	2.3×10^1	1.5×10^1	$<3.0 \times 10^0$
7	9.3×10^1	$<3.0 \times 10^0$	4.3×10^1	$<3.0 \times 10^0$	3.6×10^0	2.3×10^1	9.3×10^1	$<3.0 \times 10^0$
8	4.3×10^1	$<3.0 \times 10^0$	9.3×10^1	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.4×10^2	9.1×10^0	$<3.0 \times 10^0$
9	2.3×10^1	$<3.0 \times 10^0$	7.2×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.3×10^1	2.4×10^2	$<3.0 \times 10^0$
10	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$
11	4.3×10^1	$<3.0 \times 10^0$	3.6×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.3×10^1	3.6×10^0	$<3.0 \times 10^0$
16	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
19	4.3×10^1	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.3×10^1	$<3.0 \times 10^0$	$<3.0 \times 10^0$
21	4.3×10^1	$<3.0 \times 10^0$	9.3×10^1	$<3.0 \times 10^0$	$<3.0 \times 10^0$	9.3×10^1	2.3×10^1	$<3.0 \times 10^0$
26	4.3×10^1	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.4×10^2	3.6×10^0	$<3.0 \times 10^0$

[a]Method deviation due to failure of 45.5°C water bath. 3-Tube method data excluded from statistical evaluation.

predried LMG **(B)(b)** plate. Avoid trapping air bubbles between filter and agar. Incubate 24 ± 2 h at 35°. Count all squares containing one or more blue colonies. Include any shade of blue. Score each square as either positive (blue) or negative. Convert positive square count to MPN with the formula

$$\text{MPN} = [N \log_e(N/(N - x))]$$

Table 10b. Collaborative study results for E. coli in whole egg powder (MPN/g) by HGMF/MUG method

Coll.	E. coli, MPN/g							
	1	2	3	4	5	6	7	8
1	4.9×10^2	$<1.0 \times 10^1$	3.0×10^1	1.0×10^1	$<1.0 \times 10^1$	3.0×10^2	2.0×10^1	$<1.0 \times 10^1$
2[a]	4.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1	8.0×10^1	$<1.0 \times 10^1$
3	2.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	2.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$
5	1.1×10^2	$<1.0 \times 10^1$	4.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	$<1.0 \times 10^1$
7	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	6.0×10^1	6.0×10^3	$<1.0 \times 10^1$
8	9.0×10^1	$<1.0 \times 10^1$	7.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1	6.0×10^1	$<1.0 \times 10^1$
9	3.0×10^1	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	7.0×10^1	2.2×10^2	$<1.0 \times 10^1$
10	3.0×10^1	$<1.0 \times 10^1$	1.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	4.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$
11	4.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.3×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$
16	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.1×10^2	$<1.0 \times 10^1$
19	1.0×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	7.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$
21	5.0×10^1	$<1.0 \times 10^1$	1.7×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.2×10^2	4.0×10^1	$<1.0 \times 10^1$
26	1.5×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.2×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$

[a]See corresponding footnote to Table 10a.

where N = total number of squares and x = number of positive squares. Multiply by reciprocal of dilution factor and report as MPN of total coliform bacteria/g.

(b) *E. coli count.*—If coliforms were present in **(a),** transfer HGMF to surface of predried BMA, **(B)(c),** and incubate 2 h at 35°. Examine HGMF under longwave (366 nm) UV light in darkened room. Count all squares containing one or more large (filling ≥ 25% of square) blue-white fluorescent colonies. Do not count squares containing only pinpoint colonies or colonies fluorescing some other color. Score each square as positive (large, blue-white fluorescent colony) or negative. Convert positive square count to MPN with formula in **(a).** Multiply by reciprocal of dilution factor and report as MPN of *E. coli*/g.

Ref: JAOAC **72,** November/December issue (1989).

Results and Discussion

All reported total coliform and *E. coli* recovery data were checked for correct determination of conventional and HGMF MPN indices and for correct calculation of dilution factors, and were rounded to 2 significant figures. These data are reported in Tables 1–11. All data were converted to $\log_{10}$ for statistical analysis.

Data omitted because of significant method deviation, as well as statistical outliers, are listed below. Where these occurred, data from both the 3-tube method and HGMF method were omitted from calculation of sample means; however, only the data from the affected method were omitted from calculation of repeatability and reproducibility precision estimates. Collaborator 2 used a faulty ultraviolet lamp for the HGMF *E. coli* enumeration in raw milk. Collaborator 2 also experienced freezer failure, which resulted in incorrect storage of their raw ground poultry samples, and experienced a temperature control problem with their 45.5°C water bath. All affected data were omitted from statistical analysis as specified in footnotes to the relevant tables.

Collaborator 6 carried out the 3-tube method *E. coli* confirmations incorrectly in both the raw milk and raw ground poultry samples. Their *E. coli* data by the 3-tube method for both of these foods were excluded from statistical analysis. Collaborator 13 filtered the wrong dilution of cheese powder; their total coliform and *E. coli* data for the HGMF method were excluded from statistical analysis. Collaborator 14 did not complete the *E. coli* 3-tube analysis on samples 3, 4, 5, and 8 of the ground black pepper series. Collaborator 24 set up the black pepper samples several weeks late.

In addition to the above, the total coliform data reported by Collaborator 16 for the 3-tube method and the HGMF method in cheese powder, and for the 3-tube method in whole egg powder, as well as the *E. coli* data reported by the same collaborator for the 3-tube method in cheese powder were determined to be statistical outliers by the rank score test (3) and were excluded from statistical analysis.

Notwithstanding efforts to minimize the time interval between screening and collaborative analysis of raw milk samples, total coliform counts in all raw milks were far higher than anticipated. To recover data on this sample series, collaborators who had retained the residual samples were requested to repeat the HGMF and 3-tube method total coliform analyses at a higher dilution. Four collaborators were able to do this. These data are reported separately as "total coliform—reset" in the appropriate tables.

The data from each food product category and analysis were subjected to 3-way analysis of variance (ANOVA), and precision estimates were determined for each sample pair, as well as overall precision estimates for each food and analysis. The results of the comparison of method means, together with repeatability and reproducibility statistics, are reported in Table 13 for total coliforms and in Table 14 for *E. coli.*

Table 11a. Collaborative study results for E. coli in cheese powder (MPN/g) by AOAC 3-tube method.

Coll.	E. coli, MPN/g							
	1	2	3	4	5	6	7	8
1	1.5×10^2	4.6×10^2	$<3.0 \times 10^0$	2.4×10^2	$<3.0 \times 10^0$	1.5×10^1	9.3×10^1	3.6×10^0
2[a]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
3	1.5×10^1	2.3×10^1	$<3.0 \times 10^0$	2.3×10^1	$<3.0 \times 10^0$	9.1×10^0	7.5×10^1	3.6×10^0
4	$<3.0 \times 10^0$	7.3×10^0	$<3.0 \times 10^0$	3.6×10^0	$<3.0 \times 10^0$	9.1×10^0	3.6×10^0	$<3.0 \times 10^0$
5	9.1×10^0	2.3×10^1	$<3.0 \times 10^0$	7.3×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	4.3×10^1	3.6×10^0
7	2.3×10^1	2.3×10^1	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	4.3×10^1	2.3×10^1
8	1.5×10^1	9.3×10^1	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.5×10^1	3.6×10^0
10	9.1×10^0	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	3.6×10^0	4.3×10^1	3.6×10^0
11	9.1×10^0	9.3×10^1	$<3.0 \times 10^0$	2.3×10^1	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.5×10^1	3.6×10^0
12	2.3×10^1	9.3×10^1	$<3.0 \times 10^0$	7.5×10^1	$<3.0 \times 10^0$	4.3×10^1	2.4×10^2	2.3×10^1
13[b]	1.5×10^1	2.3×10^1	$<3.0 \times 10^0$	3.6×10^0	$<3.0 \times 10^0$	3.6×10^0	4.3×10^1	$<3.0 \times 10^0$
14	3.6×10^0	7.2×10^0	$<3.0 \times 10^0$	1.5×10^1	$<3.0 \times 10^0$	9.1×10^0	4.3×10^1	9.1×10^0
15	2.3×10^1	2.3×10^1	$<3.0 \times 10^0$	1.5×10^1	$<3.0 \times 10^0$	7.3×10^0	2.3×10^1	3.6×10^0
16[c]	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$	$<3.0 \times 10^0$
17	2.1×10^1	9.1×10^0	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	2.3×10^1	$<3.0 \times 10^0$
20	$<3.0 \times 10^0$	2.3×10^1	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^0$	$<3.0 \times 10^0$	1.5×10^1	3.6×10^0
21	9.1×10^0	2.1×10^1	$<3.0 \times 10^0$	9.1×10^0	$<3.0 \times 10^0$	1.5×10^1	2.3×10^1	9.1×10^0
26	2.3×10^1	4.6×10^2	$<3.0 \times 10^0$	2.4×10^2	$<3.0 \times 10^0$	4.3×10^1	2.4×10^2	9.1×10^0

[a]Method deviation due to failure of 45.5°C water bath. 3-Tube method data excluded from statistical evaluation.

[b]Method deviation due to incorrect dilution filtered. HGMF method data excluded from statistical evaluation.

[c]Statistical outlier for 3-tube MPN method. 3-tube MPN method data excluded from statistical evaluation.

Total coliform counts obtained by the HGMF and 3-tube methods were statistically equivalent for raw milk, raw ground poultry, whole egg powder, and cheese powder. The 3-tube method recovered significantly more total coliforms from ground black pepper than did the HGMF method. Conversely, HGMF *E. coli* recoveries from ground black pepper, whole egg powder, and cheese powder were significantly higher than 3-tube method results, and HGMF *E. coli* recoveries from raw milk and raw ground poultry were statistically equivalent to the 3-tube method.

For total coliform analysis, the HGMF repeatability variance (s_r^2) was significantly less than the corresponding 3-tube MPN s_r^2 in 4 sample pairs, significantly greater in 2, and equivalent in 8. For *E. coli* analysis, the HGMF s_r^2 was significantly less than the 3-tube method s_r^2 in 5 sample pairs, significantly greater in 2, and equivalent in 7 (*F*-test; $P < 0.05$). The repeatability results are reasonably consistent with those for a previous collaborative study (4) to compare the HGMF method to a 3-tube MPN method **(46.013–46.016)**, as well as with the in-house validation studies carried out preparatory to this collaborative study (manuscript in preparation).

Several collaborators commented that the lactose-positive (blue) color reaction on LMG agar was strong on the underside of the HGMF, but weak in the colonies. The intensity of color reaction on LMG agar is dependent on the source of aniline blue dye used in the medium. Since the completion of the collaborative study, we have surveyed a number of sources for aniline blue and have found the one specified in the HGMF method in the present report to be the most effective. Some of the black pepper samples used in the collaborative study were naturally contaminated with *Klebsiella* sp. This organism did not produce a clear-cut lactose-positive colony reaction with the dye source used in the study, which resulted in underestimation of the true total coliform population by the HGMF method. These same samples produce an easily readable lactose-positive reaction with the source of aniline blue recommended. The performance of the aniline blue dye used in the collaborative study was almost certainly the reason for the lower total coliform counts produced by the HGMF method in the black pepper.

To validate the specificity of the MUG reaction on BMA, collaborators subcultured a total of 396 MUG-positive colonies from HGMFs and subjected them to the complete confirmation protocol described. Of those colonies, 394 (99.5%) were confirmed to be *E. coli*. An additional 31 MUG-positive colonies were isolated, but the confirmation procedure was incompletely carried out. The confirmation rates of MUG-

Table 11b. Collaborative study results for E. coli in cheese powder (MPN/g) by HGMF/MUG method.

Coll.	E. coli, MPN/g							
	1	2	3	4	5	6	7	8
1	$<1.0 \times 10^1$	5.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	6.1×10^2	6.0×10^1
2[a]	5.0×10^1	6.0×10^1	$<1.0 \times 10^1$	8.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	9.0×10^1	1.0×10^1
3	1.9×10^2	3.3×10^2	$<1.0 \times 10^1$	1.4×10^2	$<1.0 \times 10^1$	4.0×10^1	2.4×10^2	3.0×10^1
4	6.0×10^1	5.0×10^1	$<1.0 \times 10^1$	3.0×10^1	$<1.0 \times 10^1$	3.0×10^1	2.0×10^1	$<1.0 \times 10^1$
5	4.0×10^1	8.0×10^1	$<1.0 \times 10^1$	3.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	9.0×10^1	1.0×10^1
7	7.0×10^1	2.0×10^2	$<1.0 \times 10^1$	4.0×10^1	$<1.0 \times 10^1$	3.0×10^1	1.2×10^2	2.0×10^1
8	1.0×10^2	1.7×10^2	$<1.0 \times 10^1$	1.3×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^2	1.0×10^1
10	1.3×10^2	2.3×10^2	$<1.0 \times 10^1$	8.0×10^1	$<1.0 \times 10^1$	2.0×10^1	2.2×10^2	6.0×10^1
11	8.0×10^1	2.7×10^2	$<1.0 \times 10^1$	7.0×10^1	$<1.0 \times 10^1$	2.0×10^1	1.6×10^2	3.0×10^1
12	3.0×10^1	1.8×10^2	$<1.0 \times 10^1$	1.7×10^2	$<1.0 \times 10^1$	6.0×10^1	2.6×10^2	2.0×10^1
13[b]	$<1.0 \times 10^2$	1.0×10^2	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.0×10^2	$<1.0 \times 10^2$
14	8.0×10^1	1.9×10^2	$<1.0 \times 10^1$	7.0×10^1	$<1.0 \times 10^1$	2.0×10^1	2.6×10^2	5.0×10^1
15	1.5×10^2	2.4×10^2	$<1.0 \times 10^1$	5.0×10^1	$<1.0 \times 10^1$	1.0×10^1	8.0×10^1	2.0×10^1
16[c]	1.0×10^1	1.2×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	3.0×10^1	1.5×10^2	$<1.0 \times 10^1$
17	3.0×10^1	2.2×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	6.0×10^1	1.8×10^2	2.0×10^1
20	1.2×10^2	4.2×10^2	$<1.0 \times 10^1$	9.0×10^1	$<1.0 \times 10^1$	3.0×10^1	2.7×10^2	3.0×10^1
21	1.1×10^2	2.7×10^2	$<1.0 \times 10^1$	1.3×10^2	$<1.0 \times 10^1$	7.0×10^1	3.2×10^2	5.0×10^1
26	7.0×10^1	2.0×10^2	$<1.0 \times 10^1$	1.3×10^2	$<1.0 \times 10^1$	3.0×10^1	3.1×10^2	4.0×10^1

[a,b,c]See corresponding footnotes to Table 11a.

positive colonies isolated from each food product are summarized in Table 15.

Numerous researchers have documented the specificity of β-D-glucuronidase activity. In 1976, Kilian and Bulow (5) surveyed a total of 615 strains of *Enterobacteriaceae* and *Vibrionaceae*. Of the 113 strains of *Escherichia coli* included in that study, 109 (96.5%) were glucuronidase positive. Only 25 (5%) of the 502 non-*E. coli* were glucuronidase positive. All 25 were *Shigella*. None of the other species of *Enterobacteriaceae* and none of the *Vibrionaceae* exhibited any glucuronidase activity. Another more recent survey by Trepeta and Edberg (6) of glucuronidase activity among 258 strains of *Enterobacteriaceae* produced similar results. Seventy-two of 75 strains (96%) of *E. coli* were positive for glucuronidase activity, whereas only 9 of 183 strains (4.9%) representing other species of *Enterobacteriaceae* were positive. The 9 positive strains consisted of 6 *Shigella* and 3 *Salmonella*.

Table 12. Replicate sample pairs prepared for collaborative study

Food	Sample[a] 1	2	3	4	5	6	7	8
Raw milk[b]	A	C	B	C	B	A	—	—
Raw ground poultry[b]	A	C	B	A	B	C	—	—
Ground black pepper	D	B	A	A	B	C	D	C
Whole egg powder	A	D	B	C	C	A	B	D
Cheese powder	B	A	D	B	D	C	A	C

[a] Pair A: high count, or naturally-contaminated lot A
Pair B: moderate count, or naturally-contaminated lot B
Pair C: low count, or naturally-contaminated lot C
Pair D: uninoculated control

[b] Naturally-contaminated product. No uninoculated control samples.

A number of researchers have also documented the specificity of β-D-glucuronidase for *E. coli* detection in naturally contaminated samples. In 1982, Feng and Hartman (7) described a multiple tube-MPN method for *E. coli* enumeration in which 4-methylumbelliferyl-β-D-glucuronide (MUG) was incorporated in LST broth. As part of their study, they determined the glucuronidase activity of 198 strains of gram-negative bacteria, including 120 *E. coli*. Of the *E. coli*, 116 strains (96.7%) were glucuronidase positive. Eight of the other gram-negative bacteria were also positive, including 6 strains of *Salmonella* and 2 *Shigella*. Their *E. coli* confirmation rate of MUG-positive tubes from naturally contaminated food and milk samples was 91%.

In 1984, Robison (8) described a presence/absence test for *E. coli* which made use of MUG in a nonselective enrichment broth. That method produced a false-positive rate of 4.8%. The author indicated that all of the false-positive results could be traced to streptococci. In that same year, Alvarez (9) reported using MUG in 3 different media for *E. coli* enumeration, namely, LST broth, VRB agar, and m-Endo broth. His reported confirmation rates ranged from 90 to 96%, depending on the test format.

Three studies were reported in 1985. Moberg (10) surveyed 1297 naturally contaminated samples and found only 18 (1.4%) false positives. These were all due to *Staphylococcus* ssp. Koburger and Miller (11) incorporated MUG into EC broth for *E. coli* enumeration in oysters. They analyzed

Table 13. Means and precision estimates by sample pair for total coliform enumeration by HGMF/MUG and AOAC 3-tube methods

Sample pair	Method	Geometric mean, count/g or mL	Precision estimates[a]			
			s_r	s_R	RSD_r, %	RSD_R, %
Raw milk						
A	HGMF	5.6×10^4	0.143	0.419	3.0	8.8
	3-Tube	1.1×10^3	0.000	0.000	0.0	0.0
B	HGMF	5.1×10^4	0.126	0.666	2.7	14.1
	3-Tube	1.1×10^3	0.000	0.000	0.0	0.0
C	HGMF	9.1×10^4	0.000	0.000	0.0	0.0
	3-Tube	1.1×10^3	0.000	0.000	0.0	0.0
Overall	HGMF	6.4×10^4	0.110	0.454	2.3	9.5
	3-Tube	1.1×10^3	0.000	0.000	0.0	0.0
Raw milk—reset						
A	HGMF	8.2×10^5	0.317	0.295	5.4	5.0
	3-Tube	5.2×10^5	0.122	0.392	2.1	6.9
B	HGMF	2.2×10^6	0.247	0.591	3.9	9.3
	3-Tube	7.5×10^5	0.365	0.344	6.2	5.9
C	HGMF	3.9×10^6	0.079	0.394	1.2	6.0
	3-Tube	1.1×10^6	0.000	0.000	0.0	0.0
Overall	HGMF	1.9×10^6	0.236	0.444	3.8	7.1
	3-Tube	7.5×10^5	0.222	0.301	3.8	5.1
Raw ground poultry						
A	HGMF	1.9×10^1	0.246	0.324	19.2	25.3
	3-Tube	2.1×10^1	0.167	0.301	12.6	22.8
B	HGMF	4.2×10^1	0.294	0.307	18.1	18.9
	3-Tube	5.1×10^1	0.393	0.397	23.0	23.2
C	HGMF	1.3×10^2	0.307	0.327	14.5	15.5
	3-Tube	1.3×10^2	0.549	0.562	26.0	26.6
Overall	HGMF	4.7×10^1	0.283	0.320	16.9	19.1
	3-Tube	5.2×10^1	0.402	0.433	23.4	25.2
Ground black pepper						
A	HGMF	2.9×10^3	0.439	0.439	12.7	12.7
	3-Tube	8.3×10^3	0.120	0.206	3.1	5.3
B	HGMF	8.3×10^1	0.630	0.734	32.8	38.2
	3-Tube	2.1×10^2	0.778	0.756	33.5	32.6
C	HGMF	1.3×10^1	0.321	0.298	28.8	26.8
	3-Tube	2.4×10^1	0.534	0.814	38.7	59.0
Overall[b]	HGMF	1.5×10^2	0.480	0.523	22.1	24.0
	3-Tube	3.5×10^2	0.549	0.652	21.6	25.6
Whole egg powder						
A	HGMF	5.9×10^1	0.269	0.440	15.2	24.8
	3-Tube	1.2×10^2	0.401	0.410	19.3	19.7
B	HGMF	4.9×10^1	0.678	0.609	40.1	36.0
	3-Tube	6.6×10^1	0.442	0.397	24.3	21.8
C	HGMF	1.1×10^1	0.126	0.121	12.1	11.6
	3-Tube	1.5×10^1	0.364	0.396	30.9	33.7
Overall	HGMF	3.4×10^1	0.427	0.439	27.9	28.7
	3-Tube	4.9×10^1	0.404	0.401	23.9	23.7
Cheese Powder						
A	HGMF	2.0×10^2	0.378	0.426	16.4	18.5
	3-Tube	1.7×10^2	0.282	0.363	12.6	16.3
B	HGMF	7.8×10^1	0.160	0.400	8.5	21.1
	3-Tube	7.8×10^1	0.406	0.370	21.4	19.5
C	HGMF	3.2×10^1	0.212	0.366	14.1	24.3
	3-Tube	2.2×10^1	0.360	0.388	26.8	28.9
Overall	HGMF	8.3×10^1	0.267	0.398	13.9	20.7
	3-Tube	6.9×10^1	0.353	0.374	19.2	20.3

[a] s_r = repeatability standard deviation; s_R = reproducibility standard deviation; RSD_r = repeatability re ative standard deviation; RSD_R = reproducibility relative standard deviation.

[b] Difference between HGMF and 3-tube method overall mean counts is statistically significant.

Table 14. Means and precision estimates by sample pair for *E. coli* enumeration by HGMF/MUG and AOAC 3-tube methods

Sample pair	Method	Geometric mean, count/g or mL	Precision estimates[a]			
			s_r	s_R	RSD_r, %	RSD_R, %
		Raw milk				
A	HGMF	3.3×10^2	0.220	0.704	8.7	28.0
	3-Tube	9.1×10^1	0.651	0.932	33.2	47.6
B	HGMF	4.4×10^2	0.070	0.739	2.6	28.0
	3-Tube	1.6×10^2	0.588	1.028	26.7	46.6
C	HGMF	2.0×10^2	0.045	0.579	2.0	25.2
	3-Tube	9.7×10^1	0.454	0.985	22.9	49.6
Overall	HGMF	5.6×10^2	0.077	0.668	2.8	24.3
	3-Tube	2.5×10^2	0.602	0.834	25.1	34.8
		Raw ground poultry				
A	HGMF	1.5×10^1	0.246	0.324	20.9	27.5
	3-Tube	8.4×10^0	0.167	0.299	18.1	32.3
B	HGMF	2.3×10^1	0.294	0.307	21.6	22.5
	3-Tube	1.6×10^1	0.393	0.397	32.6	33.0
C	HGMF	6.6×10^1	0.307	0.327	16.9	18.0
	3-Tube	3.3×10^1	0.549	0.562	36.2	37.0
Overall	HGMF	3.0×10^1	0.286	0.355	19.3	24.0
	3-Tube	1.6×10^1	0.355	0.566	29.5	47.0
		Ground black pepper				
A	HGMF	2.1×10^2	0.552	0.584	23.8	25.1
	3-Tube	3.4×10^1	0.170	0.384	11.1	25.1
B	HGMF	1.5×10^1	0.179	0.278	16.2	23.6
	3-Tube	5.0×10^0	0.308	0.359	44.1	51.4
C	HGMF	1.0×10^1	0.091	0.091	9.1	9.1
	3-Tube	3.1×10^0	0.016	0.027	3.3	5.5
Overall[b]	HGMF	3.4×10^1	0.339	0.377	22.1	24.6
	3-Tube	8.1×10^0	0.204	0.307	22.4	33.8
		Whole egg powder				
A	HGMF	5.3×10^1	0.341	0.474	19.8	27.5
	3-Tube	3.6×10^1	0.514	0.595	33.0	38.2
B	HGMF	2.8×10^1	0.707	0.670	48.9	46.3
	3-Tube	1.2×10^1	0.452	0.623	41.9	57.7
C	HGMF	1.0×10^1	0.000	0.000	0.0	0.0
	3-Tube	3.4×10^0	0.165	0.180	31.0	33.9
Overall[b]	HGMF	2.5×10^1	0.453	0.474	32.4	33.9
	3-Tube	1.1×10^1	0.406	0.508	39.0	48.8
		Cheese powder				
A	HGMF	1.6×10^2	0.230	0.301	10.4	13.7
	3-Tube	3.5×10^1	0.343	0.516	22.2	33.4
B	HGMF	5.8×10^1	0.197	0.384	11.2	21.8
	3-Tube	1.2×10^1	0.310	0.468	28.7	43.4
C	HGMF	2.6×10^1	0.211	0.301	14.9	21.3
	3-Tube	6.0×10^0	0.265	0.359	34.1	46.1
Overall[b]	HGMF	6.6×10^1	0.213	0.334	11.7	18.4
	3-Tube	1.4×10^1	0.308	0.452	26.8	39.5

[a,b] See corresponding footnotes to Table 13.

25 naturally contaminated samples. Only 1 (1%) of 103 fluorescent tubes did not confirm as *E. coli*. Petzel and Hartman (12) described a total gram-negative bacteria enumeration medium to which they added MUG for direct *E. coli* enumeration. While they did not specify a false positive rate, they observed that it was necessary to distinguish the natural dull-green fluorescence of some oxidase-positive bacteria, such as some species of *Pseudomonas*, and the bright blue-white fluorescence produced by the enzymatic breakdown of MUG. They also indicated that almost all of the false positive results due to oxidase-positive bacteria arose from colonies that developed between 24 and 48 h of incubation.

In all of the MUG procedures described to date, 1 of 3 basic approaches has been followed. Most of the procedures incorporate MUG into LST broth or EC broth. Some methods add MUG to a pour-plate medium, such as VRB agar, and some make use of MUG in a membrane filter medium. Virtually all of the methods are designed in such a way that glucuronidase activity is carried on simultaneously with the initial growth of the isolate (as opposed to a post-incubation confirmation).

Incorporation of MUG into LST has some drawbacks with respect to the interpretation of glucuronidase activity. It is noteworthy that, despite the problems outlined below, the lowest confirmation rate reported by any researchers working with naturally contaminated samples was 90%. As re-

Table 15. *E. coli* confirmation rates of MUG-positive colonies isolated from buffered MUG agar

Product	No. tested[a]	No. confirmed *E. coli*	No. incomplete tests[b]
Raw milk	66	64 (97.0%)	4
Raw ground poultry	53	53 (100%)	0
Ground black pepper	38	38 (100%)	15
Whole egg powder	71	71 (100%)	2
Cheese powder	168	168 (100%)	10
Overall	396	394 (99.5%)	31

[a] Colonies isolated and confirmation tests carried out as specified.

[b] Colonies isolated and confirmation tests incomplete or not carried out as specified.

ported by Andrews et al. (13), glass test tubes must be screened for autofluorescence before being used for this test. Poelma et al. (14) also observed that interpretation of MUG-positive tubes can be somewhat subjective, requiring use of positive and negative control tubes. Our experience indicates that this difficulty is due both to autofluorescence of the glass test tubes and to autofluorescence of the uninoculated culture medium (LST broth, for example, fluoresces quite strongly, even without MUG added to the medium). Also, Koburger and Miller (11) reported that glucuronidase activity is endogenous to oysters. This last problem can be overcome by incorporating MUG in EC broth as a confirming test rather than in LST broth.

When used in conjunction with VRB agar, MUG results in the production of a zone of fluorescence around the glucuronidase positive colony (9). On a crowded plate, interpretation of the reaction would become almost impossible. On a membrane filter, as described in the study by Alvarez (9), the fluorescence is restricted to the colony. However, the number of colonies that can be enumerated on a conventional membrane filter is very limited, and the presence of other flora on the filter could have made it difficult to isolate *E. coli* in pure culture to confirm the specificity of the MUG reaction in that study.

A further problem with MUG methods described in the scientific literature to date relates to the characteristics of the glucuronidase enzyme. The β-D-glucuronidase of *E. coli* has a pH optimum of 6.8 (Sigma Chemical Co., St. Louis, MO, 1989 Catalog, p. 648). Furthermore, 4-methylumbelliferone, the product of glucuronidase digestion of MUG, does not fluoresce significantly below pH 7.0, and fluoresces most brightly under alkaline pH conditions. During *E. coli* growth in LST, VRB, or any other lactose-containing medium, acid is produced by fermentation of lactose. This can be expected to decrease the pH of the medium below 7.0. Before *E. coli* can begin to degrade MUG, the supply of lactose in the medium must be exhausted, allowing the pH to revert to alkaline. The rate at which this alkaline reversion takes place depends, of course, on the initial numbers of lactose fermenters in the medium and the rate at which the strains present ferment lactose. This would likely explain the variation in incubation times (24–72 h) required to achieve a clear-cut, positive MUG reaction.

The HGMF/MUG method described in the present report was designed to overcome the drawbacks described above, and to optimize the detection of glucuronidase activity in the colonies. To overcome this latter problem, we separated the lactose reaction from the MUG reaction. By using MUG in a separate phosphate-buffered agar, we permit the glucuronidase reaction to take place under conditions near optimum pH, and ensure that the 4-methylumbelliferone will fluoresce brightly when released from MUG by the action of β-D-glucuronidase. We also incorporated sodium glucuronate into the total coliform medium to induce the production of glucuronidase during the initial 24-h incubation period, thus shortening the length of time necessary for development of the MUG reaction. The HGMF fluoresces a dull purple, which provides an excellent background contrast for the bright blue-white fluorescence of 4-methylumbelliferone. Carrying out the MUG reaction on the HGMF also avoids any problems relating to autofluorescence of glass tubes or endogenous glucuronidase in the sample. The reaction is confined to the colony, avoiding the problem of interpreting a zone of fluorescence, and use of the HGMF eliminates the limited colony-counting range of the conventional membrane filter. We have found it very easy to differentiate typical MUG fluorescence from the dull green produced by some pseudomonads.

Recommendation

It is recommended that the proposed hydrophobic grid membrane filter/MUG method for total coliform and *E. coli* enumeration in foods be adopted official first action as an additional method to official final action method **46.030–46.034.**

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Turbidimetric Microbiological Assay Results Calculated by a BASIC Computer Program

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A BASIC computer program was used to calculate the results of microbiological vitamin assays. The program, which incorporates the official AOAC guidelines for microbiological methods, reduces the uncertainty inherent in manual curve plotting and interpolation and minimizes the human error of repetitive calculations. Because the BASIC programming language is well suited for use on self-contained personal computers, it can be easily adapted by small laboratories.

With the advent of inexpensive, personal computers, small laboratories now have access to the advantages of computerized data processing. By handling many routine, repetitive calculations, these small, self-contained computers can save time and labor, producing end results that are frequently more consistent and accurate than manual calculations. By systematizing decisions, the element of human judgment is removed, and by automating calculations, human errors are minimized. The result is greater consistency and accuracy.

Previously, computer applications for microbiological vitamin assays involved large, expensive computer systems and/or complex equations and programs (1–5). In 1973, Brolund, Haskins, and Hudson (6) provided a computer application based on the official AOAC guidelines for microbiological methods. They used a large mainframe computer system, the IBM 360, a specialized computer language (Applied Programming Language), and one reference standard. Although the work presented here uses a similar approach, it is tailored for a small, self-contained computer system. The programming language, Applesoft BASIC, was originally designed for an Apple II computer but could easily be adapted to other small, personal computers. Data for up to 5 reference standards can be entered.

The program deals exclusively with calculations used to determine vitamin potency. The accuracy of the final results depends not only on data processing, but also on the quality of the original data and the validity of the assay. Kavanaugh and Ragheb (7) examined the sources of error in microbiological assays; other authors (8–11) have reported on the validity of the assay. Deviations from normal growth behavior can invalidate an assay, and drift, which is a discrepancy between the responses of the standard and the test sample, can result in either insufficient or excessive growth of the test sample relative to the standard. Many factors influence the quality of the final results; however, in this report the emphasis is on improving the accuracy of the calculations.

An outline is presented of the microbiological method and the resulting experimental data. A description is given of the computer program, which includes a summary of the specific steps in the analysis and describes the procedure involved in entering the initial data, calculating the standard curves, selecting appropriate curves for specific assays, and performing the final calculations.

Experimental Method and Computer Program Description

Microbiological Method

The microbiological method for vitamins is contained in *Official Methods of Analysis* (12), sections **43.167–43.174;** specific procedures for individual vitamins follow in sections **43.175–43.234.** For each standard vitamin solution, 0–5 mL of standard solution is added to individual test tubes. Similarly, 1–4 mL of test sample solution of unknown vitamin content is added to other test tubes. Water is added where necessary to bring all solutions to 5 mL. A 5 mL amount of growth medium, which includes all the nutrients necessary for growth of the appropriate microorganism, except the vitamin of interest, is then added to each tube. Standard tubes are prepared in triplicate; test sample tubes are prepared in duplicate. The tubes are sterilized, inoculated, and incubated to allow the organism to grow. Three uninoculated blanks are also prepared and are used to verify acceptably minimal growth in the inoculated blanks, which are used to establish the 100% transmittance level for the photometer. The turbidity of each of the remaining solutions is determined relative to this background level. The resulting data consist of 15 turbidity values for each standard used (5 levels

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Supporting documentation and a complete listing or a floppy disk copy of the computer program are available from the author on request.

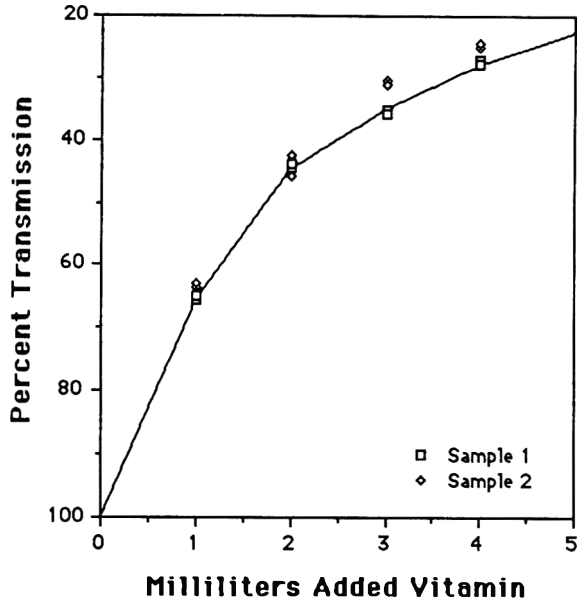

Figure 1. Point-to-point standard curve and data for 2 test samples. Sample 1 is in good agreement with the standard curve; sample 2 shows poor agreement with the standard curve.

```
MICROBIOLOGICAL ASSAY --- SAMPLE NUMBER: SAMPLE 1
 NUTRIENT SURVEILLANCE BRANCH
   TEST VALUES              9/22/88
 65.3   64.8
 44.3   43.9              ASSAY ORGANISM: L.CASEI
 35.1   35.7
 27.2   27.8
THE STANDARD IS FOLIC ACID CONC. 0.3 NG/ML STD
STANDARD VALUES
 66.7   65.6   63.9        DILUTION FACTOR: 10
 44.4   45.3   43.3
 33.8   36.2   34.4        INCUBATION TIME: 22 HRS
 28.5   27.9   26.8
 22.6   22.4   23
8 OF 8 TUBES USED IN THIS ASSAY
AVERAGE EQUIV STD CONC 1.005     .904<->1.105
AVERAGE EQUIV STD CONC OF 8 TUBES 1.005
EQUIV VOL STD CONC OF TUBES USED
1.005  1.028  1.002  1.023  .99  .969  1.026  .998
OFFICIAL ANSWER 3.015 MCG/100G      G.ANGYAL
```

Figure 2. Sample computer printout for an official answer. Sample 1, in good agreement with the standard, resulted in 8 of 8 acceptable tubes.

of added vitamin in triplicate) and 8 turbidity values for each test sample assayed (4 levels of added vitamin in duplicate).

Computer Program Description

The computer program consists of 4 distinct parts: data entry, standard curve calculations, test sample calculations, and output of results to a printer. All data are entered in response to questions in the data entry section of the program and include general, standard, and test sample data. The number of standards used can vary from 1 to 5, and the number of test samples assayed can vary from 1 to 60. The standards data include concentration factors and 15 transmittance values for each standard. The test sample data include dilution factors and 8 transmittance values for each test sample. General information, such as assay date and incubation time, is also entered.

The second portion of the program uses standard data to determine standard curves. The program averages replicate values for each volume level for each standard solution. If any value is zero, the average of non-zero values is calculated. Each set of 15 values results in a 6-point standard curve, 5 points calculated from the data, and the sixth point, representing the inoculated blanks, is 100% transmittance for 0 mL of added vitamin. Figure 1 shows a point-to-point standard curve and the data for 2 test samples.

The third portion of the program uses standard curves and test sample data to arrive at final values for the amount of vitamin present. This procedure, as outlined in *Official Methods of Analysis* (12), involves selecting an appropriate standard curve and eliminating test sample values that fall outside the cutoff limits for the standard curve. The standard curve is used to convert the turbidimetric response of test samples to corresponding volumes of standard vitamin solution. An average is determined for the replicate test samples; the 10% deviation limits are calculated around that average. Test sample values that fall outside the deviation limits are eliminated, the remaining values are averaged, and the amount of vitamin present in the test sample is calculated.

The assay is only valid between 0.5 and 4.5 mL added vitamin on a given standard curve, and the percent transmittance values corresponding to the cutoff points are calculated for each standard curve. The percent transmittance values for the largest amount of added test sample solution, 4 mL, are used to select an appropriate standard curve. If both values are inside the cutoff limit for 4.5 mL added vitamin for the lowest standard curve, then this standard curve is selected. If either value is outside the cutoff, the next higher standard curve is checked. The process is repeated with each standard curve until both values fall within the 4.5 mL bounds of some standard curve. If no standard curve is completely appropriate, the highest concentration standard curve is selected. Figures 2 and 3 show the computer output for samples 1 and 2. They include initial percent transmittance data for the test samples, 4 levels in duplicate, and for the standard curve selected, 5 levels in triplicate.

Because the assay is valid only between 0.5 and 4.5 mL added vitamin on a given standard curve, test sample values outside the 0.5–4.5 mL range are eliminated. The selected curve is used to convert each remaining percent transmittance value to the corresponding volume of standard having the equivalent percent transmittance response (i.e., the equivalent volume). The conversion is achieved by linear interpolation between points on the standard curve. The sample 1 data points are in good agreement with the standard curve in Figure 1; however, the sample 2 data points are generally high, indicating more growth than expected and lower percent transmittance values. The 2 data points for 4 mL added vitamin were eliminated because they were both above the 4.5 mL cutoff limit.

The equivalent volume of vitamin in each test sample tube is normalized by dividing by the number of milliliters of test sample solution added to the tube. The average is calculated, and the 10% deviation limits around that average are determined. Values outside the 10% limits are eliminated, and the remaining values are used to calculate the final average for the test samples. This is the average for those tubes that are within both the 0.5–4.5 mL cutoff limits and the 10% deviation limits. The amount of vitamin present is the product of the average normalized equivalent volume for the test sample, the dilution factor for the test sample solution, and the concentration factor for the standard used.

In the fourth portion of the program, the results are printed in the desired format. According to the official AOAC method, if 6 or more tubes are used to calculate the average, the result is an official answer (Figure 2); otherwise, the result is an estimate (Figure 3). The initial average, the 10% deviation limits, the final average of all retained values, the normalized equivalent volumes of all retained values, and the

```
MICROBIOLOGICAL ASSAY --- SAMPLE NUMBER: SAMPLE 2
 NUTRIENT SURVEILLANCE BRANCH
   TEST VALUES              9/22/88
 63.5   62.7
 42.5   45.9            ASSAY ORGANISM: L.CASEI
 30.3   31
 25.1   24.2
THE STANDARD IS FOLIC ACID CONC. 0.3 NG/ML STD
STANDARD VALUES
 66.7  65.6  63.9       DILUTION FACTOR: 10
 44.4  45.3  43.3
 33.8  36.2  34.4       INCUBATION TIME: 22 HRS
 28.5  27.9  26.8
 22.6  22.4  23
5 OF 8 TUBES USED IN THIS ASSAY
AVERAGE EQUIV STD CONC 1.111     1<->1.223
AVERAGE EQUIV STD CONC OF 5 TUBES 1.141
EQUIV VOL STD CONC OF TUBES USED
1.09  1.128  1.096  0  1.212  1.179  0  0
ESTIMATE ANSWER 3.424 MCG/100G      G.ANGYAL
```

Figure 3. Sample computer printout for an estimate answer shows 5 of 8 acceptable tubes. Test values 7 and 8 were outside the 0.5–4.5 mL cutoff limits for the standard curve; test value 4 was outside the 10% deviation limits for the average.

final answer are listed in Figures 2 and 3. All sample 1 data points were in good agreement and were retained. Three sample 2 data points were eliminated. Values 7 and 8 were outside the cutoff limits for the standard curve, and value 4, which was not consistent with the other data points, was eliminated for being outside the 10% deviation limits.

Discussion

The analyst's time has become increasingly important in the modern laboratory. Faster methods and quick analyses of data contribute to greater productivity. This program for the analysis of microbiological data provides many benefits, eliminating the need for repetitive manual plotting, interpolations, and calculations, which are time-consuming and prone to error. The computer program reduces initial calculation time, improves the accuracy of interpolations, and reduces the time needed to double-check results. Self-contained, personal computer systems are not subjected to the wait- and down-times frequently associated with centralized, main-frame computer systems. In addition, the printers are generally located with the terminal rather than in another location. Finally, because the BASIC programming language is easy to interpret and commonly available, it may easily be adapted to titrimetric analyses or to individual laboratory needs.

Acknowledgments

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MICROCHEMICAL METHODS

Spectrophotometric Method for Microdetermination of Nitrogen in Kjeldahl Digest

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A new spectrophotometric method for the determination of nitrogen in Kjeldahl digest has been developed. The method is based on the reaction of ammonia with acetylacetone–formaldehyde reagent in aqueous medium to yield yellow 3,5-diacetyl-1,4-dihydrolutidine with a characteristic absorption maxima at 412 nm. The effect of several experimental variables on the determination of nitrogen was studied. The method was suitable for determination of nitrogen in acidic medium without interference from the usual catalysts employed for the digestion in Kjeldahl method. Lambert-Beer's law is obeyed in the concentration range of 0.5–6.0 μg nitrogen/mL in the reaction mixture. The molar absorptivity in terms of nitrogen was 1.4×10^3 L mol^{-1} cm^{-1}. The standard deviation and relative standard deviation were ±0.0447 and ±0.896% (n = 10), respectively. The method is simple, rapid, and precise. A variety of carbocyclic and heterocyclic nitrogen compounds have been analyzed for nitrogen content. The results are comparable with those obtained by AOAC method, 47.021.

Nitrogen determination by Kjeldahl method (1) involves digestion of the sample with sulfuric acid, followed by basification of digest and distillation of ammonia produced. In the modified procedure, digestion is carried out with sulfuric acid in the presence of salt and a metal catalyst to convert all the nitrogen to an ammonium salt. The distillation of ammonia is recognized as the limiting step of the method.

As a way of circumventing the distillation step, improvements of the Kjeldahl procedure have emphasized the quantitation of ammonia by colorimetric procedures (2–8). These color reactions are carried out in alkaline medium. Precipitation of the metals used as catalyst for the digestion of organic compounds (9–13) interfere in the determination. Therefore, we wished to establish a simple spectrophotometric method for the determination of ammonia in acidic Kjeldahl digest.

The reaction of ammonia with β-ketoester and aldehyde is known to form 1,4-dihydropyridine derivative (Hantzsch reaction). This reaction has been exploited to develop a new spectrophotometric method for the determination of ammonia in Kjeldahl digest.

In present work, the digest is reacted with acetylacetone-formaldehyde reagent in the presence of sodium acetate to give a yellow reaction product having maximum absorbance at 412 nm.

The method has been applied to the determination of nitrogen in a variety of organic compounds including nitrogen heterocycles. The results are in good agreement with those obtained by AOAC method **47.021** (14) as well as with calculated nitrogen content.

Experimental

Apparatus

(a) *Micro-Kjeldahl digestion flask.*—10 and 30 mL capacity.

(b) *Spectrophotometer.*—Double beam Beckmann Model 25 equipped with 1 cm quartz matched cells.

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Reagents

Solutions were prepared volumetrically with double distilled water. All chemicals used were analytical reagent grade.

(a) *Ammonium sulfate working standard.*—1 mg nitrogen/mL. Dissolve 0.472 g ammonium sulfate (BDH) (dried previously at 105°C for 2 h) in 100 mL water. Store at 4°C.

(b) *Reagent solution.*—Mix 15 mL formaldehyde (37% w/v) (BDH) with 7.8 mL acetylacetone (SD'S) and dilute to 100 mL with water.

(c) *Sodium acetate solution.*—Dissolve 82.0 g sodium acetate (anhydrous) (BDH) in and dilute to 1 L with water.

(d) *Catalyst.*—Selenium dioxide (LOBA); mercuric oxide (yellow) (BDH); copper sulfate (BDH).

Sample Preparation

Transfer accurately weigh or pipet sample or standard nitrogen solution equivalent to ca 5.0 mg nitrogen to Kjeldahl flask. Add 1.0 g potassium sulfate, 50.0 mg yellow mercuric oxide, and 2.0 mL concentrated sulfuric acid. Heat reaction mixture to smooth boil on naked flame and then boil vigorously until solution turns colorless. Heat an additional 10 min and then cool to room temperature (ca 30°C). Dilute digest with 10 mL water and cool. Transfer solution quantitatively to 100 mL volumetric flask with the help of water and dilute to volume with the same solvent. Digest contains 50 μg nitrogen/mL solution.

Nitrogen Determination

Transfer aliquots of digest solution containing 25–100 μg nitrogen into series of 50 mL conical flasks. Add 3.0 mL sodium acetate solution and 4.0 mL reagent solution to each and mix thoroughly. Immerse flasks in boiling water bath (ca 97.5°C) for 15 min and then cool to room temperature (ca 30°C). Quantitatively transfer contents of flask to 25 mL volumetric flask with the help of water. Adjust volume to the mark with the same solvent. Measure absorbance of colored solution at 412 nm against reagent blank.

Calibration Curve

Pipet appropriate amounts of standard ammonium sulfate digest solution into individual conical flasks such that concentration of resulting solutions ranges from 0.5 to 6 μg nitrogen/mL, and proceed as in *Nitrogen Determination*, beginning "add 3.0 mL sodium acetate solution . . .". Measure absorbance at 412 nm against reagent blank. Plot absorbances at 412 nm against concentration of nitrogen/mL solution.

All sample material should be digested in a fume hood. The acetylacetone-formaldehyde reagent solution stains the skin to yellow.

Results and Discussion

Color reaction.—Ammonia in the digest solution reacts with acetylacetone-formaldehyde in presence of sodium acetate at about pH 5.5–6.0 to form the yellow compound 3,5-diacetyl-1,4-dihydrolutidine; the wavelength of maximum

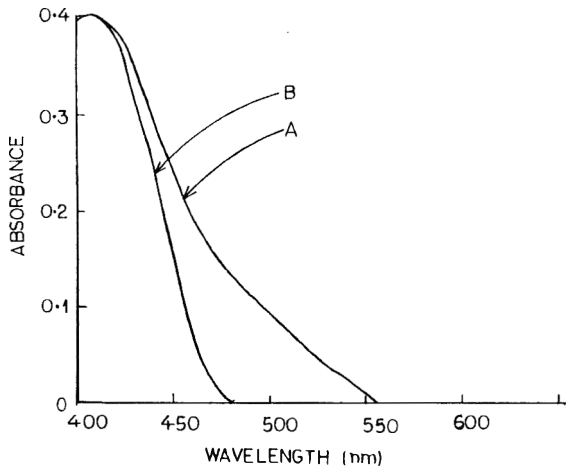


Figure 1. Absorption spectra of reaction mixture (A) and pure 3,5-diacetyl-1,4-dihydrolutidine (B).

absorbance is 412 nm (Figure 1). This color reaction forms the basis of the proposed spectrophotometric method for determination of ammonia in Kjeldahl digest.

Effect of concentration of sodium acetate (1M) solution.—A 3.0 mL sodium acetate solution (1M) was necessary for maximum color development. Increasing the concentration of sodium acetate had no adverse effect on the determination of nitrogen (Figure 2).

Effect of reagent concentration.—Color intensity was maximum in the presence of 4.0 mL reagent solution and remained constant with increased volume of reagent solution (Figure 3).

Effect of temperature.—An increase in temperature increased the rate of color development. Maximum color intensity was obtained within 15 min at about 97.5°C (reaction mixture immersed in boiling water bath) (Figures 4 and 5).

Effect of digestion catalysts.—The presence of potassium sulfate (1.0 g) alone or in combination with yellow mercuric oxide (50.0 mg), copper sulfate (50.0 mg), or selenium dioxide (50.0 mg) in the digest do not interfere in the color development (Table 1).

Linearity, optimum concentration range, sensitivity, and molar absorptivity. The Beer-Lambert curve was linear and passed through origin. The slope, intercept, and correlation coefficient obtained by linear least square treatment of the results were 0.0975, 0.0042, and 0.9999, respectively.

The color reaction obeyed Beer's law in the concentration range of 0.5 to 6.0 μg nitrogen/mL reaction mixture. The optimum concentration range for the determination as evaluated from the Ringbom plot (15) was 1–5 ppm nitrogen. The

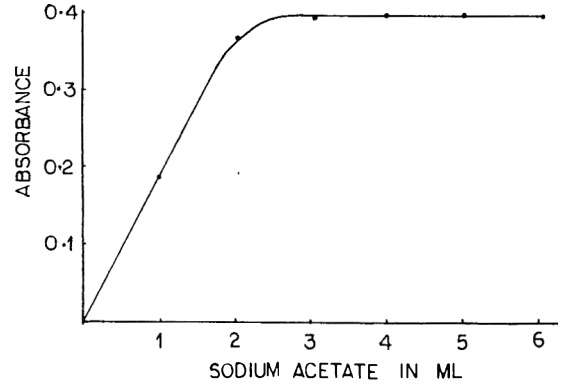


Figure 2. Effect of sodium acetate (1M) concentration on color intensity of reaction of ammonium sulfate digest with reagents.

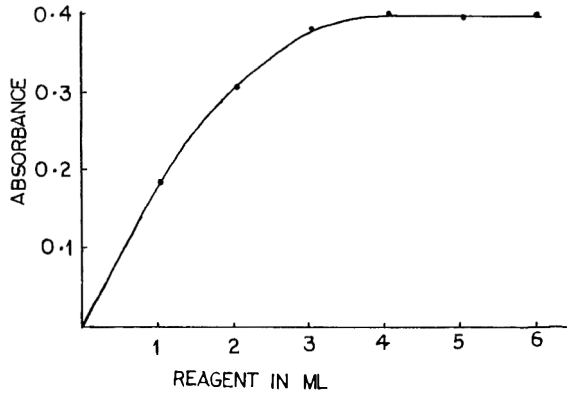


Figure 3. Effect of reagent concentration on color intensity of reaction of ammonium sulfate digest with reagents.

molar absorptivity in terms of nitrogen was 1.4×10^3 L mol^{-1} cm^{-1}, and the photometric sensitivity (16) of the color reaction was 0.01 μg nitrogen cm^{-2} at 412 nm.

Precision.—The repeatability of the procedure was tested by analyzing 10 replicates of standard ammonium sulfate solution containing 5 mg nitrogen. This statistical analysis of results obtained by the proposed method and by the AOAC method (14) are given in Table 2. Results shown in table suggest that the proposed method is as accurate and precise as the AOAC method, as indicated by the *t*-test and *F*-test, respectively.

Application of the Method

The validity of the proposed method has been tested by determining the nitrogen content of a variety of organic compounds including amines, amino acids, anilide, sulfonamides, sulfonylurea, piperidine, pyrimidine, and imidazoline derivatives having nitrogen and/or sulfur in the heterocyclic ring or side chain (Table 3). The results compare favorably with those obtained by the AOAC method (14).

Furthermore, 38 synthetic heterocyclic compounds containing different condensed pyrimidine rings, e.g., thienopyrimidines, quinazolines, and pyrazolopyrimidines (Figure 6) were analyzed by the proposed method. The estimated nitrogen value is in good agreement with the calculated nitrogen content of the compound (Table 4).

3,5-Diacetyl-1,4-dihydrolutidine is the product of the Hantzsch reaction between acetylacetone, formaldehyde, and ammonia (Figure 7). Earlier (17, 18), the reaction was used to determine formaldehyde. We expect a similar chro-

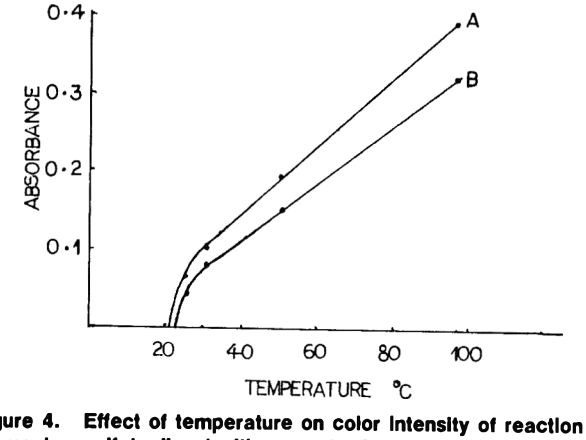


Figure 4. Effect of temperature on color intensity of reaction of ammonium sulfate digest with reagents: A, after 30 min; B, after 10 min.

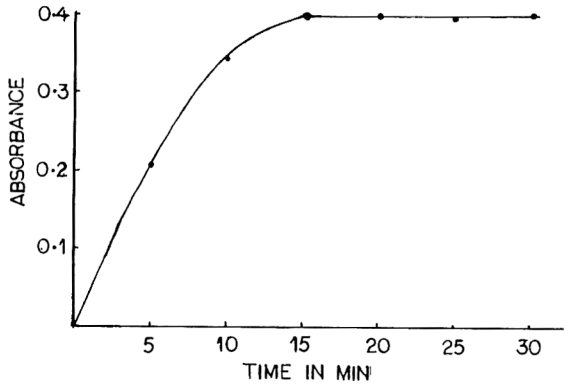


Figure 5. Effect of time of heating on color intensity of reaction of ammonium sulfate digest with reagents.

Table 1. Effect of digestion catalyst on Kjeldahl nitrogen determination

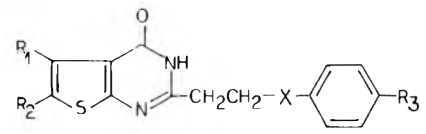
Content of digest	% N recovery (mean ± SD)[a]
H_2SO_4	100 ± 0.89
$H_2SO_4 + K_2SO_4$	100 ± 0.90
$H_2SO_4 + K_2SO_4$ + HgO (yellow)	100 ± 0.90
$H_2SO_4 + K_2SO_4 + CuSO_4$	100 ± 0.92
$H_2SO_4 + K_2SO_4 + SeO_2$	100 ± 0.91

[a] Average of 9 determinations.

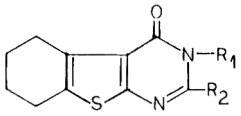
Table 2. Statistical analysis of results obtained using proposed and AOAC (14) methods for analysis of ammonium sulfate digest

Statistic	Nitrogen	
	Proposed	AOAC
Mean	4.99	4.98
Std dev.	0.0447	0.0461
N	10	10
% RSD	0.896	0.926
Variance	1.998×10^{-3}	2.125×10^{-3}
Std error of mean	0.0141	0.0146
t-Test	0.493 (2.101)[a]	
F-Test	1.064 (3.18)[a]	

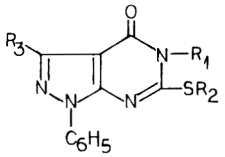
[a] 95% confidence limit.



1–3



4, 5



6, 7

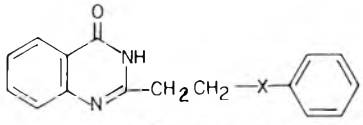


Figure 6. Structure of heterocyclic compounds.

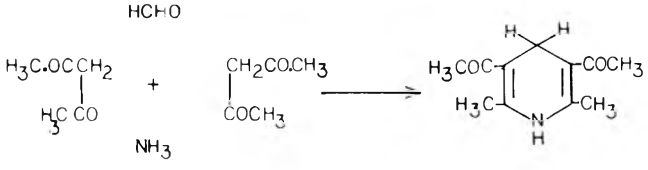


Figure 7. Hantzsch reaction.

mophore in the proposed procedure. To confirm the formation of 3,5-diacetyl-1,4-dihydrolutidine, it was synthesized under the reaction conditions. The compound was isolated and recrystallized from ethanol (mp 196–199°C; lit. (19) mp 198°C). The absorption spectrum of the product in aqueous ethanol was similar to the spectrum of chromophore obtained in the reaction; the wavelength of maximum absorbance was 412 nm (Figure 1).

The reaction mixture has a pH of 5.5–6.0. Therefore, metal ions used in digestion apparently do not precipitate; the

Table 3. Analysis of nitrogen content of organic compounds by proposed and AOAC (14) methods

Compound	Calcd	Proposed g/100 g[a]	Proposed SD	Proposed % RSD	AOAC g/100 g[a]	AOAC SD	AOAC % RSD
Paracetamol	9.25	9.28	0.041	0.442	9.20	0.058	0.630
Acetanilide	10.37	10.22	0.038	0.372	—	—	—
Sulfanilamide	16.27	16.21	0.072	0.444	—	—	—
Sulfacetamide sodium	12.12	11.94	0.099	0.829	—	—	—
Chlorpropamide	10.12	10.19	0.068	0.667	10.17	0.074	0.728
Isoprenaline sulfate	5.03	4.99	0.055	1.101	5.02	0.050	0.996
Bemegride	9.02	8.96	0.060	0.669	8.98	0.062	0.690
Trimethoprim	19.19	19.39	0.096	0.495	—	—	—
Glycine	18.65	18.86	0.071	0.376	18.85	0.088	0.467
Lysine mono-hydrochloride	15.33	14.92	0.063	0.422	14.99	0.071	0.474
Glutamic acid	9.52	9.64	0.063	0.654	9.60	0.066	0.688
Urea	46.65	46.67	0.223	0.478	46.69	0.239	0.512

[a] Average of 5 determinations.

Table 4. Analysis of heterocyclic compounds for nitrogen content

No.	R_1	R_2	R_3	X	Calcd, g/100 g	Nitrogen g/100 g[a]	Nitrogen SD	Nitrogen % RSD
1	CH_3	CH_3	Cl	S	7.99	7.96	0.066	0.829
2		$-(CH_2)_4-$	H	SO_2	7.48	7.33	0.036	0.491
3	CH_3	$COOC_2H_5$	CH_3	SO_2	6.66	6.83	0.038	0.556
4	H	$CH_2CH_2N(C_2H_5)_2$	—	—	13.77	13.70	0.087	0.635
5	C_6H_5	OH	—	—	9.39	9.40	0.070	0.745
6	p-$CH_3C_6H_4$	H	H	—	16.75	16.69	0.110	0.659
7	p-CH_3O-C_6H_4	H	SCH_3	—	14.13	14.14	0.121	0.856
8	—	—	—	S	9.92	10.16	0.053	0.522
9	—	—	—	SO_2	8.91	9.01	0.060	0.660

[a] Average of 3 determinations.

loss of ammonia by volatilization is also prevented. Thus the proposed method is simple, rapid, precise, and accurate, and does not require basification of the digest and its distillation for ammonia determination. The reagents involved are stable, inexpensive, and readily available.

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MYCOTOXINS

Enzyme-Linked Immunosorbent Assay of Aflatoxins B_1, B_2, and G_1 in Corn, Cottonseed, Peanuts, Peanut Butter, and Poultry Feed: Collaborative Study

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A direct competitive enzyme-linked immunosorbent assay (ELISA) screening method for aflatoxins at 20 ng/g was studied by 12 collaborators. Test samples of peanut butter were extracted by blending with methanol–water–hexane (55 + 45 + 100) and heating the test extracts on a steam bath; test samples of the other commodities were extracted by blending with methanol–water (80 + 20). All test extracts were filtered and the filtrates were diluted with buffer to a final methanol concentration of <30%. Each diluted filtrate was applied to a cup containing a filter with immobilized polyclonal antibodies specific to aflatoxins B_1, B_2, and G_1. Aflatoxin B_1–peroxidase conjugate was added, the cup was washed with water, and a mixture of hydrogen peroxide and tetramethylbenzidine was added. The test sample was judged to contain ≥20 ng aflatoxins/g when, after exactly 1 min, no color was observed on the filter; when a blue or gray color developed, the test sample was judged to contain <20 ng aflatoxins/g. All collaborators correctly identified naturally contaminated corn and raw peanut positive test samples. No false positives were found for controls containing <2 ng aflatoxins/g. The correct responses for positive test samples spiked at levels of 10, 20, and ≥30 ng aflatoxins/g (the ratio of B_1:B_2:G_1 was 10:1:3) were 52, 86, and 96%, respectively. The method, which is rapid and simple, has been adopted official first action for *screening* for aflatoxins at ≥20 ng/g in cottonseed and peanut butter and for aflatoxins at ≥30 ng/g in corn and raw peanuts. The procedure will require further study for poultry feed. Positive test samples may require reanalysis by an official, quantitative method.

The use of enzyme-linked immunosorbent assays as screening methods for aflatoxins has increased rapidly (1–4). A screening assay is defined as a method to rapidly and reliably analyze a large number of test samples at the designated level of interest in order to eliminate (screen) negative test samples (5). One method using microtiter wells was collaboratively studied (6, 7). The method was adopted official first action by AOAC for screening for aflatoxin B_1 in cottonseed products and mixed feed (8), and has received interim official first action approval for B_1 in corn and peanut products (7).

According to data for 3700 test samples of corn and peanuts analyzed between 1980 and 1986 by AOAC official methods, aflatoxins B_1 and G_1 were found in >40% of peanut and in 12% of corn test samples analyzed (9). The ratios of the 4 aflatoxins B_1, B_2, G_1, and G_2 were quite variable. In general, contamination levels were higher for B_1 than for G_1; B_2 and occasionally G_2 were found in trace amounts.

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This report will be presented at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO.

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

This study was carried out under the joint sponsorship of AOAC and the Commission on Food Chemistry of the International Union of Pure and Applied Chemistry (IUPAC).

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For regulatory purposes in many countries, it is necessary to determine total aflatoxins. Therefore, it is important to collaboratively study ELISA methods capable of determining total aflatoxins (B_1, B_2, G_1, and G_2).

The ELISA consists of a 2-step process: first the reaction between the antibody and the aflatoxins and then detection of the reaction by using enzymatic hydrolysis of the substrate by the aflatoxin B_1–horseradish peroxidase complex (10–12).

Another ELISA test system for aflatoxins was developed recently (personal communication, 1988, D. K. Morris, International Diagnostic Systems Corp., PO Box 799, St. Joseph, MI 49085). The specificity range of the antibodies covers aflatoxins B_1, B_2, and G_1. The procedure of the manufacturer was modified to increase the reliability for total aflatoxins (B_1, B_2, and G_1) at 20 ng/g and to broaden the applicability to include peanut butter (13). The method is rapid and simple; approximately 10 analyses can be performed in 1 h. Although some basic scientific glassware and equipment are required, these can readily be adapted to environments associated with "field" testing, such as grain storage areas. The test kit was evaluated and found to quickly sort out negative test samples (<20 ng/g) and to identify positive test samples (≥20 ng/g); results were in agreement with those obtained by official methods (14). The modified method was subjected to a collaborative study sponsored jointly by AOAC and the International Union of Pure and Applied Chemistry (IUPAC). The results of the study are reported here.

Collaborative Study

Portions of 25 lb each of ground corn, peanut butter, and raw peanuts were analyzed according to secs **26.026–26.031** and sec. **26.058** (14); cottonseed was analyzed according to secs **26.052–26.058** (14). The method of Shannon et al. (15) was used for the analyses of poultry feed. All commodities were found to contain total aflatoxins at <2 ng/g. Corn, raw peanuts, peanut butter, and poultry feed test samples were spiked in duplicate with total aflatoxins B_1, B_2, and G_1 at 30, 20, and 10 ng/g. The ratio of B_1:B_2:G_1 was 10:1:3. Test samples of cottonseed were spiked in the same manner but at 60, 20, and 10 ng/g. The artificially contaminated (spiked) test samples, controls (<2 ng/g), naturally contaminated corn (101 ng/g) and peanut meal (69 ng/g) test samples (analyzed according to secs **26.026–26.031**), and practice positive (corn, 30 ng/g) and practice negative (corn, <1 ng/g) test samples were sent to 12 laboratories in the United States, France, Canada, Denmark, Japan, and The Netherlands. Reference standards, test kits, and method directions were provided.

In the collaborative study, poultry feed samples were assayed as described for corn, raw peanuts, and whole cottonseed.

Statistical Parameters

The 95% confidence intervals were obtained by using the binomial distribution and treating duplicate results from 12 laboratories as single determinations.

The conventional within-laboratory and among-laboratory parameters are not applicable to "either/or," qualitative, or spot tests.

Aflatoxins B_1, B_2, and G_1 in Corn, Cottonseed, Peanuts, and Peanut Butter

Enzyme-Linked Immunosorbent Screening Assay

First Action

AOAC-IUPAC Method

(Applicable to screening aflatoxin B_1, B_2, and G_1 contamination in whole cottonseed and peanut butter at ≥20 ng/g and in corn and raw peanuts at ≥30 ng/g. If false negative rate of <5% is required, perform duplicate ELISAs on same test sample extract. Reanalyze positive samples by official, quantitative method.)

A. Principle

Antibodies specific to aflatoxins B_1, B_2, and G_1 are immobilized on a filter, and toxin (aflatoxin B_1) is labeled with an enzyme (horseradish peroxidase). Binding of toxin-enzyme conjugate by immobilized antibodies is inhibited by addition of free toxin present in test sample. Since fixed number of antibody reaction sites are available, enzyme activity is proportional to amount of bound toxin-enzyme conjugate. Antibody-toxin-enzyme complex concentration is inversely proportional to concentration of free toxin added. Bound enzyme catalyzes oxidation of substrate to form blue complex. Development of color indicates that test sample contains aflatoxins at <20 ng/g; no color development indicates that test sample contains aflatoxins at ≥20 ng/g.

B. Confidence Intervals

The following 95% confidence intervals for correct identification of samples positive for aflatoxin contamination were obtained in the collaborative study on this method:

Sample	*Level, ppb*	*95% Conf. Interval, %*
Cottonseed	20	79–100
Peanut butter	20	72–99
Raw peanuts	30	78–100
Corn	30	73–99

C. Specificity of Antibodies

Antibodies have specific ability to bind structurally related compounds, namely, aflatoxins B_1, B_2, and G_1. Determine specificity of purified rabbit anti-aflatoxin B_1 polyclonal antibodies by direct competitive ELISA method. Coat serially diluted antibodies on microtiter plates. Prepare standard solutions of aflatoxins B_1, B_2, G_1, G_2, and M_1; zearalenone; T-2 toxin; and deoxynivalenol, and add to individual microtiter wells. Then add solution of aflatoxin B_1 conjugated to horseradish peroxidase to each well. Add substrate solution of tetramethylbenzidine and hydrogen peroxide, and measure development of color with scanner. Least color development indicates highest reactivity of toxin-antibody reaction. Cross-reactivity to aflatoxin B_1 for antibody used in collaborative study of this method was 100, 70, 75, and <10% for aflatoxins B_1, B_2, G_1, and G_2, respectively. All other toxins tested showed no cross-reactivity.

D. Sensitivity of ELISA Reagent

Calibrate aflatoxin B_1 standard according to secs **26.004–26.010**.

(a) *Negative control test sample.*—Follow procedure in enzyme immunoassay for corn, ***J*(a)**.

(b) *Threshold-level standard.*—Used to define lower limit of determination. Dispense 100 μL working standard into test tube. Add 350 μL methanol–buffer, ***E*(d)**, (30 + 70), and mix. Follow procedure in enzyme immunoassay for corn, ***J*(a)**, steps (*2*), (*4*)-(*7*).

(c) *Positive control test sample.*—Use working standard solution; follow procedure for enzyme immunoassay for corn, ***J*(a)**, steps (*2*), (*4*)-(*7*).

Negative control test sample should develop blue color; positive control test sample should have no color development. Threshold standard should show no color development.

E. Reagents

Items **(a)**–**(h)** are available as ImmunoDot Screen (IDS) Cup, (International Diagnostic Systems Corp., 2614 Nile Ave, St. Joseph, MI 49085). Reagents from other suppliers can be used provided requirements listed below are met.

(a) *Antibody-coated solid support.*—Antibody-coated filter material attached to analytical cup made of porous polyethylene (3.2 cm diameter, 2.5 cm high, capacity 4 mL). Coated cup is specified by manufacturer to be stable for 6 months stored at 4–8°.

(b) *Aflatoxin-enzyme conjugate.*—Aflatoxin B_1–horseradish peroxidase conjugate at toxin–enzyme molar ratio of 10–15:1. Conjugate is specified by manufacturer to be stable for 6 months at 4–8°.

(c) *Wash solution.*—Phosphate-buffered saline solution. Dissolve 0.23 g $NaH_2PO_4 \cdot H_2O$, 1.95 g $K_2HPO_4 \cdot 3H_2O$, 8.70 g NaCl, 0.125 mL Tween 20 (polyoxyethylene[20]sorbitan monolaurate), and 10 mg thimerosal (ethylmercurithiosalicylic acid, Na salt), in 900 mL H_2O, adjust pH to 7.2, and dilute to 1 L.

(d) *Buffer.*—0.1% bovine serum albumin in phosphate-buffered saline solution containing 0.05% thimerosal.

(e) *Substrate solution A.*—Tetramethylbenzidine (TMB) (0.4 g/L H_2O), pH 8.3.

(f) *Substrate solution B.*—Hydrogen peroxide (0.2% H_2O_2 in 0.13% aqueous citric acid solution), pH 3.0 (Kirkegaard and Perry Laboratories, Inc., 2 Cessna Ct, Gaithersburg, MD 20879).

(g) *Methanol, hexane, and chloroform.*—Reagent grade.

(h) *Standard aflatoxin B_1.*—Approximately 28 μg as dry film.

F. Apparatus

Equipment specified is not restrictive; other suitable equipment can be substituted.

(a) *High-speed blender.*—With 500 mL jar.

(b) *Micropipet and tips.*—Recommended range 100–1000 μL; use with disposable polypropylene tips.

(c) *Glass culture (test) tubes.*—10 × 75 mm; 3 mL.

(d) *Filters.*—Whatman No. 4, or equivalent.

(e) *Timer.*—Graduated in 1 s intervals.

(f) *Carborundum boiling chips.*

G. General Instructions

Store all kit components at 4–8°. *Do not freeze.* Before use, allow 1 h for cups and reagents to reach room tempera-

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ture (23–29°). Use separate disposable pipet tip for each test solution to avoid cross-contamination. Include one negative control with each group (20 cups) of test samples. Negative control must be functioning properly (must develop *blue color* in center of cup) for test to be valid. Positive standard is provided for periodic checking or for use with each group of test samples (must show *no color* in center of cup). Threshold-level standard should also be used and must show no color development. If color develops, repeat test. Color development in more than 2 tests indicates a defective kit.

Reagents are stable 6 h at room temperature. To ensure shelf life of kit components, promptly return reagents to refrigerator after use.

Because of difficulty in monitoring 1 min timing intervals, run 1 cup at a time. As proficiency is gained, analysts can run 3 cups successively spaced at convenient time intervals for making observations.

H. Extraction of Test Sample

Note: Test sample extraction and cup testing should be performed during *same day*.

(a) *Corn, raw peanuts, and whole cottonseed.*—Weigh 50 g test portion into blender jar. Add 100 mL methanol-H_2O (8 + 2). Blend 3 min at high speed. Filter mixture and recover filtrate. Alternatively, let mixture stand 10–15 min and recover supernatant liquid.

(b) *Peanut butter.*—Weigh 50 g test portion into blender jar. Add 100 mL hexane and 250 mL methanol-H_2O (55 + 45). Blend 3 min at high speed. Filter mixture and transfer filtrate to separatory funnel. Let layers separate for 10 min. Place 20 mL lower layer in 150 mL beaker. Add minimum of 15 boiling chips and heat in stream bath or on hot plate. *Boil* 3 min and let cool.

I. Preparation of Aflatoxin B_1 Standard Solutions

(a) *Stock solution.*—Add 3 mL $CHCl_3$ to vial containing 28 μg aflatoxin B_1 standard (ca 9 ng/μL). Cap vial, mix contents, and store vial in refrigerator.

(b) *Working solution.*—Dispense 300 μL stock standard solution into vial. Add 2400 μL methanol (1 ng/μL), mix, and store solution in refrigerator. *Prepare daily.* Dispense 10 μL diluted standard (1 ng/μL) into test tube. Add 300 μL methanol and 700 μL buffer, ***E*(d)**. *Prepare ≤2 h before use.* Proceed as for diluted test extract (*Enzyme Immunoassay*, ***J*(a)**, steps (*2*), (*4*)-(*7*)).

J. Enzyme Immunoassay

(a) *Corn, raw peanuts, and whole cottonseed.*—(*1*) Allow 1 h for all reagents to reach room temperature (23–29°).

(*2*) Prepare fresh substrate in small culture (test) tube by mixing 500 μL (10 drops) substrate solution A with 500 μL (10 drops) substrate solution B for each cup being used. *Do not combine substrate solution A with substrate solution B more than 15 min before use.*

Note: Run 1 negative control cup and 1 positive standard cup each day to ensure that all reagents are functional. Threshold-level standard should be run with each set of new reagents (follow steps (*4*)-(*7*)). Negative control cup should be run by applying 100 μL (2 drops) buffer to center of cup. For positive standard cup, apply working standard to cup as in (*4*). For both cups proceed with steps (*5*)-(*7*).

(*3*) Add 200 μL test extract to 400 μL buffer, ***E*(d)**, (600 μL total).

(*4*) Thoroughly mix diluted test extract and apply one 150 μL aliquot to center of cup. *Using timer, wait 1 min and then add second 150 μL* aliquot of diluted test extract. *Using timer, wait additional 1 min before proceeding to next step.* (In summary, two 150 μL aliquots of diluted test extract are applied to cup—150 μL at a time with 1 min wait between each addition and before proceeding to next step.)

(*5*) Apply 100 μL (2 drops) enzyme solution to center of cup. Using timer, wait 1 min.

(*6*) Wash with 1.5 mL (30 drops) wash solution added dropwise. If more than 1 cup is being used, wash successively with 500 μL (10 drops) per cup 3 times.

(*7*) Add entire contents of substrate solution 1.0 mL (20 drop mixture) from each test tube to each cup. (Start timer as soon as substrate mixture is added to cup.) *Wait 1 min* and immediately observe disk (center of cup) for *blue color* development (*negative*) or *no color* development (*positive*) (see *Interpretation of Results*, ***K***).

(b) *Peanut butter.*—(*1*) Allow 1 h for all reagents to reach room temperature (23–29°).

(*2*) Prepare fresh substrate solution in small culture (test) tube by mixing 500 μL (10 drops) substrate solution A with 500 μL (10 drops) substrate solution B for each cup being used. *Do not combine substrate solution A with substrate solution B more than 15 min before use.*

(*3*) Add 500 μL test extract to 500 μL buffer, ***E*(d)**, (1000 μL total).

(*4*) Thoroughly mix diluted test extract and apply one 200 μL aliquot to center of cup. *Using timer, wait 1 min and add second 200 μL* aliquot of diluted test extract. *Using timer, wait additional 1 min and then add third 200 μL aliquot of diluted test extract before proceeding to next step.* (In summary, three 200 μL aliquots of diluted test extract are applied to cup—200 μL at a time with 1 min wait between each addition and before proceeding to next step.) Proceed as for corn, etc., steps (*5*)-(*7*) above.

K. Interpretation of Results

Observe disk (center of cup) for blue color or no color development at *exactly* 1 min after adding substrate A and B mixture.

Negative.—If disk (center of cup) turns *light blue or darker*, test sample contains total aflatoxins B_1, B_2, and G_1 at <20 ng/g (cottonseed, peanut butter).

Positive.—If no blue color is observed in disk (center of cup) and disk remains *completely white* (no color change) for at least 1 min, test sample contains total aflatoxins B_1, B_2, and G_1 at ≥20 ng/g.

Negative control.—Negative control cup *must develop blue color* in center of cup.

Positive control standard.—Positive standard cup must remain *completely white* (no color change) for at least 1 min.

Threshold-level standard.—Cup must remain completely white (no color change) for 1 min.

Note: Development at 1 min of any blue color, however faint, means aflatoxin levels are <20 ppb (negative). False negative rate may be higher for corn and raw peanuts at this cutoff level. If false negative rate of <5% is required, duplicate ELISAs must be performed on the test sample extract; otherwise, <30 ppb is considered negative for these commodities.

Ref.: JAOAC **72**, November/December issue (1989).

Results and Discussion

All 12 collaborators selected to participate completed the study and submitted data. The results are presented in Table 1. No outlier test was applied; no results were removed as outliers. However, as shown in Table 1, laboratories 1 and 8

Table 1. Collaborative results for ELISA cup method for screening aflatoxins B_1, B_2, and G_1, in corn, raw peanuts, poultry feed, cottonseed, and peanut butter[a]

Total aflatoxins, ng/g	Laboratory												Sum of positive assays	95% Confidence intervals		
	1	2	3	4	5	6	7	8	9	10	12	13		% Positive	Lower boundary	Upper boundary
Corn																
NC[b]	1	1	1	1	1	1	1	1	1	1	1	1				
NC	1	1	1	1	1	1	1	1	1	1	1	1	24	100	88	100
30	0	1	1	1	1	1	1	0	1	1	1	1				
30	1	1	1	1	1	1	1	1	1	1	1	1	22	92	73	99
20	1	1	1	1	1	1	1	0	1	1	1	1				
20	0	1	1	1	1	0	1	0	1	0	1	0	18	75	53	90
10	0	0	1	0	0	0	0	0	1	0	1	0				
10	0	1	1	0	1	0	0	0	1	0	0	1	8	33	16	55
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22
Raw peanuts																
NC	1	1	1	1	1	1	1	1	1	1	1	1				
NC	1	1	1	1	1	1	1	1	1	1	1	1	24	100	88	100
30	0	1	—[c]	1	1	1	1	1	1	1	1	1				
30	1	1	1	1	1	1	1	1	1	1	1	1	22	96	78	100
20	1	1	1	1	1	0	1	1	1	1	1	1				
20	0	1	1	0	1	1	1	0	1	1	1	1	20	83	63	95
10	0	0	1	1	1	0	1	0	1	0	1	1				
10	0	0	1	0	1	1	1	0	1	0	1	0	13	54	33	74
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22
Poultry feed																
30	1	1	1	1	1	1	1	0	1	1	1	1				
30	0	1	1	1	1	0	1	0	1	1	1	1	20	83	63	95
20	0	1	0	0	1	0	1	0	0	0	1	0				
20	0	0	1	1	1	0	1	0	0	1	1	1	11	46	26	67
10	0	0	0	1	0	0	0	0	0	0	0	0				
10	0	0	0	0	0	0	0	0	0	0	0	0	1	4	0.1	21
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22
Cottonseed																
60	1	1	1	1	1	1	1	1	1	1	1	1				
60	0	1	1	1	1	1	1	1	1	1	1	1	23	96	79	100
20	1	1	1	1	1	1	1	1	1	1	1	1				
20	1	1	1	1	1	1	1	1	1	0	1	1	23	96	79	100
10	0	0	1	0	0	0	1	1	1	1	1	1				
10	0	1	1	1	0	0	1	1	1	0	1	0	14	58	37	78
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22
Peanut butter																
30	—[c]	1	1	1	1	1	1	1	1	1	1	1				
30	1	1	1	1	1	1	1	1	1	1	1	—[d]	22	100	87	100
20	0	1	1	1	1	1	0	1	1	1	1	—[d]				
20	1	1	1	1	1	1	1	1	1	1	1	1	21	91	72	99
10	1	0	1	0	1	1	0	1	—[c]	0	1	0				
10	1	1	0	0	1	1	0	0	1	1	1	1	14	61	39	80
0	0	0	0	0	0	0	0	0	0	0	0	—[d]	0	0	0	24

[a] 1 = positive; 0 = negative.
[b] NC = naturally contaminated; corn at 101 ng/g and peanuts at 69 ng/g.
[c] Lost.
[d] Result not used; extract heated too long (30 min).

had considerably poorer results than did the other collaborators. The replicates were treated as independent measurements. In computing the confidence limits, even though 12 laboratories provided 2 estimates each, the results were treated as if they came from 24 laboratories that provided a single estimate each. The 95% confidence interval was obtained by using the binomial distribution. For this study, the 95% confidence intervals of the method for correctly identifying test samples as positive for aflatoxin contamination at 20 ng/g were 79–100 and 72–99% for cottonseed and peanut butter, respectively, and at 30 ng/g were 73–99 and 78–100% for corn and raw peanuts, respectively.

Theoretically, the upper boundary of the true positive rate at the 0 ng/g level is 22% only when 12 test samples are examined and none are found to be positive. This is a statistical calculation based on the number of test samples analyzed. When there is no aflatoxin (toxin) in the test sample competing with the toxin–enzyme conjugate, nothing prevents the

Table 2. Positive rate for all spiked (10–30 ng/g) test samples combined for ELISA cup method for screening aflatoxins B_1, B_2, and G_1

Total aflatoxins, ng/g	% Positive	95% Confidence interval: Lower boundary	95% Confidence interval: Upper boundary
Corn, peanut butter, raw peanuts, and cottonseed:			
30	96	88	99
20	86	78	93
10	52	41	62
0	0	0	6
Corn, peanut butter, raw peanuts, cottonseed, and poultry feed:			
30	92	85	97
20	78	70	85
10	42	33	51
0	0	0	5

color formation with the reagents. In this case, a negative response would always be expected on sound chemical grounds; statistics can show only that the zero response rate is included in the 95% confidence interval.

Results from all collaborators for the naturally contaminated corn and raw peanut test samples were positive for aflatoxins. At the 0 ng/g level, no false positives were reported for any commodities. For test samples spiked at ≥30 ng/g, 83–100% of the test samples were found positive, while at 10 ng/g, 4–61% of the test samples were found positive. With the exception of poultry feed, 75–96% of the test samples at a level of 20 ng/g were found positive. Only 46% of the poultry feed test samples at 20 ng/g were found positive. This lower percentage could be due to the ingredients in the feed, which interfered with the analyses by complexing or decomposing the aflatoxins.

A summary for all the commodities at the 4 spiking levels is given in Table 2. When results for the poultry feed are excluded, the percentages of test samples found to be positive at levels of 30, 20, 10, and 0 ng/g are 96, 86, 52, and 0, respectively. When feed is included in the calculation, the corresponding percentages of positives become 93, 78, 42, and 0. The upper boundary of the 95% confidence interval of identifying a 0 ng/g test sample as positive is about 6% (based on 47 test samples). The rate of false negatives for cottonseed and peanut butter at ≥20 ng/g was lower than for the other commodities. The percentage of false negatives essentially defines the limit of determination of the method.

The acceptability of a specific rate of false negatives must be based on the intended use of the method. To increase the probability of identifying a test sample containing aflatoxins at ≥20 ng/g as positive, either of 2 approaches may be taken: The original extract may be reanalyzed by the ELISA procedure, or the test sample may be analyzed in duplicate. If any of the assays is positive, the test sample is considered positive; if the test result is negative, then the test sample is negative with a 98% certainty $\{100 - [(1 - 0.86) \times (1 - 0.86) \times 100] = 100 - 1.96\}$. Reducing the percentage of false negatives by replicate analyses in general increases the percentage of false positives.

When the test sample is found to be positive by the ELISA test, then the test sample should be reanalyzed by an official, quantitative method to determine the actual contamination level if required.

Collaborators' Comments

Comments from collaborators indicated that the identification of cottonseed test samples at 10 ng/g as positive may have been due to problems with detection of the very light blue color, which was obstructed by the yellow color on the filter from the test extract. Some laboratories had difficulty in interpreting the directions for heating the peanut butter extract to boiling on the steam bath. Directions were to boil the peanut butter extract for 3 min to evaporate the hexane, which would otherwise destroy the antibodies attached to the filter. To boil the extract, the beaker must be placed in the steam bath, not on top. In general, all collaborators found the method easy to follow.

Recommendation

On the basis of the results obtained in the AOAC/IUPAC collaborative study reported here, the Associate Referee recommends that the ELISA method be adopted official first action for screening aflatoxin B_1, B_2, and G_1 contamination at ≥20 ng/g in cottonseed and peanut butter and at ≥30 ng/g in corn and raw peanuts. If a false negative rate of <5% is required, duplicate determinations must be made. Positive test samples should be reanalyzed by an official, quantitative method. The collaborative results for poultry feed did not support a recommendation for adoption.

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Comparison of Two ELISA Screening Tests with Liquid Chromatography for Determination of Aflatoxins in Raw Peanuts

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A study was conducted to evaluate the performance of 2 enzyme-linked immunosorbent assays (ELISA) for rapidly screening samples of peanuts for the presence of aflatoxin. The EZ-Screen Quick Card Test and the Afla-10 Cup Test were compared with liquid chromatography in duplicate analyses of common extracts of peanuts contaminated in the range of 0–70 ppb (ng/g). Each assay properly identified 95% of samples containing no detectable aflatoxin as negative and >97% of samples containing >10 ppb aflatoxin as positive. The card test, which had a 20 ppb detection threshold, identified as positive 32 of 34 samples in the 11–20 ppb range. This indicates that the card test might actually have a detection threshold closer to 10 ppb. Most of the errors associated with the assays occurred on samples containing <10 ppb aflatoxin. The cup and card tests identified 76 and 67% of the samples, respectively, as negative, in the range of 4–10 ppb. For samples either negative or contaminated above their detection thresholds for the assays, the methods are well suited for use as rapid screening tests.

The development of new immunochemical methods for mycotoxin analyses has progressed rapidly in recent years. In particular, enzyme-linked immunosorbent assays (ELISA) for the detection of aflatoxins in various commodities have gained popularity because of their sensitivity, specificity, and general simplicity (1). Some of the assays are designed to determine the aflatoxin concentration in a commodity to the nearest ppb (ng/g). Others are designed as screening methods with the objective of determining the aflatoxin concentration simply as greater or less than a specific amount (i.e., 20 ppb). These methods are designed for use in situations that require short analysis time, simple operation, and relatively low cost.

In the initial stage of marketing peanuts in the United States (from grower to initial buyer), the farmers' stock peanuts are graded on the basis of several factors. One of these factors is evaluation of the aflatoxin contamination of each load. This evaluation is made visually by examining a sample of peanuts from the load (ca 4000 kg) for the presence of the aflatoxin-producing fungi, *Aspergillus flavus* and *A. parasiticus* (2). Based on the findings of this visual examination, the load is presumed either to be at low risk for aflatoxin contamination because no visibly molded peanut kernels were found or at high risk because one or more visibly molded peanut kernels were found. Low risk loads proceed through normal marketing channels for edible use, and high risk loads are diverted from the edible supply to oil stock.

Although shelled peanuts are analyzed chemically to determine actual aflatoxin concentrations later in the marketing channel, various reasons have been given for not analyzing farmers' stock peanuts directly for the presence of the aflatoxins. Two of these reasons are the time and expense of conducting such analyses. However, the current environment of increased consumer demand for wholesomeness of food and stricter standards for aflatoxin tolerance warrants use of a direct analytical method. The advent of rapid ELISA screening methods could make the analysis of farmers' stock peanuts practical and improve the segregation of contaminated and noncontaminated loads at the earliest stage in the marketing channel. These assays could also be used for other commodities, such as corn and cottonseed, when rapid screening is required.

Cole et al. (3) compared an ELISA screening method with a liquid chromatographic (LC) method by analyzing common extracts of peanuts from 152 lots of farmers' stock peanuts. The ELISA procedure was designed to determine whether samples contained greater or less than 20 ppb aflatoxin. The correlation between results from the ELISA method and those from the LC method was 98.6%. However, the aflatoxin concentration in 93.5% of the samples was either <5 ppb or >50 ppb. The ELISA test in this case had a major source of error because samples contaminated between 20 and 50 ppb aflatoxin were usually identified as containing <20 ppb. Therefore, results of that study indicated that the ELISA screening method was very effective when samples were either negative or highly contaminated with aflatoxin. Results were not conclusive when samples were contaminated at a concentration near the detection threshold for the

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assay (20 ppb), in part because very few samples were contaminated in this range.

The purpose of the present study was to compare 2 commercially available ELISA screening methods with LC analyses of a large number of peanut samples contaminated in the critical 5–50 ppb range.

Experimental

ELISA Methods

Both ELISA methods were performed according to manufacturer's instructions. Each method reportedly detects aflatoxins B_1, B_2, and G_1, and required a methanol-water (80 + 20 v/v) extract of peanuts.

(a) *EZ-Screen Quick Card.*—(Kits were supplied by Environmental Diagnostics, Inc., Burlington, NC 27215.) Test has 20 ppb detection threshold and consists of card and vials containing aflatoxin–enzyme conjugate, substrate, and negative control. Card has 2 ports where antibody is located, one for sample and one for negative control. Diluted sample extract is added to sample port followed by aflatoxin–enzyme conjugate and then substrate. Same procedure is followed after negative control is added to negative control port. Results are determined by color change in 2 ports. Negative control ports become purplish blue. If sample port becomes light gray or darker, sample should contain <20 ppb aflatoxin. If sample port remains white or off-white, sample should contain ≥20 ppb aflatoxin.

(b) *Afla-10 Cup Test.*—(Kits were supplied by International Diagnostics Systems Corp., St. Joseph, MI 49085.) Test has 10 ppb detection threshold and consists of small cup with center disc, where antibody is added. Aflatoxin–enzyme conjugate, substrate, and negative control are also included. Diluted sample extract is added to disc followed by aflatoxin–enzyme conjugate and then substrate. Results are determined by color change on disc. Blue dot indicates <10 ppb aflatoxin, whereas no color change indicates ≥10 ppb. Manufacturer recommends that negative control cup be run each day to ensure that all reagents are functional.

(c) *Liquid chromatography.*—All extracts were analyzed by method of Dorner and Cole (4). LC system included 2 M6000A pumps, Model 712 WISP automatic injector, Model 730 data module (Waters Chromatography Div., Millipore Corp., Milford, MA 01757); Model LS-1 LC fluorescence detector (Perkin-Elmer, Norwalk, CT 06856); Model PCRS 510 postcolumn reactor (Applied Biosystems, Ramsey, NJ 07446); 10 cm × 8 mm Nova-Pak phenyl Radial-Pak cartridge housed in Model RCM-100 radial compression module (Waters-Millipore); and mobile phase of water–tetrahydrofuran (80 + 20). Fluorescence of aflatoxins B_1 and G_1 is enhanced by postcolumn derivatization with iodine. Detection limits for LC system: 0.1 ppb for aflatoxins B_1 and G_1 and 0.06 ppb for aflatoxins B_2 and G_2. In study of precision of method, coefficient of variation for total aflatoxins ranged from 2.7 to 4.2% (4).

Samples

To obtain quantity of naturally contaminated peanut extracts in critical contamination range (5–50 ppb), (80 + 20 v/v; methanol-water, 2mL/g) extracts of selected 1987 crop year farmers' stock peanuts were saved from another study. Initial LC analyses were performed on these extracts, and when results showed contamination in range of interest, ca 75 mL extract was stored in dark at 0°C in closed medicine bottle. Prior study had shown that naturally contaminated peanut extracts were stable for at least 3 months under these conditions (unpublished data). One hundred extracts were saved for ELISA study on this basis.

Each extract was divided in half and randomly assigned to 1 of 200 positions so that each sample was analyzed in duplicate by each ELISA method and by LC method. Duplicate analyses were not run consecutively to prevent biasing interpretation of results.

Results and Discussion

To ascertain the precision of the LC method used as the basis for comparison in this study, the coefficient of variation (CV) was calculated for each of the 100 duplicate analyses. Ten of the samples were negative for aflatoxin, and of the 90 containing aflatoxin, 17 produced the same result in both LC analyses (nearest whole ppb). Another 32 differed by 1 ppb. The mean of the 90 CVs (10 negative samples not included) was 5.5%, which indicated a low degree of variation in the LC method.

The variation within each ELISA method was about the same. In the cup test, duplicate analyses did not agree for 9 of the 100 samples. Eleven times of 100, the 2 results from the card test did not agree. The mean aflatoxin concentration of those samples was 7.0 and 9.9 ppb, respectively.

Results of the present study are summarized in Table 1, which shows the number of replications identified as positive and negative by the ELISA methods within 4 ranges of contamination. Also shown are the means of the CVs for the LC analyses within the given ranges. Although the methods supposedly had different detection thresholds, these data indicate that their actual performance was quite similar. Each method identified 95% of the samples containing no detectable aflatoxin as negative. Interestingly, the 2 ELISA methods identified the same replicate of the same negative sample as positive. In the 4–10 ppb range, the cup test identified 76% of the samples as negative and the card test identified 67% as negative. Each method identified >97% of the samples containing >10 ppb aflatoxin as positive. The card test could be considered in error on 32 of 34 replications in the 11–20 ppb range by identifying them as positive; however, the similarity in results of the 2 methods may simply indicate that the detection threshold for the card in this study was, in reality, closer to 10 ppb than 20 ppb.

A detailed presentation of the data for samples containing 4–23 ppb aflatoxin appears in Table 2. The cup test correctly identified samples contaminated at a concentration above the detection threshold for the assay (10 ppb) as positive. Most differences between the results of the LC method and those of the cup test as well as between the duplicate cup test analyses were associated with samples contaminated in the 5–10 ppb range.

Except for 1 replicate identified by LC analysis as contain-

Table 1. Numbers of positive and negative replications for each ELISA method within various ranges of aflatoxin contamination as determined by LC method[a]

Contamination range, ppb	No. of replications[b]	CV, %	Cup test		Card test	
			Pos.	Neg.	Pos.	Neg.
ND[c]	20	0	1	19	1	19
4–10	46	7.8	11	35	15	31
11–20	34	4.5	33	1	32	2
21–64	100	4.7	100	0	99	1

[a] Coefficient of variation (CV, %) is the mean of the individual CVs in the given range.

[b] Based on having the rounded mean of the LC analyses within the given range.

[c] No aflatoxin detected.

Table 2. Comparison of ELISA test results with LC results for each duplicate aflatoxin analysis of samples containing between 4 and 23 ppb aflatoxin[a]

LC method			Cup test[b]		Card test[c]	
Repl. 1	Repl. 2	CV, %	Repl. 1	Repl. 2	Repl. 1	Repl. 2
4	3	20.2	−	−	−	−
4	4	0	−	−	−	−
4	5	15.7	−	−	−	−
5	4	15.7	−	−	−	−
5	4	15.7	−	−	−	+
6	4	28.3	+	−	−	+
6	5	12.9	−	+	+	+
6	5	12.9	−	−	−	+
6	6	0	−	−	−	−
6	7	10.9	−	+	−	−
7	7	0	−	−	−	−
7	7	0	−	−	−	−
8	7	9.4	−	−	−	−
8	8	0	+	+	+	+
8	8	0	−	+	−	−
8	9	8.3	+	+	+	+
9	8	8.3	−	+	−	+
9	9	0	+	−	+	+
9	9	0	−	−	+	−
10	9	7.4	−	−	−	−
10	10	0	−	−	+	−
10	10	0	+	−	−	−
11	9	14.1	−	−	+	−
11	10	6.7	+	−	+	+
11	11	0	+	+	+	+
11	11	0	+	+	+	+
14	14	0	+	+	+	+
16	15	4.6	+	+	+	+
16	18	8.3	+	+	+	−
17	16	4.3	+	+	+	−
17	16	4.3	+	+	+	+
17	16	4.3	+	+	+	+
17	18	4.0	+	+	+	+
19	17	7.9	+	+	+	+
19	21	7.1	+	+	+	+
20	19	3.6	+	+	+	+
20	19	3.6	+	+	+	+
20	19	3.6	+	+	+	+
21	19	7.1	+	+	+	+
21	19	7.1	+	+	+	+
22	20	6.7	+	+	+	+
22	21	3.3	+	+	+	+
22	22	0	+	+	+	+
24	21	9.4	+	+	+	−

[a] Coefficients of variation (CV) are included for each of the duplicate LC analyses.
[b] + = ≥10 ppb; − = <10 ppb.
[c] + = ≥20 ppb; − = <20 ppb.

ing 21 ppb aflatoxin, the card test correctly identified all samples above its detection threshold of 20 ppb as positive. An examination of the data in Table 2 revealed that the card test identified most of the samples in the 10-20 ppb range as positive (≥20 ppb). If the detection threshold of the card test was actually closer to that of the cup test, or 10 ppb, the majority of the differences between the LC analyses and card test results and between duplicate card test analyses were also associated with samples contaminated in the 5–10 ppb range.

Because the antibodies of both assays are purported to react with aflatoxins B_1, B_2, and G_1, the LC analyses were scrutinized to determine the effect of cross-reactivity on the results obtained. Although 51% of the samples contained aflatoxin G_1, the concentrations were not high enough to have changed the result on the basis of cross-reactivity. In most samples containing aflatoxin G_1, the aflatoxin B_1 concentration was usually high enough to produce a positive result without the need of cross-reactivity with aflatoxins B_2 and G_1. Therefore, the data neither confirmed nor invalidated the assumption of cross-reactivity.

An aflatoxin screening program is designed to evaluate incoming farmers' stock peanuts so that positive loads can be properly diverted to oil stock or possible cleanup. A high degree of confidence in results is necessary since a load negative for aflatoxin would then be processed as edible food. Results from this study indicated that either the card or cup test would reliably (>95%) identify samples of peanuts containing >10 ppb of aflatoxin. Likewise, both methods were reliable (95%) in properly identifying samples that were negative for aflatoxin. Therefore, results of this study indicate the suitability of these assays for rapidly screening appropriately drawn samples for the presence of aflatoxin above a proscribed concentration.

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Determination of Aflatoxin Concentrations in Peanut Butter by Enzyme-Linked Immunosorbent Assay (ELISA): Study of Three Commercial ELISA Kits

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Sixteen United Kingdom analytical laboratories participated in an evaluation of 3 commercially available enzyme-linked immunosorbent assay (ELISA) kits for analysis of aflatoxin in peanut butter. Each laboratory was sent 3 sets of 10 randomly numbered samples of peanut butter. Each set consisted of 5 pairs of undisclosed duplicates. Four of the sets of duplicates were naturally contaminated butters with "target" aflatoxin values (estimated by liquid chromatography) between 8 and 81 μg/kg. The fifth pair was a blank peanut butter containing approximately 3 μg/kg of total aflatoxins. A statistical treatment of the results of the study is presented, together with discussion of the relative merits of the different kits.

A range of immunoassays has been developed for the analysis of several mycotoxins (1) including the aflatoxin group. Aflatoxins, the carcinogenic and teratogenic metabolites of *Aspergillus flavus* and *A. parasiticus*, can contaminate cereal crops and groundnuts and their products (2). The need for monitoring and control of aflatoxin levels in the food supply, with the consequent requirement for rapid screening of large numbers of samples, has encouraged the development of commercial ELISA kits for aflatoxin measurement.

Collaborative trials using ELISA techniques to quantify levels of aflatoxin B_1 in peanuts, corn, cottonseed and their products, and in mixed feed have been reported (3, 4). Interlaboratory studies are essential as a basis for evaluation of commercial kits and are part of the process of ensuring acceptance of the ELISA approach in the area of food contamination monitoring. This paper reports the evaluation of 3 commercially available ELISA kits manufactured in the United Kingdom but marketed worldwide for the analysis of naturally contaminated peanut butters for aflatoxin.

Analytical Study

Sixteen laboratories participated. In March 1988, each laboratory was sent one Biokits total aflatoxin kit, one Cambridge Life Sciences (CLS) Aflasure B kit, one May & Baker (M&B) Quantitox B_1 kit, 3 sets of 10 samples of peanut butter, the analytical protocol, and results sheets marked to be returned before May 1988. All laboratories received these in good condition.

Biokits Total Aflatoxin Kit

The Biokits assay, manufactured and supplied by Cortecs Diagnostics Ltd, is an indirect competitive ELISA for the quantitative determination of aflatoxins (B_1, B_2, G_1, and G_2) in nuts and other commodities at levels between 2.0 and 200.0 μg/kg. The kit consists of aflatoxin B_1 standards, aflatoxin-sensitized 96-well plate, monoclonal rat anti-aflatoxin antibody, peroxidase conjugate, and 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) substrate system plus dilution and wash buffer concentrates. Also included are a "blank" peanut meal preparation and 2 ng/mL spike solution for the preparation of internal (negative and positive) assay controls, results sheets, and graph paper. Samples are extracted using 5 mL/g of aqueous acetonitrile; standards and samples are assayed at least in duplicate (Biokits (1988) total aflatoxin assay kit for quantitative determination of total aflatoxin in nuts and nut products by enzyme immunoassay, Thames Genelink Ltd, Deeside, Clwyd CH5 2NT, U.K.).

Cambridge Life Sciences Aflasure B Kit

This kit is an immunodiagnostic kit for analysis of aflatoxin B_1 in foods. The antibody used has a 40% cross-reactivity with aflatoxin B_2. There is no reactivity to aflatoxins G_1 and G_2. The kit consists of a set of aflatoxin B_1 standards, 6 microwell strips (16 wells/strip), primary antibody, antibody conjugate, substrate reagents, washing solution concentrate, stopping solution, results sheet, and graph paper. The recommended extraction solvent is an acetonitrile–water system. It is recommended that standards and samples are assayed in duplicate (Cambridge Life Sciences (1988) Aflasure B, quantitative enzyme immunoassay for extracted aflatoxin B_1, CLS plc, Milton Rd, Cambridge CB4 4GN, U.K.).

May & Baker Quantitox B_1 Kit

This kit is a monoclonal antibody-based assay for the rapid colorimetric determination of aflatoxin B_1 only in food and feed commodities at concentrations ranging from 2 to 100 μg/kg. The kit consists of an uncontaminated ground maize reference standard, toxin-sensitized microstrips (12 × 8 wells), enzyme-antibody conjugate, various buffer concentrates, calibration table, and results sheet. The recommended extraction solvent is a water–methanol system. Duplicate analysis per sample is recommended for greater precision, although the manufacturers state that a single assay well per sample may be adequate for screening purposes (May & Baker Diagnostics (1988) Quantitox B_1, M&B Ltd, 187 George St, Glasgow G1 1YT, U.K.).

Peanut Butter Samples

Samples of peanut butter were prepared by mixing uncontaminated retail-purchased material with naturally contaminated smooth peanut butter (aflatoxin B_1 concentration approximately 200 μg/kg, assayed as described below). Uncontaminated butter (8 kg) was blended for 1 h to form sample 1. Sufficient quantity of butter 1 and the contaminated butter were blended for 1 h to make 1.55 kg each of samples 2, 3, 4, and 5. The blending procedure had been previously demonstrated (4), through the analysis of subsamples, to be sufficiently rigorous to produce homogeneous material.

Portions (10.0 ± 0.05 g) of the peanut butters were prepared and weighed into wide-neck plastic tubes. All samples were randomly coded with a 3-figure number. Each participant received 3 sets of 10 samples, which were 5 pairs of samples 1, 2, 3, 4, and 5 as blind duplicates.

Samples of each of the 5 peanut butters were analyzed for aflatoxin levels by using a post-column iodination liquid chromatographic (LC) method with immunoaffinity column

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Table 1. Order of analysis for each participating laboratory

Laboratory	Biokits	CLS	M&B
1 & 14	1st	2nd	3rd
2, 13, & 16	2nd	3rd	1st
3, 8, & 12	3rd	1st	2nd
4, 11, & 17	3rd	2nd	1st
5, 10, & 15	2nd	1st	3rd
6 & 9	1st	3rd	2nd

cleanup (5) of an acetonitrile–water peanut butter extract (manuscript in preparation).

Protocol

Each participant was instructed to carry out the study by using each of the 3 kits in the order given in Table 1. Each participant was provided with 3 report sheets requesting the concentrations of aflatoxin found, asking if the samples/kits arrived in good condition, and inviting comments regarding their opinion of the kits and the way the analyses were performed. Fourteen participants commented.

Experimental

Extraction of Peanut Butter

Each sample was extracted according to the instructions of the 3 kit manufacturers. Each recommended a different extraction procedure (detailed in Table 2), but each procedure took about the same amount of time (15–20 min). In the CLS Aflasure B procedure, extract filtering was optional; decantation or supernatant pipetting after settling could be used. Two dilutions were to be made for the M&B Quantitox B_1 kit.

Immunoassay

Immunoassay was carried out in microtiter plates using the 3 different procedures of the kit manufacturers, which are described in detail by the manufacturers. Table 3 summarizes the differences among the 3 procedures. The sample volume relates to both standards and extracts (with triplicate assays on each extract for Biokits and duplicates for the other 2 kits). Number of other additions relates to liquid additions (e.g., additions of conjugates or stopping solutions). Absorbance wavelength relates to that recommended for the microtitre plate reader. Approximate total time is time for the immunoassay only, using half the available wells, and has been evaluated by assuming sample/standard additions to the microtiter plate take 6 min, all other additions and washes take 2 min, and plate reading takes 5 min.

Calculation of Aflatoxin Concentrations

To assess aflatoxin concentrations in the Biokits and CLS procedures, a calibration curve is constructed by plotting the mean absorbance values of each of the 5 aflatoxin standards on semi-log graph paper.

In the M&B kit procedure, the mean absorbance values for the reference standard and the samples are obtained. The mean value for the reference standard is taken as 100%. The mean absorbances of the samples are expressed as a percentage of the reference standard. A calibration table provided is then used to convert the percentage values to the concentrations of aflatoxin present.

Table 2. Extraction conditions recommended by kit manufacturers

Condition	Biokits	CLS	M&B
Solvent system	acetonitrile–water	acetonitrile–water	methanol–water
Ratio (v/v)	50 + 50	60 + 40	55 + 45
Volume (mL/g)	5	2	5
Blending time (min)	2	min. of 2	1
Filter	recommended	optional	recommended
Dilution	1/25	1/20	1/10 & 1/50

Table 3. Comparison of immunoassay methodology of ELISA kits

Item	Biokits	CLS	M&B
Sample volume (μL)	50	75	100
Extract assay replication	X3	X2	X2
Number of other additions	4	4	3
Number of washes	2	2	1
Number of incubations	3	2	2
Absorbance wavelength (nm)	414	450	450
Recommendation for blanking plate reader	substrate blank well	air	air
Steps in assay	11	10	8
Approximate total assay time (min)	320	85	80

Results

Table 4 lists the concentrations of aflatoxins B_1, B_2, G_1, and G_2 present in peanut butter samples 1–5 as estimated by LC analysis. The coefficients of variation of the B_1 determinations are also shown in this table.

Tables 5–7 display the results produced by each of the 16 laboratories for the Biokits total aflatoxin kit, CLS Aflasure B kit, and M&B Quantitox B_1 kit, respectively. Also given (in Tables 5 and 6) are the number (n) of results not identified as outliers by the procedures described below.

Statistical Analysis of the Results

The statistical analysis of the analytical data produced by collaborators in the trial has proved to be difficult because a substantial number of "< x" or "> x" results were obtained for all 3 kits and because results were reported within ranges for the M&B kit. There are no agreed procedures on how such data should be treated. The approach that was taken to produce statistical data was as follows: Where "< x" and "> x" values have been reported, such values have been treated as being "= x". Where a range has been reported, the midpoint of that range has been used to produce a data point for statistical evaluation as described below.

It was decided that, because only a limited proportion of the results for the Biokits and CLS kits were "< x" or "> x" values, the results reported (in Tables 5 and 6) could be examined for evidence of individual systematic error ($P <$

Table 4. Aflatoxin concentrations (μg/kg) of peanut butter samples determined by LC analysis

	Sample				
Aflatoxin	1	2	3	4	5
B_1	2.0	8.5	15.0	40.0	65.0
B_2	1.0	1.5	2.6	5.9	9.5
G_1	0.0	2.2	3.6	4.1	6.5
G_2	0.0	0.0	0.2	0.0	0.0
Total B	3.0	10.0	17.6	45.9	74.5
Total B + G	3.0	12.2	21.4	50.0	81.0
CV, % (B_1)	3.5	9.1	6.0	14.8	5.0
n	8	3	4	3	3

Table 5. Collaborative results for total aflatoxin (μg/kg) determined by Biokits assay[a]

	Sample									
Lab.	1		2		3		4		5	
1	5.8	6.2	13.6	19	45	39	81	60	81	120
2	2.5	5.6	22	15	50	37	80	100	120	150
3	<2	<2	12	8	30	23	69	73	103	79
4	5	6	24	18	34	38	90	90	120	140
5	13.5[b]	15.5[b]	33	30	125[b]	92[b]	135	110	>200	135
6	2.7	<2	10.8	<2	23	<2	88	47	108	5
8	3.1	2.7	13.5	8.2	25.5	58	75	96	25.5	130
9	2.1	4.3	16	14	40	37	130	95	140	130
10	4	4	15	16	29	28	49	50	90	90
11	4.4	3.6	17	16.25	29	34	92.8	73.75	134	114
12	7.8	4.6	18	24	40	39	68	64	75	75
13	3.5	7	12	13	28	32	70	55	115	90
14	2	3.3	11.2	12.5	30.3	16	100	60	100	140
15	3.1	4.1	18.5	27.6	32	36	14.75	68	25.5	120
16	4.4	4.5	17.1	17.7	36	34	101	113	159	144
17	6	9	20	25	45[c]	100[c]	140	170	200	130
Mean	4.2		16.9		32.7		84.6		112.2	
r	3.7		9.7		24.8		48.4		107.7	
R	5.3		18.5		29.4		87.5		122.5	
SD	1.87		6.47		10.32		30.71		43.13	
CV, %	44.5		38.3		31.6		36.3		38.4	
n	30		32		28		32		32	

[a] Values reported as "< x" or "> x" equated to "= x."
[b] Result rejected as outlier by Dixon's test ($P < 0.05$); not used in calculations of statistical parameters.
[c] Result rejected as outlier by Cochran's test ($P < 0.05$); not used in calculations of statistical parameters.

0.05) using Cochran's and Dixon's tests progressively, by procedures described by the British Standards Institution (6). Outlying results are identified in the tables and were not used in any subsequent calculations for means, repeatabilities (r), reproducibilities (R), standard deviations (SD), and coefficients of variation (%CV) as defined by the procedures given by the British Standards Institution (6). The values are given in Tables 5 and 6. The within-laboratory variance ratios (r_1^2/r_2^2) and between-laboratory variance ratios $(2R_1^2 - r_1^2)/(2R_2^2 - r_2^2)$ were also calculated from the results obtained for these kits.

All the results for the May and Baker kit were either in ranges or "< x and > x," so we decided to produce only a simple "mean" of all the results by considering "< x" and "> x" as "= x" and by treating ranges as their mid-point ("x to y" = "x/2 + y/2"). We decided that any other statistical

Table 6. Collaborative results for aflatoxins $B_1 + B_2$ (μg/kg) determined by CLS assay[a]

	Sample									
Lab.	1		2		3		4		5	
1	2	4.8	19	12.8	64	40	100	124	>200	156
2	3.4	5.5	35	70	125	125	175	180	>200	>200
3	7	10	50	42	44	110	75	88	112	132
4	3	5	12	19	29	40	72	72	80	120
5	6.8	6.4	11.6	10.8	14	20.7	66.7	51	104	122
6	>200	1.3[b]	>200[b]	8.8[b]	>200[b]	14.8[b]	>124[b]	20.0[b]	<200	>120
8	2.5	5.6	11.2	20.8	50	32.8	92	78	184	196
9	13.4	6.6	37.2	76	110	128	>200	>200	98	>200
10	46[b]	21[b]	1	18	1	20	35	98	68	76
11	6.8[b]	45[b]	78[b]	19[b]	124	48	6.6	75	190	>200
12	4.6	0	22.4	15.2	50	33.6	88	92	140	176
13	7.8	8.4	25.6	34.4	46	38	>200	>200	>200	>200
14	2.7	12.2	14.5	11.2	20	17.2	36	42	92	142
15	3.3	8.8	9.6[c]	—	28.8	33.2	104	>100	>100	>100
16	4.5	3.6	19	26	27	42	91	94	191	162
17	1.3	0.5	8.9	1.5	15	2.7	160	117	>200	>200
Mean	5.4		24.1		49.3		103.7		151.9	
r	8.3		32.9		59.0		56.1		80.7	
R	9.5		53.3		111.4		154.5		133.0	
SD	3.4		18.6		38.9		53.8		46.5	
CV, %	62.7		77.2		78.9		51.9		30.6	
n	26		26		30		30		32	

[a] Values reported as "< x" or "> x" equated to "= x."
[b] Result rejected as outlier by Cochran's test ($P < 0.05$); not used in calculations of statistical parameters.
[c] Results reported as single figure, not used in calculations.

Table 7. Collaborative results for aflatoxin B_1 (μg/kg) determined by M&B assay[a]

	Sample									
Lab.	1		2		3		4		5	
1	0–5	0–5	6–20	6–20	21–50	21–50	21–50	21–50	>100	51–100
2	2–5	6–20	21–50	21–50	51–100	21–50	>100	>100	>100	>100
3	0	0	11–20	6–10	>20	>20	>20	>20	>20	>20
4	<5	2–5	6–10	6–10	11–20	11–20	51–100	51–100	>100	>100
5	6–20	6–20	6–20	6–20	51–100	6–20	0–5	21–50	51–100	51–100
6	0	0	6–10	6–10	6–10	6–10	11–20	>20	>20	>20
8	2–5	0	6–10	6–10	11–20	11–50	21–50	21–50	51–100	51–100
9	6–20	0–5	6–20	6–20	21–50	21–50	>100	>100	>100	>100
10	2–5	2–5	6–20	6–20	21–50	6–20	21–50	21–50	51–100	51–100
11	0	0–5	6–10	2–20	6–20	21–50	51–100	6–50	6–10	21–100
12	0	0	6–10	6–10	>20	11–20	>20	>20	>20	>20
13	2–5	2–5	6–10	6–10	6–10	6–20	21–50	21–50	21–50	>100
14	2–5	2–5	11–20	6–10	6–20	6–20	51–100	0–5	>100	51–100
15	0–5	0–5	6–20	6–20	21–50	21–50	51–100	>100	>100	>100
16	0	0/2–5	6–20	6–20	21–50	>100	>100	51–100	51–100	51–100
17	0–5	0–5	6–20	6–20	21–50	6–20	6–20	51–100	21–50	21–50
Simple "mean"	3.8		12.5		28.3		54.4		76.9	

[a] Values reported as "< x" or "> x" equated to "= x." Ranges equated to mid-point (x to y = x/2 + y/2).

procedures would be inappropriate; any conclusions based on these would probably be invalid.

Results and Discussion

Concentrations of the 4 aflatoxins present in the peanut butters were determined by LC analysis and are given in Table 4. About 80% of the aflatoxin contamination was due to aflatoxin B_1 and about 10% of the aflatoxin concentration was due to each of aflatoxins B_2 and G_1; there was only a trace of aflatoxin G_2. The aflatoxin specificity of each of the 3 immunoassay kits is different; the Biokits total aflatoxin kit assays concentrations of aflatoxins B_1, B_2, G_1, and G_2, the CLS Aflasure B kit assays aflatoxins B_1 and B_2, and the M&B Quantitox B_1 kit assays aflatoxin B_1 alone. The relative amounts of the different aflatoxins must be taken into consideration when the performance of one kit is compared with another. It is apparent that the Biokits and CLS immunoassay kits gave higher mean values of aflatoxin present, calculated from the data from the 16 participating laboratories, than the levels obtained by LC analysis. The simple "mean" values obtained from the May and Baker kit were also higher. In this paper, the LC estimation is not assumed to have provided the correct aflatoxin concentrations to which the ELISA results are to be compared. Liquid chromatography was used to provide details of sample homogeneity and the relative amounts of the different aflatoxins present.

Comparison of Mean Values

The mean values for all 5 samples for the Biokits total aflatoxin kit are closer to the LC mean values than are the equivalent mean values obtained from the CLS kit. For the 5 samples, the Biokit mean value is 1.4–1.7 times higher than the LC total aflatoxin value; for the CLS Aflasure B kit, the corresponding figures are 1.8–2.8 times the aflatoxin B_1 and B_2 level. However, comparison of the results using a null-hypothesis *t*-test suggests that the mean values for the Biokit and CLS Aflasure kits are not significantly different from each other except in the case of sample 5. This is probably because the inherent variability of the results masks any mean differences. In the case of the M&B Quantitox B_1 kit, where it is difficult to make a direct comparison of results with the other kit results, the ratios of the ELISA simple "means" to aflatoxin B_1 LC values appear to be higher than the equivalent Biokits figures for samples 1–3 but lower for samples 4 and 5.

Comparison of Precision Values

It is possible to compare the precision values obtained from the Biokits and CLS Aflasure B kits on a sample-by-sample basis. For most samples, the repeatability and reproducibility values are significantly different ($P < 0.05$) and normally less (i.e., more precise) for the Biokits results. Even where this is not so, i.e., in the case of sample 5, this may be due to the calculation procedure used with results reported as ">." For this sample, 10 CLS kit values for sample 5 were reported by participants as >200 and were treated as being equal to 200; only one >200 value was reported for the Biokits kit.

Comments of the Participants

Participants were asked to comment about the performance of the kits or any modifications from the protocol they were forced to make.

Three participants said that the M&B Quantitox B_1 kit instructions were confusing but a further 3 participants particularly noted that this kit was the quickest to use. Two participants said that they considered that the kit should be considered as semiquantitative because results are only given within ranges. One laboratory noted that the kit relies on the availability of a completely aflatoxin-free blank for accurate determination of results.

Regarding the CLS Aflasure B kit, 2 laboratories noted that the instructions were easy to follow. The main criticism of the method, by 5 participants, was that the extraction volume, at 20 mL per 10 g peanut butter, was too small.

Three participants commented that the Biokits total aflatoxin kit gave the best performance of the 3 kits tested. Two participants drew attention to the positive and negative controls provided, which act as an internal quality control. Two participants thought the instructions were poorly presented with too much detail. One laboratory did not like the long incubation periods. Four participants did not have a 414 nm filter on their plate readers and substituted the recommended wavelength by 405 or 410 nm.

Conclusion

The reproducibility values for the total aflatoxin Biokits and CLS Aflasure B kits were of the same order as the mean concentrations being determined. Results reported from 8% of the analyses for the Biokits kit and 20% of the analyses for the CLS kit had to be removed from further statistical considerations as outliers. Therefore, we conclude that, for screening purposes, single extraction and assay of samples would not be acceptable.

Analysis of the M&B Quantitox B_1 kit results was difficult because concentrations were reported within ranges. However, since some of the ranges straddle U.K. statutory limits (7), the test should be reformulated before it is used as a screening procedure.

The results of this trial indicate that the kit procedures may be useful as screening procedures but not as methods suitable to obtain results on which enforcement action may be taken.

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PESTICIDES AND INDUSTRIAL CHEMICAL RESIDUES

Determination of Linuron in Potatoes Using Capillary Column Gas Chromatography/Mass Spectrometry

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A convenient method for the determination of the *N*-methyl,*N*-methoxy-phenylurea herbicide (linuron) in potatoes has been developed. The herbicide is extracted from potatoes using a slightly modified Luke multiresidue procedure. The extract is analyzed directly by gas chromatography with cold on-column injection, using an ion trap mass spectrometer in the chemical ionization mode as the detector. Quantitation is performed using *p*-bromonitrobenzene as the internal standard. The limit of detection is 0.1 ppm. Recoveries of linuron in potatoes averaged 112 ± 6% at the 0.5 ppm level, and 110 ± 2% at the 0.2 ppm level. No linuron residues were found in 25 potato samples that were analyzed by this method. Two other *N*-methyl,*N*-methoxy-phenylurea herbicides, metobromuron and chlorbromuron, are also sufficiently stable to be determined by this method, but the *N*,*N*-dialkyl-phenylurea herbicides neburon, diuron, and monuron are too thermally unstable and degrade in the gas chromatograph.

Linuron is considered a "possible human carcinogen" by the U.S. Environmental Protection Agency (EPA) (1), which has a tolerance level of 1 ppm for linuron in potatoes. The thermal instability of phenylurea herbicides makes it difficult to analyze them by gas chromatography because of degradation to their respective isocyanates upon heating (2). Luchtefeld (3) obtained good recoveries for 6 phenylurea herbicides by using liquid chromatography (LC) with post-column photodegradation followed by derivatization. Zahnow (4) obtained good recoveries for diuron, linuron, and neburon by using LC and a photoconductivity detector. Both methods, however, specify extraction procedures different from currently used multiresidue extraction procedures. We have developed a method for linuron which uses a slightly modified version of the Luke extraction procedure (5) with gas chromatography/mass spectrometry (GC/MS) as the determinative step. This multiresidue extraction is one of the most commonly used procedures by the U.S. Food and Drug Administration.

Experimental

Reagents

Linuron (3-[3,4-dichlorophenyl]-1-methyl-1-methoxyurea), metobromuron (3-*p*-bromophenyl]-1-methyl-1-methoxyurea), chlorbromuron (3-[4-bromo-3-chlorophenyl]-1-methyl-1-methoxyurea), neburon (1-*n*-butyl-3-[3,4-dichlorophenyl]-1-methylurea), diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea), and monuron (3-*p*-chlorophenyl-1,1-dimethylurea) reference standards were supplied by EPA (Research Triangle Park, NC). Sodium chloride, anhydrous sodium sulfate, and high purity LC grade acetone, petroleum ether, and dichloromethane were purchased from Fisher Scientific Co. (Springfield, NJ). *p*-Bromonitrobenzene was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Samples

The potatoes analyzed in this study consisted of 13 samples (8 Atlantic White and 5 Superior White) collected from various farms in New Jersey, 9 Red from Florida, and 3 Standard White from California. The out-of-state varieties were obtained from supermarket warehouses in New Jersey. The untreated red potato sample used for the fortification studies was obtained from the Farsouth Growers Cooperative Association, Homestead, FL.

Instrumentation

A Varian Model 3400 gas chromatograph (Varian Associates, Walnut Creek, CA) was interfaced to a Finnigan MAT ion trap detector (Finnigan MAT, San Jose, CA) equipped with chemical ionization and controlled by an IBM PC/AT. The analyses and quantitation were performed with Finnigan Ion Trap Software Version 3.15. A splitless on-column injector held at 50°C was used, and a 2 m × 0.53 mm id deactivated fused silica pre-column was fitted between the injector and capillary column.

A 15 m × 0.25 mm id J&W DB-1 fused silica capillary column (1 μm film thickness) was temperature programmed from 50 to 300°C at 15°/min. Helium carrier gas was used at a velocity of 25 cm/s and the injection volume was 1 μL. A 15 cm syringe was used for on-column injections.

The mass spectrometer was operated in the chemical ionization mode using methane reagent at a pressure that gave a 10:1 ratio for m/z 17 to 16. The filament voltage and current were 70 eV and 80 μA, respectively. Electron multiplier gain was 10^5. Scan range was 70 to 320 atomic mass units at 1 s/scan. Transfer line and manifold temperatures were 250 and 220°C, respectively.

Preparation of Calibration Curve

Ten mg linuron reference standard was dissolved in acetone to give 10 mL of a 1 mg/mL stock solution. The stock solution was then diluted, and the appropriate amount of a *p*-bromonitrobenzene internal standard stock solution (2.5 mg/mL in acetone) was added. These standard solutions then contained 2, 5, 10, 20, and 27 ng/μL of linuron and 25 ng/μL of *p*-bromonitrobenzene. These solutions were analyzed by GC/MS with triplicate injections for each concentration level. The resulting calibration curve was used for all calculations.

Sample Analysis

The Luke procedure (5) was followed exactly up to the step where petroleum ether and acetone are added through the Snyder column, and the solution is reconcentrated. Instead, 50 mL of dichloromethane was added through the Snyder column and the solution was reconcentrated to approximately 2 mL. Then, 40 μL of the *p*-bromonitrobenzene stock solution (2.5 mg/mL) was added and the final volume was

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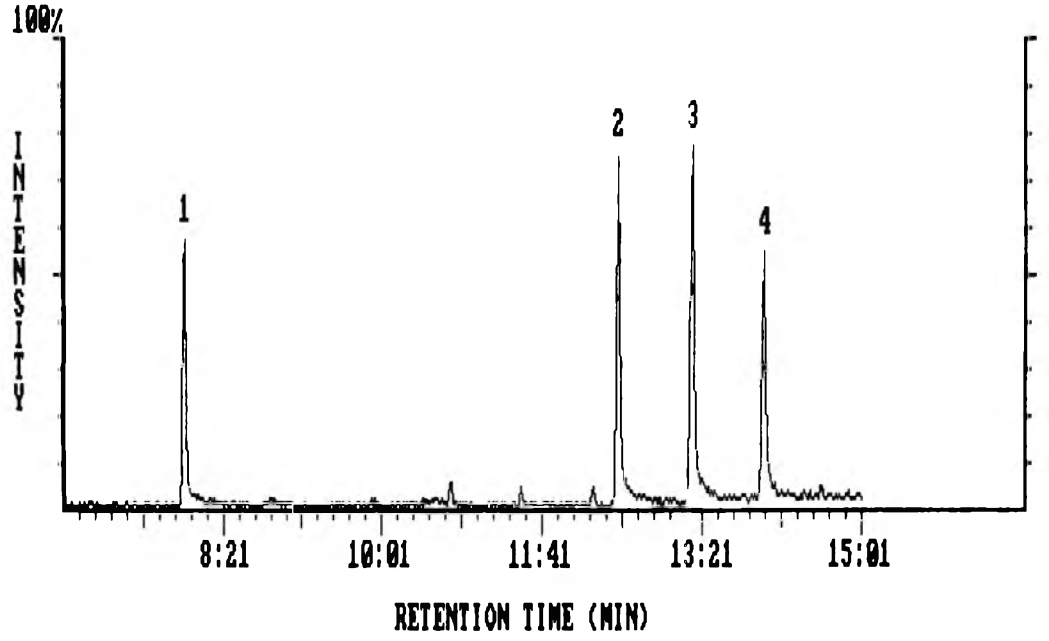

Figure 1. Total ion chromatogram of standard solution containing 25 ng/μL each of *p*-bromonitrobenzene (1), metobromuron (2), linuron (3), and chlorbromuron (4).

adjusted to 4 mL. These samples were then analyzed by GC/MS.

For the recovery studies, appropriate amounts of the linuron stock solution were added to chopped potatoes prior to extraction of the samples. The calculations were performed by the instrument software, where the relative response factor (area of m/z 249 for linuron divided by the area of m/z 202 for *p*-bromonitrobenzene internal standard) is applied to the calibration curve and the amount of linuron (ng/μL) is calculated. The amounts of linuron (ppm) in the potatoes were then calculated as in the Luke procedure (5).

Results

The chromatographic separation of a 25 ng/μL standard solution of 3 *N*-methyl,*N*-methoxy-phenylurea herbicides and the *p*-bromonitrobenzene internal standard is presented in Figure 1. The chemical ionization mass spectrum of linuron (M + 1 = 249; M + 3 = 251) is shown in Figure 2. Figure 3 shows the calibration curve for linuron, where the amount of linuron (ng) is plotted against the relative response factor. Figure 4 shows the mass chromatograms of linuron (m/z 249) and 3,4-dichlorophenyl isocyanate (m/z 188), demonstrating that very little, if any, thermal degradation occurs under our analytical conditions. Similar results (not shown) were obtained for 2 other *N*-methyl,*N*-methoxyphenylurea herbicides, metobromuron and chlorbromuron. In contrast, the *N*,*N*-dialkyl-phenylurea herbicides thermally degrade under these conditions, as demonstrated by the mass chromatograms obtained from the GC/MS analysis of neburon (Figure 5). No neburon peak (m/z 275) was observed due to decomposition to 3,4-dichlorophenyl isocyanate (m/z 188) and *N*-methyl butylamine (m/z 88).

The total ion and mass chromatograms (m/z 249 and 251) obtained from the analyses of potatoes spiked with 0.5 ppm

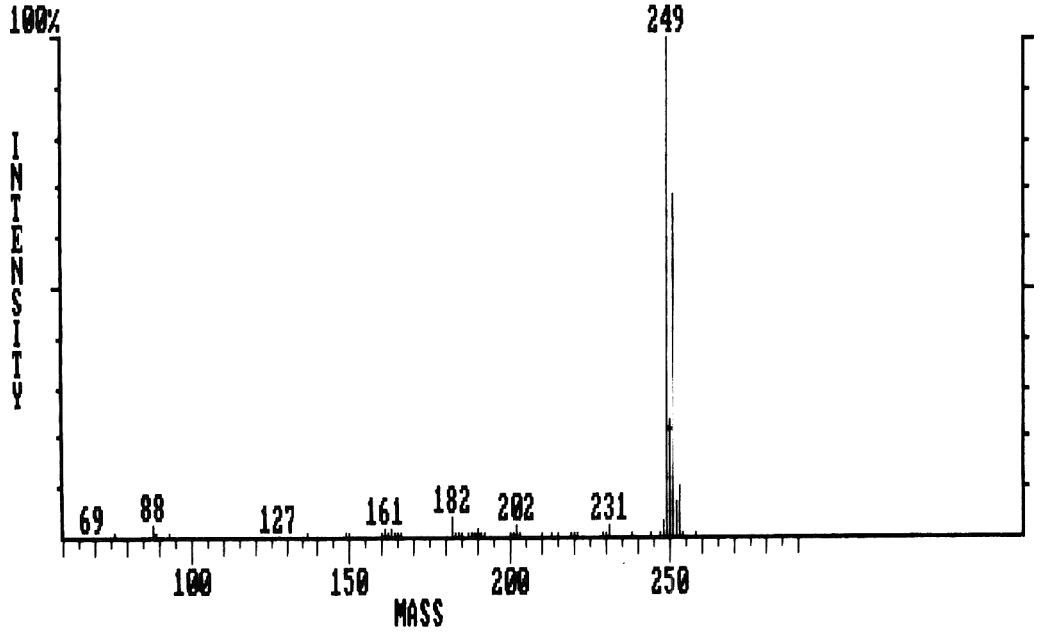

Figure 2. Chemical ionization mass spectrum of linuron (M + 1 = 249; M + 3 = 251).

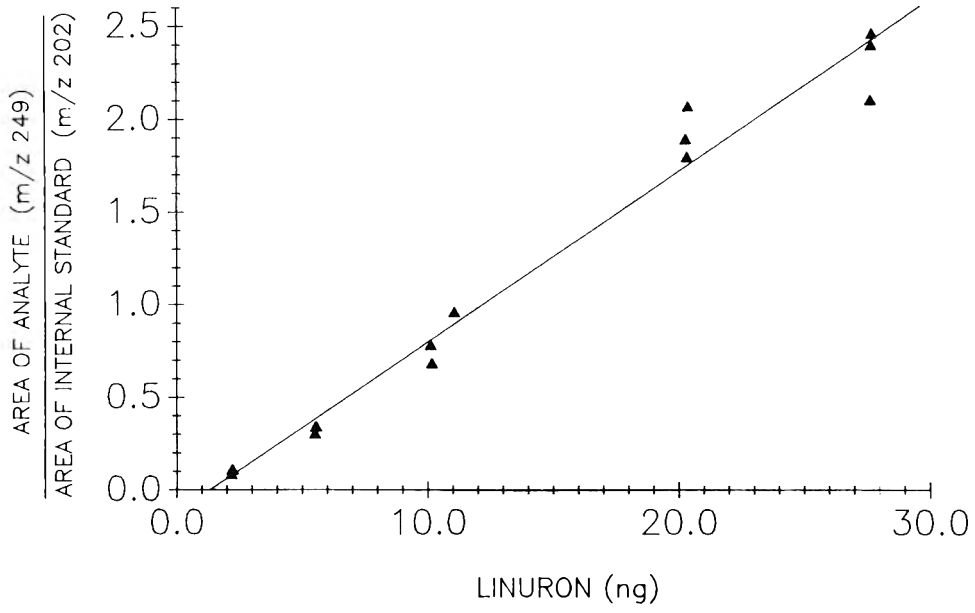

Figure 3. Calibration curve for amounts of linuron plotted against response factors.

and 0.2 ppm linuron are shown in Figures 6 and 7, respectively (retention time of linuron is 13:30 min). The large GC peaks in the total ion chromatograms consist almost entirely of materials from the potato extract. The linuron peak appears as a small shoulder on the front of the peak.

Recovery studies were performed in triplicate in the red potatoes, and gave values of 116, 115, and 106% (average = 112 ± 6%) at the 0.5 ppm level and 111, 110, and 108% (average = 110 ± 2%) at the 0.2 ppm level. About half of the positive error in the recoveries at the 0.2 ppm level is due to small contributions from chemical background at m/z 249 at the retention time of linuron. The limit of quantitation was 0.2 ppm, and the chromatographic peak for linuron became indistinguishable from background noise below 0.1 ppm (limit of detection). No linuron residues were found in the 25 potato samples that were analyzed.

Discussion

We have demonstrated that linuron can be quantified at the 0.2 ppm level in potatoes by using cold on-column injection and chemical ionization mass spectrometry. Two other *N*-methyl,*N*-methoxy-phenylurea herbicides, metobromuron and chlorbromuron, can probably be determined by this method, as well. However, no quantitative recovery studies were performed for these herbicides. Unfortunately, the *N*,*N*-dialkyl-phenylurea herbicides monuron, diuron, and neburon are too thermally unstable to be analyzed by this procedure.

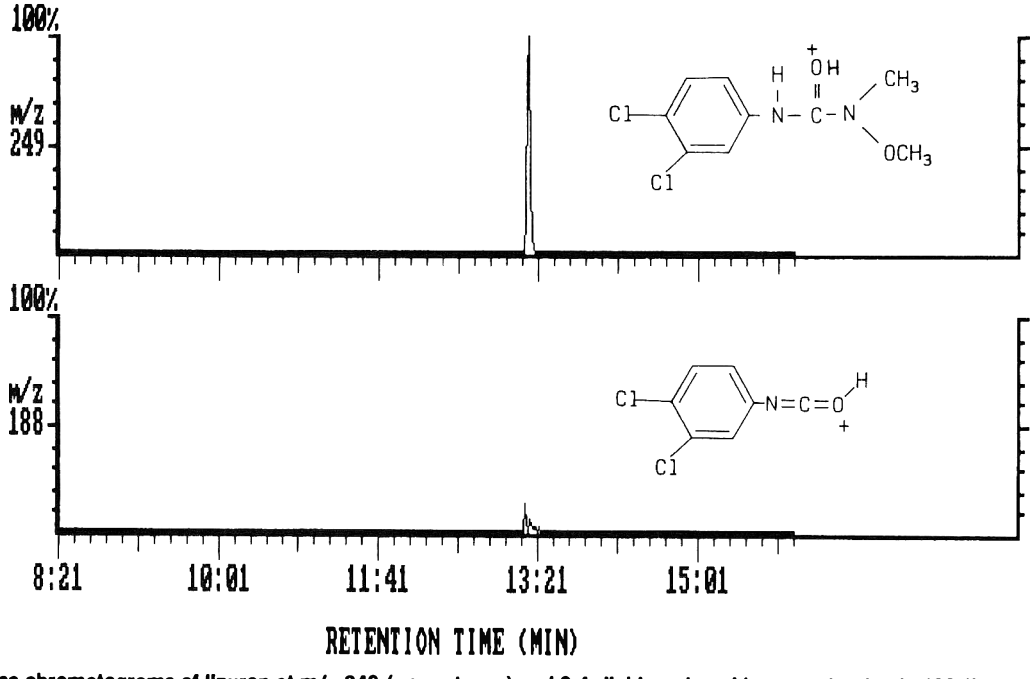

Figure 4. Mass chromatograms of linuron at m/z 249 (upper trace) and 3,4-dichlorophenyl isocyanate at m/z 188 (lower trace) obtained after injection of linuron.

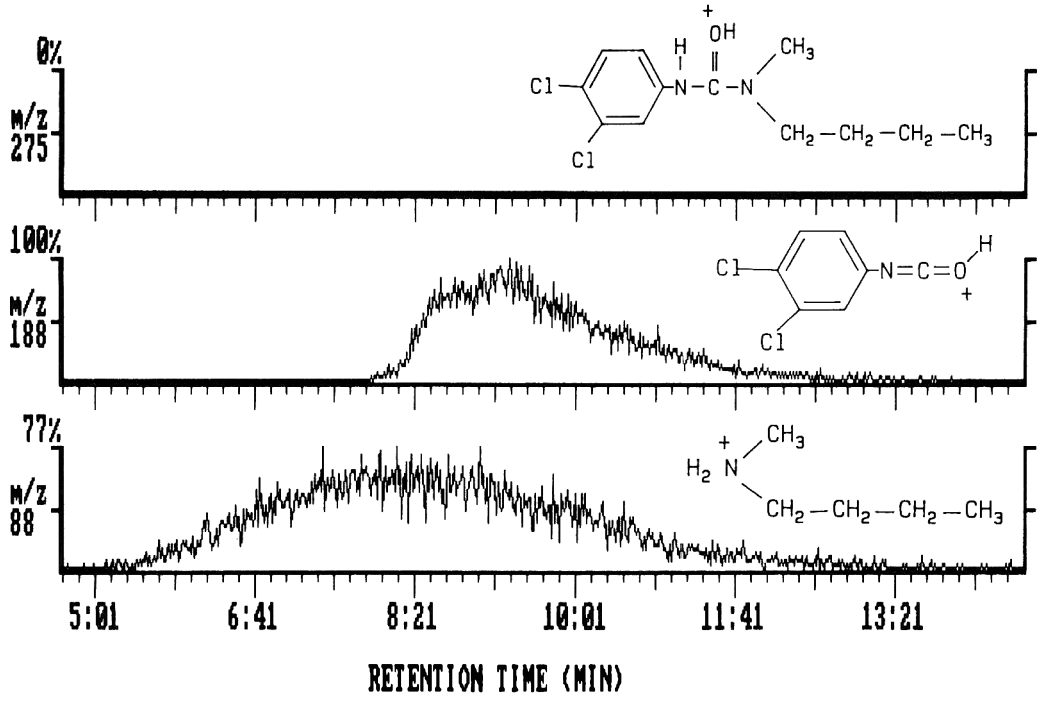

Figure 5. Mass chromatograms of neburon at m/z 275 (upper trace), 3,4-dichlorophenyl isocyanate at m/z 188 (middle trace), and *N*-methyl butylamine at m/z 88 (lower trace) obtained after injection of neburon.

The use of on-column injection is excellent for less volatile and thermally labile materials. With this type of injection, the long syringe needle deposits the sample into the column, bypassing the injection port. Thermal degradation of the materials is minimized because they are not flash-evaporated onto the column, but are volatilized as a result of temperature programming for the GC oven. The use of a megabore pre-column between the injector and column facilitates the use of on-column injection. It can easily be removed when nonvolatile sample components accumulate at the head of the column. Also, a wider syringe needle can be used because of the larger size of the column. We normally cut approximately 45 cm from the pre-column for every 5 samples injected, and a new 2 m length of column is replaced after about 20 samples. The column is then placed directly back into the injector. There is no optimum length for the pre-column, but it should be greater than approximately 50 cm.

The final step of the Luke extraction procedure was eliminated from our analyses because it was not necessary for us to rid the samples of dichloromethane, since we did not use a halogen-specific detector.

Our method for the analysis of linuron in potatoes is rapid

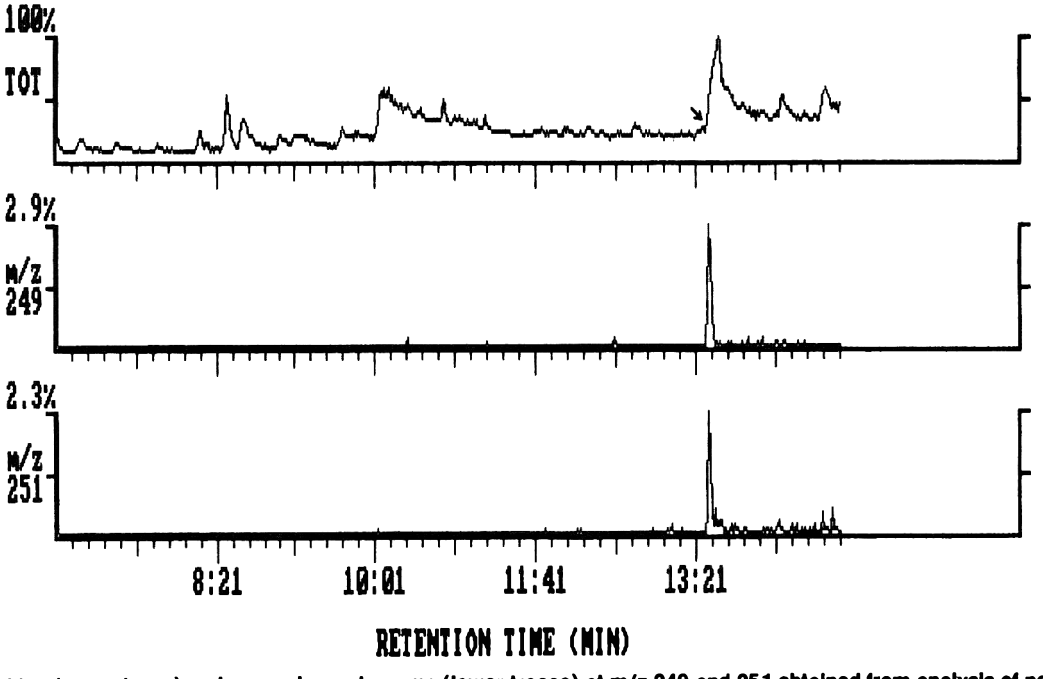

Figure 6. Total ion (upper trace) and mass chromatograms (lower traces) at m/z 249 and 251 obtained from analysis of potatoes spiked with 0.5 ppm linuron (retention time of linuron is 13:30).

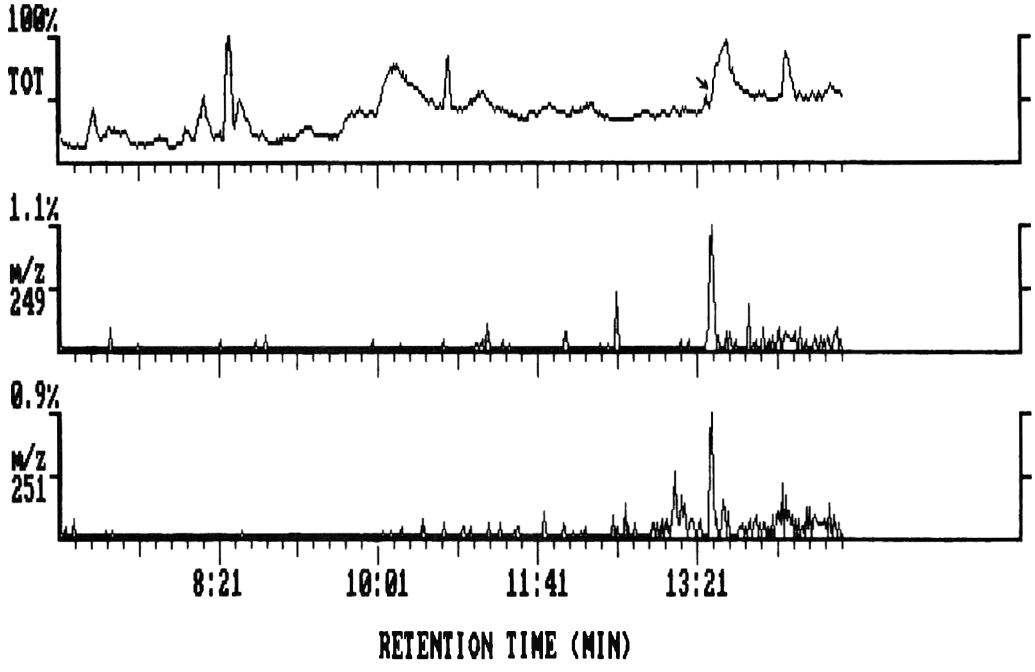

Figure 7. Total ion (upper trace) and mass chromatograms (lower traces) at m/z 249 and 251 obtained from analysis of potatoes spiked with 0.2 ppm linuron.

and gives good recoveries with reasonable precision. It requires no sample cleanup or derivatization steps and can be incorporated into the Luke multiresidue procedure.

Acknowledgments

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Liquid Chromatographic-Electrochemical Determination of Ethylenethiourea in Foods by Revised Official Method

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AOAC official method 29.119–29.125 was revised to determine ethylenethiourea (ETU) directly by a liquid chromatographic-electrochemical (LC-EC) determinative technique and to improve ETU recovery. ETU is extracted from food products with a methanol–aqueous sodium acetate solution. A portion of the concentrated filtrate is added to a column of diatomaceous earth, and ETU is eluted with 2% methanol in methylene chloride to separate it from food coextractives, which are retained on the column. The eluate is collected in a siliconized flask and evaporated, the residue is dissolved in water, and 20 μL of solution is injected onto an LC graphitized carbon column. ETU is eluted from the LC column with a mobile phase of acetonitrile–aqueous 0.1M phosphoric acid–water (5 + 25 + 70), and the eluted ETU is detected by using an amperometric electrochemical detector equipped with a gold/mercury working electrode. Recovery data were obtained by fortifying 13 food products with ETU: baked potatoes; canned applesauce, mushrooms, creamed spinach, green beans, spinach, and tomatoes; cooked fresh cabbage and frozen collards; fresh celery and lettuce; grape jelly; and powdered sugar cake donuts. Raw celery was found to cause low ETU recoveries. Average percent recoveries of ETU from the other 12 food products were 92 with a standard deviation of 12 for the low (0.05 and 0.1 ppm) fortification levels and 90 with a standard deviation of 6 for the higher (0.5 and 1 ppm) fortification levels. The limits of quantitation were 0.01 and 0.02 ppm for food products with low and high sugar content, respectively.

Ethylenethiourea (ETU) has been shown to produce thyroid cancers in rats, and when ETU was fed orally to mice, an increase in liver tumors was observed (1). The U.S. Department of Health and Human Services has listed ETU as one of the "substances that may reasonably be anticipated to be" a carcinogen (1).

ETU is used as an accelerator in the vulcanization of various elastomers, including neoprene and polyacrylate rubber. ETU is also a degradation product of the ethylenebisdithiocarbamate fungicides, i.e., mancozeb, maneb, metiram, and zineb. Over 130 million pounds of these fungicides are used yearly on agricultural crops throughout the world (2). Thus, the levels of ETU residues in agricultural crops and food products need to be determined.

In a review of procedures available for the determination of ETU residues, Bottomley et al. (3) cited 7 thin-layer chromatographic (TLC), 23 gas chromatographic (GC), and 11 liquid chromatographic (LC) methods. TLC generally does not provide the desired precision for the quantitation of ETU. GC is less than satisfactory because ETU generally chromatographs poorly on GC columns unless a derivative is formed. Unfortunately, food coextractives can adversely affect the reaction yield of such derivatives, causing low and erratic ETU recoveries. LC produces a sharp symmetrical chromatographic peak for ETU without the requirement, and disadvantages, of forming a derivative. An LC-electrochemical (EC) technique that provides the selectivity and sensitivity needed to determine ETU residues was recently reported by our laboratory (4).

The AOAC official method for ETU **29.119–29.125** (5) has been found by several investigators and this author to give low and erratic recovery of ETU (6; personal communication, J. C. Underwood, Kansas City District Laboratory, Food and Drug Administration, 1988). This method contains a procedure in which ETU is derivatized before GC determination. The present study was undertaken to determine ETU selectively without derivatization, and to increase ETU recoveries and improve their consistency. Therefore, the object of the present study was to revise the AOAC official method by taking into account recent published information and determinative techniques.

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METHOD

Reagents

(a) *Aluminum oxide.*—Fisher No. A-540 "Alumina, Adsorption," 80–200 mesh (Fisher Scientific, Pittsburgh, PA 15219).

(b) *Antifoam B solution (1%).*—Dilute Dow Corning Antifoam B emulsion (10%) (Dow Corning Corp., Midland, MI 48640) with water (1 + 9).

(c) *Solvents.*—Acetonitrile (UV grade), methanol, methylene chloride, and water. All distilled-in-glass grade (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442). Use distilled-in-glass grade wherever a solvent, including water, is required.

(d) *ETU standard solution.*—Dissolve ETU standard (Environmental Protection Agency, Research Triangle Park, NC 27711) in water to give concentration of 0.25 μg/mL, or other concentrations as needed. Store solution in actinic glassware. When not in use, store in refrigerator.

(e) *Partitioning eluant.*—2% methanol in methylene chloride. Pipet 20 mL methanol into graduated cylinder. Dilute to 1 L with methylene chloride, and mix.

(f) *Filter aid.*—Celite 545 (Manville Sales Corp., PO Box 519, Lompoc, CA 93438). Do not acid-wash; do not use acid-washed grade.

(g) *Siliconizing solution.*—Dilute "SurfaSil Siliconizing Fluid" (Pierce Chemical Co., Rockford, IL 61105) with methylene chloride (1 + 9).

(h) *Phosphoric acid solution.*—0.1M. Pipet 7 mL 85% LC grade phosphoric acid (Fisher Scientific) into 1 L graduated cylinder containing water. Dilute to volume with water, and mix.

(i) *Solid liquid/liquid column support.*—Gas-Chrom S, 45–60 mesh (Alltech Associates, Inc./Applied Science Labs, Deerfield, IL 60015). Use as is.

General Apparatus

(a) *Blender.*—Explosion-proof Waring Laboratory Blendor.

(b) *Chromatographic tubes.*—30 cm × 22 mm id Chromaflex column (size 233) with coarse porosity frit and Varibor stopcock (size 2) (No. K-420540-9042, Kontes, Vineland, NJ 08360).

(c) *Siliconized round-bottom flasks.*—Siliconize flasks in well-ventilated hood and wear rubber gloves and eye protection. Add 50 mL siliconizing solution to each new 500 mL T 24/40 round-bottom flask. Stopper and shake flask; remove stopper after shaking flask to release pressure. Shake flask 2 additional times to ensure complete siliconizing of glass surface. Pour siliconizing solution into waste container. Let flask air-dry and then cure flask surface 30 min in 105°C

Table 1. Size of analytical portion used for extraction

Food product	Weight, g
Applesauce, canned, sweetened	50
Cabbage, fresh, boiled	100
Celery, fresh	100
Collards, frozen, boiled	100
Donuts, cake, powdered sugar-coated	50
Grape jelly	50
Green beans, canned	100
Lettuce, fresh	100
Mushrooms, canned	100
Potatoes, baked	100
Spinach, canned	100
Spinach, canned, creamed, baby food	100
Tomatoes, canned	100

oven. Let flask come to room temperature. Wash flask with methylene chloride to remove unreacted SurfaSil and reaction by-products. (*Note*: After use, simply rinse flask with methanol followed by methylene chloride and then air-dry before reuse. If flask needs to be cleaned with soap and water and/or scrub brush, flask must be resiliconized. Low ETU recoveries may also indicate need to resiliconize flask.)

(d) *Filter paper.*—11 cm sharkskin (Arthur H. Thomas Co., Philadelphia, PA 19105).

(e) *Pasteur pipets.*—Borosilicate glass, 9 in. (229 mm), disposable (Fisher Scientific). Do not use soda-lime type.

(f) *Swinny filter holder.*—13 mm filter size (No. XX3001200, Millipore Corp., Bedford, MA 01730).

(g) *Membrane filters.*—Nylon-66, 0.45 μm pore size, 13 mm disc (Rainin Instrument Co., Inc., Woburn, MA 01801).

(h) *Vacuum rotary evaporator.*—Model RE Rotavapor (Brinkmann Instruments, Inc., Westbury, NY 11590). Maintain evaporation water bath temperature at 35°C. Maintain solution in condensing coils and around receiving flask at −15°C. (Refrigerated water-antifreeze solution works well.) Use vacuum pump fitted with vacuum gauge and needle valve to control vacuum in evaporator.

LC Apparatus

(a) *LC mobile phase reservoirs.*—Ultraware LC solvent reservoirs (No. K953935-1000 (1 L) and K953935-2000 (2 L), Kontes). Replace Teflon lines with 1/16 in. (1.6 mm) od × 0.040 in. (1.0 mm) id stainless steel tubing to prevent reincorporation of air (oxygen) into mobile phase. Degas solvents with helium (99.995%) purified with in-line Hydro-Purge II and Oxy-Purge traps (Alltech Associates, Inc./Applied Science Labs).

(b) *Mobile phase delivery system.*—SP8700XR LC pump (Spectra-Physics, San Jose, CA 95134).

(c) *Injector.*—SP8780XR autosampler fitted with 20 μL loop (Spectra-Physics). Use methanol for wash solution.

(d) *LC column.*—Shandon Hypercarb graphitized 7 μm spherical carbon particles, 10 cm × 4.6 mm id (Keystone Scientific, Inc., Bellefonte, PA 16823). Condition new column for 1–2 days by passing acetonitrile through column at 0.5–1 mL/min. Conditioning removes impurities and improves ETU peak shape.

(e) *Column oven.*—2080 LC forced air column oven (Varian Associates, Inc., Palo Alto, CA 94303).

(f) *Electrochemical (EC) detector.*—LC-17 thin-layer EC cell (with Au/Hg working electrode, Ag/AgCl reference electrode, stainless steel block auxiliary electrode, and 5 μm Teflon gasket) and LC-4B amperometric detector controller (No. MF-9094, Bioanalytical Systems, West Lafayette, IN 47906). Prepare Au/Hg working electrode surface as follows: To remove old amalgam, place a few drops of 6N nitric acid on electrode surface. When rusty yellow color appears, old amalgam has been destroyed. Polish gold surface first with diamond-polishing compound and then with alumina as directed by manufacturer. In tray, coat new highly polished gold surface with a few drops of high purity mercury; electrode should be totally covered. (Do not touch gold surface with pipet or other sharp object because surface may become scratched.) After 5 min, "brush" excess mercury from electrode surface with soft paper tissue. Continue "brushing" and "polishing" surface with tissue until surface is flat and without any signs of mercury "puddling." The surface should have a silver sheen (a mirror-like surface indicates excessive mercury). Let amalgam equilibrate in air atmosphere for ≥2 days before electrode is connected to cell. (If Au/Hg surface is lightly polished with dampened, soft paper tissue just before electrode is connected to cell, response to ETU seems to improve.) Connect cell to LC unit after mobile phase (see *LC Operating Parameters*) has been degassed for several hours and then passed through column to eliminate oxygen from system. Maintain flow of mobile phase at 0.5 mL/min through system and cell overnight with detector turned off. Overnight equilibration is necessary to provide stable response and low baseline noise.

(g) *Computing integrator.*—SP4200 computing integrator (Spectra-Physics). Operate at chart speed of 0.5 cm/min and attenuation of 8, and use proper peak width and peak threshold values.

LC Operating Parameters

Set pump so that solenoid switching valves produce mobile phase composition of acetonitrile–aqueous 0.1M phosphoric acid–water (5 + 25 + 70). Adjust mobile phase flow rate to 1.00 ± 0.02 mL/min. Operate column oven at 35°C. After EC cell has equilibrated overnight (see *LC Apparatus*, **(f)**), set EC detector potential to 350 mV, turn cell mode from "stby" to "on," and let baseline stabilize. Adjust microamp sensitivity setting so that 5 ng ETU produces approximately 50% full scale response on integrator paper. If LC system will not be used overnight, set mobile phase flow rate to 0.5 mL/min, turn cell to "stby," and turn detector off. If system will not be used for several days, replace mobile phase in column and tubing with 100% acetonitrile and store reference electrode in 3M sodium chloride.

Extraction

To blender jar, add 100 or 50 g (Table 1) analytical portion, 15 g sodium acetate, 20 g Celite 545, 150 mL water, and 200 mL methanol. Blend mixture 2 min at high speed. Vacuum-filter homogenate through 10–15 g bed of Celite 545 spread evenly on 2 premoistened sharkskin filter papers in 11 cm perforated Buchner funnel. Collect filtrate in 500 mL filter flask. Transfer volume of filtrate equivalent to 20% of analytical portion (mL water in analytical portion + 150 mL water + 200 mL methanol/5) to preweighed 500 mL T 24/40 round-bottom flask. Using Pasteur pipet, add 5 drops of 1% Antifoam B solution to round-bottom flask. Place 250 mL T 24/40 trap on round-bottom flask and attach to vacuum rotary evaporator. Apply vacuum slowly. After full vacuum is applied, slowly place flask in 35°C water bath. Concentrate extract to ca 12 g and then add water to bring weight to 13 g. Swirl flask to dislodge (and dissolve, if possible) any residue adhering to glass surface of flask. Proceed immediately to cleanup.

Cleanup

Add 10 g Gas-Chrom S to round-bottom flask containing concentrated extract. Stopper and shake flask. Let flask stand 2–3 min. Tap flask on cork ring to break up lumps. (Mixture should now be free-flowing and particles should not adhere to wall of flask.) Pour Gas-Chrom S into chromatographic column containing 4.5 g alumina and settle particles by gently tapping side of column. Place 0.5 cm plug of glass wool on top of Gas-Chrom S. Add 50 mL partitioning eluant to round-bottom flask. Transfer solution to column. Let liquid rapidly flow through column to remove entrained air and then adjust flow rate to 5 mL/min. Rinse flask with second 50 mL portion of eluant, and add rinse to column after last of first portion has just touched glass wool. Rinse flask with 2 additional 50 mL portions of eluant, and add each rinse to column. Collect eluate in 500 mL T 24/40 round-bottom flask that was previously siliconized. Evaporate eluate just to dryness, using vacuum rotary evaporator. Immediately pipet 4.0 mL water into flask. Swirl flask to dissolve ETU residue. Pour solution into 10 mL glass syringe containing Swinney filter holder with 0.45 μm Nylon-66 filter. Push solution through filter with syringe plunger and collect filtrate in 10 mL centrifuge tube or other suitable container. If diluted solution is needed, pipet aliquot into another container and dilute to desired volume.

Determination

Transfer solution to be injected to autosampler vials with borosilicate glass Pasteur pipet. Inject 20 μL solution onto LC carbon column, using chromatographic apparatus and parameters described in *LC Apparatus* and *LC Operating Parameters*. Measure peak area and quantitate ETU residue by comparison to peak area obtained from known amount of ETU standard injected immediately after purified extract. To ensure valid measurement of ETU residue, peak areas of purified extract and ETU standard solution should match within ±25%.

Results and Discussion

Method Refinement

The AOAC official method (5) was extensively investigated. Most methods in the literature use only methanol to extract ETU residues, whereas the AOAC method uses a mixture of water, methanol, and sodium chloride as the extraction medium. ETU is extremely soluble in both methanol and water, so both are excellent solvents for extracting ETU. The water–methanol–sodium chloride extraction solution is superior to methanol or water–methanol because it greatly reduces the coextraction of organic solvent-soluble components, i.e., chlorophyll a and b, carotenes, fats, and waxes.

The amount of Celite 545 added to the blender was increased from 10 to 20 g to increase the rate of extract filtration. The 9 cm Buchner funnel was replaced by the more commonly used 11 cm Buchner funnel to increase the rate of filtration. For slower filtering foods such as cake donuts, filtration time was decreased from 10 to 5 min. In addition, the entire extract can be added to the 11 cm funnel at one time, whereas with the 9 cm funnel, the extract had to be added in portions.

The analytical portion size for canned sweetened applesauce, powdered sugar-coated cake donuts, and grape jelly was reduced to 50 g to improve filtrate flow rate and to enable concentration of the filtrate portion to 12 g.

The AOAC method uses Gas-Chrom S as the solid support in the liquid-liquid partitioning step. The newer materials from E. Merck (Extrelut) and Analytichem (Chem Elut) were found to be inferior to Gas-Chrom S because of poor quality control of particle size. Therefore, Gas-Chrom S continues to be used in the method. The alumina performs a very important function by removing chlorophyll and other pigments and coextractives that have partitioned into the column eluant.

Antifoam B is used in place of *n*-octanol because it is more effective in reducing frothing and it is not carried through the method.

The analyst's health was also considered in evaluating the method. Chloroform, a known carcinogen that is a major component in the partitioning column eluant of the AOAC method, was replaced with methylene chloride. Also, ethanol was replaced with methanol, which can be obtained in higher purity. A 2% concentration of methanol in methylene chloride quantitatively removed ETU from the column and yet minimized the elution of coextractives.

ETU recovery from sweetened applesauce was approximately 10% when sodium chloride was used as the salting-out agent for the partitioning/cleanup step. Kobayashi et al. (7) reported low ETU recovery from a diatomaceous earth liquid-liquid partitioning procedure when the pH of the aqueous solution was low. They determined that ETU recovery was optimum for an aqueous solution of pH 7–9. Applesauce has a pH of 3.3. A 5% aqueous solution of sodium acetate has a pH of 8.9, and, thus, it has the capacity to reduce the acidic nature of the applesauce. Replacement of sodium chloride with sodium acetate increased ETU recovery from applesauce from 10 to 85%.

ETU recovery was found to be low and erratic in the vacuum rotary evaporation of the column eluate. ETU in solution is known to undergo degradation due to photolysis, especially in the presence of photosensitizing chemicals and oxygen (8). ETU residues on silica gel TLC plates have also been found to degrade in the presence of light (9). The ETU loss during the evaporation procedure was apparently caused by active sites on the glass surface of the round-bottom flask. ETU recoveries from previously used round-bottom flasks averaged 77% (range 70–81%); ETU recoveries from new flasks averaged 92% (range 87–95%). When new flasks siliconized with SurfaSil were used, ETU recoveries averaged 96% (range 94–99%). Thus, ETU recovery was optimized through treating the glass walls of the flask with SurfaSil, which reacts with the available silanol groups. Other siliconizing agents investigated did not form as permanent a bond with the glass surface.

The final aqueous solution is passed through a 0.45 μm Nylon-66 filter before injection onto the LC column. The filter not only removes particles from the solution but also removes any remaining yellow, pigmented coextractives if present. All filtered solutions were colorless.

ETU retention time was 210 s on the C-8 column originally used (4). However, in subsequent work with newer "improved" columns (better coverage of residual silanols), ETU had a retention time of 135 s. This indicates that the chromatographic separation of ETU on the C-8 columns was due to residual silanol groups. A cyclodextrin column was also investigated for the chromatographic separation of ETU. The separation mechanism is based on the inclusion of the analyte within the cyclodextrin cavity. Unfortunately, any residual methylene chloride from the partitioning column eluate is trapped in the cyclodextrin cavity, and ETU retention is decreased. Polymer columns were found to partially degrade ETU (10). In 1988, the graphitized carbon column became commercially available from Shandon Scientific Ltd, Chesh-

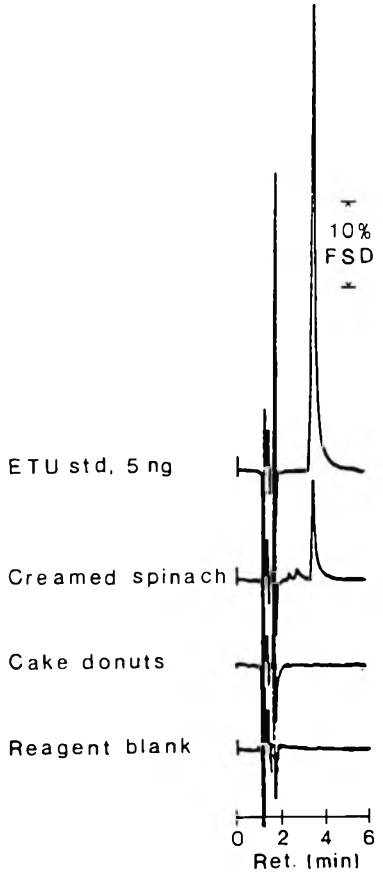

Figure 1. Example LC-EC chromatograms.

ire, U.K. ETU chromatographed well on this column, which does not have the disadvantages of the silica-based C-8 and cyclodextrin columns. Also, the carbon column is stable in the pH range of 1–14, and the phosphoric acid electrolyte solution can be added directly to the mobile phase. This eliminates the need for a post-column pump and associated hardware.

The derivatization procedure needed in the AOAC method for the GC determination of ETU is not required with the LC-EC method because ETU is determined directly. Elimination of the derivatization procedure improves ETU recovery, reduces analysis time, eliminates the need for additional reagents, and results in a more selective method for determining ETU.

Evaluation of Revised Method

Example chromatograms of a method reagent blank, 2 controls, and an ETU standard are shown in Figure 1. The chromatograms are free of extraneous peaks near the retention time of ETU, which is approximately 210 s with the carbon column. The slight tailing of the ETU peak is a function of the detector and not the carbon column (10). The creamed spinach control contains apparent ETU at a level of 11 ppb. Table 2 lists the levels of apparent ETU found in 7 of the 13 food product controls.

ETU recovery was determined by fortifying duplicate portions of the food product at a low level (0.05 or 0.1 ppm) and at a high level (0.5 or 1.0 ppm). The 13 food products were fortified just before extraction. Average percent recoveries of ETU from the fortified food products (Table 2) were 92 with a standard deviation of 12 for the low fortification levels and 90 with a standard deviation of 6 for the high fortification levels. The ETU recovery values obtained with celery were low and were omitted from the calculations of average ETU

Table 2. Recovery of ETU from fortified food products and apparent ETU levels in controls

Food product	Apparent ETU, ppb[a]	ETU rec., %[b]			
		0.05 ppm added		0.5 ppm added	
Cabbage, boiled	0	87	94	97	93
Celery	0	0	24	39	42
Collards, boiled	0	110	107	94	96
Green beans, canned	0	91	91	92	91
Lettuce	0	85	74	81	84
Mushrooms, canned	8	100	103	102	95
Potato, baked	4	98	98	94	92
Spinach, creamed, canned, baby food	11	80	85	84	87
Spinach, canned	90	128	101	91	97
Tomatoes, canned	3	88	91	86	93
		0.1 ppm added		1.0 ppm added	
Applesauce	0	85	81	80	85
Donuts	0	90	85	87	91
Grape jelly	9	84	83	82	83
Av. rec., %[c]		92		90	
SD		12		6	

[a] Confirmatory tests were not undertaken.
[b] Duplicate fortifications and analyses were performed at each level.
[c] Calculation did not include celery recovery data.

recoveries; in celery, ETU loss begins at the point of extraction and continues through the entire method. For some food products, especially canned mushrooms, cooked cabbage, and baked potatoes, the recovery of ETU was approximately 10 to 15% higher when ETU peak height was used in the calculations. This change in response was apparently due to slight changes in the chromatography of ETU in the presence of food product coextractives. These slight changes were magnified because of the very sharp chromatographic peak of ETU. The problem was eliminated by using ETU peak area in the calculations.

The effect of storage time and conditions on ETU stability in the final solution of purified extract was examined by using canned mushrooms as the food product. Storage of the final solution in a refrigerator overnight at 5°C resulted in an average ETU loss of 13% at the 0.05 ppm fortification level and an average ETU loss of 4% at the 0.5 ppm level. Storage of the final solution at room temperature overnight in the absence of light resulted in an ETU loss of 96% at the 0.05 ppm level. The specific cause of the ETU loss is currently unknown. No ETU loss was observed for standard solutions (0.025 μg ETU/mL water) stored in a similar manner. Therefore, ETU in final solutions of purified extracts must be determined without delay.

The author finds that for a series of 5 analyses, only 4 h is needed from the start of the first extraction to the completion of the final LC chromatogram.

The recommended limits of quantitation for the method are 0.01 ppm for foods with low sugar content and 0.02 ppm for foods with high sugar content. These limits are based on injections equivalent to 100 and 50 mg of the 2 food types, respectively, and on a detector response of 10% full scale deflection for 1 ng ETU.

Conclusions

The revised method effectively provides for the quantitative recovery of ETU from 12 of 13 food products tested. The

method is rapid and highly selective for the direct determination of ETU residues.

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Chemical Derivatization Analysis of Phenols. Part VI. Determination of Chlorinated Phenolics in Pulp and Paper Effluents

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Based on the in-situ acetylation procedure, a method for the determination of 31 chlorinated phenols, guaiacols, catechols, syringols, and vanillins in pulp and paper effluent samples has been successfully developed. Except for 4-chlorocatechol, this procedure provided satisfactory recovery for all phenols at 3 levels of fortification, namely, 400, 40, and 4 μg/L. The acetyl derivatives were analyzed by gas chromatography using a 30 m DB-5 capillary column interfaced to an electron-capture detector. Mass spectral abundance data for the characteristic ions of the acetyl derivatives were used for confirmation of compound identities. By operating a mass selective detector in the selected ion monitoring mode, this procedure was further extended to the monochlorinated phenolics. Using a 50 mL effluent sample, the method detection limit was 0.5 μg/L for all except the monochlorinated compounds, which had a detection limit of 1 μg/L. Several effluent samples from a Canadian paper mill were analyzed by this procedure and the results are presented.

Most pulp mills in Canada use chlorine in the bleaching process to remove the brown lignin, a natural product present in all wood pulps. Typically, bleaching is done in 5 or 6 sequential steps designated as C (chlorination), E (alkaline extraction), H (hypochlorite), and D (chlorine dioxide) stages. Effluents are produced by washing the pulp after each stage. During this process, chlorinated phenolics are formed as byproducts by electrophilic aromatic substitution, electrophilic displacement, and dealkylation/demethylation reactions occurring on lignin.

The major phenolics found in pulp and paper effluents can be classified into the following 5 groups of compounds: chlorophenols, chloroguaiacols, chlorocatechols, chlorosyringols, and chlorovanillins. Other compounds such as syringealdehydes and veratroles are also present in effluent samples in minor quantities. Chemical structures of the above compounds are given in Figure 1.

According to some reports (1-4), the type of phenolics present in the effluent depends on the type of wood being processed, and the stage in the delignification process. In general, softwood produces larger amounts of the phenolic byproducts than hardwood. Softwood effluent contains only chlorinated phenols, guaiacols, catechols, and vanillins. In addition to the above phenolics, hardwood effluents also contain chlorinated syringols and syringealdehydes. It is also noted that catechols are mostly found in the C-stage effluents while phenols, guaiacols, and vanillins are more frequently observed in the E-stage effluents. The total amount of chlorinated phenolics produced during the chlorine bleaching steps was shown to be dependent on conditions such as pH, temperature, chlorine dosage, and use of chlorine dioxide substitution (1, 4).

Recent studies have indicated that chlorinated phenolics are one of the major class of toxicants in pulp and paper effluents. The sublethal toxic effects of such effluents to fish have been reviewed (5). The 96-h LC_{50} values of various chlorinated phenolics were also reported (4). According to the latter reference, there is a trend to indicate that the toxicities of phenolics are inversely proportional to their pK_a values. Since phenols with higher level of chlorination have lower pK_a values, they are therefore more toxic than those with less chlorine substitution. To protect the fish industry and to safeguard the possibility of bioaccumulation of toxic phenols in the food chain, it is necessary to monitor pulp and paper effluent for excessive levels of chlorophenols.

Although the formation of ethyl (6), heptafluorobutyryl (7), pentafluorobenzyl ether (8), and other derivatives has been reported, chlorinated phenolics in effluent samples are most conveniently analyzed as their acetyl derivatives using an in-situ acetylation procedure (3, 9-12). This procedure has the advantage of combining the extraction and derivatization of phenolics into a single step as well as the ease of formation and stability of the derivatives. For the phenolics with 2 or more chlorine substitutions, the acetyl derivatives are sensitive to electron-capture detection so that trace levels of phenolics can be determined for environmental samples.

Recently, a collaborative study was organized for the determination of 7 phenolics (3 chlorophenols, 2 chloroguaiacols, and 2 chlorocatechols) in pulp mill effluents. While those interlaboratory results for the chlorophenols and chloroguaiacols were satisfactory, results for the chlorocatechols were poor (13).

Despite the wide use of the acetylation procedure, this method has not yet been rigorously tested for a large number of phenolics. In the present report, we shall present a validated method for the determination of 31 chlorinated phenolics

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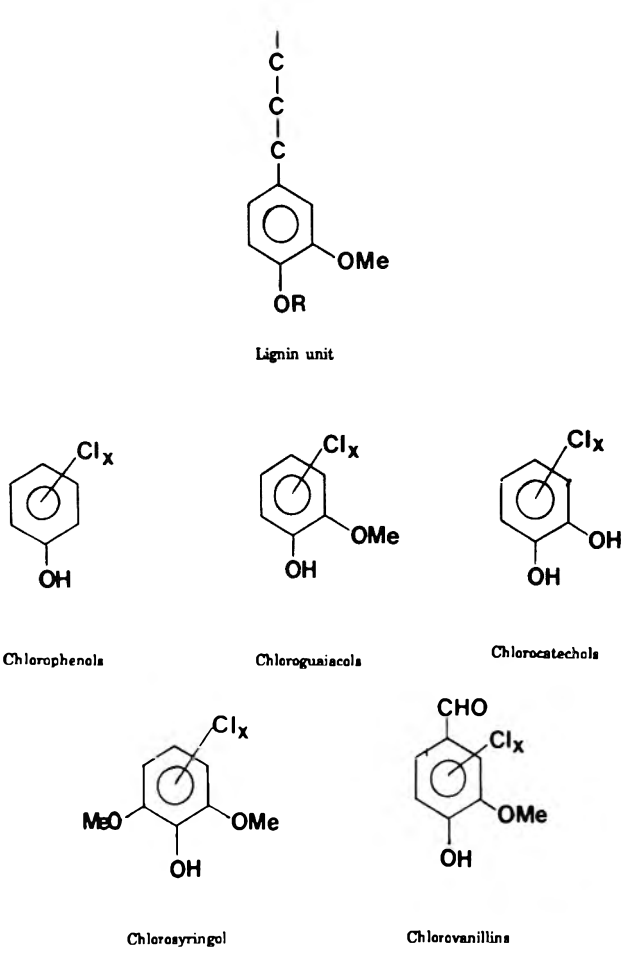

Figure 1. Chemical structures of chlorinated phenolics.

(see Figure 2 for a listing) commonly found in pulp and paper effluent samples.

(Note: For the sake of simplicity, the following abbreviations are used to denote various phenolics throughout this manuscript: P for chlorophenols, G for chloroguaiacols, C for chlorocatechols, S for chlorosyringols, and V for chlorovanillins. The numbers ahead of them are the positions of chlorine substitutions. As an example, 46G stands for 4,6-dichloroguaiacol.)

Experimental

Apparatus

Two Hewlett-Packard Model 5880A gas chromatographs were used in this study. One was equipped with an electron-capture detector, the other with a Model 5970B mass selective detector and data system. See Table 1 for chromatographic conditions.

Acquisition of Mass Spectral Data

Obtain full scan MS data by scanning MSD from m/z 40 to 350 at 1.7 scans/s and scan threshold of 1000. Use electron energy and electron multiplier voltages of 70 eV and 2000 V, respectively. For selected ion monitoring, use $[M - 42]^+$ ion for quantitation of chlorinated phenols, guaiacols, vanillins, and syringol, and $[M - 42 - 42]^+$ ion for chlorocatechols. For confirmation, monitor M^+ ion for chlorophenols, $[M - 42 - 15]^+$ ion for chloroguaiacols, chlorovanillins, and chlorosyringol, and $[M - 42]^+$ ion for chlorocatechols, and obtain ratio of quantitation and confirmation ion. Compound identity is confirmed if ratio is within ±25% compared with that from reference standard. In cases where relative abundances of confirmation ions are too low (e.g., less than 10%, see Tables 2 and 3), use chlorine isotope masses of quantitation ions instead.

Reagents

Use only distilled-in-glass, pesticide residue grade solvents and check before use for low blank values.

(a) *Purified (organic-free) water.*—Pass distilled water through Bion Exchanger composed of 2 Chemical Organosorb II (Part No. 19710) and one Chemical 3× Research II (Part No. 19720) cartridges. Alternatively, extract 1 L distilled water 3 times by stirring with 50 mL methylene chloride for 30 min. Discard organic layers. Use purified water to prepare 1.0, 1.5, and 75% (w/v) potassium carbonate.

(b) *Sodium sulfate.*—Anhydrous, reagent grade (available from BDH Chemicals). Heat 48 h at 200°C and store in clean glass bottle.

(c) *Analytical standards.*—Analytical grade (98+% purity) chlorinated phenolics. Obtain chlorophenols from Aldrich Chemical Co. (Milwaukee, WI) and other phenolics from Helix Biotech Scientific Ltd (Vancouver, BC, Canada).

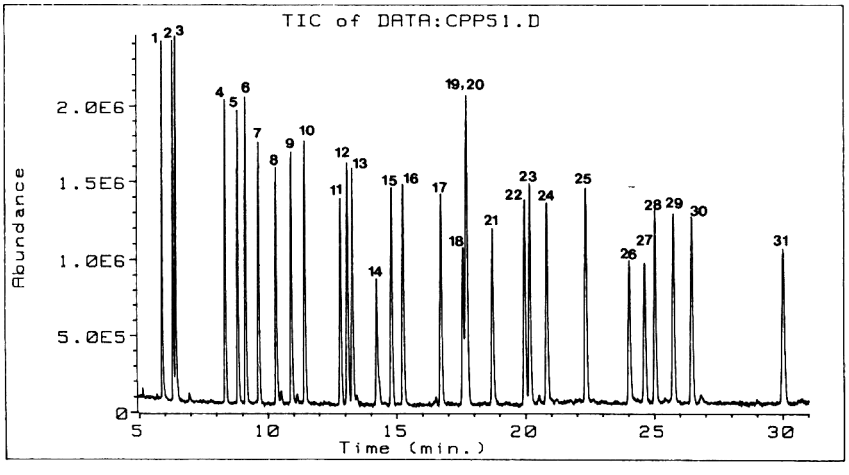

Figure 2. Total ion chromatogram of acetyl derivatives of 31 chlorinated phenolics. See Table 1 for chromatographic conditions. Peaks: (1) 2P, (2) 3P, (3) 4P, (4) 26P, (5) 24P, (6) 35P, (7) 23P, (8) 34P, (9) 4G, (10) 246P, (11) 236P, (12) 235P, (13) 245P, (14) 4C, (15) 46G, (16) 345P, (17) 45G, (18) 2356P, (19) 35C, (20) 2346P, (21) 6V, (22) 2345P, (23) 45C, (24) 345G, (25) 456G, (26) 56V, (27) 23456P, (28) 345C, (29) 3456G, (30) 345S, and (31) 3456C. Approximately 20 ng of each injected.

Table 1. Chromatographic conditions for analysis of acetyl derivatives of chlorinated phenolics

Conditions	ECD	MSD
Gas chromatograph	Hewlett-Packard 5880A	Hewlett-Packard 5880A
Detector type	Ni-63 ECD	Hewlett-Packard 5970B MSD
Column	J&W DB-5, 30 m × 0.25 mm	Supelco SPB-5, 30 m × 0.25 mm
Column temp.		
Initial, °C	70 for 0.75 mln	70 for 0.75 min
Programming rate 1	30°/min (from 70 to 120)	30°/min (from 70 to 120)
Programming rate 2	2°/min (from 120 to 180)	2°/min (from 120 to 180)
Final, °C	180	180
Detector temp., °C	300	200
Injection port temp., °C	250	250
Splitless time, min	0.75	0.75
Column head pressure, psi	15	4
Injection volume, μL	2	1
Carrier gas	helium	helium
Makeup gas and flow	argon–methane (95 + 5) 25 mL/min	none
Septum purge flow, mL/min	1.5	1.5

Prepare 1000 ppm stock solution of each individual chlorinated phenolic by dissolving 100 mg pure analytical standard in toluene or acetone and diluting to 100.0 mL in low-actinic volumetric flask. Store at 4°C in the dark.

Prepare mixed phenol stock solution by combining appropriate aliquots of individual phenols stock solutions and diluting to 100.0 mL with acetone. Prepare spiking solutions by diluting further with acetone as required. Store all solutions at 4°C in the dark.

(d) *Acetic anhydride.*—Distill reagent grade acetic anhydride 3 times and collect the 138–140°C fraction. Keep at −20°C in the dark. This will minimize decomposition of acetic anhydride to acetic acid.

Sampling

Collect grab effluent samples in 100 mL brown bottles with either Teflon-lined or aluminum foil-lined caps. Adjust pH to ca 10–12 with KOH. Keep samples at 4°C in the dark until analysis.

Extractive Acetylation of Effluent Sample

Measure 50.0 mL effluent sample in 250 mL Erlenmeyer flask. Adjust to pH 7. Add 1 mL 75% K_2CO_3, 5 mL triple-distilled acetic anhydride, and 30 mL petroleum ether (bp 30–60°C). Stopper and stir 30 min with Teflon-coated stir bar on magnetic stirrer. Separate layers in 500 mL separatory funnel and drain water sample (lower layer) back into original flat-bottom flask. Collect organic layer in 250 mL round-bottom flask. Repeat extraction/acetylation procedure twice with 30 mL petroleum ether and 1 mL acetic anhydride each time. Discard aqueous sample after third extraction. Rinse 500 mL separatory funnel twice with 10 mL petroleum ether and add rinsings to petroleum ether extracts.

Table 2. Mass number (m/z)/relative abundance (%) of some characteristic ions observed for acetyl derivatives of chlorophenols under EI conditions

Parent compd	M^+	$[M - 42]^+$	$[COCH_3]^+$
2P	170/12	128/100	43/73
3P	170/19	128/100	43/95
4P	170/11	128/100	43/59
26P	204/10	162/63	43/100
24P	204/6	162/95	43/100
35P	204/15	162/60	43/100
23P	204/14	162/81	43/100
34P	204/14	162/92	43/100
246P	238/5	196/56	43/100
236P	238/5	196/37	43/100
235P	238/6	196/40	43/100
245P	238/4	196/70	43/100
345P	238/7	196/64	43/100
2356P	272/5	230/20	43/100
2346P	272/3	230/32	43/100
2345P	272/4	230/43	43/100
23456P	306/3	264/18	43/100

Pass combined extracts through 5 cm column of anhydrous sodium sulfate in Allihn funnel into another 250 mL round-bottom flask. Wash sodium sulfate column with 25 mL petroleum ether and apply vacuum until sodium sulfate is dry. Remove funnel, add a few boiling chips to extract and attach 3-stage Snyder column to the 250 mL round-bottom flask. Wet Snyder column with 3 mL isooctane and clamp the flask securely in a heating mantle. Adjust distillation rate by heater controller and reduce sample volume to 5–7 mL.

After apparatus cools, rinse column with 3 mL petroleum ether and quantitatively transfer concentrated extract to 15 mL centrifuge tube. Further evaporate extract to 3 mL with gentle stream of nitrogen and then dilute to 5.0 mL with isooctane. Transfer about 3 mL final extract to another 15 mL centrifuge test tube containing 1 mL 1% K_2CO_3. Mix on vortex mixer ca 1 min. Pass organic layer through 9 mm disposable pipet plugged with silanized glass wool and packed with 5 cm anhydrous Na_2SO_4.

Analyze the sample extract by means of gas chromatography using electron-capture and mass selective detectors. For analysis of monochloro- phenols, guaiacol, and catechol by MSD, use final volume of 1.0 mL.

To prepare GC standard for calibration, fortify 50 mL sample of 1.5% potassium carbonate with 100 μL of an appropriate spike solution and perform extractive acetylation concurrently with effluent samples.

Results and Discussion

Due to the difference in chemical structures, the number of possible isomers at each level of chlorination is different for each class of phenolics. Among these compounds, reference standards of all 19 chlorophenols are readily available from various commercial sources. All of the 15 chloroguaiacols and 9 chlorocatechols have been synthesized and reported (14, 15); however, only 6 chloroguaiacols and 5 chlorocate-

Table 3. Mass number (m/z)/relative abundance (%) of some characteristic ions observed for acetyl derivatives of chloroguaiacol, chlorocatechols, chlorosyringol, and chlorovanillins under EI conditions

Parent compd	M^+	$[M-42]^+$	$[M-42-15]^+$	$[M-42-42]^+$	$[COCH_3]^+$
4G	200/7	158/100	143/67	—	43/61
46G	234/4	192/100	177/35	—	43/66
45G	234/5	192/100	177/63	—	43/78
345G	268/4	226/73	211/41	—	43/100
456G	268/4	226/100	211/37	—	43/100
3456G	302/2	260/46	245/17	—	43/100
4C	228/2	186/17	—	144/75	43/100
35C	262/1	220/7	—	178/22	43/100
45C	262/2	220/10	—	178/44	43/100
345C	296/1	254/6	—	212/31	43/100
3456C	330/1	288/3	—	246/15	43/100
345S	298/4	256/87	241/29	—	43/100
6V	228/8	186/100	171/9	—	43/100
56V	262/4	220/91	205/4	—	43/100

chols (see Table 3) are presently available from a commercial supplier (Helix Biotech Scientific Ltd). Similarly, only 2 chlorovanillins and one chlorosyringol (Table 3) are available from the same company. In our work all available phenolics were included into the validation work, with the exception of 25P and 234P because of GC resolution problems.

Derivatization of Phenols

In the presence of an aqueous base, each of the chlorinated phenolics readily gave a single product when reacted with acetic anhydride. Although the conversion to the acetyl derivatives was complete with 0.5 mL of the anhydride per reaction in pure or natural water samples, a large excess (total of 7 mL) of the anhydrides was required for maximum reaction of all phenolics in effluent samples. Insufficient reactant would cause low yields of the catechol derivatives, although the recoveries of the other phenolics in this study were not adversely affected. Due to the large amount of acetic anhydride used, a partitioning with 1% K_2CO_3 was required to remove this excess in the final extract. The 1% K_2CO_3 also served to neutralize any acidic compounds which were not derivatized. To minimize losses of the more volatile derivatives, the petroleum ether extract should only be evaporated in the presence of isooctane as a keeper using a 3-stage Snyder column.

Table 4. Mean recoveries and precision (standard deviations, in parentheses) of chlorinated phenolics from fortified effluent samples (no. of replicates = 6)

Phenol	Level of fortification, μg/L		
	400	40	4
2P[a]	94 (6)	92 (5)	98 (6)
3P[a]	93 (5)	91 (8)	90 (8)
4P[a]	93 (4)	91 (8)	90 (7)
26P	96 (3)	95 (8)	98 (4)
24P	96 (3)	95 (7)	99 (6)
35P	97 (3)	96 (6)	98 (8)
23P	95 (4)	95 (4)	99 (5)
34P	94 (3)	97 (4)	101 (5)
4G[a]	99 (5)	109 (8)	90 (10)
246P	97 (4)	101 (5)	88 (8)
236P	98 (5)	98 (7)	96 (9)
235P	97 (4)	95 (9)	96 (8)
245P	96 (4)	92 (6)	96 (8)
4C[a]	15 (11)	23 (17)	—
46G	93 (2)	90 (3)	88 (5)
345P	96 (4)	95 (4)	94 (9)
45G	95 (4)	91 (7)	85 (8)
2356P	100 (5)	80 (14)	93 (7)
35C	62 (10)	72 (9)	53 (5)
2346P	102 (6)	92 (14)	95 (6)
6V	98 (3)	92 (6)	103 (6)
2345P	99 (4)	91 (8)	102 (10)
45C	74 (9)	82 (9)	55 (7)
345G	99 (4)	94 (4)	111 (10)
456G	97 (5)	92 (5)	113 (8)
56V	99 (5)	90 (9)	104 (8)
23456P	93 (7)	92 (7)	104 (5)
345C	89 (8)	78 (6)	59 (7)
3456G	100 (8)	99 (6)	107 (7)
345S	94 (8)	96 (6)	102 (11)
3456C	101 (6)	72 (7)	65 (9)

[a] Results obtained by MSD.

GC Resolution

The retention times of the acetyl derivatives for all 19 chlorophenols, 15 chloroguaiacols, and 9 chlorocatechols determined on a SE-30 capillary column were reported by Knuuntinen and Korhornen (14). In our work, GC resolution of the acetyl derivatives was attempted on the following capillary columns: SPB-1, SPB-5, DB-5, and HP-5. Although the separation of the acetates was slightly different with each column, the order of elution for the derivatives was invariably the same and was consistent with the literature

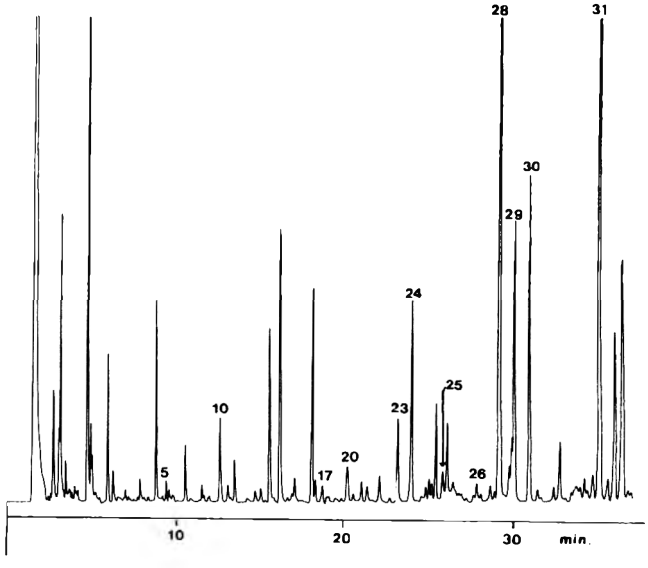

Figure 3. GC-ECD trace of acetyl derivatives in effluent sample A.

Table 5. Levels of chlorinated phenolics in effluent samples (all concentrations in μg/L)

Phenolics	Effluent[a] A	C	D
24P	5.6	6.1	ND
246P	9.9	7.9	5.1
46G	3.5	10.3	ND
45G	4.8	9.1	ND
35C	6.1	0.6	ND
2346P	5.4	3.1	1.1
6V	7.4	8.4	ND
45C	17.3	1.8	0.5
345G	17.0	25.3	2.8
456G	3.8	8.8	1.2
56V	4.4	15.2	0.9
23456P	3.1	1.3	0.6
345C	74.1	9.9	6.4
3456G	18.5	34.8	5.5
345S	27.2	5.5	0.6
3456C	77.4	17.2	10.6
Total	278.1	164.7	35.3

[a] ND = not detected.

(14). The SPB-5 and the DB-5 columns gave the best resolution of the present group of 31 compounds; both columns have the 5% diphenyl, 94% dimethyl, and 1% vinyl polysiloxane stationary phases. With these 2 columns, the acetyl derivative of 35C emerged between those of 2356P and 2346P, causing incomplete resolution. It should also be noted that the following pairs, 25P and 24P as well as 234P and 46G, did not resolve. A total ion current chromatogram of the 31 derivatives is shown in Figure 2.

GC-MSD Data

As confirmed by the following GC-MS data, the reaction of chlorinated phenolics with acetic anhydride produced the mono- (for phenols, guaiacols, syringols, and vanillins) as well as di- (for catechols) acetylated derivatives. No monoacetylated catechol was observed.

It is interesting to note that, during storage, the aldehyde group of the vanillins was gradually methylated to form the acetals when methanol was the solvent used to prepare the stock solutions. This result was supported by the observation of the molecular ions of the acetyl derivatives for the vanillin acetals. For this reason, methanol must not be used to prepare stock solutions of vanillins.

The EI mass spectrometry for the acetyl derivatives of chlorophenols (3, 16), chloroguaiacols, and chlorocatechols (3, 15) have been studied. Using a mass selective detector, all chlorophenol acetates exhibited the following characteristic ions: (*1*) molecular ion M^+, (*2*) $[M - 42]^+$, and (*3*) $[COCH_3]^+$ (m/z 43). These results affirm the findings in the previously published report (3, 16) as well as our own data (12). The base peak for all chlorophenol acetates except those of the monochlorophenols was the $[COCH_3]^+$ ion. The intensities of the molecular ions were weak, especially for the higher chlorinated congeners. Therefore, the $[M - 42]^+$ ions should be used in selected ion monitoring work for quantitative or confirmation purposes. For a listing of the characteristic ions, their mass numbers and relative abundance data for the acetyl derivatives of chlorophenols, see Table 2.

The EI mass spectra of the acetyl derivatives for the other phenolics also exhibited the above 3 characteristic ions. In addition, the $[M - 42 - 15]^+$ ion due to loss of $COCH_2$ and OCH_3 groups from the molecular ions of the guaiacols, vanillins, and 345S were also prominent. For the catechols, the characteristic $[M - 42 - 42]^+$ ions due to loss of 2 $COCH_2$ groups from the diacetylated molecular ions were present instead of the $[M - 42 - 15]^+$ ions. However, in all cases, the molecular ions were weak and not useful for selected ion monitoring work. See Table 3 for a listing of GC-MS data for the above phenolics.

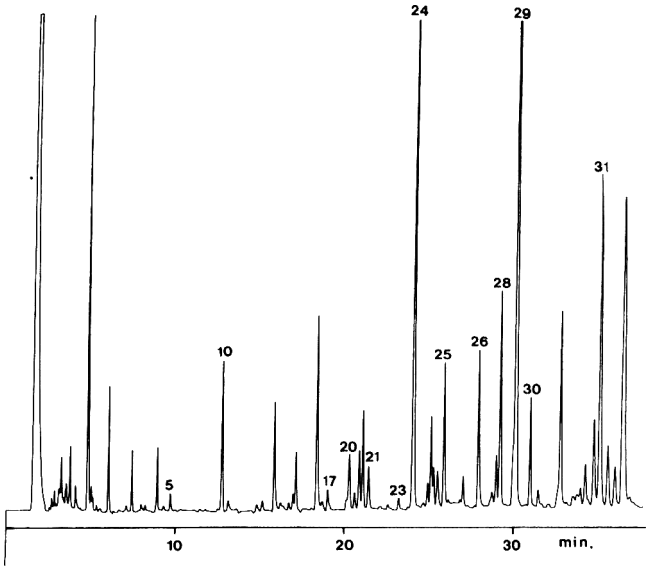

Figure 4. GC-ECD trace of acetyl derivatives in effluent sample C.

Validation Experiments

To validate the current method with authentic samples, an effluent sample from a Canadian bleached kraft mill was used. This sample was free of chlorinated phenolics except for a few compounds near the detection limit and thus it was suitable for fortification work. Since the reported phenols in effluents have a concentration range from low to high μg/L levels, this method was validated to 3 fortification levels, namely, 400, 40, and 4 μg/L. As shown in Table 4, recoveries of all phenolics except for the chlorocatechols were quantitative at all 3 levels. Other than 4C, the recoveries of catechols were generally between 70 and 90% at 400 and 40 μg/L, and were lower (ca 50–70%) at 4 μg/L. For unknown reasons, the recovery of 4C was poor at all concentrations. Thus, the present method is not suitable for the quantitative determination of 4C, and has a tendency to be biased low for the chlorocatechols at low μg/L levels. The method detection limit based on a 50 mL sample and a final volume of 1 mL was estimated to be 0.5 μg/L by ECD detection (final volume 5.0 mL) and 1 μg/L (final volume 1.0 mL) by MSD detection. If the detection of phenolics at lower concentrations is required, a larger sample should be used.

Determination of Phenolics in Effluent Samples

Several effluent samples collected from the bleach kraft mill were analyzed for chlorinated phenolics using the method described above. Sample A (Figure 3) was collected in a bleach plant and, similar to other C-stage effluents, it contained large amounts of chlorinated catechols (Table 5). The presence of high levels of 345S in this sample was consistent with the fact that this company processed both hardwood and softwood pulps. Sample C (Figure 4) was a combined effluent and it was collected just ahead of the aerated lagoon. The major chlorophenolics in this effluent sample were chlorinated guaiacols and vanillins as well as the more chlorinated catechols. Sample D was a treated effluent discharging into a

river. The total chlorinated phenolics concentration in sample D was 35.3 μg/L compared to 278.1 and 164.7 μg/L for samples A and C, respectively.

Acknowledgments

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Determination of Daminozide in Apples by Gas Chromatography/Chemical Ionization Mass Spectrometry

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A method using gas chromatography/chemical ionization mass spectrometry (GC/CIMS) for the determination of daminozide residues in apples has been developed. Daminozide was separated from the sample matrix by water extraction and cation exchange, converted to the methyl ester by treatment with HCl–methanol, and determined by GC/CIMS using succinonitrile as an internal standard. The detection level was 0.05 ppm. Recoveries were 92–104% from apples spiked at the 0.05–0.5 ppm levels. Of the 25 apple samples analyzed, only 2 were positive for daminozide (1.04 and 0.32 ppm).

Daminozide (*N*-dimethylaminosuccinamic acid) is the active ingredient in the plant growth regulator Alar. It has attracted a great deal of attention recently because its decomposition product and/or metabolite, unsymmetrical dimethyl hydrazine (UDMH), is thought to have carcinogenic activity in laboratory animals (1).

The official method for the determination of daminozide calls for alkaline hydrolysis to UDMH followed by complexing with trisodium pentacyanoamine ferroate. The complex is determined colorimetrically (2). More recent methods are based on the formation of other UDMH derivatives that can be detected by gas chromatography using electron-capture detection (3), nitrogen detection (4), or gas chromatography/mass spectrometry/selected ion monitoring (5).

Whiton (6) described a method for the direct determination of intact daminozide residues after extraction and esterification to daminozide methyl ester. That method would be of more value than presently used methods in assessing the true amount of daminozide present and, when used in conjunction with present methods for the determination of UDMH, could provide information on how much parent and metabolite/decomposition product is present in a wide variety of samples. We have used the Whiton method with some modification (longer derivatization time and chemical ionization mass spectrometry for electron ionization mass spectrometry) to analyze 25 apple samples.

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Experimental

Chemicals

(a) *Daminozide, succinonitrile, and acetyl chloride.*—Gold Label (Aldrich Chemical Co., Milwaukee, WI).

(b) *Methyl ester of daminozide.*–97% pure by gas chromatography (Uniroyal Chemical Co., Naugatuck, CT).

(c) *Cation exchange resin.*—Dowex 50 WX8, 200-400 mesh (Serva Fine Chemicals Inc., Westbury, NY).

(d) *Reagents.*—LC grade chloroform and methanol, ACS grade anhydrous sodium sulfate, hydrochloric acid, sodium bicarbonate, and sodium hydroxide (Fischer Scientific, Springfield, NJ).

(e) *HCl–methanol extracting solution.*—Prepared by slowly adding acetyl chloride to cold methanol in an ice bath and stored in a sealed vial at −15°C.

Samples

Twenty-five samples of apples (13 New Jersey-grown, 9 from New York, 1 from Pennsylvania, 1 from California, and 1 from Vermont) were collected by New Jersey Department of Health from New Jersey farms and supermarket distribution centers.

Instrumentation and Conditions

A Varian Model 3400 Gas Chromatograph (Varian Associates, Walnut Creek, CA) interfaced to a Finnigan MAT Ion Trap Detector equipped with chemical ionization (Finnigan MAT, San Jose, CA) and controlled by an IBM PC/AT was used. The analyses and quantitation were performed with Finnigan Ion Trap Software Version 3.15.

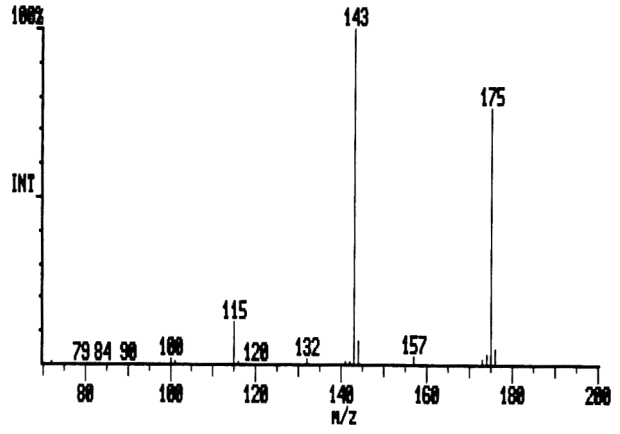

Figure 1. Chemical ionization spectrum of daminozide methyl ester. m/z 175 = $(M + 1)^+$; m/z 143 = $(M + 1)^+$—CH_3OH; m/z 115 = $(M + 1)^+$—CH_3OH—CO.

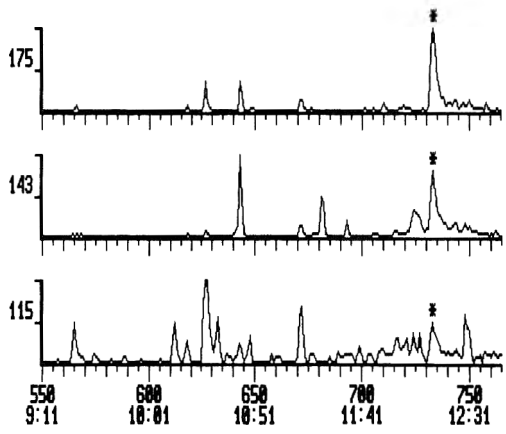

Figure 3. Mass chromatograms obtained from apple sample spiked with daminozide at the 0.1 ppm level before extraction. Asterisks indicate identical retention time.

A splitless on-column injector held at 50°C was used, and a 1.5 m × 0.53 mm id deactivated fused silica pre-column was fitted between the injector and capillary column. A 30 m × 0.25 mm id J&W DB-1 fused silica capillary column (1 μm film thickness) was held at 50°C for 2 min and then temperature programmed from 50 to 300°C at 15°/min.

Carrier gas (helium) was used at a velocity of 25 cm/s and the injection volume was 1 μL. A 15 cm syringe was used for on-column injections.

The mass spectrometer was operated in the chemical ionization mode using methane as reagent gas at a pressure that gave a 10:1 ratio for m/z 17 to m/z 16. The filament voltage and current were 70 eV and 80 μA, respectively. Electron multiplier gain was 10^5. Scan range was set at 70 to 250 mass units at 1 s/scan. Transfer line and manifold temperatures were 250 and 220°C, respectively.

Sample Preparation

Daminozide residue was extracted from apples and esterified by a procedure based on the method of Whiton (6) with some modification. Fifteen pounds of pitted apples were chopped and mixed. Twenty g samples were extracted with 40 mL water, using a Waring blender with a mini container, for 1 min at low speed. A blended sample was filtered with suction through a Buchner funnel fitted with glass-fiber filter paper and the blender container was rinsed with water, which was added to the filter. Total volume of the filtrate was kept at ca 100 mL; the exact volume was recorded.

A 2.0 mL aliquot of filtrate was transferred by volumetric pipet to a centrifuge tube containing 1 g cation exchange resin, blended on a Vortex mixer, and centrifuged, and the supernatant liquid was discarded. The resin was washed twice with 5 mL water and the washings were discarded.

The sample was mixed with 3 mL 1.0M NaOH and filtered through a plug of glass wool in a Pasteur pipet to remove resin. The filtrate was collected in a 5 mL Reacti-Vial and adjusted to ca pH 6 with 1.0M HCl. The sample was dried under a gentle stream of nitrogen at 35°C, and 0.4 mL 0.15M HCl–methanol solution was added. The residue was scraped from the wall of the vial and mixed with the reagent. The vial was capped tightly and allowed to stand overnight at room temperature (Whiton's 1 h reaction time was not sufficient to achieve good recoveries).

After standing, 1 mL 0.17M $NaHCO_3$ was added and the mixture was extracted 3 times with 1 mL chloroform using a 5 mL Mixxor Separatory Cylinder (Xydex Corp., Bedford, MA). The chloroform extracts were dehydrated by passing through anhydrous Na_2SO_4 supported in a Pasteur pipet and pooled in a 3 mL conical vial. The Na_2SO_4 was washed with an additional 1 mL chloroform, which was added to the vial. The dehydrated chloroform extract was then evaporated to ca 50 μL under a stream of nitrogen at 35°C. Five μL of 0.5 mg/mL succinonitrile internal standard in chloroform was added and the volume was adjusted to 100 μL with chloroform. The solution was mixed well and 1 μL final solution was analyzed by GC/CIMS.

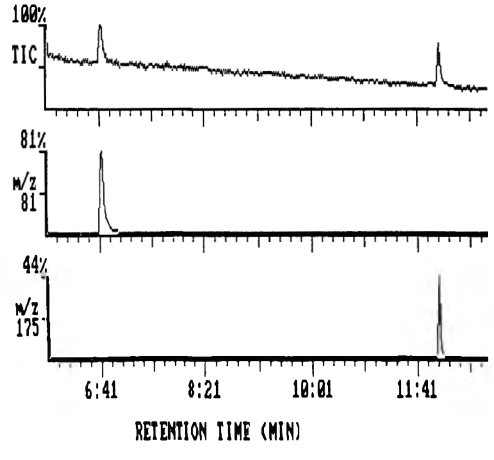

Figure 2. Total ion (upper trace) and mass chromatograms of succinonitrile (middle trace) and daminozide methyl ester (lower trace) standard mixture.

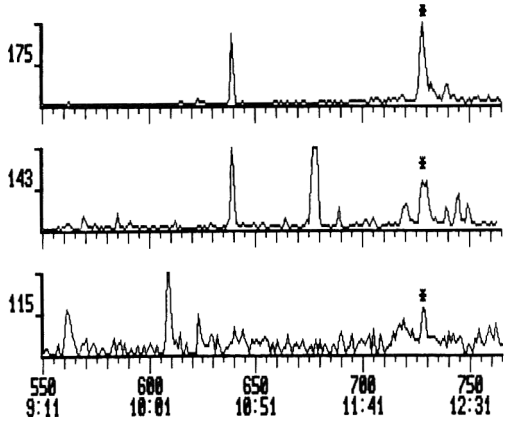

Figure 4. Same as previous figure notation except apples were spiked with 0.05 ppm daminozide.

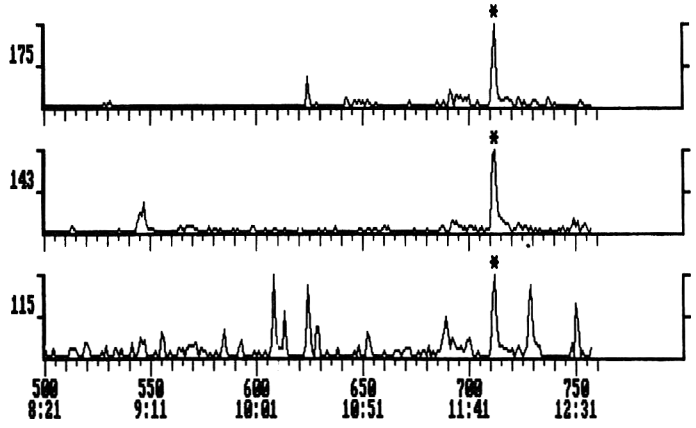

Figure 5. Mass chromatograms at m/z 175, 143, and 115 obtained from apple sample with incurred residue of 0.32 ppm. Asterisks denote identical retention time.

Preparation of Calibration Curve

A stock solution of daminozide methyl ester was prepared by dissolving 10 mg daminozide methyl ester standard in chloroform in a 100 mL volumetric flask. A series of standard solutions were prepared by adding various volumes of the stock solution to graduated tubes, adding 100 μL succinonitrile internal standard stock solution (0.5 mg/mL in chloroform), and diluting to the 2 mL mark with chloroform. These standard solutions were made to contain 0.1, 0.25, 0.5, 1, 2, 4, 5, 10, 15, 20, and 40 ng/μL of daminozide methyl ester and 25 ng/μL of succinonitrile internal standard.

Solutions were analyzed by GC/CIMS with duplicate injections at each concentration level. The area ratios of 175 (M + 1 ion of daminozide methyl ester) to m/z 81 (M + 1 ion of succinonitrile) were plotted against the amounts of daminozide methyl ester in the standard solutions on log paper. A linear calibration curve with a regression coefficient of 0.993 was obtained. In the recovery studies and sample analyses, daminozide concentration was calculated by multiplying the amount of daminozide methyl ester by the molecular weight factor, 160/174 (the molecular weights of daminozide and its methyl ester are 160 and 174, respectively).

Results and Discussion

We found that Whiton's (6) method for the determination of daminozide worked quite well if the derivatization time was increased from 1 h to overnight and if chemical ionization mass spectrometry (CIMS) instead of electron ionization mass spectrometry (EIMS) was used in the determinative step.

Figure 1 shows the chemical ionization mass spectrum of daminozide methyl ester. Figure 2 depicts the total ion and mass chromatograms of a standard solution containing 20 ng/μL of daminozide methyl ester (m/z 175) and 25 ng/μL of succinonitrile (m/z 81) internal standard. The total ion and mass chromatograms of apple samples spiked with 0.1 ppm and 0.05 ppm daminozide are shown in Figures 3 and 4, respectively. Even at these trace levels, the 3 peaks at m/z 175, 143, and 115 have sufficient signal-to-noise ratio to confirm the presence of daminozide. Figure 5 shows the total ion and mass chromatograms of an apple sample containing an incurred residue of 0.32 ppm daminozide. One other apple sample was positive (1.04 ppm). Twenty-three apple samples contained no detectable daminozide.

Apples spiked with 0.5 ppm daminozide gave recoveries of 83.1, 92.5, and 101.0% (average = 92.2%, coefficient of variation = 7.9%). At the 0.25 ppm level, recoveries of daminozide were 82.8, 113.2, and 116.4% (average = 104.1%, coefficient of variation = 14.5%). At the 0.05 ppm level, recoveries were 77.0, 86.5, and 120.0% (average = 94.5%, coefficient of variation = 23.9%).

We have demonstrated that daminozide can be quantified at the 0.05–0.10 ppm level in apples by using gas chromatography/chemical ionization mass spectrometry. Chemical ionization was used because it gives stronger molecular ions (protonated) that are of higher mass and thus less abundant in the chemical background. Also, excessive fragmentation is minimized and the intensity of the spectrum is concentrated into few ions, thus increasing the signal-to-noise ratio for the ions to be monitored and allowing the confirmation as well as quantification of daminozide at levels far below legal tolerances.

Acknowledgments

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Confirmation of Phorate, Terbufos, and Their Sulfoxides and Sulfones in Water by Capillary Gas Chromatography/Chemical Ionization Mass Spectrometry

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A gas chromatographic/mass spectrometric method capable of confirming phorate, terbufos, their sulfoxides, and sulfones in water is reported. Parents and their metabolites are separated in less than 5 min using a short capillary GC column and high carrier gas linear velocities. Positive ion chemical ionization mass spectrometry generates (M + H) ions indicative of the different molecular weights of the analytes and at least one confirmatory fragment ion for each analyte. Residues have been qualitatively confirmed at the 1 ppb level in fortified water samples from a variety of sources. Apparent residues in control water were less than 0.1 ppb.

Phorate [Thimet®, *S*-((ethylthio)methyl) *O,O*-diethylphosphorodithioate] and terbufos [Counter®, *S*-((*t*-butylthio)-methyl) *O,O*-diethylphosphorodithioate] are widely used soil insecticides. Terbufos, in particular, is highly effective for control of corn rootworms. The major metabolic route for these organophosphorus pesticides in soil is oxidation of the sulfide to the sulfoxide and sulfone (1, 2). Because of the high acute toxicity of these compounds and their metabolites to mammals (for terbufos, LD_{50} acute oral, male mouse = 3.5 mg/kg) and fish (for terbufos, LC_{50} (96 h), bluegill = 0.004 mg/L), it is extremely important to be able to monitor these compounds in water in the event of runoff, leaching, or accidental contamination.

Most residue analytical procedures for organophosphorus pesticides have used gas chromatography (GC) in conjunction with either a flame photometric detector (FPD) or a nitrogen/phosphorus detector (NPD) (3–10). Electron-capture detectors (ECD) have also been used (3, 6, 7). While packed columns were used for much of the earlier work (4, 5, 10), capillary GC has been increasingly used, especially in multiresidue screening methods (3, 6–9).

For phorate and terbufos, GC methods have been developed for total phorate-related and total terbufos-related residues by use of *m*-chloroperbenzoic acid oxidation to the oxygen analog sulfone (11–14). GC methods have been developed for phorate and its sulfoxide and sulfone (4, 15–18). Because of chromatographic difficulties with terbufos sulfoxide, it has been determined separately following column chromatographic separation and $KMnO_4$ oxidation to the sulfone (2, 19).

For a confirmatory method of analysis, structural specificity is the primary objective and the main reason that mass spectrometry (MS) has become the preferred analytical technique (20). The purpose of the present work was to develop a GC/MS confirmatory method for phorate, terbufos, their sulfoxides, and sulfones in water at the 1 ppb level. The combination of capillary GC in conjunction with chemical ionization mass spectrometry (CIMS) was capable of confirming all 6 analytes in a single analysis.

METHOD

Special Notes

Rinse all clean glassware thoroughly with water followed by acetone and let dry before using. All solvents should be distilled in glass, suitable for pesticide analyses (as supplied by Burdick & Jackson Laboratories, Inc., or equivalent). Water should be purified by a Milli-Q™ water purification system (Millipore Corp.), or equivalent.

Because of the high mammalian toxicity of phorate, terbufos, and their metabolites (see introduction), they must be handled with extreme care. Wear gloves whenever handling the pure standards.

Apparatus

(a) *Gas chromatograph.*—Varian Model 3400 equipped with splitless capillary injector liner and fitted with 4 m × 0.25 mm (0.25 μm film) DB-5 fused silica capillary column (J & W Scientific, Folsom, CA) or equivalent capillary GC equipment. Operating conditions: injector 200°C; column oven 60°C for 0.5 min, increase at 30°/min to 165°C, and hold for 1.0 min; column head pressure 7 psig He; carrier gas linear velocity 120 cm/s at 165°C; splitless injection with split open at 0.5 min. Retention times of analytes are given in Table 1.

(b) *Mass spectrometer/data system.*—Finnigan-MAT TSQ-70 operated in chemical ionization mode and set to pass all ions through first 2 quadrupoles while scanning only third quadrupole, or equivalent system. Operating conditions: GC/MS transfer line 165°C; ion source 120°C; methane source pressure 8400 mT (indicated); conversion dynodes ±5000 V; electron multiplier 1000 V; preamplifier range 10^{-8} amp/V; dwell time 20 ms/ion; total scan time ca 0.5 s/scan; switchover from monitoring phorate and terbufos to monitoring sulfoxides and sulfones ca 3.5 min. Ions monitored are given in Table 1.

Reagents

(a) *Helium.*—UHP, 99.999% minimum purity (MG Industries Gas Products, Valley Forge, PA).

(b) *Methane.*—UHP, 99.97% minimum purity (MG Industries Gas Products).

(c) *Analytical standards.*—Phorate, phorate sulfoxide, phorate sulfone, terbufos, terbufos sulfoxide, and terbufos sulfone (available from American Cyanamid Co., Agricultural Research Division, Princeton, NJ). (*1*) *Stock solutions.*—(equivalent for all analytes) 1.0 mg/mL acetone. (*2*) *Intermediate standard solution.*—20 μg/mL acetone for each analyte. Pipet 1 mL aliquot of each stock solution into 50 mL volumetric flask, dilute to volume with acetone, and mix well. (*3*) *GC/MS standard solution.*—0.5 μg/mL acetone for each analyte (equivalent to 1 ppb of each analyte in water). Pipet 2.5 mL intermediate standard solution into 100 mL volumetric flask, dilute to volume with acetone, and mix well.

Procedure

Measure 500 mL water for analysis and transfer to 1000 mL separatory funnel. Add 100 mL methylene chloride and shake vigorously 15 s. Let phases separate, and drain methylene chloride (lower layer) into 250 mL round-bottom flask. Evaporate to ca 10 mL, using rotary evaporator with water bath set at no more than 30°C. *Do not let flask contents go to dryness.*

Add second 100 mL portion of methylene chloride to separatory funnel and shake vigorously 15 s. Let phases separate, and drain methylene chloride layer into same 250 mL

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Table 1. Retention times and ions monitored for the analytes

Compound	Retention time, min:s	(M + H) ion, m/z	Fragment ions, m/z
Phorate	2:49	261	199, 75
Phorate sulfoxide	3:45	277	199
Phorate sulfone	3:48	293	247, 199
Terbufos	3:07	289	233, 103
Terbufos sulfoxide	3:59	305	187
Terbufos sulfone	4:04	321	265, 199

round-bottom flask. Evaporate to ca 10–20 mL, using rotary evaporator, and then transfer extract to 100 mL pear-shape flask. Rinse 250 mL round-bottom flask with additional 10 mL methylene chloride and transfer rinse to 100 mL pear-shape flask. Evaporate solvent just to dryness on rotary evaporator. *Do not let flask remain on evaporator after solvent has evaporated*! Dissolve residue in 1.0 mL acetone for GC/MS confirmatory analysis.

Results and Discussion

Mass spectra of organophosphorus pesticides, including several of the analytes in this study, have been reported from a number of ionization techniques including electron impact (EI) (21, 22), positive ion chemical ionization (PICI) (23, 24), and negative ion chemical ionization (NICI) (25, 26). EI of the compounds in this study generates either very weak or no molecular ions, which would serve by themselves to differentiate the analytes. While the extensive EI fragmentation is useful for confirmatory purposes, analyte response is reduced because the sample ion current is divided among numerous ions. In addition, many of the fragment ions are common to all analytes, differing only in relative intensities. Thus, accurate measurements of ion ratios and chromatographic retention times are necessary for confirmation.

PICI generates (M + H) ions indicative of the molecular weights of the analytes and minimizes, but still retains, structurally useful fragmentation information. Because of the electron-capturing properties of organophosphorus pesticides, NICI offers enhanced response when compared to PICI (25, 27). However, for these analytes, no molecular information is generated, and the fragmentation, which is indicative of only the phosphate moiety, is inadequate for a confirmatory technique (20, 27, 28). Consequently, PICI was selected as the MS ionization technique for the confirmatory method.

Methane was chosen as the reagent gas because it gave the

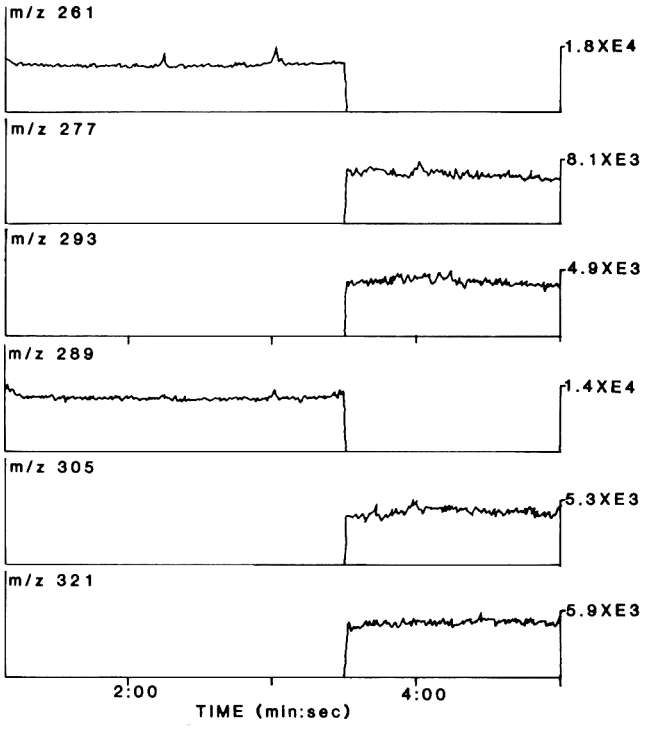

Figure 2. Extracted ion current profiles of (M + H) ions from control lake water.

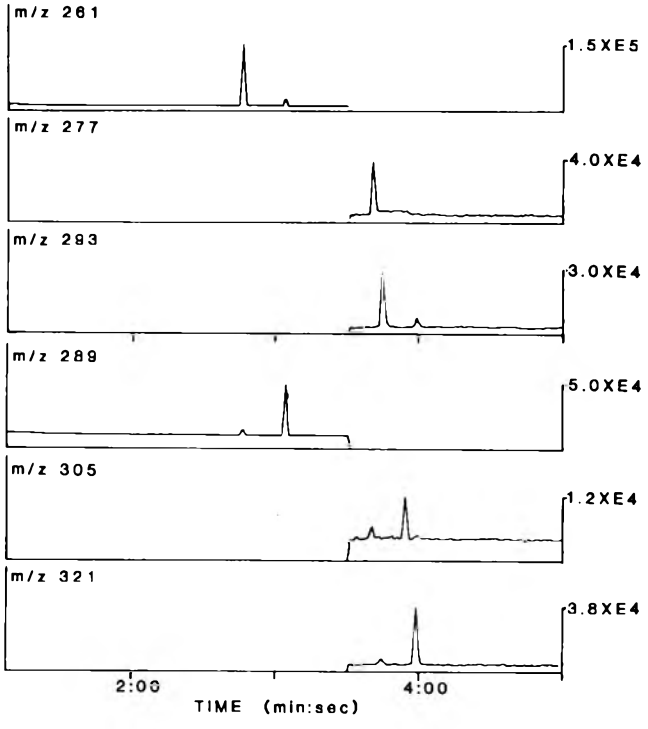

Figure 1. Extracted ion current profiles of (M + H) ions from GC/MS standard solution.

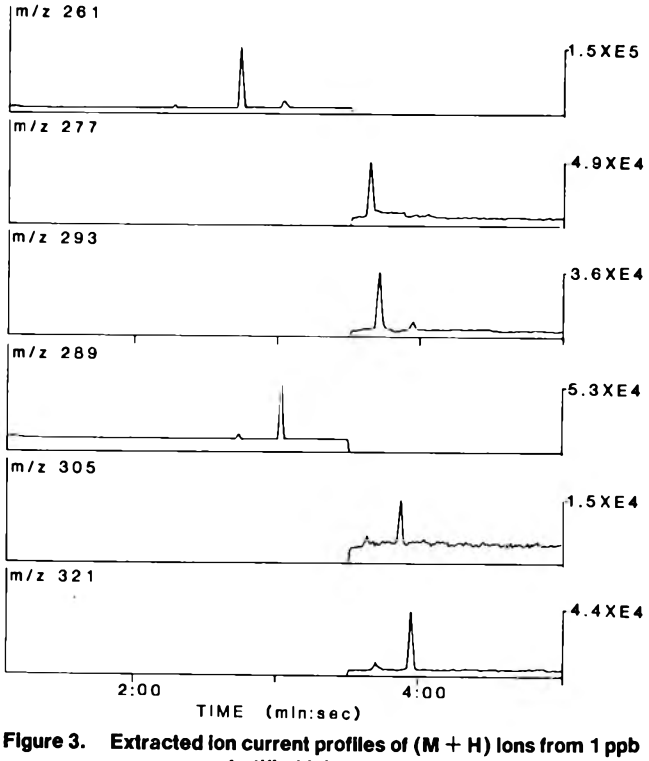

Figure 3. Extracted ion current profiles of (M + H) ions from 1 ppb fortified lake water.

Table 2. Qualitative confirmatory data for the fortified water samples

Source	Phorate		P-Sulfoxide	P-Sulfone		Terbufos		T-Sulfoxide	T-Sulfone	
	261[a] / 75	199 / 75	277 / 199	293 / 247	199 / 247	289 / 103	233 / 103	305 / 187	265 / 321	199 / 321
Std mix (3)[b]	43 (2.5)[c]	26 (5.5)	72 (14)	125 (12)	96 (24)	21 (0.6)	19 (2.6)	21 (3.1)	34 (6.1)	29 (7.6)
1 ppb Fort. reagent water −17A (3)	43	26	77	118	90	22	20	22	33	31
Std mix (15)	45 (5.8)	30 (2.7)	86 (7.5)	86 (3.2)	85 (4.3)	20 (1.3)	24 (1.2)	13 (1.1)	67 (5.5)	42 (3.8)
2 ppb Fort. reagent water −22B (3)	46	31	84	88	85	22	24	13	64	43
1 ppb Fort. reagent water −18B (1)	50	32	88	85	82	21	25	13	70	45
2 ppb Fort. reagent water −18C (1)	48	32	94	83	82	21	24	14	65	40
Std mix (4)	40 (13)	31 (1.8)	69 (20)	48 (14)	73 (4.7)	12 (4.5)	24 (2.8)	11 (1.5)	101 (19)	80 (12)
1 ppb Fort. reagent water −26D (2)	30	30	51	36	71	10	23	9.0	96	90
Std mix (10)	36 (2.1)	31 (1.1)	61 (4.0)	57 (1.4)	74 (6.1)	14 (0.8)	25 (0.9)	16 (2.3)	57 (2.9)	52 (5.5)
1 ppb Fort. reagent water −27C (2)	37	30	64	55	69	14	24	18	58	48
1 ppb Fort. reagent water −27D (2)	36	30	63	56	70	14	24	18	52	46
Std mix (4)	42 (2.9)	33 (0.5)	67 (3.1)	64 (8.4)	84 (8.2)	13 (1.4)	27 (1.0)	12 (0.6)	61 (2.4)	54 (5.9)
1 ppb Fort. well water −28D (1)	40	33	65	66	85	12	26	13	66	60
1 ppb Fort. well water −28E (1)	40	32	62	66	85	13	26	13	68	60
Std mix (4)	51 (1.7)	33 (0.8)	90 (2.5)	87 (8.8)	90 (5.2)	17 (1.0)	26 (1.3)	12 (0.8)	76 (6.8)	46 (4.5)
1 ppb Fort. lake water −33B (4)	52	33	91	84	87	18	26	13	82	48
Std mix (4)	52 (7.0)	31 (1.7)	81 (7.2)	82 (3.9)	92 (6.4)	18 (0.5)	24 (1.4)	15 (1.3)	88 (1.3)	49 (1.0)
1 ppb Fort. well water −34C (2)	49	30	83	80	96	18	24	18	93	54
1 ppb Fort. well water −34D (2)	50	30	82	78	94	18	23	17	88	50

[a] Ion ratios monitored (in percent).
[b] Number of replicate injections.
[c] Standard deviations on the standard injections for the samples.

best balance between generation of molecular weight information and retention of fragmentation information over the range of compounds analyzed. The ions monitored and the retention times of the analytes are summarized in Table 1.

The most difficult analyte, both spectrometrically and chromatographically, was terbufos sulfoxide, as has been reported by others (19, 22). Terbufos sulfoxide gave the lowest relative intensity for its (M + H) ion and the weakest overall analyte response, probably due to chromatographic difficulties. To facilitate the GC analysis of terbufos sulfoxide required the use of a short capillary column from injector to detector and high carrier gas linear velocities (29). Keeping the capillary column and GC/MS interface at or below 165°C coupled with rapid temperature programming were also essential. Under these conditions, all 6 analytes could be separated and eluted in under 5 min.

In terms of sample preparation, the only difference between this procedure and several similar ones reported previously (12–14) is the absence of PEG-400 as a keeper during solvent evaporation. As discussed by Stout et al. (28), PEG-400 is not as transparent to MS as it is to other specific GC detectors for residue analysis. Not only did the PEG-400

Table 3. Percent recoveries of phorate, terbufos, their sulfoxides, and sulfones from water[a]

Source	Phorate	P-Sulfoxide	P-Sulfone	Terbufos	T-Sulfoxide	T-Sulfone
1 ppb Fort. reagent water 17A	82.8	147.4	105.5	97.2	117.5	96.7
1 ppb Fort. reagent water 22B	92.0	110.4	105.1	108.7	113.1	98.4
2 ppb Fort. reagent water 18B	109.8	141.5	116.2	111.0	127.5	122.3
2 ppb Fort. reagent water 18C	94.7	148.3	143.6	99.9	154.8	157.2
1 ppb Fort. reagent water 26D	79.4	110.9	97.6	104.7	105.6	109.2
1 ppb Fort. reagent water 27C	96.4	103.8	96.8	100.5	97.0	104.9
1 ppb Fort. reagent water 27D	93.2	111.2	103.6	97.6	104.8	103.9
1 ppb Fort. well water 28D	93.1	86.9	101.6	97.3	94.1	94.0
1 ppb Fort. well water 28E	88.8	87.6	93.6	91.8	83.2	98.5
1 ppb Fort. lake water 33B	102.4	124.3	117.0	106.0	122.4	113.7
1 ppb Fort. well water 34C	93.4	114.8	96.4	98.1	104.9	94.2
1 ppb Fort. well water 34D	98.7	132.0	113.4	103.6	130.3	129.0

[a] Calculated from (sample response ÷ 1 ppb standard response) × 100 for the (M + H) ion of each analyte.

generate numerous large background peaks throughout the GC/CIMS chromatogram, it essentially destroyed the capillary GC column with one injection when accidentally placed in one sample extract. Consequently, as noted in the Method, it is extremely important to remove the evaporation flasks from the evaporators immediately after solvent evaporation to minimize the risk of losing the volatile parent compounds.

To test the GC/CIMS confirmatory procedure, water samples from a variety of sources were fortified at 1 ppb with each analyte, extracted, and analyzed by GC/CIMS. The extracted ion current profiles of the (M + H) ions of the analytes are shown in Figures 1, 2, and 3 for the GC/MS standard solution (equivalent to 1 ppb of each analyte in water), control lake water, and 1 ppb fortified lake water, respectively. The other water samples gave essentially identical results. The qualitative confirmatory data for the fortified water samples are presented in Table 2.

The relative intensities of the confirmatory ions from the fortified water samples are generally within one standard deviation of the mean standard intensities. The absolute standard deviations correspond, predominantly, to better than 10% relative standard deviations. These values actually understate the qualitative confirmatory capabilities of the method because each result is calculated from one day's analyses. In practice, confirmation is usually done by bracketing the sample injection with a pair of standard injections to reduce the impact of drift and instability on the ion ratios.

While quantitative data are not as important to a confirmatory method as the structural information provided, recovery data from the fortified water samples are listed in Table 3. While these recoveries are excellent for all analytes at the 1 ppb level, they unfortunately overstate the quantitative capabilities of the current methodology. As shown in Table 3 for several samples fortified at 2 ppb, the recoveries range from 94.7% to 157.2% when calculated against the 1 ppb standard. The dramatic increase in response, especially for the sulfoxides, suggests a chromatographic enhancement with larger levels of injected analytes. This assessment is supported by a linearity study which showed 13-fold (for phorate and terbufos) to 20-fold (for the sulfoxides) response increases for 10-fold increases in the injected analytes. Thus, for quantitation, separate calibration curves, several of which could be nonlinear, would be required for each analyte. This undertaking was deemed beyond the scope of the current investigation.

In conclusion, a GC/MS method capable of confirming phorate, terbufos, their sulfoxides, and sulfones at the 1 ppb level in water has been developed. The parents and their metabolites are separated in less than 5 min using a short capillary GC column and high carrier gas linear velocities. PICI(CH_4) generates (M + H) ions indicative of the different molecular weights of the analytes and at least one confirmatory fragment ion for each analyte. Residues have been qualitatively confirmed at the 1 ppb level in fortified water

samples from a variety of sources. Control water extracts were clean at the monitored ions of all analytes. Quantitative confirmations were hindered by the chromatographic nature of the analytes. Potentially, use of cold on-column capillary injection techniques might aid in producing better quantitative results by further reducing chromatographic difficulties with the analytes at residue levels.

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Simplified Extraction and Cleanup for Multiresidue Determination of Pesticides in Lanolin

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In the proposed method, a light petroleum solution of lanolin (wool fat) is adsorbed on diatomaceous earth in an Extrelut® column, and the pesticides are eluted with acetonitrile saturated with light petroleum. After evaporation to a small volume, the extract is subjected to solid-phase extraction (SPE) on a C-18 column. The acetonitrile eluate is evaporated to dryness and the residue is taken up in light petroleum. Organophosphorus pesticides are determined by temperature-programmed gas chromatography (GC) on a wide-bore column using a flame photometric detector in the phosphorus mode. Organochlorine pesticides are determined after miniaturized Florisil cleanup by classic GC on an OV-17/QF-1 packed column, using an electron capture detector. This procedure is more rapid and straightforward than the time-consuming AOAC extraction method, 29.014. Cleanup was better and the results obtained were comparable. Recoveries for 13 organochlorine and organophosphorus pesticides, frequently found in lanolin, ranged from 80 to 90%.

One of the most important raw materials for cosmetic products such as creams and ointments is lanolin, a waxy material extracted from wool. It is also called wool fat and is known to contain often high amounts of pesticide residues (1; T. Stijve (1977) *Laboratory News* **37,** 2, 4-7, internal publication, Nestec Ltd, Vevey, Switzerland).

As a matter of fact, in order to protect the wool and health of sheep, the animals are dipped dermally with insecticide formulas. Not surprisingly, lipophilic organochlorine and organophosphorus compounds are strongly retained by the lanolin. Because the presence of substantial amounts of pesticides in cosmetics, especially in creams for babies, is undesirable, this raw material must be checked. An interim limit of 40 mg/kg for total organophosphorus pesticides was recently recommended by Australian authorities (2).

Because it is rather a wax than a fat, lanolin is difficult to analyze. Cleanup according to direct elution procedures was found to be impossible, because Florisil retains very little of the lanolin waxes. The traditional solvent-partitioning step (3), also known as the back-wash procedure, is suitable, but it is time-consuming and prone to the formation of emulsions, which are difficult to eliminate. Alternatively, gel permeation chromatography (GPC), followed by cleanup on silica minicolumns may be used (1, 4).

GPC is not generally available to smaller laboratories, so we decided tc first try the solid-phase extraction (SPE) technique. We first attempted to simply extract lanolin with acetonitrile in a Polytron mixer and to pass this extract over a C-18 SPE column. Regrettably, a complete extraction required heating of both lanolin and acetonitrile before and during mixing, which is not practical.

Recently, Di Muccio et al. (5) described a single-step partition between light petroleum and acetonitrile on ready-to-use disposable Extrelut columns (filled with diatomaceous earth) for the cleanup of fatty extracts prior to the determination of organophosphorus pesticides. By applying this method to lanolin we observed that 5-10% of the material was coeluted from the Extrelut column. The purpose of the present paper is to present an improved cleanup method using successively SPE on Extrelut and on a C-18 cartridge.

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Table 1. Recoveries (%) of organochlorine and organophosphorus pesticides added to lanolin and determined by GC after extraction and cleanup

Pesticide	Added, in mg/kg 0.25		0.5		1		2		Mean	Std dev.
HCB	79	85	81	87	85	89	82	88	84.5	3.5
alpha-BHC	83	85	91	93	83	87	88	92	87.8	4.0
gamma-BHC	82	88	90	94	88	94	92	96	90.5	4.5
Dieldrin	78	84	88	92	83	87	80	84	84.5	4.5
p,p'-DDE	81	90	89	93	84	91	87	93	88.5	4.3
p,p'-TDE	81	85	84	90	79	85	85	91	85	4.0
P,P'-DDT	81	87	86	92	81	85	89	93	86.8	4.5
Diazinon	88	82	84	90	94	88	89	95	88.8	4.4
Dichlofenthion	78	84	83	87	83	89	84	90	84.8	3.8
Chlorfenvinphos	76	81	84	88	79	83	80	87	82.3	4.1
Bromophos ethyl	83	89	81	87	86	90	89	95	87.5	4.3
Ethion	79	85	79	83	82	88	87	93	84.5	4.8
Carbophenothion	78	80	77	83	78	82	82	90	81.3	4.2

Experimental

Apparatus and Reagents

(a) *Gas chromatograph I.*—Packard Model 437 A, or equivalent, with FPD operated in phosphorus mode and following column: Megabore column DB-1 (J and W Scientific, Part. No. 125-1012), 15 m × 0.53 mm id. Film thickness 1.5 μm. Initial temperature: 120°C. Initial time 1 min. Oven rise 4°C/min. Final temperature 270°C. Final time 10 min. Carrier gas, nitrogen, 4 mL/min. Make-up gas, nitrogen, 30 mL/min. Injector temperature 240°C. Detector temperature 300°C. Injection volume 2 μL.

Injection was performed using a direct flash injection liner (J and W Scientific Part. No. 210-1064), which consists of a tube with a tapered restriction at both ends. The taper at the top minimizes flash-back against the septum. The taper at the bottom automatically aligns and seals the Megabore column for efficient sample transport and helps to prevent sample contacting the column ferrule.

With this mode of injection, the sample is vaporized in the liner and swept onto the column.

(b) *Gas chromatograph II.*—Packard Model 437 A, or equivalent, with ECD and following column: 1.8 m × 4 mm id, Pyrex glass, packed with 1.5% OV-17 and 1.95% QF-1 on 100–120 mesh Chromosorb W-HP. Oven temperature 210°C. Carrier gas, nitrogen, 30 mL/min. Make-up gas, nitrogen, 30 ml/min. Detector temperature 300°C. Injector 240°C.

(c) *Evaporator.*—Rotary (Rotavapor, RE-121, Büchi CH-9230 Flawil, Switzerland), including a water bath.

(d) *Columns.*—Extrelut-3 (Merck, Cat. No. 15372). Fix disposable cannulas (Merck Cat. No. 15373) at column end as flow regulators.

(e) *Solid phase extraction cartridges.*—C-18 (J.T. Baker, Cat. No. 7020-3). Condition just before use as follows: Rinse with 2 volumes of methanol. Rinse with 2 volumes of water. Rinse with 1 volume of acetonitrile. Do not let sorbent run dry during conditioning and analysis.

(f) *Florisil.*—(Fluka, Catalogue No. 46385). Remove interfering impurities by heating overnight at 550°C. After cooling, standardize adsorbent by adding 5% by weight of water. Mix well and let equilibrate ≥6 h before use.

(g) *Solvents.*—Light petroleum (boiling range 40–60°C), acetonitrile (saturated with light petroleum), methanol, isooctane. All solvents should be residue analysis grade or redistilled in glass.

Table 2. Typical residues in lanolin (mg/kg)

Pesticide	Sample 1	2	3	4	5	6
Total BHC	4.4	3.83	0.92	1.21	0.21	2.32
Dieldrin	1.5	2.23	2.22	0.91	0.25	1.5
Total DDT	1	1.08	nd[a]	nd	0.2	nd
Toxaphene	7.5	9	20.8	4.5	0.91	45
Diazinon	37.7	15.5	32.5	0.72	nd	14.2
Dichlofenthion	5.3	8.9	2.7	12.4	nd	1.4
Chlorfenvinphos	13.8	6.6	14.8	6.7	2.64	24.2
Bromophos ethyl	4.65	9.9	0.28	10.3	nd	0.65
Ethion	nd	nd	1.15	nd	nd	0.31
Carbophenothion	0.54	1	1.17	nd	nd	1.65

[a] None detected.

Procedure

Weigh 2 g lanolin into 5 mL volumetric flask, dilute to volume with light petroleum, and shake well to mix. Transfer 2.5 mL of the solution to an Extrelut-3 column and let adsorb for 10 min.

Elute 4 times with 5 mL acetonitrile saturated with light petroleum. Collect eluates in a 100 mL round-bottom flask, add 5 mL methanol, and evaporate in the evaporator at 40°C to about 2 mL.

Transfer to a conditioned C-18 SPE cartridge using 2 mL acetonitrile to rinse the flask. Elute twice with 2 mL acetonitrile. Collect the eluates in a 25 mL round-bottom flask, add 2 mL methanol, and evaporate in the evaporator at 40°C to about 2 mL. Remove the remaining solvent under a gentle stream of clean air.

Take up the residue in small portions of light petroleum, transfer to a 5 mL volumetric flask, dilute to volume, and shake well to mix.

Evaporate a 2 mL aliquot in a vial and take up in 2 mL isooctane. Analyze for organophosphorus pesticides by GC on a megabore column using a flame photometric detector in the phosphorus mode or a thermionic detector.

Transfer the remaining 3 mL to a Florisil column (5 g deactivated with 5% water). Elute with 100 mL light petroleum–dichloromethane (4 + 1 v/v).

Evaporate in the evaporator at 30°C to about 2 mL, and then gently blow to dryness under a stream of clean air. Take up residue in small portions of light petroleum, transfer to a 5 or 10 mL volumetric flask, dilute to volume, and shake well to mix. Analyze for organochlorine pesticides by electron capture GC on a OV-17/QF-1 (1.5%/1.95%) packed column or equivalent.

Results and Discussion

The single-step partition and cleanup procedure described by Di Muccio et al. (5) was found to be unsuitable for lanolin because 5–10% of the lipid material coeluted from the Extrelut column. After a further purification on a C-18 SPE cartridge, only 10–20 mg was coextracted. The extracts thus obtained were sufficiently clean for the analysis of organophosphorus pesticides on a wide-bore column. Cleanup was clearly better than that obtained by the separatory funnel partition technique where as much as 200 mg passed into the final extract. However, for the determination of organochlorines, a supplementary cleanup, on a small column of Florisil, proved necessary.

Samples of lanolin fortified with 4 levels (0.25, 0.5, 1, and 2 mg/kg) of 13 organochlorine and organophosphorus pesticides were analyzed in duplicate. The results, listed in Table

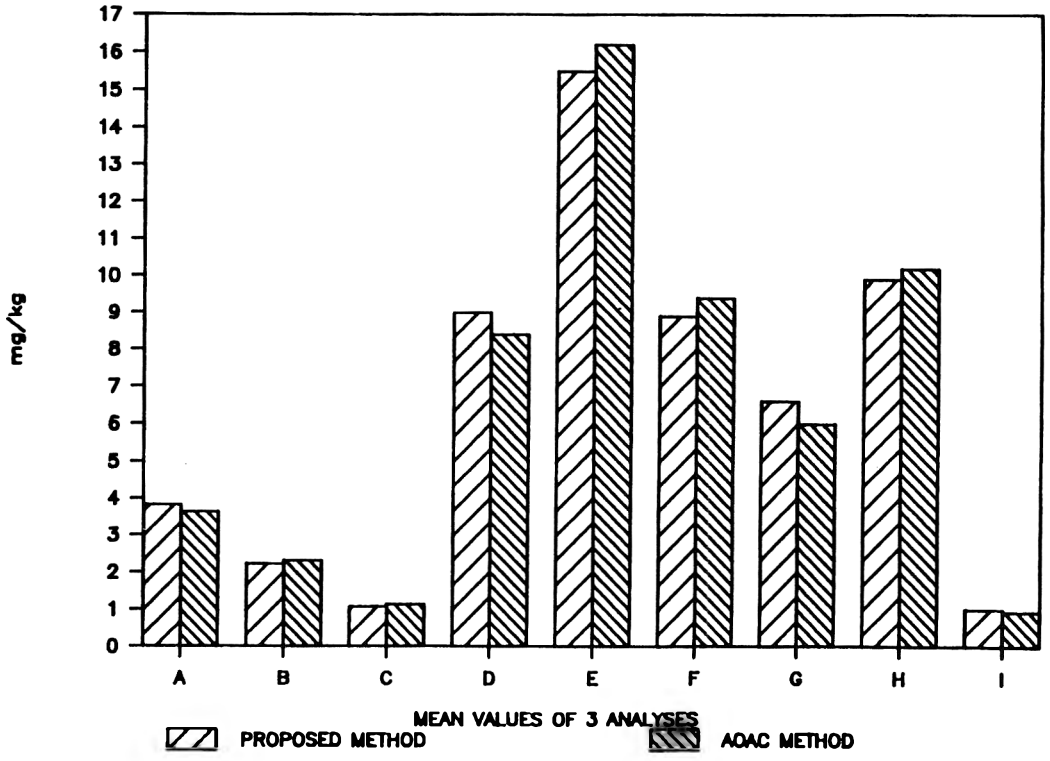

Figure 1. Comparison of methods: proposed method vs AOAC method 29.014–29.015. A = total BHC, B = dieldrin, C = total DDT, D = toxaphene, E = diazinon, F = dichlofenthion, G = chlorfenvinphos, H = bromophos ethyl, I = carbophenothion.

1, clearly indicate that recoveries ranged from about 80–90% for all the compounds tested and that the repeatability is satisfactory.

Comparative analyses performed by the proposed method and the AOAC method (3, 6) yielded essentially the same results (Figure 1).

Typical pesticide residues in lanolin are listed in Table 2. Levels are generally lower than those reported earlier (T. Stijve, 1977). Indeed we found, at that time, up to 500 mg/kg toxaphene, 73 mg/kg diazinon, and 200 mg/kg of total BHC. However, even now the contamination of some of the lanolin samples is still to be considered excessive, especially if this raw material is to be used as an ingredient of cream for babies. The present results agree rather well with those reported by other laboratories (1, 2), who also found that the levels of contamination varied considerably among the lots tested.

It is worth noting that the organophosphorus pesticides dichlofenthion and bromophos ethyl are eluted from the Florisil column with light petroleum–dichloromethane (4 + 1 v/v) together with the organochlorines.

Interestingly, whenever toxaphene was present, its peak pattern proved to be quite similar to that of the analytical standard, indicating that this acaricide was not degraded after application.

Summarizing, it can be said that the method presented is rather simple compared with other procedures for fatty substrates. It only uses small volumes of solvents, for example, only 26 mL acetonitrile compared with 120 mL in the back-wash method (3). Moreover, the method is rapid: 4 extracts can be ready for analysis of organophosphorus pesticides in about 90 min. Recoveries obtained for 13 different organochlorine and organophosphorus pesticides at various spike levels and comparative results with the back-wash extraction method suggest that it is quite suitable for routine analysis. The detection limits for the various pesticide compounds are about 5 μg/kg for organochlorines (toxaphene 500 μg/kg) and approximately 50 μg/kg for organophosphorus pesticides.

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PESTICIDE FORMULATIONS

Determination of Organic Arsenicals in Pesticide Formulations by Ion Chromatography

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The organic arsenicals, cacodylic acid and methanearsonic acid and their salts, are determined in pesticide formulations by ion chromatography. The samples are diluted in the aqueous mobile phase, injected directly into the chromatograph, and determined by non-suppressed ion chromatography. Analysis time is less than 5 min for each arsenical. The method was tested for precision, linearity, and recovery. Linearity of the methanearsonic acid gave regression and correlation coefficients of 0.9998 and 0.9999, respectively, and 0.9997 and 0.9998 for cacodylic acid. Quadruplicate analyses of 5 methanearsonic acid samples gave relative standard deviations of 0.833–3.10. Quadruplicate analyses of 4 cacodylic acid samples gave relative standard deviations of 0.648–1.41. Recovery was 100.3 ± 3.6%.

Organic arsenicals are used in pesticide formulations as selective contact herbicides. They are produced by the Meyer reaction in which biomethylation of arsenic produces cacodylic acid and methanearsonic acid (1). Acidification of these reaction mixtures, followed by appropriate isolation steps, provides cacodylic acid and methanearsonic acid. The technical material obtained from this reaction yields impure methanearsonic acid and cacodylic acid. The acids may contain arsenate impurities. Cacodylic acid contains methanearsonic acid as an additional component (1).

Official AOAC methods for organic arsenicals determine total arsenic and are unable to differentiate these compounds (2–4). Several gas chromatographic techniques have been developed for the quantitation of organoarsenic compounds (5, 6). However, the samples must be derivatized, and these products are unstable and poisonous. Several arsine reduction methods have been published but are time consuming and costly (7, 8). Methods for differentiating organic and inorganic arsenic involve multi-stage cycles of chelation-solvent extraction, phase separation, evaporation, and/or back extraction into a more suitable phase prior to quantitation by atomic absorption spectrometry.

Recently, suppressed ion chromatography has been used to separate some arsenic compounds (9–11). Conversely, high-pH eluants provide good separation and sensitivity for the direct analysis of weakly dissociated anions (12). Suppressed ion chromatography cannot be used under basic conditions that limit its applicability to the direct analysis of the weak acids, cacodylic acid, and methanearsonic acid (13). A recent method describes the analyses of arsenic oxysalts by using a potassium hydroxide-aromatic salt eluant with non-suppressed ion chromatography (13).

In the present study, a potassium hydroxide-potassium chloride eluant is used with non-suppressed ion chromatography to separate and quantitate cacodylic acid and methanearsonic acid in pesticide formulations and as manufacturing impurities.

METHOD

Apparatus and Reagents

(a) *Detector.*—Conductivity, range 1000, coarse adjustment 2, fine adjustment about 3 (Wescan Instruments, Deerfield, IL 60015).

(b) *Extractant.*—10mM KOH + 5mM KCl.

(c) *Liquid chromatograph.*—Varian Model 5060 equipped with Valco C6U injection valve with 50 μL loop, or equivalent (Varian Assoc., Palo Alto, CA 94303).

(d) *LC column.*—150 × 4.1 mm Hamilton PRP-X100, 10 μm (Hamilton Co., Reno, NV 89520).

(e) *Mobile phase.*—10mM KOH + 5mM KCl; flow rate 1.0 mL/min.

Extraction and Liquid Chromatography

(a) *Sample.—Liquids.*—Weigh equivalent of 25 mg active ingredient into 50 mL volumetric flask and dilute to volume with mobile phase. Inject 50 μL into chromatograph. *Granular.*—Weigh equivalent of 25 mg active ingredient into 125 mL Erlenmeyer flask and add 50 mL mobile phase by pipet. Shake 15 min, filter through glass fiber filter pad (Gelman Sciences, Inc., Ann Arbor, MI 48106), and inject 50 μL into chromatograph.

(b) *Standard.—Cacodylic acid.*—98.3% (Fisher Scientific). *Methanearsonic acid.*—Purified from technical material according to (1). Weigh 25 mg active ingredient into 50 mL volumetric flask and dilute to volume with mobile phase. Inject 50 μL into chromatograph. Bracket every 3 samples with standard injections.

Calculations

Use peak height as sample response and average of standard peak heights bracketing sample as standard response. Calculate as follows:

$$\text{Cacodylic or methanearsonic acid, \%} = (R/R') \times (W'/W) \times (V/V') \times \%\ \text{std purity}$$

where R and R′ = response for sample and standard, respectively; W and W′ = weight of sample and standard, respectively; V and V′ = volume of sample and standard, respectively. Here, V and V′ = 50. Calculate percent salt for acid by multiplying percent acid by factor acid to salt.

Example: For disodium methanearsonate (76.1% acid)

$$\%\ \text{salt} = \%\ \text{methanearsonic acid} \times 1.31$$

Results and Discussion

Figure 1 represents a typical chromatogram of a cacodylic acid formulation sample. All 4 cacodylic acid formulations analyzed had methanearsonic acid impurities. Figure 2 represents a calcium acid methanearsonate sample. The acids have a lower conductance than the potassium hydroxide eluant and appear as a negative peak. The polarity of the detector is simply reversed to make the peak positive for integration. The cation, which is calcium in this case, is weakly retained and elutes as a negative peak in about 1 min.

Initially, a 10mM KOH mobile phase was used without the addition of potassium chloride. Figure 3 shows the resulting chromatogram of sodium cacodylic acid under these conditions. No response was obtained at these conditions for

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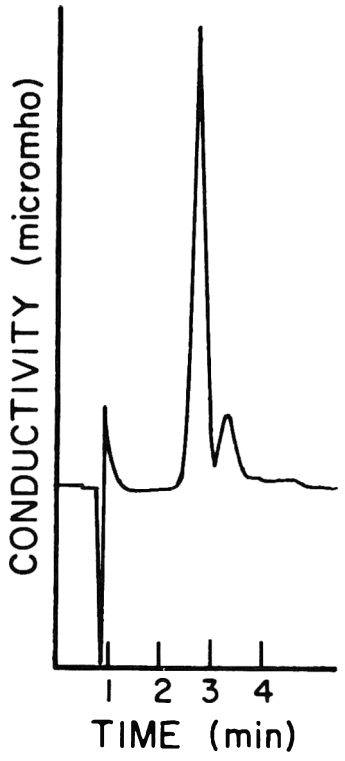

Figure 1. Typical chromatogram of cacodylic acid formulation sample. Solvent front (ca 0.8 min), cacodylic acid (ca 2.6 min), and methanearsonic acid impurity (ca 3.2 min).

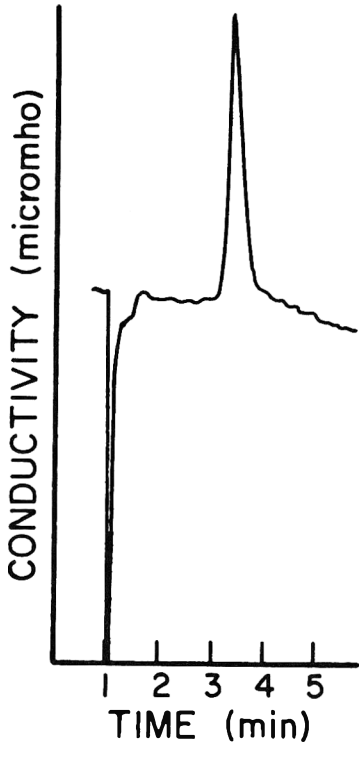

Figure 3. Chromatogram of sodium cacodylic at 10mM KOH mobile phase with 2 mL/min flow. Sodium ion (ca 1.0 min) and cacodylic acid (ca 3.5 min).

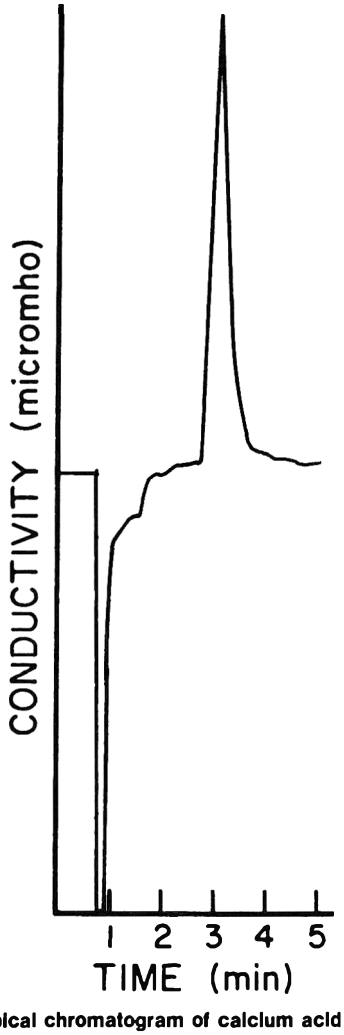

Figure 2. Typical chromatogram of calcium acid methanearsonate sample. Calcium ion (ca 0.8 min) and methanearsonic acid (ca 3.2 min).

methanearsonic acid, even when the mobile phase concentration was increased to 50mM KOH. Potassium chloride added to the mobile phase eluted the strongly retained methanearsonic acid. Thus, potassium chloride is essential for the analysis of methanearsonic acid formulations and for the identification and quantitation of methanearsonic acid impurities in cacodylic acid.

Sample solutions that sat overnight and were reinjected the next day produced chromatograms with additional peaks. Immediate injection after extraction or extraction in polypropylene volumetrics prevented this and is recommended. Linearity of the method was tested by chromatographing dilutions of the cacodylic acid standard that ranged in concentration from 0.05 to 0.7 mg/mL. Similarly, linearity of methanearsonic acid response from 0.05 to 0.8 mg/mL was established. Because of the capacity of the column, solutions over 0.8 mg/mL resulted in overload and nonlinearity. Least squares analyses of cacodylic acid data gave regression and correlation coefficients of 0.9997 and 0.9998, and 0.9998 and 0.9999 for methanearsonic acid. Recovery of diluted standard added to a sample (n = 4) was calculated to be 100.3 ± 3.6%.

Table 1 shows the results of quadruplicate analyses of methanearsonic acid in 5 formulations. Sample 88-1 contained the disodium salt. Samples 88-2, 88-3, and 88-4 contained calcium salts of methanearsonate, and sample 88-9 contained monosodium methanearsonate. Table 2 shows the results of quadruplicate analyses of cacodylic acid in 4 formulations.

Total arsenic was determined for all samples by AOAC method **36.044** (4). The percent of methanearsonic acid found by ion chromatography agreed with the total arsenic determination. However, the percent of cacodylic acid calculated from total arsenic was significantly higher than by ion chromatography. This was expected because total arsenic determinations include both cacodylic acid and methanear-

Table 1. Results of quadruplicate analyses of methanearsonic acid in pesticide formulations[a]

Formulation	Label claim, %	Found by TA, %	Found by IC, %
88-1	2.20	—[b]	2.27
			2.30
			2.26
			2.26
Av. ± SD(RSD)			2.27 ± 0.0189(0.833)
88-2	9.01	9.12	9.36
			9.20
			8.95
			8.72
Av. ± SD(RSD)			9.06 ± 0.281(3.10)
88-3	9.01	8.92	8.22
			8.45
			8.18
			8.10
Av. ± SD(RSD)			8.24 ± 0.150(1.82)
88-4	7.35	7.95	8.06
			8.32
			8.07
			8.07
Av. ± SD(RSD)			8.13 ± 0.127(1.56)
88-9	11.4	12.7	12.5
			12.1
			11.8
			12.1
Av. ± SD(RSD)			12.1 ± 0.287(2.37)

[a] TA = total arsenic; IC = ion chromatography.
[b] Sample could not be analyzed by this method.

Table 2. Results of quadruplicate analyses of cacodylic acid in pesticide formulations[a]

Formulation	Label claim, %	Found by TA, %	Found by IC, %
88-6	23.5	—[b]	LOST
			23.7
			23.6
			23.4
Av. ± SD(RSD)			23.6 ± 0.153(0.648)
88-7	0.607	0.682	0.620
			0.621
			0.629
			0.630
Av. ± SD(RSD)			0.625 ± 0.00523(0.837)
88-8	1.30	1.65	1.14
			1.14
			1.14
			1.12
Av. ± SD(RSD)			1.14 ± 0.00999(0.881)
88-10	0.617	0.837	0.792
			0.791
			0.774
			0.771
Av. ± SD(RSD)			0.782 ± 0.0110(1.41)

[a] TA = total arsenic; IC = ion chromatography.
[b] Sample could not be analyzed by this method.

sonic acid, whereas ion chromatography differentiates the 2 acids. Samples 88-1 (Table 1) and 88-6 (Table 2) contained a vermiculite carrier that interfered with the digestion step in determining total arsenic content by the AOAC method. These samples were analyzed easily by ion chromatography.

The present study shows that ion chromatography is simple, inexpensive, and efficient for the analysis of organic arsenicals in pesticide formulations. In addition, this method is much faster, safer, and more specific for the organic arsenicals than the official AOAC method. This technique offers adequate precision, linearity, and recovery for the analysis of the organic arsenicals, methanearsonic acid, and cacodylic acid.

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SAMPLE PREPARATION

Enzymatic Hydrolysis of Biological and Environmental Samples as Pretreatment for Analysis

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Four commercially available proteases were tested, in conjunction with a lipase, for efficacy in hydrolyzing 3 tissue substrates: cod fillet, chicken egg, and bovine liver. Enzymatic hydrolysis of tissues minimizes the formation of emulsions during liquid-liquid extraction and does not accelerate the decomposition of acid- or base-labile analytes. Recovery of hexane and benzene phases from the hydrolysates was also evaluated. Protease from *Streptomyces griseus* combined with lipase from *Candida cylindracea* (available commercially) produced the highest percent hydrolysis (relative to classical acid hydrolysis) in all 3 tested tissues (60–95%) and the greatest recovery of hexane (100%) and benzene (92–100%) solvent phases.

Extraction of analytes from environmental samples, particularly biological materials, can be hampered by the formation of emulsions and analyte binding to proteinaceous material. Successful control of emulsions permits the analyst to use minimum volumes of extraction solvent (critical if analyte recovery is decreased by evaporation of large volumes of solvent), allows fewer multiple extractions, and reduces the time needed for separation of the mixture after extraction. Although techniques exist to control emulsions during extraction (salt addition, alcohol added to emulsion-liquid interface, allowing mixture to stand for some time), they are not always successful, and sample pretreatment becomes necessary.

Acid or base hydrolysis can reduce emulsion formation and release bound analytes but often exposes the analytes to heated and harsh pH conditions. These techniques are unsuitable for volatile or acid/base-labile analytes.

The analysis of environmental samples for organolead compounds initiated our interest in enzymatic hydrolysis: (*1*) Emulsion control was unsuccessful with the extraction techniques discussed above. (*2*) Acid hydrolysis could not be used because organoleads are acid-labile. (*3*) Hydrolysates produced with tetramethylammonium hydroxide still exhibited high emulsion formation tendencies.

Enzymatic hydrolysis of environmental samples, using a mixture of protease and lipase, has been found to improve extraction efficiency of organolead compounds from a variety of tissues and to control the formation of emulsions (1, 2), even in the presence of benzene–hexane solvent mixtures. Enzymatic hydrolysis of food proteins has generally been used in the investigation of nutritive value (3), amino acid composition (4), and potential for food use (5).

To further investigate this alternative technique of sample hydrolysis prior to extraction of environmental samples, 4 commercially available proteases were compared for recovery of organic solvent phases and degree of hydrolysis achieved with 3 tissue substrates: cod fillet, bovine liver, and chicken egg.

A spectrophotometric method using 2,4,6-trinitrobenzenesulfonic acid, modified from a method previously reported (6), was used to determine amino nitrogen levels in the hydrolysates.

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Experimental

Reagents and Materials

(a) *Deionized water.*—18 megohms/cm (Milli-Q Water System, Millipore Corp., Bedford, MA). Used throughout.

(b) L-*Valine solution.*—L-Valine (10 mg as amino nitrogen) was dissolved in 5 mL water and stored at 4°C.

(c) *Proteases, lipase.*—Proteases isolated from *Streptomyces griseus* (type XIV), crude pancreatic (type I), *Bacillus subtilis* (type XVI), and *Aspergillus oryzae* (type XXIII), and lipase isolated from *Candida cylindracea* (type VII) were obtained from Sigma Chemical Co.

(d) *Tris(hydroxymethyl)aminomethane (tris) solution.*—Tris(hydroxymethyl)aminomethane (30.28 g) was dissolved in 500 mL water and adjusted with 6N HCl to pH 7.5 or 8.5.

(e) *Buffer A solution.*—KCl (0.37 g) and H_3BO_3 (0.31 g) were dissolved in 50 mL water and adjusted to pH 9.5 with 0.1M NaOH.

(f) *Buffer B solution.*—KH_2PO_4 (2.72 g) and 62.5 mg K_2SO_3 were dissolved in 100 mL water. K_2SO_3 was added just before use.

(g) *2,4,6-Trinitrobenzenesulfonic acid (TNBS) solution.*—TNBS (35 mg) was dissolved in 1 mL absolute ethanol. Solution was prepared daily.

Apparatus

(a) *Spectrometer.*—Spectronic 20 (Bausch and Lomb Inc., Rochester, NY).

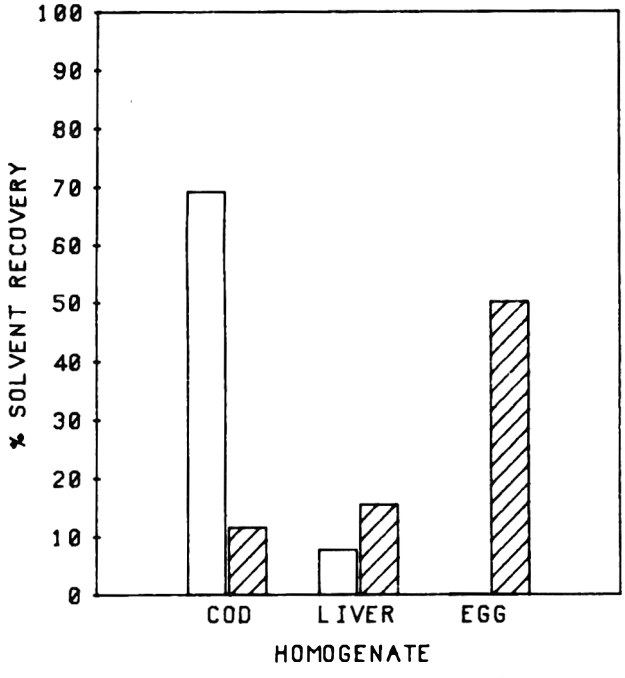

Figure 1. Solvent phase recovery from cod fillet, chicken egg, and liver homogenates: open bar, hexane; hatched bar, benzene.

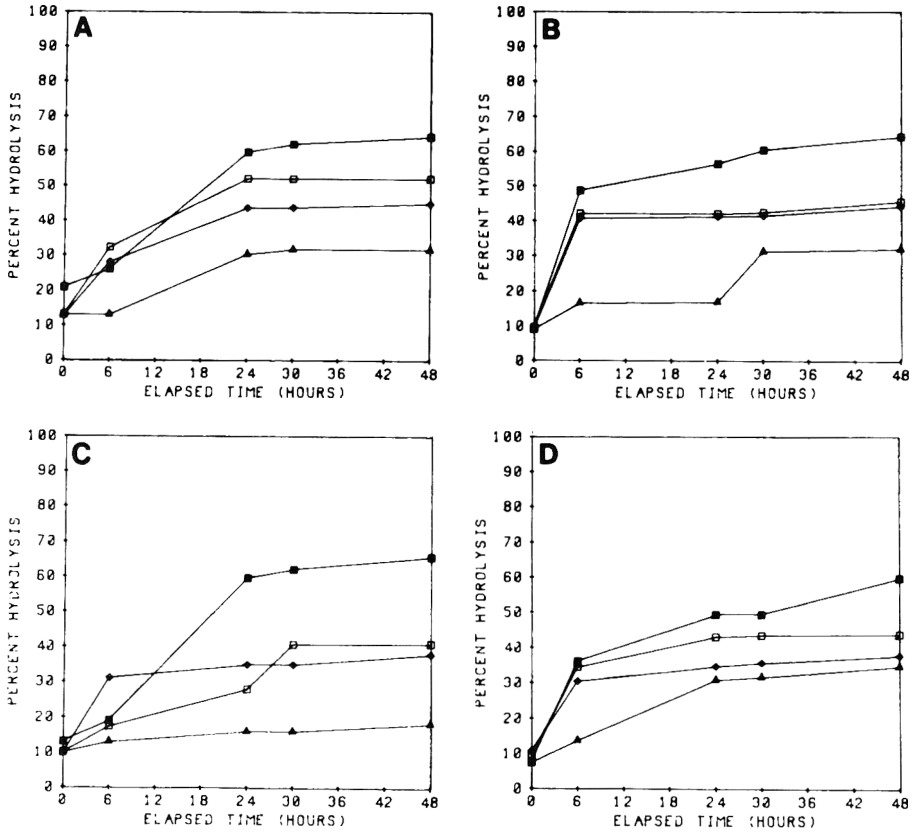

Figure 2. Percent hydrolysis of cod fillet using (■) type XIV, (△) type I, (□) type XXIII, and (◇) type XVI protease under A, pH 7.5, 37°C; B, pH 7.5, 45°C; C, pH 8.5, 37°C; and D, pH 8.5, 45°C incubation conditions.

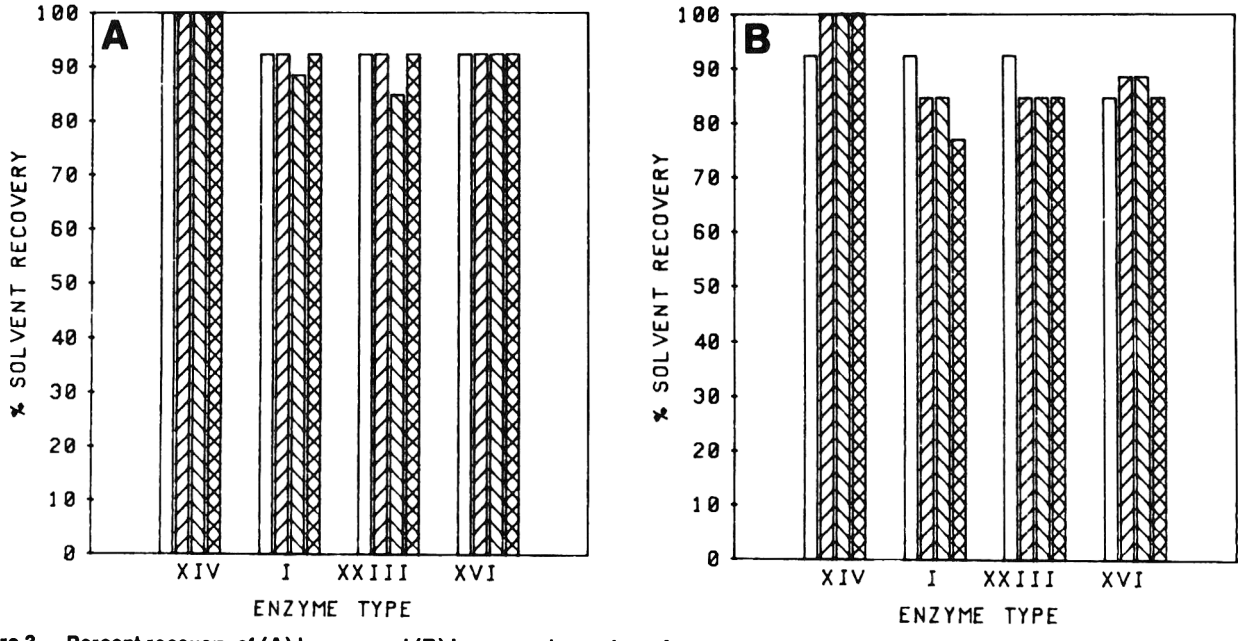

Figure 3. Percent recovery of (A) hexane and (B) benzene phases from 4 enzyme types under various incubation conditions: bars in each group, left to right: 37°C, pH 7.5; 37°C, pH 8.5; 45°C, pH 7.5; and 45°C, pH 8.5.

(b) *Rotary mixer.*—Roto-torque (Cole-Parmer Instrument Co., Chicago, IL).

(c) *Meat grinder.*—Model 244 (Moulinex, France).

(d) *Blender.*—Model 33BL73 (Waring Products Division, Dynamics Corp. of America).

(e) *Centrifuge.*—MSE Centaur 2 (MSE Scientific Instruments, Crawley, U.K.).

(f) *Culture tubes.*—Screw cap, 16 × 125 mm (Pyrex, Corning Co.).

Sample Preparation for Hydrolysis

Cod fillet and bovine liver were homogenized with a meat grinder. Chicken eggs were homogenized by blending briefly at low speed. Homogenates were stored at −20°C until hydrolyzed.

Enzymatic Hydrolysis

For each experiment, quadruplicate samples of cod fillet, bovine liver, or chicken egg homogenate (1 g) were added to

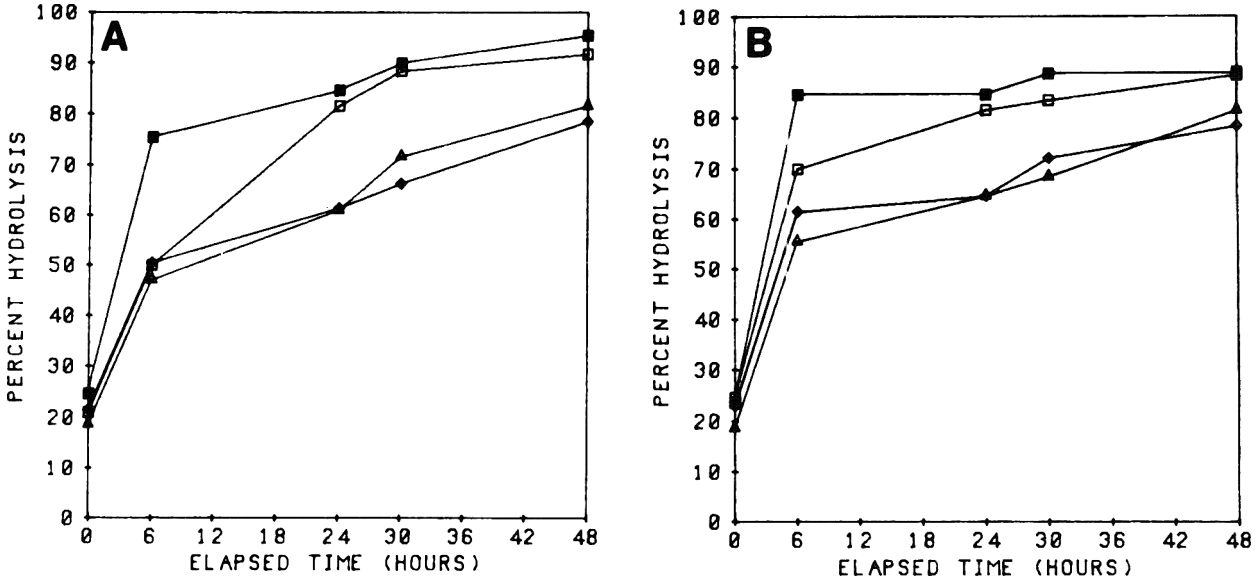

Figure 4. Percent hydrolysis of chicken egg homogenate at (A) 37°C and (B) 45°C, using (■) type XIV protease, pH 7.5; (△) type XXIII protease, pH 7.5; (□) type XIV protease, pH 8.5; and (◇) type XXIII protease, pH 8.5.

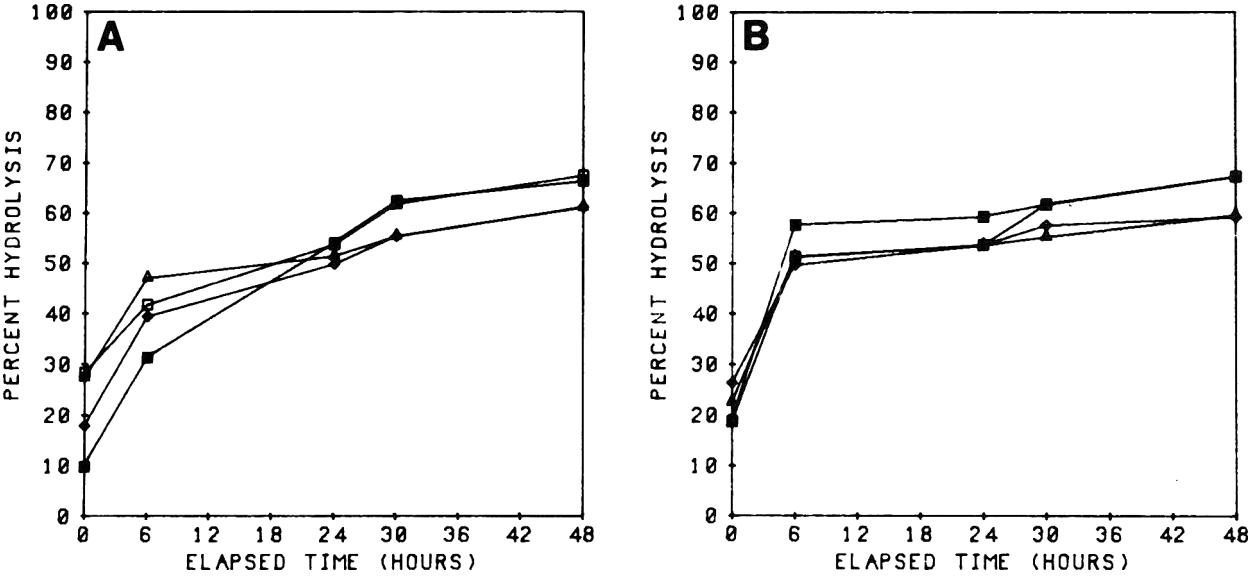

Figure 5. Percent hydrolysis of bovine liver at (A) 37°C and (B) 45°C, using (■) type XIV protease, pH 7.5; (△) type XXIII protease, pH 7.5; (□) type XIV protease, pH 8.5 and type (◇) XXIII protease, pH 8.5.

culture tubes containing 10 mL of 5% (v/v) ethanol-tris buffer (pH 7.5 or 8.5), 125 activity units (25 mg type XIV, 15.6 mg type I, 35.7 mg type XXIII, 10.6 mg type XVI) of protease, and 25 mg lipase. Samples were incubated at 37°C or 45°C and sampled (5 μL) at 0, 6, 24, 30, and 48 h to monitor degree of hydrolysis. Percent enzymatic hydrolysis was calculated by dividing the amino nitrogen value of the enzyme hydrolysate by the amino nitrogen value obtained from the acid hydrolysate (for an equivalent amount of substrate).

Acid Hydrolysis

Quadruplicate samples (50 mg) of tissue were weighed into 10 mL Pyrex glass tubes. One mL 6N HCl (constant boiling, Sequanal grade, Pierce Chemical Co.) was added to each tube. Tubes were then sealed with an air-propane gas flame after flushing with nitrogen and heated for 17 h at 140°C in a forced air circulation oven.

Amino Nitrogen Determination

Buffer A (100 μL), 5 μL sample, and 5 μL TNBS reagent were added to a 11 × 98 mm test tube. Tube contents were hand-agitated for ca 10 s and allowed to react for 10 min. Buffer B (900 μL) was added and the sample volume was adjusted to 9 mL with water. The absorbance was measured at 416 nm. Background amino nitrogen was corrected by running a suitable blank (buffer + enzymes) with all samples. A calibration curve was produced with L-valine standards run concurrently with the samples.

Solvent Phase Recovery

Hexane or benzene (2 mL) was added to duplicate 48 h hydrolysates. The samples were rotary-tumbled (65 rpm) for 10 min and centrifuged (2500 rpm) for 5 min. The height (mm) of the separated solvent phase was measured against solvent/buffer controls and expressed as percent recovery.

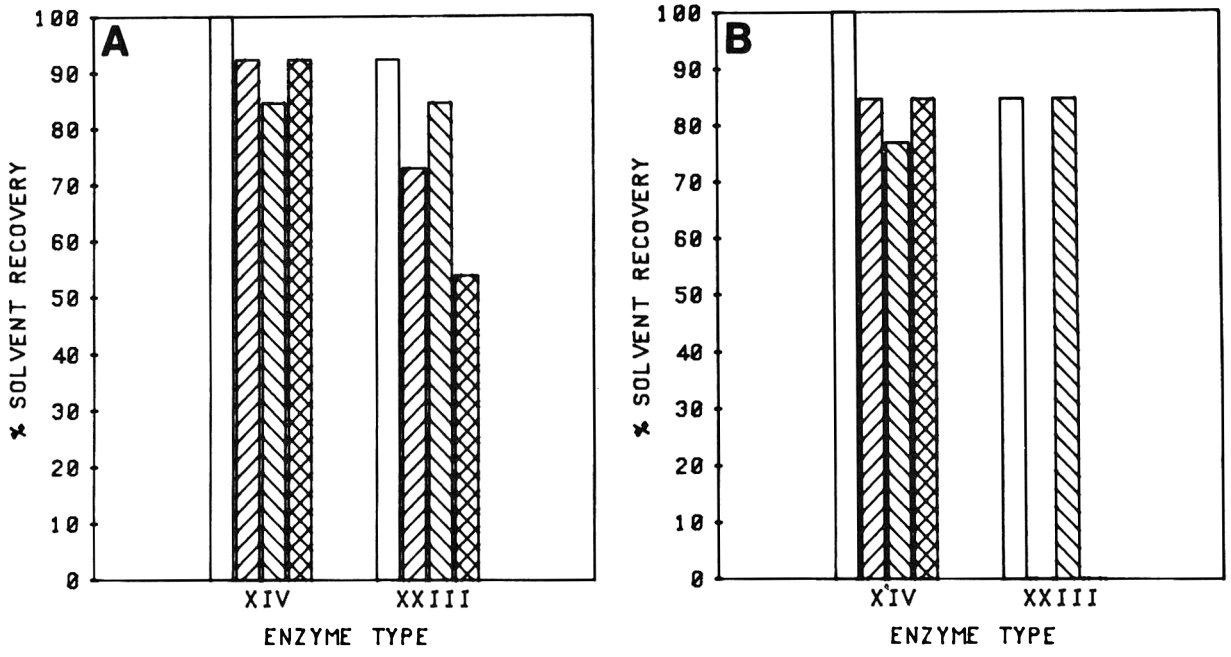

Figure 6. Percent recovery of (A) hexane and (B) benzene phases from chicken egg hydrolysates using 2 enzyme types under various incubation conditions: bars in each group, left to right: 37°C, pH 7.5; 37°C, pH 8.5; 45°C, pH 7.5; and 45°C, pH 8.5.

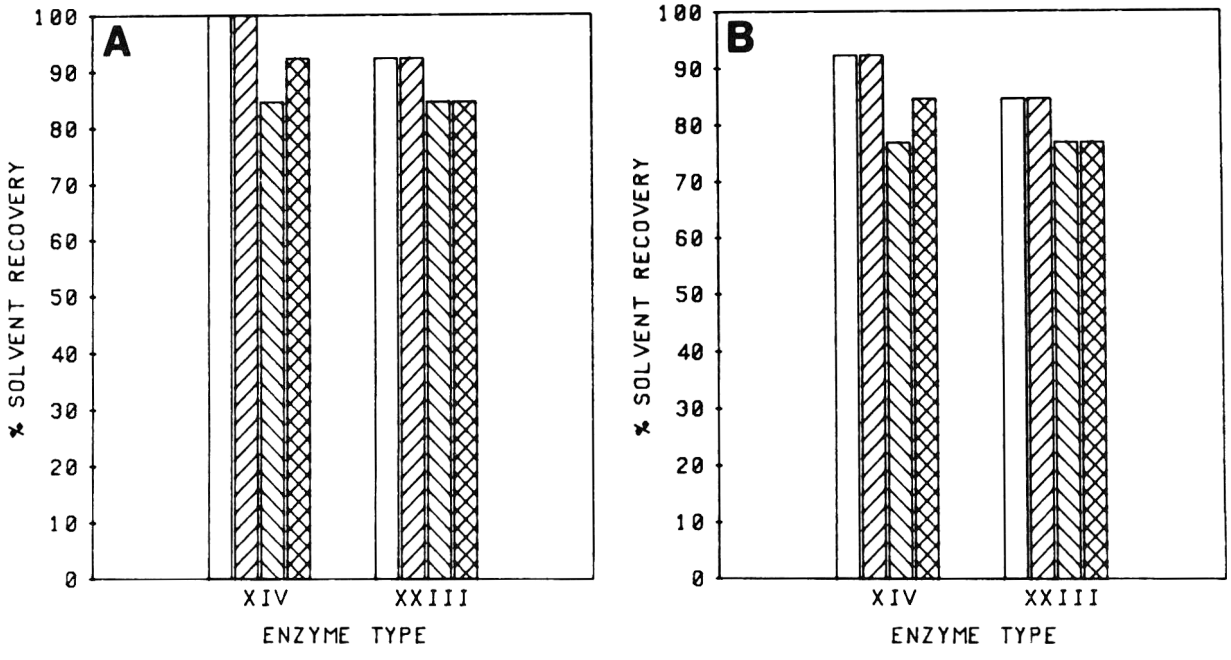

Figure 7. Percent recovery of (A) hexane and (B) benzene phases from bovine liver hydrolysates using 2 enzyme types under various incubation conditions: bars in each group, left to right: 37°C, pH 7.5; 37°C, pH 8.5; 45°C, pH 7.5; 45°C, pH 8.5.

Results and Discussion

Recovery of hexane and benzene solvent phases from untreated tissue homogenates (1 g tissue + 10 mL water) was largely unsuccessful (Figure 1) and varied widely among different tissues and solvents. It was hoped that enzymatic hydrolysis would improve solvent phase separation and reduce the differences observed.

An initial screening of the 4 proteases plus lipase was made using cod fillet homogenate under various hydrolysis conditions. The type XIV protease consistently produced the greatest percent hydrolysis (59.7–65.7%; Figure 2). Variation of the incubation temperature (37°C, 45°C) and buffer pH (7.5, 8.5) (Figures 2A, C) did not affect the relative efficacy of the tested proteases (type XIV >XXIII >XVI >I), although each protease generally performed better at pH 7.5. The higher incubation temperature (45°C) (Figure 2B, D) tended to enhance early hydrolysis results (up to 12 h), particularly for protease types XIV and XXIII, but the improvement decreased with increased hydrolysis time.

Recovery of organic solvent phases was better from cod hydrolysates prepared with type XIV protease than from the other 3 proteases (Figure 3); hexane recoveries (Figure 3A) were greater than those obtained using benzene (Figure 3B). Neither the incubation temperature nor pH consistently affected solvent phase recovery (Figure 3), although each enzyme type did show some variation. Type XXIII protease was selected for further comparison against the type XIV protease with the other substrates, chicken egg and bovine

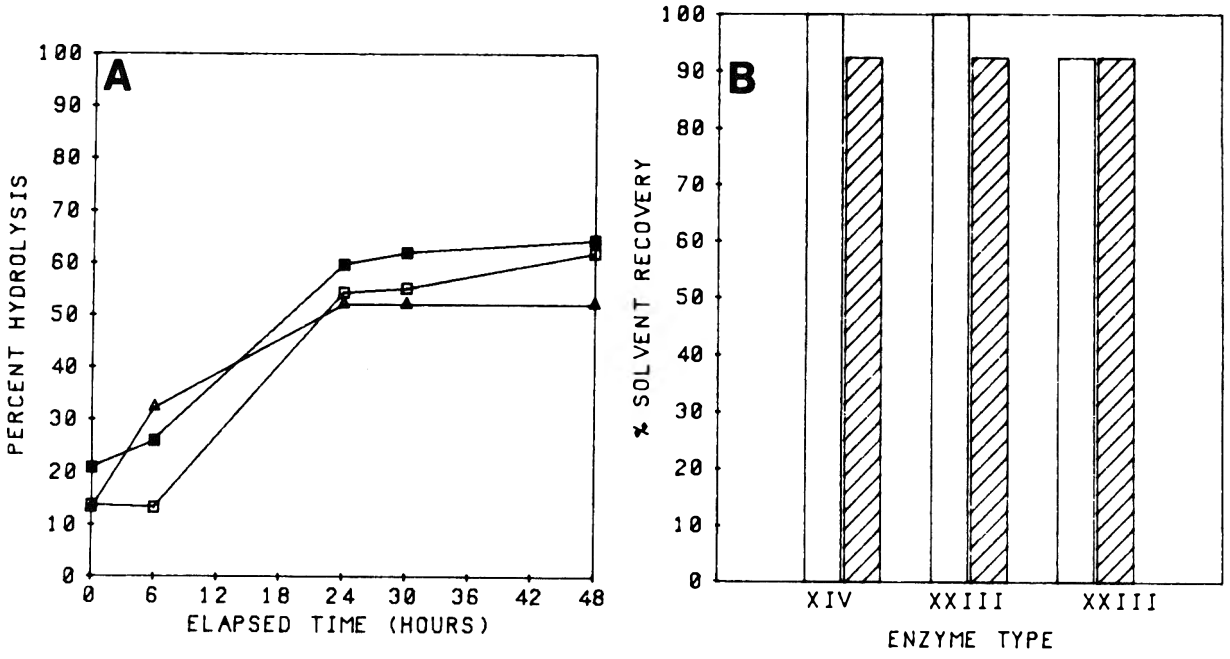

Figure 8. (A) Percent hydrolysis of cod fillet using 2 enzyme types at 37°C, pH 7.5; (■) type XIV protease (125 activity units); (□) type XXIII protease (375 activity units); (△) type XXIII protease (125 activity units). (B) Solvent phase recovery from cod fillet hydrolysates: blank boxes, hexane and diagonally lined boxes, benzene.

liver, because it produced the second highest percent hydrolysis (Figure 2), had reasonable organic phase recovery performance (Figure 3), and has a low cost per activity unit.

The type XIV protease produced higher percent hydrolysis (88.8–95.4%) in egg homogenate than the type XXIII protease (78.6–81.5%, Figure 4) under all tested hydrolysis conditions. However, bovine liver results (Figure 5) with the type XIV protease (66.3–67.3%) and type XXIII protease (59.3–61%) were quite similar.

Recoveries of solvent phases from egg homogenate (Figure 6) and bovine liver (Figure 7) hydrolysates indicate that the type XIV protease-produced hydrolysates formed less emulsion (more extractable) with hexane (Figures 6A and 7A) and benzene (Figures 6B and 7B) than the type XXIII protease. In the bovine liver series, the 45°C hydrolysates appear to be less extractable (Figure 7), whereas in the egg homogenate series, the buffer pH of 8.5 appears to lower solvent recovery particularly with benzene in the enzyme type XXIII series (Figure 6B).

Although type XIV protease performed the best of all tested proteases, the relatively high cost per activity unit makes a less expensive substitute desirable. Type XXIII protease is approximately 93% less expensive; therefore, a higher activity unit to substrate weight ratio was attempted to see if the performance of the type XIV protease could be matched. Cod fillet hydrolyzed with 3 times the activity units of type XXIII protease (Figure 8A) gave results very similar to type XIV protease results, although 48 h hydrolysis was required. Solvent recovery of hexane and benzene phases from the type XXIII protease hydrolysates also improved compared with those achieved with the type XIV protease hydrolysates (Figure 8B).

Conclusions

Recovery of hexane and benzene solvent phases from soft tissue homogenates was greatly improved after enzyme hydrolysis. Type XIV protease was the most effective type tested for percent hydrolysis achieved and solvent recovery. The less expensive type XXIII protease performed nearly as well as the type XIV protease when 3 times the number of activity units was added to the substrate. It is proposed that enzymatic hydrolysis can use as an effective sample pretreatment for hydrophobic analytes (such as pesticides and volatile contaminants), particularly if the analytes of interest are sensitive to harsh pH conditions.

Acknowledgments

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TOBACCO

Gas Chromatographic Determination of Nicotine in Environmental Tobacco Smoke: Collaborative Study

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A gas chromatographic method for determination of vapor phase nicotine in environmental tobacco smoke (ETS) was collaboratively studied by 6 laboratories. Nicotine is desorbed from XAD-4 sample tubes with ethyl acetate containing triethylamine and determined by gas chromatography with nitrogen-selective detection. Each collaborator received blind duplicate samples at each of 6 nicotine concentrations. Three concentrations were generated by spiking XAD-4 tubes with known amounts of nicotine; the remaining 3 concentrations were ETS samples obtained in a carefully controlled environmental chamber containing sidestream and exhaled mainstream smoke from 1R4F Kentucky reference cigarettes. Repeatability and reproducibility relative standard deviations ranged from 4.4 to 11.1% and from 7.0 to 11.1%, respectively, for nicotine concentrations evaluated (up to 6 μg/cu m). The method has been adopted official first action.

In recent years interest has increased in the investigation of indoor air quality. The sources of contamination that impact the quality of air in indoor environments are numerous: wood stoves and fireplaces; unvented gas and kerosene heaters, gas ranges, and oil furnaces; tobacco smoke; processed wood, carpeting, insulation, and other building materials; consumer product aerosols, cleaning agents, and pesticides; and algae and fungi from improperly maintained heating and cooling systems, to name but a few. These sources, in combination with the increasing tendency to reduce energy consumption by reducing infiltration of outside air, have resulted in a steadily increasing number of complaints and other problems apparently associated with a reduction in the overall quality of indoor air. Because these contaminants emanate from a variety of sources, a cooperative effort is needed from all organizations involved in indoor air studies so that the characterization of the indoor environments in which we live will be based on the highest quality data possible.

Because tobacco smoke has been implicated as a source impacting indoor air quality, there is a responsibility to evaluate this impact. Several compounds have been suggested as tracers of ETS in indoor air, including nicotine, carbon monoxide, and various polycyclic aromatic hydrocarbons (1) and solanesol (2). Although no single constituent is representative of the broad spectrum of compounds in ETS, nicotine is regarded by some scientists (3, 4) to be the best tracer because it is a major component of tobacco smoke and its presence in indoor air is uniquely attributable to tobacco smoking. The present report describes a collaborative study to evaluate a gas chromatographic method for determination of nicotine in ETS.

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This report was presented at the 101st AOAC Annual International Meeting, September 14–17, 1987, at San Francisco, CA.
The recommendation has been 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 at the 103rd AOAC Annual International Meeting, September 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis" (1990) *J. Assoc. Off. Anal. Chem.* 73, January/February issue.

Collaborative Study

Each of the 6 participating laboratories was provided the analytical protocol and sets of nicotine-spiked XAD-4 sample tubes as a preliminary step to the collaborative study. After several months, all laboratories reported confidence in their implementation of the protocol, and sampling for the collaborative study was scheduled. One week before sampling began, sets of 20 XAD-4 sample tubes were shipped to each laboratory for determination of nicotine desorption efficiency.

All samples for this study were generated in our research laboratory under the direct supervision of M. P. Barkdoll, Engineering Manager for International Technology Corp. He observed the preparation of spiked samples by the Associate Referee, measured flow at each sample port before and after all smoking runs, labeled and documented pertinent information for each sample, sorted all samples prior to and after sampling, retrieved samples, and prepared them for transport.

Spiked samples were generated by adding microliter quantities (Hamilton 10 μL syringe; accuracy and precision ±1%) of a solution of known nicotine concentration to the primary section of XAD-4 resin in the sample tubes. ETS concentrations were generated in a controlled environmental test chamber by delivering sidestream plus exhaled mainstream smoke (human smoking) from 1R4F Kentucky reference cigarettes. In all cases, the environmental chamber was operated in the static mode at 22°C, 50% relative humidity, with mixing fans on at 100% for sample duration of 2 h. Exact operational specifications and capabilities of this environmental chamber are available (5).

All samples were prepared in sufficient quantity to provide each collaborator with blind duplicates at each concentration. None of the personnel who assisted in the operation of the environmental chamber was involved in sample analysis so the requirement that all samples be analyzed blind was preserved. Analysts were asked to report the quantities of nicotine determined in micrograms per XAD-4 tube and submit copies of all raw data including chromatograms. The conversion of micrograms per tube to micrograms per cubic meter of air for ETS samples was made by the Associate Referee using the volumetric flow rate and sample duration data supplied by M. P. Barkdoll.

Nicotine in Environmental Tobacco Smoke
Gas Chromatographic Method
First Action

(Applicable to determination of airborne vapor-phase nicotine at 0.1–2500 μg/cu m, for 2-h sampling)

Method Performance:

$s_r = 0.10$; $s_R = 0.16$; $RSD_r = 4.4\%$; $RSD_R = 7.0\%$

A. Principle

Known volume of air is drawn at 1 L/min through sorbent sampling tube containing XAD-4 resin to adsorb nicotine present. Minimum sampling time of 1 h is recommended. XAD-4 resin is transferred to glass vial and quinoline (internal standard) is added. Nicotine and quinoline are desorbed into solvent (ethyl acetate containing 0.01% triethylamine), and an aliquot is injected into gas chromatograph equipped with thermionic-specific (nitrogen–phosphorus) detector.

B. Apparatus

(a) *Pump.*—Sampling pump calibrated for air flow rate of 1 L/min.

(b) *Sorbent sampling tubes.*—Glass, with both ends flame-sealed, 7 cm × 6 mm od and 4 mm id, containing 2 sections of 20/40 mesh XAD-4 resin (SKC Inc., Eighty Four, PA). *Note*: Tubes are available with either urethane foam spacers (Cat. No. 226-30-11-04) or glass wool spacers (Cat. No. 226-30-11-04-GWS). Latter are preferred. If tubes with foam spacers are used, chromatographic run time must be lengthened to elute relatively large amount of foam-related artifact extracted from resin. This peak does not intrinsically interfere with determination of nicotine, but, if allowed to accumulate, artifact will interfere with subsequent analyses.

(c) *Gas chromatograph.*—Temperature-programmable system equipped with either packed column inlet or split/splitless capillary column inlet, nitrogen-selective detector (NPD), and digital electronic integrator.

(d) *Column.*—30 m × 0.53 mm id fused silica capillary, coated with 1.5 μm film of 5 % phenyl methyl polysiloxane (for example, DB-5, Cat. No. 125-5032, J&W Scientific, Inc., Folsom, CA; or equivalent).

(e) *GC conditions.*—Helium carrier gas flow ca 15 mL/min (12 psig); direct injection 1–2 μL; oven temperature 150° programmed at 5°/min to 180° (run time 6 min); injector and detector temperatures 225 and 300°, respectively. Suitable detector conditions: helium make-up gas 15 mL/min; air 75 mL/min; hydrogen 3 mL/min; "bead current" sufficient to give S/N > 50 for nicotine in 0.1 μg/mL calibration standard. Approximate retention times under these conditions are 1.9 min for quinoline and 2.6 min for nicotine.

(f) *Sample containers.*—2 mL borosilicate glass vials with Teflon-lined septum closures.

C. Reagents

(a) *Ethyl acetate.*—Chromatographic quality.

(b) *Nicotine.*—Reagent grade (Cat. No. 112 4973, Eastman Kodak Co., or equivalent). (*Caution:* Nicotine is very toxic. Avoid contact with skin and eyes.)

(c) *Quinoline (internal standard).*—99+%.

(d) *Triethylamine (solvent modifier).*—99+%.

D. Preparation of Standard Solutions

Modify ethyl acetate by adding triethylamine (0.01% v/v). Use modified solvent (referred to hereafter as solvent) to prepare all standard and sample solutions.

(a) *Nicotine standard solutions.*—(*1*) *Primary solution.*—Weigh 100 mg nicotine into 100 mL volumetric flask and dilute to volume with solvent. (*2*) *Secondary solution.*—Pipet 1.00 mL primary solution into 100 mL volumetric flask and dilute to volume with solvent.

(b) *Internal standard solutions.*—(*1*) *Primary solution.*—Weigh 100 mg quinoline into 100 mL volumetric flask and dilute to volume with solvent. (*2*) *Secondary solution.*—Pipet 10.0 mL primary solution into 100 mL volumetric flask and dilute to volume with solvent.

(c) *Calibration standard solutions.*—Prepare at least 2 sets of 5 calibration solutions that cover concentration range of interest by adding, e.g., 10, 20, 50, 100, and 200 μL nicotine secondary standard solution to each of five 2 mL glass vials. Add 50 μL quinoline secondary standard solution to each vial. Add 1 mL solvent to each vial, cap, and shake vial to mix. No volume corrections are necessary for standards prepared in this fashion because weight ratio of nicotine to quinoline is constant, regardless of additional solvent volumes added.

E. Collection and Preparation of Samples

Break off both ends of XAD-4 sorbent tube; openings must be at least one-half the tube id. Position backup section of XAD-4 resin nearest pump and connect to pump with tubing. Acquire samples at flow rate of 1 L/min. Record time elapsed during sampling. If final concentration is to be adjusted to standard conditions, record temperature and barometric pressure. After sampling, cap sorbent tube with plastic caps. Samples are stable at least 2 weeks in dark at room temperature; however, freezer storage is recommended. Frozen samples are stable at least 4 weeks. Prepare and analyze 3 previously unopened XAD-4 tubes (blanks) concurrently with samples.

Transfer glass wool plug from tube inlet and primary section of XAD-4 resin to 2 mL glass vial. Transfer backup section of XAD-4 resin along with bracketing glass wool plugs to another vial. If tubes with polyurethane foam spacers are used. discard foam spacers. Add 50 μL quinoline secondary standard solution directly onto resin in each vial. Add 1 mL solvent to each vial, cap, and shake vial for 30 min.

F. Determination

Chromatograph one set of calibration standard solutions, samples, and second set of calibration standard solutions, and obtain integrated peak areas for quinoline and nicotine. Calculate nicotine-to-quinoline peak area ratio for each. Average results for all calibration solutions analyzed at a given concentration and construct calibration curve using peak area ratios as ordinate and micrograms nicotine as abscissa values. Fit calibration data to either linear or second-order least squares regression model (whichever is more appropriate for instrument used; expected $R^2 > 0.990$), and calculate weight of nicotine in each sample from nicotine-to-quinoline peak area ratio.

G. Determination of Desorption Efficiency

Transfer primary section of XAD-4 resin together with associated glass wool from 20 sample tubes to twenty 2 mL vials. Spike 5 vials with 10 μL portions of nicotine secondary standard solution, 5 vials with 20 μL portions, and 5 vials with 50 μL portions. Remaining 5 vials are blanks. Cap and store all vials in same manner as samples are handled. Add quinoline secondary standard solution, desorb with solvent, and analyze all spiked samples and blanks as described in *Determination.* Correct results obtained on spiked samples by subtracting average for blanks from average for each spiked level. Calculate desorption efficiency (DE) for nicotine as follows:

$$DE = \text{av. wt recovered } (\mu g)/\text{wt added } (\mu g)$$

Table 1. Collaborative results on GC determination of nicotine in blind duplicate samples

Coll.	Nicotine, μg/tube[a] Level 1 (0.18)	Level 2 (0.54)	Level 3 (1.25)	Nicotine, μg/cu m air sampled[b] Level 4 (1)	Level 5 (2)	Level 6 (3)
1	0.17	0.48	1.07	2.20	4.33	6.20
	0.18	0.49	1.10	2.20	—[c]	5.61
2	0.19	0.41	1.30	2.16	4.96	5.43
	0.18	0.55	1.22	2.33	4.07	5.43
3	0.16	0.48	1.05	2.01	4.19	5.71
	0.17	0.48	1.04	2.23	4.00	5.22
4	0.16	0.43	1.10	2.57	4.08	4.26
	0.17	0.45	0.89	2.42	3.21	5.46
5	0.19	0.47	1.03	2.47	4.28	5.22
	0.19	0.46	0.98	2.37	4.25	5.22
6	0.17	0.46	1.00	2.22	4.36	5.31
	0.23	0.47	1.02	2.18	4.35	5.52
Mean	0.18	0.47	1.07	2.28	4.19	5.38
s_r	0.02	0.04	0.07	0.10	0.40	0.42
RSD_r, %	11.1	8.5	6.5	4.4	9.6	7.8
s_R	0.02	0.04	0.11	0.16	0.41	0.45
RSD_R, %	11.1	8.5	10.3	7.0	9.8	8.4
Overall repeatability			0.05			0.33
Overall reproducibility			0.07			0.36

[a] Solution of known nicotine concentration spiked on XAD-4 tubes; concentration, μg/tube, in parentheses.
[b] ETS nicotine collected in environmental chamber; sampling time 2 h. Number of puffs of 1R4F cigarette in parentheses.
[c] Value reported (5.86 μg) deleted. Baseline disturbance on chromatogram.

If DE is different for 3 levels prepared (ANOVA or *t*-test, $P < 0.05$), construct plot of DE vs weight nicotine found. If DE is equivalent for 3 levels but different from 100% (*t*-test using pooled results, $P < 0.05$), use pooled arithmetic mean for DE correction.

Initially, determine DE 2 or more times until consistency is demonstrated. Thereafter, at a minimum, repeat DE determination for each new lot number of XAD-4 tubes.

H. Calculations

Correct sample results for blank as follows:

$$\text{Nicotine, } \mu g = \mu g \text{ sample} - \text{av. } \mu g \text{ blank}$$

where μg sample = weight of nicotine found in primary section of XAD-4 sample tube, and av. μg blank = average weight of nicotine found in primary sections of all blank tubes (determined in ***E***). Similarly correct nicotine found in backup section of sample tube. Then add the 2 corrected values for that tube to determine total weight (μg) of nicotine in sample (W_S).

Correct total weight of nicotine for desorption efficiency as follows:

$$\text{Nicotine (corr.), } \mu g = W_S/DE$$

where DE is obtained from plot or calculation performed earlier (if different from 100%).

Convert amount of nicotine found in XAD-4 sample tube to micrograms per cubic meter of air as follows:

$$\text{Nicotine, } \mu g/\text{cu m} = [\text{nicotine (corr.), } \mu g \times 1000 \text{ (L/cu m)}]/\text{vol. air sampled (L)}$$

where volume of air sampled (L) = flow rate (L/min) × sampling time (min).

If desired, or if necessary because of pump characteristics, adjust nicotine concentration found in sampled air to standard conditions of temperature and pressure as follows:

$$\text{Nicotine (corr.), } \mu g/\text{cu m} = \text{nicotine } (\mu g/\text{cu m}) \times (760/P) \times [(T + 273)/298]$$

where P is barometric pressure (torr) of air sampled, T is temperature (°C) of air sampled, 760 is standard pressure (torr), and 298 is standard temperature (°K).

Ref.: JAOAC **72,** November/December issue (1989).
CAS-54-11-5 (nicotine)

Table 2. Average blank values obtained on primary and backup sections of XAD-4 sample tubes for study on nicotine

Coll.	Primary blank, μg	Backup blank, μg
1	0.03	ND[a]
2	0.02	0.01
3	ND	ND
4	ND	ND
5	ND	ND
6	0.02	0.01

[a] ND = not detected.

Results

Table 1 presents the collaborative results for determination of nicotine in blind duplicate samples. Levels 1, 2, and 3 (values reported as μg nicotine per tube) correspond to the 3 concentrations of nicotine spiked on XAD-4 tubes; the "true" values were 0.18, 0.54, and 1.25 μg/tube, respectively. Levels 4, 5, and 6 (values converted to μg nicotine per cu m air sampled) correspond to the 3 concentrations of ETS nicotine collected in the environmental chamber for 1, 2, and 3 puffs, respectively, of the 1R4F cigarette. Mean values, repeatability and reproducibility standard deviations, and the corresponding relative standard deviations (RSD) are also shown.

The method sent with the samples to the collaborators described 2 columns; however, all collaborators were requested to analyze the samples on the larger diameter column. (The smaller column with operating conditions is described under *Ruggedness Test.*) Collaborator 1 analyzed each sample on both column types, with the respective differences in operating conditions. The value reported by Collaborator 1 for level 5, second sample, based on analysis with the large diameter column was 5.86 μg. Inspection of the chromatogram revealed a severe baseline disturbance at the onset of nicotine elution. On the basis of this questionable chromatogram and the apparently high response obtained, this result was deleted. (The corresponding sample amount determined on the smaller id column was 4.31 μg.) In relation to the other values for level 6, the reported value for the first analysis from Collaborator 1 also appears to be biased high; however, inspection of the chromatogram yielded no basis for excluding this result and therefore, it was retained. (The corresponding sample amount determined on the smaller id column was 5.66 μg.)

In addition to the blind duplicate pair at each concentration level, each collaborator also received 5 blank XAD-4 tubes, which had been opened, labeled, and capped in an identical manner as the samples. The reported nicotine values for the primary and backup sections of XAD-4 resin in the blanks were used to correct all sample values as stated in the method. The key to corrections made for blanks is given in Table 2.

Desorption efficiency was determined to be 100% by 4 of the 6 collaborators. However, Collaborators 3 and 4 determined the recovery of nicotine to be less than 100% and

Table 3. Key to corrections made for desorption efficiency for study on nicotine

Coll.	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
3	0.90	0.95	1.00	0.90	0.95	0.95
	0.90	0.95	1.00	0.90	0.95	0.95
4	0.85	1.00	1.00	0.92	1.00	1.00
	0.85	1.00	1.00	0.92	0.92	1.00

desorption efficiencies were calculated and applied as stated in the method. The key to corrections made for desorption efficiency is given in Table 3.

Reported values for level 3 are consistently low for 5 of the 6 collaborators compared with the "true" value of 1.25 μg. An observation made when preparing the spiked tubes most likely accounts for this discrepancy. Levels 1 and 2 were prepared by injecting 1 and 3 μL, respectively, of nicotine solution into the primary XAD-4 resin bed. Level 3 was prepared by injecting 7 μL, which saturated a small section of the resin, and resulted in contact of the nicotine solution with the glass walls of the tube. Based on the known adsorptive interactions of nicotine with borosilicate glass (see *Discussion*), we postulate that a small amount of nicotine was lost to the glass walls, which was not recovered in tube preparation because the tubes are not rinsed. As a result, the low bias in this case is attributed to sample generation and not to the collaborators' analyses. Nicotine losses during air sampling resulting from such a mechanism are insignificant. Even near the capacity limit of the tube, losses are not more than 0.5% of the total.

Comparison of the repeatability and reproducibility RSD values (RSD_r and RSD_R) reveals that virtually all of the variation noted among laboratories (reproducibility) is accounted for by the precision within a single laboratory (repeatability). In addition, analysis of variance of results in Table 1 indicates there is not sufficient evidence to indicate a difference between laboratories and also no evidence of a significant "lab × sample" interaction (confidence level = 95%). As a result, it can be concluded that the method yielded equivalent analytical results in each of the 6 collaborators' laboratories. This conclusion is further supported by application of Youden's rank sum test and the test for homogeneity of variance (6).

Discussion

Ruggedness Test

Before undertaking this collaborative study, the Associate Referee subjected the method to a ruggedness test as described by Youden and Steiner (6). To create the same type of variation that might be encountered in the collaborating laboratories, 6 variables were chosen for evaluation, including the effect of 2 analysts on the gravimetric preparation of primary standard solutions, the volumetric preparation of secondary and working standard solutions, the preparation of XAD-4 tubes for analysis, and data manipulation. We also studied the effect of the chromatographic conditions.

The analysts intentionally selected were ones who had considerable experience with the method and another with relatively no experience. The 2 analysts were arbitrarily designated A and B. The 2 methods were designated 1 and 2. Method 1 was as described in the *Method*. For method 2, the GC column was a 30 m × 0.32 mm id fused silica capillary coated with a 1.0 μm film of 5% phenyl methyl polysiloxane (DB-5, Cat. No. 123-5033, J&W Scientific), and some operating conditions were different: He carrier gas flow was at ca 4 mL/min (15 psig); split injection (split ratio ca 5:1) of 2 μL was used. Approximate retention times for the column and conditions of method 2: quinoline 3.3 min and nicotine 4.2 min.

Table 4. Eight combinations of 7 factors used to test ruggedness of GC method for nicotine in ETS

	Combination No.							
Factor value[a]	1	2	3	4	5	6	7	8
Primary nicotine (A or B)	A	A	A	A	B	B	B	B
Secondary nicotine (A or B)	A	A	B	B	A	A	B	B
Working standard (A or B)	A	B	A	B	A	B	A	B
Tube preparation (A or B)	A	A	B	B	B	B	A	A
GC method (1 or 2)	1	2	1	2	2	1	2	1
Data analysis (A or B)	A	B	B	A	A	B	B	A
G or g	G	g	g	G	g	G	G	g
Observed result	s	t	u	v	w	x	y	z

[a] See text for description of factor values.

The specific set of combinations used in this ruggedness test is shown in Table 4. Although this particular model enables evaluation of 7 factors, only 6 were chosen. The seventh, listed as G or g in Table 4, can be thought of as corresponding to a meaningless or nonexistent variation. The inclusion of such a factor is important in the final analysis because its effect on results should, of course, be trivial.

To clarify the operations in Table 4, combination No. 3 is chosen as an example. The observed result, u, was obtained from the following sequence of operations: primary nicotine standard solution prepared by analyst A; secondary nicotine standard solution prepared from this solution by analyst B; working (calibration) standard solutions prepared from this solution by analyst A; XAD-4 tubes prepared for analysis by analyst B; samples and working standards chromatographed according to method 1; data analysis performed by analyst B; trivial operation "g."

To extricate the effect of the various factor changes, the 8 determinations were split into 2 groups of 4 on the basis of one of the factors. All other factors cancel out within each group and each factor is evaluated by all 8 determinations. For example, the effect of altering G to g is determined by subtracting average (t+u+w+z)/4 from average (s+v+x+y)/4. Ideally, no effect of the factor results in a difference of zero. The relative effect of the factors were determined by obtaining and rank-ordering the 7 differences. In principle, if one factor exerts an effect, its difference will be substantially larger than those from the other factors and will be significantly different from zero. In practice, significance is virtually impossible to ascertain from a single observed result for each combination. Therefore, for this ruggedness test, 4 samples were prepared and analyzed in each combination, resulting in 4 observed results per combination. The factor differences were thus obtained as averages of the 4 determinations along with estimates of the precision of the test, thus enabling objective statistical evaluation of the factor effect. This is accomplished by a *t*-test with the hypothesis that the factor difference is equal to zero.

At this stage of the ruggedness test, only one factor (data analysis) resulted in a significant effect on observed results. This was indeed a curious finding since it had been anticipated that data analysis would be a trivial operation (similar to G/g). An audit of the data analysis for each combination by the Associate Referee revealed 2 causes. One was traced to a mistake made by the inexperienced analyst in constructing

Table 5. Evaluation of ruggedness test results

Factor	Av. diff., μg nicotine	Std dev.	P	Significant factor effect
Data analysis	0.0309	0.0331	0.16	no
Working standard	0.0191	0.0403	0.41	no
Tube preparation	0.0151	0.0256	0.32	no
GC method	0.0150	0.0436	0.54	no
G or g	0.0085	0.0254	0.55	no
Primary nicotine	0.0055	0.0504	0.84	no
Secondary nicotine	0.0001	0.0052	0.97	no

the calibration curve. The 2 primary nicotine standard solutions were prepared from 105 and 106 mg nicotine, respectively. In calculating the nicotine weight for each calibration standard solution, the experienced analyst used the actual weights whereas the inexperienced analyst used the method-specific nominal weight of 100 mg for all calculations.

The second cause was traced to a differing degree of calibration curve linearity among the groupings of analyses by combination number, resulting in more or less of a random error since, at that time, the method specified a linear calibration. It was enlightening to discover that the difference in curvature was more pronounced among sets of analyses on a single instrument than between the 2 instruments used for the different methods (otherwise, the effect would have been traced to the GC method).

After the first mistake was corrected and all data were reanalyzed on the basis of second-degree polynomial calibration curves, the average factor difference calculations, rank-ordering, and hypothesis testing were repeated. The corrected results (Table 5), rank-ordered by average factor difference absolute value, along with standard deviations, *t*-test probabilities, and conclusions at the 95% confidence level, indicate that no one variable contributed significantly to the outcome of the analytical procedure.

Modification of NIOSH Method

The method presented here is a modified version of the NIOSH method for determining nicotine (7). The NIOSH procedure was developed for occupational hazard exposure levels and, with a limit of detection of 300 μg/cu m, is not sensitive enough for determining nicotine concentrations in environmental tobacco smoke. In surveys of ETS in public places such as aircraft cabins, restaurants, and bars, for over 350 individual observations, results in 95% of the cases were < 20 μg/cu m and in 80% of the cases were < 10 μg/cu m ([8] and unpublished data). Limits of detection (LOD) and quantitation (LOQ) for this method at a sampling rate of 1 L/min are, respectively, 0.17 and 1.7 μg cu m for 1-h sample duration and 0.02 and 0.2 μg/cu m for 8-h sample duration. Both LOD and LOQ can be reduced by increasing the sensitivity of the thermionic-specific detector, although this is hardly necessary. Key factors in enabling these levels of sensitivity are the use of nonpolar capillary columns to maximize detector stability and the addition of triethylamine to the desorption solvent to eliminate adsorptive losses of nicotine in the sample vials and to ensure complete desorption from the resin (9).

These XAD-4 sorbent tubes have been used under a variety of sampling conditions including flow rates of from 10 mL/min to 2 L/min, sample durations from 1 to 480 min, and in atmospheres containing nicotine at concentrations up to 120 mg/cu m (near vapor saturation). Tube capacity is at least 300 μg nicotine with less than 5% breakthrough into the backup resin section. Under normal ETS conditions, this breakthrough is typically less than 1% with desorption efficiencies rarely less than 100%. The small tube size, compatibility with low flow-rate personal sampling pumps, and excellent analytical sensitivity make this method ideally suited to both stationary (i.e., area) and personal monitoring.

Recommendation

Overall, the data indicate excellent repeatability and reproducibility. It is recommended that this method for determination of nicotine in environmental tobacco smoke be adopted official first action.

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VITAMINS

Liquid Chromatographic Determination of Vitamin D in Infant Formula

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A method is described for the determination of vitamins $D_2 + D_3$ in milk- and soy-based infant formula. Vitamins D_2 and D_3 are extracted from the saponified sample and converted to isotachysterols with acidified butanol. Reverse-phase liquid chromatography (LC) is used to remove interferences, and total vitamin D is quantitated using normal-phase LC. Recoveries of spiked samples averaged 97.6% for milk-based infant formula, and 98.8% for soy-based infant formula. This method quantitates vitamin $D_2 + D_3$ in infant formulas containing as low as 40 IU/qt when prepared according to label direction.

Vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol) typically is added to fortify milk and infant formula at 400 IU/qt (10 μg/qt). The presence of excessive amounts of fats, proteins, and sterols including cholesterol is a major obstacle in the quantitation of vitamin D in milk. The determination of vitamin D in infant formula is even more demanding because of the presence of the wide variety of additional ingredients (1). Landen (2) reported that the coextracted components of vegetable oil in infant formulas potentially invalidate liquid chromatographic (LC) methods that have been verified for fortified milk.

A number of LC methods have been applied for the determination of vitamin D in milk and infant formula (1–10). None of the methods has yet gained wide acceptance (9). A method was reported for the determination of vitamin D in fortified milk, which involves isomerization of vitamin D to isotachysterol (11). A new method is presented here for the determination of vitamin D in infant formula by using a similar derivatization technique.

METHOD

Reagents and Standards

(a) *Solvents.*—LC grade hexane, methanol, acetonitrile, and ethyl acetate and analytical grade chloroform (Fisher Scientific Co., Springfield, NJ 07081).

(b) *Aqueous KOH solution.*—Dissolve 400 g reagent grade KOH in water, cool, and dilute to 500 mL with water.

(c) *Alcoholic KOH solution.*—Dissolve 15 g reagent grade KOH in 400 mL water and dilute to 500 mL with 95% ethanol.

(d) *Ethanolic pyrogallol solution.*—Dissolve 1.0 g pyrogallol in 100 mL 95% ethanol.

(e) *Derivatization reagent.*—10M HCl in 2-butanol (prepare by dissolving dry HCl gas in 2-butanol cooled to 0°C).

(f) *Aqueous sodium carbonate solution.*—Dissolve sodium carbonate in water to prepare saturated solution.

(g) *Vitamin D standard solutions.*—(*1*) *Stock solution:* Dissolve 100 mg crystalline D_3 (cholecalciferol) (Sigma Chemical Co.) and dilute to 200 mL with methanol. (*2*) *Intermediate solution*: Dilute 5 mL stock solution to 100 mL with methanol. (*3*) *Working standard solution*: Dilute 1 mL intermediate standard to 50 mL with methanol to provide concentration of 20 IU/mL (0.5 μg/mL). Use this standard for spiking samples.

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Apparatus

(a) *Centrifuge tubes.*—25 mL conical centrifuge tubes with glass stoppers, used for isomerization; 15 mL graduated conical centrifuge tubes with glass stoppers, used for collection of vitamin D fraction from LC cleanup step.

(b) *Vortex mixer.*—Bronwill vortex mixer, or equivalent.

(c) *LC cleanup system.*—Laboratory Data Control Constametric III pump equipped with Rheodyne 7105 syringe injection valve with 200 μL sample loop, SpectroMonitor III variable wavelength detector (LDC, Riviera Beach, FL 33404), and Hewlett Packard 3390A integrator (Hewlett Packard, Palo Alto, CA 94304). Monitor eluate at 301 nm.

(d) *LC system for quantitation.*—Similar equipment as described in **(c)**, except use sample loop of 25 μL.

(e) *Chromatographic columns.*—Supelcosil LC-18-DB, 5 μm, 25 cm × 4.6 mm id, and guard column LC-18-DB, 5 μm, 2 cm × 4.6 mm for LC cleanup system (Supelco Inc., Bellefonte, PA 16823). Spherisorb normal-phase, 5 μm, 25 cm × 2 mm id for quantitative LC system (Phase Separation, Inc., Norwalk, CT 06854).

Saponification and Extraction

Saponify and extract 25 mL sample as described earlier (11).

Isomerization

Place saponified and extracted sample or standard in 40 mL centrifuge tube and evaporate to dryness under slow stream of nitrogen. Redissolve the sample in 100 μL methanol and cool to 5°C. For standards, carry out isomerization using levels of vitamin D_3 ranging from 5 to 50 IU (0.125 to 1.25 μg) dissolved in 100 μL methanol. Add 0.6 mL derivatizing reagent to centrifuge tube containing sample or standard, shake well on vortex mixer for 15–20 s, and leave at 5°C for 3 min. Shake sample tube on vortex mixer every 30 s during this 3 min period. After 3 min, add 5 mL aqueous saturated sodium carbonate solution to destroy excess reagent and shake well on vortex mixer.

Extract isomerized vitamin D (isotachysterol) by adding 2 mL chloroform to tube and shaking well on vortex mixer. Withdraw chloroform layer and pass it through small column filled with anhydrous Na_2SO_4 (disposable pipet can be used for this purpose). Extract aqueous layer 2 more times with 2 mL chloroform each time and pass all chloroform extracts through same column. Collect all the extract in 40 mL centrifuge tube, evaporate solvent to dryness under slow stream of nitrogen, and then redissolve residue in 500 μL methanol.

LC Cleanup of Extract

Set up LC system for cleanup as detailed in apparatus section. Use following operating conditions: ambient temperature; acetonitrile–methanol (90 + 10) mobile phase; flow rate 1.8 mL/min; detector 301 nm. Inject isotachysterol standard onto column to determine its actual retention time (ca 13.0 ± 0.5 min). Inject 100 μL sample extract onto column. Because of large peak eluting just before isotachysterol peak (peak X in Figure 1B), the starting point of isota-

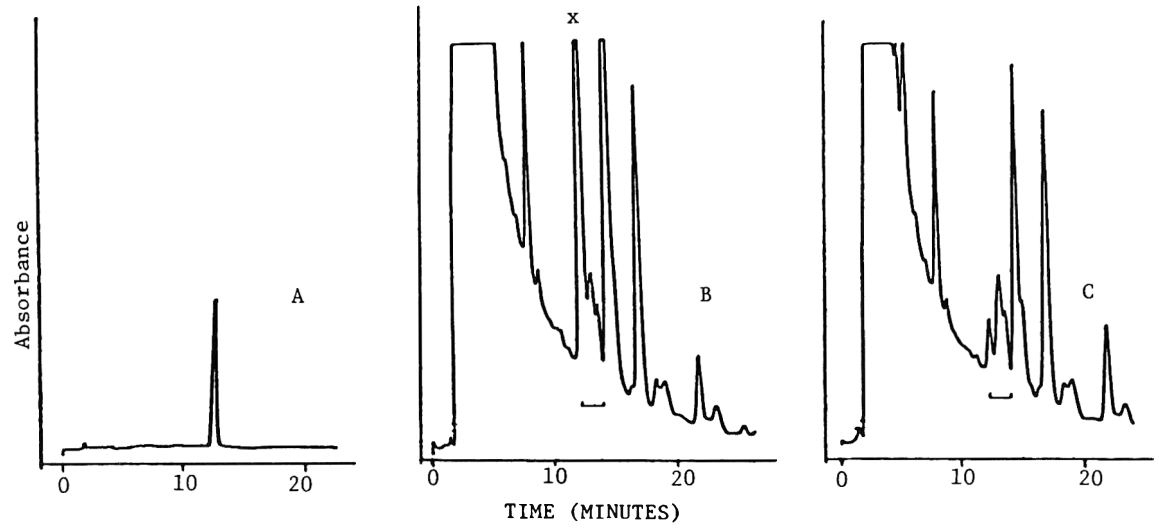

Figure 1. Chromatogram of (A) standard isotachysterol D_3; (B) milk-based liquid infant formula; (C) milk-based powder infant formula, on reverse-phase LC, Supelcosil LC-18-DB column.

chysterol peak is not clearly visible. The ending point of isotachysterol peak is, however, clearly visible. Start collecting fraction 1 min before retention time of isotachysterol and continue 30 s after isotachysterol peak returns to baseline. After collecting fraction, let flow rate continue at same rate to elute remaining peaks. All peaks elute in 25 min and next injection can be made. Evaporate collected fraction to dryness under nitrogen and redissolve residue in 250 μL hexane.

Quantitative LC

Set up normal-phase LC system as described in apparatus section. Use following operating conditions: ambient temperature; hexane–ethyl acetate–methanol (97 + 2.5 + 0.05) mobile phase; flow rate 0.7 mL/min; detector 301 nm; sensitivity 0.01 absorbance. Set attenuation at 0. Inject 10–20 μL of cleaned extract and quantitate isotachysterol by peak height.

Results and Discussion

Vitamin D in infant formulas was quantitated by determination of isotachysterol D_2 or D_3 formed by isomerization of vitamin D_2 or D_3. As pointed out in an earlier report (11), isotachysterols D_2 and D_3 have the same retention time on LC analysis; therefore, it is not possible to distinguish them. The recovery studies were carried out using only vitamin D_3. Wherever the term vitamin D is used, it signifies both vitamins D_2 and D_3.

In this method, the following steps are involved: Saponification and extraction, isomerization, cleanup using reverse-phase LC, and, finally, quantitation by normal-phase LC.

The saponification step is similar to that used in fortified milk (11). Saponification at ambient temperature proved to be advantageous in avoiding any conversion of vitamin D to previtamin D. In the present official AOAC method for vitamin D analysis in milk (7), saponification is performed at a higher temperature, which converts part of the vitamin D to previtamin D. Since quantitation of previtamin D is not possible due to interferences, a conversion factor of 1.25 is applied to quantitate vitamin D (7).

Isomerization of vitamin D to isotachysterol is performed differently from the method used for fortified milk (11). In an earlier report on fortified milk, antimony trichloride solution in chloroform was used as a derivatizing reagent (11). The isomerization of vitamin D is a rapid reaction; a slight increase in the reaction time may lead to the reduction in isomerization yield due to degradation of isotachysterol, thus making quantitation difficult.

A new reagent, 10M HCl in 2-butanol, was found to be more effective for the isomerization. The temperature, volume, and the time of the reaction are very important for quantitative conversion of vitamin D to isotachysterol D. The sample or the standard was dissolved in 100 μL methanol and cooled to 5°C. The reaction at 5°C was much slower than at room temperature and the reaction was more easily controlled. The volume of the reaction mixture was kept to 100 μL because a higher volume resulted in decreasing the yield of isomerization, presumably due to the dilution of the derivatizing reagent. At 5°C, the reaction time of 3 to 3½ min gave over 90% yield. The excess reagent was destroyed by aqueous sodium carbonate solution to stop the reaction.

The calibration plot for isotachysterol, obtained by plotting the mean peak heights vs vitamin D concentration in the 5 to 50 IU range, was linear. The correlation coefficient was 0.998.

The conversion of vitamin D to isotachysterol provided a number of advantages. Vitamin D has been found to be very sensitive to heat, light, and evaporation. Since the complete procedure is very long, there is always the possibility of losing some vitamin D during the analysis. In the proposed method, vitamin D was isomerized to isotachysterol immediately after extraction, resulting in a compound much more stable to heat, light, and evaporation. Thus the risk of losing any vitamin D was minimized. In addition, the isotachysterol has absorption maxima at 278, 288, and 301 nm and its extinction is more than double compared to vitamin D. Therefore, when monitored at 301 nm, it provided greater sensitivity as well as selectivity. A number of compounds that interfere at 265 nm do not show any absorption at higher wavelengths and thus these interferences were eliminated.

The third step was extract cleanup, which was performed using a reverse-phase LC system. Figure 1A shows a chromatogram of the standard isotachysterol D_3 on reverse-phase LC. Figure 1B shows a chromatogram of an actual milk-based liquid infant formula. A large peak (peak X, Figure 1B) elutes just before the isotachysterol peak and the isotachysterol peak elutes as a shoulder to this peak. Soy-based liquid infant formula also shows a similar chromatogram. When powdered infant formulas were analyzed on the cleanup LC system, a large peak that elutes just before isotachysterol peak was not present (Figure 1C).

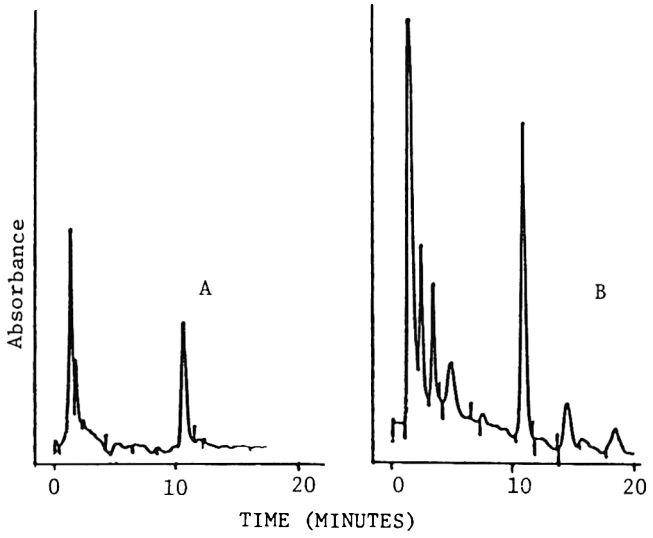

Figure 2. Chromatogram of (A) standard isotachysterol D_3; (B) milk-based liquid infant formula after cleanup on normal-phase LC, Spherisorb S3W column.

The final step involves quantitation using normal-phase LC. Standard vitamin D (10 IU) was always isomerized with every batch of samples and its peak height was used for recovery calculations. The standard or the cleaned extract was injected onto the LC column. Figure 2A shows a chromatogram of standard isotachysterol treated through the cleanup step. Figure 2B shows a chromatogram of the cleaned extract of milk-based liquid infant formula. No interfering peaks were observed, showing the efficiency of the cleanup step.

Table 1 shows the results of the vitamin D analysis of 3 commercial infant formulas. The amount of vitamin D found ranged from 98 to 103% of the declared amount in milk-based formula and from 97 to 102% in soy-based formula. All samples were spiked with standard vitamin D_3 to provide an additional 400 IU/quart. For this, 0.5 mL working standard was added to 25 mL milk. These spiked samples were analyzed to determine the accuracy of the method. The recoveries of spiked samples ranged from 94 to 101% (average 97.6 ± 2.6%) in milk-based formula and from 95 to 103% (average 98.8 ± 3.2%) in soy-based formula (Table 1).

In conclusion, the method presented represents a reliable approach for quantitative analysis of vitamin D in infant formula. This procedure has also been successfully applied to fortified milk in our laboratory. Saponification at room temperature avoids the problem of conversion of vitamin D to previtamin D. The isomerization of vitamin D to isotachysterol helps to reduce any loss due to the unstable nature of vitamin D and also increases the sensitivity and selectivity of the method.

Table 1. Results of vitamin D analysis, from fortified infant formulas and spiked recoveries[a]

Form	Label claim, IU/qt	Found, IU/qt	Recovery of spike, %
Milk-based infant formula			
Liquid	387	391 ± 9.0	94.4 ± 2.6
Liquid	387	380 ± 11.8	97.7 ± 2.7
Powder	400	410 ± 6.5	100.8 ± 3.3
Soy-based infant formula			
Liquid	387	377 ± 6.2	95.4 ± 1.8
Liquid	387	386 ± 9.9	97.8 ± 2.8
Powder	400	408 ± 10.9	103.1 ± 4.6

[a] Average ± stancard deviation, n = 3.

Acknowledgments

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TECHNICAL COMMUNICATIONS

Liquid Desorption of Organic Solvents from Industrial Sludges

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Analysis of organic solvent residues in industrial sludges is usually based on preliminary extraction of solvents from the matrix, followed by gas chromatographic determination. Liquid desorption with carbon disulfide is one of the most frequently used methods of recovery. Because of the number of parameters that can affect desorption efficiencies, we evaluated recoveries from 2 wastes with different physicochemical characteristics. Results were similar for both matrixes. The study of the kinetics of recovery showed that equilibrium was reached within about 5 min. On the other hand, specific adsorption phenomena were observed not only for strongly polar solvents even at the highest concentrations, but also for weakly polar compounds at the lowest concentrations. Water content of the matrix had little effect on recovery of the solvents examined, except for 2-ethoxyethanol, which yielded notably lower extractions when the moisture content of the matrix increased.

Analysis of organic solvent residues in industrial sludges is routinely carried out so that these waste materials can be correctly classified and subsequently disposed of in line with present regulations. The analytical method used needs to be as fast and simple as possible, and should give quantitative results. It must also be applicable to a variety of organic compounds, which may be incorporated within different matrix types at a wide range of concentrations. The method is usually based on preliminary extraction of solvents from the matrix, followed by gas chromatographic determination. The preliminary recovery step should avoid long and tedious manipulations of the sample that, over all, would make standardization of the method difficult.

Liquid desorption with carbon disulfide (CS_2) is one of the most frequently used methods of recovery, not only for sludges, but also for other types of environmental matrixes (1–9). Desorbing agents such as dimethylformamide (6, 8) or methanol in methylene chloride (10) are used less frequently. Nevertheless, many parameters that regulate the heterogeneous equilibrium must be considered: size of sludge particles in suspension, rate of release of solvents from inner to surface of particles, solid-to-liquid ratio, agitation technique and rate, temperature, etc. (11, 12). Therefore, the extraction step is the most critical one in terms of analytical reliability of the overall procedure.

The present study was undertaken to monitor the possible effect of 2 variables on the recovery of solvents from industrial sludges. They are: (*1*) the different physicochemical characteristics of sludges from different industrial plants, and (*2*) the parameters that can affect desorption efficiency.

Experimental

Sampling

(a) *Matrix selection and preparation.*—Two wastes were selected on the basis of their different physicochemical characteristics (pH, organic carbon), and were provided in large amounts by 2 different actual production units (painting with polyurethane and polyester enamels; painting with essentially polyurethane enamels, together with small amounts of polyester and nitrocellulose enamels).

(b) *Homogenization system.*—Approximately 100 g portions of each matrix were finely ground using ZK 1 blade homogenizer (Braun).

(c) *pH Determination.*—pH Determination of both wastes was performed according to ASTM method (12) using S 202 digital pH meter (SEAC) supplied with Orion RX 91-56 electrode.

(d) *Dried matrix preparation.*—Both wastes were freed of solvents and water in VV 2000 rotary vacuum evaporator (Heidolph, GFR) at 60°C to minimize possible structure modifications. Before recovery studies were performed, both matrixes were screened to 1 mm and kept in desiccator over phosphorus pentoxide.

(e) *Wet matrix preparation.*—Both dried matrixes were mixed with variable quantities of deionized water to produce sludges with 10–50% water content.

Proximate Analysis

(a) *Residual moisture determination.*—Residual moisture in 2 dried matrixes was determined by Karl Fischer titration using authomatic ABU 93 triburette, VIT 90 video titrator, and SAM 90 sample station (Radiometer, Denmark).

(b) *Organic carbon determination.*—Organic carbon content in 2 dried matrixes was determined by modification of method of Springer and Klee for compost (13): About 0.1 g waste was oxidized at 160°C with $K_2Cr_2O_7$ 1N in acidic medium (concentrated H_2SO_4), and excess $K_2Cr_2O_7$ was back-titrated with Mohr salt.

(c) *Ash determination.*—Ash content in 2 dried matrixes was determined by weighing residue obtained after treatment for 24 h at 660°C in muffle oven.

Preparation of Stock Standard Solution

Stock standard solution containing 1000 ppm each of methylene chloride, methyl ethyl ketone, ethyl acetate, methyl *iso*-butyl ketone, *iso*-butanol, *iso*-butyl acetate, toluene, *n*-butanol, *n*-butyl acetate, 2-ethoxyethanol, *p*-xylene, *o*-xylene, 2-ethoxyethyl acetate, and cyclohexanone, was prepared in carbon disulfide (J. T. Baker). Solvents were all commercially available compounds of the highest purity.

Preparation of Working Solutions

Working solution 1, containing 100 ppm each of solvents, was prepared by 1:10 dilution with CS_2 of the stock standard solution.

Working solution 2, containing 20 ppm each of solvents, was likewise prepared by 1:5 dilution of working solution 1.

Blank Test

For both wastes, 2 g dried matrix was weighed in 40 mL screw-cap glass vial with PTFE septum (Supelco, Inc.), 20

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Table 1. Main characteristics of the 2 wastes examined

Characteristic	Waste A	Waste B
Origin	spray painting cabin with dry scrubber	spray painting cabin with wet scrubber
Matrix	solid waste from polyurethane and polyester enamels	sludge from essentially polyurethane enamels
pH	5.86	8.51
Organic carbon,[a] %	24.4	18.2
Ash,[a] %	7.38	28.86

[a] Dry weight.

mL CS_2 was added, and waste-solvent mixture was occasionally shaken by hand for 1 h. After solids settled (within 1–2 min), an aliquot (about 1 mL) of clear supernatant liquid was transferred to 15 mL screw-cap glass vial with PTFE septum (Supelco, Inc.) over anhydrous sodium sulfate to eliminate traces of water, and 1 μL was injected immediately into gas chromatograph.

Recovery Tests

For both wastes, 2 g matrix was weighed in 40 mL screw-cap glass vial with PTFE septum, 20 mL stock standard solution (or working solutions) was added, and vial was occasionally shaken by hand for variable periods of time. At end of equilibration time, solids settled (within 1–2 min), and aliquot of clear supernatant liquid was processed as described in *Blank Test*.

Duplicate extractions were performed in all cases; each analysis was performed in triplicate.

All tests were carried out at room temperature (22 ± 2°C).

Gas Chromatography

Dani 6800 gas chromatograph equipped with a stainless steel column and flame ionization detector was used. 200 cm × 2 mm id column packed with 15% Ucon LB 550 X on 80–100 mesh Chromosorb W AW (Supelco, Inc.) was employed. Operating conditions: column temperature, programmed from 60°C (kept for 5 min) to 150°C at 5°/min, and maintained for 5 min; injector and detector temperature 170°C; nitrogen carrier gas flow 23 mL/min (set at 60°C); hydrogen flow 23 mL/min; air flow 200 mL/min.

Peak areas were determined by a Shimadzu C-R1A integrator.

Results and Discussion

Matrix Effect

At a preliminary stage of the research, we examined the effect of physicochemical parameters of the sludge on the recovery of solvents. For this purpose, we used 2 wastes with characteristics as different as possible (wastes A and B). Both wastes came from painting processes, because this type of matrix is likely to contain a wide range of industrial solvents that represent different classes of compounds. Sludges from other industrial processes may contain only a few solvents from a single class of compounds. The main characteristics of the 2 wastes used during the study are shown in Table 1. In particular, the 2 wastes were selected for their pH value and organic carbon content; these 2 parameters are known to play an important role in determining the extraction yields of organic compounds in environmental samples.

To eliminate water and sample interferences, the 2 wastes were first dried and freed of retained solvents. After extraction with CS_2, the 2 dried matrixes (see *Blank Test*) produced chromatograms free of detectable levels of solvents originally incorporated in the sample.

Water removal was almost quantitative; residual moisture was 0.3 and 0.4% in wastes A and B, respectively.

Solvent recovery tests were conducted on the 2 dried matrixes. The solvents selected represent the various classes of organic compounds more frequently present in industrial sludges (chlorinated hydrocarbons, ketones, acetates, alcohols, aromatic hydrocarbons, glycol ethers); selected concentrations represent levels typically found in real samples.

For handling simplicity, the method originally proposed by Dommer and Melcher (7) was chosen. Their procedure uses adsorption from solution, in line with the definition of Rudling and Björkholm (6). According to the method, the solvents under examination are dissolved in the desorbing agent and then added to the matrix. In other words, "equilibrium is reached from the other side" (6) in respect of desorption from the sludge (the spike technique).

p-Xylene was utilized as internal standard because preliminary tests using the external standard method showed that 98–103% of *p*-xylene was recovered from both wastes. The choice of a hydrocarbon as the internal standard is widespread for environmental samples, because hydrocarbons usually show no matrix interferences (5, 6, 14, 15).

Both kinetics of recovery and influence of solvent loading were evaluated simultaneously by determining extraction yields as a function of time on 3 different standard solutions (1000, 100, and 20 ppm). This simulated loadings of solvents in the matrixes of 10 000, 1000, and 200 ppm, respectively, while the waste/desorbing agent ratio (2 g/20 mL) was kept constant. Results, expressed as percent recovery of each compound with respect to the working solution, are presented in Tables 2 and 3.

A Student *t*-test was used to determine if differences existed among the recoveries obtained at the various times. For both matrixes and for the 3 loadings of solvents, recoveries were usually not significantly different at the 0.05 level. Furthermore, differences that existed did not appear to be significant; instead, they appeared to be random regarding both the solvents involved and the trend with time of recovery. The conclusion can, therefore, be drawn that both matrixes reached equilibrium within 5 min.

On the other hand, recoveries from the 2 matrixes were not quantitative even at the highest examined concentrations. This can be seen particularly for compounds with a free alcoholic group (alcohols and glycol monoethers). Specific adsorption occurred, with strongly polar compounds affected more significantly. Moreover, a dependence on loading was also observed for weakly polar compounds (ketones, acetates, chlorinated hydrocarbons, esterified glycol ethers). At the lowest concentrations, adsorption effects occurred to varying extents. Only aromatic hydrocarbons showed quantitative recoveries over the entire range of concentrations examined. Similar behavior was described for recovery of adsorbed solvents from activated carbon (5, 6). For this type of material, 2 main parameters may play an important role when specific adsorption phenomena occur. These parameters are the number and chemical nature of the adsorption sites (sur-

Table 2. Percent recoveries of industrial solvents from dried waste A, as function of equilibration time with carbon disulfide and of loading of solvents[a]

	Loading of solvents, ppm								
	10 000			1000			200		
	Recovery, %, after equilibration for								
Solvent	5 min	1 h	24 h	5 min	1 h	24 h	5 min	1 h	24 h
Methylene chloride	93.7 ± 0.9	94.5 ± 3.4	91.4 ± 0.2	94.2 ± 1.1	95.1 ± 1.4	93.4 ± 1.1	79.9 ± 3.5	86.8 ± 2.2	81.6 ± 6.0
Methyl ethyl ketone	89.4 ± 3.2	91.8 ± 2.4	89.6 ± 4.1	87.7 ± 2.6	87.9 ± 1.5	88.6 ± 1.8	80.0 ± 2.4	86.1 ± 3.0	81.4 ± 4.6
Ethyl acetate	91.5 ± 4.2	95.2 ± 1.0	94.9 ± 3.3	96.7 ± 1.1	95.5 ± 3.2	96.7 ± 3.9	87.1 ± 5.2	91.7 ± 3.5	87.3 ± 4.8
Methyl isobutyl ketone	97.3 ± 0.7	96.7 ± 0.5	97.2 ± 0.5	97.7 ± 2.2	96.1 ± 0.2	96.8 ± 1.6	94.5 ± 2.6	92.2 ± 1.3	92.8 ± 2.8
iso-Butanol	80.0 ± 1.2	80.6 ± 1.5	79.2 ± 2.2	79.1 ± 1.3	75.7 ± 0.4	75.9 ± 0.9	72.0 ± 2.0	70.2 ± 2.8	70.4 ± 2.5
iso-Butyl acetate	97.2 ± 1.7	97.5 ± 1.6	97.6 ± 0.5	97.3 ± 0.2	96.9 ± 0.6	98.4 ± 1.4	98.3 ± 3.8	94.4 ± 2.9	96.7 ± 4.5
Toluene	102.0 ± 0.8	102.2 ± 0.5	102.8 ± 1.8	101.7 ± 1.7	98.6 ± 0.3	100.8 ± 1.3	101.3 ± 3.8	98.3 ± 2.8	99.5 ± 1.5
n-Butanol	80.0 ± 1.5	79.7 ± 1.5	79.0 ± 1.4	74.9 ± 3.2	72.4 ± 0.6	73.4 ± 1.3	73.3 ± 3.7	73.3 ± 3.5	71.8 ± 2.5
n-Butyl acetate	98.4 ± 1.1	97.9 ± 0.4	98.2 ± 0.5	98.7 ± 1.1	98.3 ± 0.1	97.3 ± 1.0	95.2 ± 2.7	97.1 ± 2.6	99.4 ± 3.2
2-Ethoxyethanol	81.1 ± 1.1	79.8 ± 1.3	79.3 ± 1.1	72.6 ± 2.8	70.3 ± 1.7	71.9 ± 3.4	65.7 ± 5.8	69.0 ± 3.7	69.7 ± 4.5
o-Xylene	102.1 ± 0.4	101.8 ± 0.9	103.0 ± 2.5	100.8 ± 3.2	100.8 ± 0.1	101.0 ± 0.9	101.5 ± 3.5	99.9 ± 3.4	99.8 ± 1.9
2-Ethoxyethyl acetate	94.7 ± 1.0	96.5 ± 2.3	97.7 ± 1.0	93.0 ± 1.8	95.2 ± 1.0	94.1 ± 0.7	92.7 ± 7.6	88.1 ± 4.6	91.7 ± 8.7
Cyclohexanone	95.0 ± 0.9	94.0 ± 1.0	94.1 ± 1.0	91.6 ± 0.5	93.4 ± 1.7	93.6 ± 0.4	89.7 ± 3.4	86.0 ± 6.1	88.3 ± 6.3

[a] Average percent recovery for 6 determinations ± standard deviation. Waste/carbon disulfide ratio: 2 g/20 mL.

Table 3. Percent recoveries of industrial solvents from dried waste B, as function of equilibration time with carbon disulfide and of loading of solvents[a]

	Loading of solvents, ppm								
	10 000			1000			200		
	Recovery, %, after equilibration for								
Solvent	5 min	1 h	24 h	5 min	1 h	24 h	5 min	1 h	24 h
Methylene chloride	93.6 ± 6.1	96.0 ± 1.5	94.5 ± 2.1	97.0 ± 0.4	94.6 ± 3.3	98.6 ± 4.7	86.1 ± 4.1	89.2 ± 5.5	84.9 ± 3.1
Methyl ethyl ketone	94.7 ± 1.3	95.5 ± 0.6	92.8 ± 2.6	93.4 ± 3.0	93.5 ± 2.3	95.7 ± 1.4	90.1 ± 3.9	89.7 ± 3.6	90.2 ± 2.4
Ethyl acetate	93.3 ± 1.5	96.4 ± 0.7	97.4 ± 3.8	95.7 ± 2.1	95.5 ± 2.0	97.8 ± 2.6	90.3 ± 2.7	90.0 ± 3.5	91.4 ± 2.4
Methyl isobutyl ketone	97.7 ± 0.1	101.5 ± 0.5	97.7 ± 0.4	96.5 ± 0.7	98.0 ± 1.7	99.2 ± 0.2	95.1 ± 2.8	94.0 ± 4.6	97.3 ± 4.3
iso-Butanol	84.5 ± 1.2	85.0 ± 0.8	83.1 ± 1.4	80.8 ± 1.3	83.0 ± 0.5	84.2 ± 1.9	74.5 ± 4.1	78.1 ± 3.6	76.0 ± 3.6
iso-Butyl acetate	98.8 ± 1.5	98.6 ± 1.0	98.4 ± 1.5	96.5 ± 1.5	98.1 ± 3.8	100.3 ± 1.2	94.2 ± 4.9	95.2 ± 4.7	96.0 ± 4.1
Toluene	101.9 ± 1.5	102.2 ± 2.3	100.6 ± 0.9	100.3 ± 0.3	101.1 ± 1.8	98.5 ± 2.1	101.1 ± 2.0	99.0 ± 2.3	100.1 ± 2.4
n-Butanol	84.4 ± 0.5	83.2 ± 0.7	82.8 ± 0.9	80.2 ± 1.7	81.1 ± 1.3	81.7 ± 0.3	73.8 ± 3.7	76.0 ± 3.4	73.0 ± 3.4
n-Butyl acetate	98.5 ± 0.7	98.6 ± 1.7	98.1 ± 0.3	97.2 ± 2.6	98.3 ± 3.8	98.9 ± 0.4	93.5 ± 4.3	93.8 ± 3.1	92.1 ± 4.2
2-Ethoxyethanol	87.2 ± 1.8	85.8 ± 2.9	83.5 ± 1.3	77.8 ± 2.9	79.9 ± 4.2	79.1 ± 1.9	72.4 ± 4.7	72.7 ± 4.8	74.0 ± 3.7
o-Xylene	102.1 ± 0.7	101.9 ± 4.8	100.9 ± 1.6	97.9 ± 0.4	99.9 ± 0.6	99.6 ± 2.2	99.3 ± 2.4	99.5 ± 2.2	99.7 ± 1.6
2-Ethoxyethyl acetate	99.0 ± 3.7	99.0 ± 3.8	96.3 ± 4.7	96.5 ± 1.7	100.1 ± 4.8	97.2 ± 3.7	94.6 ± 6.6	91.6 ± 3.7	94.1 ± 6.2
Cyclohexanone	96.2 ± 1.6	97.6 ± 2.2	94.7 ± 1.8	95.2 ± 1.7	100.3 ± 0.8	95.5 ± 3.6	93.9 ± 3.4	92.5 ± 4.6	96.0 ± 4.2

[a] Average percent recovery for 6 determinations ± standard deviation. Waste/carbon disulfide ratio: 2 g/20 mL.

Table 4. Percent recoveries of industrial solvents from two wastes A and B, as function of water content of matrix[a]

Solvent	Waste A (moisture, %)						Waste B (moisture, %)					
	0	10	20	30	40	50	0	10	20	30	40	50
Methylene chloride	94.2 ± 1.1	94.1 ± 2.1	94.1 ± 1.4	95.5 ± 3.1	93.0 ± 2.3	94.9 ± 3.7	97.0 ± 0.4	92.5 ± 4.3	96.3 ± 3.1	96.6 ± 2.6	94.2 ± 1.8	96.3 ± 1.5
Methyl ethyl ketone	87.7 ± 2.6	89.0 ± 1.3	92.2 ± 3.7	87.3 ± 1.8	87.6 ± 3.5	88.3 ± 2.3	93.4 ± 3.0	91.2 ± 2.2	93.8 ± 3.5	94.2 ± 2.7	93.9 ± 2.6	92.1 ± 3.6
Ethyl acetate	96.7 ± 1.1	95.2 ± 1.1	96.3 ± 1.4	95.4 ± 1.5	94.1 ± 1.6	96.5 ± 1.4	95.7 ± 2.1	94.9 ± 0.7	97.7 ± 3.1	95.9 ± 4.5	93.9 ± 1.4	97.1 ± 3.8
Methyl isobutyl ketone	97.7 ± 2.2	97.3 ± 1.2	97.4 ± 0.7	95.4 ± 1.1	96.4 ± 0.7	98.0 ± 0.7	96.5 ± 0.7	97.2 ± 1.0	99.2 ± 3.1	100.4 ± 1.8	96.0 ± 1.8	96.9 ± 3.0
iso-Butanol	79.1 ± 1.3	75.3 ± 1.8	74.6 ± 1.1	75.1 ± 2.1	75.3 ± 2.5	76.6 ± 1.0	80.8 ± 1.3	82.3 ± 0.3	78.3 ± 3.1	80.1 ± 4.0	80.3 ± 0.8	81.4 ± 2.4
iso-Butyl acetate	97.3 ± 0.2	97.4 ± 0.6	99.3 ± 1.7	97.9 ± 1.3	98.1 ± 2.1	98.7 ± 1.0	96.5 ± 1.5	96.6 ± 1.9	98.9 ± 3.2	98.8 ± 2.8	98.9 ± 1.7	96.3 ± 0.7
Toluene	101.7 ± 1.7	99.3 ± 1.6	98.8 ± 0.4	96.7 ± 2.1	100.9 ± 2.6	100.2 ± 1.3	100.3 ± 0.3	99.7 ± 0.5	100.3 ± 2.4	98.3 ± 0.2	101.5 ± 1.6	98.2 ± 0.4
n-Butanol	74.9 ± 3.2	73.4 ± 1.4	72.1 ± 1.2	71.3 ± 3.6	72.7 ± 3.3	74.5 ± 0.9	80.2 ± 1.7	80.9 ± 1.2	79.3 ± 0.7	81.6 ± 2.8	77.6 ± 2.4	78.3 ± 3.3
n-Butyl acetate	98.7 ± 1.1	97.7 ± 0.5	101.0 ± 1.0	96.1 ± 0.4	96.4 ± 0.4	99.7 ± 0.9	97.2 ± 2.6	97.8 ± 1.2	100.7 ± 1.7	103.6 ± 3.7	97.3 ± 1.2	97.6 ± 0.7
2-Ethoxyethanol	72.6 ± 2.8	55.6 ± 3.2	41.5 ± 3.2	31.7 ± 1.7	27.9 ± 3.9	25.3 ± 1.8	77.8 ± 2.9	67.4 ± 6.2	55.7 ± 4.2	54.4 ± 3.1	43.8 ± 5.4	41.5 ± 4.8
o-Xylene	100.8 ± 3.2	99.6 ± 0.1	98.2 ± 0.5	101.4 ± 0.5	100.9 ± 0.7	100.8 ± 2.0	97.9 ± 0.4	101.4 ± 1.4	99.7 ± 0.1	99.7 ± 0.2	101.7 ± 0.2	102.0 ± 1.3
2-Ethoxyethyl acetate	93.0 ± 1.8	92.8 ± 2.3	91.5 ± 3.6	91.3 ± 2.4	92.6 ± 1.9	95.5 ± 3.3	96.5 ± 1.7	95.4 ± 4.3	99.7 ± 4.8	99.7 ± 3.5	95.5 ± 3.9	98.2 ± 3.1
Cyclohexanone	91.6 ± 0.5	91.8 ± 3.0	94.9 ± 3.5	93.0 ± 0.8	93.6 ± 1.5	95.8 ± 1.1	95.2 ± 1.7	95.2 ± 5.0	97.1 ± 2.9	96.8 ± 1.7	97.4 ± 4.6	96.2 ± 4.2

[a] Average percent recovery for 6 determinations ± standard deviation. Equilibration time: 5 min; waste/carbon disulfide ratio: 2 g/20 mL; loading of solvents: 1000 ppm each.

face oxides may be present on the matrix), as well as the properties of the desorbing agent (CS_2 may be a poor competitor with polar solvents for polar adsorption sites) (5, 6).

Effect of Moisture

To evaluate the effect of water content of the matrix, we performed recovery tests on samples with a 10–50% moisture content. This is a range usually found in real samples. Both the equilibration time (5 min) and the sludge/standard solution ratio (2 g/20 mL) were kept constant. The sludges were loaded at 100 ppm working solution so that solvents could be recovered at 1000 ppm. Results are shown in Table 4; for comparison, results for the 2 dried matrixes are also shown.

Statistical analysis of data, performed by calculating the linear regression line of the mean recoveries of each solvent and by identifying a confidence interval for the slope at the 0.05 significance level, showed that the zero slope figure falls within this interval for almost all examined solvents. Water content of the sample had little effect on recoveries of the solvents examined, except for 2-ethoxyethanol, which yielded notably lower extractions when the moisture content of the matrix increased. The water content of the sample was already observed not to influence recovery of halogenated and aromatic hydrocarbons from soils and sludges (16). On the other hand, because water is practically insoluble in CS_2, 2 phases will form and very water-soluble compounds, such as 2-ethoxyethanol, will concentrate in the polar phase. This was pointed out by Rudling and Björkholm (17) for liquid desorption of organic compounds from activated carbon that contained adsorbed water.

Conclusion

The present study shows only preliminary results. No definite conclusions can presently be drawn, because only a few of the variables that may influence desorption yields have been taken into account. Investigations on recoveries from other types of sludges, properly selected for physicochemical characteristics, are needed. The effect of solvent loading must be evaluated over the entire range of concentrations found in real samples. Neither the effect on recoveries of both analyte composition and temperature nor the optimum sludge/desorbing agent ratio have yet been determined. Extraction yields relative to other types of industrial solvents remain to be examined. It might be interesting to compare results obtained by other recovery techniques (e.g., thermal desorption) to identify the method that offers the highest yields. A complete evaluation of all the factors that play a role on desorption of solvents from industrial sludges might offer useful information in view of a standardization of the procedure.

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Effect of Gelatin on Recovery of Hair Fragments in Filth Analysis

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Prior literature has recommended the use of gelatin capsules or gelatin film for transferring spike elements to samples being examined for recovery studies. It was believed that gelatin had no effect on the recovery of filth spike elements. However, this study shows that hair recovery is lower when gelatin is present in direct trap-off procedures. Two types of gelatin capsules, gelatin film, and strips of filter paper were used to transfer spike filth elements. A comparison study employing an acid digestion and wet sieving procedure was also performed and showed that gelatin had little or no effect on the recovery of hairs using this type of procedure. An additional test was performed using gelatin added to water containing the same type of spiked filth elements. No hair fragments were recovered but all insect fragments were recovered. All recovery studies were performed using only water in the liquid phase of the trap flask extractions, with mineral oil or heptane as the flotation medium. No food product was used.

The use of gelatin capsules to transfer spike filth elements to food samples for recovery studies has been recommended for several years (1). A more recent approach uses gelatin film (2). Both of these techniques offer advantages in ease of preparing and transferring the spike material to the sample.

In a recent method development study, acceptable recoveries were obtained in preliminary trials using spikes (*Tribolium* elytral squares and mouse hair fragments) transferred on filter paper to the product. Tests were then conducted in which the same spikes were transferred in gelatin capsules to the product. In the latter case, the resulting papers were "heavy" and the recovery of hairs was significantly lower. At first, these lower recoveries were attributed to the method, but, after a number of trials with similar results, the capsules were suspected of causing the problem. Another analyst in this laboratory experienced similar problems with gelatin capsules when using a different product and trapping medium. All the procedures were direct trap-off (no wet sieving of the product).

The gelatin capsules initially used were 2-piece capsules size 00, obtained from Thomas Scientific, and designed to be burned in Parr combustion bombs. Gelatin capsules size 1 made by Eli Lily were also tried. Hair recovery was somewhat higher for the smaller Eli Lily capsules. Improved recoveries could have been due to the presence of less gelatin, a difference in the gelatin used, or both. The present study was undertaken to determine the effect of gelatin on the recovery of filth spike material.

Experimental

Three tests were made for each of the 2 types of gelatin capsules, gelatin film, and strips of filter paper. No material was used to attach the filth elements to the filter paper. The spike material consisted of 10 *Tribolium* elytral squares ca 1 sq. mm and 10 mouse hair fragments ca 2-3 mm. No product was used. Spikes were placed in the gelatin capsules, on gelatin film (ca 1 × 1/4 in.), and on strips of filter paper (ca 1 × 1/4 in.). Each of the spike carriers was added to ca 1 L water in a 2 L trap flask. Contents of the flask were boiled for ca 10 min, cooled, and trapped using 40 and 25 mL mineral oil (3, 4).

Results

An emulsion formed at the interface of the oil/water layer in the flasks containing gelatin. The larger capsule produced a greater amount of emulsion. If product had been present, this emulsion would trap product material and cause "heavy" filter papers. Recovery results were lowest for the large capsule carrier (Table 1).

Another test was conducted using a 3% HCl digestion and wet sieving procedure without product. The same spike elements were prepared for the 2 types of gelatin capsules and strips of filter paper (no gelatin). Each spike carrier was added to ca 1 L 3% HCl in a 2 L beaker. Contents of the beaker were boiled for ca 15 min and wet-sieved on a No. 230 sieve (5). The contents on the sieve were then transferred to a 2 L trap flask with water and trapped as in the previous test. The wet sieving process eliminates the gelatin. Recoveries obtained from the gelatin carriers were acceptable and compared favorably with the filter paper results (Table 2).

One test was made using heptane as the flotation medium to determine if the problem of low recoveries was limited to mineral oil. The same spike elements were placed in water containing 1 g gelatin. The same direct trapping procedure was used. All insect fragments, but none of the hair spikes, were recovered. The same problem with emulsion formation was encountered.

Hair fragments were checked for visible gelatin coating by boiling in ca 1 L water containing 1 g gelatin, mounting in water, and examining microscopically at 100-400×. Each hair fragment appeared "dirty," but it was not conclusively determined that the hair fragments were coated with gelatin.

Conclusion

The results of this study show that, in methods involving direct trap-offs, the presence of gelatin lowers hair recovery; the decrease in recovery is apparently related to the amount of gelatin present. Gelatin appears to have little or no effect on filth recovery for methods that include an acid digestion

Table 1. Comparison of recoveries for 2 types of gelatin capsules, gelatin film, and filter paper strips using direct trap-off with water and mineral oil

Transfer material	Spike[a]	Subsample recoveries 1	2	3	Av.
Capsule (size 00)	10EF	8	6	7	7
	10MH	1	1	1	1
Capsule (size 1)	10EF	6	8	7	7
	10MH	2	6	5	4.3
Gelatin film	10EF	8	9	10	9
	10MH	3	3	6	4
Filter paper	10EF	10	10	10	10
	10MH	10	9	9	9.3

[a] EF = elytral fragments; MH = mouse hairs.

Table 2. Comparison of recoveries for 2 types of gelatin capsules and filter paper strips using acid digestion and wet sieving before trapping with water and mineral oil

Transfer material	Spike[a]	Subsample recoveries 1	2	3	Av.
Capsule (size 00)	10EF	10	10	10	10
	10MH	7	7	6	6.6
Capsule (size 1)	10EF	8	10	10	9.3
	10MH	6	7	7	6.6
Filter paper	10EF	9	10	10	9.6
	10MH	7	8	7	7.3

[a] EF = elytral fragments; MH = mouse hairs.

and wet sieving step. However, if gelatin capsules are used to transfer spike filth elements, a test should be done to assure there is no effect on the recovery of filth elements for that specific product and method. Any material used to transfer or aid in the transfer of spike material should be tested for each type of product and method before it is acceptable in recovery studies.

The effect of gelatin on the recovery of insect fragments appears inconclusive. Recoveries of insect fragments on gelatin capsule carriers were somewhat lower; however, all insect fragments were recovered in the test using gelatin in water.

In this study, no attempt was made to determine the effect of gelatin on the recovery of filth from a food product. If a product containing gelatin as a listed ingredient is examined for filth, an acid digestion and wet sieving step should be employed to avoid the possibility of low hair recovery.

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Caffeine as Main Interfering Compound in Radioimmunoassay of Aflatoxin B_1 in Coffee Samples

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The content of caffeine in coffee extracts prepared for radioimmunoassay of aflatoxin B_1 was determined by gas chromatography. The extracts from coffee beans and decaffeinated coffee contained 1.76–4.60 and 0.71–0.85 g caffeine/kg, respectively. These concentrations of caffeine caused false results in radioimmunoassay of aflatoxin B_1 in the range 1.0–2.8 μg/kg for coffee beans and 0.3–0.4 μg/kg for decaffeinated coffee.

In general, thin-layer chromatography (TLC) and liquid chromatography (LC) are the techniques most commonly used to determine aflatoxins and their metabolites. However, these methods are not specific and the extensive sample cleanup is time consuming. Expensive instruments are required for LC analyses, and in TLC, large amounts of gels and solvents are used.

Immunoassays are beginning to replace conventional approaches in food analysis because of their high specificity and sensitivity, a combination that should ensure the need for minimal sample preparation and make high rates of analysis possible. Immunoassays are technically simple and can be performed at low cost (1–4). Nevertheless, a purification step can be required for quantitative and precise determination of low aflatoxin levels in crude extracts of various samples (5–8). However, only limited information exists on the feasibility of aflatoxin immunoassays in the routine analysis of food and fodder samples, with respect to possible interferences (1, 4, 7). The results presented in our earlier paper (9) show that many naturally occurring substances, e.g., derivatives of coumarin, and cinnamonic and benzoic acids, may cause interferences in radioimmunoassay of aflatoxins. Among these substances, the interference of caffeine was not studied.

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The possible occurrence of aflatoxins in coffee samples is still unclear. Some authors (10–12) proved that coffee samples usually do not contain aflatoxin B_1, because caffeine (0.5 mg/mL) inhibits its production. Therefore, potentially greater possibility for mold contamination exists in decaffeinated coffee (13). On the other hand, other observations showed formation of aflatoxin B_1 by *Aspergillus parasiticus* (14, 15) even at higher caffeine concentrations (8 mg/mL).

To elucidate differences in aflatoxin content determined by TLC and radioimmunoassay (RIA) in coffee samples (9), we analyzed crude chloroform extracts of these samples. The present study examines the interfering substance in chloroform coffee extracts prepared for aflatoxin B_1 radioimmunoassay.

Experimental

Apparatus

(a) *Gas chromatograph.*—(Model Chrom 5, Laboratory Equipment, Prague, Czechoslovakia). Equipped with flame

Table 1. Interferences of caffeine in radioimmunoassay of aflatoxin B_1

Caffeine concn expressed in	Antiserum I	Antiserum II
mg/100 μL[a]	0.02	0.01
g/kg[b]	1.7	0.7
ED_{50}, mg/100 μL[c]	0.25	0.15
Apparent affinity constants	1.2×10^3	4.3×10^3

[a] Concentration of caffeine simulating 10 pg aflatoxin B_1 in 100 μL of analyzed solution.

[b] Concentration of caffeine simulating 1 μg aflatoxin B_1 in 1 kg sample.

[c] ED_{50} = estimated dose at 50% radioligand displacement from its binding antiserum.

ionization detector and glass column (1200 × 3 mm id) packed with 1% (w/w) ethylene glycol adipate on 0.125–0.16 mm Chromaton N-AW-DMCS solid support (Lachema, Brno, Czechoslovakia). Operating conditions: nitrogen carrier gas 30 mL/min; temperatures 200 and 240°C at column and inlet (detector), respectively; sensitivity usually 1:100 to bring peaks to about half-scale deflection; recorder chart speed 60 mm/min (16).

(b) *Gas chromatograph–mass spectrometer.*—Shimadzu QP-1000 equipped with quadrupole mass analyzer operated under following conditions: fused capillary column (0.32 mm × 60 m) with SPB 1 stationary phase; oven temperatures—programmed from 75 to 320°C at 10°/min; injector port 250°C, separator 330°C, ion source 330°C; ionization energy 70 eV; helium carrier gas 1 mL/min.

Materials

(a) *Chemicals.*—Caffeine was supplied by Sigma Chemical Co. Other chemicals were analytical grade (Lachema, Czechoslovakia).

(b) *Solvents.*—Acetone and chloroform.

(c) *Samples.*—All coffee bean samples were purchased from local grocery stores.

(d) *Antisera.*—Rabbit antisera against aflatoxin B_1 as described earlier (9).

Procedure

(a) *Preparation of extracts.*—Two g coffee, ground to homogeneity on vibrating mill, was extracted in 25 mL chloroform for 30 min with continuous shaking at 20°C. The chloroform extract was filtered, and the residue was extracted again under the same conditions. The combined chloroform extracts were evaporated in a rotary vacuum evaporator. The residue for gas chromatography (GC) was dissolved in chloroform, or in acetone for RIA, and the volumes were adjusted to 2 mL.

Table 3. Radioimmunoassay of aflatoxin B_1 in coffee extract, containing 2.95 g caffeine/kg sample, and with known amount of added aflatoxin B_1

Added, μg/kg	Found, μg/kg	Rec., %
0.0	2.2 ± 0.4	—
0.5	2.8 ± 0.3	120
1.0	3.1 ± 0.4	90.0
1.5	3.6 ± 0.3	93.3
2.0	4.4 ± 0.7	110
3.0	5.1 ± 0.5	96.6
5.0	7.1 ± 0.6	98.0

(b) *Radioimmunoassay.*—Aflatoxin B_1 was assayed by using a commercial RIA kit with 125-I-aflatoxin B_1 as a radioligand, as described earlier (9).

(c) *Testing of interferences.*—The procedure described earlier was used (9).

Results and Discussion

In our previous study (9), we found that derivatives of coumarin and cinnamic and benzoic acids caused interferences in immunoassays of aflatoxin B_1. To explain the high false results obtained by RIA of coffee samples (9), we analyzed these extracts by gas chromatography. Caffeic and chlorogenic acids, which were found earlier (9) to interfere to a great extent with antisera against aflatoxin B_1, were determined in coffee extracts at a very low level. Maximal concentrations of these compounds were 0.03 g/kg sample. Significant interferences in radioimmunoassay for aflatoxin were observed at a level 10 times higher.

Results from GC analysis of the extracts showed that they contained from 0.71 to 4.60 caffeine g/kg sample. Two unidentified compounds were also present, along with some minor components.

Only caffeine shows significant interferences with antisera used in radioimmunoassay of aflatoxin B_1 (Table 1). In comparison with the compounds tested earlier (9), caffeine interferes at the lower concentration. The resulting degree of nonspecific binding is expressed by the concentration of caffeine which simulates the occurrence of 1 μg aflatoxin B_1/kg sample. The results given in Table 1 indicate that antiserum II shows more significant unspecific interactions; therefore, antiserum I was used in RIA of aflatoxin B_1.

The apparent (i.e., false) concentrations of aflatoxin B_1 caused by the occurrence of caffeine in analyzed coffee extracts are given in Table 2. The accuracy of aflatoxin B_1 determined by RIA is affected by the amount of caffeine extracted from the coffee powder. For instance, the content of caffeine in coffee beans is 7–20 g/kg (14), and when 1.7 g caffeine/kg is present in the analyzed sample, the false value of aflatoxin B_1 found will reach 1.0 μg/kg.

Table 2. Determination of caffeine in coffee extracts by gas chromatography and by radioimmunoassay for aflatoxin B_1

Sample	A Caffeine in coffee ext, g/kg, detd by GC	B[a] RIA of aflatoxin B_1 in model solns of caffeine, μg/kg	C RIA of aflatoxin B_1 in coffee exts, μg/kg	B/C, %
Coffee beans 1	2.95 ± 0.15	2.1 ± 0.2	2.2 ± 0.4	95
Coffee beans 2	4.60 ± 0.22	2.8 ± 0.2	3.0 ± 0.3	93
Coffee beans 3	1.72 ± 0.08	1.0 ± 0.2	1.2 ± 0.4	83
Decaffeinated coffee 1	0.85 ± 0.04	0.4 ± 0.1	0.4 ± 0.2	100
Decaffeinated coffee 2	0.71 ± 0.03	0.3 ± 0.1	0.3 ± 0.2	100

[a] Model solutions were prepared with concentrations determined in column A.

The accuracy of the aflatoxin B_1 radioimmunoassay was verified by the method of standard addition (Table 3). The known amount of aflatoxin B_1 in chloroform was added to the sample of coffee powder. The recovery, calculated after subtracting the false positive value 2.2 μg/kg (caused by caffeine present in the analyzed extract) from the estimated aflatoxin content, shows that RIA of aflatoxin B_1 gives reasonable results.

The present results suggest that caffeine is the main interfering compound responsible for false results in RIA of aflatoxin B_1 in crude extracts of coffee samples. Thus, only suitable purification of caffeine-containing samples, or good antisera with apparent affinity constants higher than 10^{10}, may assure reliable results in the concentration range 0–5 μg aflatoxin B_1/kg sample.

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