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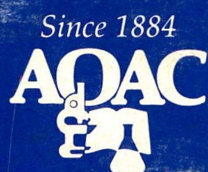

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MAY/JUNE 1989
VOL. 72, NO. 3

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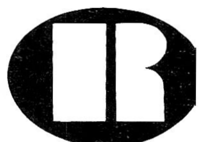

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Talanta provides a forum for the rapid publication of original research papers, preliminary communications, full reviews and mini-reviews. Other features are annotations (critical commentaries), analytical data (stability constants etc.) and letters to the editor.
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Toxic Impurities in Biological Systems . Food Additives . Pharmaceutical and Drug Analysis . Pesticides and Fungicides . Clinical Chemistry . Materials, Science and Engineering . Geochemistry . Environmental Analysis.

A Selection of Papers

JUN'ICHI TOEI (Japan), Potential of the flow-gradient function in FIA with a multifunction pump delivery system.
M PESAVENTO, A PROFUMO & R BIESUZ (Italy), Sorption of protons and metal ions from aqueous solutions by a strong-base anion-exchange resin loaded with sulphonated azo-dyes.
D MIDGLEY (UK), Combination pH electrodes of special design: temperature characteristics and performance in poorly-buffered waters.
R MORALES, C S BARTHOLDI & P T CUNNINGHAM (USA), HPLC separation of heterocyclic beta-diketonates of actinide, lanthanide and transition metals.
A PARCZEWSKI (Poland), Determination of two metals from a single potentiometric titration curve. The application of two indicator electrodes.

A Software Survey section is included in this journal.

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This publication, previously called Food Laboratory Newsletter, is distributed to 152 countries and serves laboratories engaged in chemical or microbiological analysis of food and drinking water. Its purpose is to publish brief reviews and notes of importance for food laboratories, particularly in developing regions of the world. The editors welcome questions and reports of relevance to these topics.

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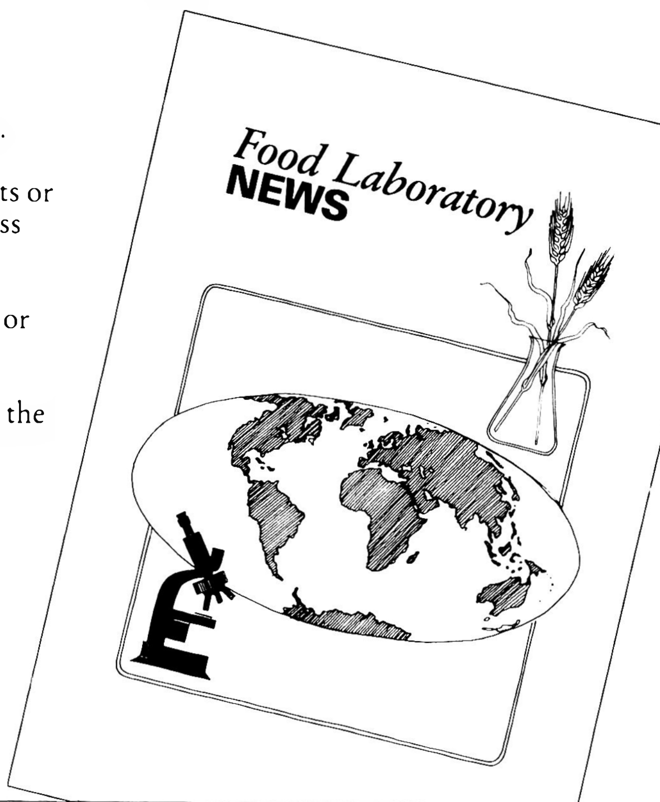

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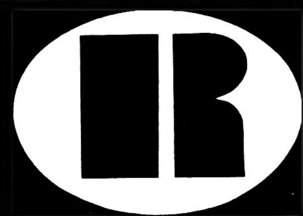

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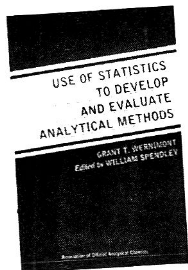

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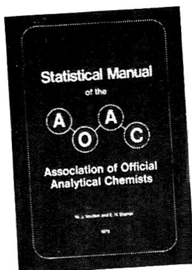

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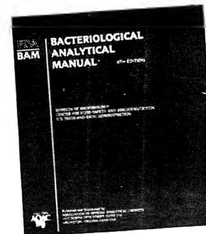

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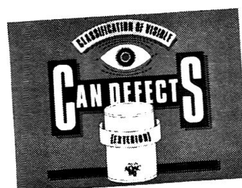

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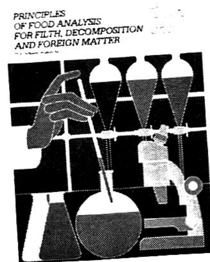

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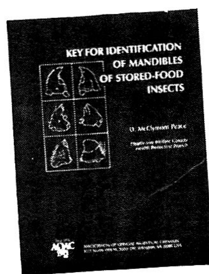

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Detection of Environmental Contaminants and Natural Toxins in Food Products of Animal Origin—Chairmen: William A. Moats and Badir Shaikh
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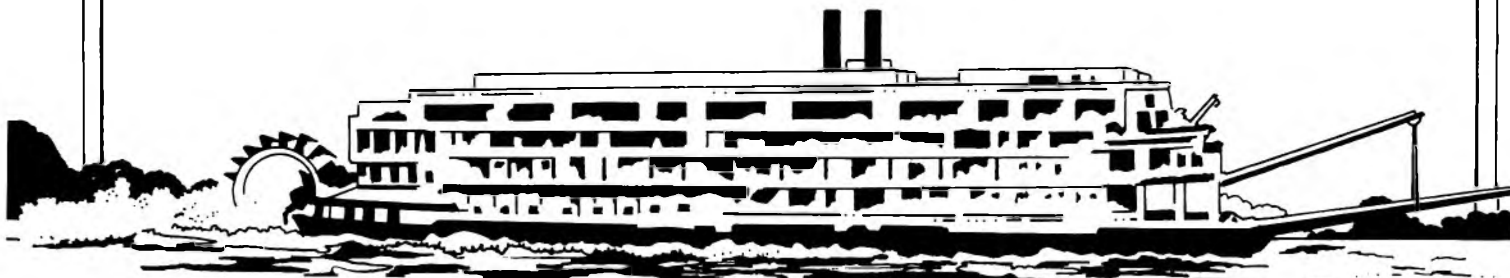
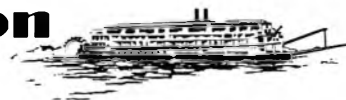


he 10_ rt AOAC Annual International Meeting and Exposition



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Please sign me up for the 103rd AOAC Annual International Meeting & Exposition, at The Clarion Hotel, St. Louis, Missouri, September 25-28, 1989.

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Early-Bird—full meeting	$130	$175	$ ______
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Education (Specify subjects and levels of degrees) ____________

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I hereby apply for membership in the Association of Official Analytical Chemists, and, if accepted, agree to abide by its rules.

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AOAC, 1989 Annual International Meeting, Suite 400-J, 2200 Wilson Boulevard, Arlington, VA 22201-3301 USA

Credit card orders may be placed by phone (703-522-3032) or FAX (703-522-5468).

Introduction to Inductively Coupled Plasma Atomic Emission Spectroscopy. By G. L. Moore. Published by Elsevier Science Publishers. PO Box 221, 1000 AE Amsterdam, The Netherlands, 1989. 340 pp. Price US $92.75/Dfl. 190.00. ISBN 0-444-43029-6.

This book introduces the analytical techniques of atomic emission spectroscopy, outlining the principles, history, and applications. It discusses spectroscopy, excitation sources, inductively coupled plasmas, instrumentation, nebulization, sample dissolution and introduction, accuracy and precision, internal standardization, plasma optimization, line selection and interferences, and inductively coupled plasma mass spectroscopy. Understanding of the material is aided by 128 illustrations, including 11 photographs. References follow each chapter, and an extensive index completes this useful work.

Chromatographic Enantioseparation: Methods and Applications. By Stig G. Allenmark. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1988. 224 pp. Price: $64.95. ISBN 0-470-21080-X.

In recent years, there has been rapid development in chromatographic methods for optical resolution, which has led to a proliferation of the number of applications of such techniques and the potential of an even greater increase in the future. The purpose of this book is to chart the accumulated knowledge of the prerequisite for chiral recognition leading to enantioseparation, and to provide a comprehensive treatment of chiral chromatography, including basic theory and methodology.

Practical HPLC Method Development. By Lloyd R. Snyder, Joseph L. Glajch, and Joseph J. Kirkland. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1988. 260 pp. Price: $45.00. ISBN 0-471-62782-8.

High performance liquid chromatography has been in use since the late 1960s. Since that time, chemists have learned much about the fundamentals of HPLC separations, but the practical application of this knowledge has lagged. This book presents the first systematic, practical scheme for developing effective HPLC separations. For the HPLC user, "Practical HPLC Method Development" simplifies and organizes the somewhat confusing aspects of controlling the many important separation variables into an easily understood pattern of method development. Specific guidelines are presented to permit users to approach a separation problem with a high probability of success, with minimum effort.

Quantitative Gas Chromatography for Laboratory Analyses and On-Line Process Control. By G. Guiochon and C. L. Guillemin. Published by Elsevier Science Publishers, PO Box 221, 1000 AE Amsterdam, The Netherlands, 1988. 798 pp. Price U.S. $165.75/Dfl. 315.00. ISBN 0-444-42857-7.

This book provides a complete discussion of all the problems involved in the achievement of quantitative analysis by gas chromatography, whether in the research laboratory, in the routine analysis laboratory, or in process control. For this reason, the presentation of theoretical concepts has been limited to the essential, while extensive explanations have been devoted to the various steps involved in the derivation of precise and accurate data. This starts with the selection of the instrumentation and column, continues with the choice of optimum experimental conditions, then calibration, and ends with the use of correct procedure for data acquisition and calculations. Finally, there is almost always a way to reduce errors and an entire chapter deals with this single issue. Numerous relevant examples are presented.

Electron Capture Negative Ion Mass Spectra of Environmental Contaminants and Related Compounds. By Elizabeth A. Stemmler and Ronald A. Hites. Published by VCH Publishers, Inc., Suite 909, 220 E 23rd St, New York, NY 10010-4606, 1988. 390 pp. Price: $65.00. ISBN 0-895-73708-6.

This book contains the ECNI mass spectra of 361 compounds selected to include the major classes of environmental contaminants, such as those found on the EPA priority pollutant list. Compound classes of environmental importance include halogenated benzenes and phenols, nitrobenzenes and the related dinitro herbicides, polycyclic aromatic hydrocarbons, DDT derivatives, hexachlorocyclopentadiene pesticides, halogenated biphenyls, dioxins, and dibenzofurans. The spectra are reported graphically. In addition, compound name, Chemical Abstract Service registry number, molecular weight, and structure are given.

Advances in Electrophoresis, Volume 1. Edited by A. Chrambach, M. J. Dunn, and B. J. Radola. Published by VCH Publishers, Inc., Suite 909, 220 E 23rd St, New York, NY 10010-4606, 1988. 441 pp. Price: $110.00. ISBN 0-895-73669-1.

The purpose of this series is to assemble from a wide variety of sources a central review data bank that is available to everyone using electrophoretic methods. It will provide a forum for authoritative voices in each specialized field of electrophoresis and serve to unify research areas whose results are published in a wide range of journals, for example those of the two most challenging substances, proteins and nucleic acids.

Analyis of Carbohydrates by GLC and MS. Edited by Christopher J. Biermann and Gary D. McGinnis. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1988. 304 pp. Price: U.S. $149.95/Outside U.S. $176.00. ISBN 0-8493-6851-0.

This textbook is a comprehensive guide to analysis of carbohydrates by gas-liquid chromatography and mass spectrometry. In addition to explaining the facets of carbohydrate analysis and their relation to each other, the text also contains in-depth reference information useful to practitioners in the field. Improvements in carbohydrate methodology during the past 6 years are also highlighted.

CRC Handbook of Data on Organic Compounds, 2nd Edition. Edited by Robert C. Weast and Jeanette G. Grasselli. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1988. Price: U.S. $2,000.00/Outside U.S. $2,350.00. ISBN 0-8493-0420-2.

The new nine-volume 2nd edition of the "CRC Handbook of Data on Organic Compounds" (HODOC II) is a massive revision of the two-volume 1st edition. With over 25,000 organic compounds covered, HODOC II not only features the most frequently used physical and chemical data but presents for the first time in a single source, extensive spectral data. Additionally, HODOC II provides 21 exhaustive indexes which allow immediate identification of a compound if one or more spectral, physical, and/or chemical properties are known.

Chemical Structure Software for Personal Computers. Edited by Daniel E. Meyer, Wendy A. Warr, and Richard A. Love. Published by American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1988. 107 pp. Price: U.S. and Canada $49.95/Export $59.95. ISBN 0-8412-1538-3.

This unique 107-page volume is a user's guide to software packages that create and use chemical structure diagrams on personal computers. It provides a wealth of practical, up-to-date information on more than 70 software packages for use in chemical word processing, graphics terminal emulation, chemical database management, molecular modeling, and special applications.

Electrochemical Detection Techniques in the Applied Biosciences, Volume 2 Fermentation and Bioprocess Control, Hygiene, and Environmental Sciences. Edited by Guy-Alain Junter. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08875-1272, 1988. 196 pp. Price: $79.95. ISBN 0-470-21227-6.

This is the second volume in a new 2-part investigation into the expanding topic of electrochemical detection techniques (EDTs), showing a wide range of techniques for the main electrochemical sensors. Volume 2 covers the important areas of fermentation and bioprocess control, and hygiene and environmental issues: the work is divided into 2 separate sections, each with its own references. In Part One of this book, the authors show how the main elements of sensor technology have remained unchanged until quite recently, often due to a lack of reliable monitoring devices. They indicate how the supply of reliable devices will encourage the demand and growth of markets, fulfilling a need in the literature for a book that outlines and explains new electrochemical devices, elucidating convention and new instrumentation. Part Two comprises a review of the multiplication, diversification, and accumulation in the environment of solid, liquid, or gaseous waste products which endanger the entire biosphere through the toxic agents they convey. The authors use electrochemical detection techniques for the determination of pollutants in foods and environmental samples, and employ the same techniques for use in conjunction with liquid chromatography.

Kinetic Methods in Analytical Chemistry. By D. Perez-Bendito and M. Silva. Published by John Wiley & Sons, Inc., 1 Wiley Dr, Somerset, NJ 08775-1272, 1988. 330 pp. Price: $74.95. ISBN 0-470-21181-4.

Reaction-rate methods, increasingly important in modern analytical chemistry, rely on recent developments in instrument design and the use of microcomputers. This book fills a gap in the literature, providing comprehensive English language coverage of the evolution of reaction-rate methods, concerning itself with every relevant aspect of kinetic methods, and presents the different methodologies on the basis of appropriate mathematical support. It fulfills 2 functions: a reference tool for researchers, and a guide for those studying the theory and application of reaction-rate methods in analytical chemistry.

UPCOMING AOAC ANNUAL MEETINGS

103rd AOAC Annual International Meeting in St. Louis, Missouri — September 25-28, 1989

104th AOAC Annual International Meeting in New Orleans, Louisiana September 9-13, 1990

Meetings

June 11-13, 1989: Midwest Regional Section Meeting, The Concourse Hotel & Convention Center, Madison, WI. Contact: David Zoromski, Wisconsin Animal Health Laboratory, 6101 Mineral Point Rd, Madison, WI 53705, telephone 608/266-2465.

June 29-30, 1989: Pacific Northwest Regional Section Meeting, Evergreen State College, Olympia, WA. Contact: Steve Pope, Environmental Protection Agency, PO Box 549, Manchester, WA 98353, telephone 206/442-0370.

June 13, 1989: Southeast Regional Section Meeting, University of Georgia, Athens, GA. Contact: Martha Hudak-Roos, National Marine Fisheries Service, 3209 Frederik, Pascagoula, MS 39568-1207, telephone 601/762-7402.

July 11-12, 1989: AOAC Short Course—Quality Assurance for Analytical Laboratories, Arlington, VA. Contact: AOAC Short Courses, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

August 2-7, 1989: 32nd IUPAC Congress, Stockholm. Sweden. Contact: 32nd IUPAC Congress, c/o Stockholm Convention Bureau, PO Box 6911, S-102 39 Stockholm, Sweden, telephone +46 8 23 09 90.

September 23-24, 1989: AOAC Short Course—Improving Sampling for Analysis of Food, Drugs and Agricultural Materials, St. Louis, MO, at the 103rd AOAC Annual International Meeting. Contact: AOAC Short Courses, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

September 23-24, 1989: AOAC Short Course—Laboratory Safety and Health, St. Louis, MO, at the 103rd Annual International Meeting. Contact: AOAC Short Courses, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

September 24-25, 1989: AOAC Short Course—Antibiotics and Drugs in Feeds Workshop, St. Louis, MO, at the 103rd AOAC Annual International Meeting. Contact: AOAC Short Courses, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

September 25-28, 1989: 103rd Annual International Meeting and Exposition. The Clarion Hotel, St. Louis, MO. Contact: Margaret Ridgell, AOAC, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

September 28-29, 1989: AOAC Short Course—Quality Assurance for Analytical Laboratories, St. Louis, MO, at the 103rd AOAC Annual International Meeting. Contact: AOAC Short Courses, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

October 1-4, 1989: International GPC Symposium. Marriott Hotel, Boston (Newton), MA. Contact: Carole Wade-Clark, Waters Chromatography Division of Millipore. 34 Maple St, Milford, MA 01757, telephone 508/478-2000.

October 18-20, 1989: Workshop on Screening Methods for Veterinary Drugs and Natural Contaminants in Food Animal Production. Capitol Hilton, Washington, DC. Contact: Alex MacDonald, Hoffmann-La Roche, Bldg 86, 4th Floor, Nutley, NJ 07110, telephone 201/235-4641.

October 18-21, 1989: 1989 Pacific Conference on Chemistry and Spectroscopy. Pasadena Hilton, Pasadena, CA. Contact: Gordon Bixler, Secretariat, American Chemical Society, 1155 16th St, NW, Washington, DC, 20036, telephone 202/872-4600.

November, 1989: AOAC Central Regional Section Meeting. Contact: Tom Bell, R&D Laboratory, 2331 Sullivant Ave, Columbus, OH 43204, telephone 614/274-6467.

November 13-14, 1989: AOAC Short Course—Quality Assurance for Analytical Laboratories, Arlington, VA. Contact: AOAC Short Courses, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301, telephone 703/522-3032.

April 8-11, 1990: 7th International Symposium on Preparative Chromatography. Gent, Belgium. Contact: M. Verzele, RUG-LOS, Krijgslaan 281 (S4), B-9000 Gent, Belgium, telephone 091-225715.

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

Harvey W. Wiley Awards Fund Contributors

The following members of AOAC have contributed to the Harvey W. Wiley Awards Fund between October 1988 and March 1989: Thomas G. Alexander, Charlie J. Barnes, Ben Borsje, Charlotte A. Brunner, Charles S. Chang, Kathleen K. Cook, Linda English, Michael G. Goergen, Herman Arthur Pierce, and Odette L. Shotwell. The Harvey W. Wiley Awards Fund was established in 1956 to honor Harvey W. Wiley, "Father of the Pure Food Laws" and a founder of AOAC. This fund supports the Harvey W. Wiley Award for the Development of Analytical Methods and the Harvey W. Wiley Scholarship Award. Contributions to sustain the Harvey W. Wiley Awards Fund will be appreciated and should be sent to AOAC.

Interim 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 I—Enzyme-Linked Immunosorbent Assay of Aflatoxins B_1, B_2, and G_1 in Corn, Cottonseed, Peanuts, and Peanut Butter, submitted by M. W. Trucksess, M. E. Stack, S. Nesheim, and A. E. Pohland (Food and Drug Administration, Washington, DC) and D. L. Park (University of Arizona, Tucson, AZ); by the Methods Committee on Residues—Liquid Chromatographic-Atomic Absorption Spectrophotometric Determination of Methyl Mercury in Seafood, submitted by W. Holak (Food and Drug Administration, New York Regional Laboratory, Brooklyn, NY); by the Methods Committee on Microbiology and Extraneous Materials—Hydrophobic Grid Membrane Filter/MUG Method for Total Coliform and *Escherichia coli* Enumeration in Foods, submitted by P. Entis (QA Laboratories, Toronto, Ontario, Canada); and by the Methods Committee on Feeds, Fertilizers, and Related Materials—(*1*) Crude Protein Determination by Generic Combustion Method Based on Current Leco Analyzers, submitted by R. A. Sweeney (Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO), and (*2*) Gas Chromatographic Determination of Nicotine in Environmental Tobacco Smoke, submitted by M. W. Ogden (R. J. Reynolds Tobacco Co., Winston-Salem, NC).

The methods will be submitted for adoption 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.

Correction

J. Assoc. Off. Anal. Chem. (1989) **72,** 197 and 313. Change **46.E03(b)** (*2*) to read: "(*2*) *Sour cream*, . . .Proceed as in (*1*) . . . with 1.0N NaOH . . .". Change **46.E03(b)** (*3*) to read: "(*3*) *Buttermilk.*—Make 1:10 diln (11 g/99 mL diln H_2O). Adjust pH to 6.6-7.2 with 1.0N NaOH (ca 0.1 mL/g sample). Mix well. . .".

Tuberculocidal Activity of Disinfectants

The Board of Directors, in session March 1, 1989, and after due consideration of previously undisclosed information, has ordered that the repeal action taken at the 1988 meeting regarding the Tuberculocidal Activity method, **4.036-4.041**, be set aside pending reconsideration at the 1989 annual meeting.

ISO Standards Published

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

- ISO 729-1988: Oilseeds—Determination of acidity of oils. $18.00.
- ISO 5943-1988: Cheese and processed cheese products—Determination of chloride content—Potentiometric titration method. $14.00.
- ISO 6391-1988: Meat and meat products—Enumeration of *Escherichia coli*—Colony count technique at 44°C using membranes. $20.00.
- ISO 6949-1988: Fruits and vegetables—Principles and techniques of the controlled atomsphere method of storage. $20.00.
- ISO 7558-1988: Guide to the prepacking of fruits and vegetables. $20.00.
- ISO 8197-1988: Milk and milk products—Sampling—Inspection by variables. $20.00.

COMING IN THE NEXT ISSUE

RADIOACTIVITY

- Gamma-Ray Spectroscopic Determination of Iodine-131 and Cesium-137 in Foods: Two Collaborative Studies—*by Edmond J. Baratta and David G. Easterly*

DRUG FORMULATIONS

- Reverse-Phase Liquid Chromatographic Determination of Clioquinol in Cream and Ointment Preparations: Collaborative Study—*by Edward J. Wojtowicz*
- Polarographic Determination of Famotidine in Dosage Forms—*by Juan A. Squella, Gladys Valencia, Igor Lemus, and Luis J. Nuñez-Vergara*
- Determination of Enantiomeric Purity of Tranylcypromine Sulfate by Proton Magnetic Resonance Spectroscopy with Chiral Lanthanide Shift Reagent—*by George M. Hanna and Cesar A. Lau-Cam*
- Determination of Diastereomeric Purity of Tranylcypromine Sulfate by Proton Magnetic Resonance Spectroscopy with Lanthanide Shift Reagent—*by George M. Hanna and Cesar A. Lau-Cam*

MYCOTOXINS

- Visual and Semiquantitative Spectrophotometric ELISA Screening Method for Aflatoxin B_1 in Corn and Peanut Products: Follow-up Collaborative Study—*by Douglas L. Park, Brinton M. Miller, Stanley Nesheim, Mary W. Trucksess, Alan Vekich, Barbara Bidigare, James L. McVey, and Lois H. Brown*
- Simulation of Aflatoxin Testing Plans for Shelled Peanuts in the United States and in the Export Market—*by Thomas B. Whitaker and J. W. Dickens*

ENVIRONMENTAL ANALYSIS

- Evaluation of Modifications to Extraction Procedures Used in Analysis of Environmental Samples from Superfund Sites—*by Cornelius A. Valkenburg, William D. Munslow, and Larry C. Butler*
- Evaluation of EPA Method 8120 for Determination of Chlorinated Hydrocarbons in Environmental Samples—*by Viorica Lopez-Avila, N. S. Dodhiwala, June Milanes, and Werner F. Beckert*

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OVERVIEW OF SAMPLING

Sampling and Sample Preparation for Detection and Quantitation of Natural Toxicants in Food and Feed

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The primary goal of a sampling plan for natural toxins, i.e., mycotoxins and seafood toxins, is to obtain a sample that accurately represents the concentrations of individual components of a given lot. Factors affecting the ability of the sampling plan to accomplish this goal include: (*1*) nature of the analyte of interest; (*2*) distribution of the analyte throughout the lot, (*3*) physical characteristics of the product, (*4*) accessibility of the product to random representative sampling, (*5*) sampling procedure, and (*6*) size of sample. Sampling plans are composed of 3 distinct components: (a) sampling, (b) sample preparation, and (c) analysis. Normally, sampling contributes the largest relative error while analysis comprises the least. Automatic, continuous stream samplers provide the most representative samples for commodities such as nuts, cottonseed, and cereal grains. Good sample preparation equipment is currently available for these commodities; the use of this equipment to obtain a representative test sample is discussed.

A well designed sampling plan will provide, within given limitations, an account of the concentration(s) of specific components/analytes for a specified lot of material. An accurate and precise estimate of the true concentration of a given component in a lot is dependent on at least 3 factors: (a) sampling, (b) sample preparation, and (c) analysis (1). Relative errors associated with these factors are by far the largest for sampling, with analysis playing a relatively minor role (2).

In the development of a sampling plan, one must consider several factors, including the purpose or potential use of the analytical result. For example, will the test result be used as a decision factor in an in-house production scheme, acceptance of a raw ingredient, final acceptance of the product prior to marketing, or as a control sample used by a regulatory agency? In addition, costs associated with taking the sample, sample preparation, and analysis must be factored into the sampling plan.

This discussion will address primarily problems associated with sampling and sample preparation. The primary objective of a sampling plan for a given lot of material is to obtain a sample of that product that accurately represents the concentrations of individual components. Whether a given sample meets this goal is dependent on a variety of factors including: (a) the nature and distribution of the contamination or analyte throughout the lot; (b) the characteristics of the product being sampled, i.e., coarse or finely ground granular material, liquid, paste, etc.; and (c) the procedure used in sampling as well as the accessibility of the product to random representative sampling. Although our discussions will focus on mycotoxins (specifically aflatoxin) and seafood contamination, the theory could apply to other analytes and commodities.

Nature and Distribution of Analyte

Soon after the discovery that aflatoxin could be a major contaminant in feed without visible evidence of mold contamination, the heterogeneous nature of the contamination was recognized. Cucullu et al. (3) reported aflatoxin concentrations ranging from a trace to 1 100 000 μg aflatoxin/kg in individual peanut kernels. Similar findings were observed with Brazil nuts, in which 5% of 100 individual kernels contained aflatoxin at levels ranging from 50 to 25 000 μg/kg (4). Individual cottonseed kernels contained aflatoxin at levels ranging from 150 to 5 750 000 μg/kg (see Table 1). Lee et al. (5) reported extremely heterogeneous distribution of aflatoxin contamination (ranging from 100 to 80 000 μg/kg) among individual kernels of corn on intact ears. Except for contamination with mycotoxins in fluid and paste products such as milk and peanut butter, mycotoxin contamination in animal feeds and human feeds is usually heterogeneous, with a reduction in this heterogeneity observed as the product is reduced in particle size, i.e., ground into flour or made into a paste.

Shellfish and finfish can become toxic through the ingestion of toxigenic, microscopic algae (dinoflagellates), which results in the accumulation of toxins in various edible tissues. Algal toxin contamination in seafoods varies from relatively uniform contamination to highly variable depending on the commodity and the source of the contaminant, i.e., shellfish vs reef fish. Since shellfish are immobile in the environment, and the source of the toxins—dinoflagellate blooms (red tide)—covers a relatively large area, the sampling plan for monitoring paralytic shellfish poisoning (PSP) in molluscs is much easier to develop than for finfish and ciguatera. For monitoring PSP contamination, samples of shellfish are collected along coastal areas and analyzed for toxicity using a mouse bioassay, and the results are a good indication of the contamination levels of the shellfish remaining in the harvesting areas. In the case of ciguatera, however, the toxigenic microscopic dinoflagellates grow in areas such as reefs, and herbivorous fish feed on these organisms, thus becoming toxic. These fish are harvested for food or fall prey to carnivores. The toxins accumulate in the fish tissues and are passed along the food chain. As the toxin passes up the food chain, it becomes concentrated in the larger fish species. The general location of the toxigenic fishing areas is usually well known by local fisherman. However, the larger fish travel over wider ocean areas and can enter fish harvesting areas that are considered safe. Then, a fisherman's catch could contain a small number of highly toxic fish. A sampling plan to be able to monitor this type of contamination must take into account the heterogeneous nature of the catch. Also, the nature or distribution of the contaminant within the individual fish is an important factor to consider. PSP toxins are water-soluble and usually concentrate in the dark (hepatopancreas) gland and are excreted once the dinoflagellate bloom dissipates. Ciguatoxins, on the other hand, are lipid-soluble and are more evenly distributed throughout the fish tissues and appear to remain in the fish over a long period of time.

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Table 1. Evidence of heterogeneous contamination with aflatoxins

Commodity	No. of individual nuts/kernels	Contamination, %	Contamination range, µg/kg	Av. concn, µg/kg	Ref.
Peanuts	visually selected suspect nuts from 2 lots	50	trace–1 100 000	112 000	3
Brazil nuts	100	5	50–25 000	—	4
Cottonseed kernels	771	18	150–5 750 000	—	6
Corn	72 analyses in each of 2 bins	—	0–376 (1st)	21	7
		—	0–332 (2nd)	15	

Physical Characteristics of the Product

Success in obtaining representative samples of food or feed for analysis is directly dependent on the physical characteristics of the product (whole kernel, flour, liquid, whole fish, etc.) and the type of the contamination (PSP, ciguatoxin, mycotoxin). Instructions for sampling most commodities are not available. The variation within a given lot can be extremely large (Table 1), and sampling error is directly proportional to the variation among individual particles in the sample, i.e., the smaller the size of the individual components the smaller the error. The effect of sample size on the variation among test sample aflatoxin concentrations has been determined for peanuts (8), cottonseed (9), and corn (10). The nature of complex food matrices has a significant influence on the analyst's ability to obtain representative samples, particularly at extremely low contaminant levels and/or when the contamination is not homogeneous throughout the lot. It is usually easier to obtain a representative sample of foods that are either liquids, e.g., milk or beer, or made into pastes or powders by grinding, e.g., smooth peanut butter or flour, than bulky foods made up of larger, irregularly shaped components, e.g., grains, whole nuts, mixed feeds, etc.

With respect to the amount of sample needed, an increase in volume usually results in an increase in the reliability of the results, i.e., reduction of the number of good lots rejected and reduction of the number of bad lots accepted. The sample size, however, must remain (a) representative of the lot, (b) manageable for sample preparation and analysis, and (c) cost effective. Table 2 presents sample sizes used by the Food and Drug Administration (FDA) for regulatory control of aflatoxin in foods and feedstuffs (Sample Schedule, Chart 6, in ref. 11). Recent advances in analytical methods, immunochemical assays for the determination of aflatoxins in agricultural commodities (12–15), can pose new problems with respect to sampling procedures. Advantages offered by these assays include simplified extraction procedures, reduced amount of test material required, shortened analytical time, and the capability to routinely screen large numbers of test samples. Any reduction in the sample size used for mycotoxin analyses will increase the relative error associated with this phase of the sampling plan.

Representative Sampling Procedures

The method of collecting a sample is very important. Ideally, to be most effective, equal portions must be taken at random points, throughout the entire lot, with a sufficient number of points sampled. Automatic stream samplers or similar devices are the most effective devices for most agricultural commodities. Care must be taken, however, to keep the amount collected manageable for subsequent preparation of the laboratory sample for analysis. Cross-cut mechanical samplers are commercially available for sampling granular or liquid products. When cross-cut mechanical sampling is not possible, e.g., bulk lots in bins, trucks, piles, etc., probe samples should be taken. Sampling probes currently in use and under evaluation in Arizona for cottonseed products include (a) Prob-O-Vac, with different style probes for short and long staple cottonseed and (b) Shop-Vac, which uses a

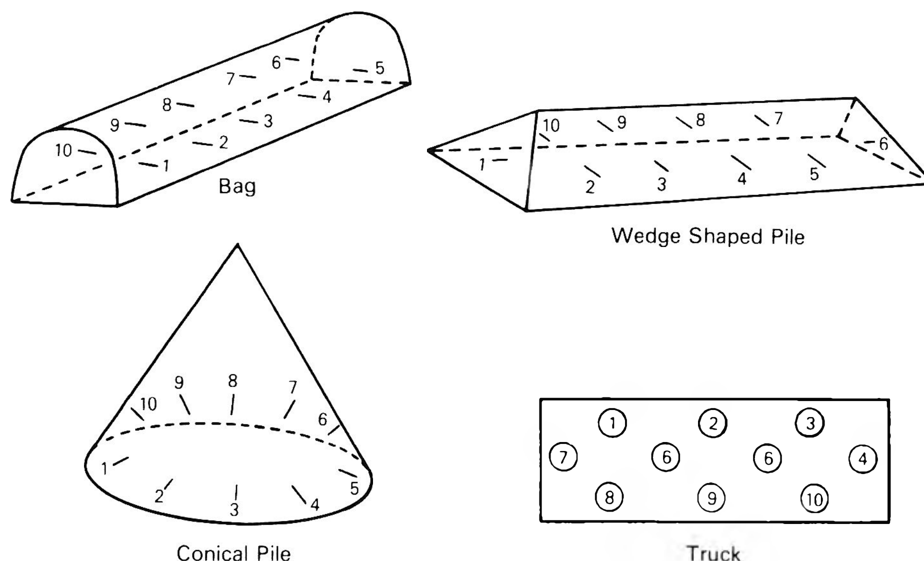

Figure 1. Sampling patterns used for sampling cottonseed for aflatoxin determination (from Arizona Commercial Feed Law, ref. 16).

Table 2. Product sample sizes used by FDA for mycotoxin determination

Product	Package type	Lot size	No. of sample units[a]	Unit size	Total sample size
			(minimum)	(minimum)	(minimum)
Peanut butter,	consumer	NA[b]	24	8 oz	12 lb
smooth	bulk	NA	12	1 lb	12 lb
Peanut butter,	consumer	NA	initial sample		
crunchy,	or bulk		10	1 lb	10 lb
Peanuts, shelled, roasted, or unroasted					
Peanuts, ground			as followup to positive analysis		
for topping			48	1 lb	48 lb
initial sample					
Peanuts, roasted	consumer		initial sample		
in-shell (only	or bulk		15	1 lb	15 lb
for domestic					
runner variety)			as followup to positive analysis		75 lb
			75	1 lb	(composite)
Tree nuts (except	consumer	NA	initial sample		
in-shell Brazil	or bulk		10	1 lb	10 lb
and all pistachio			as followup to positive analysis		
nuts in import			50	1 lb	50 lb
status), in-shell shelled slices, or flour					
Tree nuts, paste			12	1 lb	12 lb
			(minimum)	(minimum)	(minimum)
Brazil nuts, in-	bulk	<200 bags	20	1 lb	20 lb
shell (in import		201–800 bags	40	1 lb	40 lb
status)		801–2,000 bags	60	1 lb	60 lb
			(minimum)		
Pistachio nuts	bulk	≤75,000 lb	20% of units		shelled 25 lb
(in import status)					in-shell 50 lb
			(minimum)		
Pistachio nuts	bulk	75,000–	20% of units		shelled 50 lb
(in import status)		150,000 lb			in-shell 100 lb
Corn, shelled,	consumer	NA	10	1 lb	10 lb
meal, flour, or grits	or bulk				
Cottonseed	bulk	NA	15	4 lb	60 lb
			(minimum)	(minimum)	(minimum)
Oilseed meals, peanut meal, cottonseed meal	bulk	NA	20	1 lb	20 lb
Edible seeds, pumpkin, melon, sesame, etc.[c]	bulk	NA	50	1 lb	50 lb
Ginger root, dried,	bulk	"n" units	n		15 lb
whole, ground	consumer	NA	16	16 × 1 oz	1.0 lb
Milk, whole, low	bulk	NA			10 lb
fat, skim	consumer	NA	10	1 lb	10 lb
Small grains, e.g., sorghum, wheat, barley	bulk	NA	10	1 lb	10 lb
Dried fruit	consumer	NA	50	1 lb	50 lb
e.g., figs	or bulk				
			(minimum)	(minimum)	(minimum)
Mixtures containing commodities susceptible to mycotoxin contamination					
Commodity particles	consumer	NA	50	1 lb	50 lb
relatively large	or bulk		10	1 lb	10 lb
Commodity particles finely ground					

[a] To be collected from random sites in the lot.

[b] NA = Not applicable.

[c] Optional sampling program for seeds or dried fruits with a low incidence of contamination: Take initial 10 × 1 lb sample. If any aflatoxin is detected, resample 50 × 1 lb for a determination of contamination on which to base a regulatory judgment.

Table 3. Sample preparation equipment for mycotoxin analysis of selected commodities

Equipment[a]	Commodity	Sample weight	Subsample weight, g	Comments
A = Dickens-Satterwhite subsampling mill	peanut kernels	22 kg	1100	Use screen with 3.2 mm openings. Solvent-extract entire subsample or make slurry (see Waring blender).
	corn kernels	5 kg[b]	500	Use screen with 3.2 mm openings. Solvent-extract entire subsample or grind entire subsample to pass 1 mm opening and extract 50 g portion (see Wiley mill).
	cottonseed (dehulled)	9 kg[b]	200	Use screen with 1.6 mm openings. See Bauer attrition mill for dehulling process.
B = Hobart vertical cutter mixer (VCM)	pistachio nuts (in-shell)	23–68 kg	200	Grind all samples for about 3 min. Sample weight depends on lot size. Compute aflatoxin concentration based on 100 g kernels in 200 g subsample.
	pistachio kernels	12–34 kg	200	Add equal weight of oyster shells to facilitate grinding. Treat sample same as in-shell nuts.
	Brazil nuts	9–27 kg	200	Treat same as pistachio nuts.
	Brazil nut kernels	5–14 kg	200	Treat same as pistachio kernels.
	almonds (in-shell)	23 kg	100	Treat same as pistachio nuts except subsample will consist of 50–60% kernels, depending on variety.
	almond kernels	14 kg	50	Usually almonds may be ground without addition of oyster shells or other grinding aid. When grinding aid is necessary, subsample weight should compensate for it.
C = Wiley mill (std Model 4)	coarsely ground corn cottonseed, whole	500 g	50	Use screen with 1 mm openings to grind 500 g subsample from Dickens-Satterwhite mill.
D, E, F = Hammer mill (Model 10 with 3 hp motor)	corn kernels peanut meals copra coconut	5 kg	100	Use screen with 1.3 mm openings. Use shop-type vacuum cleaner to collect discharge from mill (W. W. Grainer 8 gal. Shop-Vac Cat. No. 22982). Use Kol Mixal M-58 1/4 to blend ground sample for 3 min, then remove 100 g subsample for analysis.
G = Bauer attrition mill	cottonseed cereal grains peanuts, meal and kernels	18 kg	9000	After treatment, separate kernels from hulls by screening and grind all kernels with Dickens-Satterwhite mill.
H = Waring blender	comminuted peanuts fish products egg products pistachio nuts (with oyster shells)	1100 g	55	See indicated references on production of slurry to provide finer comminution of material and thus justify solvent extraction of smaller quantity of material.
I = Retsch ultracentrifugal mill	raw peanuts, peanut cakes, pellets cottonseed corn, mixed feed	500 g	50	Several different size screens available.
J = Romer mill	corn	2.5 kg	125	Can vary opening in collection chute to vary subsample weight.

[a] Listing of these sources is for information only and does not imply endorsement of these sources over other sources of similar equipment.
A = Federal State Inspection Service, PO Box 3050, Albany, GA 31706.
B = Hobart Corp., World Headquarters, Troy, OH 45347.
C = Arthur H. Thomas, Third and Vine St, Philadelphia, PA 19105.
D = C. S. Bell Co., PO Box 291, Tiffin, OH 44883.
E = W. W. Grainger, Inc., 5959 Howard St, Chicago, IL 60648.
F = ManuFab, Inc., 3737 3rd St, NE, Minneapolis, MN 55421.
G = The Bauer Bros Co., 1717 Sheridan Ave, Springfield, OH 45500.
H = Dynamics Corp. of America, Rt 44, New Hartford, CT 06057.
I = Brinkmann Instruments, Inc., Cantiaque Rd, Westbury, NY 11590.
J = Romer Labs, Inc., PO Box 2095, Washington, MO 63090.
[b] This sample weight provides about the same sampling accuracy as the three 22 kg samples presently used for peanuts in the United States

smaller diameter probe (1 in. vs 3 in.) (J.H. Paulson, Office of Arizona State Chemist, Mesa, AZ, private communication, 1988). Figure 1 shows the sampling patterns used by the State of Arizona for sampling large lots of cottonseed for aflatoxin determination (16). The maximum lot size permitted under this program is 100 tons. For this program all lots must be sampled by taking at least 10 equally spaced probes. All probes must penetrate at least 50 in. (127 cm), picking up approximately 3 lb (1.36 kg) product each time. The total sample must be ≥30 lb (13.6 kg). If, after 10 probe samples are taken, the sample is <30 pounds, it must be discarded and a new set of 10 or more probes must be taken. When the lot to be analyzed consists of bagged material, ideally, samples should be taken while bags are being filled or emptied. When this is not possible, randomly selected bags should be sampled either by taking "grabs" of opened bags or by using small triers or probes. The distribution of the contaminant within an individual bag must be considered.

Preparation of Test Portion

Most of the time, the amount of sample material taken from a lot is more than is required for analysis; therefore, it is necessary to thoroughly mix this material and subdivide it using mechanical dividers or by applying the "quartering" technique to obtain the laboratory sample. A simple shop-built device has been used for subdividing pistachio nuts (17). Large, irregularly shaped products, i.e., grains, nuts, etc., must be reduced in particle size and blended to distribute the analyte as evenly as possible in the product prior to subsampling. For mycotoxin analyses, it is recommended that the degree of size reduction for dry commodities must allow the product to pass a No. 20 sieve.

The next step in the scheme is to prepare the laboratory sample for analysis. Several pieces of equipment used in comminuting and preparing test samples for analysis are listed in Table 3 (18). Comminuted material should be subdivided into analytical test samples using a riffle-type divider or similar device. The Dickens-Satterwhite (19) and Romer (20) mills automatically take test samples of approximately the size needed for analysis. When commodities are comminuted in a Hobart vertical cutter-mixer, a paste is often formed, which is usually sufficiently homogeneous for an analytical sample. The Waring blender is very versatile and can be used for many materials, particularly for preparation of a slurry to provide finer comminution (21-23). In addition, the ultracentrifugal mill has been demonstrated to be applicable to a wide range of uses, from cereal grains to peanut meal pellets and dehulling cottonseed (without screen) (D. L. Park, unpublished data). Chapter 26 in *Official Methods of Analysis* (24) provides an excellent section on sample preparation.

Selected Sampling Plans

The sampling and analysis program for aflatoxins in peanuts in the United States is well documented (8, 25-27) and techniques used in this program can be used for other commodities and analytes. Since there is no practical way of determining the actual ("true") concentration of a given contaminant except by extraction of the entire lot, the sampling plan is a practical means of minimizing the risk to the consumer due to the acceptance of bad lots (2). The plots of these risks (probabilities) vs the concentration of the contaminant is called an operating characteristics curve, which will vary according to a particular sampling plan and will have to be determined for each commodity and analyte. The operating characteristics curve for aflatoxin is presented in Figure 2 (2).

The State of Arizona's sampling program for aflatoxin in cottonseed (16) is designed to handle large volume lots, oftentimes with unusual configurations, that require analysis. The maximum lot size permitted under this program is 100 tons. Should this approach be inadequate, i.e., the lot is too large or inaccessible for the probe to adequately penetrate the commodity and obtain a representative sample of the lot, specific sampling procedures must be developed. Additional sampling plans have been developed which can be used as guidelines including those developed by the Federal Republic of Germany for aflatoxins (1, 28), the U.S. Department of Agriculture for residue testing in meat (29), the Nebraska State Agricultural Laboratory for supplements in animal feeds (M. L. Hasselberger, Nebraska State Agricultural Laboratory, Lincoln, NE, personal communication, 1987), and the well established plans provided by the U.S. Department of Defense for food products (30, 31). Sampling procedures have been developed for monitoring microbial contamination in foods (11, 32) and air and water (33-36). Several international organizations have published instructions on sampling techniques (37-39).

Sampling Plan Costs

Often costs associated with sampling, sample preparation, and analysis are substantial. In addition to the cost of the material analyzed, there are expenses for personnel involved in the collection of the sample, preparation of the test portion, and the analysis itself. Equipment necessary for each of these operations can require a sizeable investment, i.e., mills, blenders, laboratory glassware and instrumentation, etc. If samples are analyzed at a location other than the sampling site, shipping cost of the test material must be considered. The largest potential cost, should the sample not be representative of the lot, is selling or purchasing the product at below

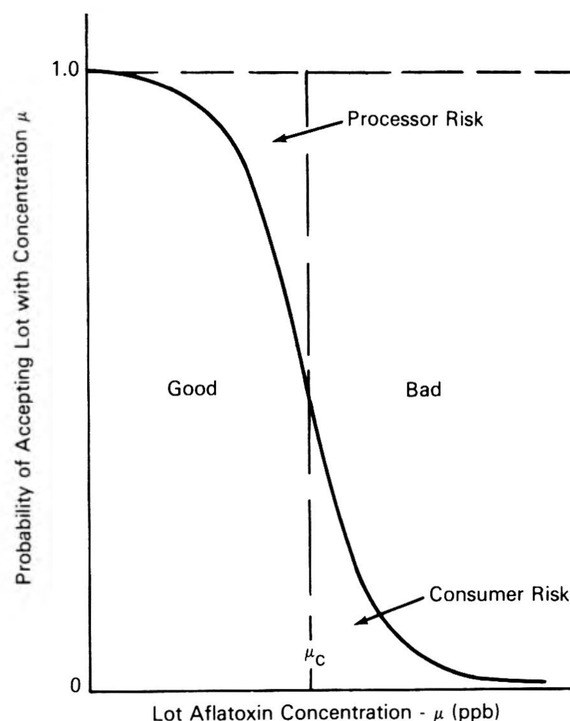

Figure 2. Typical operating characteristics curve for evaluating aflatoxin testing programs. (Reprinted from ref. 2 with permission from the publisher.)

or above the true economic value. This error could be enormous should the product reach the retail market and later be found to be unacceptable by either the consumer, manufacturer, or regulatory agency responsible for that product.

Conclusions/Summary

The diversity in the types of materials to be sampled, analytes of interest, sampling situations, and newer methods of analysis highlight the complexity of preparing sampling plans. More research is required to determine the role that rapid immunochemical methods will play in the analytical scheme and the appropriateness of a smaller sample size with an increase in the number of analyses vs the traditional procedure used. Also, the relative distribution of other analytes in target commodities, if economically important, must be determined in order to develop suitable sampling plans. Sampling plans are available for some commodities and analytes, i.e., aflatoxin contamination in peanut products, and these can be used as a guide for other products and contaminants. With respect to seafoods and seafood toxin contamination, shellfish monitoring programs can be used as a model, provided the characteristics of the contamination are similar. Ciguatera toxin(s) contamination in reef fish, however, highlights the need to do more research to determine the nature and distribution of the toxin in affected fish species before an appropriate sampling/monitoring program can be established. Unfortunately, 100% sampling is not a practical possibility.

Costs can be relatively large and should be considered when developing the plan. The potential cost should be a major consideration in cases where results of the analysis play a role in determining the market value of the commodity. This is irrespective of the actual costs incurred in sampling, sample preparation, and analytical procedures.

Organizations such as AOAC can play lead roles in the establishment of guidelines for sampling and sample preparation procedures. These guidelines should be organized according to commodity, analyte, type of container, and lot size. The sampling procedure should also be able to address (a) the concentration of the analyte based on a representative sample and (b) the degree of variation of the analyte concentrations within the lot being sampled.

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Sampling in the Analytical Scheme

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The general principles of sampling in the analytical scheme, sampling definitions, and sampling planning are considered. Statistical considerations are stressed with attention to sampling by attributes and variables and the use of sampling control charts. Sampling techniques, records and chain-of-custody procedures, sample handling, laboratory sampling, and sample preparation for analysis, as well as reasons and causes of sampling errors, are discussed.

Four basic elements must be considered in solving analytical problems: (*1*) sample collection; (*2*) sample preparation for analysis; (*3*) sample analysis; and (*4*) report preparation. These elements are both independent and system interactive. Sampling, in particular, and sample preparation for analysis are often the least considered steps in solving analytical problems in spite of the fact that they may be the most significant factors and the largest source of error.

Sampling is a complex subject. Quackenbush and Rund (1) recognized that "... sampling presents one of the oldest basic problems in regulatory work. The problem transcends all products with which the AOAC concerns itself: foods, drugs, feeds, fertilizers, pesticides, residues, etc." They then pointed out that "... the basic reason for the problem is heterogeneity (variable distribution of the component of interest within the product)." Certainly heterogeneity is one of the principal reasons, but there are others that may contribute to the problem.

A considerable amount of information on sampling is available in the technical literature: Kratochvil and Taylor (2) cite an extensive list of annotated sampling references. The information may be found under the product or analyte name, or it may be incidental to other problems. Acceptance sampling is extensively covered in many references but is not usually applied to materials investigations. Many studies have also been conducted and published on mycotoxin sampling and sampling plans (3–11), and on microbiological sampling, especially on sampling for determination of *Salmonella* (12–17).

Several national and international consensus standards organizations, such as the American National Standards Institute (ANSI), International Organization for Standardization (ISO), Association of Official Analytical Chemists (AOAC), International Dairy Federation (IDF), and the International Commission on Microbiological Specifications for Foods (ICMSF) of the International Union of Microbiological Societies (IUMS), have published texts or individual commodity monographs or "standards" on sampling. Monograph listings can be found in catalogs of these organizations (18–20).

The Codex Committee on Methods of Analysis and Sampling (CCMAS) of the Codex Alimentarius Commission has, for several years, studied the relationship of a Codex Standard and the method of sampling. As a result of its deliberations, the Committee published "General Principles for the Establishment or Selection of Codex Sampling Procedures" together with "General Principles for the Establishment or Selection of Codex Methods of Analysis" (21).

Sampling Definitions

In general, sampling can be defined as the act or process of selecting a representative portion of material, in some manner, to represent a larger body of material, presumably for testing or analysis. No universally accepted definition for this term or other nomenclature in this area exists. Sampling can mean one thing to a statistician and have some other connotation to a technician collecting a sample, an analyst examining a sample, or an administrator determining if a sample meets the requirements of a law or a contract. Definitions of terms have always been a matter of concern in sampling, and efforts are being made to clarify the situation. For example, the International Union of Pure and Applied Chemistry (IUPAC) has prepared a document "Nomenclature of Sampling in Analytical Chemistry" (22) intended "... to furnish concepts, terms, and definitions in the field of sampling relevant to analytical chemistry and that are generally applicable regardless of what sampling objective, commodity, location, quantity, or form is involved." The Codex Committee on Methods of Analysis and Sampling has also addressed the problem in a paper, "Instructions on Codex Sampling Procedures" (23). The Codex publication is intended to guide Codex Committees in applying the general principles for establishment or selection of Codex sampling procedures. These definitions are, in some instances, different from those of IUPAC, and several terms, not defined by IUPAC, are covered.

Sampling Planning

Most sampling is done for a specific purpose, and the purpose may dictate the nature of the sampling approach. Inspection and sampling are necessary in production, processing, manufacturing, or regulatory control. During the growing season, crops must be inspected and sampled to ascertain pesticide residue levels, insect damage, disease, maturity, and adherence to grade or quality standards or specifications. At food packaging houses, sizing, sorting, and weighing provide desirable and necessary information (40).

For processed food, there is economic sampling of raw materials, correlation of raw product quality with processed product quality, control to ensure adherence to process requirements, and inspection and sampling to determine such factors as fill of container, net weight, and organoleptic and microbiological quality.

For drugs, the quality of raw materials is important. Batch variations must be established, specifications must be defined, and in-line and finished articles must be analyzed for conformance with in-house specifications and regulatory requirements.

Regulatory samples are collected to determine if a commodity is safe for consumption, wholesome, esthetically acceptable, contaminated with another product, contaminated with a deleterious substance, or in compliance with labeling claims or tolerance requirements. Consideration must also be given to the need for representative sampling or selective sampling, homogeneity of the product, budget including sample and travel costs, personnel requirements, suitability of sampling equipment, program objectives, and the level of quality control necessary. All of these factors, and others, must be assessed and brought together in a sampling plan or program.

The IUPAC paper, "Nomenclature of Sampling in Ana-

lytical Chemistry" (22), defines a sampling plan as "a predetermined procedure for the selection, withdrawal, preservation, transportation, and preparation of the portions to be removed from a lot as samples, whereby mathematical treatment of the test values or observations yields an estimate for the concentration of an analyte or for a property determined with a degree of uncertainty at a specific confidence level. Such a plan includes the designation of the numbers, location, and size of the portions, and instructions for the extent of compositing and for reduction (in amount and fineness) of the portions to a laboratory sample and to test portions. It may also contain acceptance criteria." The document then points out, "Some sampling plans do not include more than instructions for the statistical selections of portions to be removed." Other definitions are more or less broad in concept and may include such items as management policy, objectives, and procedures for producing quality information or data on the various commodities to be sampled.

Regardless of the definition, the sampling plan serves several important purposes: It requires thorough consideration of numerous elements before the plan is written; it requires agreement among the persons concerned with the plan; it serves as a reliable reference source; it provides the means for operating on a planned basis; it may be used as a training source; and it can be a foundation for comparison of performance and objectives.

Various approaches to sampling planning are used depending on the purpose of sampling, type and size of organization or agency, management approach, and the autonomy of the units in the organizational structure. A new product manufactured commercially in a complex process may require a sampling planning group composed of production managers, engineers, laboratory representatives, statisticians, quality experts, and others. Usually a plan is developed by a principal investigator in collaboration with others. In some situations, especially when interdisciplinary aspects are involved, the persons responsible for certain operations may each develop a portion of the plan. The separate parts are then coordinated by the principal investigator. A statistician may cooperate with the principal investigator and with each of the other plan participants. In some organizations where strong central control exists, a planning unit may be responsible for all planning. Programs produced in such a unit are likely to be formal, following an established format. Programs tend to be well written and detailed, they use statistical approaches, and performance and results are reviewed with feedback into programs. This approach may stifle the initiative of some sampling personnel and may be resented by others as bureaucratic control. Programs prepared in decentralized units tend to lack uniformity, are less detailed, and may lack attention to statistical and other disciplinary considerations. On the other hand, they may be more practical and acceptable to operating personnel. In either of the above situations, resources and cost constraints may become critical factors in the planning process and may lead to reduced sampling schedules and departures from statistical or other desirable considerations.

Plan format is important because it provides a standard model of the major items that need consideration in formulating the sampling plan. The plan should be a no-nonsense "how to do it" document that establishes the required, approved, and authorized procedures for accomplishing the program's objectives. It should consider the interrogatives of who, what, when, where, why, and how.

The ISO monograph, identified as ISO/TC34, ISO/DIS 7002.2, "Agricultural Food Products—Layout for a Standard Method of Sampling from a Lot" (20), suggests a format that deserves serious consideration. It certainly can serve as a starting point or check list for developing a suitable sampling plan for most commodities. The headings, from sections in the monograph, are as follows:

(1) Title
(2) Introduction
(3) Scope
(4) Field of application
(5) References
(6) Definitions
(7) Principle (of the method of sampling)
(8) Administrative arrangements
  (8.1) Sampling personnel
  (8.2) Representation of parties concerned
  (8.3) Health, safety, and security precautions
  (8.4) Preparation of the sampling report
(9) Identification and inspection of the lot prior to sampling
(10) Sampling equipment and ambient conditions
(11) Sample containers and packing
(12) Sampling procedures
  (12.1) Sample size
  (12.2) Taking of the sample
  (12.3) Preparation of bulk samples and reduced samples
  (12.4) Selection of samples of prepackaged products
(13) Packing, sealing, and marking of samples and sample containers
  (13.1) Filling and sealing of sample containers
  (13.2) Marking
  (13.3) Packing samples for storage and/or transportation
(14) Precautions during storage and transportation of samples
(15) Sampling report
  (15.1) Administrative details
  (15.2) Details of unit packs or enclosure containing the lot
  (15.3) Material sampled
  (15.4) Marking and sealing of samples
(16) Annexes

In the production of a plan, each item in the ISO proposal need not be addressed, but each item requires consideration.

Sampling Methods and Statistical Considerations

In many sampling programs, statistical approaches are not always given the attention they deserve. The old and often used square root sampling method is now being discarded because it has questionable application. Percentage sampling systems that specify a fixed percentage of a lot, say 5 or 10%, do not provide the quality protection often assumed.

Uncertainty is an inherent part of all measurements and evaluations. Sample-to-sample differences are inevitable. Statistical sampling theory provides the means to analyze the relationship between a lot or population and the sample drawn from it. Statistics can be used to estimate population parameters (variance, correlation) from a knowledge of corresponding sample quantities (24). One of the goals of a statistical approach to sampling is to identify causes of variation, to evaluate their significance, and to draw conclusions or make inferences from the sample to the population. Simple and intuitively acceptable statistical techniques, however, are desirable. Presentation of evidence before a court or to a producer that a product is defective or in violation of a law or contract is more convincing if the statistics used are understandable to all persons concerned.

Probability Sampling

A number of sample selection methods are used in survey sampling of food, drugs, and agricultural commodities. These can be grouped into 3 broad types: probability sampling, nonprobability sampling, and bulk sampling.

Probability sampling is used when a representative sample is desired, and the sample is selected in accordance with the principles of statistical sampling and probability. Three ap-

proaches can be used: simple random sampling, stratified random sampling, and systematic sampling. In simple random sampling (24), any portion or any unit has an equal chance of being selected by using a random selection system, e.g., a random number table, a bowl of numbered chips, or a random number die. The method is not the same as selecting units haphazardly. In stratified random sampling (22), the lot is first stratified or subdivided into sub-lots or sub-parts, and a simple random sample is selected from each stratum in accordance with its size relative to the entire lot. The purpose of taking a stratified sample is to obtain a more representative sample than would result from simple random sampling. This procedure can be used, for example, when the lot is known to be made up of different codes of canned goods, and it is desirable to learn about the composition of the lot, as well as individual codes. In systematic sampling (24), units are selected on a systematic basis by selecting the first unit at random and then every 5th or 10th item or some other number thereafter. The spacing can be chosen on the basis of the size of the lot and the predetermined number of units to be taken. Bias can exist in the selection process. Previewing the lot can influence the spacing among units drawn, especially when defective units are obvious.

Nonprobability Sampling

Nonprobability sampling is used when a representative sample cannot be collected or it is not desired to do so. In certain types of adulteration as rodent or insect contamination in a commodity, the objective may be to highlight the adulteration rather than to obtain a representative sample that would dilute the objectionable characteristics of the material. Three general approaches are used in this type of sampling: judgment sampling, convenience sampling, and restricted sampling.

In judgment sampling, the sampler uses personal judgment and experience in selecting units or portions from the lot. The process is non-uniform, and statistical techniques cannot be used to judge the product. In convenience sampling, a sample is chosen on the basis of accessibility, expediency, cost, or other reasons not directly concerned with statistical sampling parameters. In restrictive sampling, a sample is taken of a portion of the lot that is readily accessible, e.g., sampling a carload of grain or fertilizer when the sampler cannot reach certain areas in the car, or sampling cans in a warehouse when it would be difficult to reach cans in the bottom back part of a large stack. In some situations, it might be possible to take a restricted random sample on a portion of the lot.

Bulk Sampling

Bulk sampling (24, 25) poses special problems. This type of sampling involves the taking of a sample in some fashion from a lot of material that does not consist of discrete, identifiable, or constant units. Bulk materials may be gaseous, liquid, or solid in form, may be homogeneous or segregated, and may require sampling in static or dynamic situations. Because of the complex nature of bulk sampling, planning may require the assistance of a statistician. When a sampling plan of this type is designed, such questions as the following must be addressed: How many increments should be taken? What size should they be? Where in the pile or stream should increments be collected? What sampling device should be used? Should a composite be made? How should the sample be reduced to a reasonable size for delivery to the laboratory?

Acceptance Sampling

Acceptance sampling (26) differs from survey sampling. Acceptance sampling involves the application of a predetermined plan to decide whether a lot of goods meets defined criteria of acceptance. The risks of accepting "bad," or rejecting "good" lots are stated in conjunction with one or more parameters, e.g., quality indices of the plan. Statistical plans can be designed to regulate the probabilities of rejecting good lots or accepting bad lots.

The 2 broad categories in acceptance sampling are inspection or sampling by attributes (27), in which the unit of product is classified simply as defective or nondefective, or the number of defects in the unit of product is counted with respect to a given requirement or set of requirements; and inspection or sampling by variables (28), where a specified quantity characteristic or a unit of product is measured on a continuous scale, e.g., pounds, inches, feet per second, and a measure is recorded.

Horwitz et al. (29) provide examples of both attribute and variable sampling plans in a rather concise statement: ". . . in performing an examination for net weight, every unit that weighs one pound or over is accepted and every unit that weighs less than one pound is rejected. When the quality of the lot is determined by the number or fraction of defectives in the lot, the sampling is said to be by 'attributes.' If the actual weights are averaged, a standard deviation [is] calculated, and the acceptability of the lot determined on the basis that the 'average' meets or exceeds the declared weight with no unreasonable shortages as judged by the standard deviation, the sampling is said to be by variables."

Lot Sampling Plans

Single, double, multiple, or sequential sampling plans can be used. In a single sampling plan, a single sample of "n" units is taken and if the number of defectives found is equal to or less than the acceptance number, the lot is accepted. If the number is equal to or more than the rejection number, the lot is rejected. In a double sampling plan, a sample of units is taken and a decision is made to accept, reject, or take a second sample if no decision can be made from the first sample. The cumulative results of the first and second samples are then used to determine the acceptability of the lot. Similar reasoning applies in multiple plans. In sequential sampling (22), units, increments, or samples are taken one at a time or in successive predetermined groups until the cumulative result of their measurements, as assessed against predetermined limits, permits a decision to accept or reject the lot or to continue sampling. The number of determinations may or may not be set in advance.

Operating Characteristic Curves

Operating characteristic (OC) curves are used extensively in acceptance sampling. The OC curve shows the relationship between the quality and percent of lots expected to be acceptable for the quality characteristic inspected. In other words, the OC curve is a graph of lot defectives against the probability that the sampling plan will accept the lot. Figure 1 (24) shows an OC curve for an ideal sampling plan where all lots 3.0% or less defective will be accepted 100% of the time, and all lots more than 3.0% defective will be rejected 100% of the time. Figure 1 also shows an OC curve for an actual sampling plan where rejecting a good lot or accepting a bad lot is a risk.

United States Military Standard—105D (MIL-STD-105D), "Sampling Procedures and Tables for Inspection by

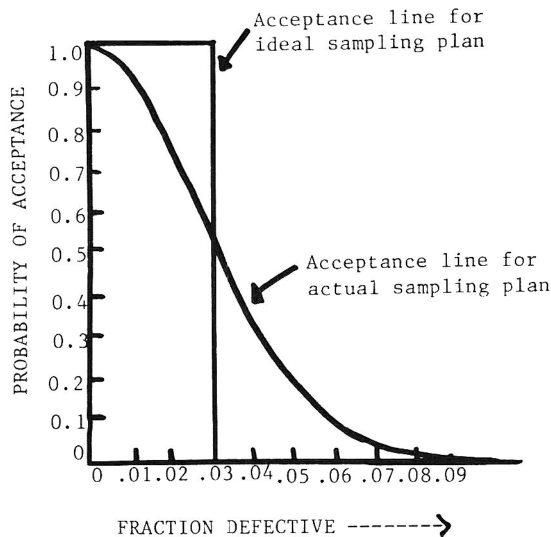

Figure 1. OC curves for ideal and actual sampling plan.

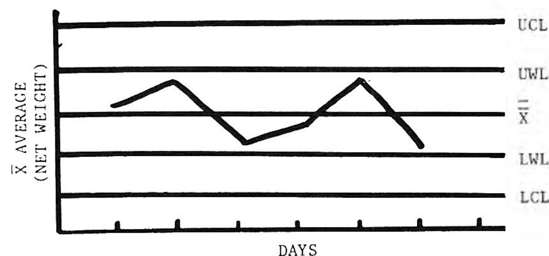

Figure 2. $\bar{X}$-chart for net weight.

Attributes" (27), has gained international recognition as a "standard" for inspection or sampling by attributes. It contains a variety of tables and operating characteristic curves. It provides 7 inspection levels covering varying levels of discrimination, i.e., "tightness" or "steepness" of OC curves and 3 levels of inspection in terms of severity of inspection, i.e., "normal," "tightened," and "reduced." The usual procedure is to start with a normal inspection plan and, if the quality of the lot is shown to be poor, the inspector or sample collector switches to a tightened level plan. If the quality is shown to be good, a reduced level plan is used. The sampling plans in MIL-STD-105D are applicable, but not limited to, end items (finished products), components and raw materials, operations, supplies in storage, maintenance operations, data records, and administrative procedures. The plans are intended primarily for sampling continuing series of lots or batches, but they can be used on single lots or batches.

MIL-STD-414, "Sampling Procedures and Tables for Inspection by Variables for Percent Defective" (28), is also universally accepted for examining lots by variables. It presents curves for a variety of plans, but the plans do not match MIL-STD-105D.

Both MIL-STD-105D and MIL-STD-414 standards should be consulted before an attributes or variables plan is initiated. This can save a great deal of development time. If a suitable plan cannot be found in MIL-STD-105D to fit a particular situation, one can be prepared. Wiesen (26) and Puri (24) suggest procedures that can be followed.

A number of published sampling plans have special application for lot-by-lot sampling or inspection in attribute sampling. They can be applied when rejected lots can be 100% inspected, when production is continuous and inspection is not destructive, or when quality level is high and inspection is costly or destructive. An extensive discussion of these plans and others is made by Wiesen in the *Quality Control Handbook* (26).

Control Charts

Control charts are a useful tool in tracking quality control efforts in process sampling and end product sampling, as well as in laboratory measurements. These charts provide the means to display data in a form that compares variability of test results with the average or expected variability of small groups of data (30, 31). The results of tests are plotted on the vertical axis of the chart in units of the test results against time on the horizontal axis in hours or days. Each chart can provide a means to determine trends and lack of randomness, and can trigger timely diagnosis and feedback for appropriate action to correct unsatisfactory conditions. Control limits (UCL (upper), LCL (lower)) are often set at ± 3 standard deviations, and warning limits (UWL, LWL) are often set at ± 2 standard deviations. Standard deviations of ± 2 in a normally distributed variable will provide a distribution range of 95.5%, and standard deviations of ± 3 will provide a distribution range of 99.7%. Figure 2 shows a variable control chart for mean: X-chart for net weight with upper and lower warning and control limits.

Sampling control charts (24), are generally classified in 2 main groups: control charts for attributes and control charts for variables. Attribute control charts are used to plot fraction defective (p-chart), number defective (np-chart), and number of defects (c-chart). Variable control charts are used to plot mean (X-chart), range (R-chart), and standard deviation (sigma-chart).

Sampling Techniques

Every material to be sampled presents a challenge. It is not possible to provide 100% confidence that any given sample, even when taken in conformance with a sound statistical sampling plan, will represent the lot being sampled. Although random sampling is the most reliable approach, in reality, implementation of the sampling plan depends in some measure on the skill and expertise of the sample collector.

Samples are useful for their intended purpose when they are taken in a manner consistent with generally recognized good sampling techniques or good sampling practices. Sound sampling technique means more than just collecting a representative sample (32). It calls for: (*1*) inspecting lots prior to sampling; (*2*) accurate reporting of the condition of the lot at the time of sampling; (*3*) maintaining sample integrity; (*4*) using sampling devices that have been found to be suitable for the particular commodity; (*5*) taking adequate precautions in packing and delivering the sample to the analyzing laboratory; and (*6*) use of suitable containers and adequate preservation of the commodity if it is not prepackaged.

A number of procedures or guidelines should be followed, especially in finished product and/or regulatory sampling (32):

(*1*) A predetermined sampling plan is followed in sampling.

(*2*) As a general rule, sample collection is entrusted only to trained personnel.

(*3*) Samples are taken from original, previously unopened shipping cases or containers.

(*4*) Frozen product samples are kept frozen, and refrigerated product samples are "iced" prior to delivery to the laboratory.

(*5*) Representative sampling is used unless there is justification to do otherwise.

(*6*) Lots are restored to original conditions, and not allowed to be slack-filled or short weight.

(*7*) Care is exercised to avoid contamination or cross contamination of lots from the use of unclean or non-sterilized equipment.

(*8*) If dealer balances or scales are used in weight determinations, they are checked for accuracy and sensitivity.

(*9*) Plastic bags are avoided as containers for foodstuffs to be examined for pesticide residues.

(*10*) Bulk drugs in tablet, capsule, or powder form are placed in tight, light-resistant containers to avoid adverse effects of moisture and light.

(*11*) A suitable label is affixed to each case or bulk container showing that a sample was taken (some warehousers or wholesalers may object to this practice).

(*12*) A duplicate or photographic copy of labels on containers is submitted as part of the collection record.

Sampling Equipment

A variety of commercially available devices and powered equipment can be used for sampling commodities in the plant and in commercial channels. The physical form of the product, and its state of homogeneity, can affect the choice of the sampling device. A number of firms around the world manufacture devices that range from disposable plastic tubes to hand-held probes (triers) and pneumatic samplers.

Bin and bag cereal probes are double tube brass or stainless steel, with various diameters, lengths, and numbers of openings. Special purpose triers for sampling seeds, rice, or nuts can be used. These are sharp-pointed, hollow probes of lengths from 5 to 13 in. and diameters from $1/2$ to $1\,1/2$ in. The sharp point is used to puncture bags with minimum damage, and the sampled product is allowed to pour through the hollow tube into the sample receptacle.

For grain sampling, long partition type probes with separate compartments can be used. The sample is emptied from the probe onto a sampling canvas for inspection of grain quality in each of the compartments (33).

Butter and cheese samplers are smooth and tapering with a sharp end and edge to cut through the product. Power drilling augers and cylindrical hollow probes are available with a saw-tooth end for sampling frozen foods.

As a last resort, a sample collector can utilize the "grab" method to sample dry products that are boxed, bagged, or in piles by grabbing handfuls of product. This technique can be justified when the product is homogeneous, but few products are. The procedure is questionable at best, but it may be the only choice possible in some situations.

Records

Attainment of quality in sampling requires a system of creating, using, storing, and disposing of records. Records are essential in establishing facts about who, what, when, where, and how. For quality assurance purposes, or when litigation is a possibility as a result of sampling, then chain-of-custody procedures (34) must be followed. Chain-of-custody can be defined as procedures and traceable records that demonstrate an unbroken control over or custody of a sample from collection through its final disposition. The chain-of-custody concept establishes that the sample came from the source at which it was collected. It also verifies that the sample was not contaminated, altered, or tampered with during collection, handling, shipment, and analysis.

All persons in the analytical system need to understand the importance of records and why each record must reconcile with every other with no disagreements. Management must provide the system, arrange for safe storage, and dispose of samples and records when their retention is no longer necessary. For each sample, records to be maintained should include, but not be limited, to the following: (*1*) project plan or program; (*2*) sample collection assignment (if assignments are prepared); (*3*) sample collection report; (*4*) field examination report or field measurements; (*5*) carrier bills, invoices, post office receipts, and so forth; (*6*) sample identification tags or stickers; (*7*) seals; (*8*) laboratory accountability record; (*9*) analytical report (in notebook or on work sheet); (*10*) photographs, sample label and labeling, instrument charts, and so forth; (*11*) action papers (report to customer, legal action papers, and so forth); (*12*) sample disposition record.

Sample Preservation, Preparation, and Transport

It is axiomatic that a sample is not worth testing or analyzing unless it arrives at the laboratory in "good order" (1). Ideally, a sample should be examined promptly after collection, but rarely is this possible. Therefore, precautions are required to avoid or prevent sample change in composition before analysis. This then calls for the use of suitable containers to hold the sample and to protect it from moisture loss or gain, oxidation, contamination, decomposition, or breakage during shipment.

Some foods are perishable, while other foods, as well as drugs and agricultural commodities, have the potential to deteriorate if they are not preserved or held under conditions to slow the process. Many foods and drugs have substances added to them during production to preserve or to extend their shelf lives. Generally these latter products do not need further preservation, but they might need some temperature control. Acetic acid, ascorbic acid, propionic acid, or sorbic acid and some of their salts, as well as sulfur dioxide, sodium sulfite, alcohol, and formaldehyde are commonly used to preserve certain types of samples after collection (32). When samples are treated with these chemicals, containers must be clearly labeled to show their presence. Obviously, if a preservative is likely to interfere with an analysis, it is not to be used.

Samples collected for insect infestation, or suspected of such adulteration, need to be fumigated immediately to stop the continued increase in the infestation. Chloroform is the fumigant of choice; paradichlorobenzene can also be used. If it is necessary to show the presence of "live" infestation, the addition of a fumigant is contraindicated. Refrigeration (icing) and freezing (dry ice) are suitable preservative procedures for commodities that cannot or should not be preserved or fumigated by chemical means.

Aseptic sampling and sample preservation require special techniques and specially treated containers and sampling equipment. Whenever possible, unopened containers are collected. When bulk containers are encountered, samples must be drawn aseptically and packaged and handled under conditions that will prevent multiplication or undue reduction in the bacteriological population. Equipment and containers used must be clean, sterilized, and of suitable material and size. Samples require prompt delivery to the laboratory with original storage conditions maintained as closely as possible.

Special packaging and labeling of packages are necessary to protect samples and personnel from contact with dry ice or from released carbon dioxide. Adequate quantities of icing

materials and dry ice must be used to provide adequate preservation for the time it takes for the sample to be delivered to the laboratory. Compliance with shipping regulations must be observed. Suitable storage is also required once the sample reaches the laboratory. Even during what appears to be proper storage, especially during long-time storage, changes in composition of some constituents can occur. Experimentation may be indicated in some situations to determine the extent of change. Sometimes a control sample can be fortified or spiked to assist in deciding on the extent of change (29).

Laboratory Sampling and Sample Preparation for Analysis

Almost without exception, the sample delivered to the laboratory is greater in quantity than the amount required for analysis, and it is likely to require treatment to achieve some degree of homogeneity. In both cases, some care is necessary during sample preparation to prevent changes in composition.

Another problem occurs when more than one unit is submitted. The question that must be resolved is whether a composite should be made or individual units should be analyzed. Compositing can best be justified when homogeneity is not a significant problem. Compositing saves analytical time, but it does not establish the variability of the sample or the parent lot. If the total number of determinations must be fixed, multiple independent units are preferred over replicate aliquots from a single sample. If only a single analysis is possible, a composite sample is indicated over a single random sample (35). Other factors that influence sampling for analysis and the procedure used in sample preparation are nature of the analyte of interest, distribution of the analyte, chemical and physical characteristics, size, and homogeneity or heterogeneity.

Reduction in volume of a dry particulate material can be achieved by coning and quartering, or rolling and quartering, or by use of a splitter, such as a riffle (36). A variety of implements and machines are available for sample disintegration, such as mills, grinders, and cutters. Care in their use is desirable to prevent loss of dust and change in product composition through the partial separation of components. Screening can be used to improve the efficiency of disintegration and to attain homogeneity. Loss of moisture during manipulations can be minimized by keeping samples covered with plastic or aluminum foil. Pick up of moisture by cold products is avoided by allowing samples to reach room temperature before preparation begins.

As a general guide, food samples should be analyzed in the form in which they are commonly consumed (29). Inedible portions, such as peel, nut shells, and fish bones should be removed and discarded prior to analysis, and suitable notes and quantitative data should be recorded on how the sample is prepared.

Trace metals analyses present problems, e.g., metals distribution can be unequal between liquid and solid phases in canned vegetables and canned fruits. Most people do not consume the vegetable brine, but the liquid portion of the canned fruits is generally eaten. Obviously, this irregular distribution of metals can pose problems for the analyst in establishing the level of the metal residue in the product, and for administrators concerned with setting tolerances for metals in canned foods (37).

One of the most difficult sampling situations for lot and laboratory sub-sampling is encountered in trying to obtain a representative sample for aflatoxin analysis in raw agricultural commodities. Aflatoxin contamination has a highly erratic distribution. The contamination is heterogeneous, with a reduction in heterogeneity as the food or feed is reduced in particle size. In early studies of the occurrence of aflatoxin in agricultural products, the sampling and subsampling errors were shown to be as high as 90% of the total analytical scheme (38). After recognizing the high level of variability between and within lots, researchers began to use larger and larger samples in an effort to improve results. For peanuts, the sample size started at 1 kg, and the size increased when more reliable results were insisted upon by food producers who were aware that increasing sample size reduced the number of good lots rejected and the number of bad lots accepted.

At the present time in the United States, the sample taken from a lot of shelled peanuts is 144 lb—three 48 lb samples—with portions taken at random from the lot. In the laboratory, the sample is examined by sequential analysis with the first 48 lb sample ground in a sub-sampling mill and test portions examined in duplicate. If the average of the test portions is below the established tolerance (set by the Food and Drug Administration), the lot is passed. If the average is above the acceptance level, the lot is rejected. If the finding falls between the 2 figures, the second 48 lb sample is comminuted, and the analysis is repeated. A decision is then made on the combined findings. If a decision cannot be made to accept or reject the lot, the third 48 lb sample is prepared and assayed, and the cumulative figures are used as the basis for acceptance or rejection. For a finely comminuted bulk lot of smooth peanut butter, the sample size is much reduced. Generally, 12 sample units of 1 lb each are taken at random and submitted to the laboratory for analysis.

The example of aflatoxin just cited points out dramatically the need for attention to lot sampling and laboratory subsampling. While this is an extreme case, the necessity for attention to sampling cannot be ignored or treated indifferently. A good quality control program can help to reduce sampling uncertainty.

Sampling Errors

Sampling errors may occur for a variety of reasons and causes. Errors can be minimized or controlled if planners and operating personnel understand the causes and sources of error. This calls for appropriate attention to such factors as (39): (*1*) sampling source (site, time, shipment identification); (*2*) method of sampling (technique and equipment); (*3*) sample preservation (use of preservatives, temperature control); (*4*) sample preparation (identification, proper containers, packing); (*5*) storage of samples (prior to and following analysis); (*6*) personnel errors (inadequate training, carelessness); (*7*) sample preparation for analysis (size reduction, homogeneity); (*8*) records (chain-of-custody, carelessness, loss).

Conclusion

Many laboratories consider sampling beyond their control. This attitude must change. It can change if laboratory management and analysts understand the complexity of the sampling process and then exert their special knowledge to influence and improve all aspects of sampling.

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ALCOHOLIC BEVERAGES

Spectrofluorometric Determination of Histamine in Wines and Other Alcoholic Beverages

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The spectrofluorometric determination of histamine in wines, other alcoholic beverages, and vinegars is described. Histamine is extracted with *n*-butanol, transferred to hydrochloric acid, and subjected to a condensation reaction with *o*-phthalaldehyde (OPT). The method was tested for sensitivity (0.03 ppm limit of detection and 0.08 ppm limit of determination), precision (6.4% CV for a content of 1.25 ppm and 19.5% CV for a content of 0.25 ppm), accuracy (97.1%), recovery (90.6–96.9%), and lack of interference by histidine. The method can be applied to wine, must, beer, champagne, cider, vermouth, and vinegar with satisfactory results.

Histamine is a biogenic amine found in many animal and plant tissues as well as in various foods and beverages. It is generally accepted that histamine presence in food products is due to microbial activity on its precursor amino acid, histidine. This activity may be related to the fermentation processes involved in food production or to processes of deterioration and spoilage.

In wines, a relationship has been established between histamine appearance and the yeasts used in the alcoholic fermentation, between histamine and malo-lactic fermentation bacteria (1–3), and even between histamine and contaminating microorganisms (3, 4), basically *Enterobacteriaceae* (Klebsiella and Proteus).

The study of histamine in wines is interesting from both a technological and a toxicological point of view. From a technologic viewpoint, it should be noted that certain authors have related high levels of biogenic amines, in particular, histamine, to a low quality index of the product or to a defective elaboration (5, 6). The toxicological interest in histamine in foods is based on the development of direct toxicological problems such as "histamine poisoning" (7–9), and indirectly related problems such as interaction with monoamine oxidase-inhibiting drugs (MAOI) (10, 11), interaction with alcohol (1, 11), and interaction with other nonvolatile amines (1, 6, 12). An increase in the toxic effect of histamine has been noted in all of these interactions.

As a result, some national governments have implemented legal regulations or, at least, recommended a maximum histamine content of certain foods. These recommendations apply to fish and marine products in which the content of this amine is used as an index of microbial spoilage (7–9). Certain recommendations for wines also exist.

Therefore, it is important to know the biogenic amines content of food in general and, in particular, the histamine content of wines, considering that it is a widely consumed product in many countries and histamine content may be regulated in the future.

To date, a wide range of methods for the identification and/or analytical determination of histamine in foods has been described. For wine, the number of methods is lower: biological methods (4), thin-layer chromatographic methods (1, 5), spectrofluorometric methods (3, 13, 14), and liquid chromatographic methods (6, 15, 16) are available.

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In the present paper, we propose a method for the spectrofluorometric determination of histamine, based on previous techniques (17, 18) which have been modified for wines, other alcoholic beverages, and vinegars. The amine is isolated by liquid-liquid extraction in separatory funnels, thus eliminating the need for ion-exchange resins or centrifugation. The proposed method is simple, relatively rapid, and low-cost. Data are provided in support of its performance in terms of sensitivity (detection and determination limits), precision, recovery, accuracy, and lack of interference by histidine.

METHOD

Apparatus

(a) *Spectrofluorometer.*—Kontron SFM-25, with Kontron 800 printer/plotter.

(b) *Test-tube shaker.*—Heidolph-reax 2000.

(c) *pH meter.*—Crison digit 501.

Reagents

(a) *o-Phthalaldehyde (OPT) solution.*—Merck, 1% (w/v) in methanol.

(b) *Histamine standard solutions.*—(*1*) *Stock solution*: 1 mg/mL (1000 ppm) as free base. Accurately weigh 165.6 mg histamine dichloride (Merck) into 100 mL volumetric flask and dissolve and dilute to volume with 0.1N HCl. (*2*) *Intermediate solution*: 30 ppm. Pipet 3 mL stock solution into 100 mL flask and dilute to volume with 0.1N HCl. (*3*) *Working solutions*: Dilute intermediate solution to 0.5, 1.0, 1.5, 2.0, and 15 ppm (w/v).

Standard Wine

Some analytical tests were carried out using a simulated and synthetic wine of known composition relatively similar to the composition of authentic wine. The standard wine was prepared on the basis of data available on the composition of Spanish wines (19), as follows: 127 mL ethanol, 8.02 g glycerol, 1.85 g glucose, 3 g tartaric acid, 1.35 g citric acid, 0.5 g acetic acid, 1 g succinic acid, 170 mg *o*-phosphoric acid, 1.80 g tannic acid, 0.3 mL acetaldehyde, 10 mL anthocyanine grape extract (Enocolor Liquid, EEC-163/Reggiana Enocianina), and histidine and histamine required in each case. The mixture was diluted to 1000 mL with water and adjusted to pH 3.5 with NaOH.

Extraction and Separation

Pipet 5 mL wine into 250 mL separatory funnel and add 10 mL 0.4N $HClO_4$, ca 8 mL 1N NaOH (to obtain pH 12–13), and ca 4 g NaCl (to saturation). Extract 5 times with successive 25–30 mL portions of *n*-butanol. Shake each extraction 2 min. Collect all butanolic phases in a second separatory funnel and wash with 10 mL 1N NaOH saturated with NaCl. Remove aqueous phase. Add 25 mL petroleum ether to butanolic phase, shake, and let separate. Remove liberated

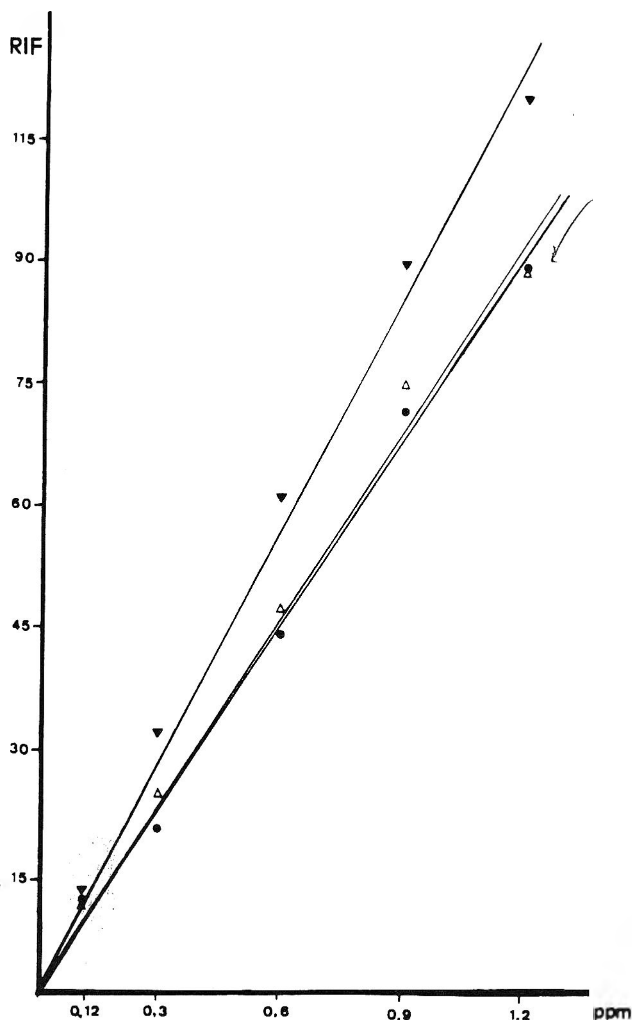

Figure 1. Graphic representation of matrix interferences in relative intensity fluorescence of histamine. ▼——▼ in 0.1N HCl; Δ——Δ in acidic extract from water; ●——● in acidic extract from standard wine without histamine.

aqueous phase. Extract histamine from butanolic phase in separatory funnel with 5 portions of 10 mL 0.1N HCl. Collect aqueous-acid phases in 50 mL volumetric flask. Add 30–40 mL petroleum ether to butanolic phase and add liberated aqueous phase to 50 mL volumetric flask. Adjust aqueous extract to 50 mL with 0.1N HCl.

Preparation of Solutions for Calibration Curve

Into five 5 mL volumetric flasks, pipet 1 mL 0.1N HCl, 1 mL standard solution containing 0.5 ppm histamine, 1 mL standard solution containing 1.0 ppm histamine, 1 mL standard solution containing 1.5 ppm histamine, and 1 mL standard solution containing 2 ppm histamine. Dilute each to 5 mL with aqueous-acid extract obtained from wine.

Condensation Reaction

Transfer 2 mL of each calibration solution to test tubes. Add 4 mL 0.1N NaOH and shake. Add 0.2 mL OPT solution immediately, mix thoroughly, and let stand 5 min for reaction. Add 2 mL 0.2M citric acid to each tube and shake.

Spectrofluorometric Determination

Record fluorescence intensity using excitation wavelength of 340 nm and emission wavelength of 425 nm. Set instrument to zero with 0.1N HCl blank, to which all the reagents used in condensation reaction with OPT have been added.

Table 1. Precision of method for determination of histamine content of authentic wines

Detn	Histamine content, ppm	
	White wine	Red wine
1	0.30	1.25
2	0.25	1.15
3	0.25	1.35
4	0.35	1.25
5	0.20	1.30
6	0.30	1.35
7	0.20	1.15
8	0.25	1.35
9	0.30	1.15
10	0.20	1.25
$\bar{X}$	0.25	1.25
SD (n − 1)	0.05	0.08
CV, %	19.5	6.4
CL (95%)	0.25 ± 0.04	1.25 ± 0.06

Also, a qualitative verification is done by comparing excitation and emission spectra with those of histamine standard solution.

Calculations

Calculate histamine content of wine from intercept of calibration curve on horizontal axis. Point of intersection corresponds to concentration of histamine (C_1) in 5 mL of final solution, without added histamine (4 mL aqueous acid extract and 1 mL 0.1N HCl). Calculate concentration of histamine in wine (C) from calibration curve as follows:

$$C, \text{ppm} = (C_1 \times 50)/4$$

Results and Discussion

Because of interferences, the histamine content in wine must be calculated by means of calibration curves obtained

Table 2. Accuracy of method applied to standard wine

Histamine content, ppm	Histamine found, ppm		Accuracy, %		Average, % ± SD (% CV)
0.30	0.25	0.27	83.5	90.0	96.2 ± 9.5
	0.29	0.28	96.7	93.5	(9.87)
	0.31	0.33	103.0	110.0	
0.80	0.81	0.77	101.2	96.2	99.5 ± 3.7
	0.83	0.77	103.7	96.2	(3.75)
1.60	1.40	1.38	87.5	86.2	91.8 ± 9.6
	1.70	1.40	106.2	87.5	(10.4)
2.40	2.30	2.40	100.0	95.8	94.4 ± 5.2
	2.26	2.10	94.2	87.5	(5.5)
4.00	3.50	3.95	87.5	98.7	94.4 ± 5.4
	3.71	3.95	92.7	98.7	(5.7)
8.00	7.15	8.30	103.7	89.4	94.5 ± 5.7
	7.15	7.70	89.4	96.2	(6.0)
12.00	12.50	11.60	107.5	96.7	101.0 ± 4.6
	11.70	12.50	104.2	97.5	(4.6)
20.00	21.50	20.40	107.5	102.0	101.6 ± 6.5
	20.90	18.50	104.5	92.5	(6.4)
40.00	42.10		105.2		102.5 ± 5.2
	41.30		103.2		(5.1)
Overall accuracy, %			97.1		
SD (n − 1)			7.0		
CV, %			7.2		

Variance analysis: $F_{(exp)} = 1.13$ $F_{(tab)} = 3.23$ ($P = 0.05$; DF = 8; 27)

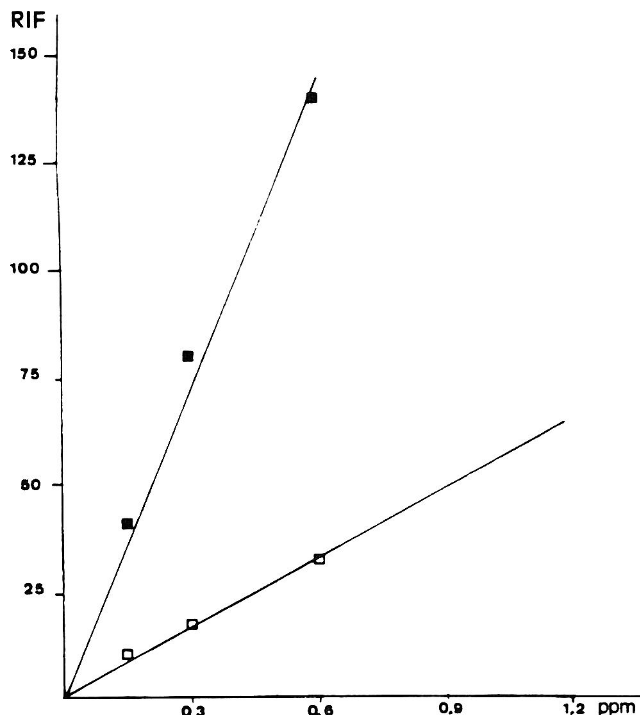

Figure 2. Relative intensity fluorescence of histidine and histamine complexes with OPT. ■——■ Histamine ($y = 231.8x + 4.4$; $r = 0.9966$; $P < 0.001$); □——□ histidine ($y = 52.38x + 1.0$; $r = 0.9976$; $P < 0.001$).

by adding histamine standard solutions to the final extract of the test wine. The interference was verified by comparing relative intensity fluorescence (RIF) obtained from standard solutions of histamine in 0.1N HCl, histamine dissolved in the acidic solution obtained by extraction from water, and histamine dissolved in the acidic extract of a standard wine without histamine. Figure 1 shows that RIF values of histamine standard solutions dissolved in HCl are higher than RIF values for the same concentration of histamine in the final solutions extracted from water and/or standard wine without histamine. Apparently, interferences in the matrix diminish the fluorescence of the OPT-histamine complex.

The correlation between histamine concentration and RIF value is acceptable in the concentration range 0 to 1.5 ppm ($r = 0.9994$; $P < 0.001$). The fluorescent histamine-OPT complex is stable at least 30 min.

Table 3. Recovery of method applied to authentic wines

	Recovery, %	
Detn	White wine[a]	Red wine[a]
1	89.3	97.0
2	90.0	95.7
3	90.0	96.8
4	90.0	90.1
5	99.3	90.1
6	89.3	97.6
7	89.3	98.1
8	92.0	93.3
9	91.3	100.3
10	90.7	94.3
$\bar{X}$, %	91.1	95.3
SD (n − 1)	3.01	3.37
CV, %	3.3	3.5

Variance analysis: $F_{(exp)} = 3.65$ $F_{(tab)} = 4.41$ ($P = 0.05$; DF = 1; 18)

[a] Histamine content was 0.25 ± 0.05 ppm for white wine and 1.25 ± 0.08 ppm for red wine.

Table 4. Recovery of method for authentic wines with different histamine contents

	White wines		Rosé wines		Red wines	
Sample	ppm	%	ppm	%	ppm	%
1	0.20	88.0	0.20	88.0	0.40	90.5
2	0.30	91.5	0.25	101.1	0.45	101.0
3	0.40	98.5	0.30	85.0	0.75	88.5
4	0.55	89.0	0.40	96.0	1.32	97.0
5	0.70	95.5	0.50	88.5	2.65	98.0
6	1.15	84.5	0.50	87.0	3.80	92.0
7	2.60	96.5	1.25	92.0	4.30	87.0
8	2.75	98.0	1.60	90.0	6.00	92.5
9	4.60	93.5	3.20	93.0	12.50	97.5
10	4.80	90.0	4.00	85.5	12.90	93.0
$\bar{X}$, %	92.5		90.6		93.7	
SD (n − 1)	4.7		5.0		4.5	
CV, %	5.0		5.6		4.8	

To determine the sensitivity of the method, the detection limit (DL) and the determination limit (DtL) were calculated (20). Ten extractions from standard wine without histamine and the corresponding reactions with OPT were carried out, and the results were calculated. The DL was 0.03 ppm and the DtL was 0.08 ppm. We tested this DtL by applying the method to a standard wine with 0.1 ppm histamine. For 3 determinations, the average content was 0.11 ppm, with a standard deviation, $S_{n-1} = 0.03$. This corresponds to an accuracy of 110 ± 30% which, according to the FDA (U.S. Food and Drug Administration) criteria set out by Horwitz (21), is an acceptable level. The excitation and emission spectra obtained coincided qualitatively with those of histamine.

To examine method precision, 10 determinations of histamine in an authentic white wine and 10 in an authentic red wine were carried out. The determinations were performed on successive days but with the same reagents and apparatus. The results obtained from each determination are shown in Table 1. The coefficients of variation were acceptable, according to the Horwitz formula (21), for both wines.

The accuracy of the method was tested using standard wines with 9 different but known concentrations of histamine: 0.3, 0.8, 1.6, 2.4, 4.0, 8.0, 12.0, 20, and 40 ppm. The results of this study are shown in Table 2. Analysis of variance (22) showed no significant statistical differences ($P = 0.05$). Thus, the overall accuracy of the method can be established at 97.1% with a coefficient of variation of 7.2%.

Recovery from authentic wines was calculated by using the standard additions method: 15 μg histamine was added to 5 mL aliquots of 2 wines (one white and one red). A second calibration curve is necessary to calculate recovery, and it must be verified that the slopes of both curves (wine $y = 141.1x + 12.9$, $r = 0.9991$, $P < 0.001$; added wine $y = 138.8x + 43.4$, $r = 0.9999$, $P < 0.001$) are equal. We tested this equality by analysis of covariance with a degree of significance of $P = 0.05$ ($F_{exp.} = 0.5875$; $F_{tab.} = 5.99$; DF = 1; 6). Ten determinations were carried out for each wine. The results obtained are shown in Table 3. By analysis of variance ($P = 0.05$), we verified that there were no significant statistical differences in the recovery values for the 2 types of wine.

We also determined the recovery of the method for different authentic wines (white, rosé, and red) with different histamine contents. The results were satisfactory as can be

Table 5. Interference of histidine in determination of histamine in standard wine

Histamine content, ppm	Histidine content, ppm	Relative intensity fluorescence[a]
1	0	38.75 ± 1.91
1	28.5	39.50 ± 1.00
1	120.0	37.50 ± 1.29

[a] N = 3.

observed in Table 4. Thus, our results demonstrate no significant differences in recoveries for the various types of wine. Similarly, we concluded that the values obtained for the accuracy of the method and those obtained for its recovery do not differ. This demonstrates the validity of the standard wine which we used in testing the method.

The amino acid histidine, which can be found with histamine in wines, likewise reacts with OPT to form a fluorescent complex with maximum fluorescence at the same wavelengths as histamine (Figure 2). Therefore, this amino acid must be eliminated during extraction. To test our method for this interference, we carried out an assay in which histidine was added to a standard wine, along with 1 ppm histamine, at 28.5 and 120 ppm. These values exceed the expected histidine content of wine. From 3 tests, we observed no effect from the presence of histidine (Table 5).

Finally, the method was applied to other alcoholic beverages (beer, champagne, vermouth, cider), musts, and vinegars. The recovery obtained with these samples is presented in Table 6. The results were likewise satisfactory.

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Table 6. Recovery of method for other alcoholic beverages and vinegars

Sample	No. of samples	Range of histamine content, ppm[a]	Recovery, %	CV, %
Beer	24	0.30–4.90	94.5 ± 2.63	2.78
Champagne	10	tr –3.30	92.0 ± 4.53	4.90
Cider	5	tr –1.40	95.7 ± 6.60	6.90
Must	10	0.20–0.30	86.8 ± 5.87	6.78
Vermouth	10	0.20–2.65	92.4 ± 6.28	6.80
Vinegar	10	0.20–17.30	96.9 ± 7.36	7.60

[a] tr = trace.

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CHEMICAL CONTAMINANTS MONITORING

Survey of Lead in Canned Evaporated Milk

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A Food and Drug Administration survey of lead in canned evaporated milk conducted in fiscal year 1985/86 found a mean level of 0.006 μg Pb/g. This level is much lower than that found in previous surveys and is attributed to the use of nonlead-soldered cans for packaging evaporated milk.

The Food and Drug Administration (FDA) has a continuing objective to reduce the lead content in the diet, especially in that of very young children, who are more susceptible to the effects of lead. Domestic evaporated milk manufacturers previously used vent-hole-type lead-soldered cans, which frequently contributed lead to the product. Evaporated milk is occasionally fed to young children as a substitute for mother's milk or processed infant formula products. Therefore, evaporated milk is one possible source of lead in the diet of young children. FDA, in cooperation with the manufacturers of evaporated milk, has sought to reduce the lead contribution from this source.

Since 1972, the evaporated milk industry has undertaken comprehensive programs to reduce the level of lead in their products. These efforts, coordinated by the Evaporated Milk Association, included evaluating milk throughout the processing line, evaluating the can-making process (can materials, can-forming construction), and establishing critical control points and incorporating them into quality control programs. These efforts have resulted in significant reductions of lead levels and culminated when the industry switched from vent-hole lead-soldered cans to forge-welded or wire-welded cans in 1986.

In 1973, FDA initiated a quality assurance program with the evaporated milk industry to ensure compliance with a temporary FDA guideline of 0.5 μg/g for lead in evaporated milk. In 1974 FDA proposed a tolerance of 0.3 μg/g for lead in evaporated milk (1). The proposed tolerance was based on the levels attained by the evaporated milk industry and an FDA fiscal year (FY) 1974 survey (2) of major evaporated milk producers. In 1979, FDA published an advance notice of proposed rulemaking on lead in foods (3). In this document, FDA stated its intention to withdraw its 1974 proposed tolerance for lead in evaporated milk in favor of establishing an appropriate action level that would take into account the further reductions of the amounts of lead in evaporated milk.

The FY 1974 survey of evaporated milk (2) found that 83 samples from 10 producers of evaporated milk had a mean lead level of 0.12 μg/g. An FDA FY 1981 survey (4) collected evaporated milk and infant formula samples in concert with the FDA FY 1981 Total Diet Study. The 104 samples of evaporated milk from the FY 1981 survey had a mean lead level of 0.06 μg/g. Infant formula samples had mean lead levels of 0.012 and 0.026 μg/g for 110 ready-to-feed products and 30 concentrated products, respectively. At the time of the FY 1981 survey, the infant formula industry had partially converted from lead-soldered cans to 2-piece drawn steel or wire-welded cans. The evaporated milk industry was then using vent-hole lead-soldered cans.

In FY 1985/86, FDA conducted a survey of canned evaporated milk to determine the levels of lead in domestic evaporated milk packed in nonlead-soldered cans. A brief description of the survey and its findings are reported here.

Experimental

Sample Collection

Samples of evaporated milk were collected by FDA investigators from all 12 known evaporated milk manufacturing sites representing 5 different manufacturers. Four samples were collected from each of 11 sites and 5 samples were collected from one site. Each sample was taken from a different lot and consisted of 3 wire-welded 12 oz cans.

Analysis

All samples were analyzed by the FDA New Orleans District Laboratory. One can from each laboratory sample was analyzed for lead by a dry ash-differential pulse anodic stripping voltammetric method (5). The remaining 2 cans were kept in reserve for confirmatory analysis if the lead level exceeded 0.1 μg/g. Because of the relatively simple evaporated milk matrix, the method was modified as described here to enhance the lead quantitation limit.

After the outside of the can was cleaned and the can was shaken, the can was opened and a 25 g test portion of evaporated milk was weighed into a quartz vessel. The test portion was dried in an oven at 110–120°C and then ashed at 500°C in a furnace. The ash was dissolved by adding 1 mL concentrated nitric acid and 15 mL water and gently warming the mixture. The solution was quantitatively transferred to a 50 mL volumetric flask and diluted to volume. A 10 mL aliquot of the solution was transferred to an electrolysis cell for direct quantitation of lead by the method of standard additions with a 2–5 min deposition time.

Table 1. Results from FY 1985/86 survey of lead in canned evaporated milk[a]

Sampling site[b]	Found, μg Pb/g[c]					Mean, μg Pb/g
A	0.006	0.008	0.015	0.008		0.009
B	0.007	0.006	0	0.006		0.005
C	0	0	0.008	0.007		0.004
D	0.007	0.007	0	0.006		0.005
E	0.006	0.017	0.005	0.007		0.009
F	0.008	0.009	0.008	0		0.006
G	0.008	0	0.006	0.006		0.005
H	0.009	0.012	0.005	0.012	0	0.008
I	0.019	0.011	0.009	0.010		0.012
J	0.006	0	0	0.011		0.004
K	0	0	0.007	0.006		0.003
L	0	0	0	0		0
Overall mean						0.006

[a] Forty-nine samples, each from a different lot, were analyzed.
[b] Five different manufacturers were represented.
[c] Results below the limit of quantitation (0.005 μg Pb/g) are reported as zero.

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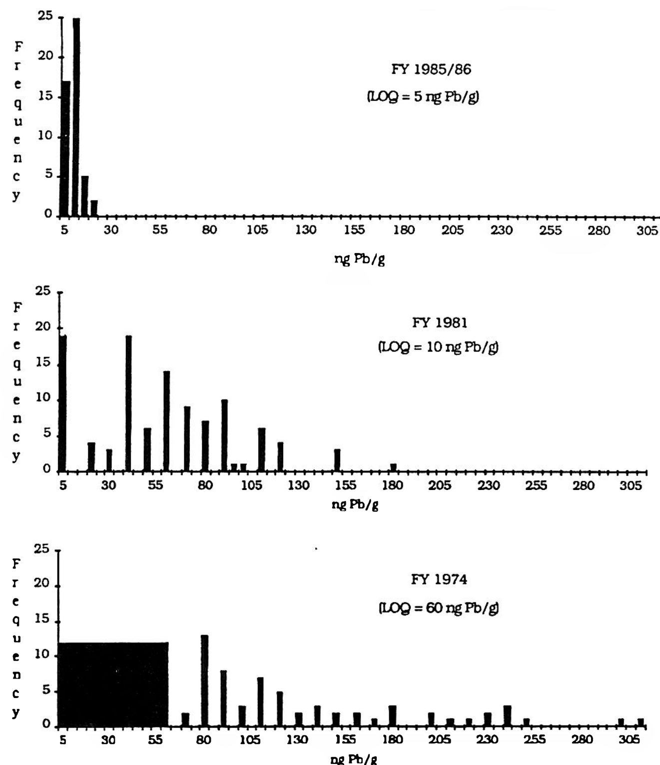

Figure 1. Frequency distribution of lead levels in canned evaporated milk from FDA surveys; LOQ = limit of quantitation.

Results

The mean lead level in the 49 samples analyzed was 0.006 μg/g. The highest lead level found was 0.019 μg/g. Lead in 15 samples was below the quantitation limit of 0.005 μg/g (10 times the standard deviation of the blank); a value of zero was used for these samples in computing the mean lead level (using zero was not materially different from using one-half the quantitation limit). Individual results and means are listed by sample site in Table 1. The mean recovery of lead from canned evaporated milk spiked at 0.1 μg Pb/g was 99% (n = 5), and agreement was good between duplicate analyses.

Discussion and Conclusion

The mean finding of 0.006 μg Pb/g is 1/20th of the mean finding obtained in the FY 1974 evaporated milk survey (0.12 μg Pb/g) and 1/10th of the mean finding in the FY 1981 evaporated milk survey (0.06 μg Pb/g). The highest finding of 0.019 μg Pb/g is less than 1/10th of the 0.3 μg Pb/g tolerance proposed by FDA in 1974. A frequency distribution for each FDA survey is presented in Figure 1. The findings indicate that the conversion to wire-welded cans has greatly reduced the level of lead in evaporated milk.

The levels found in FY 1985/86 in evaporated milk packaged in wire-welded cans are comparable to levels of lead found in infant formula that were reported to FDA by the Infant Formula Council in 1982 and found in the FDA FY 1981 infant formula survey. These infant formula studies did not identify the type of can seam; however, at the time of the studies, only a small percentage of infant formula was packaged in lead-soldered cans, and many of these cans had an interior coating protecting the infant formula from the lead solder.

Mean lead levels in market milk are generally less than 0.005 μg/g (6). When the concentration factor for milk/evaporated milk, which is approximately 2 (7), is considered, the lead levels in evaporated milk arising from the milk itself are generally less than 0.010 μg/g. The mean finding of 0.006 μg Pb/g in evaporated milk is consistent with this projected level, indicating that the process of producing evaporated milk is not a major contributor of lead.

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DAIRY PRODUCTS

Differential Characteristics of Fatty Acids in Cheese from Milk of Various Animal Species by Capillary Gas Chromatography

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The kind of milk used in the manufacture of cheese has been identified by analysis of the fatty acids. The milk fat is extracted from the cheese and saponified. The methyl esters of the fatty acids are prepared and determined by capillary column gas chromatography. Seven major fatty acids are separated and quantitated, namely, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, and C18:1. Many of the 21 simple ratios that can be formed from these 7 quantities are characteristic of the type of milk from which the fatty acids were obtained. The method allows the identification of cheese prepared with the milk of cows, buffalo, sheep, or goats. Substitution or adulteration of milk can also be detected.

Cheeses made wholly or in part from cow's milk are subject to import quota restrictions (1). Cheeses made solely with other milk, for example, from water buffalo, sheep, or goats, are not controlled in this manner. Also, the duty imposed on importations of cheeses may depend on the milk used in their preparation. Chemists in Customs laboratories must, therefore, analyze cheeses for the type of milk used in their manufacture, especially for the presence of cow's milk. Polyacrylamide gel electrophoresis has been used to distinguish the milk from buffalo (2, 3), sheep (4–6), or goats (7, 8) from cow's milk. An alternative method which is less laborious and allows easier quantitation is gas chromatography (GC). Differentiation is based on differences in the fatty acid profiles of milk from cows (9–15), buffalo (2, 3, 9, 10, 16–18), sheep (9–12, 16, 19, 20), and goats (10, 11, 16, 18). The acids are prepared by saponification of the extracted fat from the cheese and then determined as the methyl esters. Previous workers invariably used packed columns for separation of the methyl esters of the fatty acids and usually studied no more than 2 types of milk. Identification was based on comparison of the percentages of the fatty acids and, in some cases, on comparison of one or a few of the ratios of these percentages.

In the present work, all types of milk of concern were analyzed by capillary column gas chromatography. All independent ratios of major fatty acid concentrations were calculated, which allowed the detection of the addition of cow's milk to other types, as well as the identification of the milk in buffalo and cow's milk cheeses and often also in sheep and goat's milk cheeses. Commercial and a few certified cheeses were used to perform this work.

Experimental

Apparatus and Reagents

(a) *GC column.*—30 m × 0.25 mm id SP-2340 (Supelco, Inc., Bellefonte, PA 16823) fused silica column, film thickness 0.20 μm.

(b) *Gas chromatograph.*—H-P (Hewlett-Packard, Avondale, PA 19311) Model 5890 equipped with flame ionization detector. Operating conditions: initial column temperature 30°C, hold for 4 min, then program at 4°/min to 250°C, and hold at final temperature for 10 min; injector 250°C; detector 275°C; nitrogen carrier gas flow 30 mL/min; hydrogen 30 mL/min; and air 300 mL/min.

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(c) *Integrator.*—H-P Model 3390.

(d) *Blender.*—Waring Model 7911S 2-speed commercial blender.

(e) *Heater.*—Blok Heater (Supelco, Inc.), Cat. No. 3-3315.

(f) *Tetramethylammonium hydroxide.*—Prepare 25 mL of 2M stock solution in methanol using "Baker Grade" tetramethylammonium hydroxide 5-hydrate and reagent grade methanol.

(g) *GC standard.*—Mix fatty acid methyl esters (Alltech Associates, Inc., Deerfield, IL 60015) to approximate composition of samples to be analyzed.

(h) *Cheese samples.*—Imported samples submitted for Customs examination and claimed to be made from milk of cows, buffalo (Mozzarella), sheep (Pecorino Romano and Feta), or goats. Also several Italian Mozzarella cheeses obtained by U.S. government officials and certified to be made from milk of water buffalo.

Procedure

Inject 0.2 mL GC standard solution to establish retention data and verify that chromatograph is operating properly. Mix 5 g portion of cheese with 100 mL dichloromethane for 30 s in blender. Filter mixture under suction into filter flask containing small amount of anhydrous sodium sulfate. Decant filtrate into evaporating dish and evaporate almost to dryness on steam bath. Transfer 10 μL residue to 1 mL crimp-top vial and add 1 mL 2M tetramethylammonium hydroxide in methanol. Heat sealed vial on heating block until content is completely dissolved. Inject 0.2 mL solution into chromatograph. Acquire peak area percentages for major components and directly calculate all possible independent ratios.

Results and Discussion

Chromatograms for the various types of milk were very similar and exhibited 7 major peaks with areas of about 1% or greater and accounting for more than 80% of the total peak area on average. A typical chromatogram is shown in Figure 1 with peak identification obtained from retention times of standard samples. Response factors obtained with the calibration standards were very close to unity. Subsequent evaluation of GC data from cheese samples was based on area percentages. Peaks for the C4:0 and C6:0 acid esters, accounting for about 3% of the total peak area on average, were ignored because of likely errors due to the relatively high volatility of these methyl esters (15). Most of the remaining peak area could be attributed to 16 peaks which averaged from 0.2 to 1.5%. The data for these peaks were usually too variable to provide useful information. Ranges and averages for peak areas corresponding to the fatty acids C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, and C18:1 are listed in Table 1 for the 4 different kinds of milk. Only a few ranges for cow's milk concentrations do not overlap at all with those for other types of milk, i.e., C10:0 for sheep milk and C8:0 and C10:0 for goat milk. Some other ranges show only slight overlap

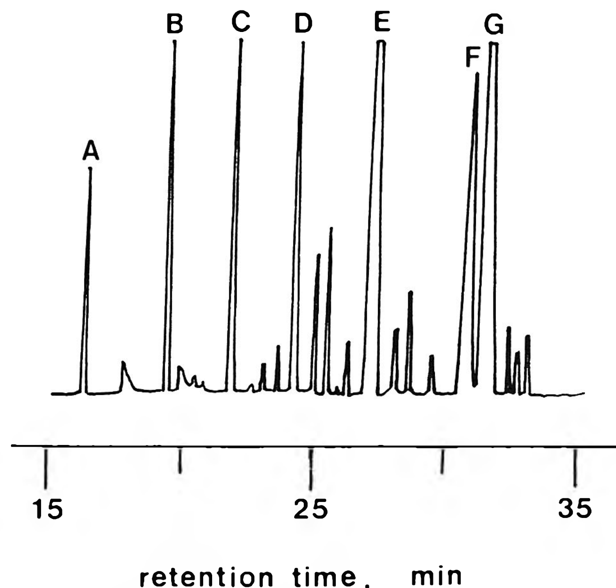

Figure 1. Typical chromatogram of methyl esters of fatty acids of milk in cheese. Peak identification: A, caprylate (C8:0); B, caprate (C10:0); C, laurate (C12:0); D, myristate (C14:0); E, palmitate (C16:0); F, stearate (C18:0); G, oleate (C18:1).

between cow's milk acid concentrations and the corresponding ones for other types, for example, C10:0 for buffalo and C8:0 for sheep milk. Most of these parameters, however, are too similar to permit distinguishing buffalo, sheep, or goat milk from cow's milk.

On the other hand 21 independent ratios can be formed from the 7 concentrations and these allow for much more reliable identification of the type of milk present in a cheese. The ranges and averages obtained are given in Table 2. Unlike cheeses claimed to be made from other kinds of milk, there is no reason to suspect that those said to be manufactured from cow's milk are not genuine. The data shown for the 28 cow's milk cheeses analyzed also agree reasonably well with those in the literature (10, 12, 14) and are, therefore, considered satisfactory criteria for the identification of cow's milk.

Buffalo Milk

The data in Table 2 for cheeses claimed to be made with milk from buffalo were obtained from 22 samples. Comparison of the ranges found for the cow's milk cheeses and for these samples shows 2 ranges, for ratios C14:0/C10:0 and C18:0/C10:0, which do not overlap at all. Additional ratios can also be used to aid in the identification. For at least 20 of the 22 samples, the ratios C16:0/C10:0, C14:0/C12:0, C16:0/C12:0, and C18:0/C12:0 fall outside of the ranges for cow's milk given in Table 2. These 6 ratios will, therefore, serve to distinguish cheeses made from the milk of cows and buffalo. Furthermore, for 16–17 of these 22 samples, the ratios C18:0/C8:0 and C18:1/C10:0 also fall outside the ranges for cow's milk; these can be considered secondary criteria, which in addition to the 6 previous primary criteria, can be used to identify buffalo milk cheeses. Two of these 22 samples have been certified to be manufactured with buffalo milk only. All 8 distinguishing ratios were outside the cow's milk ranges by more than 20% for these 2 samples. It is concluded that all 22 samples contain buffalo milk and that the 8 ratios mentioned in this section suffice to identify samples containing such milk.

Sheep Milk

Data for sheep milk cheeses given in Table 2 result from the analysis of 34 samples claimed to be made from sheep milk. It is easier to distinguish sheep and cow's milk than to distinguish buffalo and cow's milk. Of the 21 ratios, 7 are outside the ranges of those for cow's milk for all 34 samples, namely, C12:0/C8:0, C14:0/C8:0, C16:0/C8:0, C12:0/C10:0, C14:0/C10:0, C16:0/C10:0, and C18:1/C10:0. For 28 and 25 of these samples, respectively, the C18:0/C10:0 and C18:1/C8:0 ratios also are outside the ranges for cow's milk. These 2 ratios can serve as secondary criteria for the identification of sheep milk cheeses. It is concluded that all 34 samples contain sheep milk and that identification can be made by means of the 9 ratios cited herein.

Goat Milk

Of the milks under consideration, goat milk is easiest to distinguish from cow's milk, as seen by a comparison of the fatty acid ratios in Table 2. Goat milk response ratios resulted from the analysis of 15 samples. Of the 21 ratios, 10 do not overlap at all and 2 others overlap only slightly. The former are C10:0/C8:0, C12:0/C8:0, C14:0/C8:0, C16:0/C8:0, C18:1/C8:0, C12:0/C10:0, C14:0/C10:0, C16:0/C10:0, C18:0/C10:0, and C18:1/C10:0; the 2 latter are C18:0/C8:0 and C14:0/C12:0. All 15 samples are considered to contain goat milk as claimed.

Adulterated Cheeses

Occasionally, cheeses have been analyzed and found to contain milk other than that claimed. Table 3 presents fatty acid response ratios for a number of these samples. For buffalo milk cheeses, we have found 6 fatty acid concentration ratios that serve as primary criteria and 2 others that are secondary criteria for distinguishing such cheeses from those that are made with cow's milk. For the first 2 samples listed in this table, at least 6 of these 8 ratios fall outside the buffalo and generally within the cow's milk ranges. It is concluded

Table 1. Fatty acid peak area response ranges and averages

	Cheeses made with milk from							
	Cows		Buffalo		Sheep		Goats	
Acid	Range, %	Av., %	Range, %	Av., %	Range, %	Av., %	Range, %	Av., %
C8:0	0.72–1.40	1.04	0.57–1.05	0.79	1.21–2.72	1.94	2.07–2.78	2.32
C10:0	2.20–3.37	2.80	1.24–2.38	1.66	3.76–8.20	6.01	7.53–9.89	8.61
C12:0	2.63–4.22	3.30	1.39–3.08	2.18	2.53–5.34	3.67	3.48–4.77	4.16
C14:0	9.31–13.3	10.6	8.41–12.4	9.96	6.57–12.5	9.47	8.09–11.6	9.94
C16:0	22.6–36.1	27.7	26.4–34.5	30.5	13.8–28.9	21.7	22.2–31.4	26.0
C18:0	6.80–13.2	10.6	9.00–18.0	13.2	6.83–13.7	10.4	7.56–12.6	9.87
C18:1	19.9–28.8	24.6	18.3–31.8	25.0	18.4–28.1	23.8	18.5–25.1	21.9

Table 2. Fatty acid peak area response ratio ranges and averages

	Cheese samples made with milk from							
	Cows		Buffalo		Sheep		Goats	
Ratio	Range, %	Av., %	Range, %	Av., %	Range, %	Av., %	Range, %	Av., %
C10:0/C8:0	2.05–3.10	2.73	1.77–2.35	2.07	2.56–3.89	3.23	3.26–4.15	3.72
C12:0/C8:0	2.85–4.22	3.17	2.43–3.46	2.76	1.44–2.73	1.94	1.47–2.24	1.80
C14:0/C8:0	7.68–12.5	9.93	10.5–19.7	13.3	3.48–6.86	5.12	3.15–5.12	4.31
C16:0/C8:0	19.7–37.9	27.2	25.7–55.4	37.3	6.81–18.0	12.1	8.49–13.0	11.1
C18:0/C8:0	5.48–14.9	10.4	12.4–21.9	17.0	3.05–9.45	5.70	3.09–5.50	4.27
C18:1/C8:0	15.3–33.1	24.2	23.6–43.5	32.0	8.38–20.4	12.9	7.95–10.9	9.49
C12:0/C10:0	1.02–1.40	1.18	1.09–1.51	1.32	0.52–0.73	0.61	0.44–0.57	0.48
C14:0/C10:0	3.10–4.67	3.84	5.02–7.17	6.06	1.18–2.23	1.62	0.94–1.31	1.16
C16:0/C10:0	7.66–13.1	10.0	13.0–23.9	18.2	2.42–5.85	3.78	2.58–3.72	3.03
C18:0/C10:0	2.46–5.08	3.79	6.39–10.9	8.21	0.88–3.11	1.85	0.83–1.67	1.16
C18:1/C10:0	5.96–12.2	8.99	11.1–20.0	15.4	2.55–5.72	4.07	1.95–3.19	2.56
C14:0/C12:0	2.62–4.03	3.26	3.94–5.33	4.55	2.16–3.58	2.64	2.14–2.75	2.39
C16:0/C12:0	6.10–10.7	8.48	9.55–17.7	12.9	4.16–9.39	6.13	5.28–8.16	6.29
C18:0/C12:0	1.93–4.37	3.23	3.62–9.33	6.01	1.33–4.93	3.03	1.68–3.52	2.41
C18:1/C12:0	5.61–10.6	7.87	8.06–15.1	11.5	3.49–9.32	6.69	3.88–6.70	5.39
C16:0/C14:0	2.15–3.00	2.60	2.09–3.59	2.86	1.82–2.76	2.30	2.37–2.78	2.57
C18:0/C14:0	0.56–1.34	1.01	0.73–1.81	1.26	0.55–1.70	1.14	0.71–1.44	1.01
C18:1/C14:0	1.67–3.09	2.36	1.60–3.69	2.48	1.50–3.62	2.57	1.64–3.10	2.23
C18:0/C16:0	0.19–0.52	0.39	0.26–0.87	0.47	0.24–0.64	0.49	0.24–0.56	0.39
C18:1/C16:0	0.66–1.27	0.91	0.58–1.27	0.87	0.64–1.52	1.13	0.66–1.13	0.85
C18:1/C18.0	1.26–3.10	2.31	1.46–2.69	2.01	1.65–3.02	2.31	1.90–2.57	2.20

that these 2 samples contain cow's milk. For sample 3, only 2 of the primary and one of the secondary criteria fall outside the buffalo and within the cow's milk ranges. However, 4 of the other 5 distinguishing ratios fall at or near the lower ends of the buffalo and also within the cow's milk ranges. It is believed that this sample contains at least some cow's milk.

For sheep milk cheeses, identification is based primarily on the values of 7 ratios; 2 other ratios provide additional but less definite evidence of the nature of the milk used. For sample 4, three primary criteria and one secondary criterion fall outside the sheep milk ranges, while one additional primary one is at the very end of the sheep milk range and all of these are in or close to the cow's milk ranges. This sample is believed to contain at least some cow's milk. For the other 2 samples claimed to be made with sheep milk, all 7 primary criteria are outside the sheep milk ranges and within or near the cow's milk ranges. These cheeses are considered to be made from cow's milk. For the 3 samples supposedly manufactured from goat milk, at least 10 of the 11 main distinguishing ratios are not in the goat milk ranges. For samples 7 and 8, most of these ratios are within the cow's milk ranges and it is concluded that these samples are made from cow's milk. For sample 9, the ratios that fall outside the goat milk ranges are either in the cow's milk ranges or between the ranges for these 2 types of milk. This sample may contain some goat milk in addition to cow's milk.

This method provides a simple and rapid means for identifying the type of milk used in the manufacture of cheeses. It is also possible to qualitatively detect the adulteration of cheese by substitution or mixing of different kinds of milk.

Table 3. Fatty acid ratios for cheese found to contain cow's milk

	Cheese samples purported to be made with milk from								
	Buffalo			Sheep			Goats		
Ratio	1	2	3	4	5	6	7	8	9
C10:0/C8:0	2.44	2.42	2.80	5.71	2.42	2.35	2.35	2.50	2.47
C12:0/C8:0	2.69	2.77	3.31	4.15	3.67	2.75	2.78	3.03	1.67
C14:0/C8:0	9.63	6.63	13.1	9.33	8.78	9.58	9.62	10.4	5.71
C16:0/C8:0	27.8	26.9	36.3	23.5	19.8	27.4	28.3	30.3	13.6
C18:0/C8:0	10.9	8.19	12.8	4.88	8.09	5.83	8.71	6.52	10.1
C18:1/C8:0	26.4	21.1	29.6	20.7	17.7	18.1	22.5	18.7	18.3
C12:0/C10:0	1.10	1.15	1.18	0.73	1.52	1.17	1.18	0.86	0.67
C14:0/C10:0	3.94	2.74	4.67	1.63	3.63	4.08	4.09	4.17	2.31
C16:0/C10:0	11.4	11.1	13.0	4.10	8.20	11.7	12.0	12.1	5.49
C18:0/C10:0	4.46	3.39	4.58	0.85	3.34	2.48	3.70	2.60	4.08
C18:1/C10:0	10.8	8.74	10.6	3.62	7.32	7.70	9.55	7.47	7.42
C14:0/C12:0	3.58	2.39	3.96	2.25	2.39	3.48	3.46	3.45	3.43
C16:0/C12:0	10.4	9.69	11.0	5.65	5.41	9.97	10.2	10.0	8.13
C18:0/C12:0	4.05	2.95	3.88	1.18	2.20	2.12	3.13	2.16	6.04
C18:1/C12:0	9.82	7.61	8.96	4.98	4.82	6.58	8.08	6.18	11.0

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Rapid Determination of Total Cholesterol in Homogenized Milk

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A rapid method that is amenable to automation has been developed for the determination of total cholesterol in homogenized milk. The milk sample is saponified in ethanolic KOH in the presence of an internal standard, cholestane. Cholesterol and the internal standard are then isolated by solid-phase extraction on a nonpolar adsorbent and eluted with organic solvent. The evaporated extract is derivatized and analyzed by capillary gas chromatography. Average recovery of cholesterol acetate added to milk prior to saponification was 95%. The average relative standard deviation for repeated analyses was 2%. The limit of detection for this method is 2 mg/100 g. Twenty samples can be analyzed by one analyst in a normal work day if the gas chromatograph is equipped with an autosampler. This method has been compared with a modified AOAC method for the determination of total cholesterol. At a confidence level of 95%, no difference was observed between the 2 methods.

Many methods have been published in the last 3 decades for the determination of cholesterol. Naito and David (1) and Zak (2) reviewed the available methodology in great detail. The determination of cholesterol often involves an organic solvent extraction. Components of the extract can be further separated by thin-layer chromatography (3, 4) or can be analyzed directly by colorimetric determination (5), enzymatic determination (6, 7), liquid chromatography (8-11), or gas chromatography (12, 13). Since no saponification step is involved, the cholesterol esters and free cholesterol naturally present in the sample are extracted together. Depending on the experimental parameters, one can measure the cholesterol esters, the free cholesterol, or both. An alternative approach, used frequently in the study of food composition, includes a saponification step before the organic phase extraction. The esters are hydrolyzed during saponification to free cholesterol, and then the organic solvent extracts the total cholesterol. Because repeated extractions are necessary for complete extraction of cholesterol, and because the samples must be extracted individually, the procedure is time consuming and has little potential for automation.

Due to the increasing interest in the possible link between arteriosclerotic diseases and cholesterol, a rapid method is necessary to assist in the research toward a low-cholesterol or cholesterol-free diet; many samples may be generated during such a project. Developments in the area of an enzymatic determination of cholesterol are extremely encouraging. In these procedures, the enzyme cholesterol esterase replaces the chemical saponification of the esters, and then the cholesterol oxidase oxidizes cholesterol to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide reacts with phenol and 4-aminoantipyrine in the presence of a peroxidase to form a dye which is easily measured spectrophotometrically. The entire process is carred out batchwise, in disposable tubes, enabling a tremendous increase in sample throughput. However, a positive error is possible due to turbidity, and a negative error is likely if species are present which will deplete the amount of hydrogen peroxide.

The present communication describes an alternative to the enzymatic method and the solvent extraction method for total cholesterol in homogenized milk. Saponification is carried out as described by Kovacs et al. (14). Many samples can be saponified simultaneously because saponification is carried out on a small scale in culture tubes. Instead of isolation by repeated extraction, cholesterol and the internal standard are isolated by solid-phase extraction on a nonpolar adsorbent, such as C18 packed in disposable polypropylene tubes (Bond-Elut). The isolates are then eluted in batches from the sorbent, dried with sodium sulfate, derivatized with BSTFA containing 10% TMCS, and analyzed by gas chromatography. All glassware used is disposable. The amount of solvent used in the extraction is reduced by 90% compared to the methods using solvent extraction. This method is also safer

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with respect to fire hazard and explosion because a mixture of methanol and chloroform is used as the solvent instead of ether. In addition to cost reduction for each analysis and the safety factor, the method lends itself to automation with subsequent gain in productivity, and avoids some of the interferences that may affect the enzymatic methods.

Experimental

Apparatus

(a) *Capillary column gas chromatographic system.*—Hewlett-Packard Model 5890 equipped with split injector and 30 m × 0.25 mm id fused silica capillary column, DB 1 (J & W Scientific), with 0.25 μm film thickness. Column temperature, 245°C programmed at 5°/min to 285°C and held at that temperature for 17 min; helium carrier gas at 1 mL/min measured at 160°C; helium make-up gas at 18 mL/min; detector gases, hydrogen at 50 mL/min and air at 300 mL/min; injection port temperature, 280°C; detector temperature, 300°C; split ratio, 1:25.

(b) *Culture tubes.*—16 × 125 mm with Teflon-lined screw caps.

(c) *Test tubes.*—Disposable, 16 × 125 mm.

(d) *Solid-phase extraction cartridges.*—C18 adsorbent (Bond-Elut), 6 mL (Analytichem International, Harbor City, CA 90710).

(e) *Solid-phase extraction vacuum manifold.*—Supelco (Bellefonte, PA).

(f) *Heating block.*—Reacti-Therm III™ heating/stirring module (Pierce Chemical Co., Rockford, IL).

Reagents

(a) *Cholesterol standard.*—Prepare ethanol solution containing 1.4 mg/mL of cholesterol (>99% purity, Sigma Chemical Co., St. Louis, MO). Store at room temperature and prepare fresh weekly.

(b) *Cholestane internal standard.*—Prepare ethanol solution containing 1.4 mg/mL of cholestane (>99% purity, Aldrich). Prepare fresh weekly and store at room temperature.

(c) *Potassium hydroxide solution.*—Dissolve 80 g AR grade KOH pellets in 50 mL water.

(d) *Hydrochloric acid solution.*—7.5N. Dilute 125 mL concentrated HCl to 200 mL with water.

(e) *Ethyl alcohol.*—Dehydrated and 200 proof.

(f) *Chloroform.*—Contains 1% ethanol as preservative (Burdick and Jackson).

(g) *Methanol.*—LC grade (Burdick and Jackson).

(h) *Mobile phase.*—5% (v/v) methanol in chloroform.

(i) *Derivatization agent.*—BSTFA containing 10% TMCS (Regis Chemical Co., Morton Grove, IL).

Preparation of Calibration Standard

Pipet 1 mL each of cholesterol and cholestane standard solutions into culture tube fitted with Teflon-lined screw cap. Evaporate mixture to dryness under nitrogen and derivatize with 1 mL BSTFA containing 10% TMCS. Inject 2 μL calibration standard between every 5 samples.

Preparation of Samples

Accurately weigh homogenized milk samples (1 ± 0.1 g) into culture tubes fitted with Teflon-lined screw caps. Pipet 1 mL aliquot of internal standard solution into each sample followed by 3 mL ethanol and 1 mL KOH solution. Place ½ in. stirring bar into each tube. Seal culture tubes and saponify contents 1 h on heating/stirring block at 100°C behind safety shield. Let cool 5 min, and transfer 1 mL aliquot from each tube to disposable test tubes. Add 0.4 mL aliquot of 7.5N HCl into each test tube and mix on vortex mixer. Remove drop of each solution, using capillary tubes, and test with pH paper to assure that pH is between 2 and 5. If not, adjust with an extra drop of HCl solution or dilute KOH solution.

Add 1 mL water to each mixture with vortex-mixing to dissolve potassium chloride formed during acidification. Transfer each solution to C18 cartridges which have been conditioned with 5 mL methanol, followed by 5 mL water. (Do not discard pipets and test tubes at this point.) When all solutions have been transferred onto cartridges, which are attached to vacuum manifold, adjust vacuum line to pressure of 10 in. Hg. Let solutions elute dropwise. When all solutions are eluted, dry cartridges by pulling air through them for at least 5 min.

Discard eluates and replace receiving tubes inside vacuum manifold with clean culture tubes. Rinse test tubes saved previously with three 1 mL portions of 5% methanol in chloroform, transferring each rinse with original Pasteur pipets to corresponding cartridges. Collect each rinse solution through cartridges in culture tube. Place an additional 12 mL mobile phase directly onto each cartridge so that total of 15 mL eluate is collected in each tube.

Dry each solution with 4 g anhydrous granulated Na_2SO_4 for at least 15 min on mechanical shaker. Transfer supernatant liquid into clean culture tube, evaporate under nitrogen to dryness, and derivatize 20 min at 70°C with 0.3 mL BSTFA containing 10% TMCS. Inject 2 μL of each sample into gas chromatograph. (Perform steps involving methanol and chloroform in fume hood due to toxicity of both solvents.)

Results and Discussion

This rapid method was used to analyze 45 homogenized milk samples. Each milk sample was also analyzed by a modified AOAC method. In the latter method, behenyl alcohol was used as an internal standard and was added prior to the saponification and extraction. In the unmodified AOAC method (15), 5a-cholestane is used instead and is added after saponification and extraction. When the modified AOAC method was used to determine the cholesterol level in National Institute of Standards and Technology Standard Reference Material 1563, composed of cholesterol added to coconut oil at a level of 64.2 mg/100 g, recovery was 99–101%. The results of the analyses on homogenized milk using both the rapid method and the modified AOAC method are listed in Table 1. Using the Student *t*-test to analyze the data, no difference between the 2 methods was observed at a 95% confidence level when used to examine milk samples with a cholesterol level of 2–12 mg/100 g.

Even though saponification is carried out at 100°C for 1 h, no detectable degradation was observed for either cholesterol or cholestane. A 1 mL aliquot of cholesterol acetate in ethanol was spiked into 4 different samples prior to saponification. The recoveries of the acetate spiked at levels of 16 and 130 mg/100 g and the relative standard deviations for replicate analyses of each sample are tabulated in Table 2. An average recovery of 95% and an average relative standard deviation of 2% were obtained using the short method. The limit of detection of this method is 2 mg/100 g when sample size is 1 g. The sample size cannot be increased because of the limited volume of the culture tube. Below the level of 2 mg/100 g, the signal-to-noise ratio is too low for an accurate determination.

To obtain reproducible results, the solid-phase adsorbent,

Table 1. Analysis of homogenized milk for cholesterol content by rapid method and modified AOAC method

Sample	Cholesterol content, mg/100 g	
	Rapid method	Modified AOAC method
F04320	11.33	11.07
F04321	10.70	10.44
F04322	6.40	6.17
F04323	4.00	3.79
F04797	11.47	11.39
F04798	10.40	10.96
F04799	11.14	11.28
F04800	11.40	11.13
F04801	10.87	11.47
F04802	11.49	11.38
F04803	11.10	11.47
F04804	11.16	11.15
F04805	10.59	10.78
F04806	9.65	10.53
F04807	10.87	10.97
F04808	10.09	10.89
F04810	11.57	10.97
F04982	11.85	11.04
F04983	7.21	8.26
F04984	11.78	11.35
F04985	11.77	11.62
F04986	11.03	11.24
F04987	11.41	11.67
F04988	10.38	11.49
F04989	11.05	11.60
F04990	9.49[a]	11.07
F05146	9.27	10.26
F05147	10.41	9.58
F05148	10.59	10.09
F05149	9.69	9.68
F05150	10.00	8.89
F05151	9.29	9.91
F05152	8.48	9.19
F05153	8.47	10.03
F05154	9.23	8.71
F05156	10.10	9.54
F05157	11.05	10.89
F05158	10.87	11.33
F05159	11.18	10.70
F05160	10.16	9.98
F05459	9.84	10.65
F05460	10.87	8.70
F05461	2.27	2.07
F05689	11.44	10.90
F05690	11.28	10.90

[a] Average of 3 determinations.

Table 2. Recoveries of cholesterol from spiked, homogenized milk samples by rapid method

Sample	Cholesterol content, mg/100 g
F03590	10.90
	10.60
	10.80
	10.60
	10.70
Mean	10.72
RSD, %	1.08
F03590 spike	27.20
Rec., %	96.6
F04990	9.25
	9.35
	9.96
Mean	9.48
RSD, %	2.9
F04990 spike	138.37
Rec., %	96.0
F05149	9.74
	10.20
Mean	9.97
RSD, %	2.3
F05149 spike	18.09
Rec., %	91.0
F05969	11.47
	11.65
	11.62
	11.44
	11.56
Mean	10.77
RSD, %	0.7
F05969 spike	21.10
Rec., %	95.4
Overall mean RSD, %	2.0
Overall mean rec., %	95.0

C18, must be properly solvated with methanol and then water. Even after the solvation procedure, if the adsorbent is allowed to dry under a strong vacuum prior to the adsorption of the samples, loss of cholesterol and cholestane is significant.

The solid-phase extraction cartridge used in the study contains 1 g adsorbent. The capacity for strongly adsorbed species on C18 is about 5 mg/100 mg of adsorbent. If the adsorbent is overloaded, the compounds of interest may elute prematurely. In the milk sample, overloading is not a major problem because of the low fat content in the milk. The sample size must be kept below 1 g to avoid diluting the ethanolic base significantly and consequently reducing the efficiency of the saponification. To ensure that both the cholesterol and cholestane remain dissolved in solution until the solid-phase extraction step, it is also crucial to maintain a high percentage of ethanol in which both compounds are soluble. In addition, the aliquots from the saponified samples must be adjusted to between pH 2 and 5. The presence of ionic surfactant formed from the potassium hydroxide and the fatty acids generated during the saponification of the lipids may radically alter the properties of the sorbent. Acidifying the samples before the solid phase extraction suppresses ionization of the fatty acids to minimize interference with the adsorption of cholesterol and cholestane. The chromatograms of homogenized milk and and coconut oil spiked with cholesterol indicate that the sample extraction and purification by C18 is sufficient to provide a sample relatively free of interference (Figures 1 and 2, respectively).

A milk sample with a cholesterol level of 11 mg/100 g exhibited an erroneous result of 18 mg/100 g when only the supernatant liquid of an acidified aliquot with no added water was used for the determination of cholesterol. Water must be added to facilitate the adsorption of the nonpolar analytes such as cholestane and cholesterol onto the nonpolar adsorbent. After the addition of water, a small amount of cholesterol and cholestane precipitate from the solution. The rinsing of the test tubes and the quantitative transfer of the rinse onto the sorbent are extremely important steps to assure complete recovery of both analytes.

Except for using a maximum sample size of 0.5 g, this

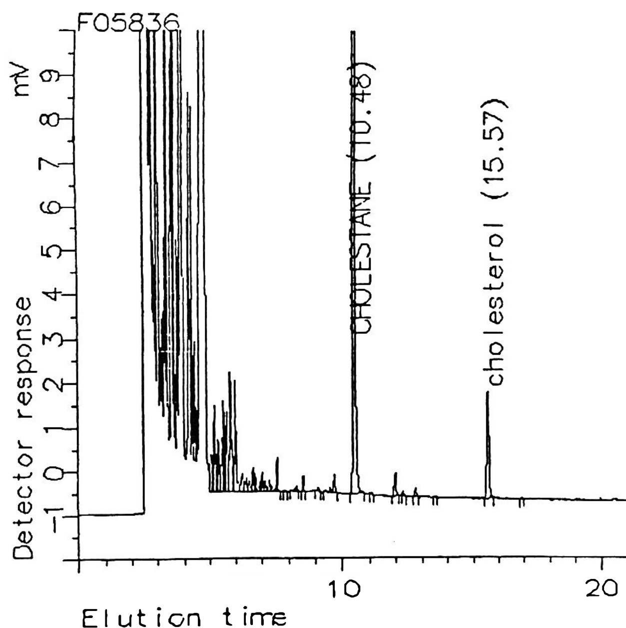

Figure 1. Separation of cholesterol in milk.

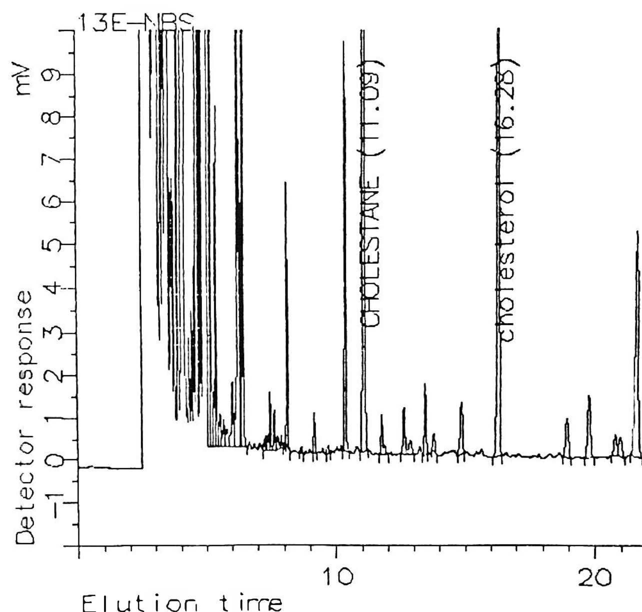

Figure 2. Separation of Standard Reference Material No. 1563.

Table 3. Cholesterol analyses of samples other than milk

Type of sample	Cholesterol content, mg/100 g	
	Short method	Modified AOAC method
Salad dressing	42.47	39.32
	39.27	39.00
Dehydrated yogurt	76.00	75.00
Yogurt	7.82	7.33
Cream cheese	101.77	102.30
Pasta	91.41	90.00
NIST Ref. Std	66.00	64.70

method has been extended without modification to other samples such as salad dressing, mayonnaise, yogurt, cream cheese, pasta, and the reference standard mentioned earlier. The results are tabulated in Table 3. The difference between the data from the fast and modified methods is about 0.5-4%. Even though the data are insufficient to make a statistical comparison of the 2 methods, the new, rapid method has great potential to become the method of choice for measuring cholesterol in various food matrixes.

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Ion Chromatographic Determination of Lactose, Galactose, and Dextrose in Grated Cheese Using Pulsed Amperometric Detection

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A method is presented to determine lactose, galactose, and dextrose in grated cheese using ion chromatography with pulsed amperometric detection (IC/PAD). The method is specific, sensitive, rapid, and precise. Sugar levels as low as 0.01% are detectable, and recoveries ranged from 114% at the 0.1% level to 93.3% at the 2.0% level. The precision expressed as coefficient of variation (CV) was 2.3% for lactose, 2.1% for galactose, and 3.1% for dextrose. Lactose, galactose, and dextrose levels are given for 45 authentic hard cheeses and 26 authentic soft cheeses.

Sugars in food products can easily be quantitated by numerous AOAC methods (1) including copper reduction (sec. **16.297**), gas chromatography (GC) of silyl derivatives (sec. **22.078**), and liquid chromatography using refractive index detection (LC/RI) (sec. **31.145**). However, no official AOAC method exists to quantitate lactose and its hydrolysates, galactose and dextrose, in grated cheese.

The quantitation of sugars is important both from a manufacturing and a regulatory standpoint. Sugars can be used to monitor curing and aging procedures in an attempt to minimize browning caused by the Maillard reaction (2) between various sugars and protein. The presence of sugars can also be used to detect adulteration of grated cheese with cheese whey and other cheese varieties (3). The standard of identity for grated cheese (4) permits the use of both hard and soft cheeses, provided the product is labeled accordingly. Soft cheese may contain sugars whereas hard cheese contains little or insignificant levels (5). The presence of sugars in products labeled as grated hard cheese would indicate in most cases the presence of soft, unaged cheese.

The AOAC copper reduction method for lactose in processed cheese can be used for grated cheese and is sensitive to about 0.1%. However, the method is time consuming and, because numerous sugars reduce copper, the method is nonspecific. GC methods, although specific, are also time consuming and are subject to matrix interferences. The LC/RI method (3) used until recently by the author was adequate but dilution factors limited the detection limits to about 1%. Matrix interferences in the galactose/dextrose region and poor resolution between galactose and fructose made all these sugars difficult to identify and quantitate.

Ion chromatography using pulsed amperometric detection (IC/PAD) (Dionex Corp., Tech. Note 20, 1987) was investigated as a quantitative technique. The procedure is specific and is 100 times more sensitive than conventional LC/RI procedures (6). A method using IC/PAD is presented in the present paper along with analytical data on authentic cheeses.

METHOD

Apparatus

(**a**) *Chromatograph.*—Dionex 4000i ion chromatograph.

(**b**) *Detector.*—Dionex pulsed amperometric detector with gold electrode. Detector voltages: E1 = 0.10 V, T1 = 300 ms; E2 = 0.60 V, T2 = 120 ms; E3 = −0.80 V, T3 = 300 ms. Range = 30 K.

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(**c**) *Integrator.*—Spectra Physics 4100 computing integrator. Input = 0.1 V; attenuation = 8; offset = −110. Integrate by peak area.

(**d**) *Column.*—Dionex AS6A column with AG6A guard column.

(**e**) *Filter.*—0.45 μm (Gelman Sciences, ACRO LC25, or equivalent).

Reagents

(**a**) *NaOH solution.*—50% (Fisher Scientific, SS254, or equivalent).

(**b**) *Mobile phase A.*—Water. Degas deionized distilled water by bubbling He gas through water at 100 mL/min for 0.5 h/L water.

(**c**) *Mobile phase B.*—0.2M NaOH. Weigh 16 g 50% NaOH (**a**) into 1 L volumetric flask. Dilute to volume with mobile phase A (**b**).

(**d**) *Concentrated sugar standards.*—2% lactose, 1% galactose, 1% dextrose. Individual sugars available from Aldrich Chemical Co. Dry individual sugars 12 h at 60°C under vacuum. Weigh 2.000 g lactose, 1.000 g galactose, and 1.000 g dextrose into single 100 mL volumetric flask. Dilute to volume with water. Add 2–3 drops of chloroform as preservative.

(**e**) *Working standards.*—40 ppm lactose, 20 ppm galactose, 20 ppm dextrose. Dilute 2 mL concentrated standard (**d**) to 1 L with water. Prepare daily.

Operating Conditions

Maintain He pressure over mobile phase reservoirs to prevent CO_2 absorption. Establish flow of 0.8 mL/min with 18% mobile phase B and 82% mobile phase A. Galactose and dextrose elute with nearly baseline separation in 6–7 min. Lactose elutes at approximately 16 min. Sweep absorbed CO_2 from column by increasing mobile phase B to 100% after lactose elution. Maintain for 3 min and return to original conditions. Maintain original conditions for 3 min prior to next injection. If baseline rises more than 20% during gradient, polish gold electrode according to manufacturer's instructions.

Sample Preparation

Prepare cheese samples according to sec. **16.258** (1). Mix thoroughly and pass through No. 8 sieve. Weigh, to nearest mg, 3 g cheese into 250 mL beaker. Add 100 mL water and heat with stirring on hot plate just to boil. Cool to room temperature and transfer to 100 mL glass-stopper cylinder. Dilute to volume and mix. Transfer 5 mL to 25 mL volumetric flask. Dilute to volume and mix. Filter through 0.45 μm filter prior to chromatography. Final dilution represents approximately 3 g sample in 500 mL assay and is based on individual sugar levels of 1% galactose and dextrose and 2% lactose in cheese. If sugar levels exceed these, dilute assay accordingly.

Chromatography

Integrate peaks by peak area. Inject 50 μL standards and samples. Repeat 1 or 2 injections of standard until retention

times stabilize. Determine concentration of sugars in assay by comparing peak area of sugar in assay with corresponding peak area in standard. Repeat standard after 5 samples. Calculate % sugars in cheese as follows:

$$S = (Z \times D)/(W \times 20)$$

where S = sugar content in %; Z = concentration (ppm) of sugars in assay; D = dilution factor of filtered assay if needed; and W = weight of cheese in g. Report sugar levels to 0.01%.

Results and Discussion

The New York State Food Laboratory first became interested in the analysis of sugars in grated cheese when certain samples were found to be adulterated with whey solids. Lactose as determined by LC/RI analysis (3), using an amine-based column, was used as the index of adulteration. The limited sensitivity of the procedure did not hinder the detection of whey solids in adulterated products since lactose is a major component. Difficulty was experienced in interpreting data when the sugar content was well below the 1% level. Copper reduction indicated the presence of sugars ranging from 0.1 to 1.6%. However, LC/RI indicated that almost none of these cheeses contained lactose at levels in excess of 1%.

The presence of the hydrolysates of lactose, galactose, and dextrose was a logical explanation. However, a large peak with similar retention times to dextrose was found in many cheese assays, despite the fact that no reducing sugars (i.e., dextrose) were present. Salt, a common ingredient in hard cheese, has been reported to cause interference with dextrose analysis performed with amine-based columns (7). Further analytical work revealed that galactose and fructose were not resolved on this column. The problems encountered with matrix interferences and poor resolution made quantitation of galactose and dextrose difficult.

The use of IC/PAD resolved many of these problems. Ion chromatography provides a powerful separatory tool where isocratic separations of many complex mixtures of sugars can be obtained simply by adjusting the mobile phase strength. Galactose, dextrose, fructose, and lactose are easily separated (6). Electrochemical detection using a gold electrode provides a highly sensitive tool for detecting hydroxyl groups common to sugars. Sensitivities approaching 30 ppb are reported (6). The combination provides a fast, selective, and sensitive means of analyzing a variety of matrixes for sugars. For this method, a simple aqueous extraction followed by dilution proved to be adequate. Figure 1 is a series of chromatograms of standards, an authentic hard cheese, and a spiked hard cheese. It demonstrates the selectivity and sensitivity of the method. Galactose, dextrose, and lactose are easily identified with background levels in hard cheese approaching 0.01%.

A few minor problems were encountered during the evaluation of the IC/PAD technique. First, the gold electrode must be polished periodically. After its cleaning and throughout its use, the sensitivity gradually increases. This increase can be as much as 10% during a day's use. This change in sensitivity can be offset by injecting standards after every fifth sample. Second, the resolving power of the column increases as mobile phase strength decreases. The absorption of dissolved carbonates and other matrix components adversely affects the resolving power of the column. Most can be removed by flushing the column with stronger mobile phase after each analysis. After many months of use, the resolving power of the column will be reduced. A decrease in the initial strength of the mobile phase can restore the resolution. Finally, the capacity of the column appears to be about 200 ppm. Assays with sugar levels in excess of this tend to have reduced retention times. The use of peak area for quantitation compensates for this change but if sugar levels are very high, dilution is necessary for accurate peak identification.

Tests to determine linearity, recoveries, and precision were conducted on the method. A series of standards ranging from 5 to 60 ppm galactose and dextrose and from 10 to 120 ppm lactose were injected, and both peak areas and peak heights were determined. A linear regression analysis was performed for each sugar. All sugars quantitated by peak area for these ranges were linear with correlation coefficients >0.9999. Galactose and, to a lesser extent, dextrose, were linear when

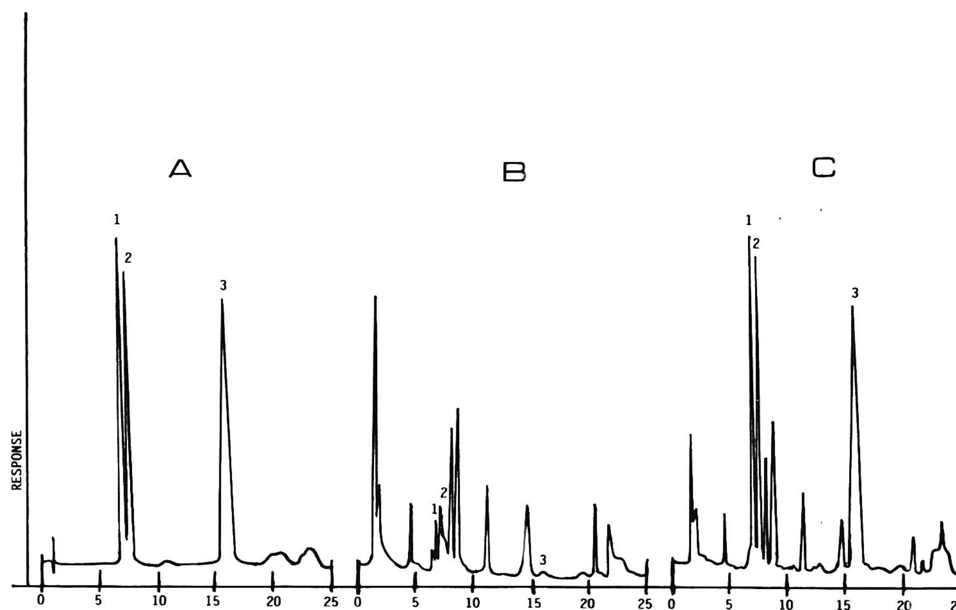

Figure 1. (A) Sugar standard containing 10 ppm galactose (1), 10 ppm dextrose (2), and 20 ppm lactose (3). (B) Authentic Parmesan containing 0.02% galactose, 0.03% dextrose, and >0.01% lactose. (C) Spiked authentic Parmesan containing 0.17% galactose, 0.17% dextrose, and 0.33% lactose.

Table 1. Recoveries[a] of sugars added to 3 g grated cheese

Statistic	Galactose			Dextrose			Lactose		
Added, %	0.100	0.500	1.000	0.100	0.500	1.000	0.200	1.000	2.000
Recovered, %	0.108	0.512	0.960	0.114	0.518	0.967	0.201	1.007	1.867
Recovery, %	108.0	102.4	96.0	114.0	103.6	96.7	100.5	100.7	93.3

[a] Average of 2 determinations.

Table 2. Study of precision on naturally occurring sugars in grated cheese

Statistic	Lactose	Galactose	Dextrose
Range	1.72–1.86	0.55–0.58	0.72–0.78
Mean[a]	1.78	0.56	0.75
Std dev.	0.041	0.012	0.023
CV, %	2.3	2.1	3.1

[a] Mean of 8 determinations.

quantitated by peak height but lactose was not. The ranges studied correspond to levels of up to 1% galactose and dextrose and up to 2% lactose in cheese. Additional dilution steps are necessary to extend this range.

Recovery studies were performed on 2 grated cheeses with insignificant levels of sugars. The cheeses were spiked at 3 different levels by adding sugar solutions to 3 g cheese and proceeding with the method. Recoveries were determined for one set of cheese spikes on one day and for the second set on the next day. The results are shown in Table 1. Recoveries ranged from 93.3 to 114.0% with values in excess of 100% at the lowest levels spiked.

The precision of the method was evaluated by determining sugars naturally present in a grated cheese. A sample was analyzed 8 times. The results are shown in Table 2. The coefficients of variation (CV) ranged from 2.1 to 3.1% and indicate an acceptable level of precision.

Literature sources (5, 8) indicate that no lactose is present in ripened cheese but few data are available on galactose and dextrose. Forty samples of authentic bulk hard cheese were obtained and analyzed. The samples consisted of 9 imported Parmesan, 9 domestic Parmesan, 17 imported Pecorino Romano, and 5 domestic Romano. The results are shown in Table 3. There were no levels of sugars in excess of 0.03% in any authentic hard cheese except one. A domestic Romano contained 0.45% galactose and no dextrose or lactose. The explanation for this one sample was found after conversations with cheese manufacturers. They indicated that alternative salting procedures were being tried for the manufacture of hard cheese and that cheese made by these procedures contained galactose. Six domestic Parmesan cheeses were obtained from 2 manufacturers that used alternative manufacturing procedures. The results, found in Table 1, indicate that galactose can be present in hard cheese made by alternative procedures. However, there were no levels of lactose or dextrose in excess of 0.03%.

Twenty-three samples of fresh soft cheese were also obtained and analyzed. The samples consisted of 8 whole milk Ricotta, 6 whey Ricotta, 5 Mozzarella, and 4 washed curd. In addition, one processed American, one Provolone, and one Swiss were analyzed. The results are shown in Table 4. Lactose was the only sugar present in excess of 0.07% in the Ricotta cheese. Of these, 4 whole milk Ricotta and 2 whey Ricotta were placed in cheese cloth and dried at room temperature to approximately 20% moisture. They were then grated and stored at room temperature for one week and reanalyzed. The results in Table 4 show that the level of lactose

Table 3. Sugar analysis (%) of grated hard cheese

Variety	No. analyzed	Galactose		Dextrose		Lactose	
		Range	Av.	Range	Av.	Range	Av.
Imp. Parmesan	9	0.00–0.03	0.01	0.00–0.01	0.01	0.00–0.01	0.00
Dom. Parmesan	9	0.00–0.03	0.01	0.00–0.01	0.00	0.00–0.01	0.00
Pec. Romano	17	0.00–0.02	0.02	0.00–0.01	0.01	0.00–0.01	0.01
Dom. Romano	4	0.00–0.01	0.01	0.00–0.01	0.01	0.00	0.00
Dom. Romano[a]	1	—	0.45	—	0.00	—	0.00
Dom. Parmesan[b]	6	0.00–0.56	0.18	0.00–0.03	0.01	0.00	0.00

[a] Domestic Romano suspected of being made by alternative salting procedure.
[b] Domestic Parmesan made by alternative salting procedure.

Table 4. Sugar analysis (%) of grated soft cheese

Variety	No. analyzed	Galactose		Dextrose		Lactose	
		Range	Av.	Range	Av.	Range	Av.
Milk Ricotta	8	0.00–0.05	0.01	0.00–0.01	0.00	1.04–3.02	2.07
Whey Ricotta	6	0.00–0.07	0.03	0.00–0.02	0.01	0.24–3.64	2.15
Dried Ricotta[a]	6	0.00–0.46	0.24	0.00–0.33	0.18	1.02–3.82	2.53
Mozzarella	5	0.00–1.19	0.47	0.00	0.00	0.00–1.36	0.31
Washed curd	4	0.01–0.02	0.02	0.00	0.00	0.00	0.00
Proc. American	1	—	0.08	—	0.01	—	1.20
Provolone	1	—	0.04	—	0.02	—	0.00
Swiss	1	—	0.01	—	0.01	—	0.00

[a] Dried in laboratory.

increased due to moisture loss but, more important, the levels of galactose and dextrose increased. In all cases, galactose exceeded dextrose. Analysis of the remaining cheeses showed that, when sugars were present, either lactose or galactose predominated. Only one Mozzarella had levels of both lactose and galactose in excess of 0.10%. No dextrose levels in excess of 0.02% were found.

Summary

Sugar levels in grated cheese can easily be determined at levels about 0.01% using IC/PAD. The method is fast, accurate, and precise. Grated cheese made from hard cheese contains insignificant levels (<0.10%) of lactose and dextrose. However, if hard cheese made by alternative manufacturing procedures is used, significant levels (>0.10%) of galactose may be present. Grated cheese containing significant levels of lactose and dextrose contains cheese other than hard cheese.

Acknowledgments

Appreciation is expressed to the New York State Department of Agriculture and Markets for their help in this study. Appreciation is also extended to Universal Food Corp., Milwaukee, WI, and Mid-America Farms, Springfield, MO, for providing hard cheese made by alternative salting procedures.

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DRUG FORENSICS

Simple, Rapid, and Portable Chromatographic Tetrazolium Reduction Method for Detection of Potassium Cyanide in Medicinal Drugs and Confectionery

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A simple, rapid, and portable paper chromatographic method for detection of potassium cyanide in medicinal drugs and a few confectionery samples is described. Potassium cyanide is extracted in methanol and concentrated. Acetone-water-1.5% EDTA (4 + 5.5 + 0.5) mixture is used as the solvent system for paper chromatography. The KCN chromatograms appear as pink spots on paper due to reduction of the chromogenic salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride; phenazonium methosulfate is a catalyst. Microgram amounts of KCN can be separated and detected in the laboratory or the marketplace because of the simplicity of the method.

Potassium cyanide, a deadly poisonous compound, can be abused as a homicidal and suicidal agent. Tylenol caplets laced with potassium cyanide caused the death of 7 persons in the United States who had ingested the caplets (1). A simple and rapid method is needed for both a spot test (nonchromatographic) and a qualitative detection method that could be carried out in the drug store or in the laboratory with a minimum requirement of apparatus.

The method presented here is a nonenzymatic chromatographic method unlike the simple, portable, chromatographic-enzymatic methods previously reported for detection of organophosphates and heavy metal compounds of mercury, copper, cadmium, and silver (2, 3). The present report describes an appropriate solvent system, an extraction method, and the chemical reaction for the detection of potassium cyanide in some medicinal drugs (tablets, caplets, and capsules) and a few confectionery products.

METHOD

Apparatus and Reagents

(a) *Medicinal drugs.*—Caplet forms: (*1*) acetaminophen, codeine (Tylenol®, McNeilab, Inc., Fort Washington, PA 19034); (*2*) phenyl propanolamine, noscapine, paracetamol (Contac-CC®, Eskayef Pharmaceuticals, India). Tablet forms: (*1*) paracetamol (Fepanil®, Citadel Fine Pharmaceuticals Ltd, India); (*2*) pheniramine maleate (Avil®, Hoechst, India); (*3*) multivitamin (Hexavit® [sugar-coated], Indian Drugs and Pharmaceuticals Ltd); (*4*) prednisone, theophylline, ephedrine hydrochloride, phenobarbitone (Cortasmyl®, Roussel); (*5*) acetylsalicylic acid (aspirin, Nicholas Laboratories, India); (*6*) B complex (Becosules®, Pfizer, India); (*7*) chlorpheniramine maleate (Zeet®, Alembic Chemical Works Ltd, India). Confectionery: (*1*) cocoa-flavored sugar candy (Nutrine, India); (*2*) mentholated sugar candies (Halls®, Warner Hindustan Ltd, India); (*3*) Strepsils® (Makson Pharmaceuticals, India); (*4*) Vicks® (Richardson Hindustan Ltd, India).

Fortify each drug with potassium cyanide (Loba-Chemie Indo Austranal Co., India), either dissolved in water or in powder form. For aqueous solution, use syringe to dispense 40 mg 0.5 mL on 10 tablets or caplets, by poking through lid if possible or open lid of container and dispense KCN uniformly. In powder form, dispense 40 mg KCN directly on tablets. Shake container well for uniform mixing.

Fortify confectionery samples with 4 mg KCN in powder form for each candy or peppermint. Repack the confectionery samples in the same cover paper. Keep all KCN-fortified samples for 2 weeks at laboratory temperature of 30°C.

(b) *Potassium cyanide standard.*—Dissolve KCN in methanol, 1 mg/mL.

(c) *Chromogenic reagent.*—Mixture of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) and phenazonium methosulfate (PMS). Prepare INT (Loba-Chemie Indo-Austranal Co., India) at 0.4% in water and PMS (BDH, Poole, UK) at 0.1% in water. Mix 10 + 2.

(d) *Solvent mixture for paper chromatography.*—Acetone (E. Merck, India), water, and 1.5% ethylenediamine tetraacetic acid (E. Merck, India) in water. Mix 4 + 5.5 + 0.5.

(e) *Filter paper.*—Whatman No. 3. Cut 7.5 × 2.5 cm strips.

Extraction Procedure

Scrape surface of medicinal drugs (tablet and caplet) with sharp blade. Transfer scraped powder to separate test tubes containing 2 mL methanol. Alternatively, transfer 10 tablets (fortified with KCN solution) to conical flask containing 10 mL methanol. Shake contents gently 2–3 min, decant methanol extract into petri dish, and evaporate to 2 mL under air circulator. Wash each drug container with 3 mL methanol to extract KCN adhering to walls. This extract is essential because most of the KCN (powder) particles adhere to the walls of the container.

For confectionery products, take surface swabs of samples with cotton buds soaked in methanol. Dip these swabs in methanol (2 mL) and squeeze swabs with forceps.

Use all of the methanol extracts for chromatographic detection. Prepare control samples of methanol extracts of unfortified medicinal drugs and confectionery products.

Paper Chromatography

Spot 10 μL methanol extracts of experimental and control medicinal drugs and confectionery, the container wash, and standard KCN solution on separate Whatman No. 3 chromatographic paper strips (7.5 × 2.5 cm) 2 cm above the base with fine microcapillary. Minimize spot-spreading by applying extracts in μL aliquots and drying with hair dryer or gentle breeze. Develop strips (2–4) to 7 cm by ascending chromatography by placing strips in 12 × 4 cm glass jars containing solvent mixture.

Remove the strips, air dry, and spray with chromogenic reagent. Spray chromogenic mixture as fine mist, just wetting paper without any leaching. Place paper strips either on glass plate in 60°C air oven or on glass slide warmed with cigarette lighter for 3–4 min. Observe strips for appearance

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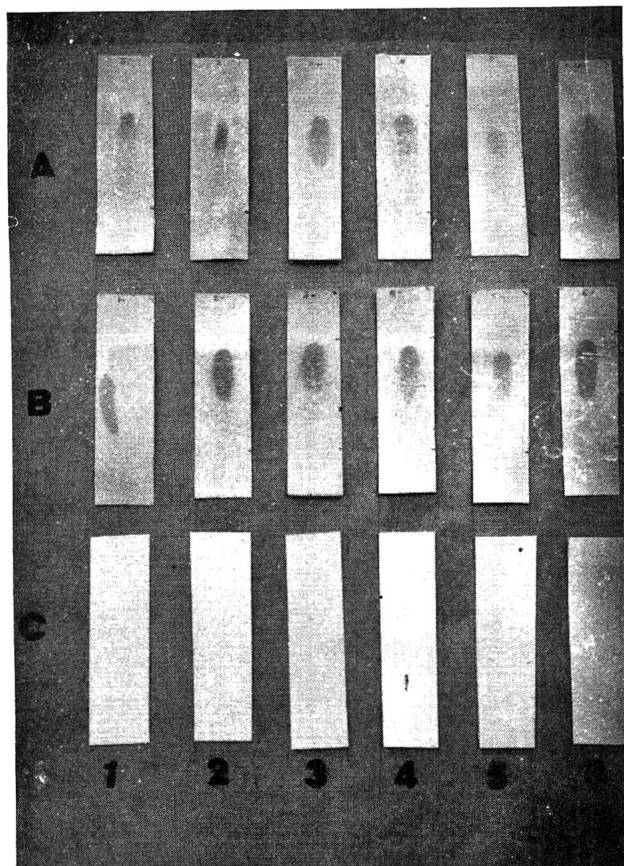

Figure 1. (A) Chromatograms of potassium cyanide from methanol extracts of 1, prednisone, theophylline, ephedrine hydrochloride, phenobarbitone; 2, phenyl propanolamine, noscapine, paracetamol; 3, paracetamol; 4, acetaminophen, codeine; 5, pheniramine maleate; 6, multivitamin tablets. (B) Chromatograms of potassium cyanide from methanol extracts of containers corresponding to 1–6. (C) Control methanol extracts corresponding to tablets 1–6 without KCN.

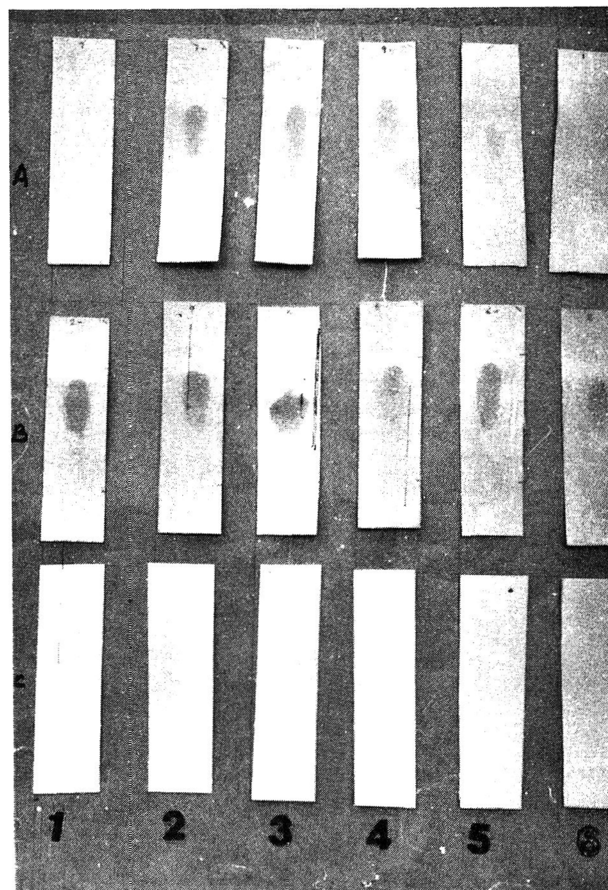

Figure 2. (A) Chromatograms of potassium cyanide from methanol extracts of 1, acetylsalicylic acid (tablet); 2–5, confectionery; 6, chlorpheniramine maleate (tablet). (B) Chromatograms of potassium cyanide from methanol extracts of containers corresponding to 1–6. (C) Control methanol extracts of tablets and confectionery (1–6) without KCN.

of pink chromatograms at R_f 0.75 ± 0.13 cm for presence of KCN.

Mark pink chromatograms. After 30 min, background will also develop light pink color due to photochemical reaction; hence, original chromatograms appearing at 4–5 min must be marked. Compare chromatograms of samples with those of KCN standards. Controls should show no chromatograms.

Caution: Do not inhale dust of pharmaceutical drugs or vapors of methanol extracts.

Results and Discussion

Methanol (100% pure) is used to extract KCN because most of the medicinal formulation and base remain intact during gentle shaking with methanol in the conical flask. The methanol extract along with suspended particles is decanted into a Petri dish and evaporated to 2 mL. This extract is used for chromatography.

To determine whether the medicinal ingredients have an artifactual effect on the KCN chromatogram, methanol extracts of corresponding medicinal drugs and confectionery (control) which are not fortified with KCN were also analyzed. None of the control drugs or confectionary products showed a reaction with the chromogenic reagent.

Acetone–water–1.5% EDTA (4 + 5 + 0.5) was the best solvent system compared with various other solvent systems tried. The chromatograms are compact with no trailing and no residue at the baseline.

In the chemical reaction, the tetrazolium salt (INT) is an electron acceptor; after reduction, it is converted to formazan, a pink product (5–8). Originally, this reaction was transferred to a thin-layer chromatographic plate and Whatman No. 3 filter paper (2–4) for heavy metal analysis. The nonreduction of tetrazolium salt (INT) due to dehydrogenase inhibition from heavy metal compounds caused white zones against the background of pink formazan color. In the chemical reaction of the present method, KCN, a reducing agent, is converted in the presence of water to HCN; the latter reduces INT to formazan in the presence of PMS, which quickly transfers the reducing equivalents ($2H^-$) (9) from HCN to INT.

Guilbault and Kramer (10) showed the reduction of triphenyl tetrazolium chloride (TTC) to formazan by cyanohydrin (cyanide is allowed to react with *p*-nitrobenzaldehyde to produce an active reductant, cyanohydrin) with regeneration of cyanide. INT, a highly sensitive tetrazolium salt compared to TTC (5) in accepting electrons, is used here for the first time to detect KCN on chromatographic paper along with PMS. Thus, wherever KCN is present, the pink chromatograms are formed at R_f 0.75 ± 0.13 cm in the specific solvent system. No pink chromatograms are formed with the extracts of controls. Any reducing ingredients in the medicinal or confectionery products might react; however, in the 12 samples tested, such reducing agents were not detected. Even if reducing agents are present, the R_f values might be different from that for KCN.

The method can be used either in the laboratory or in the

field. In the field, the glass plates can be warmed with a cigarette lighter or a wax candle to speed the chemical reaction on the filter paper. Thus, the method can be applied for instant testing. The limit of detection by chromatographic analysis is 5 μg. By spot test, i.e., without chromatographic resolution, 0.1 μg can be detected.

The paper chromatographic method reported is suitable for detection of cyanide in medicinal drugs. A few TLC (thin-layer chromatographic) methods are reported for analysis of laboratory cyanide samples with qualitative and semiquantitative detection limits of 20 and 40 μg, respectively (11, 12). A number of sophisticated, but cumbersome instrumental methods, have been reported, e.g., colorimetry (13), spectrophotometry (14), gas chromatography (15, 16), flow injection analysis (17), pulse polarography (18), voltammetry (19), atomic absorption spectroscopy (20), and ion chromatography (21). Such methods are specific, accurate, and very sensitive but could not be used in small laboratories or as a field test due to cost and complexity.

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DRUG FORMULATIONS

Derivative Spectrophotometric Determination of Clotrimazole in Single Formulations and in Combination with Other Drugs

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First (D_1) and second (D_2) derivative spectrophotometric methods are presented for the determination of clotrimazole after its acid hydrolysis. Mixtures of clotrimazole with azidamfenicol and dexamethasone have been assayed using D_2 measurement at 302 nm after acid hydrolysis for clotrimazole, D_1 measurement at 288 nm for azidamfenicol, and D_1 measurement at 436 nm after reaction with phenylhydrazinium sulfate for dexamethasone. Reproducible results with relative standard deviations of <2% are obtained. The proposed method has been successfully applied to the analysis of creams, topical solutions, and vaginal tablets.

Use of ultraviolet spectrophotometry in the analysis of clotrimazole has been limited due to low absorptivity of clotrimazole ($A^{1\%}_{1\,cm}$ = 22 at 260 nm in methanol) and the absence of characteristic bands in the absorption spectrum (1). Various colorimetric methods based on the reaction of clotrimazole with bromothymol blue (2) or perchloric acid and their application in biological fluids after either solvent extraction (3) or extraction followed by thin-layer chromatography (4) have been reported. Titrimetric methods involving titrants such as perchloric acid (5), picric acid (6), and sodium lauryl sulfate (5, 7) require high clotrimazole concentration.

Although different chromatographic techniques like thin-layer chromatography (3), gas chromatography (8), and liquid chromatography (9) have been used to assay clotrimazole, the corresponding instruments are not always available in many laboratories.

Clotrimazole is stable in alkaline medium but hydrolyzes in acid medium to (2-chlorophenyl)-diphenyl methanol plus imidazole (10).

Recently, derivative spectrophotometry has found wide application for correction of spectral interferences or resolution of spectral overlapping (11, 12). Such a method can also be used for single component assay in the presence of different matrixes (13–15). Simultaneous multicomponent assays (16–19) using a derivative spectrophotometric method are still limited.

First and second derivative spectrophotometric methods are described in the present report for the assay of clotrimazole after acid hydrolysis in single formulations and in combination with azidamphenicol and dexamethasone.

Experimental

Apparatus and Reagents

(a) *Spectrophotometer.*—Perkin-Elmer Model 550S UV-Vis spectrophotometer with fixed slit width (2 nm) and Hitachi Model 561 recorder. Record spectra of test and reference solutions in 1 cm quartz cells over the range 440–220 nm. Suitable settings are as follows: scan speed 60 nm/min (clotrimazole) and 120 nm/min (azidamfenicol and dexamethasone); chart speed 60 mm/min; derivative mode $D_1 = dA/d\lambda$ (first derivative) and $D_2 = d^2A/d\lambda^2$ (second derivative); ordinate maximum and minimum ±0.2 clotrimazole (D_1) or azidamfenicol (D_1) and ±0.05 clotrimazole (D_2) or dexamethasone (D_1); response time 4 s.

(b) *Phenylhydrazinium sulfate reagent* (*20*).—Dissolve 65 mg phenylhydrazinium sulfate in sufficient volume of mixture of 170 mL H_2SO_4 and 80 mL water to produce 100 mL.

(c) *Commercial samples.*—(*1*) Canesten® cream, Canesten® topical solution, and Canesten® vaginal tablets—labeled to contain 10 mg/g, 10 mg/mL, and 100 mg/tablet, respectively (Alex. Co., Egypt, and Bayer, GFR). (*2*) Baycuten® cream—labeled to contain 10 mg clotrimazole, 10 mg azidamfenicol, and 0.4 mg dexamethasone/g (Alex. Co., Egypt, and Bayer, GFR).

(d) *Authentic drugs.*—Clotrimazole, azidamfenicol, dexamethasone—pure (Alex. Co., Egypt).

Preparation of Standards

Drug solutions of clotrimazole, azidamfenicol, and dexamethasone.—Prepare individual standard solutions to contain 100 mg of each drug in 100 mL methanol. Also prepare azidamfenicol and dexamethasone standard solutions in methanol to contain 100 mg/mL.

Solution of hydrolytic product of clotrimazole (*equivalent to 50 μg/mL of clotrimazole*).—Transfer 2.5 mL (equivalent to 2.5 mg) from standard solution of clotrimazole to 50 mL volumetric flask and evaporate to dryness on boiling water bath. Add 20 mL HCl, heat on boiling water bath 90 min, and cool to room temperature (≃25°C). Dilute to volume with methanol.

Preparation of Standard Calibration Curves

(a) *For azidamfenicol and clotrimazole.*—Prepare 2 separate concentration sets from azidamfenicol standard solution (100 mg/mL) and clotrimazole previously hydrolyzed with acid (50 μg/mL). In the first set, pipet 2–5.5 mL (in 0.5 mL steps) from azidamfenicol standard solution into a series of 25 mL flasks. In the second set, pipet 1–4.5 mL (in 0.5 mL steps for D_2 measurement and in 1 mL steps for D_1 measurement) from hydrolyzed clotrimazole standard solution in 25 mL volumetric flasks. Dilute the contents of all flasks in the 2 sets with methanol. Measure absolute values of D_1 at 288 nm (for azidamfenicol) and D_1 and D_2 at 302 nm (for clotrimazole).

(b) *For dexamethasone.*—Transfer different volumes of 1–4.5 mL (in 0.5 mL steps) of dexamethasone standard solution (100 μg/mL) to different 25 mL volumetric flasks. Dilute volume in each flask to 5 mL with methanol. Add 10 mL phenylhydrazinium sulfate reagent to each flask and mix well. Place in 60°C water bath for 20 min; then cool immediately to room temperature (ca 25°C). Measure absolute values of D_1 of each solution at 438 nm, using reagent blank.

Assay of Laboratory-Prepared 3-Component Mixtures

(a) *For clotrimazole and azidamfenicol components.*—Dilute dexamethasone standard solution with methanol to

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Table 1. Preparation of synthetic mixtures containing clotrimazole, azidamfenicol, and dexamethasone

Drug to be detd in mixt.	Concn range, µg/mL in mixt.		
	Clotrimazole	Azidamfenicol	Dexamethasone
Clotrimazole	3–9	4–9	0.12–0.4
Azidamfenicol	12–20	1–22	0.2–1.2
Dexamethasone	200–540	120–500	10–30

contain 4 µg/mL. Into 25 mL volumetric flasks, transfer different volumes of clotrimazole and azidamfenicol standard solutions (each of 100 µg/mL, prepared above) and also different volumes of the 4 µg/mL standard solution of dexamethasone in ratios represented in Table 1. Dilute to volume with methanol and measure absolute values of $D_1 \pm D_2$ at 302 nm (for clotrimazole) and D_1 at 288 nm (for azidamfenicol).

(b) *For dexamethasone component.*—Prepare separate stock solutions of clotrimazole and azidamfenicol in methanol, each containing 104 µg/mL and also of dexamethasone in methanol containing 100 µg/mL. Into 25 mL volumetric flasks, transfer different volumes of the above stock solutions in the ratios represented in Table 2. Continue the procedure as described under preparation of calibration curve for dexamethasone, beginning "Dilute volume . . .".

Preparation of Sample Solution

(a) *Topical solution.*—Accurately transfer volume of sample equivalent to 10 mg clotrimazole to 100 mL volumetric flask, and dilute to volume with methanol.

(b) *Creams and vaginal tablets.*—Transfer weight of cream or finely powdered tablets equivalent to 10 mg clotrimazole to 30 mL screw-cap centrifuge tube. Extract with two 20 mL aliquots of methanol by vigorous mechanical agitation for 10 min, followed by centrifugation. Combine and filter methanolic extracts into 100 mL volumetric flask. Dilute to volume with methanol.

Procedure

Transfer 25 mL from each of the above prepared solutions [(**a**) and (**b**)] into 50 mL volumetric flasks and evaporate to dryness on boiling water bath. Add 20 mL concentrated HCl, heat on boiling water bath 90 min, and cool to room temperature. Dissolve and dilute to volume with methanol. Measure

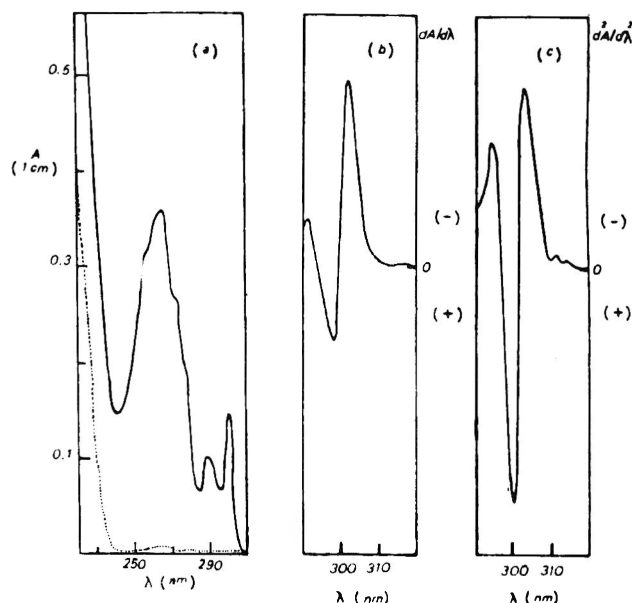

Figure 1. (a) Zero order absorption spectra of 6 µg/mL of clotrimazole in methanol before (---) and after hydrolysis (—); (b) first derivative spectra; (c) second derivative of hydrolytic products.

absolute values of D_1 and D_2 of each solution at 302 nm against solvent blank.

Determination of azidamfenicol in cream.—Transfer weight of cream equivalent to 10 mg azidamphenicol. Extract as described under clotrimazole creams. Transfer 8 mL extract to 25 mL volumetric flask and dilute to volume with methanol. Measure absolute value of D_1 at 288 nm against solvent blank.

Determination of dexamethasone in cream.—Transfer weight of cream equivalent to 5 mg dexamethasone (ca 12.5 g cream) to 250 mL stopper conical flask. Extract 3 times, each with 50 mL aliquot of methanol, by vigorous mechanical shaking for 10 min and transfer by washing through double filter paper to another 250 mL conical flask. Reduce volume of combined methanolic extracts and washings to about 30 mL by evaporation on boiling water bath. Cool to room temperature (ca 25°C) and transfer quantitatively to 50 mL volumetric flask with subsequent dilution, using methanol. Transfer 3 mL extract to 25 mL volumetric flask. Complete as described under dexamethasone assay, beginning "Dilute volume to 5 mL . . .".

Table 2. Preparation of laboratory-prepared mixtures of clotrimazole (Ct), azidamfenicol (Az), and dexamethasone (Dx)

Mixt. No.	Volume taken from different standard solutions								
	For Ct determination			For Az determination			For Dx determination		
	Ct[a]	Az[a]	Dx[b]	Ct[a]	Az[a]	Dx[b]	Ct[c]	Az[c]	Dx[d]
1	0.75	2.00	2.50	5.00	2.50	7.50	0.81	0.78	1.50
2	1.00	2.25	2.00	4.50	3.00	5.00	0.78	0.81	2.00
3	1.25	1.50	1.50	4.00	3.50	4.00	0.75	0.75	2.50
4[e]	1.50	1.50	1.50	4.00	4.00	4.00	0.75	0.75	3.00
5	1.75	1.50	1.50	4.00	4.50	4.00	0.75	0.75	3.50
6	2.00	1.00	1.25	3.50	5.00	1.25	0.60	0.30	4.00
7	2.25	1.25	0.75	3.00	5.50	2.50	0.30	0.18	4.50

[a] Standard solution of 100 µg/mL.
[b] Standard solution of 4 µg/mL.
[c] Standard solution of 104 µg/mL.
[d] Standard solution of 100 µg/mL.
[e] Laboratory prepared mixture of the 3 drugs in the same ratio as in the commercial preparation.

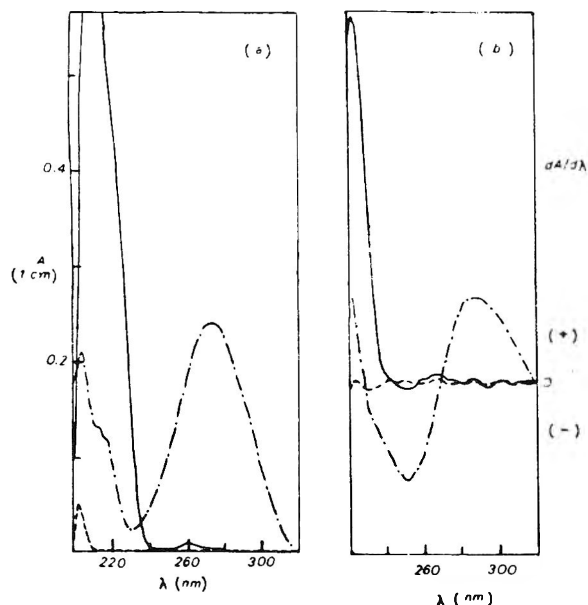

Figure 2. (a) Absorption spectra of 8 μg/mL of clotrimazole (—), 8 μg/mL of azidamfenicol (- • -), and 0.32 μg/mL of dexamethasone (---) in methanol; (b) their corresponding first derivative spectra.

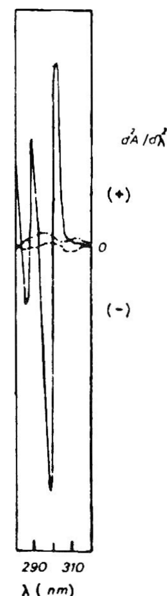

Figure 3. Second derivative spectra of 6 μg/mL of clotrimazole (—), 6 μg/mL of azidamfenicol (- • -), and 0.24 μg/mL of dexamethasone (---) in methanol after acid hydrolysis procedure.

Results and Discussion

Figure 1a shows the zero order UV spectra of clotrimazole and its acid-induced hydrolytic products in methanol. After hydrolysis, a characteristic peak appears at 264 nm with a hyperchromic effect (apparent $A^{1\%}_{1\,cm}$ = 600). The first and second derivative spectra of the hydrolytic products (Figures 1b and 1c) show a series of characteristic peaks from which the only wavelength at 302 nm has been selected for both D_1 and D_2 (analytical wavelength).

Figure 2a shows the zero order absorption of clotrimazole, azidamfenicol, and dexamethasone in concentration ratio (1:1:0.04) equal to their existence in commercial preparations. Figure 2b shows their corresponding first derivative curves. From this figure, it is clear that azidamfenicol can be determined by direct D_1-peak amplitude measurements at 288 nm. Clotrimazole has been determined in the mixture after its acid hydrolysis and subsequent measurement of D_2-peak amplitude at 302 nm (Figure 3). Other components in the mixture show no contribution after hydrolysis process (Figure 3).

As a minor component in the test mixture, dexamethasone cannot be assayed using derivative spectrophotometry. But the color reaction with phenylhydrazinium sulfate reagent (20) optimized the dexamethasone assay. A calibration graph correlating D_1 measurement at 438 nm and dexamethasone concentration (in a range of 8–22 μg/mL) has been constructed.

For clotrimazole and azidamphenicol, the correlation between D_1 or D_2 for the former, and D_1 for the latter, and their concentrations (in the range stated in Table 3) was linear with a negligible intercept.

Regression analysis using the method of least squares was done for the slope (b), intercept (a), and correlation coefficient (r) values (Table 3). Separate determinations at different concentration levels were carried out for each drug to assess the reproducibility. The coefficient of variation was less than 2%, indicating good reproducibility.

To prove the validity and applicability of the proposed methods, 7 synthetic mixtures were prepared with different proportions of drug components (Table 2) and analyzed for each drug using the proposed procedures. The results obtained were both precise and accurate (Table 4).

Commercial pharmaceutical preparations (creams, topical solutions, and vaginal tablets) of clotrimazole in single dosage forms and in combination with other drugs were assayed using the proposed method and the official titrimetric method (5). The 2 methods gave concordant results (Tables 4 and 5).

The results obtained show the high reliability, sensitivity, and reproducibility of the proposed methods, which require simple extraction and direct measurements.

Table 3. Assay parameters for derivative spectrophotometric determination of clotrimazole, azidamfenicol, and dexamethasone

Drug	Concn range, μg/mL	Derivative mode	Selected λ, nm	Regression equation[a] a (intercept)	b (slope)	r	CV,[b] %
Clotrimazole	2–10	D_1[c]	302	−0.0030	12.259	0.9999	0.22
		D_2[c]	302	−0.1071	11.429	0.9999	0.44
Azidamfenicol	8–22	D_1	288	−0.1964	3.595	0.9996	1.19
Dexamethasone	6.7–30	D_1[d]	438	−0.3277	2.392	0.9998	1.25

[a] f(λ) = a + bC, where f(λ) = D_1 or D_2 (measured in mm), whenever applicable, and C is concentration in μg/mL.
[b] Five separate determinations (at least).
[c] Measured after hydrolysis.
[d] Measured after derivatization.

Table 4. Recovery results[a] of clotrimazole, azidamfenicol, and dexamethasone in laboratory synthetic mixtures and cream

	Laboratory mixture[b]		Cream	
Concn taken, μg/mL	Recd, μg/mL	Rec., %	Recd, μg/mL	Rec., %
Clotrimazole[c]				
3	2.9	96.7	2.9	96.7
4	4.0	100.0	4.0	100.0
5	5.0	100.0	5.1	102.0
6	6.1	101.7	6.1	101.7
7	7.1	101.4	7.1	101.4
8	8.1	101.3	8.1	101.3
9	9.2	102.2	9.2	102.2
Mean		100.5		100.8
SD		±1.86		±1.93
Azidamfenicol				
10	10.0	100.0	10.0	100.0
12	12.2	101.7	11.9	99.2
14	14.0	100.0	14.1	100.7
16	15.7	98.1	15.9	99.4
18	17.9	99.4	18.3	101.7
20	20.0	100.0	20.1	100.5
22	21.8	99.1	21.9	99.6
Mean		99.8		100.2
SD		±1.10		±0.88
Dexamethasone[d]				
10.0	9.9	99.0	9.9	99.0
13.3	13.5	101.5	13.7	103.0
16.7	16.8	100.6	16.4	98.2
20.0	20.1	100.5	19.5	97.5
23.3	23.1	99.1	23.3	100.0
26.7	26.6	99.6	27.0	101.1
30.0	30.0	100.0	30.4	101.3
Mean		100.0		100.0
SD		±0.90		±1.93

[a] Recovery from added or nominal drug content.
[b] Concentration range as listed in Table 1.
[c] Measured after hydrolysis.
[d] Measured after derivatization.

Table 5. Recovery results[a] of clotrimazole[b] as single component in different dosage forms

Proposed method, D_1 (302 nm)			Official method (5)		
Concn, μg/mL		Rec., %	Concn, μg/mL		Rec., %
Taken	Recd		Taken	Recd	
Cream					
2	2.0	100.0	200	201.2	100.6
6	5.9	98.3	200	196.9	98.5
7	7.0	100.0	200	204.9	102.5
8	7.9	98.8	200	200.5	100.3
9	8.9	98.9	200	203.5	101.8
10	10.0	100.0			
Mean		99.3			100.7
SD		±0.76			±1.54
Vaginal tablets					
3	3.0	100.0	1000	1004.2	100.4
5	5.0	100.0	1000	996.7	99.7
6	6.0	100.0	1000	1007.9	100.8
7	7.1	101.4	1000	1034.0	103.4
9	9.1	101.1	1000	1037.7	103.8
10	10.1	100.0			
Mean		100.4			101.6
SD		±0.65			±1.85
Topical solution					
4	4.0	100.0	200	200.5	100.3
6	6.0	100.0	200	197.6	98.8
7	7.1	101.4	200	204.2	102.1
8	8.1	101.3	200	201.2	100.6
9	9.1	101.1	200	199.7	99.9
10	10.0	100.0			
Mean		100.6			100.3
SD		±0.70			±1.20

[a] Recovery from added or nominal drug content.
[b] Measured after hydrolysis.

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Determination of Size Distribution of Fat Globules in Intravenous Fat Emulsions by Photon Correlation Spectroscopy

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A photon correlation spectroscopy method has been developed to characterize the size distribution of fat globules in intravenous fat emulsions (IFE) in terms of mean diameter, standard deviation of the distribution, and percentage of large particles outside the distribution. Mean fat globule diameters of samples of all IFE products available in Canada were about 0.3 μm, similar to values reported in the literature. The methodology is sufficiently sensitive to detect the presence of 5% by weight of 2 μm polystyrene microspheres in an intravenous fat emulsion. The effect of changes in instrument settings and variables on the results has been evaluated.

Intravenous fat emulsions (IFE) are an aqueous suspension of 10 or 20% soybean and/or safflower oil, an emulsifying agent which is usually a mixture of egg phosphatides, and glycerol for isotonicity. The individual fat globules have diameters in the range 0.1–0.5 μm and are meant to mimic endogenous chylomicrons (1). The measurement of mean diameters and distributions of fat globules in IFE by photon correlation spectroscopy (PCS) has been reported by several authors (2–7). However, we did not find a comprehensive method supported by an evaluation specific to IFE of experimental and instrumental variables and the mode of calculation on the results. In the present paper, the method is preceded by an overview of the theory of the instrument. Results obtained on marketed products are given, as well as evidence of sensitivity of the method to changes in particle size distribution and instrumental and experimental variables.

Theory

The general theory of photon correlation spectroscopy has been described elsewhere (8–10) and only a brief overview will be given here. The technique is based on measurement of the time-dependent fluctuations in laser light scattered from particles in suspension or molecules in solution. The scattered light fluctuates as a result of the constant (Brownian) random motion of the suspended particles. The instrument analyzes the intensity of the fluctuations to obtain a translational diffusion coefficient, D, and calculates the diameter of the particles by using the Stokes-Einstein equation:

$$D = kT/3\pi\eta d$$

where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium, and d is the spherical diameter of the particles.

The Coulter Model N4 photon correlation spectrometer used in this work treats scattering data by unimodal analysis or size distribution processor (SDP) analysis, at the option of the operator. Unimodal analysis is a cumulant process which interprets the data as a single set of particles with a log Gaussian distribution. The instrument calculates the mean diameter and standard deviation of the particles. SDP analysis uses a constrained regularization of linear equations program (11). The constraint forces the result to be positive and regularization effectively smooths the distribution and provides the simplest solution. In the case where it is not possible to differentiate mathematically between a unimodal and bimodal distribution, a unimodal solution is chosen. The dust term in the SDP analysis represents slowly decaying components of the autocorrelation function. These are due to large particles, gas bubbles, and convection currents in the sample. Histograms of the intensity and weight distribution and tables of results which include the diameter, relative amount, and the standard deviation of the histogram components from the mean value of each peak are provided. The instrument initially calculates an intensity vs fat globule size distribution from which a fat globule weight size distribution is determined. This last calculation uses the Mie equation (8) and requires the complex refractive index, as well as the angle of scatter. If the complex refractive index is not known, the instrument uses an approximate value. The intensity of light scattered from particles with diameters larger than approximately 0.2 μm is an oscillating function with angle and diameter dependence. For example, 0.9 μm particles give maxima in scattering intensity at 52 and 87° and a minimum at 110°, whereas, 40 nm particles scatter light with equal intensity at all angles (12).

Stock and Ray (11) compared 6 mathematical methods for the analysis of PCS data and, although none was ideal, the method similar to SDP analysis was the most reliable for analyzing experimental data. They recommended that at least 2 independent methods of data analysis be used to confirm the calculated distributions. Because of the complex and varied data treatments available for PCS experiments, it is important that instrument manufacturers inform the user of the mode of calculation used, and that researchers report their experimental procedures in detail.

METHODS

Apparatus

(a) *Photon correlation spectrometer.*—Model N4, equipped with 128 channels, detector set at 90°, sample compartment thermostatted at 20°C, and software version 9.1 (Coulter Electronics, Hialeah, FL 33010). Calibrate according to procedure provided by manufacturer. Instrument was connected to computer (Model IIe, Apple Computer, Cupertino, CA 95014) via RS-232 interface for data manipulation and storage.

(b) *Fluorescence cells.*—Optical glass (Hellma Ltd, Toronto, Ontario, Canada).

Reagents and Chemicals

(a) *Polystyrene microspheres.*—Latex suspensions: 0.040 and 2.02 μm (Coulter Electronics).

(b) *Intravenous fat emulsions.*—(Abbott Laboratories, Montreal, Quebec H4P 1A5; Pharmacia (Canada) Inc., Dorval, Quebec H9P 1H6; Alpha Therapeutic Corp., Los Angeles, CA 90032; and Travenol Laboratories Inc., Deerfield, IL 60015).

(c) *Phosphate buffer.*—Transfer 50 mL 1M potassium dihydrogen phosphate solution and 39.1 mL 1M sodium hydroxide solution to 200 mL volumetric flask, dilute to volume with distilled, filtered water, mix, and pass through 0.22 μm membrane filter (Millipore, Bedford, MA 01730).

(d) *Buffered water.*—Pass water through ion-exchange cartridges, Model NANOpure II system with Organicfree cartridge (Barnestead, Boston, MA 02132) and filter

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Table 1. Example of raw data from single sample run[a]

	Unimodal analysis		SDP analysis			
Run	Diam., nm	SD[b]	Diam., nm	SD	Amount, %[c]	Dust, %
1	284	79	318	150	100	0
2	286	64	316	46	100	0.9
3	290	62	316	46	100	0
4	286	64	316	46	100	0.9
5	289	65	316	46	100	0
6	286	67	306	46	100	1
7	282	77	456	480	100	0
8	285	67	309	100	100	0
9	284	75	303	48	100	3
10	285	71	304	47	100	2
Mean	286	69	326	105		0.8
RSD, %	1	9	14	29		132

[a] Sample P 20% fat.
[b] Mean of standard deviations of individual particle distributions.
[c] Weight percent total solids represented by first peak.
None of the runs represented in this table yielded double peaks.

through 0.22 μm filter (Millipore). Add 5 mL phosphate buffer to each liter to give final pH of 7.8.

(e) *Standard preparations.*—A: Dilute 40 nm polystyrene microsphere latex suspension with buffered water to ca 6 μg/mL. B: Add polystyrene spheres with nominal mean diameter in range 1.8–2.2 μm to diluted IFE to final concentration of 10% polystyrene spheres in total solids content of 20 μg/mL.

(f) *Test preparation.*—Transfer amount of IFE equivalent to 10 mg fat to 10 mL volumetric flask, dilute to volume with buffered water, and mix. Transfer 50 μL of this solution to sample cuvet containing 3.5 mL buffered water, cap cuvet, and invert 10 times to mix contents.

System Suitability

Using method given in *Procedure*, measure mean particle diameter of standard preparation A. Mean diameter by cumulant (unimodal) analysis must fall between 35 and 45 nm with relative standard deviation <3% for 10 consecutive runs of 400 s. Perform 10 runs of 400 s each with standard preparation B and analyze results by constrained regularization (SDP) or similar method. Resulting distributions must be either single peaks with mean dust level >2% or double peaks.

Procedure

Mix IFE sample by inverting container 25 times. Transfer amount equivalent to 10 mg fat to 10 mL volumetric flask, dilute to volume with buffered water, and mix. Transfer 50 μL of this solution to cuvet containing 3.5 mL buffered water, cap cuvet, and invert 10 times to mix contents. Place cuvet in PCS instrument and allow to equilibrate to 20°C for 15 min. Do 10 scattering experiments (runs) at instrument angle of 90°. Calculate mean fat globule diameter by unimodal and SPD data treatment procedures. Report mean diameters and standard deviations with their associated relative standard deviations and mean percentage of calculated dust.

Results and Discussion

The method requires that 10 runs be made on each sample. These are done without opening the sample cuvet or removing it from the photon correlation spectrometer. Each individual run gives diameters and standard deviations by the unimodal and SDP methods of calculation. Histograms of the distributions are also given. Results obtained from a typical experiment of 10 individual runs are given in Table 1 and histograms of a single, typical run are shown in Figure 1. The data in Table 1 show that no individual run can be relied on to characterize a sample. For example, compared with most runs in Table 1, Run 7 gave an unusually large diameter and standard deviation, and Run 1 a large standard deviation, both by the SDP method. Individual runs are subject to random instrumental and sample variations. Calculation of the standard deviation is critically dependent on an accurate determination of the baseline (13) and the dust level, which varies considerably from run to run.

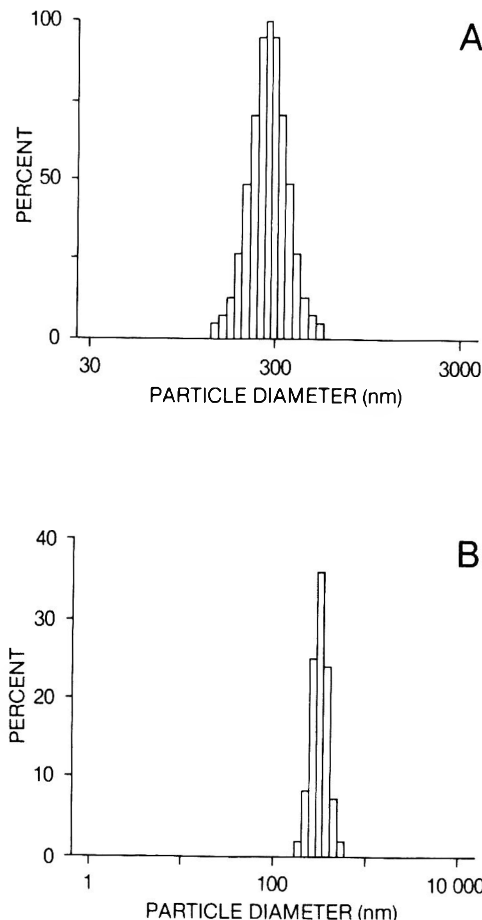

Figure 1. Typical fat globule size distributions by photon correlation spectroscopy showing distribution curves calculated by unimodal (A) and SDP (B) methods.

Representative samples of commercial IFE products, available in Canada from 4 manufacturers, were analyzed by the method described in this paper (Table 2). These include 10 and 20% emulsions, some of which were beyond their expiry date when analyzed. Mean fat globule diameters of all samples, by both methods of calculation, were between 234 and 367 nm, with most less than 300 nm. The results show that fat globules in the 20% emulsions are significantly larger than in the 10% emulsions (Tables 2 and 3). Diameters calculated by the SDP method are usually about 15% larger than those calculated by unimodal analysis. No generalizations can be made about differences between expired and unexpired samples. Mean fat globule diameters reported in the literature range from 184 to 400 nm (1–6). These are in general agreement with results obtained by transmission electron microscopy (14), sedimentary field flow fractionation (15), and fiber optic Doppler anemometry (16).

Undisturbed IFEs develop a concentrated, cream-like layer in the upper part of the container after a few months.

Table 2. Photon correlation results for commercial IFE products

Sample	Fat, %	Expiry date	Unimodal analysis		SDP analysis		
			Diam., nm[a]	SD, nm[b]	Diam., nm	SD	Dust, %
P	10	05/86	242(1)	54(2)	281(1)	73(1)	0.2(176)
P	20	12/84	243(1)	55(9)	282(1)	72(2)	0.2(179)
Q	10	09/87	234(1)	52(11)	272(4)	77(36)	0.2(240)
Q	20	04/87	300(1)	74(22)	312(2)	58(33)	2.1(190)
R	10	06/85	263(1)	71(7)	290(5)	79(40)	1.7(108)[c]
R	20	12/87	272(1)	74(8)	303(7)	90(40)	1.5(127)
S	10	01/85	247(1)	66(8)	292(4)	72(15)	1.0(108)[d]
S	20	12/86	314(1)	86(8)	367(17)	161(85)	1.2(175)
T	10	11/88	276(1)	74(10)	312(8)	88(58)	1.3(157)
U	10	05/89	252(1)	60(6)	291(3)	65(11)	0.6(146)
U	20	08/89	286(1)	69(9)	326(14)	106(129)	0.8(132)

[a] Values in parentheses are relative standard deviations (%).
[b] Mean of standard deviations of individual particle distributions.
[c] One doublet in SDP analysis: 32 nm (60%) and 300 nm (40%).
[d] One doublet in SDP analysis: 283 nm (81%) and 1780 nm (19%).

Samples taken from this layer were analyzed and compared with results obtained after the total contents of the container were mixed. Because of the concentration difference, twice the volume of the mixed IFE was taken for dilution and analysis, although the analysis is not dependent on concentration (see below). Globules in the cream layer are from 8 to 67% larger than in the mixed sample as a whole (Table 3). The tendency for large globules to segregate in the cream layer is manufacturer specific and does not appear to be related to the expiry date of the sample.

Sensitivity of Methods to Changes in Particle Size Distribution

An effective standard for fat globule size distribution must not only yield values for the mean diameters, but must also respond to abnormalities in the fat globule distribution of the product under test. The sensitivity of the method to bimodal distributions was demonstrated by measurements on mixtures of polystyrene spheres of different diameters and by adding polystyrene spheres to IFE samples.

Polystyrene microsphere latex suspensions used had mean particle diameters of 0.040, 0.17, 0.31, 0.50, 2.02 and 5.0 μm (Coulter Electronics); 0.30 and 3.0 μm (Eastman Kodak, Rochester, NY 14650); 0.46 μm (Sigma Chemical Co., St Louis, MO); and 0.895 μm Standard Reference Material (SRM) 1690 (National Institute of Standards and Technology, Gaithersburg, MD 20899). The solids content of the polystyrene latexes were determined by evaporation to dryness at 60°C. Values found were 2.33% for Coulter (L)3000, nominal mean diameter 0.31 μm and 2.51% for Kodak (L)1, nominal mean diameter 2.0 μm. Samples for PCS measurement were prepared by diluting the latexes to a final total solids concentration of 14 μg/mL.

Particle size distribution measurements were made for mixtures of 0.31 and 2.0 μm polystyrene spheres over the entire range from 100% 0.31 μm spheres to 100% 2.0 μm spheres. Scattering levels were 119 000 and 228 000 counts/s, respectively. Results of analyses by the unimodal and SPD methods are given in Table 4. Thus, 9 runs on the sample containing 5% polystyrene gave a mean unimodal diameter of 380 nm and a mean peak standard deviation of 119. SPD analysis of the same 9 runs represented the data as 5 runs with a single peak at 387 nm and 4 runs with bimodal peaks at 320 and 1023 nm. Mean diameters calculated by unimodal analysis were a linear function (r^2 = 0.997) of the weight percent of the 2 components in the mixture (Table 4). By SPD analysis, as the proportion of the larger diameter component of the mixture increases, the analysis yields an increasing proportion of doublet peaks. For example, at 1% of the larger component, there are 2 doublets in 9 runs, but at 10% there are 8 doublets in 10 runs and at 20%, all results are reported as doublets. For this mixture the technique was sensitive to 1% of the larger particles in the suspension.

Well characterized fat emulsions containing globules of different sizes and distributions are not available. Attempts to prepare such emulsions by controlled agglomeration of fat globules in IFEs by freeze-thaw cycles or by addition of divalent calcium and magnesium ions were unsuccessful. Therefore, the sensitivity of the method to particles outside the globule size distribution in IFE was investigated by adding polystyrene spheres to IFE. Globule size diameter data from mixtures of 2.0 μm polystyrene spheres and IFE show that both the diameter and peak width increase as the percentage by weight of polystyrene spheres increases (Table 5). The SDP analysis of a bimodal mixture may yield results in 1 of 3 forms: a single peak with a high percentage of dust, a single, broad peak with no dust, or 2 peaks. Data for both

Table 3. Mean globule diameters and standard deviations of IV fat emulsions

Sample (% fat)	Mixed[a]	Unimodal analysis		SDP analysis		
		Diam., nm[b]	SD, nm[c]	Diam., nm	SD	Dust, %
P(10)	N	280(1)	70(16)	306(5)	69(43)	1.0(137)
	Y	242(3)	63(12)	284(8)	81(21)	0.7(193)
P(20)	N	330(1)	91(9)	376(5)	154(42)	1.1(145)
	Y	282(1)	73(9)	309(3)	81(50)	0.7(178)
Q(10)	N	407(1)	122(5)	497(9)	166(14)	1.2(141)
	Y	283(1)	79(11)	337(9)	141(36)	0.1(364)
Q(20)	N	438(1)	130(6)	550(16)	240(40)	1.5(154)
	Y	329(1)	91(6)	349(7)	120(57)	1.6(145)
R(10)	N	357(2)	104(12)	415(17)	162(35)	1.3(145)
	Y	268(1)	78(3)	304(7)	110(48)	1.6(117)
R(20)	N	385(1)	119(4)	545(18)	309(69)	1.4(280)
	Y	285(2)	76(12)	327(14)	113(92)	1.3(151)
S(10)	N	275(3)	72(6)	307(6)	82(57)	0.9(140)
	Y	242(3)	60(13)	280(2)	74(5)	0.8(143)
S(20)	N	347(1)	97(6)	397(11)	42(15)	0.9(194)
	Y	313(2)	83(7)	338(3)	23(49)	1.2(158)

[a] N = analysis of cream layer before mixing; Y = after mixing.
[b] Values in parentheses are relative standard deviations (%).
[c] Mean of standard deviations of individual particle distributions.

Table 4. Mean diameters and standard deviations of polystyrene sphere mixtures[a]

		Unimodal analysis		SDP analysis					
				First peak		Second peak			
PS[b]	No. of runs	Diam., nm[c]	SD, nm[d]	Diam., nm	SD	Diam., nm	SD	First peak, %[e]	Dust, %
0	10	308(1)	66(26)	312(2)	46(0)	—	—	100	2(99)
1	7	330(4)	89(9)	397(27)	181(103)	—	—	100	3(103)
	2			319	52	6600	930	40	3(141)
5	5	380(5)	119(8)	387(10)	140(20)	—	—	100	5(34)
	4			320(3)	62(25)	1023	215	49	0(0)
10	2	420(4)	154(6)	406	150	—	—	100	14(5)
	8			339(11)	80(57)	2580(58)	573(70)	42	1(280)
20	10	686(49)	245(5)	467(24)	179(22)	2510(30)	710(36)	18	1(316)
50	9	1099(5)	399(3)	354(21)	105(83)	1910(22)	511(48)	8	2(229)
100	7	1743(3)	484(9)	1790(13)	400(60)	—	—	100	4(66)

[a] Mixtures of polystyrene spheres, mean diameters 0.31 and 2.0 μm, in latex suspensions.
[b] Weight percent of 2.0 μm polystyrene spheres in total solids.
[c] Values in parentheses are relative standard deviations (%).
[d] Mean of standard deviations of individual particle distributions.
[e] Percent of total solids represented by first peak.

types of single peaks have been combined (Table 5). At low levels of 2.0 μm polystyrene spheres, SDP analysis usually yielded a single peak with a high percentage of dust. The dust term is used in SDP analysis to account for slowly decaying components of the autocorrelation function which may result from large particles, gas bubbles, or convection currents. As the level of polystyrene spheres increases, the instrument is more successful in determining the bimodal character of the suspension. For example, of 10 runs at the 5% polystyrene level, 9 gave a single peak with an increased mean dust level and 1 gave a double peak (Table 5).

The sensitivity of the method to polystyrene spheres of other diameters is given in Table 6. The addition of 50% 0.33 μm polystyrene spheres to an IFE had little effect on the results; these spheres were of similar diameter to the fat globules themselves. This shows that the instrument is responding to the particle size distribution and not some coincidental factor, such as refractive index or particle agglomeration. Mixtures containing 10% of 0.99 and 2.95 μm polystyrene spheres yielded a significant number of doublet peaks at the 10% level (Table 6). The larger 2.95 μm particles are near the upper limit of photon correlation spectrometry, where Brownian movement of the massive 3 μm particles is sluggish.

Instrumental and Experimental Variables

The robustness of the method was investigated by changing the instrument settings and experiment variables.

Run time.—This is the period over which data are collected and is usually about 400 s. Given sufficient scattering intensity, the percentage error in calculated diameter is inversely proportional to the square root of the run time. Samples containing large particles and those with bimodal distributions require longer run times to achieve satisfactory reproducibility.

Sample time.—The sample time is the time delay between products used in the calculation, usually about 40 μs for IFE. It can be thought of as the time interval between measurements of the intensity of the scattered light and should be chosen to match the speed of the particles undergoing Brownian motion. The sample time may be entered manually, calculated by the instrument from a manually entered estimate of the particle diameter, or calculated by an autoranging function of the instrument. The latter method was used in this paper. At an angle of 90°, entering diameters of 40, 170, and 900 nm led to sample times of approximately 5.2, 22, and 118 μs. As the angle decreases from 90° the sample time increases. For the 170 nm standard, the sample times, calculated from estimated diameters, for angles of 63.2, 29.8, 22.9,

Table 5. Mean diameters and standard deviations of IFE-polystyrene sphere mixtures

		Unimodal analysis		SDP analysis					
				First peak		Second peak			
PS[a]	No. of runs	Diam., nm[b]	SD, nm[c]	Diam., nm	SD	Diam., nm	SD	First peak, %[d]	Dust, %
0	10	234(3)	52(9)	272(1)	77(2)	—	—	100	0.2(214)
5	9	237(1)	63(12)	284(4)	96(33)	—	—	100	0.9(109)
	1			274	80	2590	820	80	
10	6	243(2)	75(5)	243(2)	89(5)	—	—	100	8.0(19)
	1			287	100	2650	800	79	
20	6	263(2)	89(5)	289(8)	86(31)	—	—	100	8.0(19)
	3			272(24)	145(36)	1316(81)	387(100)	56	
50	7	397(10)	broad[e]	311(16)	123(44)	4056(60)	1106(58)	21	
100	7	1740(5)	510(9)	1893(21)	433(45)	—	—	100	5.0(76)

[a] Weight percent in total solids of 2.0 μm polystyrene spheres (Kodak) in Sample D, 10% fat, diluted to 20 μg/mL.
[b] Values in parentheses are relative standard deviations (%).
[c] Mean of standard deviations of individual particle distributions.
[d] Weight percent of total solids represented by first peak.
[e] Coulter software term for unusually large SD.

Table 6. Mean diameters and standard deviations of IFE–polystyrene sphere mixtures: effect of varying sphere diameter

				SDP analysis					
		Unimodal analysis		First peak		Second peak			
PS[a]	No. of runs	Diam., nm[b]	SD, nm[c]	Diam., nm	SD	Diam., nm	SD	First peak, %[d]	Dust, %
0	10	242(3)	60	280(2)	74	—	—	100	
0.33 μm spheres: Sigma (L)66F-0059									
50	10	292(3)	67(10)	310(3)	44(26)	—	—	100	1.4(119)
100	10	339(2)	70(14)	338(2)	68(13)	—	—	100	0.4(246)
0.99 μm spheres: Sigma (L)17F-0003									
10	5	240(1)	64(8)	237(7)	97(15)	—	—	100	0.1(223)
	5			146(41)	24(65)	488(58)	70(65)	59	0.8(224)
50	10	313(1)	110(0)	177(19)	34(29)	626(37)	97(20)	31	3.8(101)
100	10	1040(2)	280(7)	1038(6)	136(69)	—	—	100	3.1(85)
2.95 μm spheres: Sigma (L)105F-0711									
10	8	234(1)	60(5)	287(9)	136(71)	—	—	100	0.6(182)
	2			196	46	1000	149	100	0
50	6	261(2)	90(3)	275(4)	107(35)	—	—	100	9(19)
	4			216(20)	65(29)	1246(48)	252(76)	35	
100	10	2739(8)	918(13)	2740(22)	834(116)	—	—	100	15(78)

[a] Percentage by weight of polystyrene spheres in IFE, Sample D, 10% fat.
[b] Values in parentheses are relative standard deviations (%).
[c] Mean of standard deviations of individual particle distributions.
[d] Weight percent of total solids represented by first peak.

15.6, and 11.0° were 40, 168, 284, 588, and 1203 μs, respectively. Thus, both larger particle diameters and lower angles of detection require increased sample time. The sample temperature and diluent viscosity also affect the sample time and are therefore held constant.

Sample concentration.—The concentration should be chosen to obtain scattering intensity in the manufacturer's recommended range of 50 000 to 1 000 000 photon counts/s, usually between 1 and 200 μg/mL. The intensity of scattered light is also dependent on the angle of scatter and the volume, shape, and flexibility of the particles. The instrument provides a means of checking the scattering intensity at 90°. Counts below the recommended range result in a decreased signal-to-noise ratio, and high counts may result in saturation of the electronics. High concentrations of sample result in particle–particle interaction and in photons being scattered by more than 1 particle. When the concentration of 170 nm polystyrene microspheres was varied from 2.5 to 200 μL of latex suspension in 3.5 mL water, the photon counts increased from 16 000 to 1 310 000, but the unimodal diameter remained within the range from 171 to 179.4 nm, with a mean of 175.7 nm, based on 7 runs. The reproducibility of the method was further evaluated using the 170 nm standard at a sample preparation which gave about 500 000 counts/s. The mean unimodal diameter for sets of 4 determinations per sample using 4 different samples over 4 consecutive days was 175.3 nm with an RSD of 1.3 and for 5 sets of 4 determinations per sample within 1 day was 177.9 with an RSD of 0.6%.

Angle of detection.—The photon correlation spectrometer used for this work was equipped with a multiangle detector (90, 63.2, 29.8, 22.9, 15.8, or 11.6°). Particle diameters and distributions of 7 polystyrene sphere latexes ranging from 0.04 to 3.0 μm were measured at the 6 angles. Mean diameters calculated by the unimodal procedure are compared to the nominal diameters in Table 7. In all cases the results depend on the scattering angle, especially for the larger particles. A similar pattern emerges when the mean diameters are

Table 7. Mean unimodal diameters at various scattering angles

	Diameter, nm[b]					
Nominal diam., nm[a]	90.0°	63.2°	29.8°	22.9°	15.6°	11.0°
40	42.6(0.2)[c]	43.1(0.6)	44.3(1.1)	44.0(2.4)	46.3(2.3)	44.8(5.6)
170	174(0.5)	172(0.8)	185(1.8)	169(2.8)	199(6.5)	188(1.4)
300	354(1.0)	373(2.4)	396(1.5)	372(16)	443(1.8)	424(7.0)
310	319(1.6)	346(2.6)	357(5.5)	341(4.0)	389(2.9)	359(8.4)
460	495(1.1)	512(1.8)	343(2.2)	487(2.5)	364(16)	379(20)
898	912(1.3)	958(2.2)	907(2.3)	1198(12)	var.[d]	598(14)
2020	2050(2.4)[c]	2115(1.9)	2031(2.2)	2754(11)	var.	1876(74)
3000	2645(2.0)	2986(2.3)	var.	3282(8.6)	var.	var.

[a] Samples were diluted with filtered water then sonicated 15–30 s.
[b] Diameters are means of 5 determinations except where indicated. Values in parentheses are relative standard deviations (%).
[c] Mean of 10 determinations.
[d] var. = variable results, i.e., results included both large positive and negative values for diameters in 1 set of runs.

Table 8. Mean SDP diameters at various range settings[a]

Range setting, nm	Diam., nm[b]	SD, nm[c]	Dust, %
1-10 000	309(2)	69(29)	0.4(137)
10-10 000[d]	307(0.8)	81(37)	0.2(163)
100-10 000[e]	310(10)	129(48)	0.3(73)
3-3000	286(4)	108(29)	0.4(223)
30-3000	304(7)	105(48)	0.2(234)

[a] Data are means of 5 runs. IFE sample was Mfr R, 20% fat.

[b] Values in parentheses are relative standard deviations (%).

[c] Mean of standard deviations of individual particle distributions.

[d] One doublet peak in set of 10 runs.

[e] Two doublet peaks. Set limit of 100 nm encroaches on lower end of fat globule distribution.

calculated by the SDP method. The results at 90° are in closest agreement with the nominal diameters of the microspheres. All IFE data presented in this report were obtained at 90°.

Diameter range.—The particle size range over which the SPD calculation is made can be set between 1 and 10 000 nm. Changes in the range (Table 8) result in changes to the calculated diameter, but these are small compared with the diameter. The range used was 1-10 000 nm.

Conclusions

The objective of this work has been to develop methodology to serve as the basis of a test method and specifications for fat globule diameters and fat globule size distributions in IFE. We have shown that mean fat globule diameters determined by the method described agree with previous results obtained by PCS and other techniques. The method is sensitive to particles outside the size distribution of fat globules in IFE and is not unduly sensitive to sample and instrumental variables.

Fat globules in IFE can be characterized by their mean diameter and diameter distribution. The mean globule diameters in all products examined were well below 500 nm. This would appear to be an appropriate upper value from the manufacturing point of view and the range is physiologically sound. Particles with diameters greater than approximately 7 μm become trapped in the capillary bed of the lungs due to mechanical filtration (17, 18). Abnormally broad fat globule distributions are revealed by large standard deviations of the mean diameter, determined by either the unimodal or SDP calculations, double peaks indicative of a bimodal distribution, or high dust levels. Indications of abnormality would be standard deviations over 150 nm, double peaks, or dust levels above 2%.

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Liquid Chromatographic Determination of Miconazole Nitrate in Creams and Suppositories

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A rapid method has been developed for the determination of miconazole nitrate in creams and suppositories. The sample is dissolved in ethanol, diluted in acetonitrile–water (1 + 1), and injected onto a C18 column. The mobile phase consists of 55% acetonitrile, a triethylammonium phosphate buffer, and an ion-pairing agent. The total run time is less than 4 min, and the active ingredient is determined using absorbance detection at 214 nm. The mean recovery of miconazole from spiked placebo samples was 99.7 ± 0.7% for the cream samples at the 2% level and 98.8 ± 0.3% for the suppository samples at the 4% level.

Miconazole nitrate, 1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-1*H*-imidazole mononitrate, is widely used as a topical antifungal agent at a level of 2% in creams and 4% in suppositories. The current USP method for miconazole nitrate (1) requires extractions using chloroform, evaporation to dryness, crystallization from chloroform-pentane, and re-solution in chloroform–methanol. The compound is finally determined by gas chromatography using flame-ionization detection.

A search of the literature suggested that several methods (2–5) based on reverse-phase liquid chromatography would be simpler and less time consuming than the USP method. These methods generally have run times in excess of 5 min and/or use reagents such as THF in the sample preparation. In addition, the published methods do not consider possible co-elution of BHA with miconazole.

In the procedure presented here, ethanol is used to extract the miconazole, the total run time is less than 4 min, and a wavelength of 214 nm is used to give increased response over the usual wavelengths of 230 and 254 nm.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Model 6000A pump, or equivalent, operating at 2.0 mL/min; WISP autosampler, or equivalent; 15 cm × 4.6 mm μBondapak C18 analytical column incorporating C18 Guard Pak; Model 441 fixed wavelength detector, or equivalent, operating at 214 nm and 0.05 AUFS; and Model 730 Data Module, or equivalent (all equipment from Waters Associates, Inc.).

(b) *Chemicals and reagents.*—Organic-free water was prepared by passing reverse osmosis water through a Milli-Q™ water purification system (Millipore Co., Bedford, MA 01730). LC quality triethylamine, LC quality acetonitrile, reagent grade phosphoric acid, reagent grade ethanol (Fisher Scientific Co.); miconazole nitrate (Sigma Chemical Co.); 1-octanesulfonic acid Na salt (Kodak).

(c) *Buffer solution.*—Dilute 100 mL triethylamine to ca 900 mL with water, add 80 mL 85% phosphoric acid, mix cautiously, cool to room temperature, and dilute to 1 L with water.

(d) *Mobile phase.*—Dissolve 1.00 g 1-octanesulfonic acid Na salt in ca 300 mL water, add 10.0 mL buffer solution and 550 mL acetonitrile, and dilute to 1 L with water. Mix well and pass solution through 0.45 μm membrane filter.

(e) *Stock standard solutions.*—Accurately weigh ca 80 mg miconazole nitrate into 100 mL volumetric flask. Dissolve in ethanol, dilute to volume with ethanol, and mix well to prepare stock solution. Dilute stock solution 10:100 with ethanol to prepare intermediate solution. These solutions are stable for 1 week.

Standard Preparation

Dilute intermediate standard solution 5:100 with acetonitrile-water (1 + 1). Prepare this solution fresh daily.

Sample Preparation

Accurately weigh sufficient sample to contain 8 mg miconazole nitrate into 100 mL volumetric flask. Add ca 80 mL ethanol and cautiously warm on steam bath to dissolve sample. Cool to room temperature, dilute to volume with ethanol, and mix well. Dilute 5:100 with acetonitrile-water (1 + 1). Filter portion through 0.45 μm membrane filter before injection.

Procedure

Inject equal volumes (e.g., 20 μL) of standard and sample solutions. Duplicate injections of standard solutions should differ by less than 2%. Calculate results by using the following formula:

$$\text{Miconazole nitrite, \% (w/w)} = (A/A') \times (C'/C) \times 100$$

where C′ = concentration of standard solution in mg/100 mL; C = concentration of sample solution in mg/100 mL; A = area of sample solution miconazole peak; and A′ = area of standard solution miconazole peak. Report final results to 0.01%.

Results and Discussion

The absorption spectrum of miconazole (Figure 1) was obtained using a Hewlett-Packard Model 1040A diode array system. This scan shows that miconazole does not have significant absorbance at 254 nm, but as the wavelength decreases, the absorbance increases. We chose 214 nm to allow use of the fixed-wavelength zinc lamp detector. Analysts in possession of a multi-wavelength detector may prefer to work at 230 nm. At 230 nm, the miconazole response is approximately 70% that of the response at 214 nm, and baseline stability may be slightly improved.

Initial chromatographic work was done using a mobile phase consisting of 550 mL acetonitrile and 10.0 mL buffer solution diluted to 1 L with water. Unfortunately, in this mobile phase, miconazole and BHA co-eluted; octanesulfonic acid ion-pairing agent was added to give increased retention to miconazole. The chromatographic scan of BHA, miconazole, and BHT is shown in Figure 2.

The acetonitrile concentration of the mobile phase may be adjusted to compensate for differences in column length. For example, a mobile phase containing 60–65% acetonitrile rather than 55% acetonitrile gives excellent separation between BHA and miconazole when a 30 cm column is substituted for a 15 cm column. In addition, the detector attenuation may be adjusted to yield an optimum response.

To test response linearity, a calibration curve was constructed in the range of 0.04–0.60 mg miconazole/100 mL; the correlation coefficient (r) was found to be 0.9993.

With respect to precision, this method does not use an internal standard, and, therefore, quantitation depends on

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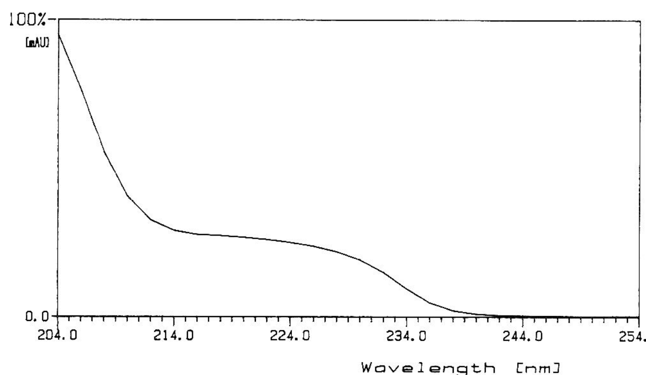

Figure 1. Absorption spectrum of 0.80 mg miconazole/100 mL mobile phase.

injection volume reproducibility. This reproducibility must be checked by injecting the standard solution several times and determining the relative standard deviation of the resulting peak areas. This standard deviation is always less than 0.5% when the equipment is working properly. It is recommended that the analyst perform this type of test if the difference between duplicate injections is more than 2%.

Table 1. Recovery of miconazole added to 400 mg portions of placebo cream and to 200 mg portions of placebo suppository

Added, mg	Cream		Suppository	
	Found, mg	Rec., %	Found, mg	Rec., %
0.8	0.800	100.0	0.936[a]	117.0[a]
	0.768	96.0	0.821	102.6
	0.765	95.6	0.808	101.0
	0.795	99.4	0.815	101.9
	0.776	97.0	0.841	105.1
	0.800	100.0	0.813	101.6
Av.	0.784	98.0	0.820	102.4
SD	0.016		0.013	
CV, %	2.04		1.59	
4.0	3.987	99.7	4.071	101.8
	4.043	101.1	4.186	104.7
	4.059	101.5	4.059	101.5
	4.016	100.4	4.166	104.2
	4.038	101.0	4.019	100.5
	3.995	99.9	4.034	100.9
Av.	4.023	100.6	4.089	102.3
SD	0.028		0.070	
CV, %	0.70		1.71	
8.0	7.970	99.6	7.982	99.8
	7.949	99.4	8.001	100.0
	8.078	101.0	8.010	100.1
	7.938	99.2	8.016	100.2
	6.456[a]	80.7[a]	7.959	99.5
	7.944	99.3	7.954	99.4
Av.	7.976	99.7	7.987	99.8
SD	0.058		0.026	
CV, %	0.73		0.33	
12.0	12.016	100.1	11.766	98.1
	12.005	100.0	11.806	98.4
	11.976	99.8	11.887	99.1
	12.005	100.0	11.856	98.8
	11.979	99.8	11.788	98.2
	11.987	99.9	11.857	98.8
Av.	11.995	99.9	11.827	98.6
SD	0.016		0.047	
CV, %	0.13		0.40	

[a] Value discarded.

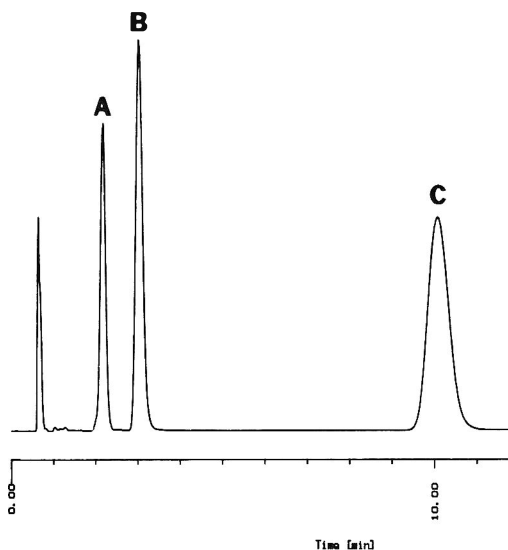

Figure 2. Chromatogram of standards: A, BHA, 1.00 mg/100 mL; B, miconazole, 0.80 mg/100 mL; C, BHT, 2.00 mg/100 mL.

Spiking recoveries were done by adding known quantities of miconazole to placebo samples. Recoveries from both the cream and the suppository showed good precision and a slight departure from linearity. This departure is not a problem because the method requires that the sample weight be taken to match the response of the standard. These data are shown in Table 1.

Assay values for a miconazole cream were 1.98, 2.00, 2.00, 2.01, 2.02, and 2.00% by weight, with average of 2.00, standard deviation of 0.013, and coefficient of variation of 0.65%. Assay values for a miconazole suppository were 3.96, 4.01, 4.13, 3.91, 3.95, and 3.98% by weight, with average of 3.98, standard deviation of 0.80, and coefficient of variation of 2.01%. Although both samples are well within specification, the suppository shows a larger coefficient of variation than does the cream. This may be due to the smaller sample weight taken in the case of the suppository or possibly a less uniform distribution of miconazole within the suppository.

To test the variation in results by different analysts, a cream sample was assayed independently by 3 different analysts. Each analyst ran the assay on 6 replicates as shown in Table 2. When all 18 results were combined, the average assay value was 1.969 and the standard deviation was 0.017, resulting in a coefficient of variation of 0.86%. An analysis of variance indicated that there was no statistically significant difference between the analysts at the 95% confidence level.

To attempt to determine the stability indicating quality of the method, samples of creams and suppositories were subjected to various stress conditions. (A) The required sample

Table 2. Comparison of miconazole assay values (% by wt) of commercial sample of miconazole nitrate cream by 3 analysts

	Analyst 1	Analyst 2	Analyst 3
	1.970	1.962	1.993
	1.976	1.959	1.966
	1.980	1.984	1.968
	1.986	1.962	1.961
	1.985	1.958	1.950
	2.012	1.951	1.958
Av.	1.985	1.957	1.966
SD	0.015	0.006	0.015
CV, %	0.76	0.31	0.76

weight (400 mg cream or 200 mg suppository) was heated in a 100 mL volumetric flask at 85°C for 72 h. (B) Then, 1.0 mL of 2N ethanolic NaOH and 10 mL ethanol were added to the required sample weight. The solution was heated on a steam bath for 10 min and then neutralized with 1.0 mL of 2N ethanolic HCl. (C) Next, 1.0 mL of 2N ethanolic HCl and 10 mL ethanol were added to the required sample weight. The solution was heated on a steam bath for 10 min and then neutralized with 1.0 mL of 2N ethanolic NaOH. (D) Finally, 0.5 mL of 1N aqueous $KMnO_4$ and 10 mL ethanol were added to the required sample weight. The solution was heated on a steam bath for 10 min.

The stressed samples were carried through the method. Then, using the Hewlett-Packard 1040A diode array system, spectral scans of the miconazole peaks from the stressed samples were superimposed on the spectral scan of the miconazole standard. In all cases, the spectral scans were nearly identically superimposed. This stress testing suggests—but does not confirm—that the method is stability indicating. We feel that to do so would require subjecting samples to a long-term stability study and then verifying peak purity by scan superposition.

This method is designed for levels of miconazole in creams and suppositories in the 2–4% range and not for trace or residue levels.

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Determination of Sulfamethazine in Milk

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A simple, relatively rapid liquid chromatographic method has been developed for the determination of sulfamethazine (SMZ) in milk at levels in the low ppb range. The method is based on extracting SMZ from milk with chloroform, evaporating the chloroform, dissolving the residues in hexane, extracting into buffers, and chromatographing the buffer solution. The method has been shown to determine levels as low as 5 ppb reliably. Levels ≥7 ppb have been confirmed by gas chromatography/mass spectrometry after derivatization of extracts from fortified, incurred, and shelf milk. Intralaboratory recoveries and percent coefficients of variation are satisfactory. Sulfadimethoxine and sulfaquinoxaline can also be determined by the method. Application of the method to other dairy products is being investigated.

Sulfamethazine (SMZ), an antimicrobial commonly used in the swine and veal industries, is rapidly gaining nationwide attention. A recent study by the National Center for Toxicological Research indicates that SMZ may be a thyroid carcinogen (1). Claims have been made that SMZ is widely used in the dairy industry and that SMZ residues are found in milk (2).

SMZ residues have been determined by thin-layer chromatography (TLC) (3, 4), gas chromatography (GC) (5-7), and liquid chromatography (LC) (8-11), as well as by other methods (12, 13). Analytical methodology reported for SMZ through 1980 has been critically reviewed (12, 13). Many of the reported methods require elaborate preliminary workups or suffer from relatively low sensitivities, i.e., ≥30 ppb. We have developed a simple, relatively rapid LC procedure for determining sulfamethazine in the low ppb range. It is based on extracting SMZ from milk with chloroform in a separatory funnel, evaporating the chloroform, and dissolving the fatty residue in hexane. SMZ is concentrated 10 fold during the final extraction steps; it is extracted into an aqueous potassium phosphate solution, injected onto an LC system, and detected by UV absorption at 265 nm.

Experimental

Equipment

(a) *Liquid chromatograph*—Equipped with Perkin-Elmer Model LC-95 UV/Vis detector (Perkin-Elmer Corp., Instrument Div., Norwalk, CT 06056) and Supelco Model LC-18-DB column, 250 × 4.6 mm id (Supelco, Inc., Bellefonte, PA 16823). 2 cm guard column (Supelco) and 0.5 μm precolumn filter (Supelco) precede the column.

(b) *Rotatory evaporator*.—Buchi, Laboratory Techniques Ltd, Flawil, Switzerland.

(c) *Vortex mixer*.—Genie Scientific, Fountain Valley, CA 92708.

(d) *Polypropylene plastic tubes*.—50 mL (Fisher Scientific).

(e) *Eppendorf pipettors*.—10 mL, 1 mL, 100 μL (Brinkmann Instruments, Inc., Westbury, NY 11590).

(f) *Fluted filter paper*.—Schleicher & Schuell Cat. No. 588, 12.5 cm (Schleicher & Schuell GmbH, Dassel, FRG).

(g) *Nylon-66 filter* (*N66*).—0.4 μm porosity (Supelco, Inc.)

Reagents

(a) *Sulfamethazine standard*.—Sigma Chemical Co. Cat. No. S-6256 (Sigma Chemical Co., St. Louis, MO 63178).

(b) *Potassium dihydrogen phosphate*.—LC grade.

(c) *Methanol*.—LC grade.

(d) *Purified water*.—Distilled and deionized.

(e) *Chloroform*.—Distilled in glass (Burdick & Jackson, Muskegon, MI 49442).

(f) *Hexane*.—LC grade.

Solutions

(a) *LC solutions*.—*Potassium dihydrogen phosphate, 0.1M* (*PDP solution*).—Dissolve 27.2 g potassium dihydrogen phosphate in water, dilute to 2 L, mix, and filter through N66 filter. *Mobile phase*.—Dilute 600 mL N66-filtered methanol to 2 L with PDP solution, and mix thoroughly. *Flush solution*.—Dilute 1200 mL methanol to 2 L with water, mix, and filter through N66 filter. All LC solutions are stored at room temperature and have an expiration date of 3 months. All water is distilled and deionized.

(b) *Standard solutions*.—*Master solution*.—Weigh 100 mg sulfamethazine standard at room temperature in glass weighing boat and transfer to 100 mL volumetric flask, dissolve in methanol, dilute to volume with methanol, and mix thoroughly. *Intermediate solution, 10 000 ng/mL sulfamethazine solution*.—Measure 1.0 mL master solution with 1.0 mL volumetric pipet into 100 mL volumetric flask, dilute to volume with water, and mix thoroughly. *Fortification solution, 1000 ng/mL sulfamethazine solution*.—Transfer 10 mL intermediate solution to 100 mL volumetric flask with 10 mL volumetric pipet, dilute to volume with water, and mix thoroughly. All standard solutions have an expiration date of 3 months after date of preparation of master solution. Store all standard solutions below 10°C.

Standards

(a) Use 1000 ng/mL fortification solution as 100 ppb standard.

(b) Prepare 200 ng/mL solution (equivalent to 20 ppb standard) by diluting 20 mL fortification solution to 100 mL with water, and mix thoroughly.

(c) Prepare 100 ng/mL solution (equivalent to 10 ppb standard) by diluting 10 mL fortification solution to 100 mL with water, and mix thoroughly.

(d) Prepare 50 ng/mL solution (equivalent to 5 ppb standard) by diluting 5 mL fortification solution to 100 mL with water, and mix thoroughly.

Samples

Milk is stored below 10°C. However, milk that will not be analyzed within a few days is subdivided into about 50 mL volume lots in polypropylene plastic tubes and stored below −40°C. Raw bulk tank milk, shelf milk, or thawed frozen

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Table 1. Recoveries of sulfamethazine added to milk at 0, 5, 10, and 20 ppb and incurred in milk[a]

5 ppb		10 ppb		20 ppb		Incurred
Rec., ppb	Rec., %	Rec., ppb	Rec., %	Rec., ppb	Rec., %	Found, ppb
3.99	79.8	7.51	75.1	14.89	74.5	4.46
4.23	84.6	7.22	72.2	15.07	75.4	4.64
4.40	88.0	7.98	79.8	15.19	76.0	4.29
3.47	69.4	7.51	75.1	14.07	70.4	4.35
4.05	81.0	7.92	79.2	13.84	69.2	4.52
Av.	80.6		76.3		73.1	4.45
SD	7.0		3.2		3.1	0.14
CV, %	8.7		4.2		4.2	3.1

[a] SMZ not detected in the 5 control samples.

milk is gently mixed before sampling. Fortified milk is prepared from milk that has been analyzed and found free of SMZ. Milk portions fortified at 5, 10, and 20 ppb are prepared by adding 50, 100, or 200 μL fortification solution for each 10 mL milk sample and by mixing thoroughly.

Analytical Methodology

First, place fluted filter paper into 75 mm funnel, wash paper with 5 mL chloroform, and discard chloroform. Place 100 mL pear-shape flask under funnel as receiver. Next, pipet 10 mL milk sample into 125 mL separatory funnel, and add 50 mL chloroform. Shake milk and chloroform mixture vigorously for 1 min, and then carefully vent through stopper. Shake for 1 min, vent, and let phases separate for 1 min. Again, shake for 1 min, vent, and shake for 1 min. Vent and let phases separate for a minimum of 5 min. Draw off chloroform, and filter it into pear-shape flask. Rinse filter paper twice with 5 mL chloroform, and collect washings in pear-shape flask.

Evaporate chloroform solution in pear-shape flask just to dryness on rotatory evaporator at 32 ± 2°C. Add 5 mL hexane to flask, stopper, and dissolve residue by agitating vigorously on vortex mixer about 1 min. Immediately add 1.0 mL PDP solution to hexane in flask, agitate vigorously on vortex mixer 3 or 4 times at intervals of approximately 1 min. over a minimum of 15 min. Using Pasteur pipet, transfer aqueous layer. Store sample in glass test tube or autoinjector vial. Sample is now ready for injection.

Chromatographic Conditions

With UV detector at 265 nm, inject 100 μL of both standards and samples with isocratic flow of mobile phase at 1.5 mL/min. Set run time at 15 min with 1 min equilibration between runs; adjust column heater to 35.0 ± 0.2°C. Use flush solution to rinse autoinjector and to clean up LC system and column. Quantitate compounds by measuring peak heights. Chromatograph at least 3 levels of standards and use linear regression analysis of the standard curve to calculate concentrations. Each ppb SMZ in milk results in 1 ng/mL in final PDP extract injected onto LC system. Standard concentrations in ng/mL are, thus, equivalent to ppb level of SMZ in milk, uncorrected for recovery. Typical retention time for SMZ is approximately 5 min.

Results and Discussion

Percent recoveries from an intralaboratory study using raw bulk tank milk are presented in Table 1. One example from an incurred milk (12) study is also included in Table 1.

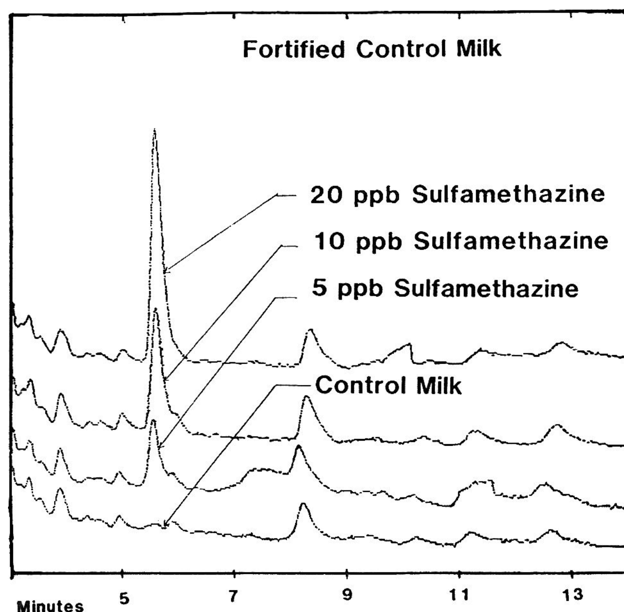

Figure 1. Chromatograms of control milk and control milk fortified at 3 levels.

Figure 1 shows chromatograms of control milk and control milk fortified at 3 levels. The tracing from 0 to 3 min was not recorded because the dynamic range of the integrator will not simultaneously accommodate the relatively large negative inflection at the injection front and the low signal levels of the analyte. We confirmed SMZ in spiked and incurred milk by the gas chromatographic/mass spectrometric method of Matusik et al. (5, 7). Both raw bulk tank milk and pasteurized, homogenized milk, with fat content ranging from 1 to 4%, have been successfully examined using this method. Application of the method to other dairy products is being investigated.

At such low residue levels, cross contamination becomes a major concern (10). To eliminate this possibility, all previously cleaned glassware is rinsed before use with 1N HCl, then thoroughly rinsed with water, and finally rinsed with methanol.

Two critical steps, venting during extraction and contact time during extraction with the PDP solution should be carefully noted. Venting through the stopper is required because venting through the stopcock often caused clogging with milk solids, resulting in sample loss. Also, a contact time of at least 15 min is required for optimum transfer of sulfamethazine from the organic to aqueous phase.

The initial stages of an interlaboratory study of the method are under way. Preliminary results have already shown that sulfadimethoxine (retention time 18 min) and sulfaquinoxaline (retention time 21 min) can be determined by this method by increasing the run time to about 30 min.

Acknowledgments

The authors thank the following members of their laboratory for assistance: Herbert F. Righter who dosed the cow and supplied the incurred milk; Michael H. Thomas for helpful discussions and suggestions; and Valerie B. Reeves for intralaboratory validation of the method.

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Direct Competitive Enzyme-Linked Immunosorbent Assay for Sulfamethazine Residues in Milk

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A direct competitive enzyme-linked immunosorbent assay (ELISA) is described for the detection and estimation of sulfamethazine residues in milk. Samples are cleaned up rapidly by acidifying and centrifuging the milk, adjusting the supernatant liquid to pH 7.0, and centrifuging again. The supernate is then assayed using set points to estimate sulfamethazine levels in the sample in the range of 1 ppb to 1 ppm. Multiple samples of milk can be screened in 1.5-2 h by this ELISA method.

Sulfonamide residues may occur in milk for any of several reasons, such as use in mastitis therapy, deliberate feeding, inadvertent feeding, or use of sulfamethazine-containing boluses to prevent infection in cows that have calved. Brady and Katz (1) found that 64% of milk sampled in the New York City area, central New Jersey, and eastern Pennsylvania contained one or more antibiotic/antimicrobial residues; sulfonamide residues appeared in 42% of the samples. Collins-Thompson et al. (2) and Wehr (3) found similar frequencies of residues in milk. In a limited survey in 10 U.S. cities, the U.S. Food and Drug Administration found that over 50% of the milk samples contained measurable amounts of sulfonamide residues (unpublished milk survey, Food and Drug Administration, Washington, DC, 1988).

Because sulfamethazine has been shown to cause neoplasms in mice and rats (4), a rapid screening assay is needed to ensure that milk is as free of sulfamethazine residues as possible. With the exception of the receptor assay (5), no procedures are available to screen milk for antibiotic/antimicrobial residues. Systems currently used to analyze products for sulfonamide residues include gas chromatography (6-9), liquid chromatography (10-15), colorimetry (16-18), and tandem mass spectrometric (MS) systems (19, 20). These systems are time consuming and labor intensive, and require extensive sample cleanup. Except for procedures that use MS confirmation, these analyses are relatively nonspecific and can detect and measure only about 0.1 ppm sulfonamide residue. The receptor assay can detect residue levels of 10 ppb. Confirmation by other procedures is usually desirable.

Although a direct competitive enzyme-linked immunosorbent assay (ELISA) was developed for sulfamethazine residues in the blood of swine, no similar assay has been developed for milk (21). The procedure presented herein utilizes an ELISA system which provides excellent sensitivity, a specificity similar to that for enzymatic systems, speed, and practicality.

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METHOD

Equipment

(a) *Enzyme immunoassay reader.*—Model EL307 (Biotek Instruments, Inc., Burlington, VT), or equivalent.

(b) *Centrifuge.*—Sorvall RC-3, (Sorvall Inc., Norwalk, CT).

(c) *Microtiter plates.*—NUNC polystyrene 96-well (Vanguard International, Neptune, NJ).

(d) *Spectrapore membrane tubing.*—Molecular weight cutoff 12 000-14 000 (Spectrum Medical Industries Inc., Los Angeles, CA).

Chemicals

(a) *For substrate and conjugate preparation.*—Tween 20; 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); bovine serum albumin Fraction V (BSA); rabbit serum albumin Fraction V; ovalbumin Grade II (OA); horseradish peroxidase Type VI (HRP); and glutaraldehyde Grade II (Sigma Chemical Co., St. Louis, MO).

(b) *Protein-A-agarose.*—(Pierce Chemical Co., Rockford, IL).

(c) *Complete and incomplete Freund's adjuvant.*—(Difco Laboratories, Inc., Detroit, MI).

(d) *Sulfamethazine (SMZ).*—(U.S. Biochemicals, Cleveland, OH).

(e) *Dioxane.*—(Fisher Scientific Co., Fairlawn, NJ).

All other chemicals used were reagent grade or better.

Reagents

Use deionized water throughout.

(a) *Phosphate buffer (PB).*—*(1) pH 7.0.*—Mix 390 mL 0.2M NaH_2PO_4, 610 mL 0.2M Na_2HPO_4, and 1000 mL water. *(2) pH 7.2.*—Mix 190 mL 0.2M NaH_2PO_4, 810 mL 0.2M Na_2HPO_4, and 1000 mL water.

(b) *Phosphate buffer and saline (PBS).*—*(1) pH 6.8.*—Mix 51 mL 0.1M KH_2PO_4, 49 mL 0.1M Na_2HPO_4, and 100 mL 0.15M NaCl. *(2) pH 7.0.*—Mix 390 mL 0.2M NaH_2PO_4, 610 mL 0.2M Na_2HPO_4, and 1000 mL 0.15M

NaCl. (*3*) *pH 7.2*.—Mix 480 mL 0.1M KH_2PO_4, 1520 mL 0.1M Na_2PO_4, and 2000 mL 0.15M NaCl.

(**c**) *Normal saline*.—Dissolve 8.5 g NaCl in 1 L water.

(**d**) *Coating buffer*.—0.15M bicarbonate buffer, pH 9.0. Add 2.93 g $NaHCO_3$ and 1.59 g Na_2CO_3 to 1 L water.

(**e**) *Washing solution*.—0.2% Tween 20 in PBS, pH 7.2.

(**f**) *Blocking solution*.—1% ovalbumin in PBS, pH 7.2. Dissolve 1 g crude ovalbumin in 100 mL PBS, pH 7.2. Filter solution through Whatman No. 1 paper and store filtrate at 4°C.

(**g**) *Enzyme-labeled conjugate* (*SMZ:HRP*).—Dilute 400 μL sulfamethazine-horseradish peroxidase conjugate (see *Preparation of Sulfamethazine Conjugates* (**c**)) in 9.6 mL 1% OA-PBS, pH 7.2 (**i**).

(**h**) *ABTS substrate*.—(*1*) *0.05M citrate buffer*.—Add 9.6 g citric acid to 500 mL water. Adjust pH to 4.0 with 1N NaOH and dilute to 1 L. (*2*) *ABTS stock solution*.—Dissolve 45 mg ABTS in 15 mL water.

Prior to use, add 5 μL 30% H_2O_2 to 11 mL pH 4.0 citrate buffer (*1*) and 1 mL ABTS stock solution (*2*).

(**i**) *Stopping reagent*.—Add 63 g citric acid monohydrate and 1 g sodium azide to 1 L water.

(**j**) *Sulfamethazine stock solution*.—10 mg/mL. For preparation of standard curve. Weigh 10.0 mg sulfamethazine into 10 mL volumetric flask and add 5 mL 0.1M PBS, pH 7.2. Add 1 mL 1N NaOH to dissolve drug, and then add 1 mL 1N HCl to lower pH to ca 7.2. Dilute to volume with PBS. Prepare standard solutions just before use.

(**k**) *Sulfamethazine conjugation solution*.—5 mg/mL. Add 5.0 mg sulfamethazine to 0.50 mL normal saline. Add 100 μL 1N NaOH to dissolve drug; pH is lowered to approximate neutrality when 100 μL NaOH is added. Adjust final volume to 1.0 mL by adding 0.30 mL physiological saline.

(**l**) *Carbonate-bicarbonate buffer*.—pH 9.5. Mix 13.0 mL 1.0M Na_2CO_3, 37.0 mL 1M $NaHCO_3$, and 150 mL water.

Animals

New Zealand female white rabbits (Davidson Mills Farms, South Brunswick, NJ).

Preparation of Sulfamethazine Conjugates

(**a**) *Sulfamethazine-bovine serum albumin conjugate*.—Dissolve 350 mg sulfamethazine and 600 mg bovine serum albumin in 75 mL phosphate buffer-dioxane (2 + 1). Add 0.35 mL 25% glutaraldehyde to mixture. Stir solution 3 h at room temperature. Dialyze against phosphate buffer, pH 7.0, for 6 days with 2 buffer changes each day using Spectrapore membrane tubing. Lyophilize dialyzed solution and store light orange conjugate in desiccator at 4°C (22). Use this conjugate to immunize rabbits for antibody production.

(**b**) *Sulfamethazine-rabbit serum albumin conjugate*.—Prepare a similar conjugate by using 350 mg sulfamethazine and 600 mg rabbit serum albumin as a solid phase antigen to coat ELISA plates for identification of antisulfamethazine antibody.

(**c**) *Sulfamethazine-horseradish peroxidase conjugate*.—Prepare enzyme-labeled conjugate of sulfamethazine and horseradish peroxidase by using 2-step glutaraldehyde procedure of Avrameas (23) as follows: Dissolve 10.0 mg horseradish peroxidase in 0.2 mL 0.1M PBS pH 6.8 containing 1.25% glutaraldehyde. Incubate mixture overnight at room temperature and dialyze against normal saline (3 changes) using Spectrapore membrane tubing to remove free glutaraldehyde. Dilute dialyzed solution to 1.0 mL with normal saline. Add sulfamethazine conjugation solution to activated HRP solution and add 0.10 mL carbonate-bicarbonate buffer, pH 9.5. Let mixture stand 24 h at 4°C. To this solution, add 0.10 mL 0.2M lysine. Incubate mixture 2 h at room temperature. Dialyze mixture against PBS, pH 7.2, using Spectrapore membrane tubing. Store solution at −20°C.

Antiserum Production

Immunize rabbits with sulfamethazine-bovine serum albumin conjugate by intradermal-intravenous route (23). Determine serum titer by ELISA (21, 24).

Purify antibodies by ammonium sulfate precipitation followed by further purification using protein-A-agarose affinity chromatography column (A. D. Voller, D. Bidwell, & A. Bartlett, "The Enzyme Linked Immunosorbent Assay (ELISA)," Dynatech Laboratories, Inc., Chantilly, VA, 1979). Verify antibody activity in fractions by ELISA. Pool fractions that show maximum activity, and store at −80°C in small portions (0.5 mL).

Determination

(**a**) *Preparation of microtiter plates*.—Dilute purified antibody 1/20 in coating buffer. Add 50 μL diluted antibody to wells of 96-well microtiter plate. Place microtiter plate in 40°C drying oven for 4 h. Remove microtiter plates and wash each well 3 times with 250 μL PBS-Tween to remove any unbound antibody. Add 300 μL 1% OA-PBS to each well. Incubate 30 min at 37°C to complete blocking reaction. Wash wells 4 times with PBS-Tween to remove any remaining unbound blocking agent.

(**b**) *Preparation of standards*.—Supplement another set of milk samples with sulfamethazine to contain final concentrations of 0.0, 1.0, 10, 100, and 1000 ng sulfamethazine/mL milk.

Mix samples well and acidify with 0.5N HCl to pH 4.5 (pH meter). Place acidified milk samples in 250 mL centrifuge bottles and centrifuge at 6000 rpm (3200*g*) for 20 min. Decant supernatant liquid and readjust pH to 7.0 (pH meter) with 1N NaOH. Centrifuge pH-adjusted solutions as previously described. Use these extracts to prepare standards in assay.

(**c**) *Enzyme immunoassay*.—Mix 100 μL enzyme-labeled conjugate with 100 μL cleaned-up milk sample or milk standard. Add 50 μL aliquots of each mixture to each of duplicate wells for both standards and samples. Incubate plates 1 h at 37°C. Wash plates 8 times with PBS-Tween to remove any unbound reactants. Add 100 μL substrate and incubate 15 min at 37°C. Add 100 μL stopping reagent to each well. Measure absorbance of each well at 405 nm using EIA reader. Average the absorbance measurements for samples and for standards. There should be 3 replicates for each standard; for each, calculate average and determine lower value of 95% confidence limits as follows:

$$\text{Set point} = \text{av. absorbance} - (\text{std dev.} \times 2.484)$$

Factor 2.484 is derived from dividing *t*-value for 2 degrees of freedom by square root of 3 (number of determinations).

If absorbance of milk sample is less than set point for concentration being used as lower limit of estimation, sample contains concentration of sulfamethazine greater set point concentration.

Results and Discussion

Immunological determination of sulfonamide residues in milk offers several advantages. Antibodies are rather specific

Table 1. Sensitivity and repeatability of the direct competitive ELISA for sulfamethazine residues: absorbance values for standards and reagents

		Sulfamethazine concn, ng/mL							
Repl.	Day	1000	100	10	1.0	0.10	0.05	0.01	0.00
1	1	0.368	0.375	0.492	0.494	0.600	0.860	0.905	1.012
1a	1	0.319	0.422	0.487	0.496	0.497	0.802	0.888	1.060
Av.		0.344	0.399	0.490	0.495	0.548	0.831	0.896	1.086
2	2	0.366	0.384	0.390	0.530	0.641	0.936	0.935	1.031
2a	2	0.294	0.361	0.508	0.596	0.622	0.775	0.890	1.068
Av.		0.330	0.373	0.449	0.553	0.631	0.855	0.912	1.050
3	3	0.306	0.340	0.388	0.524	0.645	0.830	0.836	1.114
3a	3	0.372	0.386	0.469	0.469	0.545	0.772	0.920	1.004
Av.		0.339	0.369	0.429	0.497	0.595	0.806	0.878	1.059
4	4	0.297	0.372	0.405	0.455	0.634	0.961	0.936	0.975
4a	4	0.342	0.381	0.402	0.497	0.692	0.690	0.888	0.956
Av.		0.320	0.377	0.403	0.476	0.663	0.820	0.912	0.965
Av. *A* at each concn		0.333	0.379	0.443	0.505	0.609	0.829	0.899	1.040
Std dev.		0.011	0.013	0.036	0.033	0.049	0.020	0.016	0.052
CV, %		3.17	3.54	8.28	6.57	8.05	2.44	1.78	5.03

Correlation = 0.998 over concn range 1.0–1000 ng SMZ/mL
0.989 over concn range 0.1–1000 ng SMZ/mL
0.908 over concn range 0.05–1000 ng SMZ/mL
0.930 over concn range 0.01–1000 ng SMZ/mL

reagents and react almost exclusively with those structures against which they were raised. The sulfamethazine molecule was derivatized at the N-4 position and coupled with a bridging agent to the bovine serum albumin. For this reason, reactivity is unrelated to the aromatic amine function. The antibodies raised react to the substituted sulfonic acid portion of the molecule and, hence, react equivalently to both sulfamethazine and sulfamerazine. With sulfanilamide, the antibodies react at a level of 5% of the reaction with sulfamethazine; the antibodies do not react with sulfathiazole, other sulfonamides, procaine penicillin, chlortetracycline, or *p*-aminobenzoic acid. The equivalent reaction with sulfamerazine and sulfamethazine is not surprising since the molecules differ by one methyl group. Sulfamerazine is rarely used, if at all, in dairy animals, so the procedure can be considered reasonably specific for sulfamethazine.

This screening procedure allows rapid estimation of the concentration of sulfamethazine in milk samples by establishing the absorbances for various levels of concentration.

Table 1 shows the absorbance values obtained for the assay system over the range 0.01–1000 ng sulfamethazine/mL using standards and reagents. Correlation over the 10^5-fold range was 0.930; correlation over the range 1–1000 ng sulfamethazine/mL was excellent. The latter is an extremely useful range for residue measurement because it corresponds to 1 ppb–1 ppm. Standard response lines determined on 4 different days using different reagents (excepting the antibody) yielded good reproducibility. The coefficients of variation for the concentrations of 0.01–1000 ng sulfamethazine/mL ranged from 1.78 to 8.28% and between 3.17 and 8.28% over the range 1–1000 ng sulfamethazine/mL. These data are averages for 2 wells on 4 different days. For determinations on single wells, coefficients of variation were considerably greater; similarly, using 3 wells lowered the CV values fur-

Table 2. Sensitivity and repeatability of absorbance values at 405 nm in direct competitive ELISA for sulfamethazine in milk

		Sulfamethazine added, ng/mL				
Repl.	Day	1000	100	10	1	Blank
1	1	0.243	0.367	0.502	0.558	0.776
1a	1	0.223	0.398	0.600	0.614	0.706
Av.		0.233	0.382	0.551	0.586	0.741
2	2	0.275	0.398	0.544	0.553	0.637
2a	2	0.265	0.344	0.505	0.571	0.630
Av.		0.270	0.371	0.524	0.562	0.634
3	3	0.224	0.406	0.542	0.567	0.571
3a	3	0.204	0.346	0.480	0.550	0.630
Av.		0.214	0.376	0.511	0.559	0.600
Av. *A* at each concn		0.239	0.376	0.529	0.569	0.658
Std dev.		0.028	0.006	0.020	0.569	0.073
CV, %		11.92	1.46	3.86	2.60	11.09
Range of 95% conf. limits		0.239 ± 0.069	0.376 ± 0.015	0.529 ± 0.050	0.569 ± 0.037	
Absorbance set point		0.170	0.361	0.479	0.532	

Table 3. Adherence of direct competitive ELISA for sulfamethazine in milk to calculated set point (*A* at 405 nm)

	Sulfamethazine added, ng/mL				
Detn	1000	100	10	1	Blank
1	0.256	0.366	0.520	0.549	0.649
2	0.250	0.406	0.529	0.545	0.626
Calculated set point	0.170	0.361	0.479	0.532	

ther but not sufficiently to warrant the extra effort (data not shown).

Table 2 shows the range and repeatability of the absorbance measurements for direct ELISA in milk samples supplemented with sulfamethazine at levels from 1 to 1000 ng sulfamethazine/mL (equivalent to 1 ppb to 1 ppm). Repeatability between days as measured by coefficients of variation were between 1.46 and 11.92%; this range is quite typical for ELISA-based assays. By calculating the 95% confidence limits for the absorbance at each level of supplementation, it is possible to establish the lower limits of measurement for a concentration. The calculated absorbance values for each of the concentrations used are shown in Table 2. To establish the validity of the set point determination, assays were performed on 2 different days on milk supplemented with levels from 1 to 1000 ng sulfamethazine/mL. Table 3 shows the 2-well average absorbance for each level of supplementation. None of the determinations performed exceeded the lower range of values calculated for the respective concentrations. These analyses, 2 at each concentration, are far from a definitive statistical study, yet, none of the 8 supplemented milk samples yielded absorbances that exceeded the set points for the concentrations.

There is an obvious difference in the absorbance measured for a given concentration in milk vs PBS. The milks were obtained from commercial sources and were assayed to ensure that no residues were present. However, the lower range of detection using a Bratton-Marshall determination was limited, 25–50 ppb. If the milk samples did indeed contain sulfamethazine residues, then the differences could be explained. This is a very strong possibility because sulfonamide residues are being found with relatively high frequency in market milk. At the same time, milk is a complex mixture which might react with the reagents in a nonspecific fashion to cause lower absorbance values. Without solid evidence to support either or both possibilities, both sources should be considered contributory.

In comparison, the receptor assay is reported to be capable of routinely detecting 10 ppb sulfamethazine (5). That assay, which is used to screen milk for several families of antibiotics/antimicrobials, must be performed in a laboratory. A field assay is being developed to determine sulfonamides on the farm (private communication, S. Charm, 1988). FDA has available a chemical assay procedure using a chloroform extraction followed by liquid chromatographic determination which appears to be capable of measuring <10 ppb sulfamethazine. This procedure is labor intensive, and only about 8 assays can be completed in a day. Furthermore, the assay must be carried out in a laboratory (J. D. Weber & M. D. Smedley, unpublished data, 1988).

The direct competitive ELISA can be used for estimating sulfamethazine in multiple samples of milk in 1.5 to 2 h. For small laboratories or those not equipped with a microtiter plate reader, visual determinations of color intensity can be used to estimate the levels of sulfamethazine contamination in milk samples.

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EXTRANEOUS MATERIALS

Extraction of Light Filth from Spirulina Powders and Tablets: Collaborative Study

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Results are reported for a collaborative study of a method for the extraction of light filth from spirulina (a blue-green alga) powder and tablets. A 50 g portion of either powder or tablets is dispersed in water, and then boiled with dilute HCl solution. Hairs and insect fragments are isolated by wet sieving on a No. 230 sieve, flotation with mineral oil, and washings of the mineral oil in a percolator. Average recoveries by 12 collaborators for tablets and powders were 70.6 and 70.2%, respectively, for 10 rat hair spikes and 68.3 and 84.4%, respectively, for 20 insect fragment spikes. The method has been approved interim official first action.

Spirulina, a blue-green alga found in alkaline lakes, is used as a food supplement by many people. Spirulina is being commercially produced in the United States, Mexico, Japan, Israel, and Taiwan. The most common retail forms of the product are powders, tablets, and capsules, although flake and granular forms are also produced. Spirulina may also be added to pasta and candy bars or used in the coating of multiple vitamins.

Because this alga is produced in open ponds and is thus susceptible to contamination, a method was developed to detect light filth in spirulina powders and tablets. The method consists of dispersing 50 g spirulina powder or tablets in water, boiling with dilute HCl solution, and wet sieving on a No. 230 screen. The residue is transferred to a beaker with water and brought to a boil. Mineral oil is added to the mixture, which is magnetically stirred and transferred to a percolator where the product is removed and the mineral oil is filtered and the filth is counted.

Collaborative Study

The study included thirty-six 50 g test portions each of spirulina tablets and powders. Three of each form were sent to 12 collaborators. All test samples were spiked with 10 rat hairs (about 1 mm long) and 20 insect fragments (abdominal squares or rectangles of *Musca domestica*, the common house fly, about 0.5 sq mm). The collaborators were instructed to report their analytical times and to return the extraction papers so that their results could be checked by the Associate Referee.

Light Filth in Spirulina Powders and Tablets

Flotation Method

Interim First Action

Method Performance (expert's counts in parentheses):

Rat hairs, 10 added:

Tablets:

s_r = 2.6(2.7); s_R = 3.2(2.8); RSD_r, % = 41.9(38.0); RSD_R, % = 51.6(39.4)

Powders:

s_r = 2.5(1.8); s_R = 3.0(2.4); RSD_r, % = 39.7(25.7); RSD_R, % = 47.6(34.3)

Insect fragments, 20 added:

Tablets:

s_r = 3.7(3.1); s_R = 5.4(4.7); RSD_r, % = 30.8(22.6); RSD_R, % = 45.0(34.3)

Powders:

s_r = 2.7(3.4); s_R = 5.0(4.4); RSD_r, % = 16.7(20.1); RSD_R, % = 30.9(26.0)

A. *Principle*

Spirulina powder or tablets are dispersed in water, boiled with dilute HCl, and wet sieved on a No. 230 screen. Residue is brought to boil in water, then mineral oil is added. After stirring, the product is removed by washings in the percolator, mineral oil is filtered, and filth is counted.

B. *Pretreatment*

In 1 L beaker, thoroughly mix with spoon 50 g spirulina powder or tablets with portions of 500 mL tap water, reserving amount to rinse spoon.

(a) *Powders.*—Proceed to isolation.

(b) *Tablets.*—Magnetically stir on hot plate at low setting until tablets are *completely* dispersed. Proceed to isolation.

C. *Isolation*

Note: Use tap water throughout isolation step.

Add 0.3 mL antifoam A solution, **44.003(e)**, and then add 15 mL HCl in increments with magnetic stirring on cool hot plate to disperse foam; if no foam occurs, add remaining HCl. Bring mixture to boil on hot plate and boil 30 min with magnetic stirring, **44.002(n)**. Clean beaker sides with rubber policeman. Gently sieve (No. 230), **44.005(a)**, beaker contents with hot water (>50°) until washings are clear. Use water to quantitatively transfer residue back to 1 L beaker, and then fill beaker to 500 mL with water. Bring mixture to boil with magnetic stirring, but *do not boil.* (**Caution**: Boiling some tablet mixtures may cause gummy residue to form on beaker sides.) Immediately add 150 mL mineral oil, **44.003(p)**, and magnetically stir, **44.005(c)**, 10 min on cool hot plate. Let stand 5 min. Add beaker contents to percolator, **44.002(h)**(*2*), containing ca 250 mL H_2O. Rinse beaker and stirring bar with hot water (>50°) and add washings to percolator. Retain beaker. Fill percolator to 1700 mL with water. Stir percolator contents with glass rod 2–3 s, then rinse rod into percolator with hot water (>50°). Let stand 3

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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 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," *J. Assoc. Off. Anal. Chem.* (1990) 73, January/February issue.

Table 1. Collaborative results for recovery of 10 rat hair spikes (blind triplicates) from spirulina tablets and powders

Coll.	Tablets			Powders		
1	5 (6)[a]	0 (2)	2 (7)	2 (8)	1 (3)	4 (7)
2	8 (9)	5 (6)	9 (7)	5 (6)	4 (6)	6
3	9	5 (7)	9	10	10 (8)	8
4	11 (9)	10	6 (7)	9 (10)	10	7
5	8	7 (8)	7	4 (5)	5 (6)	6 (7)
6	4 (7)	1 (3)	3 (9)	3 (5)	2 (4)	4 (6)
7	14 (15)	3 (2)	2 (4)	8	3 (7)	10 (9)
8	10	7 (8)	9	5	8	6 (9)
9	7	8	8 (9)	6	9	4
10	6 (10)	7 (6)	8 (10)	9 (10)	10	9 (10)
11	6	6	5	8 (9)	7	9
12	1 (4)	0	5	13 (0)	3 (7)	1 (4)
$\bar{x}$	6.2 (7.1)			6.3 (7.0)		
$\bar{x}$, %	61.4 (70.6)			63.2 (70.2)		
s_r	2.6 (2.7)			2.5 (1.8)		
s_R	3.2 (2.8)			3.0 (2.4)		
RSD_r	41.9 (38.0)			39.7 (25.7)		
RSD_R	51.6 (39.4)			47.6 (34.3)		

[a] Associate Referee counts are in parentheses if different from those of collaborator.

Table 2. Collaborative results for recovery of 20 insect fragment spikes (blind triplicates) from spirulina tablets and powders

Coll.	Tablets			Powders		
1	14 (13)[a]	0 (6)	6 (9)	16 (15)	10	14
2	14 (18)	15 (16)	17 (20)	19	16	14 (16)
3	14 (18)	6 (16)	11 (17)	20	19	19
4	13	9 (11)	20	19 (20)	18 (17)	21 (20)
5	15 (18)	19	19 (18)	16 (15)	21 (20)	20
6	9 (15)	4 (11)	8 (12)	8 (10)	6 (7)	14 (17)
7	68[b] (17)	44[b] (13)	18[b] (8)	26[b] (19)	33[b] (18)	32[b] (18)
8	6 (10)	10 (14)	10 (12)	20	19 (20)	20
9	14	18 (19)	14	19	20	20
10	17 (13)	16 (9)	17 (18)	17 (18)	18 (19)	19 (20)
11	14 (17)	16 (17)	15 (14)	19 (18)	17	17
12	3 (4)	1	11 (8)	2 (0)	13 (19)	5 (12)
$\bar{x}$	12.0 (13.7)			16.2 (16.9)		
$\bar{x}$, %	59.8 (68.3)			81.0 (84.4)		
s_r	3.7 (3.1)			2.7 (3.4)		
s_R	5.4 (4.7)			5.0 (4.4)		
RSD_r	30.8 (22.6)			16.7 (20.1)		
RSD_R	45.0 (34.3)			30.9 (26.0)		

[a] Associate Referee counts are in parentheses if different from those of collaborator.

[b] Outlier by Dixon test; not included in calculations.

min. Drain percolator to 250 mL level. Repeat fill and drain until lower aqueous phase is clear or free of suspended material. Drain oil layer into retained beaker, then rinse glass rod into beaker with isopropanol. Wash percolator sides successively with hot water (>50°), isopropanol, and 1% Na lauryl sulfate, **44.003(i)**. Boil beaker contents 2 min (without boiling over). Immediately filter beaker contents and wash beaker successively with hot water (>50°), isopropanol, and 1% Na lauryl sulfate; filter washings. Examine paper microscopically at 30×.

Ref.: JAOAC **72**, May/June issue (1989).

Results and Discussion

Results for recoveries of rat hairs are given in Table 1. A Student's *t*-test based on the Associate Referee's counts showed no significant difference ($P > 0.05$) between the mean percentage recoveries for tablets and powders. The slightly higher and less variable Associate Referee's counts reflect better recognition of the spike by the Associate Referee than by the collaborators. Collaborators 1 and 6, and to a lesser degree Collaborator 12, overlooked rat hair spikes consistently.

The data for insect fragment recoveries are summarized in Table 2. Unlike rat hairs, there was a significant difference between the mean percentage recoveries for tablets and powders ($P < 0.05$). One problem that arose with the tablets, and reduced the counts, was breakage of the spikes by contact with the product during shipping. Since the resulting pieces were smaller than the specified spike size, all collaborators, with the exception of Collaborator 7, did not count small pieces of shattered spike material. Counting of smaller pieces by Collaborator 7 resulted in inflated numbers that were outliers by the Dixon test (W. J. Youden and E. H. Steiner, *Statistical Manual of the AOAC* (1975) AOAC, Arlington, VA) and were not included in the statistical analysis. Examination of the collaborators' papers showed that some shattered spike material, although not of the specified size, should be counted. Therefore, the Associate Referee included only fragments with at least 2 straight edges and greater than one-half of the intact fragment in the counts. Thus, 23.2% of the Associate Referee's 68.3% average count (Table 2) were insect fragments using this criterion. Since 68.3% represents an artificially low number, an in-house study, using the same lots of each product and the same spikes as were used in the collaborative study, was conducted to demonstrate that insect fragment recoveries were comparable to those obtained for powders. The results (Table 3) support the conclusion that 68.3% was lower than would be expected. Insect fragment recoveries were 94.5% for tablets and 98.5% for powders. The standard deviations were different, but the coefficient of variation of 3% for insect fragments from powders was exceptionally low for a particulate filth method. The performance parameters obtained fall well within acceptable limits for such methods.

The collaborators took an average of 3.3 h (range 2–5.8 h) to perform the extraction and 1.1 h (range 0.1–3 h) to count the plates.

Some collaborators had dirty and/or excessive numbers (>2) of papers for 1 or 2 of the 3 tablet test portions. A cause of excessive numbers of papers for Collaborators 3, 4, 7, 9, and 11 was clogged filter papers. The reason for this was that, in the filtration step, bringing the beaker contents to a full boil did not sufficiently reduce the viscosity of the mineral oil. To remedy this, the method was changed from "Bring beaker contents to a full boil without boiling over." to "Boil beaker contents for 2 min (without boiling over)." A second reason for large numbers of papers with tablets was excessive amounts of trapped product. The solution to this problem is less clear because of inconsistencies in the results. For example, Collaborators 3, 7, 8, and 10 experienced problems with excessive trapped product for 2 test portions (av. 5.75 papers), yet each had a third portion which required only 1 or 2 papers. The order in which the clean test portions were examined did not indicate that performance of the method improved with analyst experience. Insufficient removal of all fine particles is a potential factor, but all collaborators stated that they performed the sieving procedures as directed.

An important observation was made by Collaborators 7, 8, and 10, who noted the incomplete dissolution of the tablets, even after the 30 min acid digestion and sieving. Collaborator 10 could, in fact, associate specific test portions of undis-

Table 3. In-house results for recovery of 10 rat hair (RH) and 20 insect fragment (IF) spikes from spirulina tablets and powders

	Tablets		Powders	
Repl.	RH	IF	RH	IF
1	9	21	5	19
2	10	20	9	20
3	10	17	9	20
4	9	19	10	20
5	10	20	10	21
6	7	19	10	19
7	10	20	6	20
8	9	17	9	20
9	10	20	6	19
10	7	17	9	20
$\bar{x}$	9.1	19	8.3	19.8
$\bar{x}$, %	91	94.5	83	98.5
SD	1.3	2	1.8	0.6
CV, %	14.3	10.5	21.7	3

solved tablets with dirty papers. It is conceivable, then, that undissolved tablets could disintegrate further during the "Bring mixture to boil . . ." period before adding the mineral oil, and as a consequence fine material that would have been removed by sieving was present for trapping in the mineral oil. The postcollaborative in-house study confirmed this. In 4 of 10 replications, ≥2 papers were required. Incompletely dissolved tablets were noted in 2 of these replicates and the amount of product on the papers was noticeably greater than for the other 2 replicates. To emphasize the need for total dissolution of the tablets, the word "completely" is italicized in the pretreatment section for tablets.

For powders, 34 of 36 test portions required only 1 filter paper, although the debris on Collaborator 1's paper could have been spread onto 2 papers. Of the 2 test portions needing > 1 paper, only Collaborator 11 had excessive debris, while Collaborator 3 had filtering problems.

Recommendation

On the basis of acceptable analyte recoveries and postcollaborative clarifications of the method to emphasize tablet dissolution and sieving techniques, both of which should result in more uniformly clean papers with tablets, it is recommended that the proposed method for extraction of light filth from spirulina powders and tablets be adopted official first action.

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FEEDS

Improved Turbidimetric Assay of Tylosin in Premix and Animal Feeds Not Containing Urea

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A turbidimetric method is described for the determination of tylosin in premix and animal feeds not containing urea. This method includes several modifications of existing tylosin turbidimetric and AOAC plate assays to remove interferences from the feed, to concentrate low levels of tylosin, and to reduce the variability of the assay results. An acidic alumina column cleanup step has been incorporated into the method to remove interferences from feed ingredients. A disposable C18 column was used to concentrate the tylosin from low-level feeds, and the use of a larger analytical sample size has decreased the variability of the assay results. Average recoveries of tylosin added to chicken and swine rations were 98 and 101%, respectively.

Tylosin is the major macrolide antibiotic produced by a strain of the actinomycete, *Streptomyces fradiae*. The phosphate salt of tylosin is marketed as Tylan® premix (tylosin, Elanco) for disease contol and/or improved feed efficiency in swine, poultry, and cattle feeds.

The use of a microbiological turbidimetric assay for tylosin in feeds has been described (1-4) as an alternative to the AOAC plate assay (5). This turbidimetric procedure has been used routinely by Eli Lilly and Co. laboratories, as well as many other laboratories. Several problems have been identified with the turbidimetric method, especially when feeds containing tylosin levels less than 100 g/ton are assayed. These problems included assay interferences from feed components and other feed additives, plus the relatively high variability of the assay results. In addition, the turbidimetric assay for tylosin did not exhibit sufficient sensitivity for feeds containing less than 10 g tylosin activity per ton. In the past, these samples still required the use of the plate assay, which can be very labor-intensive and less precise than the turbidimetric assay for tylosin.

An additional problem has been identified with the assay of cattle feeds containing urea. The presence of urea results in the formation of a tylosin urea adduct which is not active in the standard tylosin method. A hydrolysis step is required to free the tylosin activity for assay. An alternative method for the analysis of feeds containing urea will be available in the near future (manuscript in preparation).

The revised turbidimetric assay discussed here includes steps to remove assay interferences and reduce variability of assay results, and outlines a simple procedure to concentrate tylosin from feeds containing low levels of tylosin.

METHOD

Apparatus

(a) *Autoturb® system*.—Microbiological assay system (Elanco) including diluter module, water bath (37°C), and reader module.

(b) *Water bath*.—80°C, or steam sterilizer.

(c) *Feed grinder*.—Brinkmann centrifugal grinding mill (3 mm screen), or equivalent.

(d) *Feed mixer*.—Hobart Model C100T, or equivalent.

(e) *Chromatographic columns*.—Glass, plain, ca 19 mm id × 500 mm, with glass wool plug; or Analytichem International 75 mL reservoir with frit (20 μm pore).

(f) *Gyratory shaker*.—New Brunswick Scientific, Model G-33, or equivalent.

(g) *Filter paper*.—Whatman No. 4, or equivalent; Schleicher and Schuell prepleated filters, or equivalent.

(h) *Spectrophotometer*.—Spectronic 20, or equivalent.

(i) *Extraction apparatus*.—Sep-Pak, or equivalent.

Reagents

(a) *Solvent*.—Reagent grade methanol.

(b) *Phosphate buffers*.—Prepare pH 8 and pH 7 phosphate buffers according to **42.204**(b) and (c), respectively (5).

(c) *Extraction solution*.—Methanol-pH 8 phosphate buffer (1 + 1 v/v).

(d) *Chromatographic phases*.—Sep-Pak C18 cartridges (Waters Associates) and Alumina Woelm A, Akt. 1 (acidic alumina).

(e) *Tylosin reference standard*.—Dry standard material 3 h in 60°C vacuum oven. Accurately weigh dried standard to obtain 1000 μg tylosin activity/mL. Dissolve in methanol (1 mL methanol/10 mg standard) and dilute to volume with pH 7 phosphate buffer (stock standard solution).

(f) *Standard solutions*.—On day of assay, pipet 5 mL stock standard solution into 50 mL volumetric flask. Add 25 mL methanol and dilute to volume with pH 8 phosphate buffer. Prepare chromatographic column with ca 20 mL acidic alumina and pour contents of volumetric flask over column. Prepare dilutions of column effluent, using methanol-phosphate buffer pH 8 (1 + 1) as diluent, to contain 2.0, 3.0, 4.0, and 5.0 μg tylosin activity/mL.

Microorganism and Media

Maintain *Staphylococcus aureus* ATCC 9144 on Antibiotic Medium 1 slants. Use slants to inoculate Difco Antibiotic Medium 3. Incubate 16-18 h at 37°C on gyratory shaker. Filter Antibiotic Medium 3 through prepleated paper. Inoculate Antibiotic Medium 3 with ca 20 mL inoculum/L.

Rations

Compositions of basal chicken and swine rations are listed in Table 1.

Feed Sampling

Collect composite sample from bulk feed, whether it be in a bag, feed bunker, or feed bin. Use a feed scoop, probe, or other suitable sampling device to collect subsample (at least 500 g) from minimum of 3 locations with bulk feed. Submit entire sample to assay laboratory for analyses.

Feed Sample Preparation

Finely mill all feed samples and thoroughly mix prior to assay. Grind feed samples in mill with 3 mm screen. Mix entire feed sample for 10 min in mixer.

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Table 1. Composition (%) of poultry and swine rations

Ingredient	Poultry	Swine
Ground yellow corn	53.42	69.90
Soybean meal	31.73	26.00
Animal fat	2.83	——
Fish meal—menhaden	5.00	——
Corn distillers dried solubles	4.00	——
Dicalcium phosphate	1.28	1.90
Ground limestone	0.62	0.80
Vitamin premix	0.50	0.50
Salt (NaCl)	0.30	0.50
Trace minerals premix	0.10	0.10
Methionine hydroxy analog	0.17	——
Selenium premix	0.05	0.15
L-Lysine	——	0.15

Extraction and Cleanup

(a) *Premix.*—Extract 10 g premix sample with 200 mL extraction solution in suitable container, such as Mason jar, by mixing on gyratory shaker for 1 h. Filter extract through No. 4 paper. Prepare chromatographic column with ca 20 mL acidic alumina and pour 50 mL extract over column. Collect effluent and dilute to assay level of 3.0–4.0 μg tylosin activity/mL.

(b) *Feeds.—Tylosin levels greater than 100 ppm:* Extract 50 g feed sample with 200 mL extraction solution by mixing on gyratory shaker for 1 h. Filter extract through No. 4 paper. Prepare chromatographic column with ca 20 mL acidic alumina and pour 50 mL extract over column. Collect effluent and dilute to assay level of 3.0–4.0 μg tylosin activity/ mL.

Tylosin levels less than 100 ppm and greater than 10 ppm: Extract 100 g feed sample with 400 mL extraction solution by mixing on gyratory shaker for 1 h. Filter extract through No. 4 paper. Prepare chromatographic column with ca 20 mL acidic alumina and pour 50 mL extract over column. Collect effluent and dilute to assay level of 3.0–4.0 μg tylosin activity/mL.

Tylosin levels less than 10 ppm: Extract 100 g feed sample, filter, and clarify on acidic alumina column as described above. Dilute 25 mL effluent from acidic alumina column with 25 mL phosphate buffer, pH 8. Condition C18 cartridge by pumping 10 mL methanol through cartridge, followed by 10 mL phosphate buffer, pH 8. Pass diluted extract through C18 cartridge. Rinse sample vessel with buffer and add rinse to C18 cartridge. Elute tylosin from C18 column with methanol into volumetric glassware and dilute to volume. Dilute concentrated sample to assay level of 3.0–4.0 μg tylosin activity/mL.

Quantitation

The Autoturb automated turbidimetric system has been previously described (6). Calculations for sample results may be performed by computer, using appropriate programming, or may be determined according to the following: Average turbidities of each pair of duplicate tubes (0.10 and 0.15 mL loops) for both sample and standards. Prepare 2 dose-response curves with averaged turbidities of standard curve levels, one each for 0.10 and 0.15 mL loops. Determine corresponding tylosin level in each sample directly from corresponding dose-response curves by entering curve via turbidity reading and determining concentration. Obtain potency of sample assayed by multiplying average of the 2 tylosin concentrations, determined from the 2 dose-response curves, by dilution factor of sample.

Table 2. Extraction of tylosin from Tylan 40 premix

Extraction soln	Range, g/lb[a]	Mean, g/lb	CV, %
Methanol + buffer[b]	40.0–47.5	43	5.3
Methanol (200 mL)	37.1–42.3	40	4.1
Methanol-water (9 + 1)	40.1–43.9	41	3.2
Methanol-buffer (1 + 1)	35.2–44.8	41	3.8
Methanol-water (1 + 1)	40.0–43.0	41	2.3
Buffer	37.5–40.5	39	2.4
Water	35.3–39.4	37	3.4

[a] Twelve analyses for each range: 3 weighings per day for 4 days.
[b] 100 mL methanol, mix for 30 min; 100 mL pH 8 phosphate buffer, mix for 30 min.

Results and Discussion

Previous tylosin methods (1–4) and the current AOAC method (5) require that tylosin be extracted from premix and feeds with hot pH 8 phosphate buffer solution. This step was necessary when tylosin was gelatinized, but is no longer required by the revised method. Tylosin can be extracted from premix and feed by a variety of extraction solutions as outlined in Table 2. Tylan 40 premix (40 g/lb) was extracted with 200 mL of the indicated extraction solution. The ranges, means, and coefficients of variation for the 12 determinations suggest that tylosin is easily extracted from premix.

Most laboratories which have evaluated this revised method have adopted the use of methanol–pH 8 phosphate buffer (1 + 1), or methanol–water (1 + 1), because these diluents were routinely used to dilute standard solutions. The use of methanol–water (9 + 1) solvent possesses another advantage; this extraction solvent could be used with feeds containing both Tylan and Rumensin® (monensin sodium, Elanco), resulting in a single extraction of sample for 2 separate analyses. A solution of buffer or water alone is not recommended for the extraction of tylosin because the lower recoveries observed and the many water-soluble components which are co-extracted with the tylosin. Methanol alone as the extraction solution required an additional dilution of the extract prior to Autoturb analyses to reduce the methanol concentration.

The removal of matrix interference by the use of an acidic alumina column is demonstrated in Table 3. A nonmedicated chicken ration (Table 1) resulted in a positive bias of the assay on the order of 50–60 ppm. This high bias was observed with several other rations, including swine and cattle feeds. The interference was removed by purification of the extract with an acidic alumina column, but was not removed with either basic or neutral alumina. In addition, the bias was not observed when the extract was concentrated on a C18 column prior to assay. The recovery of tylosin from tylosin-fortified swine and chicken feeds ranged from 95 to 101.4%. The feed components responsible for the high bias observed in the

Table 3. Removal of matrix interference by alumina column

Ration	Alumina column	Tylosin activity, μg/mL	Observed activity, μg/mL
Chicken	—	0.0	50–60
Chicken	basic	0.0	15–65
Chicken	neutral	0.0	3–10
Chicken	acidic	0.0	< 2[a]
Chicken	acidic	10.0	9.5
Chicken	acidic	50.0	50.5
Swine	acidic	20.0	20.3
Swine	acidic	100.0	101.4

[a] Test sensitivity.

Table 4. Identification of feed components responsible for observed assay interference[a]

Feed component[b]	Alumina column	Tylosin activity, μg/mL	Observed activity, μg/mL
SOM	—	0.0	< 2[c]
SOM	acidic	0.0	< 2
SOM	acidic	3.0	3.1
CDDS	—	0.0	< 2
CDDS	acidic	0.0	< 2
CDDS	acidic	3.0	3.0
FM	—	0.0	25–30
FM	acidic	0.0	< 2
FM	acidic	3.0	3.1
AF	—	0.0	25–35
AF	acidic	0.0	< 2
AF	acidic	3.0	3.1

[a] Each result is the average of 3–6 determinations.
[b] SOM = soybean oil meal; CDDS = corn distillers dried solubles; FM = fish meal; AF = animal fat.
[c] Test sensitivity.

chicken ration were identified by evaluating each component of the ration for observed bias. Fish meal and animal fat were the major components of the chicken ration that contributed to the observed interference (Table 4). The addition of the acidic alumina column purification of feed extracts removed the observed interference. The recovery of tylosin from tylosin-fortified fish meal and animal fat averaged 103%.

The effect of analytical sample size on the precision of the tylosin assay is demonstrated in Table 5. Studies were performed with Tylan 40 premix and swine rations containing 20 and 100 g tylosin activity/ton. Previous tylosin methods (2–4) recommend the use of 20–50 g analytical feed samples. These data indicate that a 100 g analytical sample could reduce the observed variation for low-level feeds by nearly 50%. A 50 g sample is recommended for feeds containing greater than 100 ppm tylosin, and a 10 g sample is recommended for premix assays. Accuracy and precision data for the method are listed in Table 6. Two analysts performed the assays independently over 2 time periods. Accuracy data

Table 5. Effect of analytical sample size on tylosin assay precision

Sample	Sample size, g	CV, %[a]
Tylan 40 premix	5	2.5
(40 g/lb)	10	1.8
	20	1.6
Swine ration	50	24.0
(20 g/ton)	100	12.5
Swine ration	20	10.3
(100 g/ton)	50	8.8

[a] N = 3 weighings per day for 3 days.

Table 6. Accuracy and precision of the revised tylosin assay

Ration	N	Anticipated level[a]	Observed mean	CV, %
Chicken	32	10 g/ton	8.9 g/ton	18.2
	36	10 ppm	9.5 ppm	6.6
	35	50 g/ton	49.3 g/ton	11.4
	36	50 ppm	50.5 ppm	6.3
Swine	35	20 g/ton	19.3 g/ton	15.1
	35	20 ppm	20.3 ppm	8.2
	36	100 g/ton	99.6 g/ton	11.1
	36	100 ppm	101.4 ppm	5.3
ICS[b]	36	3 ppm	3.04 ppm	5.6

[a] g/ton = feed containing Tylan premix; ppm = feed fortified with tylosin standard.
[b] ICS = Internal control standard.

were obtained by fortifying chicken and swine rations with tylosin standard to the desired levels. The precision data were obtained by medicating the chicken and swine rations with Tylan premix. Recoveries of tylosin ranged from 95 to 101.5%. Coefficients of variation ranged from 11.1 to 18.2% for the medicated feeds.

The relatively high coefficients of variation associated with the analysis of medicated feeds, as opposed to the recovery samples, indicate that the nature of the collection, handling, and processing of the sample prior to assay may be a major factor in the observed variation of the assay result. As stated previously, a recommendation has been made to collect a composite sample from the bulk feed. In the laboratory, milling and mixing of the samples prior to assay can also greatly affect the observed variation. Another recommendation is to report the final result as a mean of the analysis of multiple weighings. One such process would be to analyze 2 weighings a day for 2 days and report an average of the 4 results. This process will result in increased confidence in the reported values.

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Determination of Ergosterol in Cereals, Mixed Feed Components, and Mixed Feeds by Liquid Chromatography

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A sensitive, rapid, reproducible, and reliable liquid chromatographic (LC) method is described for determination of ergosterol in feedstuffs. The sample is saponified directly and the saponified mixture is extracted with *n*-hexane. Ergosterol is determined without further purification or cleanup steps by using a liquid chromatograph with a 250 × 4.6 mm column packed with LiChrosorb Si 60, 5 μm, and a high pressure column prefilter. The ultraviolet detector is set at 282 nm. The limit of detection was 0.1 ppm; recovery ranged between 96.7 and 102.2%. Diode array technology is used for identification and peak purity control. Under strong UV irradiation (254 nm) and oxygen or nitrogen atmosphere ergosterol was converted almost quantitatively to ergocalciferol. Under the described conditions of the method, ergosterol proved to be stable. Ergosterol was determined in cereals, mixed feeds (e.g., for swine and poultry), and their components of plant and animal origin. It was not found in carcass meal, meat-and-bone meal, citrus pulps, or molasses; only traces were detected in fish meal.

Mold invasion of foods and feedstuffs is generally quantified by using microbiological methods such as plate counting and determination of infection rate (percentage of seed that yields fungi after surface disinfection) (1, 2). These methods are time consuming, poorly reproducible, and, in the case of infection rate, not applicable to mealy samples. The most important disadvantage of these methods is their failure to assess nonviable mycelia, and plate counting may reflect only the content in viable mold spores, and not in nonsporulating mycelia (1, 3, 4).

Within the past years, chemical methods have been developed which avoid these disadvantages because they are based on the determination of mycelial constituents such as chitin and ergosterol.

Chitin is a polymer of *N*-acetyl-D-glucosamine and is one of the most frequently occurring polymers in fungal walls. It is found in mycelia and spores of fungi, but also in the exoskeleton of insects. It can be assayed by colorimetric measurements of glucosamine released by acid (5, 6), alkaline (7, 8), or enzymatic hydrolysis, by measurement of chitosan (9, 10) produced by incomplete hydrolysis of the polymer, or by estimation of total hexosamine using ion-exchange column chromatography (11). The disadvantages of these methods are their low sensitivity and reproducibility, as well as interference by glucosamine or acetyl glucosamine of bacterial, plant, or insect origin.

Seitz et al. (12, 13) proposed ergosterol as a measure of fungal growth. It is the predominant sterol of fungi (molds and yeasts) except in certain aquatic phycomycetes and rust fungi (14–16), and plays an essential role as a component of cell membranes and other cell constituents (14, 17). It is probably formed by some bacteria, but, compared to fungi, only in very small amounts (18). In plants, it is not formed or, if so, only in traces; the main sterols of plants are β-sitosterol and stigmasterol (19, 20). Some members of Gramineae contain low concentrations of cholesterol; animals usually contain this compound as the major sterol (20). Ergosterol has been analyzed by colorimetry, thin-layer and gas chromatography, infrared and ultraviolet spectroscopy, and liquid chromatography (LC) (21–24). The latter method has allowed for considerable reduction in the time required for sterol analysis, without sacrificing efficiency of sample recovery and resolution. The correlation of mycelium dry weight with ergosterol is better than the correlation with chitin (13, 25, 26).

So far, ergosterol has been determined in cereals. Seitz et al. (12, 13) used a procedure consisting of methanol extraction, saponification, reextraction with petroleum benzine, and liquid chromatography. We have found that LC conditions described by these authors cannot be applied to mixed feed components such as rapeseed meal, carcass meal, fish meal, and others, or to mixed feeds. This prompted us to develop a LC method which can be used for ergosterol assay in these commodities as well. Zill et al. (27) described a method for the extraction of bound ergosterol from fungal mycelia by direct saponification followed by *n*-hexane extraction. These authors found that the yield of ergosterol was increased considerably compared to the procedure described by Seitz et al. (12). Therefore, we applied direct saponification to agricultural commodities. In the present paper, this procedure is described, and results are given with respect to sample handling, endurance of LC columns, recovery of ergosterol, and identification by diode array detection. We also studied the stability of this compound.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Constametric III metering pump; Spectro monitor TMD variable wavelength detector; CI-10 integrator; Model SEK plotter (LDC/Milton Roy, Riviera Beach, FL). Floppy 2031 LP (Commodore Business Machines, Inc., Frankfurt, FRG).

(b) *Diode array detector.*—2140 Rapid Spectral Detector; LKB Wavescan EG software (LKB Produkter AB, Bromma, Sweden). Nelson Spectral Search software (Nelson Analytical, Inc., Cupertino, CA). IBM PC AT (IBM Corp.).

(c) *Sample injector.*—Model 7125 syringe loading sample injector with 20, 50, and 100 μL sample loops (Rheodyne, Inc., Cotati, CA).

(d) *Sample syringes.*—25 and 100 μL (Hamilton, Bonaduz, Switzerland).

(e) *LC columns.*—LiChrosorb Si 60, 250 × 4.6 mm, 5 μm (Merck, Darmstadt, FRG). Hypersil MOS, 125 × 4.6 mm, 5 μm (Shandon Southern Products Ltd, Cheshire, UK).

(f) *High pressure column prefilter.*—Prefilter disc, 2 μm (Scientific Systems, Inc., State College, PA).

(g) *UV light box.*—UV-Betrachter Series 29230, 254 and 366 nm. Sylvania F8T5/BLB, steril air 6-9, 220 V, 0.04 W (Camag, Berlin, FRG).

(h) *Blender.*—Moulinette S (Moulinex, France).

(i) *Rotary shaker.*—Gerhardt GmbH & Co. KG, Bonn, FRG.

(j) *Rotary evaporator with heating bath.*—Rotavapor-R 110 (Büchi AG, Flawil, Switzerland).

(k) *Heating block.*—TCS-Metallblock-Thermostat, Vapotherm Mobil I (Labortechnik Barkey, Bielefeld, FRG).

(l) *Ultrasonic unit.*—Sonorex TK 52 (Bandelin Electronic, Berlin, FRG).

(m) *Water bath.*—GFL, Burgwedel, FRG.

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(n) *Filters for extraction.*—Filter paper circles, 90 mm diameter; folded filters, 150 mm diameter (Schleicher & Schuell, Dassel, FRG).

Reagents

(a) *Solvents for LC.*—LC grade (Rathburn Chemicals Ltd, Walkerburn, Scotland).

(b) *Mobile solvents for LC.*—Methanol-water (95 + 5); *n*-hexane-isoamyl alcohol (95 + 5).

(c) *Chemicals and other solvents.*—Merck, Darmstadt, FRG.

(d) *Standards.*—Ergosterol, ergocalciferol, cholesterol, β-sitosterol, campesterol, stigmastanol, stigmasterol (Sigma Chemical Co., St. Louis, MO).

Extraction

Blend 30 g representative sample (particle size ca 0.3 mm) each for extraction method A and B.

Extraction method A: (Sample extraction modified from that described by Zill et al. (27).) Weigh 20 g into 500 mL round-bottom flask. Add 75 mL methanol, 50 mL ethanol, and 10 g KOH. Reflux exactly 30 min at 80°C. Cool mixture and filter sample with suction through fluted paper into 300 mL Erlenmeyer flask; rinse reflux flask and filter with additional 25 mL methanol. Transfer mixture to 250 mL separatory funnel and rinse flask with 25 mL water.

Extraction method B: (Sample extraction is similar to that described by Seitz et al. (12, 13).) Weigh 20 g into 250 mL screw-cap bottle. Add 75 mL methanol and extract 10 min on rotary shaker. Filter sample with suction through fluted paper into 500 mL flask; rinse bottle and filter with additional 25 mL methanol. To filtrate, add 10 g KOH and 25 mL ethanol and reflux exactly 30 min at 80°C. To dilute KOH, shake from time to time. Cool mixture and transfer to 250 mL separatory funnel. Rinse flask with 25 mL water.

Shake saponified mixture from A or B ca 1 min with 50 mL, and then 40 mL portions of *n*-hexane. In a second experiment, shake with portions of petroleum benzine (60–80°C). Let stand 15 min to allow complete separation of layers. Filter upper layer (*n*-hexane or petroleum benzine) over anhydrous Na_2SO_4 into 250 mL round-bottom flask. Rinse original container and filter with additional 5 mL *n*-hexane or petroleum benzine. Discard lower layer. Evaporate combined *n*-hexane or petroleum benzine extracts (up to 40°C bath temperature) to ca 2 mL, and transfer quantitatively to 5 mL snap-cap bottles. Use heating block and gentle stream of nitrogen at 40°C to evaporate to dryness. By brief sonication, dissolve residue in 1-5 mL solvent, depending on concentration of ergosterol expected. For normal-phase system, use *n*-hexane for dissolution; for reverse-phase system, use methanol.

Liquid Chromatography

Recrystallize ergosterol twice from absolute ethanol and dry under vacuum 3 h at room temperature. Prepare 2 stock solutions of ergosterol in absolute ethanol and in *n*-hexane, each containing 1000 and 50 μg/mL.

Adjust flow rate of mobile phase to 2.0 mL/min for LiChrosorb Si 60 column, or 1.5 mL/min for Hypersil MOS. Set wavelength of UV detector at 282 nm. Select "external standard method" and "valley-to-valley" integration and set integration parameters as required. Perform chromatography at room temperature. Inject 20 μL ergosterol analytical standard (50 ng/μL) as calibration amount (1000 ng) for computation of concentrations. Recheck calibration from time to time.

Inject 10–100 μL sample solution, within linear response (10–2500 ng, see *Results and Discussion*). On LiChrosorb Si 60 column, retention time of ergosterol is about 3.9 min; on Hypersil MOS column, retention time is about 2.5 min.

Identification, Peak Purity Control, Spectral Search

Use diode array technology for identification and peak purity control. Inject sample solution to enable (via IBM PC) continuous spectral acquisition over complete UV spectrum during elution. For positive ergosterol identification, use spectrum, isogram, and/or topogram mode. For purity control, acquire spectra on upslope, apex, and downslope of peak. Differences in curve shape indicate presence of an impurity. For further identification of unknown peaks, compare spectra to library, custom-created from Wavescan files.

Recovery Experiments

Blend about 30 g sample. Weigh 20 g blended sample into 250 mL round-bottom flask and add known amount of ergos-

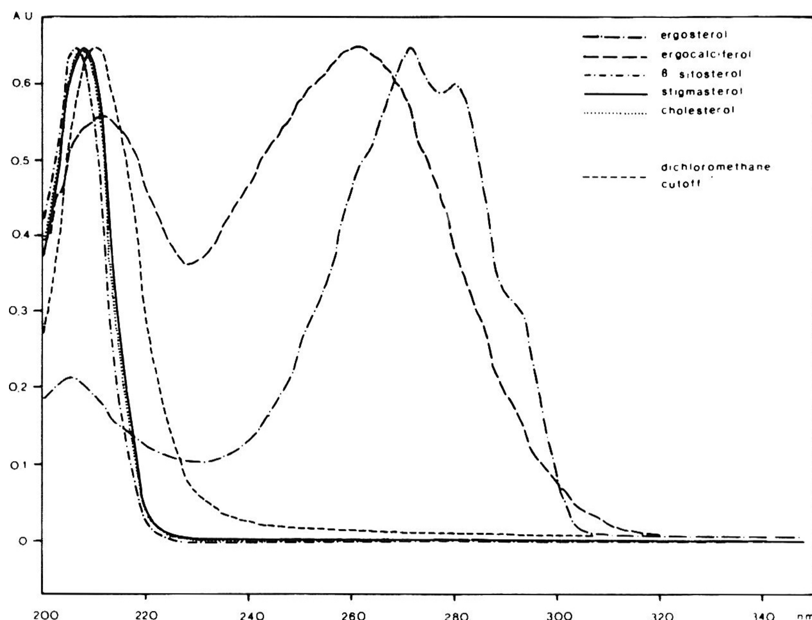

Figure 1. Absorption spectra of different sterols and cutoff of CH_2Cl_2.

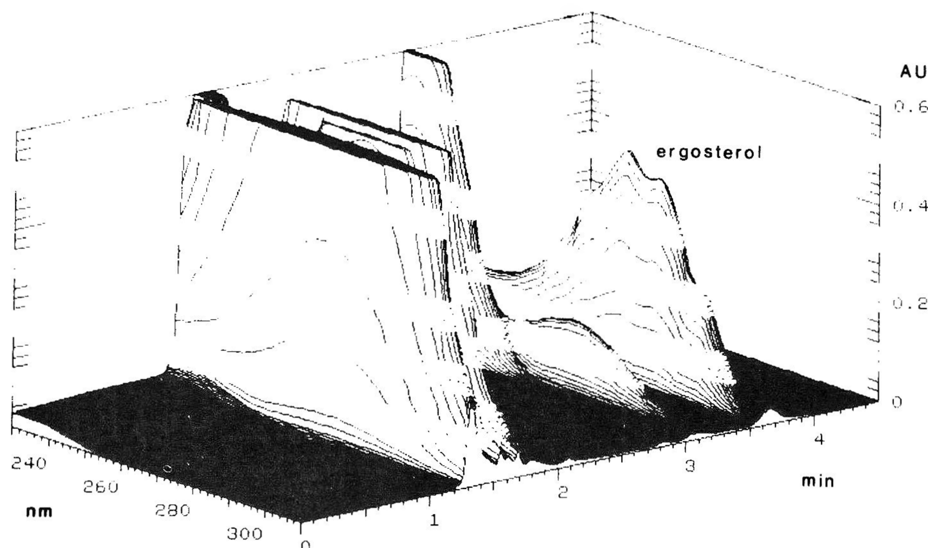

Figure 2. Topogram drawn in spectral mode (diode array detection) of malt sprout extract.

terol in ethanolic solution (e.g., 500 ng). Extract according to procedure A and determine recovery.

Stability Experiments

Select parameters to test stability of ergosterol with regard to sample handling and analytical procedure. For stability experiments, use standard ergosterol solution, kernels and flour of cereals (wheat, barley, oats), and sample extracts. Enclose solid samples in thin leaves of aluminum and sheets of polyethylene; contain liquids in snap-cap bottles of glass and polypropylene. Determine effects of vigorous agitation, temperature (4°C, 40°C), oxygen, illumination, and ultraviolet irradiation (UV light box, 254 nm, 366 nm), and 2 or more of these parameters combined.

Results and Discussion

The UV absorbance spectrum of ergosterol can be used for identification and confirmation (12, 28). Sterols with conjugated double bonds ($\Delta^{5,7}$-unsaturation) are characterized by strong absorbance at high UV wavelengths; those lacking this configuration, such as β-sitosterol, stigmasterol, and cholesterol, absorb only weakly above 240 nm (28, 29) (Figure 1). The specific UV absorbance spectrum of ergosterol with maxima at 282, 271, 293.5, and 262 nm differs from that of all other sterols, including ergocalciferol (vitamin D_2) (Figure 1).

To control purity of the ergosterol peak received from the UV detector, we use diode array technology. Various possibilities of manipulating the results of a personal computer allow detection of the interfering substances masking the presence of ergosterol. In Figure 2, a topogram of malt sprout extract drawn in the spectrum mode demonstrates the separation (see time and wavelength axis) which is required for accurate quantification. With regard to spectrum, isogram, and topogram mode, the solvents in the mobile phase should exhibit UV absorbance cutoffs below 210 nm. For this reason, methylene chloride is not favorable (cutoffs: water < 190 nm, *n*-hexane 190–210 nm, methanol 205–210 nm, petroleum benzine and isoamyl alcohol about 210, methylene chloride 230–245 nm).

For determining ergosterol in cereals, the resolution of reverse-phase columns, e.g., packed with Hypersil MOS (C8 chains), is completely satisfactory (Figure 3). However, Hypersil MOS column as well as LC column types and separation conditions described by Seitz et al. (12, 13), are not applicable to mixed feeds and their components other than cereals. This is due to interfering peaks which do not allow a reliable integration of the ergosterol peak and hence prevent accurate quantification (Figure 4). Unsatisfactory performance was also observed using the columns LiChrosorb RP-18, 10 μm, 250 × 4 mm (Merck, FRG); Superspher RP-18, 4 μm, 250 × 4 mm (Merck, FRG); Hypersil ODS, 5 μm, 125 × 4.6 mm (Shandon, England); and appropriate mobile solvents.

We have found that the use of a normal-phase column

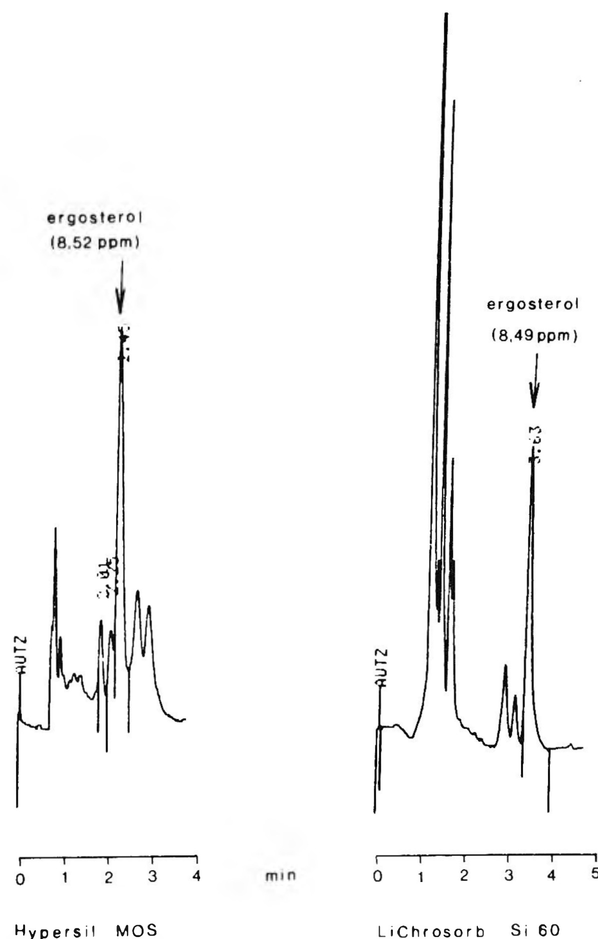

Figure 3. Determination of ergosterol in barley: LC columns Hypersil MOS and LiChrosorb Si 60, injection volume 20 μL each.

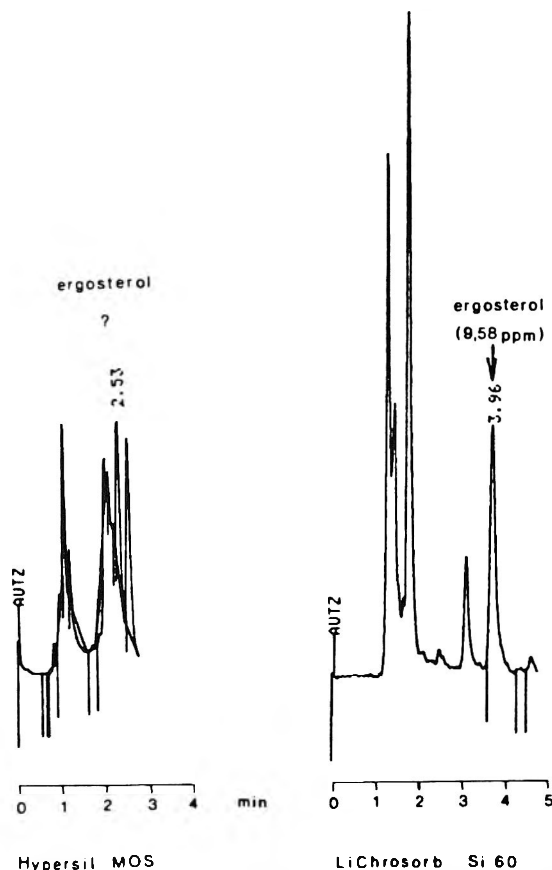

Figure 4. Determination of ergosterol in molded mixed feed for pigs: LC columns Hypersil MOS and LiChrosorb Si 60, injection volume 20 μL each.

(LiChrosorb Si 60, 5 μm, 250 × 4.6 mm) is well adapted to the determination of ergosterol not only in cereals, but also in other mixed feed components and mixed feeds. Values obtained by this method for mixed feed components and mixed feeds are from 1.6 to 2.7 as high as those found with a Hypersil MOS column (extraction method B). For cereals the difference between the 2 column types is within the repeatability of the method (Figure 3).

In agricultural commodities, ergosterol can be converted to ergocalciferol (see below) by sunlight, e.g., by hay making. This leads to erroneous values if the Hypersil MOS column is used since this column does not separate the 2 substances. The molar extinction coefficient (ϵ) of ergocalciferol (18300 at λ(max) 265 nm) is considerably higher than that of ergosterol (11900 at λ(max) 282 nm). Therefore, the formation of ergocalciferol from ergosterol and an incomplete separation of these 2 substances simulate a higher ergosterol content than is actually present. To avoid this overestimation, we prefer the normal-phase column LiChrosorb Si 60, 5 μm, 250 × 4.6 mm for determination of ergosterol in all agricultural commodities.

Ergosterol is detected with a variable wavelength spectrophotometer, operated at 282 nm. The limit of its detection is 10 ng injected into the liquid chromatograph, and 0.1 ppm for the method. The linear range for the estimation of ergosterol is from 10 to 2500 ng. It is nearly the same for LiChrosorb Si 60 and Hypersil MOS.

Seitz et al. (12) and Cahagnier et al. (30) have recommended various cleanup procedures prior to LC determination. We have tested some purification methods employing thin-layer chromatographic plates (pre-coated TLC plates SIL G-25HR, Macherey-Nagel, Düren, FRG), silica gel

Table 1. Increase of ergosterol value (%) determined for method A compared to method B

Sample	Increase, %[a]
Cereals:	
Wheat	35.0
Barley	27.6
Oats	45.0
Corn	55.7
Mixed feed components:	
Soybean meal (toasted)	12.4
Soybean flakes (toasted)	325.7
Sunflower meal	205.5
Wheat bran	257.7
Wheat red dogs	131.8
Malt sprouts	112.3
Maize gluten feed	183.7
Maize gluten	139.3
Maize germ meal	90.2
Manioc meal	151.7
Rapeseed meal	372.6
Field peas	76.6
Broad beans	165.9
Coconut extraction meal	178.6
Palm kernel extraction meal	93.7
Beet pulp	62.5
Beet pulp with molasses	191.0
Linseed cake	279.1
Grass meal	137.3
Alfalfa meal	314.7
Mixed feeds:	
Complete feed for fattening pigs	173.7
Complete feed for pregnant sows	99.9
Complete feed for lactating sows	117.3
Pig starter	133.2
All-mash feed for layers	172.4
All-mash feed for chickens	172.8
All-mash feed for broilers	148.5
All-mash feed for pullets	149.5
Protein supplementary feed for layers	104.6

[a] Each value is the mean of 4 or more samples analyzed in duplicate (full method).

glass columns (silica gel 60, 70-230 mesh ASTM, Merck, Darmstadt, FRG), or Sep-Pak silica and C-18 cartridges (Waters Associates, Inc., Milford, MA). These procedures proved to be time consuming, unsuitable for routine use, and leading to some loss of ergosterol. Both extraction methods described above did not require any purification step prior to LC. Likewise, the use of precolumns packed with normal-phase or reverse-phase materials was not advantageous.

In order to extend lifetime and to ensure high performance of the analytical LC column, we use an in-line high pressure column prefilter which protects against particles introduced with the sample. The filter discs are made of stainless steel. After about 200 injections, the back pressure may increase and their low-cost replacement is due.

After about 400-500 injections, the analytical packing should be regenerated by a special ablution procedure. To resolve particulates and sample constituents that irreversibly bind to the column sorbent, and to restore particles to primary swelling, the LC columns are eluted successively with the following solutions of a flow rate of 1.5 mL/min: 30 mL tetrahydrofuran, 30 mL methanol, 30 mL tetrahydrofuran, 30 mL dichloromethane, and 30 mL *n*-hexane (31).

With regard to sample extraction and practical experiments, both columns withstand more than 1000 injections.

Testing the effect of the extraction solvents *n*-hexane and

Table 2. Reproducibility of ergosterol determination by method A

Extract of portion	Retention time, min		Ergosterol, mg/kg dry matter	
	Wheat	Broad beans	Wheat	Broad beans
1	3.96	3.93	7.99	1.03
2	3.96	3.93	7.63	1.05
3	3.93	3.93	7.69	1.04
4	3.96	3.94	7.82	1.03
5	3.92	3.95	7.79	1.01
X	3.946	3.936	7.784	1.032
SD	0.0195	0.0089	0.1381	0.0148
CV, %	0.49	0.23	1.77	1.43

petroleum benzine on the yields of ergosterol, there was no difference in extraction method B. However, for extraction method A, the yields with *n*-hexane were higher by about 10.4–32.9% than with petroleum benzine. Compared to extraction method B (methanol extraction prior to saponification and reextraction with petroleum benzine), method A (direct saponification with *n*-hexane extraction) yielded a higher ergosterol content with all feedstuffs currently investigated. For individual samples, the yield increased by 25–71% with 4 cereals, 12–377% with 20 mixed feed components, and 67–257% with 9 types of mixed feeds for swine and poultry. Means are shown in Table 1.

Recovery ranged from 96.7–102.2% with extraction method A.

Reproducibility of method A was tested with wheat and broad beans. A representative sample was blended and divided into 5 homogeneous portions. Each portion was extracted and 20 μL of the extract was injected into the liquid chromatograph. As shown in Table 2, the standard deviation of the sample expressed as a percentage of the sample mean is very low, indicating a good reproducibility.

Some modifications of the extraction step of method A (32, 33) proved not to be successful. An acid hydrolysis (6M HCl) of the sample followed by alkaline saponification, yielded products that no longer absorbed at 282 nm, nor did they exhibit the characteristic spectrum of ergosterol. These products might be formed by rearrangements, degradations, or other reactions. A mild acid hydrolysis (0.1M HCl) for labilization of sterols did not increase the ergosterol value significantly.

Ergosterol is unstable under the influence of oxygen and UV light. Visible light might produce bisteroids in the absence, and ergosterol peroxide in the presence of oxygen. Under UV irradiation, a great number of products regularly arise from ergosterol. The principal reaction product is ergocalciferol, which is probably formed via several active intermediates, e.g., previtamin D_2. Minor by-products might be lumisterol and tachysterol (20, 34).

This poses the question of ergosterol stability during sample handling and analysis. During storage of a stock solution (initial content: 1 mg/mL ethanol absolute) in the dark at 4°C under air for 7 days, there was no measurable decrease of ergosterol. This was also true for storage under oxygen at 40°C and under daylight (in glass tubes and polypropylene snap-cap bottles); all these conditions were combined with and without vigorous agitation. These results indicate that, under the given conditions of analysis, no ergosterol is lost. This is in accordance with the recovery of ergosterol of about 100%.

When the stock solution was stored in the dark at 4°C under air for 100 days, a decrease of 23% was observed. Because of this instability, it is necessary to determine ergosterol concentration again prior to each LC run. Likewise, when wheat kernels were stored in the dark at 4°C under air for 2 years, ergosterol content decreased by about 30%. This means that samples which can not be analyzed for ergosterol immediately should be stored in the freezer.

Ergosterol proved to be extremely unstable both in stock solution (under oxygen and nitrogen) and in naturally molded material (under air) during strong UV irradiation (254 nm).

As can be seen from Figure 5, about 97% of ergosterol in the stock solution disappeared within 16 h. Under the conditions employed, the conversion rate to ergocalciferol under both nitrogen and oxygen atmosphere was about 100%.

Figure 6 represents ergosterol decomposition in naturally molded kernels and flour of wheat, barley, and oats. Samples

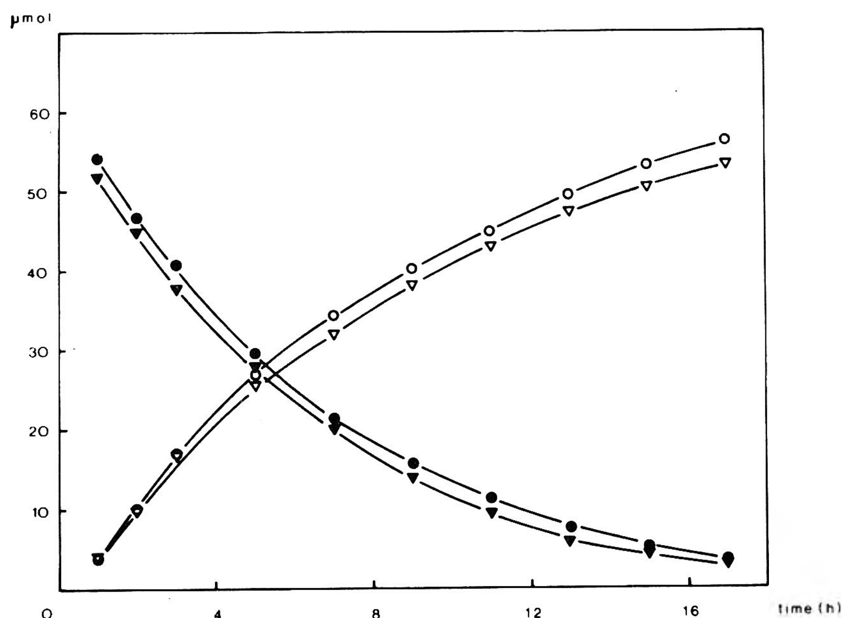

Figure 5. Decrease of ergosterol (full symbols) and increase of ergocalciferol (open symbols) in stock solution (initial content: 62.5 μmol/L) under UV irradiation (254 nm). Solution was kept in polypropylene snap-cap bottles under nitrogen (circles) or oxygen (diamonds) atmosphere at room temperature. Each value represents concentration in separate bottle.

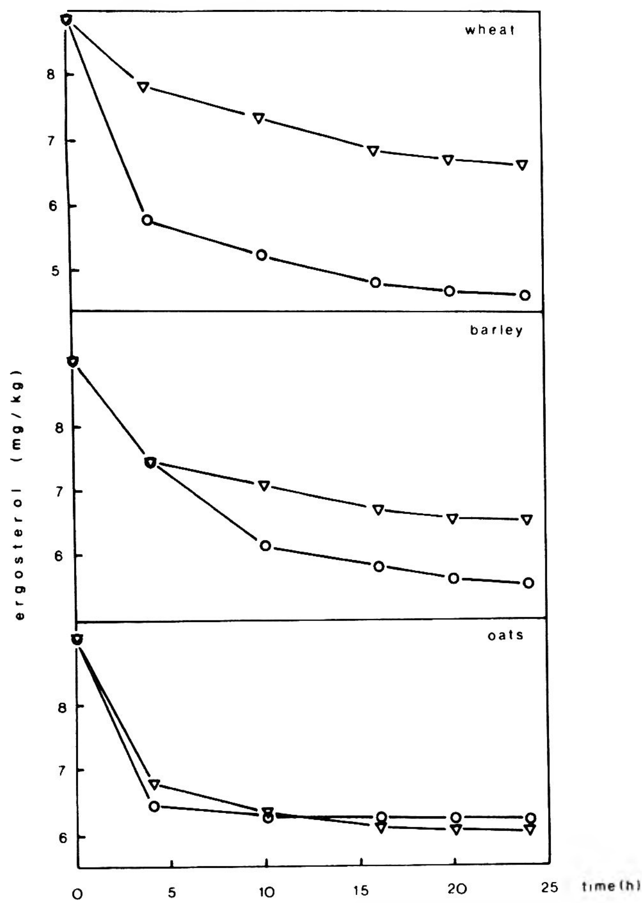

Figure 6. Decrease of ergosterol (mg/kg dry matter) in wheat, barley, and oats (diamond = kernels; circle = flour) under UV irradiation (254 nm). Naturally molded samples were enclosed in polyethylene sheets (layer about 0.5 cm) and stored at room temperature under air.

were enclosed in polyethylene sheets and stored at room temperature under air. Decomposition rate was less in kernels than in wheat and barley flour but not in oats flour. This was possibly due to UV absorbance by the spelts of the oats. Decrease in decomposition rate during irradiation can be explained by the absorbance of UV in superficial layers.

Because of the instability of ergosterol under shortwave UV, sunlight illumination might cause a loss of this compound during production and storage of feedstuffs. It is known that hay making under direct sun strongly favors the production of ergocalciferol, compared to drying of grass at high temperature. Preliminary results of our investigations have shown that, under diffuse daylight, ergosterol in cereals decreases only slightly. This, along with modern methods of harvesting (combines, etc.), suggest only a slight decrease of ergosterol during harvest and storage.

Employing the method described (direct saponification, *n*-hexane extraction, and LC with LiChrosorb Si 60 column), we encountered no problems in detecting and quantifying ergosterol in cereals (wheat, barley, oats, corn), in a diversity of mixed feed components of plant origin, in some mixed feeds for swine and poultry, and in CCM silage. Ergosterol was not found in carcass meal, meat-and-bone meal, citrus pulps, and molasses; only traces were detected in fish meal. All samples were obtained from either farms or a feed factory, and exhibited a good mycological quality. Ergosterol was also determined in samples inoculated with different mold species.

The entire ergosterol analysis requires about 4 h for 3 samples, all done in duplicate along with a separate blank. Thus, the degree of mold invasion of a feedstuff sample can be assayed much more rapidly than by a biological method such as plate count.

Because different extraction methods, LC columns, sample handlings, etc., lead to different ergosterol values, its determination must be considered as a conventional method. Therefore, values can only be interpreted if the same methods have been used.

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Determination of Water-Insoluble Cell Walls in Feeds: Interlaboratory Study

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A collaborative study was conducted to test a new rapid procedure for determination of water-insoluble cell wall (WICW) content in feeds. In the method, starch is solubilized near boiling temperature with Termamyl, a heat-stable alpha-amylase, and proteins are solubilized at 40°C with sodium dodecylsulfate and Pronase. Then, the organic matter of the residue is determined by incineration. Three hours were required to treat 12 different samples, including solubilization treatments, filtrations, and rinses. Eleven unknown products including 9 common feedstuffs of various origin and 2 mixed diets for poultry were analyzed by 7 analysts in France. Coefficients of variation ranged from 2.3 to 6.1%. The results were compared to those for water-insoluble dietary fiber (WIDF), total dietary fiber, and neutral detergent fiber. Agreement was best with the water-insoluble dietary fiber procedure. For most samples, the ratios of WIDF/WICW ranged from 0.981 to 0.842. The differences between WICW and WIDF values correspond to cell wall protein which is accounted for in WICW, but not in WIDF.

Water-insoluble plant cell walls (WICW) include water-insoluble nonstarch polysaccharides, lignin, and cell wall proteins, according to previous works performed on pure cell wall materials (1-5). This definition differs slightly from that given for water-insoluble dietary fiber which includes water-insoluble nonstarch polysaccharides and lignin, but not cell wall proteins (6-8). Complete solubilization of non-cell wall proteins is not needed in the dietary fiber determination because a correction for total residual protein is applied (8), according to the definition of dietary fiber (6).

Complete solubilization of non-cell wall proteins is required for the gravimetric determination of water-insoluble cell walls (3, 4). However, exhaustive solubilization of non-cell wall proteins is difficult and requires long treatments (3-5, 9). The use of a detergent, first introduced by Van Soest (10), speeds up the protein solubilization (5) and shortens the procedure. However, the neutral detergent fiber, determined according to Van Soest and Wine (10), does not include the water-insoluble pectic substances which, in dicotyledonous plant materials, represent a major part of water-insoluble cell walls (4). This is due to the use of ethylenediaminetetraacetate (EDTA), a chelating agent able to solubilize the water-insoluble pectic substances (10), in Van Soest and Wine's procedure. The use of sodium dodecylsulfate (SDS), the detergent introduced by Van Soest, has been reassessed recently for the determination of water-insoluble cell walls (5). The combination of SDS and Pronase was able to solubilize nearly all non-cell wall proteins within only 1 h (5). This deproteinizing step was the basis of the new procedure presented here.

Termamyl, first introduced by Theander and Aman (11), was used in the present procedure and permitted very rapid starch solubilization. These modifications introduced for solubilization of both protein (5) and starch (11) allowed development of an accurate and rapid method for determination of water-insoluble cell walls in feeds. Such a method, convenient for routine analysis, was needed by feed manufacturers for practical application of the new system of energy value prediction for poultry diets, which is based on the determination of water-insoluble cell walls (12). The new procedure presented here required 3 h to treat 12 different samples, including solubilization treatments, filtrations, and rinses.

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Interlaboratory Study

Two kg of each of the 9 ingredients were ground using an ultracentrifuge rotary mill (F. Kurt Retsch GmbH & Co. KG, Haan, Rheinland, GFR) provided with a 0.5 mm mesh sieve. Peas were subsequently reground by ball-milling (Dangoumeau Apparatus with 150 mL cell; Prolabo S.A., Paris) for 10 min. Ground samples were stored in sealed plastic bags at +4°C before mailing.

Blind samples of the 9 ingredients and 2 diets were sent in screw-cap sealed plastic flasks to each of the 7 analysts for determination of water-insoluble cell wall (WICW) contents. Analyst 6 also determined total dietary fiber (TDF), water-insoluble dietary fiber (WIDF) (8), and neutral detergent fiber (NDF) (13). For this purpose, an additional pea sample was sent to analyst 6: This sample was only ground to 0.5 mm and was specified for determinations of TDF, WIDF, and NDF. These methods do not require that mature starchy legume seeds have to be reground by ball-milling before analysis. In all cases, the analysts did not regrind samples before analysis.

The 11 samples were as follows: (*1*) Whole corn grains. (*2*) Whole wheat grains. (*3*) Whole barley grains. (*4*) Whole sorghum grains. (*5*) Cassava roots (82.67% starch on dry matter basis). (*6*) Partly dehulled soybean meal, solvent-extracted (53.94% crude protein on dry matter basis). (*7*) Undehulled rapeseed meal, 0 type, solvent-extracted (39.55% crude protein on dry matter basis). (*8*) Whole seeds of mature white flowered peas (*Pisum sativum*). Samples 1-8 were obtained from Institut Technique des Céréales et des Fourrages (ITCF, Boigneville, France). (*9*) Dehulled full-fat white lupin meal was obtained from Institut National de la Recherche Agronomique (INRA, Nantes, France). (*10*) Diet 1, for poultry, was prepared by INRA (Tours, France) by mixing 49% ground (0.5 mm) corn (sample 1), 19% ground (0.5 mm) soybean meal (sample 6), 20% ground (0.5 mm) peas (sample 8), 7% low-fat meat meal (Française Maritime S.A., Concarneau, France), and 5% animal fat (Salmon S.A., Moisdon-la-Rivière, France). (*11*) Diet 2, for poultry, was prepared by INRA (Tours, France) by diluting diet 1 with 10% calcium carbonate and 3% dicalcium phosphate (Coopérative Agricole La Tourangelle, Tours, France).

Method

Principle

Ground samples are treated first with heat-stable alpha-amylase in nearly boiling buffer (pH 5.6) and then with protease in detergent (sodium dodecylsulfate) solution near neutral pH at 40°C to solubilize starch and protein. The residue is filtered, suspended in chloroform-methanol (2 + 1, v/v), filtered again, and washed with acetone. After drying, the residue is weighed and incinerated for ash determination. WICW corresponds to the residue corrected for its ash content.

Apparatus

(a) *Balance.*—Analytical, with 0.1 mg precision.

(b) *Tubes.*—40 mL round-bottom, with 25 mm internal diameter.

(c) *Magnetic bar.*—Teflon®-coated, round-ended, cylindrical, 15 mm long and 8 mm wide.

(d) *Magnetic stirring and heating apparatus.*—Dry heating aluminum block with holes 1 mm wider than external diameter of tubes, (b), provided with magnetic stirring device to continuously stir suspensions in tubes, (b). Stirring/heating module from Pierce Chemical Co., Rockford, IL.

(e) *Fritted glass crucible.*—Porosity 2 with 40-100 μm pores, 30 mL volume, and 30 mm internal diameter. Before each use, crucibles are cleaned in reverse position with nitric acid-water mixture (50 + 50, v/v) or solution of 40 g potassium dichromate in 1 L sulfuric acid-water mixture (50 + 50, v/v), and then thoroughly rinsed with water. About 0.5 g Celite 545 is added to crucible before samples are filtered.

(f) *Vacuum filtration system.*—Filter flask provided with clamp designed to tightly connect fritted glass crucible, (e). Vacuum pump is fitted to filter flask for filtration. Vacuum pump is replaced by low pressure pump for back-bubbling. Funnel, with ≥ 200 mL capacity, is fitted to glass crucible during filtration of aqueous solutions. Filtration modules "Fibertec" "M," or "E" from Tecator AB (Höganäs, Sweden) were used.

(g) *Drying oven.*—104°C and desiccator.

(h) *Incinerator.*—500°C.

Reagents

(a) *Methanol.*—Analytical grade.

(b) *Acetone.*—Analytical grade.

(c) *Chloroform-methanol mixture.*—Mix 1 L chloroform and 500 mL methanol.

(d) *Acetate buffer, pH 5.6.*—Dissolve 2.8 mL glacial acetic acid and 61 g sodium acetate trihydrate in ca 800 mL water; dilute to 1 L with water.

(e) *Phosphate buffer, pH 7.5.*—Dissolve 7.5 g sodium dihydrogen phosphate dihydrate and 90 g disodium hydrogen phosphate dodecahydrate in ca 800 mL water; dilute to 1 L with water.

(f) *Detergent solution, pH 7.5.*—Dissolve 33 g sodium dodecylsulfate in ca 800 mL phosphate buffer pH 7.5, (e), and dilute to 1 L with phosphate buffer pH 7.5, (e).

(g) *Heat-stable alpha-amylase solution.*—Termamyl 120 L from *Bacillus licheniformis* (Novo A/S, Copenhagen, Denmark). Store enzyme solution at +4°C after each use.

(h) *Protease.*—Pronase from *Streptomyces griseus* (Boehringer Mannheim GmbH, Mannheim, GFR). Store enzyme powder at +4°C after each use.

(i) *Refined alimentary vegetable oil.*—From soybean, corn, sunflower, groundnut, or rapeseed.

(j) *Diatomite.*—Celite 545.

Sample Preparation

Grind sample to pass 0.5 mm mesh sieve. Regrind samples of mature starchy legume seeds (peas, beans, etc) by ball-milling for 10 min, using 5-10 g of 0.5 mm ground sample for 150 mL volume cell.

Determination

Conduct entire procedure with a blank along with the samples.

Weigh 1 g sample, accurate to 0.1 mg, into 40 mL tube provided with magnetic bar. Weigh 0.5 g for samples expected to contain more than 40% WICW. Slowly add water up to 5 mL with continuous stirring, using conventional magnetic stirrer. Stir until complete disappearance of clumps. If necessary, use spatula to break clumps and then rinse spatula into tube, using no more than 1 mL water. Successively add 1.5 mL acetate buffer, 100 μL alpha-amylase solution, and 50 μL vegetable oil (anti-foaming agent), and stir briefly.

Place tube in block of magnetic stirring and heating apparatus stabilized at 100°C. Immediately and with stirring, add 18 mL nearly boiling water. Maintain incubation and stirring for 10 min after suspension reaches 93°C. At 5 min, add 100 μL alpha-amylase solution.

Remove block containing tubes and place in water bath at ambient temperature for cooling. Meanwhile, cool stirring and heating apparatus by placing in empty unheated block.

When temperature in tubes reaches 44-47°C, place block containing tubes in stirring and heating apparatus. Start magnetic stirring. Successively add 10 mL detergent solution pH 7.5 and 10 mg protease dissolved in 2 mL phosphate buffer pH 7.5. Maintain incubation 1 h at 40°C under magnetic stirring.

Add water to glass crucible containing diatomite and filter to obtain an even bed of diatomite.

Filter uncooled suspension through crucible, using vacuum suction. Let filtration proceed for 2 min before applying back-bubbling if necessary for improvement of slow filtration. Rinse precipitate 3 times with water as follows: Fill the crucible with ca 30 mL water, apply bubbling for homogenization, and filter with suction. Add ca 30 mL methanol to residue, apply bubbling, and then filter with suction. Fill crucible with ca 30 mL chloroform-methanol mixture, homogenize with spatula, and let stand 5 min. Complete with chloroform-methanol mixture during this time and mix with spatula if passive filtration of chloroform-methanol mixture is rapid. Apply suction and rinse 3 times with acetone as follows: Fill crucible with ca 30 mL acetone, homogenize contents with spatula, and filter with suction. Apply longer suction (at least 5 min) for last filtration to remove nearly all acetone. Thoroughly disperse acetone-dried residue with spatula.

Dry crucible containing residue in 104°C drying oven for 1 h. Cool crucible in desiccator under vacuum for 25 min and weigh crucible containing residue. Place crucible in incinerator at temperature lower than 100°C. Let temperature increase to 500°C and maintain at least 2 h. Decrease temperature of incinerator to 100°C over more than 1 h. Place crucible in desiccator under vacuum for 25 min, and weigh again.

Calculation

WICW, % = [(mg crucible containing assay residue − mg incinerated crucible) − (mg crucible containing residue of blank − mg incinerated crucible of blank)] × (100/mg sample)

Table 1. Collaborative results of WICW determination in feeds (% by weight)

Analyst	Sample[a] 1	2	3	4	5	6	7	8	9	10	11
1	9.09	8.84	13.01	10.16	—	15.97	32.20	11.97	18.22	10.30	8.96
	9.49	8.89	13.49	10.32	—	—	32.43	11.20	—	10.40	9.19
2	9.10	9.10	13.80	10.40	4.40	14.90	32.00	10.90	19.60	11.40	10.50
	—	—	—	—	—	—	—	11.00	—	—	—
3[b]	10.40	9.50	14.30	13.70	4.00	15.30	32.10	—	19.00	12.60	10.90
	11.00	9.50	14.60	13.80	4.20	15.90	32.20	—	19.80	12.70	11.00
4	9.33	9.27	14.06	10.44	4.18	14.17	31.65	11.18	18.40	11.64	9.26
	9.44	9.28	14.15	10.61	4.27	14.69	32.29	11.45	19.31	11.72	10.12
5	8.68	8.78	13.32	9.96	4.51	14.99	31.03	10.66	20.32	14.77[c]	9.36
	8.99	9.00	13.80	10.04	4.56	15.19	31.70	10.79	20.91	15.34	9.62
6	8.96	9.07	13.68	9.43	4.28	15.11	30.99	11.23	17.83	11.09	9.55
	9.03	9.14	13.73	9.61	4.29	15.18	31.31	10.99	17.89	11.20	9.57
7	8.67	8.55	13.50	9.66	4.08	13.64	30.35	11.13	18.01	10.28	9.26
	8.88	8.70	13.69	9.73	4.30	14.93	30.60	10.96	18.22	10.51	9.72
Mean, % WICW by wt											
	9.06	8.97	13.66	10.03	4.32	14.88	31.50	11.12	18.87	10.95	9.56
Reproducibility											
S_x	0.282	0.245	0.330	0.406	0.157	0.636	0.730	0.35	1.143	0.618	0.451
CV_x, %	3.1	2.7	2.4	4.1	3.6	4.3	2.3	3.1	6.1	5.6	4.7

[a] See text.
[b] Results of this analyst were not retained for statistical calculation. Suspensions were not agitated by magnetic stirring.
[c] Dixon outlier; results not retained for statistical analysis.

Results and Discussion

All analysts carried out the analysis on most samples. According to the responses given in the questionnaire, analyst 3 agitated the suspensions with an alternative shaking in a water bath instead of magnetic stirring as described in the procedure; the data for this analyst were not retained for statistical analysis (Table 1).

For diet 1, the results of analyst 5 were not retained for statistical analysis; the data were outliers according to the Dixon test (14). The CVs of WICW determinations were about 4%, and did not exceed 6.1%.

It is noteworthy that analyst 3 found the highest values in 5 samples (samples 1-4 and 11), demonstrating that agitation is an important factor which needs to be standardized to avoid excess variation. In a preliminary experiment, it was observed that alternative shaking in a water bath could lead to higher values, especially with pea samples. Pea samples (0.5 mm ground) submitted to different types of agitation exhibited different concentrations of cell wall residue. Analyses of pea cell wall residues showed that variation in their concentration was due to variation in starch interference. Lastly, it was also observed that regrinding 0.5 mm ground samples by mall-milling was necessary for some pea samples to achieve complete solubilization of starch during preparation of cell wall residue.

The 11 samples under study covered a wide range of WICW content from 4.32% (cassava root) to 31.50% (rapeseed meal). The starch content of the 11 samples, measured by the EEC polarimetric procedure (15), varied from 4.65% (rapeseed meal) to 71.09% (cassava root). Their crude protein (N × 6.25) content varied from 3.03% (cassava root) to 46.21% (soybean meal). Their lipid content measured as ether extract according to the EEC procedure (15) ranged from 0.54% (cassava root) to 7.69% (white lupin meal). Two of the 11 samples (samples 6 and 7) were heat-processed feed. According to a previous study (4), it can be expected that the cell wall compositions varied widely according to samples.

Diet 2 was prepared by diluting diet 1 with 13% minerals. The WICW content of diet 2 calculated (9.53%) from the diet 1 value (10.95%) was very close to the measured value (9.56%), indicating that the added minerals did not affect the

Table 2. Comparison among methods of determination of fiber fraction of feed (% by weight)

Method	Sample 1	2	3	4	5	6	7	8	9	10	11
WICW[a] (present study)	9.06	8.97	13.66	10.03	4.32	14.88	31.50	11.12	18.87	10.95	9.56
WIDF[b] (8)	9.38	8.60	13.38	9.16	3.79	12.92	26.52	17.27	18.22	10.43	9.38
WIDF (8) (not corrected[c])	11.14	10.14	14.85	13.78	4.09	21.95	38.77	21.25	24.32	14.19	12.72
NDF[a] (13)	8.77	9.46	13.55	10.01	3.36	8.22	22.42	9.04	6.51	9.12	8.05
TDF[b] (8)	9.29	8.69	15.26	9.25	4.91	14.46	29.05	17.37	18.04	11.64	9.91
TDF (8) (not corrected[c])	10.79	10.02	16.69	13.54	5.34	25.38	41.39	22.71	28.91	16.85	13.43

[a] According to procedure, values are not corrected for undegraded residual crude protein.
[b] According to procedure, values are corrected for undegraded residual crude protein.
[c] Not corrected for undegraded residual crude protein.

accuracy of the procedure. In a preliminary experiment, it was observed that mixing maize oil into soybean meal or peas up to 40% of the mixture did not change the WICW content of either, showing that the procedure was efficient in the removal of lipids.

Analyst 6 measured the content of total dietary fiber (TDF) and water-insoluble dietary fiber (WIDF) (8), and the content of neutral detergent fiber (13) in the 11 samples. WIDF contents were measured by applying the destarching and deproteinizing steps of the TDF procedure (8) and then filtration and rinsing with water, without addition of ethanol to the suspensions, so that the water-soluble dietary fiber was not recovered in the residues.

Comparison of methods (Table 2) shows that most of the WICW values were slightly higher than the WIDF values; this difference has to be related to cell wall protein accounted in WICW values, but not in WIDF values. According to Albersheim (1), proteins represent minor components of cell walls (<10%). The differences observed between WICW and WIDF values are in good agreement with this figure. However, high protein contamination of cell wall residues (up to 30% of the residue) can be expected for the samples containing condensed tannins (4, 5) and thus, in such cases, an overestimation of WICW should not be excluded. Therefore, for the samples containing condensed tannins, it can be of interest to determine the amount of undegraded residual protein on a duplicate sample, in the same way as that described in the TDF procedure (8). The WIDF contents calculated without applying correction for undegraded residual crude protein cannot be used for estimation of WICW; such values would be overestimated in most cases (Table 2) because the amounts of undegraded residual proteins were generally high with the WIDF procedure, especially with the protein-rich samples.

For peas (sample 8), the low value of WICW, as compared to the WIDF value, probably resulted from a higher efficiency of the WICW procedure for starch solubilization. This higher efficiency may be related to grinding by ball-milling, which is recommended for mature starchy legume seeds in the WICW procedure but not in the WIDF procedure (8). This also may be related to magnetic stirring, which is a requisite in the WICW procedure but not in the WIDF procedure (8). Alternative shaking in a water bath was used by laboratory 6 for determination of WIDF and TDF. This difference between WICW and WIDF pea values cannot be accounted for by a greater solubilization of nonstarch polysaccharides with the WICW procedure, because in such a case, this difference would also have been observed with similar materials such as soybean meal (sample 6) and white lupin meal (sample 9).

The NDF procedure led to values similar to those of WICW except for dicotyledonous samples (samples 5–11), because of solubilization of water-insoluble pectic substances in the NDF procedure (4). Water-insoluble pectic substances may represent a large part of water-insoluble cell walls isolated from dicotyledonous plant materials (4).

WICW contents were similar to TDF contents for most samples since the amounts of water-soluble fiber [calculated according to the difference (TDF − WIDF)] were generally low. As expected, barley (sample 3), which is known to be rich in water-soluble fiber (16), exhibited a higher TDF value compared to WICW.

The WICW procedure has also been tested for its nutritional significance. In a previous study (12), the apparent metabolizable energy value corrected for nitrogen retention (AMEn) was measured on 48 diets with adult cockerels. These 48 diets stored at 4°C in sealed plastic bags were analyzed for their content of WICW, according to the present procedure. Two multiple regression equations based, respectively, on gross energy (GE), crude protein (CP), and WICW and on ether extract (EE), ash (As), and WICW were calculated for predicting the AMEn values of poultry diets. These equations were as follows (data expressed on dry matter basis):

$$\text{AMEn, kcal/kg} = 0.9362 \times \text{GE (kcal/kg)} - 15.38 \times \text{CP (\%)} - 25.16 \times \text{WICW (\%)}^{1.2}$$

$r^2 = 0.9683$; residual standard deviation = 53 kcal/kg (n = 48)

$$\text{AMEn, kcal/kg} = 3985 + 47.02 \times \text{EE (\%)} - 44.62 \times \text{WICW (\%)} - 53.07 \times \text{As (\%)}$$

$r^2 = 0.9601$; residual standard deviation = 61 kcal/kg (n = 48)

Both equations are very similar to those obtained with a previous WICW method (12), and gave practically the same residual standard deviations as those previously found (12).

Thus, the accuracy of the AMEn prediction for poultry diets is not changed when this new method is used instead of the previous one (12).

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Liquid Chromatographic Determination of Residual Reactants and Reaction By-Products in Polyethylene Terephthalate

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A precipitation procedure and liquid chromatography (LC) were used to measure the residual reactants and reaction by-products in polyethylene terephthalate (PET) polymers and food packages. The polymer is dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol/methylene chloride and then precipitated with acetone. The filtered solution is evaporated almost to dryness, and the concentrate is diluted with dimethylacetamide for LC analysis. Recoveries for terephthalic acid (TA), bis(2-hydroxyethyl) terephthalate (BHET), and the PET cyclic trimer averaged 95, 104, and 98%, respectively. The residual levels of TA, BHET, monohydroxy ethylene terephthalic acid, and the PET cyclic trimer were measured in commercial resins and food packages.

The use of polyethylene terephthalate (PET) as a food-packaging polymer is expanding rapidly. Until recently, it has been used primarily in the production of soft drink bottles. Now, many of the newer PET packaging products are employed to heat or cook food. For example, PET is used for hot sandwich wraps and dual ovenable trays that may be heated in microwave or conventional ovens. A metallized PET film used for susceptor packaging rapidly reaches temperatures exceeding 204°C (400°F) when heated in microwave ovens (1). Susceptor packaging is used for cooking, browning, and crisping foods. The use of a polymer in such harsh environments is not without disadvantages. Residual compounds in the polymers exposed to such temperatures have an increased potential for migrating into the food contained in the polymeric packaging. Because some of the migrants may be potentially harmful to the consumer, food-packaging materials are regulated by the U.S. Food and Drug Administration (2).

To estimate exposure to potential migrants from polymeric food-packaging materials, the identities and concentrations of the possible migrants are determined. Migrants from PET items typically consist of initial reactants, including monomers, and reaction by-products such as low molecular weight oligomers. Although several analytical methods have been developed for PET (3–8), they are generally designed to determine oligomer content or molecular weight distribution or to characterize the prepolymer (7). They do not measure initial reactants.

The present paper describes an analytical method for the determination of several low molecular weight terephthaloyl moieties in PET. These include terephthalic acid (TA), bis(2-hydroxyethyl) terephthalate (BHET), monohydroxy ethylene terephthalic acid (MHET), and cyclic tris(ethylene terephthalate). The method also appears suitable for determining dimethyl terephthalate (DMT) and the homologous series of cyclic oligomers from the tetramer to the cyclic nonamer.

In the method described here, the polymer is dissolved and separated from the low molecular weight residues by a precipitation and filtration procedure similar to that used by Hudgins et al. (6). The residual compounds in the resulting solution are then determined by liquid chromatography (LC). External standards are used for quantitation.

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Experimental

Apparatus

(a) *Chromatographic system.*—Hewlett-Packard Model 1090 solvent delivery system (Hewlett-Packard, Analytical Group, Palo Alto, CA 94303) equipped with Rheodyne Model 7010 20 µL sample injector (Rheodyne, Inc., Cotati, CA 94928) and Rheodyne Model 7125 sample injector with Brownlee C_8, 30 × 4.6 mm, 5 µm guard column (Brownlee Labs, Inc., Santa Clara, CA 95050) as sample loop; Waters Model 480 Lambda Max variable wavelength detector (Waters Chromatography Div., Millipore Corp., Milford, MA 01757) operated at 254 nm; and Nelson Analytical Model 3000 chromatography data system (Nelson Analytical, Inc., Cupertino, CA 95014) run on IBM AT computer.

(b) *LC column.*—Rainin Microsorb C_8, 5 µm, 250 × 4.6 mm (Rainin Instrument Co., Inc., Woburn, MA 01801).

(c) *Filtration system.*—Millipore 47 mm glass filter holder with stainless steel screen and 0.5 µm polytetrafluoroethylene filters (Millipore Corp., Bedford, MA 01730).

(d) *Evaporative concentrator.*—Kuderna-Danish, with 10 mL collection tube, 125 mL flask, and 3-ball condenser column (Kontes, Vineland, NJ 08360).

Reagents

(a) *Solvents.*—LC grade acetonitrile, acetone, and methylene chloride (Burdick & Jackson, Muskegon, MI 49442).

(b) *1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP).*—(Sigma Chemical Co., St. Louis, MO 14508).

(c) *Terephthalic acid (TA).*—(Sigma Chemical Co.)

(d) *Water.*—Deionized, distilled, obtained from Milli-Q water purification system (Millipore Corp.).

(e) *PET cyclic trimer.*—(Eastman Chemical Products, Inc., Kingsport, TN 37662).

(f) *N,N-Dimethylacetamide.*—Practical grade (Eastman Kodak Co., Rochester, NY 14650).

(g) *Bis(2-hydroxyethyl) terephthalate (BHET).*—(Polysciences Inc., Warrington, PA 18976).

(h) *Dimethyl terephthalate (DMT).*—(Polysciences Inc.).

(i) *Acetic acid, glacial.*—(J. T. Baker Inc., Phillipsburg, NJ 08865).

(j) *LC mobile phase.*—*Solvent A:* Water-acetonitrile-acetic acid (85 + 15 + 0.25). *Solvent B:* Acetonitrile-water (85 + 15). Linear gradient programmed as follows at flow rate of 1.5 mL/min: from 5 to 60% B in 8 min; from 60 to 70% B in 8 min; from 70 to 100% B in 1 min; 100% B for 4 min; from 100 to 5% B in 1 min.

Precipitation of PET

Weigh ca 0.3 g polymer into 125 mL Erlenmeyer flask; add 30 mL HFIP-methylene chloride (30 + 70), and let

Table 1. Recovery of terephthalic acid (TA), bis(2-hydroxyethyl) terephthalate (BHET), and polyethylene terephthalate (PET) cyclic trimer from PET

Compound	Added, ppm	Rec., ppm	Rec., %	Av. rec. ± SD, %
TA	4.57	3.98	87	
	4.56	4.71	103	
	4.57	4.38	95	95 ± 8
BHET	6.32	6.34	100	
	6.29	6.58	105	
	6.32	6.84	108	104 ± 4
PET cyclic trimer	24.4	23.8	98	
	27.9	28.0	100	
	24.1	22.8	95	98 ± 2

polymer dissolve. (*Note:* HFIP-methylene chloride is a hazardous mixture and should be used in a fume hood.) Add Teflon magnetic stir bar, and stir rapidly; slowly add 25 mL acetone dropwise from buret to generate very fine polymer precipitate. Filter precipitated polymer through Millipore filtration system, and rinse Erlenmeyer flask with two 10 mL portions of methylene chloride, using rinsings to wash precipitate twice. Transfer filtrate to Kuderna-Danish evaporative concentrator, and evaporate almost to dryness on steam bath; dilute concentrate to 5.0 mL with dimethylacetamide for LC analysis. To determine cyclic trimer, dilute 5.0 mL solution 100-fold by diluting 100 μL aliquot to 10 mL with dimethylacetamide.

Quantitation

All components of PET (except MHET) were quantitated by external standard calibrations based on linear regression analysis of integrated areas (or peak height in case of cyclic trimer) for at least 5 standard solutions. All standard solutions were prepared in dimethylacetamide and ranged in concentration from 0.4 to 70 ppm. Because pure standard for MHET was not available, we assumed that MHET and BHET had same response factor for quantitation. Retention time of MHET was confirmed by LC analysis of synthetic mixture of BHET and MHET prepared by acid-catalyzed esterification of ethylene glycol and TA.

Recovery Studies

Recovery experiments were performed by spiking dissolved polymer with 0.5 mL standard solution containing between 20 and 40 ppm each of TA, BHET, and cyclic trimer. PET in fortified solution was precipitated, and resulting solution underwent LC analysis. Because cyclic trimer is generally present in PET at relatively high concentration of approximately 1%, less polymer (ca 0.01 g) was used for recovery experiments. Table 1 presents recovery results. Table 1 shows good recoveries and illustrates reproducibility of method.

Results and Discussion

The chromatographic separation of PET residual compounds achieved by our chromatographic system using the C_8 reverse-phase column is illustrated in Figure 1. Chromatograms obtained for a standard-component mixture and a commercially available bottle-grade PET are plotted. Figure 1 shows that the standards are completely resolved and that the corresponding residues in the commercial material are well separated and quantifiable. The identities of several

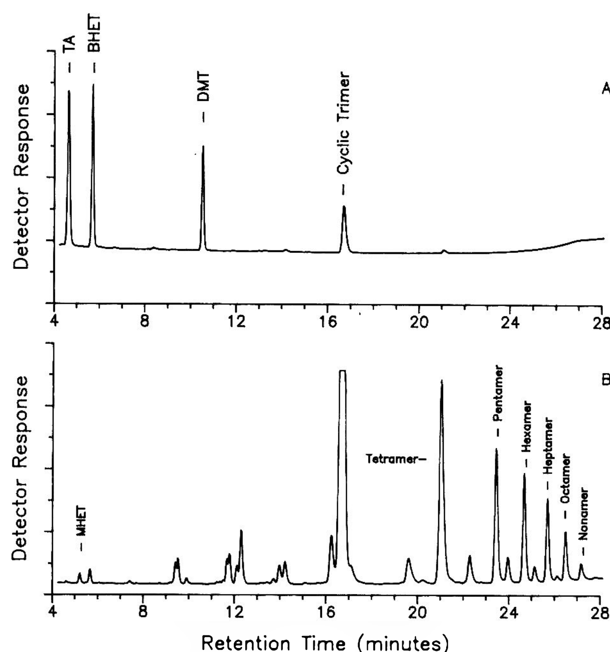

Figure 1. Selected liquid chromatograms obtained for (A) standard solution of 1.46 ppm TA, 1.38 ppm BHET, 0.840 ppm DMT, and 0.826 ppm PET cyclic trimer and (B) a commercially available PET material. Oligomer assignments from the hexamer to the nonamer are tentative. Abbreviations: TA = terephthalic acid; BHET = bis-(2-hydroxyethyl) terephthalate; DMT = dimethyl terephthalate; PET = polyethylene terephthalate.

peaks in the chromatogram of the PET extract have not yet been confirmed. It is believed that the peaks in the 7–14 min retention region are linear oligomers and oxidation products formed in the polymerization process. The peaks in the 17–26 min retention region are probably a homologous series of cyclic oligomers; the first major peak after the cyclic trimer is probably the cyclic tetramer. The series continues through the cyclic nonamer. The minor peaks between the major peaks of the cyclic oligomers are probably another homologous series beginning with the cyclic trimer ether, as described by Hudgins et al. (6). Results obtained from the LC-mass spectrometric analysis of compounds responsible for several of these peaks are consistent with the molecular weights of the cyclic oligomers from the trimer to the pentamer. We plan to confirm the identity of these compounds and to examine the migration of these components in future studies.

The results of the LC analysis of several commercially available PET materials are shown in Table 2. Each value is the average of 3 determinations per item. Under the LC conditions described here, the typical quantitation limit of the method for the cyclic trimer in standard or test solution is at least 50 ng/mL, which is equivalent to 0.8 μg/g in the polymer when 0.3 g polymer is analyzed. The remaining terephthalic acid moieties exhibit similar absorptivities with the UV detector. Of course, if necessary, the quantitation limit can be improved by increasing the portion taken for analysis. The results for TA and the cyclic trimer agree very well with published values (6, 9); however, no information has been reported for MHET and BHET. DMT has not been found in any of the commercial materials analyzed so far, and recovery studies for DMT have not yet been completed. Also, because of the lack of standards, no recovery studies for the higher molecular weight cyclic oligomers have been conducted, and mass spectrometric confirmations are still in progress.

Table 2. Compounds found (ppm) in commercially available polyethylene terephthalate (PET) materials[a]

Item	TA	MHET	BHET	PET cyclic trimer
Beverage bottle	6.9 (0.6)[b]	34.4 (1.5)	49.1 (1.3)	9592 (193)
CPET microwavable tray	3.7 (0.2)	12.5 (0.4)	18.2 (0.7)	7951 (557)
Commercial resin A	2.9 (0.2)	6.4 (0.9)	8.0 (0.9)	8968 (777)
Experimental film	4.8 (0.5)	20.2 (0.6)	36.2 (0.9)	8100 (438)
Commercial resin B	14.3 (1.4)	47.4 (4.9)	44.9 (3.8)	11032 (184)

[a] Abbreviations: TA = terephthalic acid; MHET = monohydroxy ethylene terephthalic acid: BHET = bis(2-hydroxyethyl) terephthalate; CPET = crystallized PET.
[b] Values in parentheses are standard deviations for 3 replicate analyses.

In summary, a precipitation-LC method has been developed that is capable of measuring residual reactants and reaction by-products in PET. The method has several advantages: (*1*) No derivatization of TA is needed for its determination; (*2*) one liquid phase results from the addition of acetone to the HFIP–methylene chloride solvent during precipitation, eliminating liquid/liquid partitioning problems; (*3*) because this method, unlike liquid extraction methods, dissolves the polymer, the residual compounds do not remain encapsulated in the polymer, and the analysis is therefore more reliable; (*4*) the method is capable of monitoring compounds having a wide range of molecular weights, i.e., from TA to oligomers of high molecular weight.

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Optimized Monier-Williams Method for Determination of Sulfites in Foods: Collaborative Study

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A collaborative study was conducted of the Food and Drug Administration (FDA)-optimized Monier-Williams method for determining sulfites in foods. Twenty-one industry and government laboratories participated in the study, which was jointly sponsored by the National Food Processors Association and FDA. Familiarization samples were shipped to each collaborator. Collaborators were permitted to proceed to the main study only after they demonstrated ability to perform the method to ensure that the study tested the performance of the method itself and not that of the individual laboratories. The study design involved 3 food matrixes (hominy, fruit juice, and protein [seafood]). Each matrix was prepared at 3 sulfite levels—the regulatory level, half the regulatory level, twice the regulatory level—and as a blank. All test samples were analyzed as blind duplicates, which gave each collaborator a total of 24 test portions. Collaborative recoveries gave a reproducibility (among-laboratories) coefficient of variation that ranged from 15.5 to 26.6% for sulfite determined as SO_2 by weight in the 3 foods at the 10 ppm level. The optimized Monier-Williams method has been approved interim official first action to replace the AOAC modified Monier-Williams method, 20.123–20.125.

Sulfites in various forms have been added to foods for centuries. Only recently has the widespread use of sulfites in foods become an issue of health concern. It was found that certain individuals exhibited adverse reactions to sulfite residues in foods. This prompted the U.S. Food and Drug Administration (FDA) to require labeling of products containing determinable levels of sulfites. Ten parts per million of sulfur

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The method was approved interim official first action by the General Referee, the Committee on Foods I, and the Chairman of the Official Methods Board and will be submitted for adoption official first action at the 103rd AOAC Annual International Meeting, Sept. 25–28, 1989, at St. Louis, MO. Association actions will be published in "Changes in Official Methods of Analysis," *J. Assoc. Off. Anal. Chem.* (1990) **73**, January/February issue.
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dioxide was the critical level defined by regulation for foods analyzed by the FDA-modified Monier-Williams procedure (1).

The AOAC Monier-Williams method (2) has been the method of choice for many years and has been the reference method with which new methods have been compared for accuracy and precision. Many modifications exist for application to particular matrixes. However, for most matrixes the Monier-Williams method, although time consuming, has proved the most reliable. The Monier-Williams method measures free sulfite in foods, plus a reproducible portion of the bound sulfites, such as the carbonyl addition products.

The relatively minor procedural changes made by FDA in the AOAC Monier-Williams method (2) permit quantitation of sulfites at the 10 ppm level in foods. These minor modifications do not change the chemistry of the method but do establish additional specifications to achieve a lower level of determination. For example, the concentration of the titrant has been reduced by a factor of 10 to permit more accurate measurement of the volume. The risk of interfering substances reaching the hydrogen peroxide trap by aerosolization, co-distillation, or steam distillation has been reduced because values are specified for condenser coolant temperature, reflux ratio, and nitrogen flow. The elimination of the hot condenser step not only reduces operator time but also decreases the likelihood that interfering substances will reach the trap by steam distillation.

Although only minor changes were made in the AOAC method and it had been successfully subjected twice to a 3-laboratory ruggedness test, there were no multiple laboratory data to demonstrate the validity of the method at the 10 ppm level. Therefore, the FDA-optimized method was subjected to a full AOAC collaborative study to further challenge the method, since the procedure was to be used by many laboratories, on many matrixes, to determine low levels (parts per million) of sulfite. In addition, to initially test the performance of the FDA-optimized Monier-Williams method and to identify heretofore unknown sources of variability or errors which should be corrected, both an intralaboratory study and an interlaboratory study were performed before the collaborative study was conducted.

In the intralaboratory study, 3 chemists, 2 of whom had no prior knowledge of the method, performed the analyses independently. The study design involved 3 food matrixes (fruit juice, shrimp, and hominy) each at sulfite levels of 10 and 30 ppm. All test samples were analyzed as blind replicates, which were randomly coded by an independent preparer.

A statistical evaluation of the results from the intralaboratory study established that the procedure is capable of determining sulfites in foods at a level of 10 ppm with an overall coefficient of variation ranging from 3.8 to 9.8% (av. = 7.0%). These estimates of the coefficient of variation are consistent with those already well established for regulatory analytical methods for the 10 ppm region. In particular, the empirical formula of Horwitz (3), based on over 300 collaborative assays and using AOAC methods, predicts an overall coefficient of variation of about 10% at the 10 ppm region. Note that estimates from the intralaboratory study are below this value. As expected, the estimate for the coefficients of variation for the 30 ppm level were lower, ranging from 4.9 to 7.2% (av. = 5.7%).

The interlaboratory study included 3 participating laboratories. The study design involved 4 materials: hominy fortified with 21.5 ppm sulfite, hominy fortified with 42.8 ppm sulfite, shrimp fortified with 26.3 ppm sulfite, and shrimp fortified with 40.0 ppm sulfite. All test portions for analysis were supplied as blind replicates, which were randomly coded by an independent preparer.

Because sulfites are so reactive with air and food matrixes, and because they lack the stability for distant transport, the portions were fortified with sodium hydroxymethylsulfonate (HMS), a stable source of sulfite and the bisulfite addition product of formaldehyde. This compound is structurally similar to some combined forms of sulfite in foods.

A statistical evaluation of the test results established that the method is capable of determining sulfites in foods at a level of about 10–20 ppm with an average overall coefficient of variation of 6.0%.

Collaborative Study

Twenty-one industry and government laboratories participated in the study. Familiarization samples were first analyzed by each laboratory. No collaborator was permitted to proceed to the main study until ability to perform the method was demonstrated (recovery ≥ 80% HMS).

The study design included 3 food matrixes (hominy, fruit juice, and protein [seafood]) each at 3 levels of sulfite, including the regulatory level (about 0.5X, X, and 2X, where X is the regulatory level). Blank determinations were also included. All test samples were analyzed as blind duplicates, which were randomly coded by an independent preparer. Each of the 21 collaborating laboratories analyzed 24 test portions. Separate portions of the fortified matrixes were analyzed throughout the study to observe any breakdown of the sulfite during storage.

Sample Preparation and Stability Studies

The reactive nature of sulfite required careful control of the preparation and storage of analytical samples. Hominy and seafood were each blended in a Hobart mixer to ensure uniform distribution of sulfite throughout the composite and formation of any reaction products before the test portions were packaged.

For example, the 5 ppm hominy composite was prepared by adding 0.60 kg sodium sulfite/hominy premix (321 ppm as sulfur dioxide) to 8.7 kg unsulfited hominy. After extensive blending, the composite was found to contain 3.9 ppm sulfite. The composite was further enriched with sufficient sodium sulfite to increase the sulfite level by 2 ppm. The analytical results for this composite averaged 4.88 ppm.

The test portions were packed in quantities of about 75 g each for single determinations. The test portions were randomly analyzed over a 3 week period to monitor their stability before shipment to the collaborators. The hominy showed no significant loss in sulfite content. The seafood and juice exhibited some degree of instability. The 5 ppm seafood samples had reacted completely with the matrix and were removed from the study; the 10 and 20 ppm test portions dropped to approximately 50% of the initial sulfite values. Similar instability was noted in the juice portions. At the end of this test period (about 3 weeks), we concluded that the test samples were sufficiently stable to proceed with the main study. Since the sulfite value had reached a plateau, it was reasonable to assume stability over the 2 weeks of the study. With the exception of the juice fortified with HMS and the hominy, the products did exhibit some instability, which may have contributed to the variability of some results.

Test portions were shipped frozen to the collaborators, who were instructed to maintain the products below −10°C in a freezer without a self-defrosting cycle until analysis.

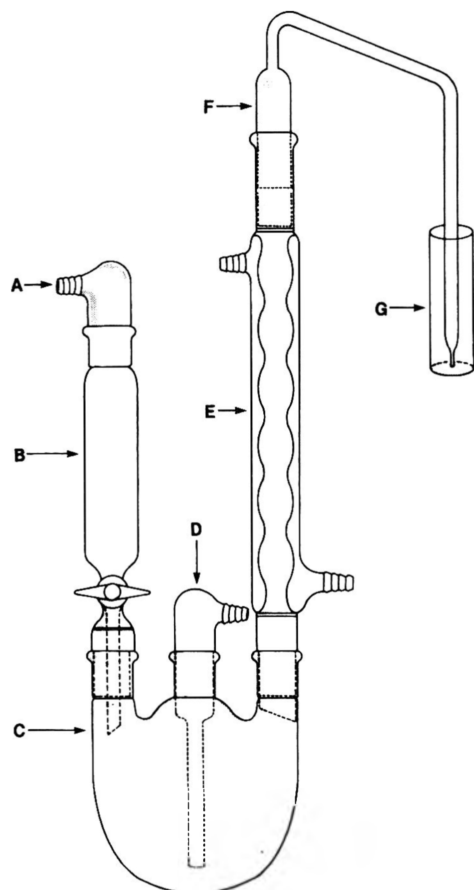

FIG. A—Apparatus for optimized Monier-Williams method: A, inlet adapter; B, separatory funnel; C, round-bottom flask; D, gas inlet tube; E, Allihn condenser; F, bubbler; G, vessel

Sulfites in Foods

Optimized Monier-Williams Method

Interim First Action

(Applicable to determination of ≥ 10 ppm sulfites in foods. Applicable in presence of other volatile sulfur compounds; not applicable to dried onions, leeks, and cabbage.)

Method Performance:

Hominy, 9.17 ppm sulfites:

$s_r = 1.33$; $s_R = 1.42$; $RSD_r = 14.5\%$; $RSD_R = 15.5\%$

Fruit juice, 8.05 ppm sulfites:

$s_r = 1.36$; $s_R = 1.62$; $RSD_r = 16.9\%$; $RSD_R = 20.1\%$

Protein (seafood), 10.41 ppm sulfites:

$s_r = 1.47$; $s_R = 2.77$; $RSD_r = 14.1\%$; $RSD_R = 26.6\%$

A. Principle

Method measures free sulfite plus reproducible portion of bound sulfites, such as carbonyl addition products, in foods. Test portion is heated with refluxing 1N HCl to convert sulfite to SO_2. Stream of N_2 introduced below surface of refluxing solution sweeps SO_2 through water-cooled condenser and, via bubbler attached to condenser, into 3% H_2O_2 solution, where SO_2 is oxidized to H_2SO_4. Sulfite content is directly related to generated H_2SO_4, which is determined by titration with standardized NaOH solution. For verification, sulfate can be determined gravimetrically as $BaSO_4$.

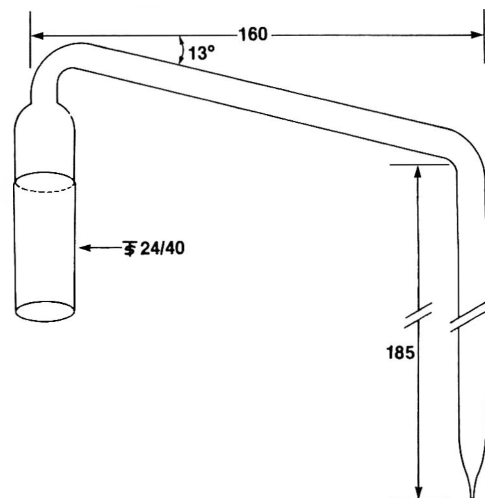

FIG. B—Enlarged diagram of bubbler for Monier-Williams apparatus (lengths in mm)

B. Apparatus

(a) *Distillation apparatus.*—(*Note:* In this method, back pressure inside apparatus is limited to unavoidable pressure due to height of 3% H_2O_2 solution above tip of bubbler (F). Keep back pressure as low as possible to avoid loss of SO_2 through leaks. Use thin film of stopcock grease on sealing surfaces of all joints except joint between separatory funnel and flask. Clamp together each joint to ensure complete seal throughout analysis.) Assemble apparatus (Fig. **A**) which includes: (*1*) Inlet adapter (A) with hose connector (Kontes K-183000). Adapter provides means of applying head pressure above solution. Use of pressure-equalizing dropping funnel is not recommended because condensate, perhaps containing SO_2, is deposited in funnel and side arm. (*2*) Separatory funnel (B), ≥ 100 mL capacity. (*3*) Round-bottom flask (C), 1 L, with three 24/40 tapered joints. (*4*) Gas inlet tube (D) (Kontes K-179000) of sufficient length to permit introduction of N_2 within 2.5 cm of bottom of flask. (*5*) Allihn condenser (E) (Kontes K-431000-2430), jacket length 300 mm. (*6*) Bubbler (F), fabricated from glass according to dimensions in Fig. **B**. (*7*) Vessel (G), ca 2.5 cm id and 18 cm deep.

(b) *Buret.*—10 mL (Kimble Glass, Inc., No. 17124-F) with overflow tube and hose connections for Ascarite tube or equivalent air-scrubbing apparatus to permit maintenance of CO_2-free atmosphere over standardized 0.010N NaOH.

(c) *Chilled water circulator.*—Chill condenser with coolant, such as methanol-water (20 + 40 v/v), maintained at ≤ 15°. Circulating pump Neslab Coolflow 33 (Neslab Instruments, Inc., PO Box 1178, Portsmouth, NH 03801), or equivalent, is suitable.

C. Reagents

(a) *Aqueous hydrochloric acid.*—4N. For each analysis, prepare 90 mL solution by adding 30 mL HCl to 60 mL deionized (18 megohm) water.

(b) *Methyl red indicator.*—Dissolve 250 mg methyl red in 100 mL ethanol.

(c) *Standardized titrant.*—0.010N NaOH. Certified reagent may be used (Fisher SO-5-284). Standardize solution with reference standard KH phthalate.

(d) *Hydrogen peroxide solution.*—3%. For each analysis, dilute 3 mL ACS reagent grade 30% H_2O_2 to 30 mL with deionized (18 megohm) water. Just prior to use, add 3 drops

methyl red indicator and titrate with 0.010N NaOH to yellow end point. If end point is exceeded, discard solution.

(e) *Nitrogen.*—High purity, used with regulator to maintain flow of 200 mL/min. To guard against oxygen in N_2 gas, use GC-type trap (Oxy-Purge N [Applied Science], or equivalent).

Alternatively, oxygen-scrubbing solution, such as alkaline pyrogallol, in gas-washing bottle (Kimble Glass, Inc.) may be used. Prepare trap as follows: (*1*) Add 4.5 g pyrogallol to trap. (*2*) Purge trap with N_2 for 2–3 min. (*3*) Prepare KOH solution by adding 65 g KOH to 85 mL H_2O. **Caution:** Heat is generated. (*4*) Add KOH solution to trap while atmosphere of N_2 is maintained in trap.

D. Sample Preparation

(a) *Solids.*—Transfer 50 g food, or quantity that contains 500–1500 μg SO_2, to food processor or blender. Add 100 mL ethanol-water (5 + 95 v/v) and briefly grind mixture. Continue grinding or blending only until food is chopped into pieces small enough to pass through standard taper 24/40 joint of flask (C).

(b) *Liquids.*—Mix 50 g test sample, or quantity that contains 500–1500 μg SO_2, with 100 mL ethanol-water (5 + 95 v/v).

Note: Carry out sample preparation and analysis as quickly as possible to avoid loss of labile forms of sulfite.

E. System Preparation

Using apparatus assembled as shown in Fig. **A**, position flask (C) in heating mantle controlled by power-regulating device (rheostat), and add 400 mL H_2O to flask. Close stopcock of separatory funnel (B) and add 90 mL 4N HCl to separatory funnel. Begin N_2 flow at 200 ± 10 mL/min. Initiate condenser coolant flow at this time. To vessel (G) add 30 mL 3% H_2O_2, which has been titrated to yellow end point with 0.010N NaOH. After 15 min, apparatus and water will be thoroughly deoxygenated and prepared test portion may be introduced into system.

F. Sample Introduction and Distillation

Remove separatory funnel (B) and quantitatively transfer test portion in aqueous ethanol to flask (C). Wipe tapered joint clean with laboratory tissue, quickly apply stopcock grease to outer joint of separatory funnel, and return separatory funnel to flask. Nitrogen flow through 3% H_2O_2 solution resumes as soon as separatory funnel is reinserted into appropriate joint in flask. Examine each joint to be sure that it is sealed.

Use rubber bulb equipped with valve to apply head pressure above HCl in separatory funnel. Open stopcock in separatory funnel and let HCl flow into flask. Continue to maintain sufficient pressure above acid solution to force solution into flask. Stopcock may be closed, if necessary, to pump up pressure above acid, and then opened again. Close stopcock before last 2–3 mL drains out of separatory funnel to guard against escape of SO_2 into separatory funnel.

Apply power to heating mantle. Use power setting that causes 80–90 drops/min of condensate to return to flask from condenser. Let contents of flask boil 1.75 h, and then remove vessel (G).

G. Determination

(a) *Titration.*—Titrate contents of vessel (G) with 0.010N NaOH to yellow end point that persists ≥20 s. Compute sulfite content, expressed in μg SO_2/g food (ppm), as follows:

$$SO_2\text{, ppm} = (32.03 \times V_B \times N \times 1000)/wt$$

where 32.03 = milliequivalent weight of SO_2; V_B = volume (mL) of NaOH of normality N required to reach end point; 1000 = factor to convert milliequivalents to microequivalents; wt = weight, g, of test portion introduced into 1 L flask.

(b) *Gravimetric determination.*—Optional. Following titration, rinse contents of vessel (G) into 400 mL beaker. Add 4 drops 1N HCl and excess of filtered 10% $BaCl_2$ solution, and let mixture stand overnight. Wash precipitate by decantation 3 times with hot water through weighed gooch crucible. Wash with 20 mL alcohol and 20 mL ether, and dry at 105–110°.

$$SO_2\text{, ppm} = (\text{mg } BaSO_4 \times 274.46)/\text{g sample}$$

(c) *Blank determination.*—Determine blank on reagents both by titration and gravimetrically, and correct results accordingly.

H. Recovery Assays

To become familiar and proficient with method before routine use, analyze food test portions containing known amounts of sulfite. Perform analysis in manner that precludes any loss of sulfite by oxidation or reaction with components in food. Since sulfites are reactive with air and food matrixes and lack stability, fortify portions with stable source of sulfite, not sodium sulfite or similar salts. Sodium hydroxymethylsulfonate (HMS), which is bisulfite addition product of formaldehyde and is structurally similar to some combined forms of sulfite in foods, is useful for preparing stable fortified test materials.

For analysis, transfer 50 g prepared sample of sulfite-free food to Monier-Williams flask. Add aliquot of aqueous solution of HMS sodium salt. Analyze solution immediately.

HMS recoveries of ≥80% from food matrixes fortified at 10 ppm are recommended to ensure accurate analytical data.

Ref: JAOAC **72**, May/June issue (1989).

CAS-7446-09-5 (sulfur dioxide)

Results and Discussion

Twenty-one laboratories concerned with the analysis of foods for sulfites participated in the collaborative study. A preliminary study included all participating laboratories to determine competence with the method. Results of these analyses indicated that all laboratories permitted to proceed with the main study were reasonably familiar with the method.

For the main study each of the participating laboratories analyzed 24 test portions as blind duplicates. Recovery studies of sulfite from fruit juice fortified at the 10 ppm level with HMS averaged 80.5%.

Sulfite data submitted by 21 laboratories are tabulated in Tables 1–3. A statistical summary of the data with outliers excluded according to the criteria in ref. 4 is shown in Table 4 and is depicted diagrammatically in Figure 1.

The major focus of this study was determination of the reproducibility of the method for quantitation of sulfites in foods at the 10 ppm level. An examination of the recovery data indicates reproducibility (among-laboratories) coefficients of variation that ranged from 15.5 to 26.6% for sulfite determined at the 10 ppm level in hominy, fruit juice, and protein (seafood).

Table 1. Collaborative results for analysis of sulfited hominy by optimized Monier-Williams method

Coll.	Sulfite content, ppm[a]							
	Blank		5		10		20	
1	0.44	0.38	5.27	5.40	9.80	9.48	14.83	—[b]
2	1.60	—[b]	6.17	6.57	6.01	10.40	18.12	14.97
3	0.18	0.34	4.11	0.52	9.06	4.46	9.64[c]	0.25[c]
4	0.22	0.14	5.75	5.95	17.03[c]	11.60	18.97	11.63
5	1.20	0.56	4.93	4.90	8.85	8.37	16.13	15.97
6	0.30	0.21	4.48	4.63	8.69	9.17	15.22	15.00
7	0.86	0.80	3.90	1.53	8.29	8.43	13.64	15.15
8	0.00	0.00	4.38	4.00	9.19	9.59	15.04	13.86
9	0.33	0.29	4.75	4.54	8.63	8.29	15.20	14.57
10	0.75	3.22[c]	4.95	7.82	10.15	12.72	15.78	17.82
11	10.45[c]	0.86	5.71	45.28[c]	58.25[c]	11.39	—[b]	89.21[c]
12	0.20	0.22	3.70	4.11	8.61	9.57	15.28	15.57
13	0.20	0.33	4.70	4.91	9.96	8.50	14.69	15.44
14	2.15[c]	2.94[c]	4.31	4.46	8.95	9.16	7.36	14.47
15	0.65	1.72	3.97	9.86[d]	11.60	14.19[c]	14.19	15.32
16	0.73	0.68	1.12	3.72	7.47	8.84	11.87	11.68
17	0.43	0.65	5.16	5.36	8.34	10.97	15.26	15.06
18	0.65	1.08	5.38	5.33	10.39	10.68	14.79	15.75
19	0.70	0.37	8.02	7.37	8.09	9.83	15.64	14.32
20	0.58	0.96	6.15	5.76	9.80	10.82	16.56	16.26
21	0.97	0.68	5.74	5.36	10.13	10.30	15.92	16.52

[a] Sodium sulfite used for all fortifications.
[b] No result reported: breakage, incorrect treatment, or deviation from procedure.
[c] Grubbs outlier.
[d] Cochran outlier.

Collaborator Comments

Collaborators were encouraged to submit any comments, suggestions, criticisms, or descriptions of difficulties pertaining to the method that they considered important. No responses were received from any of the collaborators about the procedure, which indicated ease and capability in using the method. Comments were received in regard to packaging of the test samples because holes and actual breaks in the plastic bags had occurred, causing leakage or total loss of product. Other participants reported extending reflux time by an additional hour, using water rather than ethanol to rinse the funnel, forgetting to add HCl to the test portion, not deoxygenating the H_2O_2 trap before titration, using a Pasteur pipet in place of the recommended bubbler, and condenser failure.

One collaborator observed that the H_2O_2 trap attained a deeper shade of yellow after the nitrogen had bubbled

Table 2. Collaborative results for analysis of sulfited fruit juice by optimized Monier-Williams method

Coll.	Sulfite content, ppm[a]									
	Blank		5		10		20		10[b]	
1	1.19	1.44	3.29	3.93	7.43	8.13	19.55	18.78	8.12	7.13
2	2.01	2.46	3.38	3.59	6.50	9.76	18.14	16.94	10.69	8.04
3	0.59	12.86[c]	2.09	1.95	10.74	6.59	18.52	8.23[c]	8.12	6.91
4	0.94	1.02	3.42	3.74	8.67	7.89	26.77	22.70	9.24	9.27
5	1.34	1.74	2.48	2.89	7.16	5.66	18.37	20.24	9.46	9.27
6	0.77	0.71	2.83	3.33	6.69	7.38	20.49	20.36	8.44	8.12
7	1.36	1.33	1.77	2.62	6.34	5.60	18.01	16.98	6.98	7.64
8	0.27	0.00	2.91	1.89	4.86	5.78	14.13	16.10	5.16	5.22
9	1.21	0.90	2.77	2.62	7.05	4.85	19.11	18.81	7.67	7.49
10	1.49	2.36	2.95	3.00	7.60	6.81	19.91	19.66	9.52	9.98
11	2.46	1.77	5.79	2.38	7.64	9.05	18.11	13.31	9.53	8.38
12	1.24	0.78	3.43	2.48	6.59	7.46	18.26	18.63	7.53	7.62
13	0.80	1.70	2.17	2.49	7.43	6.58	14.50	18.25	7.57	12.17
14	0.64	1.73	2.59	2.78	6.78	6.27	16.83	18.03	8.98	6.30
15	1.91	2.48	5.04	4.15	7.04	8.01	13.73	14.80	8.67	10.07
16	0.68	1.70	0.34	3.06	7.10	6.34	13.30	16.02	2.68	7.89
17	0.72	0.95	1.75	2.24	4.93	5.80	18.00	17.29	6.81	7.15
18	1.58	1.87	3.24	3.31	7.21	7.37	16.70	19.41	8.73	7.87
19	0.84	1.23	0.22[d]	—[e]	4.81	2.63	19.27	14.19	5.64	8.10
20	1.69	1.19	3.86	4.51	7.39	6.82	20.26	18.09	8.20	8.78
21	1.23	1.36	3.45	3.48	6.94	7.52	16.83	15.91	7.88	8.67

[a] Sodium sulfite used for all fortifications except for 1 set of duplicates fortified at 10 ppm level. See footnote *b*.
[b] Sodium hydroxymethylsulfonate used to fortify this set of duplicates.
[c] Grubbs outlier.
[d] Cochran outlier.
[e] Sample lost.

Table 3. Collaborative results for analysis of sulfited protein (seafood) by optimized Monier-Williams method

Coll.	Sulfite content, ppm[a] Blank		5		10	
1	0.73	0.69	6.56	6.16	11.85	13.01
2	3.94	2.95	6.49	7.47	11.47	11.98
3	0.89	0.57	1.99[b]	1.19	2.52	12.05[b]
4	1.03	0.99	6.09	8.93	9.81	10.66
5	1.27	1.00	8.40	5.78	8.78	8.85
6	0.93	0.95	6.62	6.45	11.03	11.86
7	5.42[c]	1.79	5.37	3.68	9.26	7.61
8	0.41	0.62	7.14	7.37	15.92	19.63
9	1.00	1.23	5.41	5.07	10.59	12.10
10	1.67	1.69	6.83	5.98	7.77	6.47
11	2.86	2.28	25.59[b]	11.80	10.01	15.42
12	0.18	0.77	5.26	6.37	9.30	9.02
13	0.76	0.76	7.80	5.97	10.07	10.51
14	1.11	1.41	7.69	5.35	10.45	10.91
15	3.86	2.77	6.29	5.82	11.28	14.67
16	2.27	2.35	8.00	6.14	5.50	7.51
17	1.00	1.47	5.51	5.46	6.77	8.79
18	—[d]	1.76	6.47	5.59	7.90	7.52
19	1.35	1.72	8.26	7.50	10.50	8.22
20	1.96	1.53	7.85	8.96	8.95	11.86
21	1.35	1.27	7.82	9.67	11.84	11.24

[a] Sodium sulfite used for all fortifications.
[b] Grubbs outlier.
[c] Cochran outlier.
[d] Sample discarded; deviation from procedure.

Table 4. Statistical summary of collaborative results for determination of sulfite in foods

Food	Target concn, ppm	Av. rec., ppm	RSD_r[a], %	RSD_R[b], %
Hominy	5	4.88	20.0	31.6
	10	9.17	14.5	15.5
	20	15.41	10.0	10.0
Fruit juice	5	2.87	27.2	36.5
	10	6.89	15.8	20.3
	10[c]	8.05	16.9	20.1
	20	17.87	9.4	14.8
Protein (seafood)	5	6.67	15.3	19.2
	10	10.41	14.1	26.6

[a] Repeatability (within-laboratory) relative standard deviation.
[b] Reproducibility (among-laboratories) relative standard deviation.
[c] Fortified with sodium hydroxymethylsulfonate.

through it for 15 min. That analyst recommended that the color of the H_2O_2 solution be adjusted by dropwise addition of the titrant after 15 min of bubbling.

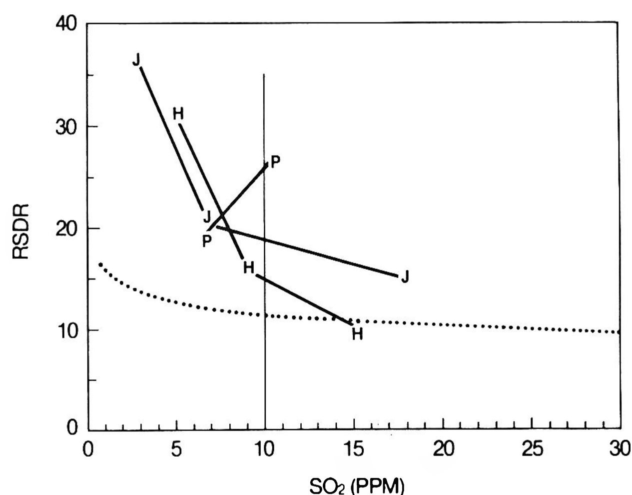

Figure 1. Plot of statistical data summarized in Table 4. H = hominy; J = fruit juice; P = protein (seafood); dotted line = historically expected reproducibility (RSD_R).

Recommendations

It is recommended

(*1*) That the method be adopted official first action for the quantitation of ≥ 10 ppm sulfites in foods.

(*2*) That laboratories become familiar and proficient with the method before using it on a routine basis. HMS recoveries of ≥80% from food matrixes fortified at the 10 ppm level are recommended to ensure accurate sulfite analytical data.

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Determination of Sulfites in Foods by Simultaneous Nitrogen Purging and Differential Pulse Polarography

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An improved technique has been developed for determination of sulfites in food by differential pulse polarography. A Teflon™ sleeve is fitted to the dropping mercury electrode capillary so that SO_2 is purged from the sample and simultaneously detected at peak potential. Bound sulfite in the sample is released at room temperature by addition of base in the absence of oxygen. For some foods, the prepared sample was passed through a Sep-Pak C-18 cartridge to remove naturally occurring sulfur compounds so that only added sulfite is measured. The level of detection was approximately 1 μg SO_2/g. Results agreed with those obtained by the optimized Monier-Williams method for a variety of foods.

The addition of sulfites to foods has become an important safety issue because of a number of reported adverse reactions in hypersensitive individuals. This has prompted government agencies to review the use of sulfites as preservatives in foods and to issue new regulations. The addition of sulfites to fresh fruits and certain vegetables is now prohibited by the U.S. Food and Drug Administration (FDA), and a label declaration requirements has been issued for detectable sulfites in other food products. The regulation has defined "detectable" as 10 ppm SO_2 as determined by the optimized Monier-Williams method (1).

Sulfites in some foods are not reliably determined by the Monier-Williams method because degradation compounds such as hydrogen sulfide interfere in the analysis (R. L. Madl, Ralston Purina Co., St. Louis, MO, personal communication, 1986). Furthermore, brussel sprouts, cabbage, garlic, soy isolates, etc., contain naturally occurring compounds that release SO_2 during the assay procedure (Office of Regulatory Affairs-Office of Regional Operations, U.S. Food and Drug Administration, Rockville, MD, personal communication, 1987). Consequently, many other more sensitive and specific methods have been proposed, including ion chromatography (2), flow injection analysis (3), use of Ellman's reagent (B. L. Madison, AOAC Task Force on Sulfites, Washington, DC, 1985), enzymatic determination, ion exclusion chromatography (4), and differential pulse polarography (DPP) (5).

The DPP method has been collaboratively studied (5) and was adopted by AOAC for determination of total sulfite (5). In principle, the DPP method uses the same procedure for isolating sulfite from the sample as does the Monier-Williams method, i.e., purging with nitrogen, but a simplified apparatus is used, with detection by DPP. Both free and total sulfites are measured. The sample is blended with 5% alcohol to minimize the oxidation of sulfite by atmospheric oxygen. Free sulfite is released from the sample at pH 1.5 by purging with nitrogen at room temperature; the free sulfite is then trapped in a pH 5.2 acetate buffer and polarographed. Subsequently, bound sulfite is similarly released from a more strongly acidified sample by heating; the bound sulfite is then trapped in the same aliquot of the buffer and polarographed as total sulfite.

We have recently developed a new technique in which volatile electrochemically active species are purged with nitrogen and simultaneously measured at peak potential by differential pulse polarography. This has been achieved by modifying the dropping mercury electrode capillary with a Teflon™ sleeve. This technique provides a useful tool for observing the rate of evolution of the species from the sample. As a result, the quality of analysis is significantly improved.

The present paper describes the application of this technique for the determination of sulfites in foods. During the course of the study, other improvements to the original method were also developed. For example, an efficient procedure for releasing bound sulfite has been developed, which permits rapid determination at room temperature. Sep-Pak™ C-18 cartridges have also been found useful for differentiating between added sulfite and naturally occurring compounds that release sulfur dioxide. A variety of foods have been analyzed, and the results of the proposed technique have been compared to those of the Monier-Williams method.

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METHOD

Apparatus

(a) *Polarographic analyzer.*—Capable of DPP analysis. Equipped with 3-electrode cell arrangement, i.e., dropping Hg electrode, Pt wire counter electrode, and Ag-AgCl reference electrode; Model 264A (EG&G Princeton Applied Research Corp., Princeton, NJ 08543), equipped with Model 303 static Hg drop electrode (SMDE) and Model RE0089 X-Y recorder, or equivalent. Strip chart recorder is recommended for recording rate of SO_2 evolution. Suggested polarographic conditions: initial potential, −0.50 V; final potential, −0.85 V; modulation amplitude, 50 mv; scan rate, 5 mv/s; drop time, 1 s; mode, differential pulse; range, 10 μamp. Constant voltage operation: same as above except that scan rate was set at 0 and voltage was manually set at peak potential with sulfite standard in deaerated electrolyte-trapping solution.

(b) *Purge-trap apparatus.*—Connect to polarographic cell through hole of electrode support, adjacent to capillary (see Figure 1). Secure Teflon tubing to plunger with tape. Position tubing so that it does not interfere with operation of capillary.

(c) *Capillary sleeve.*—Cut 47 mm diameter Millipore LS Mitex (PTFE) type, 5 μm filter into 25 × 37 mm rectangle. Roll lengthwise onto unconnected capillary and fuse into cylinder by momentarily pressing on hot plate set at medium heat. Teflon sleeve that is formed can be moved along capillary and set at any position. In operation, set sleeve to extend below the capillary tip by ca 2 mm.

(d) *Tank nitrogen.*—Oxygen-free. Pass nitrogen through alkaline pyrogallol solution prepared as follows: add 20 mL 1N NaOH to 25 × 200 mm test tube with inlet and outlet tubing, and purge with nitrogen for 5 min. Then add about 1 g pyrogallol and reconnect.

(e) *Homogenizer.*—Polytron (Brinkmann Instruments, Inc.), or equivalent.

(f) *Glass tubes.*—Borosilicate, 25 × 200 mm.

(g) *Micropipets.*—50 μL.

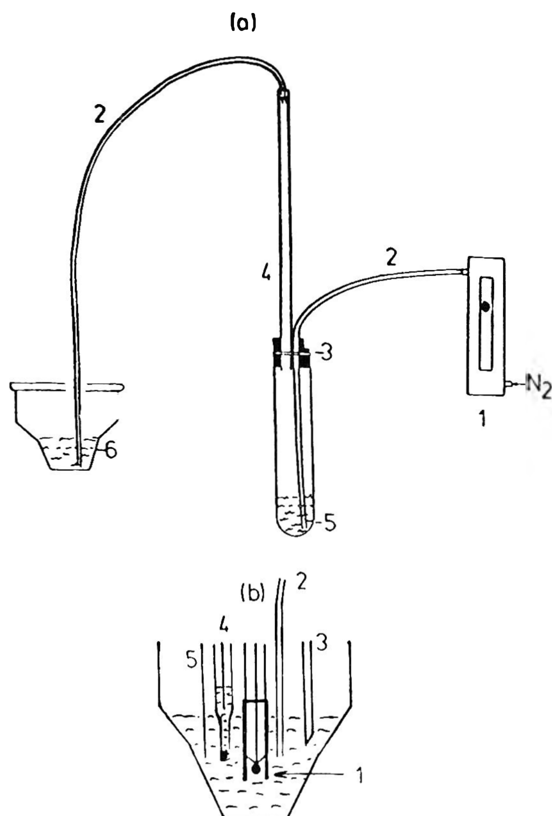

Figure 1. Purge-trap apparatus for SO_2 determination: (a) 1, nitrogen inlet; 2, pyrogallol scrubbing solution; 3, flow meter; 4, glass tubing, 1.0 cm id × 40 cm; 5, sample test tube, 25 × 200 mm; 6, Teflon tubing, 2 mm id; 7, polarographic cell with Teflon sleeve on capillary. (b) Enlarged view of capillary with Teflon sleeve.

Reagents

(Use ACS reagent grade chemicals unless otherwise indicated, and distilled or deionized water.)

(a) *Alcohol.*—5% v/v.

(b) *Sulfuric acid.*—H_2SO_4 (1 + 1).

(c) *Sodium sulfite (Na_2SO_3) standard.*—Determine purity as follows: Accurately weigh ca 250 mg Na_2SO_3 into exactly 50 mL 0.1N iodine solution in glass-stopper flask. Let solution stand 5 min at room temperature. Add 1 mL HCl, and titrate excess iodine with 0.1N $Na_2S_2O_3$, using 1% starch solution as indicator (1 mL 0.1N I consumed = 6.302 mg Na_2SO_3).

(d) *Sulfite standard solution.*—200 μg SO_2/mL. Dissolve 0.1968 g Na_2SO_3, adjusted for % purity, in 500 mL 5% alcohol (0.1968 × 100/x, where x = % assay). Prepare fresh daily.

(e) *Ammonium acetate buffer.*—2M. Add 77.1 g ammonium acetate to 500 mL graduated cylinder. Add ca 400 mL water, and mix to dissolve. Add 57 mL acetic acid, dilute to 500 mL with water, and mix.

(f) *Electrolyte-trapping solution.*—Dilute 2M ammonium acetate buffer with equal volume 5% alcohol.

(g) *Silicone defoamer.*—Dow Corning Antifoam A, or equivalent.

(h) *Sodium hydroxide.*—NaOH, 50% w/v.

(i) *Thymol blue indicator.*—0.25% (w/v) in 0.01N NaOH.

Sample Preparation

Use open-pan balance (Mettler p1200, sensitivity 10 mg per division, or equivalent) to weigh representative sample (1-10 g) into 200 mL Erlenmeyer flask. Add 5% alcohol so that total weight of mixture is 100 g. Stopper and mix or, if necessary, homogenize to obtain fine suspension. Complete sample preparation quickly to minimize oxidation of sulfite by atmospheric oxygen.

Calibration

Assemble apparatus as shown in Figure 1. Add ca 10 mL 5% alcohol and 0.1 mL H_2SO_4 (1 + 1) to sample tube. Adjust nitrogen flow to 0.5 L/min and rinse apparatus by inverting sample tube and let acidified 5% alcohol run out and collect in beaker placed under exit tubing. Continue to purge with nitrogen in order to blow excess liquid from tubing. Add 10.0 mL 5% alcohol and 0.1 mL H_2SO_4 (1 + 1) to sample tube and 10.0 mL electrolyte-trapping solution to dry polarographic cell. Initiate run; while nitrogen flow is 0.5 L/min, set scan rate and purge time at 0 and 2 min, respectively. Voltage is manually set at peak potential. At end of purge cycle, instrument will advance to "scan" cycle. Adjust position of recorder pen near lower portion of paper. Without changing other conditions, remove Neoprene rubber stopper, together with reflux tubing, and add 50 μL sulfite standard. Quickly reinsert rubber stopper, and observe signal. Shake tube occasionally with up and down motion so that liquid touches rubber stopper. When signal reaches maximum value, 5-6 min, terminate run. Similarly, add 3 additional 50 μL aliquots of standard solution and record signals. Prepare calibration curve, μg SO_2 vs maximum μ/amp. (50 μL standard = 10 μg SO_2. Calibration curve exhibits slight curvature toward concentration axis.)

Determination

(Use same conditions as for *Calibration*). Rinse apparatus as described. Add 10 mL electrolyte-trapping solution to dry polarographic cell. Add 0.1 mL H_2SO_4 (1 + 1), 0.1 mL thymol blue indicator, and sufficient 5% alcohol to sample tube so that when sample aliquot is added, total volume of mixture is ca 10 mL. Some silicone defoamer may be added to side of sample tube if sample is expected to foam. Initiate run with purge time set at 2 min. Place 10 mL syringe with 20-gauge needle on open-pan balance, and set weight at 0. Withdraw prepared sample aliquot, and record weight. When instrument advances to scan cycle, inject sample into tube through indented rubber stopper. Shake tube occasionally with up and down motion so that liquid touches rubber stopper. When signal reaches maximum value, 5-6 min (free SO_2), inject 50% NaOH dropwise with 1 mL syringe and 21-gauge needle. Inject 50% NaOH until solution turns blue, pH ca 9.6. Reacidify by injecting 1 mL H_2SO_4 (1 + 1). When signal reaches maximum value, 5-6 min (bound SO_2), terminate run. Obtain μg SO_2 from calibration curve, and calculate amount in sample, μg/g.

Results and Discussion

The principle of the technique depends on a rapid release of SO_2 from the sample by purging with nitrogen into the electrolyte-trapping solution. The signal is simultaneously recorded by differential pulse polarography at a constant voltage set to peak potential. The method modification involved placing a Teflon sleeve onto the dropping mercury electrode capillary to shield the mercury drops from the turbulence produced by the purging gas (Figure 1). Since SO_2 is measured by differential pulse polarography, the proposed method is just as effective as the conventional tech-

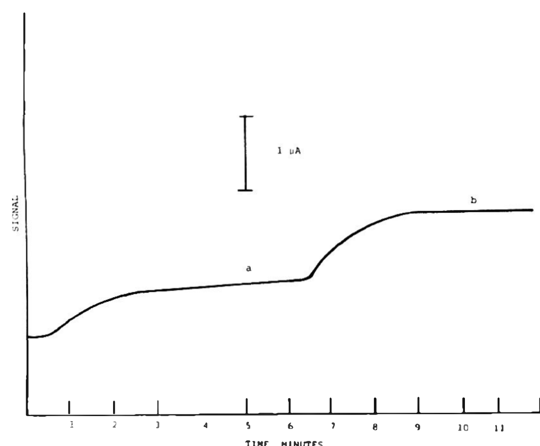

Figure 2. Signal profile of solution containing (a) free and (b) bound sulfite.

nique in rejecting the double-layer charging current. Furthermore, after all SO_2 has been purged from the sample, the voltage may be scanned in the usual manner to obtain a differential pulse polarogram, which may be useful in providing additional specificity for sulfite.

Free sulfite is readily released from the sample which is acidified to pH 1–2. Bound sulfite is first treated with NaOH in order to dissociate the sulfite-aldehyde adducts. This procedure is more efficient than heating with acid (4). However, our first attempts were not totally successful; some samples yielded low sulfite results because of the presence of oxygen in the solution. This difficulty was overcome by adding reagents while the sample was being purged with nitrogen.

Figure 2 shows a typical signal profile obtained when both free (a) and bound (b) sulfites are present in the sample. As shown by the maximum signal, each species is flushed out of the sample in approximately 5 min.

Table 1 shows the results of sample analysis by the proposed method for both free and total sulfites, as well as by the optimized Monier-Williams method for total sulfite. In general, agreement between the 2 methods for total sulfite was excellent. Some samples, however, produced different results by the 2 methods. For the instant potato sample, a higher value was obtained by the proposed method (483.1 µg/g) than by the Monier-Williams method (439.0 µg/g). Apparently, more SO_2 was released by the alkaline treatment used in the proposed method than by the Monier-Williams acid treatment. This results from added sulfite, not from naturally occurring compounds that release SO_2; unsulfited fresh potato did not yield any sulfite. Similar findings have been reported by others (4).

Table 1. Analysis of SO_2 (µg/g) in food samples by DPP and Monier-Williams methods

Sample	DPP		Monier-Williams
	Free	Total	total
Wine cooler (orange)	<1	67.9	72.1
Wine cooler (citrus)	<1	45.8	45.5
Minced clams (canned)	188.9	206.0	203.8
Instant potatoes	339.0	483.1	439.0
Shrimp A	17.2	97.8	103.0
Shrimp B	27.0	58.3	56.4
Dried apricots	403.8	2147.4	2228.1
Grape juice	6.1	49.0	49.1
Indian salad	<1	<1	5.2
Fresh potatoes	<1	<1	1.3
Gelatin	<1	<1	6.6
Mustard	<1	<1	14.2
Fresh turnips	<1	<1	22.8[a]
Crushed red pepper	<1	<1	2.2

[a] Hydrogen sulfide detected in gas phase.

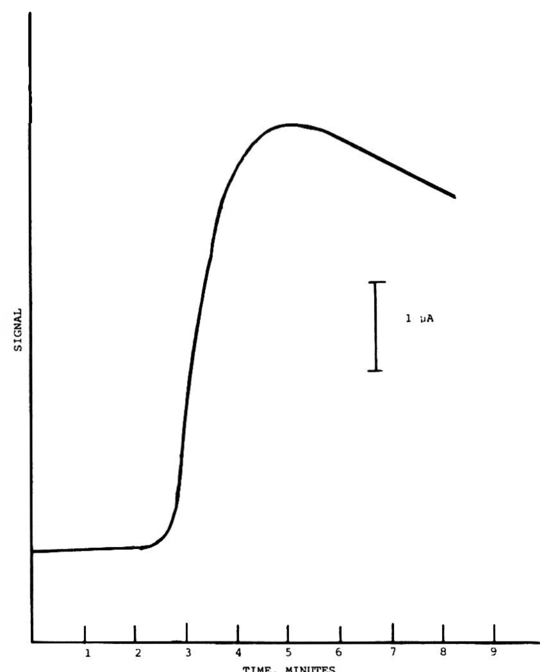

Figure 3. Signal profile for citrus wine cooler sample.

Originally, the citrus wine cooler sample yielded a lower value by the proposed method than by the Monier-Williams method. This can be explained from the signal profile shown in Figure 3. The signal for bound sulfite does not remain constant, but decreases. A similar signal profile was obtained when the sulfite standard was prepared in 0.1% formaldehyde. Apparently, volatile carbonyl compounds may be purged from the sample and react with sulfite in the electrolyte-trapping solution. When the sample weight was reduced from 1.0 to 0.1 g, a "normal" signal profile was obtained, and the result (45.8 µg/g) agreed with that for the Monier-Williams method (45.5 µg/g).

Table 1 shows that no sulfite was found by the DPP method in Indian salad, fresh potatoes, gelatin, mustard, fresh turnips, and crushed pepper. The Monier-Williams method yielded a positive result for sulfite in these samples. The highest value was 22.8 µg/g in fresh turnips. These samples had not been sulfited, so the positive results of the Monier-Williams method must, therefore, have been due to sample degradation and volatilization of interfering compounds. This was confirmed for the turnip sample by placing a lead acetate paper strip within the gas stream preceding the trap in the Monier-Williams procedure. The test paper became black indicating the presence of hydrogen sulfide. Hydrogen sulfide interferes in the Monier-Williams method because it is oxidizing to sulfuric acid.

Figure 4 shows the signal profile for the garlic sample, which yielded a signal profile that did not reach a plateau, but which continued to increase beyond the expected 5 min. This indicates the release of SO_2 from naturally occurring compounds such as alliin and allicin (6). To confirm this, an aliquot of the sample suspension was filtered (Whatman No. 42) and passed through a Sep-Pak C-18 cartridge; no sulfite was detected by DPP analysis. Apparently, naturally occurring nonpolar sulfur compounds are adsorbed on a C-18 cartridge. On the other hand, sulfites present in foods as a result of sulfatization should be forms of the free sulfite or

Table 2. Determination of SO_2 (μg/g) in food samples and recovery of added SO_2

Sample	DPP		C-18 cartridge treatment		Monier-Williams total
	Free	Total	Free	Total	
Fresh garlic	<1	106.4[a]	<1	<1	112.9
Fresh garlic spike (20 000)[b]	14 615	16 538	15 865	16 827	16 442
Recovery, %		82.2		84.1	81.6
Fresh onion	<1	1.4	<1	1.4	4.5
Fresh onion spike (2000)[b]	51.0	326.5	54.9	322.4	429.5
Recovery, %		16.3		16.1	21.3
Shrimp	<1	<1	<1	<1	<1
Shrimp spike (22.2)	13.3	19.6	8.9	15.5	19.7
Recovery, %		88.3		69.8	88.7
Applesauce	<1	<1	<1	<1	<1
Applesauce spike (60.0)	49.1	57.3	37 8	55.6	56.3
Recovery, %		95.5		92.7	93.8
Dried onion	<1	9.2	<1	6.2	42.1
Dried onion spike (600)	<1	93.4	<1	82.8	125.3
Recovery, %		14.0		12.8	13.9

[a] After purging 5 min.
[b] After standing overnight.

aldehyde adducts (7). Since these compounds are polar, they should not be adsorbed on a C-18 cartridge. These cartridges have been used previously for removing compounds responsible for extraneous peaks in ion chromatography (8). The present use of the C-18 cartridge indicates the possibility of differentiating between added sulfite and naturally occurring compounds that release sulfur dioxide. This hypothesis was tested by using several samples and spikes. The results are shown in Table 2. The garlic and onion samples were spiked at relatively high levels of sulfite because much sulfite became irreversibly bound in these foods and after a short time, none could be recovered. The results indicate, however, that naturally occurring sulfur compounds were adsorbed on C-18 cartridges (garlic), whereas compounds from added sulfite were not adsorbed and were detected (Table 2). The data show that only the garlic sample yielded SO_2 above 10 ppm from naturally occurring sulfur compounds by the DPP method. The Monier-Williams method is not applicable to onions, but these samples have been analyzed by the latter method to provide a basis for comparison.

The level of detection by the proposed method was approximately 1 μg SO_2, which corresponds to 1 μg/g, if a 1 g sample is taken. The relative standard deviation was 2.5% (crab meat, 202.5 μg/g) and 7.0% (dried onions, 9.2 μg/g).

The variability of some results indicates that the recoveries depend on the degree of interaction between sulfite species and the food components (Table 2). As previously reported (7), free sulfite is an equilibrium mixture of SO_2, bisulfite, and sulfite. The relative concentration of the species is a function of the pH of the solution. Bound sulfite can be reversibly and irreversibly bound. Examples of reversibly bound sulfite are carbonyl compounds such as sugars and aldehydes, which react with bisulfite to form 1-hydroxy alkyl sulfonates. These substances dissociate in base, or upon heating with an acid, releasing sulfite, or SO_2, respectively. Irreversibly bound sulfite is formed when the sulfite species react with alkenes or aromatic compounds to form sulfonic acids. The DPP and Monier-Williams methods, as well as other commonly used methods, measure only free and reversibly bound sulfites. For these reasons, recoveries were matrix-dependent as expected and were similar to the Monier-Williams method (Table 2).

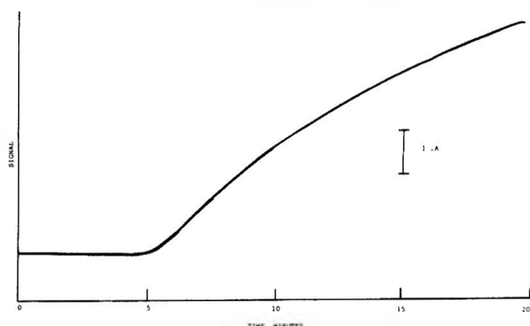

Figure 4. Signal profile for garlic sample.

In summary, the differential pulse polarographic determination with simultaneous nitrogen purging offers a number of advantages for the determination of sulfites in foods. Monitoring the rate of SO_2 evolution is a very efficient analysis because evolution and completion of SO_2 release from the sample is recorded. The signal profile indicates interferences so that corrective action can be taken, if necessary. The release of bound sulfite at room temperature minimizes sample degradation and volatilization of interfering compounds. C-18 cartridges were useful for removing certain naturally occurring compounds that release sulfur dioxide so that only added sulfite could be measured. Results of the proposed and Monier-Williams methods agreed well for a variety of foods. Differences can be explained from the experimental data and from the chemistry of sulfur-containing compounds. The method is very rapid; both free and bound sulfites can be determined in 10 min.

Although the experimental design is quite simple, details of the technique should be closely followed because of the peculiarity of the chemical and physical properties of SO_2. Acid conditions are maintained within the apparatus to minimize absorption of SO_2 by water droplets. The sample tube should be occasionally shaken during purging to wash down the water droplets and release any absorbed SO_2. The sample should be analyzed without delay since sulfites are prone to oxidation by atmospheric oxygen.

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FOOD COMPOSITION

Gravimetric Determination of Ash in Foods: NMKL Collaborative Study

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A gravimetric method for the determination of ash was collaboratively studied in 14 laboratories. The food is ashed at 550°C to constant weight and the ash is determined by weighing. Seven samples of various food commodities with estimated ash contents varying between low and high (0.07–8.0 g/100 g) were included in the study. The relative standard deviations for reproducibility varied, ranging from 1.0 and 1.3 for ash contents of 7.2 and 8.0 g/100 g, to 11 ± 1% for low ash contents of 0.07 and 0.27 g/100 g.

For labeling purposes, food laboratories need simple methods for the determination of the ash content of foods. Usually the ash content is needed in connection with corrections for ash when the energy content of foods is calculated. Ashing methods should be simple and preferably generally applicable to all kinds of foods. When this study was initiated, no validated method for the determination of the ash content of foods in general was available. The ICC standard No. 104, for example, applies only to cereals and cereal products. Various AOAC methods (1) exist, but refer to specified materials, not to food in general. Therefore, a simple general method was set up within NMKL and subjected to a collaborative study in 14 laboratories in 4 Nordic countries.

General applicability of the method was taken into account when test materials to be included in the study were chosen. Samples with ash contents ranging from low (less than 0.1 g/100 g) to relatively high (8 g/100 g) were selected. Also, samples of high fat content and high carbohydrate content were included. The materials included in the study were: maize starch (voluminous ash), marmalade (high carbohydrates content, low ash content), mayonnaise (high fat content, low ash content), rolled oats (high carbohydrates content, intermediate ash content), feta cheese (high protein content, high ash content), dry sausage (high fat content, high ash content), and milk powder (slag forming).

Collaborative study

For each of the 7 test materials (Table 1), ca 2 kg was purchased. Each material was carefully mixed, and the homogeneity of the material was tested by analyzing samples in triplicate. Each of 16 collaborators received samples of each of the 7 materials and was asked to perform duplicate ash determinations on all samples by using the method described below.

METHOD

Principle

Samples are ashed to constant weight in a muffle furnace at 550°C. If necessary, samples are dried prior to ashing.

Apparatus and Reagents

(a) *Muffle furnace.*—Controlled to ±5°C for ashing at 550°C.

(b) *Infralamps.*—250 watt or other equipment for drying samples.

Preparation of Sample and Determination

Homogenize the food. With accuracy of 0.1 mg, weigh test sample corresponding to ca 5 g dry matter into tared crucible of porcelain, quartz, nickel, or platinum, which previously has been heated for not less than 30 min at 550°C and cooled to room temperature in a desiccator. Dry sample under infralamp or using other equipment, e.g., electric bath. Continue to heat until sample shows initial browning.

Dry materials containing sugar (e.g., marmalade and honey) very carefully and, to avoid blazing up during subsequent ashing, continue to heat until sample is black or completely dry. Treat sample with high fat content in the same manner to avoid splashing fat out of crucible.

Place crucible in muffle furnace at 550 ± 5°C for 16–20 h until appearance of grey white ash. Alternatively, to separate drying and ashing, place crucible in programmable oven at room temperature and regulate heating to a speed that does not cause blazing up of sample. When analyzing dry samples, place the crucible directly in muffle furnace at 200°C and turn temperature switch to 550°C.

Cool crucible in desiccator and weigh with 0.1 mg accuracy. Repeat ashing to constant (±1.0%) weight.

Ash from foods with high sugar content may be very light and voluminous. Take care when removing crucible from oven and desiccator. Ash may be carefully wet with water, the water may be evaporated under infralamp, and ashing may be continued for 0.5 h.

Foods with high phosphate content, such as milk powder, may give an ash of constant weight even if complete ashing has not been achieved, because of presence of uncombusted particles immersed in the ash. In such cases, dissolve ash in water, evaporate water, and continue ashing at 550°C. Check ash for residual carbon particles.

Calculation

$$\text{Ash, g/100 g} = [(a - c)/(b - c)] \times 100$$

where a = final weight (g) of crucible and ash; b = weight (g) of crucible and sample; c = weight (g) of empty crucible.

Remarks

This method describes a simple procedure for determination of ash content in foods for use when calculating energy content of foods. Its collaborative study was not designed to determine suitability for elemental analysis of the resulting

[1] Address correspondence to this author.
This method was accepted as an official NMKL method at the 42nd Annual Meeting of the Nordic Committee on Food Analysis, 1988.
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Table 1. Collaborative results (g/100 g) for determination of ash

Coll.	Maize starch	Marmalade	Mayonnaise	Rolled oats	Feta cheese	Sausage	Milk powder
1	0.078	0.230	0.510	1.82	6.38	6.89	8.06
	0.076	0.250	0.500	1.85	6.35	6.81[a]	8.07
2	0.020	0.270	0.700	1.81	6.11	7.14	8.03
	0.020[a]	0.250	0.620	1.75	6.10	7.12	8.02
3	0.070	0.310	0.600	1.85	6.16	7.13	8.07
	0.070	0.300	0.600	1.82	6.19	7.13	8.06
5	0.070	0.290	0.610	1.79	5.95	7.11	7.91
	0.060	0.290	0.630	1.81	6.03	7.24	7.92
6	0.072	0.260	0.610	1.81	6.22	7.20	7.92
	0.079	0.270	0.610	1.80	6.16	7.32	7.92
7	0.073	0.280	0.600	1.83	6.05	7.18	8.04
	0.074	0.280	0.540	1.83	6.09	7.19	8.04
8	0.067		0.610	1.77	6.58	7.27	8.16
	0.065		0.600	1.78	6.34	7.18	8.16
9	0.067	0.280	0.590	1.84	5.96	7.12	8.12
	0.068	0.300	0.580	1.92	5.98	7.18	8.14
10	0.075	0.260	0.610	1.84	5.80	7.21	8.01
	0.083	0.270	0.590	1.84	5.72	7.16	7.88[b]
11	0.062	0.319	0.585	1.78	6.19	7.13	8.07
	0.057	0.328	0.553	1.76	6.23	7.00	8.1
12	0.063	0.225	0.375	1.77	5.77	6.93	7.86
	0.065	0.232	0.411[c]	1.76	5.55	6.82[a]	7.84
13	0.070	0.310	0.630	1.77	6.00	6.99	8.06
	0.050	0.290	0.550	1.79	6.05	7.18	7.99
14	0.066	0.278	0.548	1.82	6.30	7.11	7.91
	0.053	0.223	0.603	1.79	6.30	7.22	7.92
15	0.040	0.260	0.560	1.76	6.09	7.09	7.87
	0.040[a]	0.260	0.540	1.74	6.29	7.21	7.84

[a] Outlying result flagged by the pair value Grubbs test.
[b] Outlying result flagged by the Cochran test.
[c] Outlying result flagged by the single value Grubbs test.

ash. Despite this fact, the ash may be used for the determination of certain elements. This ashing method can not, however, be used if determinations of lead and cadmium are to be made on the sample, since these metals require an ashing temperature lower than 550°C. If the ash is to be used for the determination of elements such as sodium, potassium, and iron, platinum crucibles must be used; porcelain glaze may react with metals. Also, platinum crucibles should be used for subsequent determinations of sulfates. If chloride is to be determined, either porcelain or platinum crucibles should be used.

Results and Discussion

Results were received from 15 participants. Collaborator 4 had taken amounts of test sample significantly lower than prescribed in the method and was designated a "procedural deviate." All results from this participant were excluded from further calculations. Results, excluding those of Collaborator 4, are listed in Table 1.

The results were tested for outliers according to the IUPAC 1987 recommendations (1). The Cochran test for removal of laboratories showing extreme variances indicated

Table 2. Statistical results for collaborative study on determination of ash in foods (g/100 g)

Material	Maize starch	Marmalade	Mayonnaise	Rolled oats	Feta cheese	Sausage	Milk powder
No. of labs after eliminating outliers	12	13	13	14	14	12	13
No. of outlying labs removed	2	—	1	—	—	2	1
Mean	0.0680	0.274	0.588	1.807	6.105	7.159	8.000
	(0.0626)		(0.574)			(7.116)	(8.000)
Repeatability SD (s_r)	0.0058	0.013	0.029	0.023	0.078	0.072	0.017
	(0.0048)		(0.029)			(0.073)	(0.029)
Repeatability rel. SD (RSD_r)	8.6	4.7	4.9	1.3	1.3	1.0	0.21
	(8.7)		(5.0)			(1.0)	(0.37)
Repeatability value, r ($2.8 \times s_r$)	0.0164	0.037	0.081	0.064	0.22	0.20	0.047
	(0.0152)		(0.081)			(0.20)	(0.082)
Reproducibility SD (s_R)	0.0081	0.029	0.042	0.040	0.23	0.075	0.10
	(0.0162)		(0.069)			(0.16)	(0.10)
Reproducibility rel. SD (RSD_R)	11.9	10.6	7.2	2.2	3.8	1.0	1.3
	(25.8)		(12.0)			(2.2)	(1.3)
Reproducibility value, R ($2.8 \times s_R$)	0.0226	0.081	0.12	0.11	0.63	0.21	0.29
	(0.0453)		(0.20)			(0.44)	(0.28)

the result from Collaborator 10 for milk powder to be outlying at P = 0.01. The single value Grubbs test for removal of results with extreme averages signalled the result from Collaborator 12 for mayonnaise as an outlier at P = 0.01. The pair value Grubbs outlier test at P = 0.01 was positive for the 2 lowest results for maize starch and for the 2 lowest results for the sausage sample. Recycling the tests for outliers did not flag additional outliers. Outlying results are indicated in Table 1 and were excluded from calculations of the statistical parameters of the method. Not more than 2 results of 14 were removed for any sample, which is well below the IUPAC 1987 recommended maximum of 2 of 9.

The precision parameters of the method were calculated according to the guidelines for Collaborative Study Procedure (2) and are given separately for each of the 7 test materials in Table 2. The repeatability relative standard deviations varied from 0.2% for a material with an ash content of 8 g/100 g, to 8.6% for a material with a mean of 0.068 g/100 g. The reproducibility relative standard deviations also varied; the highest, between 10 and 12%, occurred for ash contents of 0.07 and 0.27 g/100 g. For ash contents over 0.5 g/100 g, the reproducibility relative standard deviation was, in all cases, less than 8%; for ash contents between 1.8 and 8.0 g/100 g, it ranged between 1.0 and 3.8%.

None of the collaborators reported any difficulties with the performance of the determinations. The results support applicability of the method to foods over a wide range of ash content. The precision of the method is quite sufficient for its intended use, i.e., to determine the correction for ash content to be used in calculations of the energy content of foods. With minor modifications for certain commodities, as stated in the method, the method is applicable to all foods.

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National Consumer Agency of Denmark, Copenhagen, Denmark

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Swedish University of Agricultural Sciences, Uppsala, Sweden

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METALS AN OTHER ELEMENTS

Determination of Arsenic and Selenium in Whole Fish by Continuous-Flow Hydride Generation Atomic Absorption Spectrophotometry

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A combined wet chemical and dry ash digestion and use of a continuous-flow hydride generator coupled with a flame-heated quartz cell enabled the simple, precise, and highly automated atomic absorption determination of arsenic and selenium in tissues of whole fish. Percent relative standard deviation averaged 4% for each element; method detection limits (μg/g dry wt) were about 0.06 for arsenic and 0.04 for selenium. Digestion of samples proceeded with little operator attention and without perchloric acid. Analysis for arsenic as As(V) simplified sample preparation but care had to be exercised to avoid interferences from high concentrations of selenium.

Due to gradual improvements in recent years, hydride generation atomic absorption spectroscopy has gained wide acceptance for the determination of the potentially toxic elements arsenic and selenium. Hydride generators are generally classified as either batch or continuous-flow systems, and each has advantages. With batch-type generators, for which hydrides are formed after the rapid addition of excess reductant to a constant sample volume, small sample volumes can be used, detection limits are generally reduced, and response is less dependent on valence state for some elements. With continuous-flow generators, in which reductant is continuously mixed with sample solutions by means of a peristaltic pump, precision is improved, interferences are reduced, and automation is facilitated.

Tissue samples are usually prepared for analysis by hydride generation atomic absorption by one of 2 basic methods: wet digestion with perchloric acid in combination with other mineral acids (1-8), or dry ashing with magnesium nitrate (2, 3, 9-12). Traditionally, digestion with perchloric acid has been the accepted method, even though constant attention by the operator is required to prevent analyte losses through foaming and charring, etc. But despite contradictory reports about its reliability (13), the dry ash procedure is becoming more widely used because of its simplicity and safety.

Few reports have been published on the determination of both arsenic and selenium in biological samples by hydride atomic absorption. Fiorino et al. (6) successfully determined these elements in fish tissue, using a nitric, sulfuric, perchloric acid digestion and a specially constructed semi-automated, batch-type generator. Unfortunately, the digestion required constant operator attention, the analysis was only partly automated, and the authors did not report spike recoveries. Agemian and Thomson (1) used a similar digestion, but analyzed digestates with an automated continuous-flow generator. However, the generator manifold included up to 17 individual pump tubes, several mixing coils, and 2 heating baths. Tam and Lacroix (9) determined arsenic and selenium in various food products by using a dry ash digestion and manual hydride generation analysis. Recoveries of spikes, although deemed satisfactory, tended to be low for selenium and somewhat high for arsenic.

References to trade names or manufacturers do not imply U.S. Government endorsement of commercial products.

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We report on the use of a commercially available continuous-flow hydride generator coupled with a flame-heated quartz cell for determination of arsenic and selenium in homogenized, whole-body fish by atomic absorption spectroscopy. Preparation of samples by a combined wet chemical and dry ash digestion requires little operator attention and no special perchloric acid fume hood.

METHOD

Apparatus

(a) *Spectrophotometer.*—Perkin-Elmer Model 5000 with electrodeless discharge source lamps; Perkin-Elmer 3600 data station and Model 056 chart recorder; air-acetylene burner with cell mounting bracket.

(b) *Hydride generator.*—Varian VGA-76.

(c) *Quartz absorption cell.*—Varian fitted with Perkin-Elmer graphite cooling rings and heated by air-acetylene flame (extra lean).

(d) *Muffle furnace.*—Thermolyne Model F-A1740.

(e) *Hot plate.*—Fisher Scientific Model 610T.

Reagents

(a) *Nitric acid.*—Reagent grade, 70-72% (J.T. Baker) further purified by sub-boiling distillation.

(b) *Hydrochloric acid.*—Reagent grade, 37% (J.T. Baker) further purified by sub-boiling distillation.

(c) *Magnesium nitrate hexahydrate.*—ACS grade (Alfa Products Inc.).

(d) *Sodium borohydride.*—98% purity, pellet (Alfa Products Inc.).

(e) *Sodium hydroxide.*—98% purity, pellet (Alfa Products Inc.).

(f) *Anti-foam agent.*—DB-110A (Dow Corning).

(g) *Methanol.*—Glass distilled.

(h) *Atomic absorption standards.*—1 mg/mL (J.T. Baker).

(i) *Water.*—Greater than 10 megohm-cm (Culligan reverse osmosis/ion-exchange).

Digestion

Transfer ca 0.5 g lyophilized and homogenized tissue to 100 mL beaker, wet with 2-3 mL methanol, and add the following: 5 drops of anti-foam agent, 10 mL 40% (w/v) magnesium nitrate hexahydrate, and 10 mL nitric acid. Cover with watch glass, place on hot plate, and reflux on low heat (70-80°C) overnight. Increase temperature to ca 200°C, tip watch glass to speed evaporation, and heat sample to dryness (ca 4 h). Reposition watch glass, transfer to cold muffle furnace, ramp temperature to 500°C over 3-4 h, and hold 2-4 h. After sample has cooled, add 20 mL 50% HCl, replace watch glass, and gently boil 1 h on hot plate. Readjust volume of cool sample to 20 mL with 50% (v/v) HCl. Quantitatively transfer beaker contents to tared polyethylene bottle and dilute to 102.0 ± 0.5 g (100 mL) with deionized water.

Determination

Set up spectrophotometer according to manufacturer's recommendations with background correction enabled (reduces noise due to air peaks), using peak hold mode and triplicate 5 s readings. For arsenic, set wavelength at 193.7 nm and slit at 0.7 nm (HIGH). For selenium, use 196.0 nm and 2.0 nm (HIGH). Fill acid reservoir of hydride generator with sub-boiled HCl. Prepare reductant of 0.6% $NaBH_4$ and 0.5% NaOH, and analyze samples for selenium by comparison to Se(IV) standards (0–8 ng/mL, prepared in 10% (v/v) HCl). For arsenic determination, prepare filtered solution of 1% $NaBH_4$ and 0.5% NaOH, and analyze samples by comparison to As(V) standards (0–10 ng/mL in 10% HCl). Dilute sample digestates to reduce selenium concentration to less than 10 ng/mL prior to arsenic determination to minimize interference.

Results and Discussion

Accuracy and Precision

Results from analyses of reference materials, sample spikes, and replicate samples, spanning more than 2 years and several hundred fish samples, are summarized in Tables 1–3. Measured values for NIST Standard Reference Materials agreed well with certificate values. Method limits of detection (LOD), determined by the formula LOD = $3[(SD_B^2 + SD_S^2)^{1/2}]$ where SD_B and SD_S are standard deviations of procedural blanks and low-level samples, respectively, averaged 0.06 μg/g dry weight for arsenic and 0.04 μg/g for selenium. We did not determine arsenic in NIST Bovine Liver because it falls below the limit of quantitation (3.3 times LOD) for this method. In addition to NIST Standard Reference Materials, we analyzed an "in-house" reference material—NFCRC striped bass (*Morone saxatilis*)—which is a lyophilized, cryogenically pulverized, whole-body specimen and is, therefore, a better matrix match for our typical whole-fish sample. The accepted range for this material was determined from multiple determinations by several independent laboratories. Method precision (Table 2) has been consistent; the percent relative standard deviation for triplicate determinations averages about 4% for each element. Recoveries from digested sample spikes (Table 3) for both organic and inorganic analyte forms are essentially complete; however, selenomethionine recovery runs slightly low (average of 92%). This is further discussed in *Digestion*, below.

Table 1. Accuracy of measured concentrations of arsenic and selenium in biological reference materials (μg/g dry weight)

Material	n	Concentration	
		Measured[a]	Reported[b]
Arsenic			
NIST SRM 1566 Oyster	19	13.8 ± 0.7	13.4 ± 1.9
NIST RM 50 Tuna	32	3.06 ± 0.13	3.3 ± 0.4
NFCRC Bass[c]	30	3.52 ± 0.21	3.2 ± 0.5
Selenium			
NIST SRM 1566 Oyster	27	2.02 ± 0.08	2.1 ± 0.5
NIST RM 50 Tuna	61	3.60 ± 0.14	3.6 ± 0.4
NFCRC Bass[c]	58	2.20 ± 0.12	2.3 ± 0.2
NIST SRM 1577 Liver	21	1.05 ± 0.03	1.1 ± 0.1

[a] Mean ± std dev.
[b] 95% confidence interval about mean.
[c] NFCRC Bass = National Fisheries Research Center "in house" whole-body striped bass (non-certified material, accepted range based on interlaboratory round-robin analysis).

Table 2. Average percent relative standard deviation of arsenic and selenium concentrations determined by triplicate sample preparation and analysis

Element	n[a]	Rel. std dev., %	
		Range[b]	Mean, SD[c]
As	39	0.4–12.8	4.5, 3.2
Se	61	0.6–9.4	3.6, 2.0

[a] Total number of triplicate sample sets prepared and analyzed.
[b] Range of percent relative standard deviations for each triplicate analysis set.
[c] Overall mean and standard deviation for percent relative standard deviations of triplicate analyses.

Digestion

The digestion procedure of May (10) was modified slightly to increase the consistency of results. A nitric acid wet digestion before ashing (2, 3, 11) helped to disperse the sample evenly in the $Mg(NO_3)_2$ "cake" and eliminated the fluffing out of sample during ashing. It also provided for additional oxidation at low temperature that might reduce losses of volatile analyte forms. We eliminated the addition of magnesium oxide to the ashing aid solution because it offered no improvement in recoveries and was often contaminated with arsenic. Although this procedure is longer than most, almost no operator attention is needed; also, since no perchloric acid is required, it is a relatively safe procedure.

As noted in the previous section, losses of arsenic and selenium are minimal with this digestion. Recoveries of selenium as the amino acid selenomethionine, were somewhat low, however. Apparently, this form of selenium is not completely oxidized in the preliminary nitric acid step and a small percentage is lost through volatilization. Preliminary tests in which a small volume (2 mL) of hydrochloric acid was added to samples (3) indicated that complete recovery of selenomethionine is possible. The method with this minor modification included is to be further studied.

Analysis

A general requirement of the hydride atomic absorption technique is that the analyte be in a specific valence state. Selenium must be in the +4 oxidation state for conversion to the hydride. Samples are typically oxidized to convert all selenium forms to the +6 state and then boiled with hydrochloric acid, which reduces selenium to +4. Arsenic may be determined as either +3 or +5, but the +5 state has a slower rate of hydride formation and gives a much lower response with most continuous flow systems (14, 15). We obtained inconsistent results for arsenic with potassium iodide prereduction (6, 8, 9, 14), as sample constituents apparently altered the rate of reduction unpredictably; also, potassium

Table 3. Recoveries of arsenic and selenium spiked into whole-body fish tissue

Analyte	Range of effective spike concns, (μg/g dry weight)	Spike form	n	Recovery, mean %, SD
As	0.5–5.0	As_2O_3	41	102, 5
As	0.5–5.0	$(CH_4)_4AsI$	47	102, 9
Se	0.5–25.0	Na_2SeO_4	79	97, 6
Se	0.5–40.0	*d-l*-selenomethionine	59	92, 7

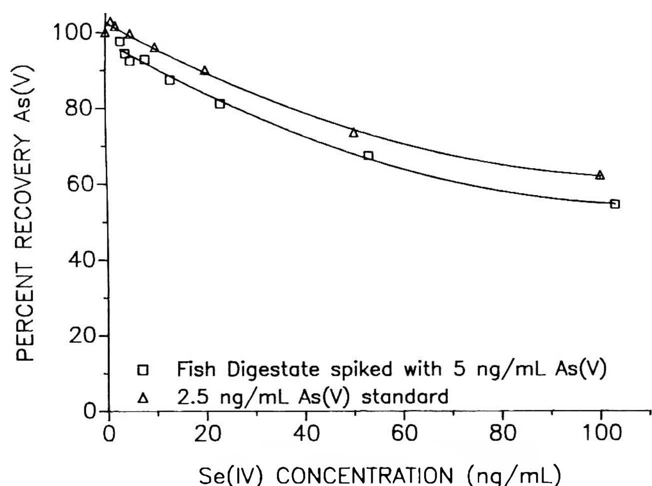

Figure 1. Effect of Se(IV) on As(V) determination by hydride generation atomic absorption.

iodide is difficult to flush out of system components and is detrimental to other determinations; and finally, degradation of the quartz cell is accelerated when potassium iodide is used. Therefore, we analyzed arsenic without reduction (boiling with hydrochloric acid does not affect As(V)), in spite of reduced sensitivity, to simplify the procedure.

The response for As(V) can be improved by increasing borohydride concentration, but at the expense of increased noise and matrix interferences. We used a filtered 1% borohydride solution chilled in an ice bath during analysis to reduce excess hydrogen gas formation. Also, the flame used to heat the cell was kept at minimum fuel flow to reduce the cell temperature and thereby increase the analyte residence time and decrease turbulence. (An added benefit is longer cell life.) Reports for electrothermally heated cells have suggested 850-900°C as optimum for arsenic and selenium determinations (2). With a thermocouple probe, we measured interior cell temperature at 930°C for a flame with acetylene at 1.2 L/min (near the minimum required to sustain a flame) and air at 15 L/min. With a fuel flow of 1.5 L/min, the interior cell temperature increased to 985°C and signals were lower and considerably noisier. Graphite cooling rings attached to the cell gave a quieter signal, as did removal of the flame shield—especially at slightly higher fuel flows.

There are many reports of chemical interferences with hydride atomic absorption, especially for samples containing high concentrations of transition metals (14, 16-19). Interferences are more severe for methods in which concentrations of borohydride are relatively high and concentrations of hydrochloric acid are low (14, 17). We have analyzed several hundred tissue samples by this procedure; as judged by the results of standard additions, we have encountered no significant interferences (10% or greater) for selenium determinations. For arsenic, interferences were detected only in the small percentage of samples that contained relatively high concentrations of selenium. Quantitation of this interference with increasing additions of Se(IV) indicated that concentrations above 10 ng/mL caused significant interference on arsenic (Figure 1). In agreement with Welz and Melcher, who used a batch-type generator (20), the degree of interference by Se(IV) was similar on either trivalent or pentavalent arsenic. Attempts to eliminate the selenium interference were largely unsuccessful. Use of copper to inhibit SeH_2 formation (20) proved impractical with the continuous flow system, because the rapid buildup of copper metal in lines degraded performance. Dissolution of the ash with perchloric in lieu of hydrochloric acid to keep selenium in the unreactive higher oxidation state was only partly successful, as about 50% of the selenium was still measured as Se(IV). Because most of our sample analyses involve both arsenic and selenium, we dilute sample digestates after determining selenium so that the selenium concentration is below 10 ng/mL, to minimize the interference on arsenic. This method has proven to be simpler and more reliable than the potassium iodide reduction method.

There are obvious limitations with simple dilution for samples low in arsenic and extremely high in selenium. Fortunately, the high sensitivity of the hydride technique allows for considerable dilution of samples. In extreme cases, the method of additions may be necessary.

One problem with analyses of hydride atomic absorption is the chronic corrosion of laboratory equipment caused by fumes from the high concentrations of hydrochloric acid used. This problem is reduced with the continuous flow generator because concentrated hydrochloric acid can be metered into sample solutions during analysis to reduce chemical interferences (14), without using large amounts of acid for each sample digestate.

In conclusion, both arsenic and selenium can be accurately determined in fish tissue with minimal operator attention by the described method. We are presently modifying our flame autosampler to accommodate the long read delays that are required with the continuous-flow generator, and thus totally automating the analysis.

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METHOD EVALUATION

Criteria for the Detection of Analytes in Test Samples

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Analytical control must be carried out by validated methods. Validation of methods for all possible situations, i.e., for all kinds of analytes in many different matrixes, by interlaboratory studies of meticulously described methods is unworkable because of the financial burden, the lack of analytical capacity, and the impossibility of standardizing highly sophisticated instrumentation between laboratories. Yet methods, particularly those used in regulatory control, must be validated. To overcome this dilemma, we suggest another approach for validation of quantitative analytical methods, viz., the application of certain "criteria." Such criteria are presented here for a number of analytical methods, including low and high resolution mass spectrometry, infrared spectroscopy, gas and liquid chromatography, thin-layer chromatography, spectrometric detection after TLC and LC, as well as general consideration of the whole analytical procedure. The criteria for a given method must be adopted by a board of experts for that method. Then, once an analysis fulfills the adopted criteria of the applied method, the presence of the investigated analyte can be taken as proved within the limits of ambiguity of the method. The criteria are already incorporated in a European Communities Commission Decision for detecting residues of substances having hormonal or thyrostatic action; criteria are submitted for a Commission Decision on analysis of residues of organic compounds in general.

Analytical results serve a purpose, which can usually be defined in terms of a decision to be made. Examples are classification of a material according to value, or a decision about a safety action required. The nature of the decision determines the requirements of the analysis, both quantitative ones, e.g., limits of detection, and qualitative ones, e.g., degree of certainty that the identification of a compound is unambiguous.

In trade, in regulatory control, and particularly in cases of dispute, the results of chemical analysis must be unambiguous and interpretable in only one way. To achieve this certainty, the usual practice has been to describe an analytical method in great detail. This is done in the standard methods of ISO and in numerous international and national commodity and standardization organizations. It is necessary to describe methods in terms of parameters such as specificity, accuracy, precision (repeatability and reproducibility), limit of detection, sensitivity, practicability, and applicability. To characterize the merits and parameters of methods, extensive investigations and collaborative studies have to be carried out.

To achieve the utmost certainty, an exact experimental protocol for the method is described in great detail and is considered validated only after a successful collaborative study. However, the requirement of exact formulations of analytical procedures has some drawbacks, which are well recognized: (*1*) A standardized method, by virtue of its rigidity, cannot accommodate new developments, and is likely to become old-fashioned. (*2*) The behavior of reagents and equipment may vary over time, forcing adaptation of the method. (*3*) Modern methods, involving highly sophisticated equipment or materials, may operate according to unique procedures which are not transferable to other laboratories. (*4*) The information present in an analytical result may not be clearly accessible. As an example of the latter, an infrared spectrum of an analyte, isolated from a sample matrix, will naturally differ from the reference spectrum, and confound the certainty of the presence of the analyte in the sample.

A complication for qualitative analyses, in particular for residue analyses, is that some of the parameters stated above are irrelevant (e.g., repeatability and reproducibility). This is the reason for so few standard qualitative methods. Yet unambiguous results are needed in qualitative analyses as well.

Therefore, we searched for another approach. In particular, a new approach was urged in The Netherlands because, in the past, court cases on analytical control of veterinary drugs had to admit some false-positive results (1). Although only a few cases were involved, the impact was enormous. Not only was the government condemned to pay claims for damages, but since then, The Netherlands authorities and courts have mistrusted chemical residue analyses in general.

An analytical result was acceptable only after thorough validation of the applied method by interlaboratory tests. As an example, the analysis of one analyte in one matrix (DES in urine) could be validated only after the method was meticulously described and an interlaboratory study was conducted with 5 laboratories and 4 methods on 302 urine samples of 24 bulls, at a total cost of $250 000 (2–4). Because many analytes and many matrixes are possible in analytical control, it became clear that this type of validation was impractical and unworkable in general, and too expensive in terms of costs and laboratory resources.

Yet methods had to be validated before they were acceptable in court. To solve this dilemma, we approached the validation of methods by another direction, viz., by formulating "criteria" for each method to be validated. For a positive conclusion ("the presence of the analyte is proved in the sample examined"), the analytical result has to fulfill the criteria specified for the detection method applied. When consensus is achieved about the criteria, the conclusion holds, irrespective of the details of the analytical procedure leading to the analytical result. And although analytical procedures applying highly sophisticated techniques are not easily transferable to another laboratory in exactly the same way, thus making collaborative studies virtually impossible, the same criteria can be applied to the ultimate identification method.

To obtain a good and acceptable analytical result, 2 requirements have to be fulfilled: (*1*) the method has to be adequate (quality of the method); and (*2*) the execution has to be adequate (quality of the laboratory). The classical approach to the first requirement is to describe the method meticulously. In our approach, this description is replaced by

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setting exact requirements to the output of the analytical operation. Likewise, the classical approach uses collaborative studies for combined testing of the quality of the method and the quality of the laboratories. In our approach, collaborative studies are superfluous for validation of criteria for identification.

Of course, the proposed criteria will not automatically guarantee the quality of analytical chemical results. Only a thorough, management-supported local and super-organizational quality assurance program will ensure proper execution of validated methods. To control the performance of laboratories, interlaboratory studies still remain necessary.

The criteria for each identification method must be developed and adopted by a board of experts for that identification method.

Criteria are presented here for (radio)immunoassay, gas chromatography (GC), thin-layer chromatography (TLC), liquid chromatography (LC), LC and TLC with UV spectrum identification, GC with mass spectrometric (MS) identification, and infrared spectroscopy (IR), as well as general considerations for the entire analytical procedure (5).

This new approach does not imply that an exact experimental protocol (standard operating procedure, SOP) for a method of analysis should no longer be mandatory. In the framework of good laboratory practice, each laboratory has the responsibility of writing such protocols for its own methods of analysis that are used in analytical quality control. The degree of specificity has to be known (6), and the results of the analysis should be related to this specificity (7). When the method is improved or altered, a new SOP must be written within the laboratory.

The criteria stated below for mass spectrometry are extrapolated for general application from statements accepted by a Dutch working group of mass spectroscopy experts. The criteria for infrared spectroscopy have been accepted as reliable for the identification of analytes in test samples by a Dutch-Belgian group of experts on infrared spectroscopy. The criteria have been adopted for detecting residues of substances having hormonal or thyrostatic action in EEC control programs (8), with force of law in the European Communities, and have been submitted for a Commission Decision on analysis of residues of organic compounds in general.

We realize that this paper represents a first attempt to supply some reasonable guidelines for identifications of residues. We, therefore, call for comments or reactions otherwise.

Definitions Regarding the Presence of an Analyte

Analyte: A component of a test sample, the presence of which has to be demonstrated. The term "analyte" includes derivatives formed from the analyte during the analysis wherever this is applicable.

Standard material: A well defined substance in its highest attainable purity to be used as a reference in the analysis.

Positive result: The presence of the analyte in the sample is proved, according to the method, when the general criteria, and the criteria specified for the relevant detection method, are fulfilled.

Negative result: If not all of the general criteria and the criteria specified for the relevant method are fulfilled, the analysis, according to the method, is not able to prove the presence of the analyte in the sample. Nor does the result of the analysis provide proof for the absence of the analyte in the sample.

Limit of decision: The lowest analyte content which, if actually present, will be detected with reasonable statistical certainty and can be identified according to the criteria of the method.

Co-chromatography: The purified test solution prior to the chromatographic step is divided into 2 parts: (a) one part is chromatographed as such; and (b) the standard material of the analyte that is to be identified is added to the second part, and this mixed solution of analyte and standard material of analyte is chromatographed. The amount of added standard material should be about equal to the estimated or expected amount of the analyte.

General Considerations for the Whole Analytical Procedure

General criteria for the whole procedure.—The method must have been proved to be able to distinguish the analyte from all known interfering materials in the appropriate matrix. The physical and chemical behavior during the analysis of the analyte should be indistinguishable from that of the corresponding standard material in the appropriate matrix.

General criteria for separation techniques.—Reference samples containing known amounts of analyte must be carried through the entire procedure simultaneously with each batch of test samples analyzed. Alternatively, an internal standard may be added to test samples. Appropriate reference samples having a content of standard material close to that of the expected analyte content of the samples (9) must be subjected to the same derivatization and cleanup.

Criterion for the off-line physical and/or chemical preconcentration, purification, and separation, if applied.—The analyte should be in the fraction that is characteristic for the corresponding standard material in the appropriate matrix material.

Criterion for on-line separation, if applied (e.g., GC).—The analyte should elute at the retention time which is characteristic for the corresponding standard material in the appropriate matrix material.

Quality Requirements for Determination of Analyte by Radioimmunoassay (RIA) (*4, 8, 10–13*)

The working range of the calibration curve must be specified and in general must cover a concentration range of at least one decade. Control samples must be included in each assay. Results for concentration levels of zero and at lower, middle, and upper parts of the working range must be in line with those of previous assays. At the limit of decision, the within-run coefficient of variation for the control samples must be less than 0.15. A minimum of 6 calibration points is required, adequately distributed along the calibration curve. The recovery must be controlled and specified. If logit-log transformation of the original data is applied, the within run coefficient of correlation of the calibration curve must be at least 0.985. The calibration must have its highest precision around the limit of decision. Adequate quality control parameters have to be in line with those of preceding assays, e.g., Bo/T, NSB, and slope and intercept of the calibration curve. (Compliance with these quality requirements does not exclude the possibility of false positive results originating from systematic errors such as antibody cross reactivities (10) and interference from nonrepresentative sample material (14, 15).)

Criteria for Identification of Analyte by Gas Chromatography (GC) and Liquid Chromatography (LC) (*16*)

The analyte should elute at the retention time which is characteristic for the corresponding standard material. The nearest peak maximum in the chromatograph should be sep-

arated from the designated analyte peak by at least one full width at half maximum height. For identification, additional co-chromatography in the chromatographic stage is mandatory; as a result, only the peak presumed to be due to the analyte should be intensified, and the width at half maximum height should be within ± 10% of the original width. This requirement may be taken as fulfilled whenever the retention times are identical with 10% of the peak width at half maximum height.

Criteria for Identification for Analyte by (High Performance) Thin-Layer Chromatography (TLC, HPTLC) (17, 18)

The R_f value(s) of the analyte should agree with the R_f value(s) characteristic for the standard material. This requirement is fulfilled whenever the R_f value(s) of the analyte is (are) within 3% of the R_f value(s) of the standard material under the same conditions. The visual appearance of the analyte should be indistinguishable from that of the standard material. The center of the nearest spot to that due to the analyte should be separated from it by at least half the sum of the spot diameters. For identification, additional co-chromatography in the TLC step is mandatory. As a result, only the spot presumed to be due to the analyte should be intensified; a new spot should not appear, and the visual appearance should not change. For confirmation, 2-dimensional TLC is mandatory.

Criteria for Identification of Analyte by Liquid Chromatography with Full Spectrum Detection (LC-SP) (19)

The maximum absorption wavelength in the spectrum of the analyte should be the same as that of the standard material within a margin determined by the resolution of the detection system. For diode array detection, this is typically ± 2 nm. The spectrum of the analysis should not be visually different from the spectrum of the standard material for those parts of the 2 spectra with a relative absorbance larger than 10%. This criterion is met when the same maxima are present and at no observed point the difference between the 2 spectra is more than 10% of the absorbance of the standard material. For identification, co-chromatography in the LC step is mandatory. As a result, only the peak presumed to be due to the analyte should be intensified.

Criteria for Identification of Analyte by (High Performance) Thin Layer Chromatography with Full Spectrum Detection (TLC-SP, HPTLC-SP)

The R_f value(s) of the analyte should agree with the R_f value(s) characteristic for the standard material. This requirement is fulfilled when the R_f value(s) of the analyte is (are) ± 3% of the R_f value(s) of the standard material under the same conditions. The visual appearance of the analyte should be indistinguishable from that of the standard material. The center of the spot nearest to that due to the analyte should be separated from it by at least half the sum of the spot diameters. For identification, additional co-chromatography in the TLC step is mandatory. As a result, only the spot presumed to be due to the analyte should be intensified; a new spot should not appear. The maximum absorption wavelength in the spectrum of the analyte should be the same as that of the standard material, within a margin determined by the resolution of the detection system. The spectrum of the analyte should not be visually different from the spectrum of the standard material.

Criteria for the Identification of Analyte by Gas Chromatography-Mass Spectrometry (GC-MS)

GC criteria.—An internal standard should be used if a material suitable for this purpose is available. It should preferably be a stable isotope-labeled form of the analyte. The ratio of the GC retention time of the analyte to that of the internal standard, i.e., the relative retention time of the analyte, should be the same as that of the standard analyte, within a margin of ± 0.5%. If this requirement is not fulfilled, or if no internal standard is used, then identification of the analyte must be proved by using co-chromatography. In the case of co-chromatography, the retention time of the analyte added to the sample must coincide with the retention time of the analyte already present in the sample.

Criteria for GC-low resolution MS.—The intensities of at least 4 diagnostic ions must be measured. If the compound does not yield 4 diagnostic ions with the method used, then identification of the analyte should be based on the results of at least 2 independent GC-LRMS methods with different derivatives and/or ionization techniques, each producing 2 or 3 diagnostic ions. The molecular ion should preferably be one of the 4 diagnostic ions selected. The relative abundances of all diagnostic ions monitored from the analyte should match those of the standard analyte. The relative intensities of the diagnostic ions detected, expressed as a percentage of the intensity of the base peak, must be the same as those for the standard analyte within a margin of ± 10% (EI mode) or ± 20% (CI mode).

Criteria for GC-high resolution MS; fragmentography.—To be classified as high resolution measurements, the accuracy of mass setting should be equal to or better than 3 parts per million. The relative abundance of 3 or more diagnostic ions must be the same as for the standard analyte within a margin of ± 10% (EI mode).

Criteria for GC-high resolution MS; accurate mass plus low resolution natural isotope (4, 20, 21).—To be classified as high resolution measurement, the accuracy of mass determination must be equal to or better than 3 parts per million. The m/z value of the diagnostic ion should be equal to the theoretical value of the corresponding standard analyte. If measurements of a single diagnostic ion does not fulfill the criterion for specificity, then the natural isotope abundance ratio of the diagnostic ion should be measured with low resolution. This ratio should be equal to the theoretical value within a specified margin (typically ± 5%). If an unambiguous elemental composition cannot be derived according to this procedure, an additional diagnostic ion should be measured accordingly.

Criteria for Identification of Analyte by Infrared Spectrometry (22, 23)

Definition of adequate peaks.—Adequate peaks are absorption maxima in the IR spectrum of a standard material, fulfilling the following requirements: The absorption maximum appears in the wavenumber range 1800–500 cm^{-1}. The intensity of the absorption is not less than: (*1*) a specific molar absorbance of 40 with respect to zero absorbance and 20 with respect to peak base line, or (*2*) a relative absorbance of 12.5% of the absorbance of the most intense peak in the region 1800–500 cm^{-1} when both are measured with respect to zero absorbance, and 5% of the absorbance of the most intense peak in the region 1800–500 cm^{-1} when both are measured with respect to their peak base line. Although adequate peaks according to (*1*) may be preferred from a theoretical point of view, those according to (*2*) are easier to determine in practice.

A minimum of 6 adequate peaks is required in the IR spectrum of the standard material. If there are less than 6 adequate peaks, then the IR spectrum at issue cannot be used as a reference spectrum. The number of peaks in the IR spectrum of the standard material, within a margin of ± 1 cm^{-1}, is determined.

IR criteria.—Absorption must be present in all regions of the analyte spectrum which correspond with an adequate peak in the reference spectrum of the standard material. The "score," i.e., the percentage of the adequate peaks found in the IR spectrum of the analyte, shall be at least 50. When there is no exact match for an adequate peak, the relevant region of the analyte spectrum must be consistent with the presence of a matching peak. The procedure is applicable only to absorption peaks in the sample spectrum with an intensity of at least 3 times the peak-to-peak noise.

Conclusions

Criteria can be defined for validation of analytical methods. They offer an attractive solution for the dilemma that control methods of analysis must be validated, but that validation, especially for modern highly sophisticated methods, by interlaboratory studies is impractical.

Criteria have been defined here so as to prevent false-positive results, viz., to prevent errors of type II (small β). Therefore, the criteria are very sharp. This implies that the risk for errors of type I increases (large α). When the aim of the analysis is different, i.e., for screening purposes (where preventing of false negative results is required), other criteria can be defined to ensure reliability of that type of conclusion.

The criteria represent a first step for a new way of thinking about validation of analytical methods. We hope that they will open a discussion, and eventually lead to an objective foundation of the reliability of methods of analysis.

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MYCOTOXINS

Mycotoxin Analysis by Fast Atom Bombardment Tandem Mass Spectrometry

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Positive fast atom bombardment tandem mass spectrometry is demonstrated to be an effective technique for determination of crude aflatoxins and sterigmatocystin-related compounds. The molecular ion was selected by the first system and bombarded to produce characteristic daughter ions that could be used to identify mycotoxins in mixtures and with the same molecular weight.

Mycotoxins are secondary metabolites of various fungal species. Many of them contaminate foods and feeds. Natural contamination often occurs in trace amounts, 10^{-9} g/g sample. Minicolumn chromatography, thin-layer chromatograhy (TLC), and liquid chromatography (LC) have become the most useful analytical techniques for mycotoxins. Enzyme-linked immunosorbent assay (ELISA) has also been reported (1-4).

Analyses for aflatoxins are particularly difficult for oily material such as peanuts and corn products. Many steps are required to prepare samples for identification of mycotoxins at low levels. Detection is also complicated by false-positive results. In such cases, mass spectrometry (MS) has proved to be an extremely valuable technique for unambiguous identification of mycotoxins.

Recent publications have described field desorption MS (5), electron impact MS (6), and negative ion chemical ionization MS (7). Although the confirmation of mycotoxins by mass spectrometry has become the preferred method, samples must still undergo extensive cleanup before analysis. Furthermore, these techniques are inadequate for reliable determination when the sample is contaminated with many mycotoxins or with compounds showing coincident MS signals. We describe here the identification of mycotoxins in such cases by positive fast atom bombardment tandem mass spectrometry (FAB-MS/MS).

Experimental

Chemicals

Aflatoxins were secured from Makor Chemicals Ltd, Jerusalem, Israel, as authentic samples. Aflatoxin B_1 (25 ppm) and aflatoxin B_2 (6.2 ppm) were extracted from peanuts (100 g; received from Nagoya City Health Research Institute) by the Japanese official method for analysis of aflatoxins in peanuts (8). Sterigmatocystin-related compounds were obtained from T. Hamasaki, Tottori University, Japan, as pure compounds isolated from mycelial mats of fungi (9).

Mass Spectrometry

A JEOL JMS-DX 705L tandem quadrupole mass spectrometer was used for FAB-MS/MS analysis. Two JEOL JMS-DX300 instruments are combined through EB-EB geometry with an He collision chamber interface to obtain daughter ions caused by the collisional activation. The mass spectrometer was calibrated using the masses of Ultramark ions for positive FAB-MS/MS.

The matrix for FAB measurements was a mixture of glycerin and 3-nitrobenzyl alcohol. Mycotoxin standard (less than 1 μg) was dissolved in 10 μL solvent (dioxane, chloroform, or dimethyl sulfoxide), a 2 μL aliquot was mixed with matrix (ca 10 μL), and the mixture was introduced into the first mass spectrometer where it was irradiated with a xenon gun at 10^{-6} torr for ionization in an electric field with 3 kV acceleration. The molecular ion (M + 1) of each mycotoxin was selected from the first system and bombarded with He gas to produce daughter ions which were subsequently analyzed by the second system. The data of each measurement were averaged by accumulation about 10 times with a computer.

Results and Discussion

General Features of Single Stage Mass Spectrometry

Figures 1A and 1B show the positive FAB/MS spectra of aflatoxin B_1 (MW = 312) and aflatoxin B_2 (MW = 314), respectively. Figures 1C, 1D, 1E, and 1F show those of versicolorin A, *O*-methylsterigmatocystin, 5,6-dimethoxysterigmatocystin, and nidurufin, respectively. The spectra showed the molecular ion (M + 1) in appreciable abundance at the highest m/z. The molecular ion peak was stable longer than 10 min with very little fragmentation. This technique as well as other techniques of MS (FD, NICI, and EI) can be used with good success when the sample is very clean. However, for crude samples (Figure 1G), these techniques cannot be used reliably to confirm the presence of a certain mycotoxin.

Tandem Mass Analysis

The molecular ion was selected by the first mass system for collision with He and subsequent fragmentation. The resulting daughter ions were analyzed by the second mass system in the accumulation mode. This technique improved sensitivity and specificity over single stage mass spectrometry. Tandem mass spectrometry allowed unambiguous identification and confirmation of a mycotoxin in much cruder samples.

Figures 2A and 2B show the positive FAB-MS/MS spectra of aflatoxins B_1 and B_2, respectively. Both show consistent fragmentation behavior, with the loss of m/z 15($-CH_3$), 28 ($-CO$), 44($-CO_2$), and 72. The positive FAB-MS/MS spectrum of aflatoxin B_1 is better than the spectrum obtained with NICI-MS/MS. The molecular ion peak can be detected in the former system as the most intense peak. FAB-MS/MS also gives more information because of the many daughter ions of very high intensities; NICI-MS/MS produces fewer daughter ions (10).

Because of the more detailed fingerprint characteristics from the daughter ions, FAB-MS/MS can be used to identify the compounds with the same molecular weight. Versicolorin A has the same molecular weight as *O*-methylsterigmatocystin (MW = 338), but showed a completely different fragmentation pattern (Figures 2C and 2D). In addition, 5,6-

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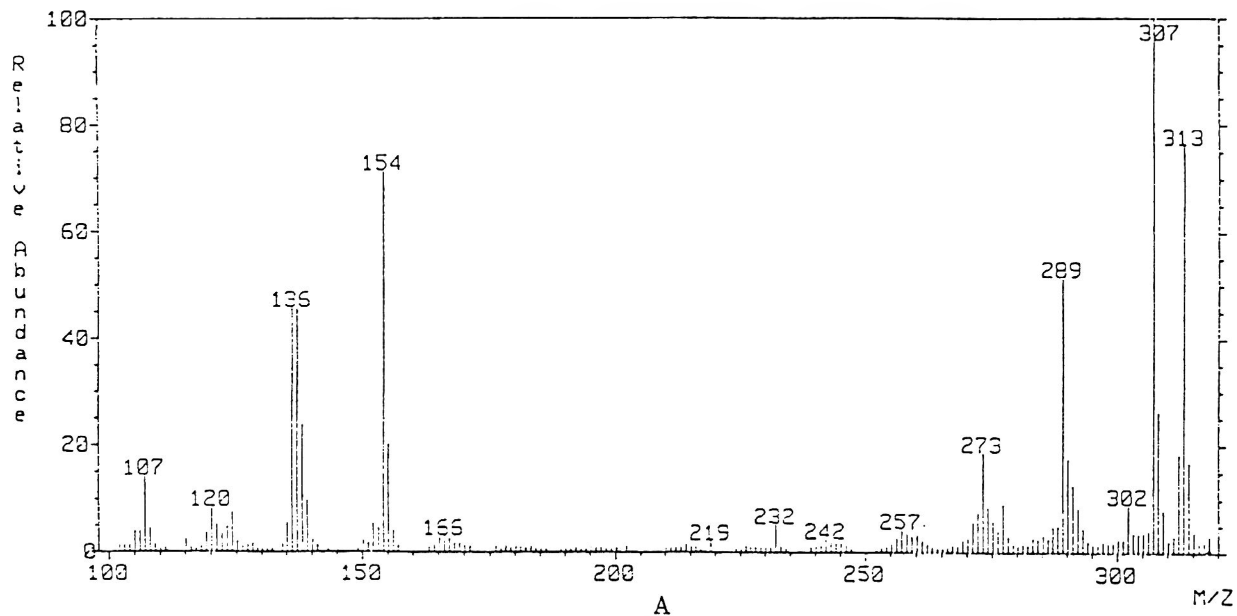

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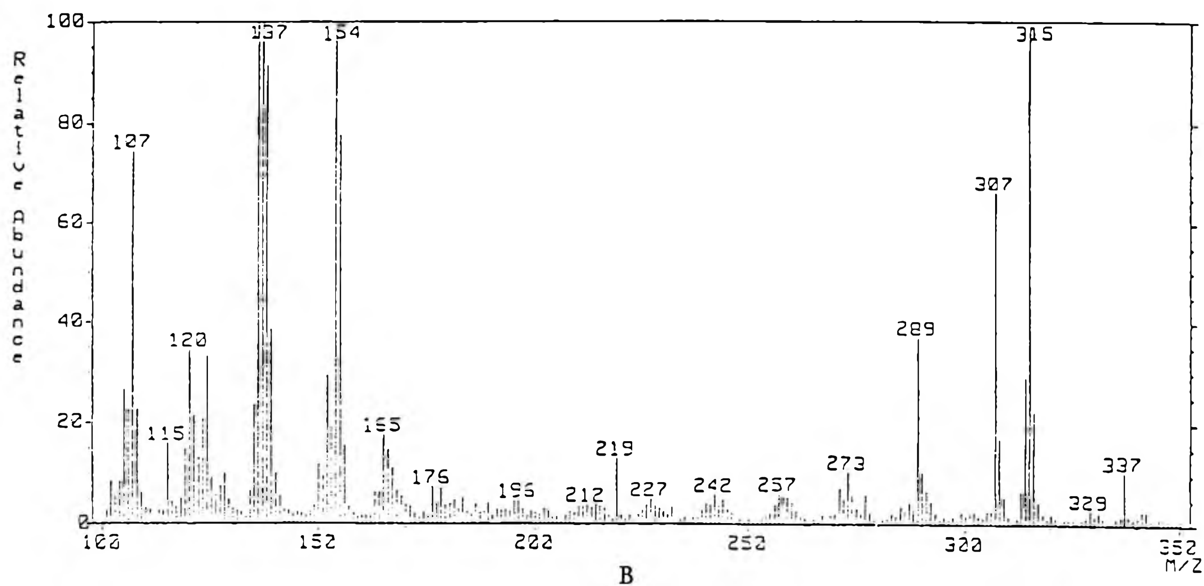

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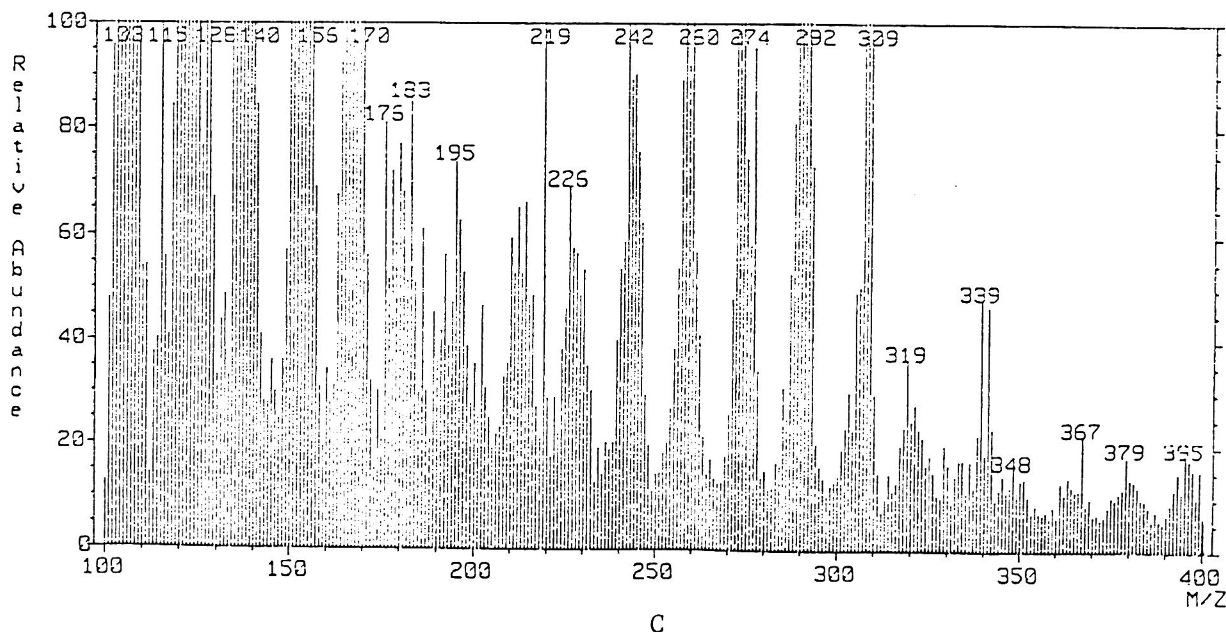
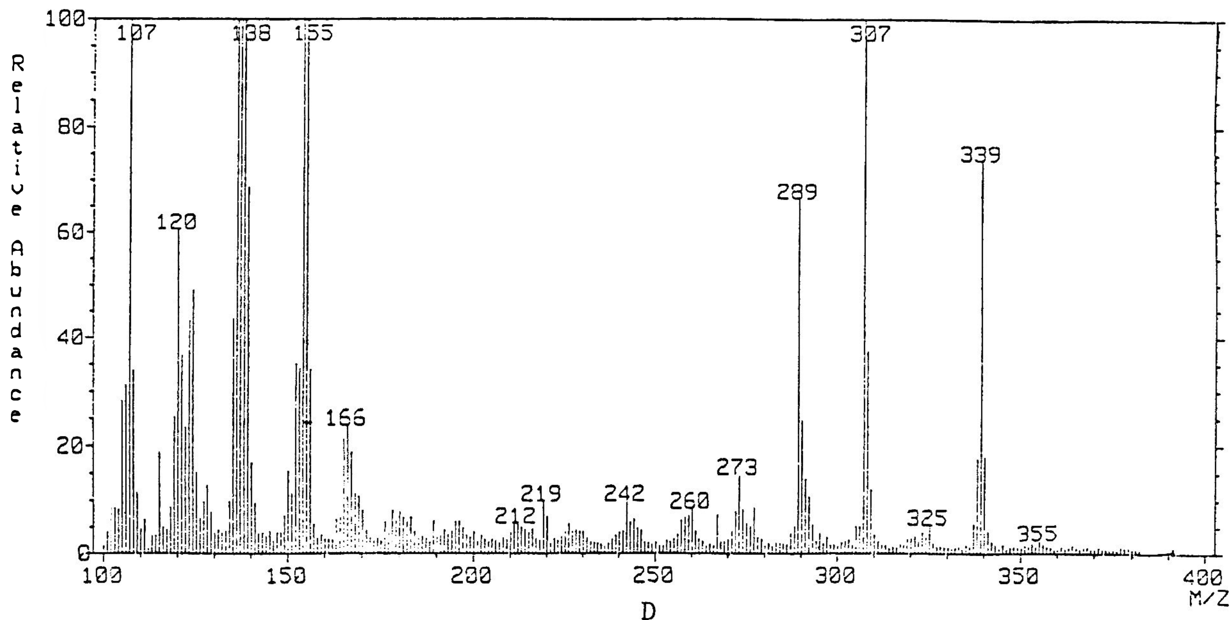


C

Figure 1 **Continued on next page**



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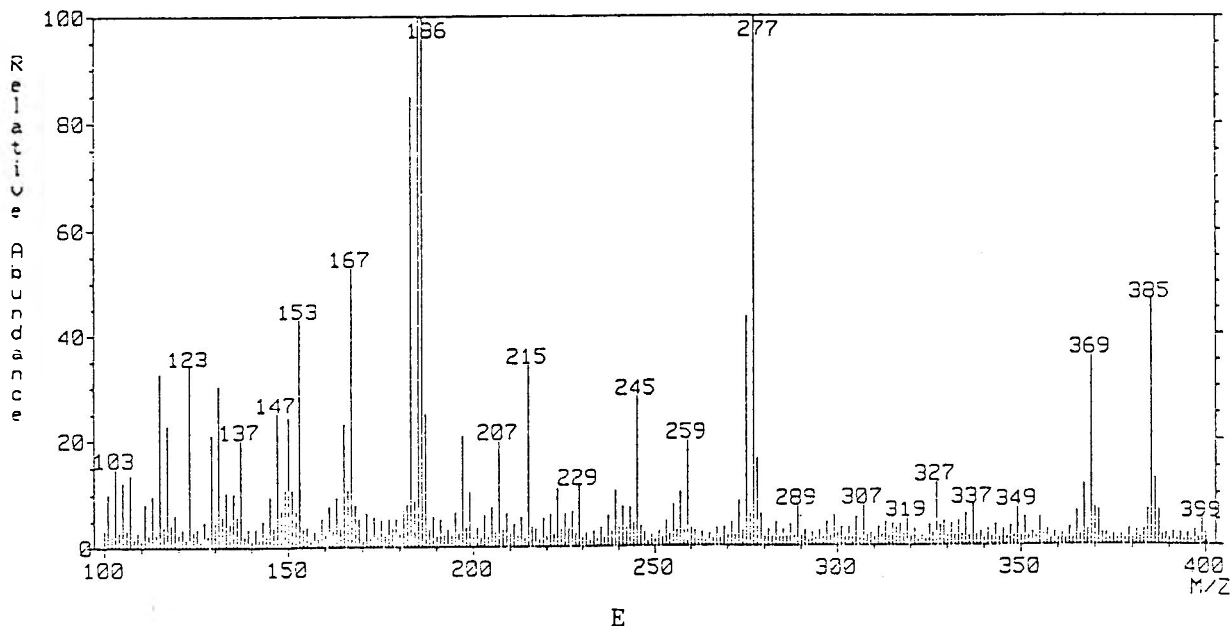

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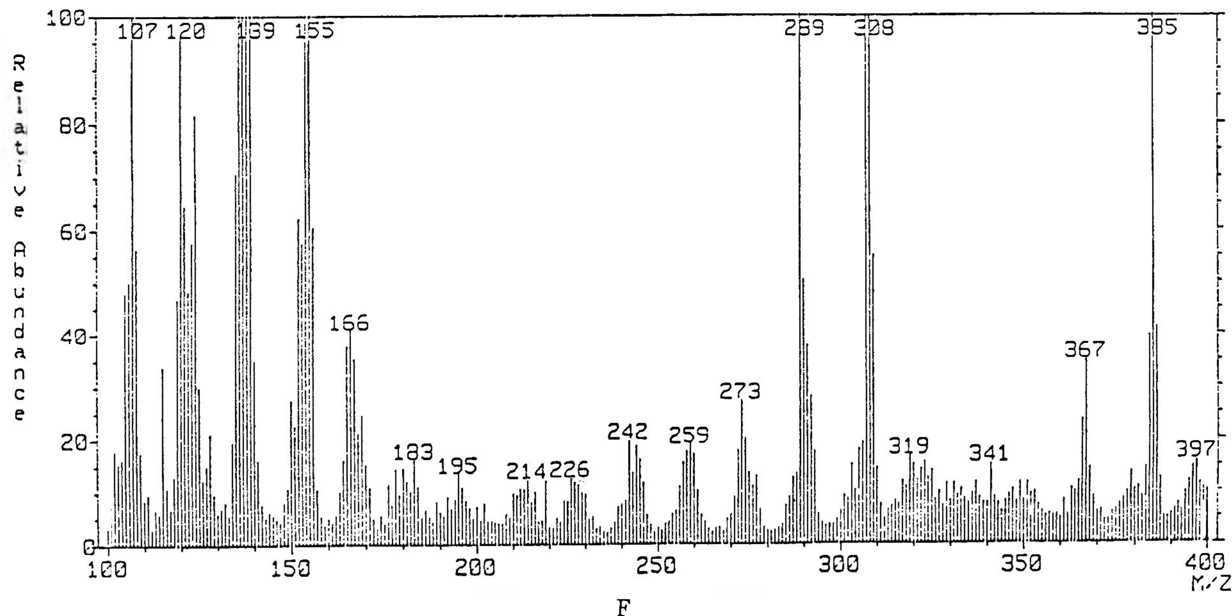

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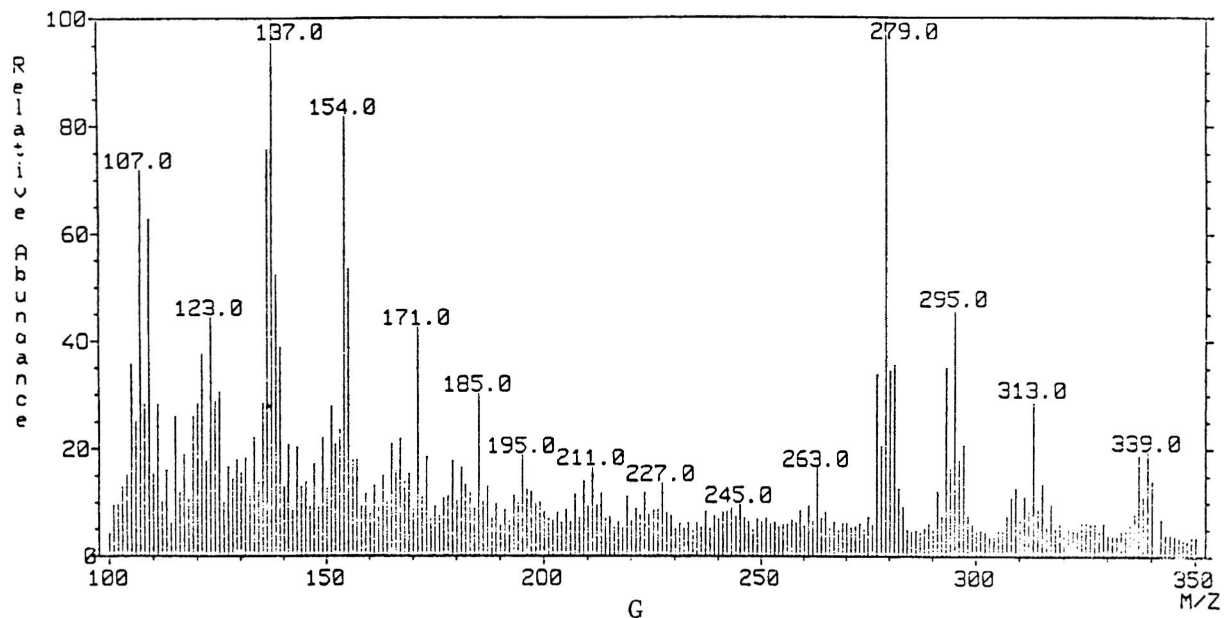
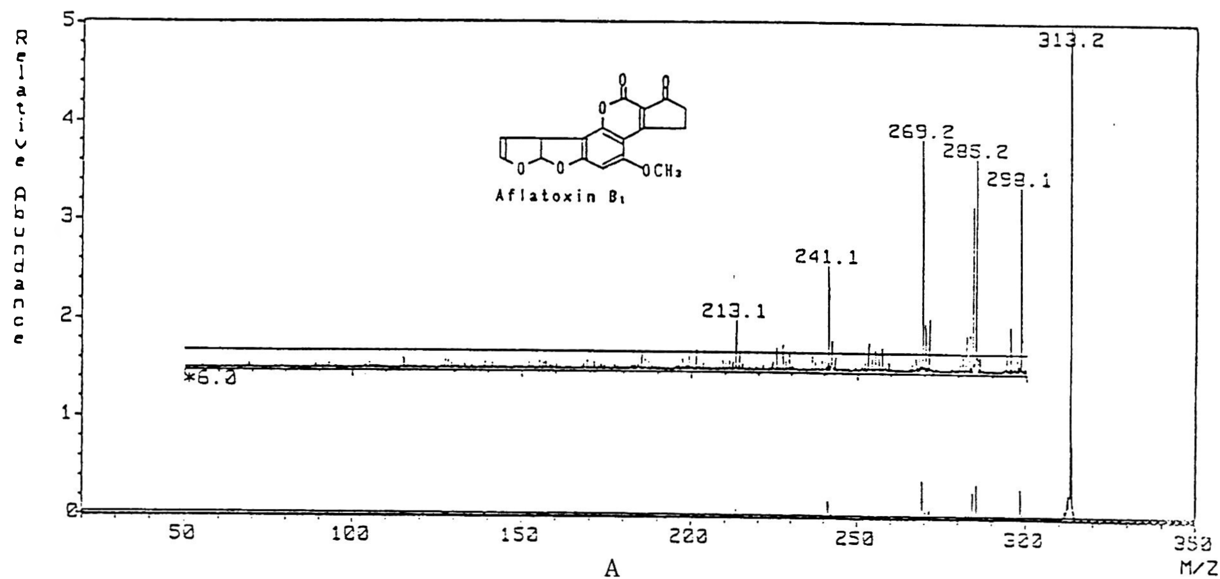


Figure 1. Positive FAB-MS spectra of A, aflatoxin B_1 (MW = 312); B, aflatoxin B_2 (MW = 314); C, versicolorin A; D, *O*-methylsterigmatocystin; E, 5,6-dimethoxysterigmatocystin; F, nidurufin; G, peanut extract.



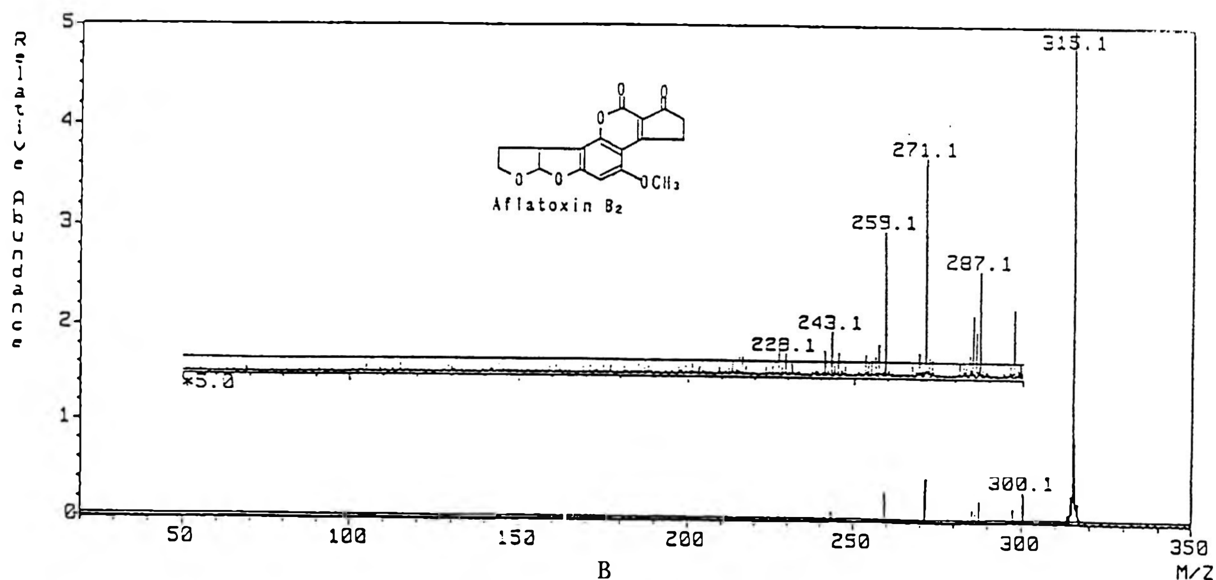
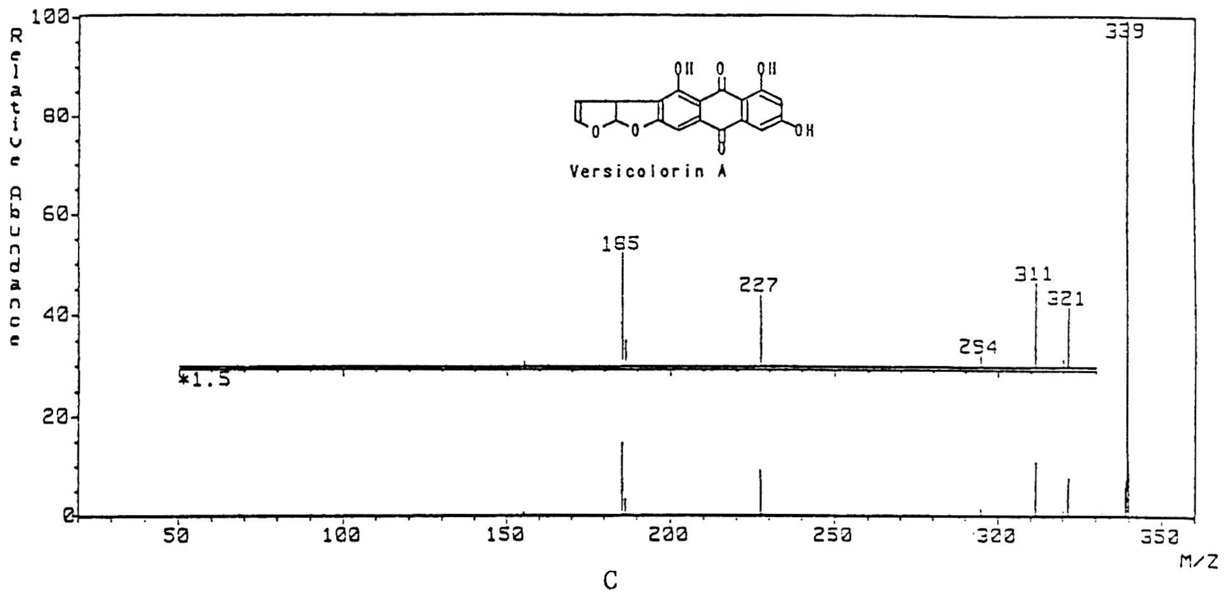


Figure 2 **Continued on next page**



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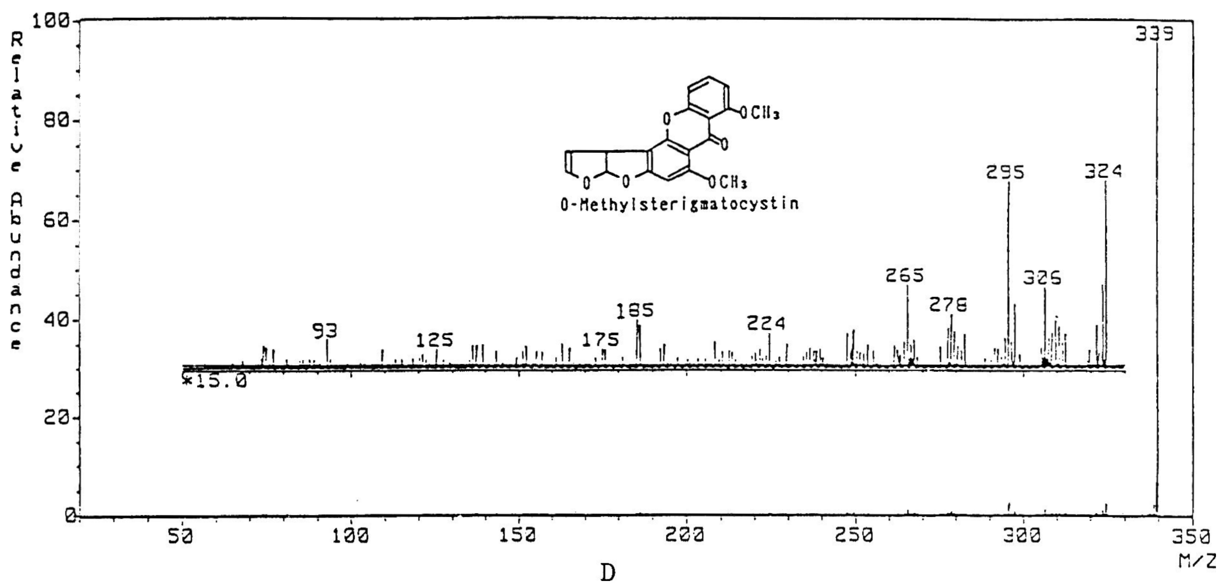

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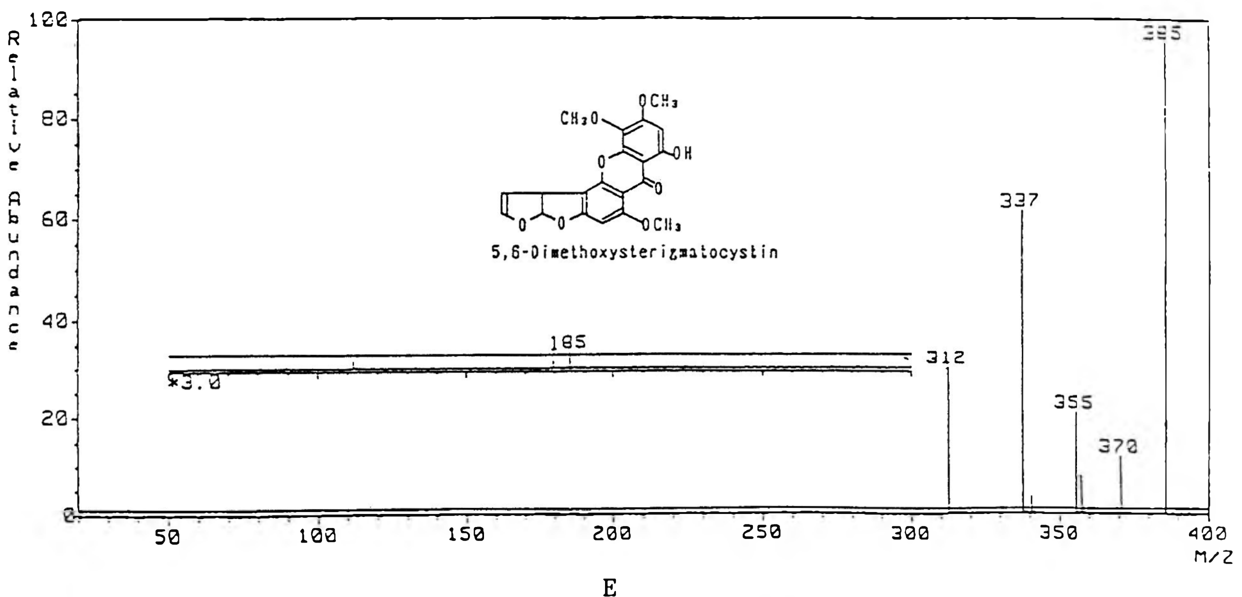

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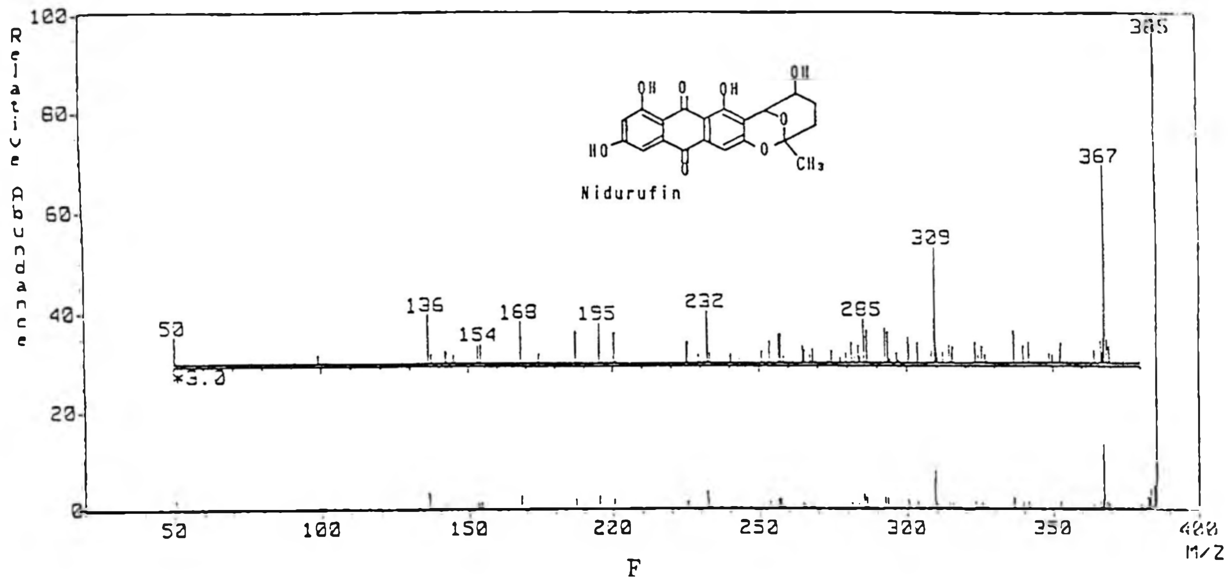

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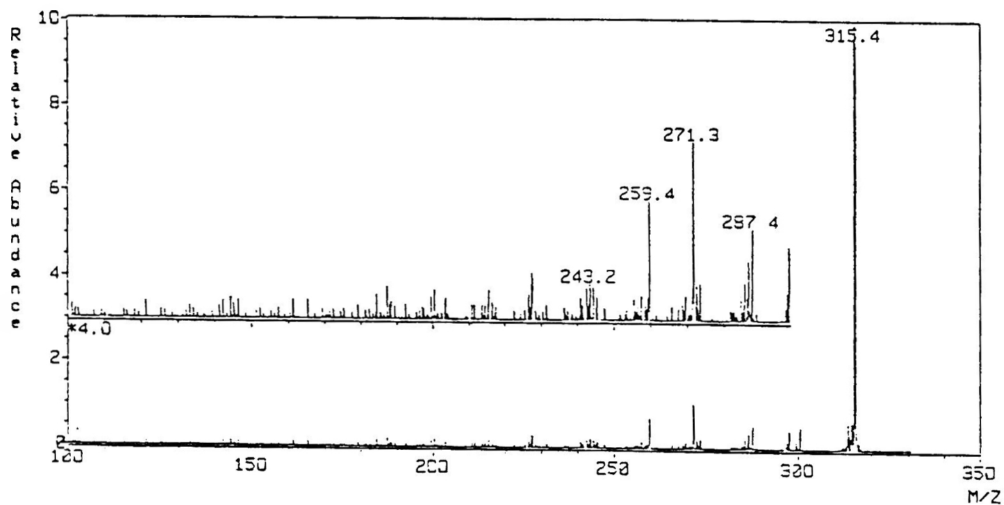

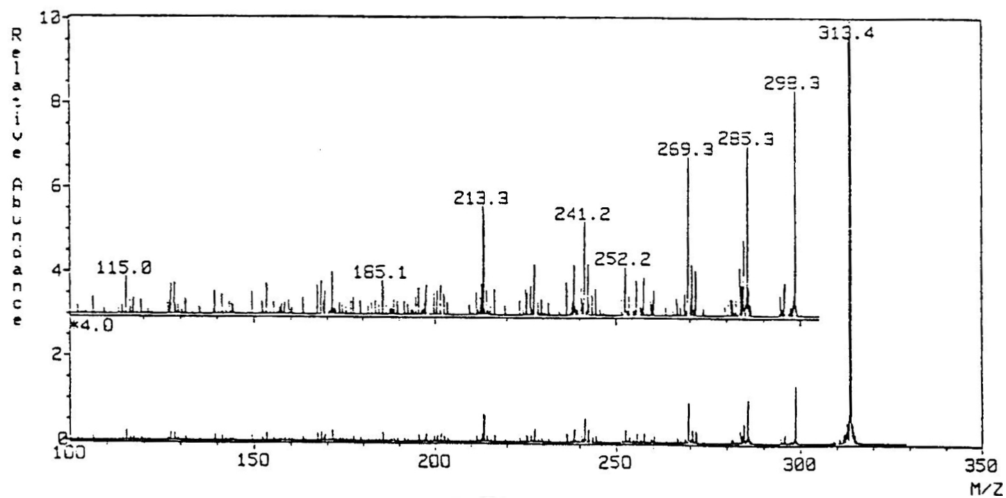

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Figure 2. Positive FAB-MS/MS spectra of A, aflatoxin B_1; B, aflatoxin B_2; C, versicolorin A (MW = 338); D, *O*-methylsterigmatocystin (MW = 338); E, 5,6-dimethoxysterigmatocystin (MW = 384); F, nidurufin (MW = 384); G, peanut extract.

dimethoxysterigmatocystin and nidurufin (MW = 384) could be distinguished unambiguously (Figures 2E and 2F).

It is often necessary to analyze mixtures of mycotoxins in naturally contaminated foods and feeds. For these situations, tandem mass spectrometric analysis is applicable for identification and confirmation of structure without rigorous purification (Figure 2G).

Acknowledgments

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OILS AND FATS

Determination of Iodine Value by Bromine/Instrumental Neutron Activation Analysis

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A new microanalytical method has been developed to measure iodine value (IV) of oils and fats. Bromine vapor was used to saturate the ethylenic double bonds, and reacted bromine was determined by instrumental neutron activation analysis. The method was applied to measure the iodine values of 7 commercially available vegetable oils: almond oil, sunflower oil, peanut oil, soy oil, sesame oil, corn oil, and olive oil. No significant difference was observed between the iodine value determined by the proposed method and that determined by an officially approved (Hübl) method. Bromine measurements can be performed up to 150 days after bromination with no significant variation in iodine value; thus, availability of an irradiation facility on the premises is not a limitation. No corrosive and toxic reagents are required, and the method is faster than the official methods. The method is also applicable to measuring iodine values of free or esterified fatty acids.

Selective hydrogenation of unsaturated lipids is a common ocedure used for the production of shortenings and margarine fats. Measuring the degree of unsaturation for both the reactant and the product is crucial for producing shortenings and margarine fats of selected texture. The unsaturation value is also a good indicator for detecting adulteration of vegetable oils with highly saturated animal fats and mineral oils. Halogenation methods are, by far, the most common methods for measuring the degree of unsaturation of oils and fats.

Measurement of iodine value, based on the principle of adding a halogenating reagent to oils or fats, followed by titrating the residual unused reagent, was reported almost a century ago (1-6); some of these early methods are still in use. However, most of the early methods suffer from one or more of the following limitations: (*i*) limited reagent lifetime (some reagents need to be prepared 24 h before the analysis and are stable only 48 h); (*ii*) extended analysis time (reagents need to be in contact with the sample for a considerable length of time, up to 48 h); (*iii*) the need for relatively large samples (up to a 1 g sample should be used in applying Wijs, Hübl, or Hanus methods); and (*iv*) the use of toxic chemicals (e.g., $HgCl_2$) and corrosive solvents (e.g., glacial acetic acid). With the development of modern instrumentation, several modifications to iodine value determinations have been introduced with the main goal of shortening the time needed for preparing reagents and/or completing the analysis (7-17).

The objective of the present study is to develop a new microanalytical method to measure IV of oils and fats, whereby bromine vapor is used to saturate the ethylenic double bonds in the oil sample. The quantity of bromine reacted is determined by instrumental neutron activation analysis (INAA). Because access to a neutron source may be limited, we studied the stability of the brominated species. We found that the bromination step may be performed in one laboratory and then the bromine measurements, done by INAA, may be performed in another, remote facility.

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Experimental

Apparatus

(**a**) *Neutron source.*—TRIGA Mark-I nuclear reactor operates at steady state operating power of 250 kW, with thermal neutron flux of ca 2×10^{12} n cm^{-2} s^{-1}.

(**b**) *Gamma-rays detector.*—Detection system consists of high-purity germanium coaxial detector (EG&G ORTEC Model 20109) coupled to 4096 multichannel analyzer (EG&G ORTEC Model 7010). Detection system measures resolution of 1.90 keV full width at half maximum for 1332 keV line of ^{60}Co, with relative efficiency of 20%. Data acquisition and data reduction were controlled by local computer equipped with LSI/11 microprocessor (EG&G ORTEC Model 1152).

(**c**) *Bromination chamber.*—Consists of 1 L air-tight glass vessel. Filter paper can be suspended from 2 glass hooks descending from stopper (Figure 1). Sufficient bromine vapor in the vessel was assured by keeping the level of bromine liquid at ca 1 cm height from base of vessel.

(**d**) *Sample holder.*—Specially fabricated to hold filter paper flat over detector during gamma-rays counting procedure (Figure 2).

Reagents

(**a**) *Potassium bromide.*—From Johnson Matthey, Inc., with certified purity of 99.998%. Standard bromine solution containing 100 μg Br/μL was prepared.

(**b**) *Liquid bromine.*—Obtained from Fisher Scientific.

(**c**) *Almond, sunflower, peanut, soy, sesame, corn, and olive oils.*—Manufactured in the United States and purchased from local supermarket.

(**d**) *Oleic acid.*—Obtained from Fisher Scientific.

(**e**) *Polyethylene vials.*—Obtained from Olympic Plastic, Inc.

(**f**) *Filter paper.*—4.3 cm diameter (Micro Filtertion System).

Procedure

(**a**) *Determination of bromination time.*—To determine optimum bromine-exposure time required to reach complete bromination, a series of experiments were performed where 2-100 mg sunflower oil was smeared onto the center of the filter paper and then suspended in the bromination chamber for periods ranging from 10 to 180 s. The filter paper was then suspended in a clean laminar flow hood for 20-30 min to remove excess (unused) bromine. Blank experiments were performed in an identical manner, but no oil was applied. The radioactive concentration of ^{80}Br in becquerel per mg oil was calculated and normalized to that for the 180-s experiment (for irradiation procedure, see part (c) below). We concluded that 90 s exposure is sufficient to achieve complete bromination (Figure 3).

(**b**) *Determination of IV.*—For IV measurements, ca 2-18 mg oil was smeared onto filter paper, the paper was suspended in the bromination chamber for 90 s, and excess bromine

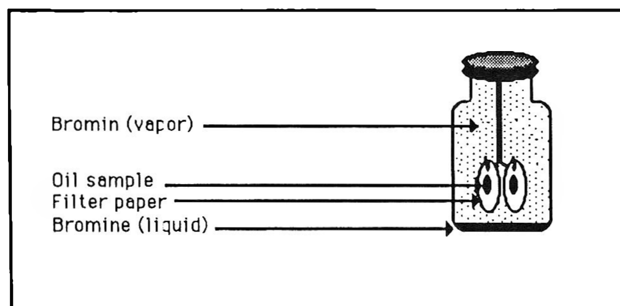

Figure 1. Brominating chamber.

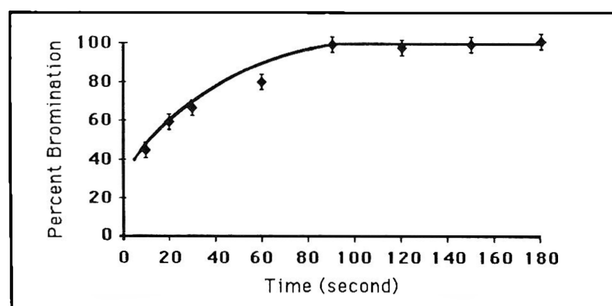

Figure 3. Bromination rate.

was removed as previously described. A 50 μL standard Br solution was applied to the center of similar filter paper and air-dried in a contamination-free environment. Blank experiments were performed as mentioned before.

(c) *Irradiation procedure.*—Filter paper containing the treated sample (blank or standard) was sandwiched between polyethylene films and thermally sealed. The assembly was then inserted into a 2 dram high-purity polyethylene vial, and bromine content of the brominated species was determined by INAA via the nuclear reaction $^{79}Br(n,\gamma)^{80}Br$. Parameters pertaining to that reaction are given in Table 1. After irradiation, the assembly was taken out of the polyethylene vial and stretched flat in the sample holder, and the emitted gamma rays were counted for 120 s at fixed geometry.

(d) *Precision and accuracy of the method.*—At least 4 independent measurements were performed on each oil sample to confirm the repeatability of the method. The weight of the samples varied in each experiment to assure the applicability of the method over a considerable range of weights. The accuracy of the method was confirmed by determining IV of the same oils by the Hübl standard method as described in IUPAC *Standard Methods of the Oils, Fats, and Derivatives* (18).

(e) *Stability of brominated species.*—To ensure applicability of the proposed method for laboratories that do not possess an irradiation facility, a set of experiments were designed to determine stability of the brominated species over a 5 month period. Thus, the bromination step on oleic acid and sunflower oil was carried out as described earlier and bromine concentration was determined at 1, 15, 30, 45, 60, 90, and 150 days from bromination.

Results and Discussion

The weight and calculated iodine values for the 7 oils and oleic acid are shown in Table 2. Because IV is defined as the number of grams of iodine reacted per 100 g sample, whether or not the halogen used is iodine, the weight of iodine equivalent to the weight of bromine reacted with 100 g sample can be calculated as follows:

$$\text{Iodine value} = (126.9/79.9) \times 100 \times (W_{Br}/W_{oil}) = 158.8\ R$$

where R is the weight ratio of reacted bromine (W_{Br}) to oil (W_{oil}), 126.9 is the atomic weight of iodine, and 79.9 is the atomic weight of bromine. As shown in Table 2, the smallest sample weight used in this study was 1.95 mg. However, because the minimum detection limit for INAA is approximately 0.003 μg Br, a 3–5 ng oil (with IV of 100–150) may be sufficient for IV determination.

To study the accuracy of the proposed method, the same oils used in the study were analyzed by the Hübl standard method (18); the results are also shown in Table 2. The iodine value of oleic acid is similar to that obtained in another study (16) by the Kaufmann method (91.0 ± 1.8) and by bromine selective electrode (89.1 ± 1.8). Also, the iodine values for the 7 oils examined fall within the expected range given by AOCS (19). Statistically, no significant difference was observed between the results obtained by the neutron activation analysis method and the Hübl method (applying Student's *t*-test at 95% probability).

To study the stability of the formed brominated species, the bromination step was performed on oleic acid and on sunflower oil. Iodine value was then determined at 1, 15, 45, 60, 90, and 150 days from bromination; results are shown in Table 3. For oleic acid, the difference between results after one day (89.1 ± 1.3) and after 150 days (85.8 ± 3.1) does not significantly vary from zero (3.3 ± 3.4). The same conclusion is also valid for sunflower oil, where the difference for iodine values determined after 150 days (131.2 ± 5.4) and after one day (128.0 ± 4.2) does not significantly vary from zero (3.2 ± 6.8). These results indicate the stability of the brominated species over considerable periods (5 months); thus, the proposed method has the flexibility to be carried out by a laboratory with no irradiation facility on the premises.

In conclusion, the proposed method provides the following advantages: (*i*) it can be applied to trace quantity of lipids; (*ii*) neither corrosive solvents nor toxic chemical reagents are required; (*iii*) the analysis is free from reagent stability-time constraints; and (*iv*) most important, the reaction time is shortened and results can be obtained faster (typically days

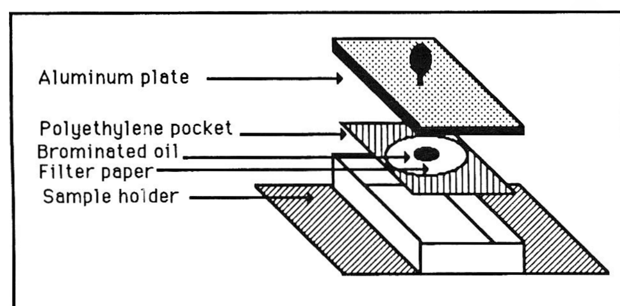

Figure 2. Schematic view of sample holder.

Table 1. Experiment parameters for determination of bromine by instrumental neutron activation analysis

Nuclear reaction used	$^{79}Br(n,\gamma)^{80}Br$
Indicator radionuclide	^{80}Br
Half-life (min)	16.8
Neutron flux (n cm^{-2} s^{-1})	~ 2 × 10^{12}
Time of irradiation (min)	1
Time of decay (min)	5–10
Time of counting (min)	2
γ-Rays monitored (keV)	616, 639, 656

Table 2. Determination of iodine value of oleic acid, olive oil, peanut oil, almond oil, corn oil, soy oil, sunflower oil, and sesame oil by proposed INAA method and Hübl standard method

	INAA		Hübl		
Sample	Weight, mg	Iodine value[a]	Weight, mg	Iodine value	Other studies
Oleic acid	3–15	90.0 ± 1.9 (4)	211–257	89.1 ± 1.4 (4)	89.1 ± 1.8[b]
Olive oil	3–17	82.9 ± 0.93 (4)	285–322	82.5 ± 0.19 (5)	80–88[c]
Peanut oil	2–6	92.7 ± 2.1 (6)	97–118	92.5 ± 2.6 (5)	84–100[c]
Almond oil	3–7	95.7 ± 2.0 (4)	121–147	95.5 ± 0.26 (4)	93–105[c]
Corn oil	2–18	127.0 ± 2.0 (4)	142–385	126.6 ± 1.1 (5)	109–133[d]
Soy oil	4–10	127.5 ± 2.4 (4)	110–135	125.6 ± 0.76 (7)	120–141[c]
Sunflower oil	2–5	129.9 ± 2.8 (5)	80–115	128.6 ± 3.0 (6)	125–136[c]
Sesame oil	3–8	108.6 ± 0.7 (4)	111–161	107.8 ± 1.5 (5)	103–116[c]

[a] Number of replicates in parentheses.
[b] Adapted from ref. 16.
[c] AOCS value, adapted from ref. 19.
[d] Adapted from ref. 19.

Table 3. Iodine values of oleic acid and sunflower oils determined at different times after bromination

	Iodine value	
Days from bromination	Oleic acid	Sunflower oil
1	89.1 ± 1.3	128.0 ± 4.2
15	88.9 ± 4.6	130.7 ± 7.3
45	87.1 ± 2.5	132.7 ± 5.1
60	86.8 ± 2.3	130.1 ± 2.8
90	86.9 ± 2.2	130.5 ± 2.6
150	85.8 ± 3.1	131.2 ± 5.4

by Hübl method, hours by Hanus method, and minutes by the proposed method).

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Levels of Polychlorinated Biphenyls and Pesticides in Bluefish Before and After Cooking

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Similar levels of polychlorinated biphenyls (PCBs), pesticides, and fat were found in 20 correlated uncooked and cooked (baked) bluefish fillets. Fillets averaged 2.5 ppm PCBs as Aroclor 1254 (whole basis) before cooking; after cooking, with the oil drippings and skin discarded, the average PCB level was 2.7 ppm. Although PCBs, lipophilic pesticides, and fat were lost along with oil drippings and skin that were discarded after cooking, the moisture loss in the fillets during cooking compensated for these weight losses almost completely. After the fillets were cooked and the oil drippings and skin were discarded, the PCB content of the fillets was 27% lower on the average.

The National Oceanic and Atmospheric Administration, the Food and Drug Administration (FDA), and the Environmental Protection Agency recently conducted the *1984-86 Federal Survey of PCBs in Atlantic Coast Bluefish* (1, 2). In that survey, no composite or individual fish in the 300 mm or less and the 301-500 mm fork length categories at any site sampled exceeded the FDA tolerance of 2 ppm for polychlorinated biphenyls (PCBs). However, some samples in the greater than 500 mm fork length category exceeded this tolerance at every site sampled during the survey. For that survey, raw fillets with the skin on and scales off (3) were analyzed because both the PCB tolerance and pesticide residue limits are based on the raw commodity. As a follow-up to that survey, the effects of cooking (baking) on PCB levels in bluefish were determined and are reported here in order to assist in defining the PCB level in bluefish as ingested by a large segment of the bluefish-consuming public.

Experimental

Apparatus

(**a**) *Oven.*—Caloric electric convection oven (Raytheon, Caloric Div., Topton, PA 19562).

(**b**) *Gas chromatograph.*—Varian 6000 (Varian Associates, Sunnyvale, CA 94089) equipped with ^{63}Ni electron capture detector and 6 ft × 2 mm glass column containing 5% OV-101 on Chromosorb W (HP). Column flow, 30 mL N_2/min. Temperatures: column 200°C; injector 205°C; detector 300°C.

(**c**) *Computing integrator.*—Varian 402 data system (Varian Associates).

Sample Collection

Twenty bluefish (*Ponatomus saltatrix*) were collected during September and October 1986; 17 were from Buzzards Bay, MA, 2 (samples 8 and 17) were from New Bedford, MA, and one (sample 2) was from Plymouth, MA.

Sample Preparation

One fillet from each bluefish was analyzed raw with skin on and scales off, and the other fillet from each bluefish was baked with skin on and scales off.

Fillet was baked skin side up in uncovered baking pan of appropriate size. Fish was supported by baking rack in bottom of pan. Oven was preheated to 325°F. Baking pan was placed in oven, and fish was baked until it was no longer translucent and flesh flaked readily. Total baking time was recorded, and baked fish was removed from oven. (Average baking time was about 1 h.) As soon as fish was sufficiently cool, edible portion of fillet was removed. Edible portion of cooked bluefish at this point included all muscle tissue but no oil in the pan or skin. Baking rack had holes to let oil drippings drain into pan below. Oil from baking pan was placed in glass container and identified with sample number of fish. These oil drippings were analyzed separately for PCBs. Before baked fillet was analyzed, skin was removed and discarded.

Determination

Samples were analyzed according to *Pesticide Analytical Manual, Vol. I* (3) secs 211.13f(1), 211.14a, and 211.14d. Each PCB residue was quantitated by comparing total area of residue peaks to total peak area from appropriate Aroclor(s) reference materials. Only those peaks from residue that could be attributed to chlorobiphenyls were used. (This methodology uses petroleum ether extraction, acetonitrile–petroleum ether partition, and Florisil column chromatography before the gas chromatographic determination.)

Quality Assurance

An uncooked fillet was fortified with 1.7 ppm Aroclor 1254; recovery was 95%. An oil sample was fortified with 2.8 ppm Aroclor 1254; recovery was 76%. A method reagent blank showed no apparent PCBs or pesticides.

Additional recoveries from bluefish fortified with Aroclors and pesticides were performed by our laboratory for the work reported in ref. 1. Eight recoveries from bluefish fortified with Aroclor 1254 averaged 86% (range 76-102%). A total of 25 recoveries were performed for all pesticides detected; the lowest recovery was 71% for bluefish fortified with 1.0 ppm *p,p'*-DDT, and the highest was 110% for bluefish fortified with 0.20 ppm octachlor epoxide.

Results and Discussion

Table 1 shows the results of the PCB and lipid analyses of the 20 bluefish fillets before and after cooking. Aroclor 1254 was used as the reference standard for determining the PCB residues because the residues found in the fillets most closely matched this Aroclor. The limit of quantitation was 0.3 ppm PCBs; the limit of detection was 0.1 ppm PCBs. Table 1 shows similar levels of PCBs and fat in the uncooked and cooked fillets. The mean PCB level (whole basis) in the uncooked fillets was 2.5 ppm (range, 1.0-7.2 ppm). The mean PCB level (whole basis) in the cooked fillets was 2.7 ppm (range 0.7-4.9 ppm), or 8.0% greater than in the uncooked fillets. The mean % fat in the uncooked fillets was 11.8% (range 3.9-19.0%). The mean % fat in the cooked fillets was 13.3% (range 1.8-22.0%), or 12.7% greater than in the uncooked fillets. The mean PCB levels (fat basis) in the uncooked and cooked fillets were 21 and 20 ppm PCBs, respectively. The mean PCB level (fat basis) for uncooked fillets was 5.0% greater than for cooked fillets.

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Table 1. Results of PCB and lipid analyses of bluefish before and after cooking

Sample	PCBs, ppm (whole basis)		Lipid content, %	
	Raw	Cooked	Raw	Cooked
1	2.4	2.6	8.1	8.9
2	1.2	1.4	19.0	21.0
3	5.2	4.8	8.5	9.8
4	2.2	2.6	6.3	6.7
5	7.2	7.1	17.1	21.3
6	1.0	0.7	3.9	1.8
7	1.8	1.9	10.8	11.7
8	2.5	3.0	15.5	17.8
9	1.6	1.3	7.7	9.3
10	3.3	4.0	17.3	21.3
11	2.1	2.0	15.3	16.6
12	3.6	4.1	15.4	16.9
13	4.3	4.9	18.8	22.0
14	1.7	2.4	10.6	12.0
15	1.5	1.6	6.9	6.5
16	1.4	1.2	4.3	7.1
17	1.1	0.8	9.9	13.9
18	1.3	2.8	17.5	19.7
19	1.7	2.2	14.7	14.7
20	2.2	2.0	7.5	7.4
Mean	2.5	2.7	11.8	13.3
Mean (fat basis)	21	20		

Table 2. Bluefish fork lengths and weights of raw fillets with skin, cooked fillets without skin, oil drippings, and cooked skin

Sample	Fork length, mm	Wt raw fillets, g	Wt cooked fillets, g	Wt oil drippings, g	Wt cooked skin, g
1	640	583	404	16.2	23.8
2	780	1114	798	74.3	59.8
3	660	714	498	30.5	30.8
4	610	507	337	19.1	23.6
5	670	792	561	48.7	28.9
6	610	405	240	1.6	33.4
7	780	836	588	45.6	34.1
8	850	1091	748	60.2	70.0
9	830	815	554	53.8	32.6
10	750	824	590	49.6	37.9
11	770	793	543	44.5	38.4
12	750	793	566	26.9	50.2
13	760	891	644	36.4	49.2
14	820	798	533	30.7	42.9
15	780	872	600	79.1	37.8
16	800	974	678	91.9	63.8
17	840	754	515	41.3	30.8
18	800	823	571	41.1	42.7
19	760	740	494	49.6	44.7
20	750	763	514	38.4	49.3
Mean	750.5	794.1	548.8	44.0	41.2

Table 2 shows the fish fork lengths and the weights of raw fillets with skin, cooked fillets without skin, oil drippings, and cooked skin. The data in Table 2 show that uncooked fillets on the average lost 30.9% of their weight during cooking to oil drippings (5.5%), to moisture loss (20.2%), and to discarded skin (5.2%). From Tables 1 and 2, uncooked fillets were calculated to have lost on the average 27% of their PCB weight as a result of the cooking process and the discarding of the oil drippings and skin. PCBs can be vaporized during baking and can be discarded in the oil drippings and skin, which can be PCB-rich. Thus, for this study, cooking (baking) substantially reduced the PCB weight in the bluefish. The relatively large loss of moisture during cooking compensated for the PCB and oil loss and resulted in similar ppm PCB and % fat levels in the uncooked and cooked fillets.

Six of the 20 oil drippings had quantifiable PCB levels

Table 3. Pesticides (ppm, whole basis) found in bluefish before and after cooking

Sample	trans-Chlordane		cis-Chlordane		*p,p'*-DDT		*p,p'*-TDE		Dieldrin		Hexa-chloro-benzene		trans-Nonachlor		*p,p'*-DDE		α-BHC		Octa-chlor expoxide	
	BC[a]	AC[a]	BC	AC	BC	AC	BC	AC	BC	AC	BC	AC	BC	AC	BC	AC	BC	AC	BC	AC
1	T[b]	0.02	0.02	0.02	T	T	T	T	T	T	T	T	T	T	0.07	0.04	T	T	T	T
2	T	T	0.02	0.03	T	T	T	0.06	T	T	T	T	T	T	0.12	0.14	T	T	T	T
3	0.03	0.05	0.05	0.04	T	T	0.06	0.06	T	0.02	T	T	0.02	T	0.10	0.06	T	T	T	T
4	T	0.03	0.02	0.02	T	T	0.04	0.07	T	T	T	T	T	T	0.18	0.08	T	T	T	T
5	0.03	0.05	0.04	0.07	T	T	0.05	0.11	0.04	0.05	T	T	0.02	T	0.20	0.13	T	T	T	T
6	T	T	T	T	T	T	T	T	T	T	T	T	T	T	0.09	0.03	T	T	T	T
7	T	0.03	T	0.03	T	T	T	0.05	T	T	T	T	T	0.02	0.12	0.10	T	T	T	T
8	T	0.05	0.03	0.06	T	T	0.04	0.10	0.02	0.04	T	T	T	T	0.31	0.24	T	T	T	T
9	0.02	0.03	0.04	0.03	T	T	0.05	0.07	T	T	T	T	T	T	0.07	0.16	T	T	T	T
10	0.06	0.09	0.08	0.11	0.04	0.06	0.14	0.16	0.03	0.05	T	T	T	T	0.12	0.15	T	T	T	T
11	0.03	0.03	0.04	0.04	T	T	0.09	0.09	T	T	T	T	T	T	0.10	0.10	T	T	T	T
12	T	0.02	0.02	0.04	T	T	T	0.05	T	T	T	T	0.03	0.03	0.15	0.18	T	T	T	T
13	0.04	0.03	0.06	0.06	T	T	0.09	0.09	0.04	0.04	T	T	0.03	0.03	0.18	0.20	0.01	0.01	T	T
14	T	T	0.03	0.03	T	T	0.03	0.03	T	T	T	T	0.02	T	0.10	0.12	T	T	T	T
15	T	T	0.02	0.02	T	T	0.03	0.04	T	T	T	T	T	T	0.08	0.09	T	T	T	T
16	0.02	0.02	0.03	0.04	T	T	0.04	0.06	T	T	T	T	T	T	0.09	0.08	T	T	T	T
17	0.04	0.04	0.06	0.06	T	T	0.09	0.10	T	T	T	T	T	T	0.08	0.08	T	T	T	T
18	0.04	0.04	0.06	0.06	T	T	0.09	0.10	T	T	T	T	T	T	0.09	0.10	0.01	0.01	T	T
19	0.04	0.04	0.05	0.04	T	T	0.08	0.07	0.03	T	T	T	T	T	0.09	0.07	T	T	T	T
20	T	0.02	0.02	0.03	T	T	0.04	0.05	T	T	T	T	T	0.03	0.07	0.08	T	T	T	T
LQ[c]	0.02		0.02		0.04		0.03		0.02		0.01		0.02		0.02		0.01		0.02	

[a] BC = before cooking; AC = after cooking.
[b] T = trace (from 0.5 LQ to LQ).
[c] LQ = limit of quantitation; limit of detection = 0.5 LQ.

(mean, 8.1 ppm; range 1.5–30 ppm). The other 14 oil drippings gave results of none detected or trace levels (levels between the limit of detection and the limit of quantitation); these 14 oil drippings were dark brown solids or viscous liquids. It is postulated that the PCB levels of these oil samples were originally much greater and that the PCBs were vaporized from these oil drippings.

Although the primary purpose of this study was to ascertain the effect of cooking on PCB levels in bluefish, the bluefish were also analyzed for pesticide residues. Ten pesticides were found in both the uncooked and the cooked fillets. Table 3 lists these pesticides, their limits of quantitation, and the results of the pesticide analyses of uncooked and cooked fillets. Limits of detection were about one-half the limits of quantitation. Table 3 shows similar pesticide levels in uncooked and cooked fillets. The relative differences between individual pesticide levels in the uncooked and cooked fillets were greater than these for the ppm PCB and % fat levels. This is at least partly due to the low levels of pesticides found in the uncooked and cooked fillets (trace–0.31 ppm); most of the pesticide levels are reported with only one significant figure.

In conclusion, this study showed a significant weight loss (27%) for PCBs in bluefish after the fillets were cooked and the oil drippings and skin were discarded. Thus, this study demonstrated that the intake of PCBs from cooked (baked) bluefish and probably from other baked fish may be significantly lower than the results of the analyses of the raw commodity would indicate. In this study, similar concentrations (ppm, %) of PCBs, pesticides, and fat were determined before and after cooking. The moisture loss in the fillets during cooking compensated for the loss of the PCBs, pesticides, and fat on a concentration basis.

Acknowledgment

The help of the Massachusetts Division of Marine Fisheries in collecting the bluefish samples is acknowledged.

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Determination of Fluoroacetate in Biological Matrixes as the Dodecyl Ester

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A new method for the quantitative determination of fluoroacetate in biological samples was applied to a number of avian samples. Fluoroacetate is isolated as its potassium salt by ion-exchange chromatography and directly converted to its dodecyl ester, using a novel derivatization procedure. The ester is quantified by capillary gas chromatography with a flame ionization detector for the range 1.0–10.0 μg/g and by selected ion monitoring GC/mass spectrometry for the range 0.01–1.00 μg/g. Recoveries from 1 g chicken muscle were about 80%. The method was applied to the determination of fluoroacetate in the crop, stomach, liver, heart, intestine, and breast muscle of 5 Zebra finches (*Peophila guttata*) that had been fed millet containing 9 μg/g of sodium fluoroacetate. Despite a wide variation in dose, the levels in organs and tissues were approximately 1 μg/g except for heart tissue which was about 2 μg/g. The presence of interfering peaks at low levels necessitated the use of selected ion monitoring GC/MS when sample weights were less than 1 g or when levels were less than 1 μg/g. Samples can be analyzed within hours of receipt; therefore, the method is suitable for routine use in a diagnostic laboratory.

Sodium fluoroacetate (compound 1080) is used widely for control of rodents and certain predators. It is highly toxic with an acute LD_{50} value for oral doses in dogs of 0.05–1.0 mg/kg body weight (1).

Analytical techniques currently available for determining this compound in biological samples include use of ion-selective electrode detection after pretreatment of sample to release fluoride (2, 3), liquid chromatographic (LC) determination of the *p*-nitrobenzyl derivative (4), and gas chromatographic (GC) determination of the ethyl, *n*-propyl, or pentafluorobenzyl ester (5, 6).

The chromatographic techniques, although more specific and sensitive than fluoride detection, require extensive sample preparation to remove naturally occurring interferences such as acetic, propionic, and butyric acids. Sensitive detection for LC techniques requires derivatization to introduce a chromophore, and derivatization is also required for GC determination to reduce polarity. Derivatization and isolation of fluoroacetic acid is complicated by high volatility, which leads to evaporation losses when solvents are removed, and poor solubility in aprotic organic solvents because of its highly polar nature (pKa = 2.68). In addition, most chromatographic procedures previously reported suffer from interferences and low recoveries. Lengthy extraction procedures aimed at removing interfering substances are the major cause of these low recoveries.

In the present work, fluoroacetic acid was isolated from biological matrixes as its potassium salt by ion-exchange chromatography. Because the salt is involatile, evaporation of the ion-exchange eluate without loss of analyte was possible. A novel derivatization procedure was then employed to directly convert the salt to the dodecyl ester; low volatility of the ester again enabled evaporation of solvent without loss of derivative. The kinetics and mechanism of this esterification reaction will be the subject of a separate report.

The high recovery of fluoroacetate from biological matrixes (80%) afforded by this technique enables its detection in samples weighing less than 1 g, an essential requirement when organs from Australian native species are studied (7, 8). With this amount of sample, gas chromatography with flame ionization detection is used for analysis in the range of 1–10 μg/g, and selected ion monitoring GC/MS is used for the range 10–1000 ng/g. The method requires only standard

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laboratory equipment for sample preparation and, compared to published procedures, is rapid; analysis can be completed within a few hours of receipt of samples. The present paper describes the improved procedure and its application to a number of avian samples.

Experimental

Reagents

(a) *Formate buffers.*—(*1*) Adjust 0.5M HCOOH to pH 3.7 with 3.6M NaOH. (*2*) Dilute 0.5M buffer 1:10 with water to give 0.05M buffer pH 3.8.

(b) *Ion-exchange column.*—0.1 g DEAE-Sephadex A-25 (Pharmacia, Upsala, Sweden) (9) in 0.8 × 5 cm polypropylene column (polypropylene Econo-columns, Bio-Rad Laboratories, Richmond, CA).

(c) *Esterification reagent.*—160mM 1-bromododecane (Tokyo Chemical Industry, Tokyo, Japan) in dimethyl acetamide (distilled at 0.1 torr and 70°C) containing 0.8mM 1, 4, 7, 10, 13, 16-hexaoxocyclooctadecane (Sigma Chemical Co., St Louis, MO). **Caution:** This reagent is a powerful alkylating agent and as such may be carcinogenic. Avoid contact with skin and inhalation of vapors.

(d) *Internal standard.*—4mM methyl tetradecanoate (Polyscience, Niles, IL) in hexane.

(e) *Sodium fluoroacetate.*—97% (E. Merck, Darmstadt, FRG).

(f) *Solvents.*—Nanograde hexane, dichloromethane, and acetonitrile (Mallinckrodt, St Louis, MO).

Apparatus

(a) *Gas chromatography.*—Packard 438 gas chromatograph with flame ionization detector and Model 510 compact flow capillary system. Fused silica open tubular column (20 m × 0.3 mm id) coated with SE 54. Column temperature programmed from 140 to 230°C at 6°/min; hydrogen carrier gas at 58 kPa; inlet split ratio 10:1; injector and detector 250°C. Signal from detector recorded on digital integrator programmed to calculate peak area of dodecyl fluoroacetate proportional to that of internal standard.

(b) *Gas chromatography/mass spectrometry.*—Hewlett-Packard 5985A quadrupole GC/MS system incorporating capillary GC and valved capillary GC/MS interface. Fused silica 15 m × 0.32 mm id capillary columns, 0.5 μm film BP5 (SGE, Melbourne, Australia). GC column temperature programmed from 140 to 230°C at 6°/min; carrier gas at 9 psi; GC/MS interface at 250°C, ion source.

Caution: Sodium fluoroacetate, fluoroacetic acid, and its ester derivatives are potent poisons. Do not ingest, contaminate skin, or inhale vapors. Perform all evaporation and derivatization steps in fume cupboard.

Calibration Curve for Sodium Fluoroacetate

Equilibrate 4 DEAE-Sephadex A-25 columns with 10 mL 0.05M sodium formate buffer. Then load 10, 30, 50, and 70 μL lmM $CH_2FCOONa$ onto columns, to give 1.0, 3.0, 5.0, and 7.0 μg respectively. Wash columns with 0.5 mL water and elute each with three 0.5 mL aliquots of 0.2M KCl. Pool the 3 eluted fractions, and evaporate to dryness under stream of nitrogen at 80°C. Esterify fluoroacetate in each eluate as given below.

Extraction of Fluoroacetate from Biological Matrixes

Weigh 1.0 g stomach contents, muscle, or liver tissue into glass tissue grinder and grind 3 min with Teflon pestle. Wash pestle and contents of grinder into centrifuge tube with 5 mL water and centrifuge 5 min at 2400 rpm. Load entire supernate directly onto DEAE-Sephadex column; wash tube with 0.5 mL water and add wash to column. Elute fluoroacetate with three 0.5 mL aliquots of 0.2M KCl into single 10 mL culture tube and evaporate to dryness under stream of nitrogen at 80°C. Esterify fluoroacetate as given below.

Esterification of Fluoroacetate

To dried residue, add 0.5 mL esterifying reagent. Crush solid residue with glass rod and heat mixture 60 min at 120°C. When cool, add 3 mL hexane and then 3 mL water, and mix on vortex shaker. Separate hexane phase and transfer to vial containing internal standard solution. Evaporate hexane extract to dryness under nitrogen at room temperature. Dissolve residue in 100 μL hexane, and use 1 μL aliquot for capillary gas chromatographic analysis.

Isolation of Dodecyl Fluoroacetate using Activated Florisil

Weigh 3.0 g activated (600°C, 6 h) Florisil into 1 cm id glass column fitted with sintered glass disc. Tap gently and elute column initially with 10 mL hexane; discard this fraction. Dilute hexane solution obtained from esterification step to 1 mL with hexane and quantitatively transfer onto column. Elute with 25 mL dichloromethane and collect eluate in 50 mL beaker as fraction 1. Elute column again with 10 mL 2.5% acetonitrile in dichloromethane as fraction 2. Finally, elute column with additional 10 mL 2.5% acetonitrile in dichloromethane and collect this fraction as fraction 3. Evaporate each fraction to dryness under filtered, compressed air or nitrogen on 80°C water bath. Resuspend residue in hexane, transfer to vial containing 10 μL internal standard solution, reduce to dryness, and resuspend in 100 μL hexane. Analyze 1 μL aliquot of this solution by capillary gas chromatography.

Gas Chromatography

Calculate amount of fluoroacetate (X) in sample by substituting relative area value for Y in equation derived from regression analysis of calibration data: $Y = 0.1609X + 0.019$. Thus, amount of sodium fluoroacetate = $Y - 0.019/0.1609$ μg.

Selected Ion Monitoring Gas Chromatography/Mass Spectrometry

Use same sample preparation procedure described above except use lower concentration of internal standard, 0.05mM methyl tetradecanoate. Monitor ions m/z 61.1 for dodecyl fluoroacetate and m/z 74.1 for methyl tetradecanoate with cycle time of 50 ms for each mass ion. Divide peak area for dodecyl fluoroacetate by peak area for methyl tetradecanoate. Calculate amount of sodium fluoroacetate in sample by substituting relative area value so obtained for Y in the equation derived from regression of calibration data: $Y = 1.354X + 0.018$. Thus, amount of sodium fluoroacetate = $Y - 0.018/1.354$ μg.

Results and Discussion

At each of the 3 steps in this analytical procedure, i.e., extraction using ion-exchange chromatography, elution of bound fluoroacetate from ion-exchange column, and derivatization, there was potential for loss of analyte. Quantitative extraction of fluoroacetate from biological tissues relied on its solubility in the aqueous solution obtained after sedimentation of the finely ground tissue. A weak anion-exchange resin

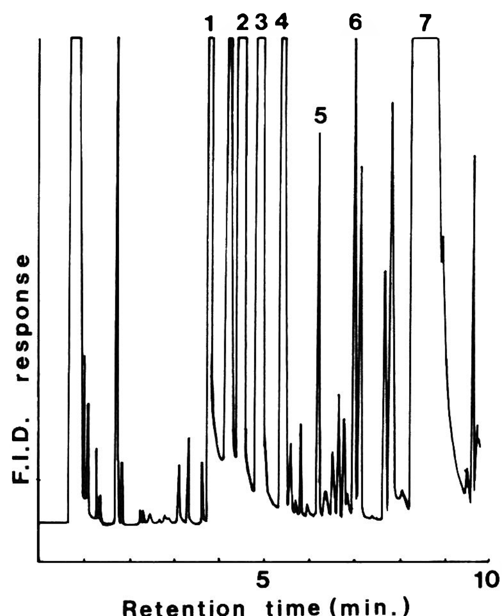

Figure 1. Chromatogram of derivatized extract from ground chicken muscle (1 g), with 5 μg sodium fluoroacetate added. 1 = 1-dodecanol, 2 = dodecene, 3 = 1-bromododecane, 4 = dodecyl acetate, 5 = dodecyl fluoroacetate, 6 = methyl tetradecanoate (internal standard), 7 = dodecyl lactate.

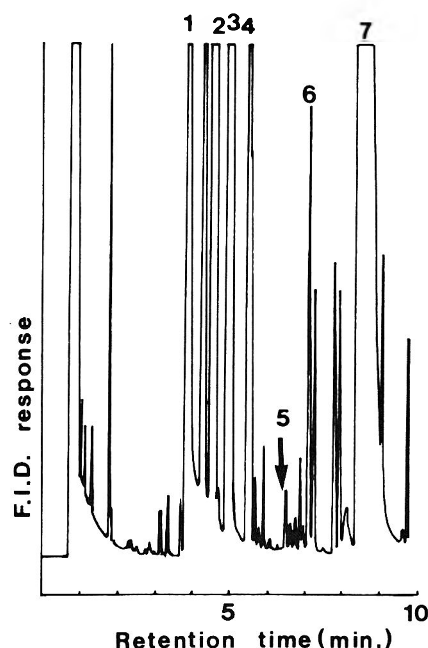

Figure 2. Chromatogram of derivatized extract from ground chicken muscle (1 g): 1 = 1-dodecanol; 2 = dodecene; 3 = 1-bromododecane; 4 = dodecyl acetate; 5 = elution position of dodecyl fluoroacetate among several closely eluting compounds and minor co-eluting components; 6 = methyl tetradecanoate (internal standard); 7 = dodecyl lactate.

was used so that only strong acids and proteins in the aqueous solution were retained on the column. Fluoroacetate was not found in the wash eluate even when the column was loaded with 5 mL extracts, provided the extract pH was 4 or higher. Soluble protein in the extract appeared to collect on the top of the ion-exchange column and was not eluted when the column was developed, except when the extract was unusually concentrated.

The calibration procedure was designed to control sample losses caused by incomplete elution from the ion-exchange column or incomplete derivatization. Standard solutions containing 1.0, 3.0, 5.0, and 7.0 μg 1080 were applied to the ion-exchange column, eluted, and derivatized using procedures identical to those specified above for sample extracts. The mean area ratios (n = 2) were 0.190, 0.501, 0.796, and 1.164, respectively. Linear regression analysis gave slope = 0.1609, y-intercept = 0.019, and correlation coefficient = 0.9988. These data show that response was linear over the range 1–7 μg sodium fluoroacetate. The low value for the y-intercept indicates lack of significant interference in this range. Recovery of fluoroacetate was then determined by adding sodium fluoroacetate at 4 different levels to 1 g samples of ground chicken muscle and liver. Samples were extracted and fluoroacetate was measured as detailed above. Results (Figure 1 and Table 1) show that 80% of the added fluoroacetate was recovered.

Table 1. Recovery of 1080 from spiked biological specimens (chicken liver and muscle)

Sample (1 g)	1080 added, μg	Replicates (n)	Rec. %[a]
Chicken liver	1.0	5	80
Chicken muscle	5.0	4	79
Chicken muscle	7.5	2	76
Chicken muscle	10.0	2	77

[a] Percentage recovery calculated by dividing amount detected by amount added and multiplying result by 100.

This method was then applied to the determination of fluoroacetate in organs and muscle from 5 Zebra finches that had been fed millet containing 10 μg/g sodium fluoroacetate. We found that, despite the variation in dosage, tissue levels were generally about 1 μg/g except for heart tissue which was about 2 μg/g. However, some of the results were unexpectedly high; for example, the value determined for the crop, which contained mostly undigested seed, of bird 11 was 12.6 μg/g, which was higher than the seed concentration. In addition, some chromatograms had closely eluting components, indicating the possibility of interference in samples yielding less than 1 μg equivalent of sodium fluoroacetate (Figure 2). The results of GC/MS analyses of control samples showed that most of the interfering components were hydrocarbons originating from the 1-bromododecane; the selected ion monitoring (SIM) GC/MS assay was developed which effectively eliminated this interference.

The mass spectrum of dodecyl fluoroacetate (Figure 3) contains an abundant characteristic ion at m/z 61. This ion presumably originates through alpha cleavage from the carbonyl group and thus contains the fluorine atom. Although a relatively low mass ion, it was not present in any of the interfering hydrocarbons and so was used for selected ion monitoring. The ion at m/z 79 has the formula $[RCOO + 2H]^+$ and results from the rearrangement of 2 hydrogen atoms, while the ion at m/z 91 is due to cleavage of the C—C bond beta to the ester function to give $[RCOOH_2]^+$ (10). Since all 3 ions (i.e., m/z 61, 79, 91) arise from the acid moiety of the ester; all must be present for confirmation of the identity of dodecyl fluoroacetate.

With this technique, dodecyl fluoroacetate was observed as a small peak on the tail of a large peak of dodecyl acetate, which also had an ion at m/z 61 due to $[RCOO + 2H]^+$ (Figure 4). The dodecyl acetate arose from traces of acetic acid in the dimethylacetamide reagent. Despite the incomplete chromatographic resolution, the peak areas and retention times were sufficiently reproducible to enable quantita-

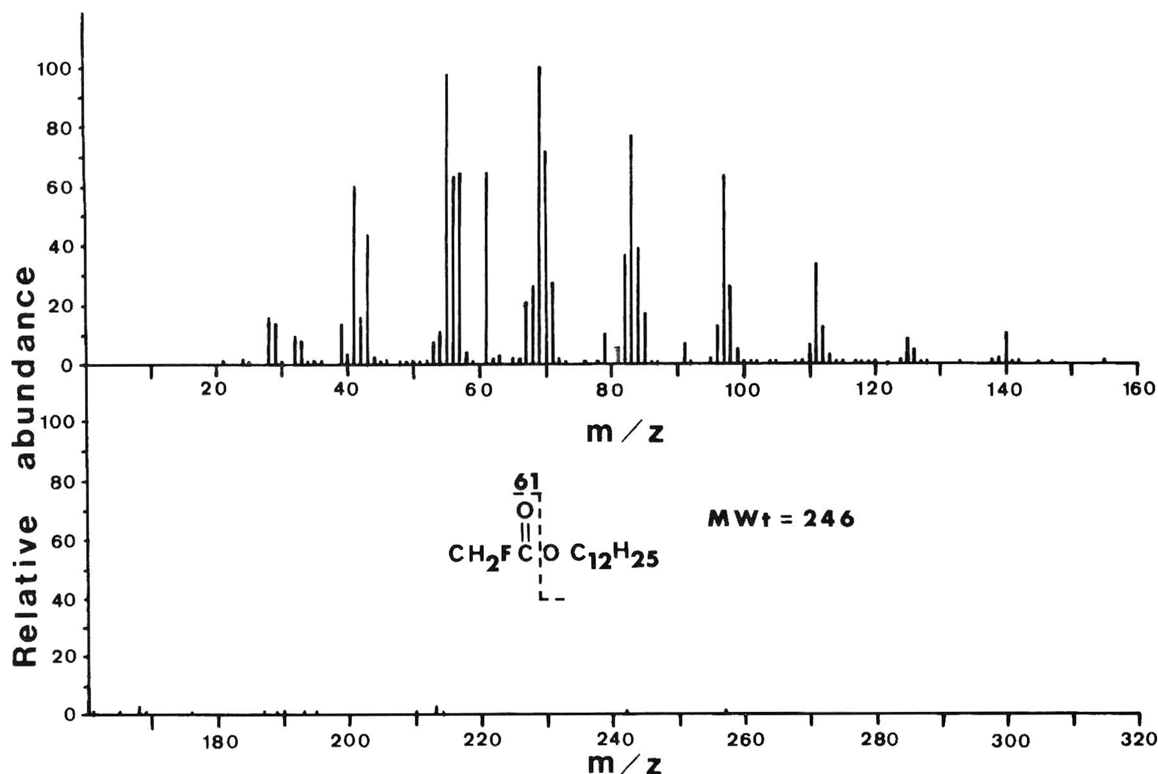

Figure 3. Mass spectrum of peak 5 from chromatogram in Figure 1. Characteristic ions are m/z 61, 79, and 91. Ion series at m/z 41 + 14 n (where n = 0–7) arises from dodecyl chain. No EI molecular ion.

tion of the extremely small amounts of dodecyl fluoroacetate obtained from sample weights of less than 1 g. The enhanced sensitivity of this technique enabled accurate determinations to be made in the range 10–500 ng with a practical limit of detection of about 100 ng per sample. Mean area ratios (n = 2) for 0.01, 0.05, 0.10, and 0.50 μg 1080 were 0.024, 0.092, 0.155, and 0.694, respectively. Linear regression analysis gave slope = 1.354, y-intercept = 0.018, correlation coefficient = 0.9988. These data show that response was linear over the range, but the relatively high value for the y-intercept indicated that reduced precision was obtained at the lower end of the range.

All samples were re-analyzed using the selected ion monitoring assay and, although most results were in agreement with the GC determinations, some samples showed significant differences, especially those that had unusually high values by GC alone. The results of both the GC and SIM GC/MS determinations are given in Table 2.

Many laboratories do not have access to GC/MS equipment; therefore we investigated whether the interfering components could be removed using Florisil column chromatography. The solution normally analyzed by GC, which contained the ester product, the alkylating agent, and byproducts, was applied to the activated Florisil column. The presence of a large amount of dodecyl bromide and its byproducts in the solution enhanced the elution of dodecyl

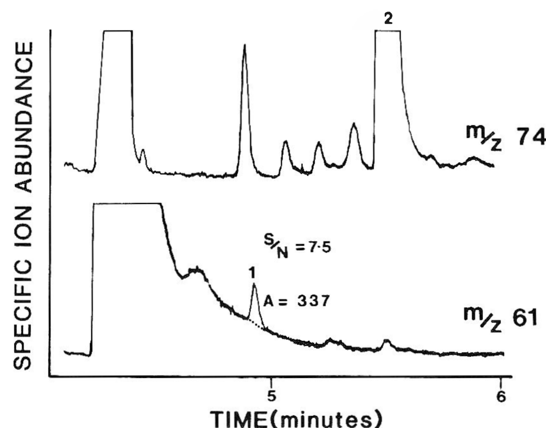

Figure 4. Selected ion chromatogram of derivatized extract from heart (0.13 g) of bird 14. Sodium fluoroacetate concentration of sample was calculated to be 1.91 μg/g, giving total amount of analyte equal to 248 ng. Determination was more specific and gave lower result than capillary gas chromatography with FID. 1 = dodecyl fluoroacetate, 2 = methyl tetradecanoate (internal standard).

Table 2. Fluoroacetate levels in organs and muscle tissue from 5 Zebra finches after their ingestion of grain containing compound 1080

Bird (dose, μg/g)[a]	Method[b]	Concn of sodium fluoroacetate, μg/g[c]					
		Crop	Stomach	Liver	Heart	Intestine	Muscle
11	GC	12.6	1.0	2.0	2.7	N/S	0.6
(3.4)	SIM	3.60	1.64	1.53	2.16	N/S	0.71
12	GC	N/S	0.5	0.8	2.4	2.7	0.6
(7.9)	SIM	N/S	0.95	1.37	2.07	1.37	1.17
13	GC	3.4	1.7	0.7	3.9	0.8	1.0
(4.5)	SIM	3.57	N/A	1.05	0.96	1.16	0.82
14	GC	1.6	0.5	2.0	6.0	3.9	0.9
(3.1)	SIM	1.13	1.15	0.75	1.91	1.64	0.71
15	GC	N/S	2.0	1.5	4.0	1.6	0.5
(1.9)	SIM	N/S	1.61	1.13	1.93	1.10	0.53

[a] Dose calculated by product of amount of seed consumed by concentration of 1080 in seed (9 μg/g) by reciprocal body weight.

[b] GC = Sample analyzed by capillary gas chromatography without prior Florisil column chromatography. SIM = Sample analyzed by selected ion monitoring GC/MS without prior Florisil column chromatography. N/S = Not sampled. N/A = Not analyzed.

[c] Sample weights ranged from 0.06 to 0.40 g for organs and 0.41 to 0.84 g for muscle tissue.

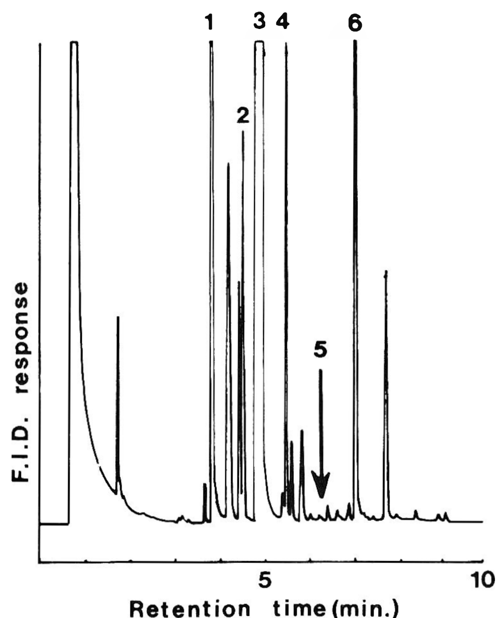

Figure 5. Chromatogram of first fraction (25 mL CH_2Cl_2) eluted from Florisil column loaded with derivatized extract from ground chicken muscle (1 g) containing added sodium fluoroacetate (5 µg): 1 = 1-chlorododecane; 2 = dodecene; 3 = 1-bromododecane; 4 = dodecyl acetate; 5 = retention time of dodecyl fluoroacetate; 6 = methyl tetradecanoate (internal standard). Interfering components were eluted in this fraction.

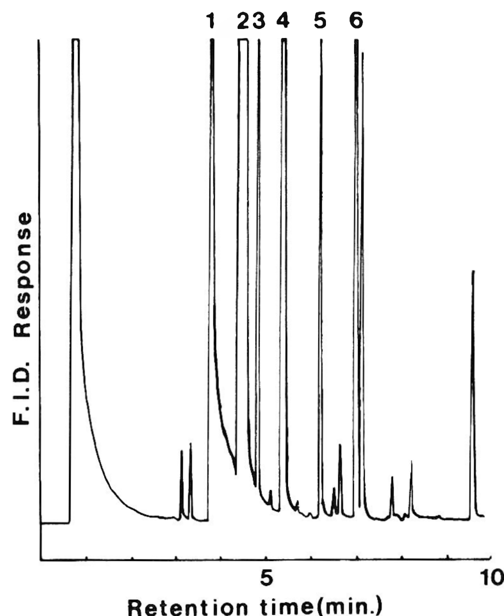

Figure 6. Chromatogram of second fraction (10 mL 2.5% CH_3CN in CH_2Cl_2) eluted from Florisil column after loading derivatized extract from ground chicken muscle (1 g) containing added sodium fluoroacetate (5 µg): 1 = 1-dodecanol; 2 = dodecene; 3 = 1-bromododecane; 4 = dodecyl acetate; 5 = dodecyl fluoroacetate; 6 = methyl tetradecanoate (internal standard).

fluoroacetate. In a 2-step fractionation, the interfering components were first removed by elution with dichloromethane, and then dodecyl fluoroacetate was eluted with 2.5% acetonitrile in dichloromethane. The first fraction (Figure 5) contained the least polar components dodecyl chloride, dodecene, most of the excess dodecyl bromide, dodecyl acetate, and most of the minor interfering components. In addition to dodecyl fluoroacetate, the second fraction (Figure 6) contained mainly dodecanol, dodecene, and dodecyl acetate.

Eight samples previously analyzed by both GC alone and SIM GC/MS were subjected to this fractionation procedure. A well resolved peak in fraction 2 corresponding to dodecyl fluoroacetate was observed in only 3 of these. For these 3 samples, the result was comparable to that obtained by SIM GC/MS. However, the majority of the samples did not give useful results, so it must be concluded that, for small sample weights of animal tissue where the sodium fluoroacetate levels are likely to be 1 µg/g or less, SIM GC/MS must be used for reliable results.

Despite the improvements in sensitivity and accuracy for this method, we have been unable to detect fluoroacetate in a few bovine and ovine samples where 1080 poisoning was strongly implicated. The possibility that the entire dose was converted to fluorocitrate in these large animals and was thus not detected cannot be ruled out and should be investigated further (11).

Acknowledgments

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PESTICIDE FORMULATIONS

Rapid Gas Chromatographic Method Using Nitrogen-Phosphorus Detection for *N*-Nitrosodimethylamine in 2,4-D and MCPA Herbicide Formulations

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A simple and rapid analytical method has been developed for the determination of *N*-nitrosodimethylamine (NDMA) in amine salts of phenoxy herbicide formulations of 2,4-D and MCPA, plus mixtures of these with mecoprop and dicamba amine salts. Sample preparation consists of direct extraction using pre-packed disposable extraction tubes eluted with dichloromethane followed by cleanup on a disposable silica gel mini-column using ethyl acetate as eluting solvent. Samples are injected on-column for gas chromatography with a Megabore fused silica column; the NDMA is measured by a thermionic specific detector (TSD) that is selective for nitrogen-phosphorus (NP). A detection limit of 0.1 μg/mL was easily attainable without any concentration step because the solvent volume is minimal. TSD and thermal energy analyzer (TEA) results have been compared and confirmed by gas chromatography/mass spectrometry. Recovery studies were performed as well as a reproducibility study on one of the 2,4-D formulations.

A simple and rapid analytical method has been developed for the determination of *N*-nitrosodimethylamine (NDMA) in amine salts of phenoxy herbicide formulations of 2,4-D and MCPA, plus mixtures of these with mecoprop and dicamba amine salts. *N*-Nitroso compounds are of some concern since they have been found to be carcinogenic in certain laboratory animals (1, 2) and have been reported in a wide range of substrates including cooking oil (3), beer (4), and drugs (5). Recently, trace levels of *N*-nitrosodiethanolamine have been reported by Wigfield and Lanouette (6) in diethanolamine formulations of 2,4-D. Wigfield and McLenaghan (7, 8) observed NDMA in laboratory-prepared dimethylamine salts of 2,4-D, mecoprop, and dicamba and observed an increase of NDMA in commercial amine formulations after long-term storage at elevated temperature. Hindle et al. (9) also report trace levels of NDMA in amine formulations of 2,4-D.

Nitrosamines have generally been analyzed by gas or liquid chromatography using a thermal energy analyzer (GC-TEA or LC-TEA). Hindle et al. (9) report a limit of detection of 0.006 ppm for NDMA in 2,4-D formulations analyzed by GC-TEA, while other workers have used a variety of methods including thin-layer chromatography, mass spectrometry (MS), and gas chromatography with Hall or Coulson conductivity detection (10). Maybury and Grant (11) analyzed *N*-nitrosodipropylamine (NDPA) in trifluralin by using gas chromatography with a nitrogen-phosphorus selective detector (NPD). The TEA and MS detectors are both expensive systems which may not be readily available for all laboratories. Since NPDs and TSDs are frequently available in pesticide quality control laboratories, the present study was undertaken with a view to finding a simple and practical screening method for NDMA in 2,4-D and MCPA amine formulations.

METHOD

Instrumentation

Varian Model 6000 gas chromatograph equipped with a Varian 8000 automated injector and interfaced with a Vista 402 chromatograph data system as instrument controller and for data reduction was used.

Injectors 1 and 2 were modified to permit on-column injection by installation of Megabore™ adapter kits (J & W Scientific, Inc.) and were fitted with Megabore 15 m × 0.53 mm id columns with 1 μm film coating of DB-225.

Column 1 was connected to a thermionic specific detector (TSD). Column 2 was interfaced with a Model 543 TEA (Thermo Electron Corp., Waltham, MA).

Injector 3 was a temperature-programmable cold on-column capillary system connected to a 30 m × 0.32 mm id capillary column with a 1 μm coating of DB 225 and interfaced with a flame ionization detector (FID).

Operating conditions: injectors 1 and 2 (common heater block) 190°C; ionization detectors (common block) 200°C. Column oven program: initial 70°C, hold 5 min, program at 8°/min to 110°C, hold 2 min, post-program 150°C, hold 5 min. Gas flow rates: helium carrier gas 2–3 mL/min with helium make-up gas for TSD at 30 mL/min; hydrogen 4.5 mL/min TSD, 25 mL/min FID; air 175 mL/min TSD, 300 mL/min FID.

TEA conditions: heated interface to gas chromatograph 200°C; pyrolysis tube 500°C; oxygen flow, optimized as per instrument manual with CTR gas stream filter (Thermedics catalog No. 10220) in-line prior to TEA reaction chamber.

MS conditions: Varian 3700 gas chromatograph with Varian Model 1095 on-column capillary injector connected by direct capillary coupling to VG ZAB-2F mass spectrometer; 30 m × 0.32 mm id capillary column with 1 μm coating of DB 225; resolution 5000; trap current 200 μA; source 210°C.

Reagents

(a) *N-Nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) standard solutions.*—(*1*) *Stock solution.*—1.0 mg/mL. Dissolve 100 mg neat analytical standard (Thermedics, Inc., Woburn, MA) in absolute ethyl alcohol (Consolidated Alcohols Ltd, Toronto, Ontario) and dilute to 100 mL with absolute alcohol, using low actinic volumetric flask. Mix well.

(*2*) *Working solution.*—0.01 mg/mL. Make serial dilutions of stock solution, first 10 mL to 100 mL with absolute alcohol, and then 1 mL to 10 mL with purified water (Milli-Q System, Millipore Corp., Bedford, MA).

(*3*) *Calibration solution.*—50 ppb. Dilute 25 μL working solution to 5 mL with ethyl acetate. Use this solution to calibrate gas chromatograph.

(*4*) *Internal standard (NDEA) solution.*—Prepare in absolute alcohol and dilute to 1 μg/mL.

(b) *Solvents.*—All glass-distilled (Caledon Laboratories Ltd, Georgetown, Ontario). Store ethyl acetate in glass-stoppered container over bed of molecular sieve.

(c) *Anhydrous sodium sulfate.*—(Anhydrous-granular Baker). Prepare by drying in 120°C oven. Store in desiccator cabinet containing indicating Drierite.

(d) *Silica gel.*—(Keiselgel 60, E. Merck, Darmstadt,

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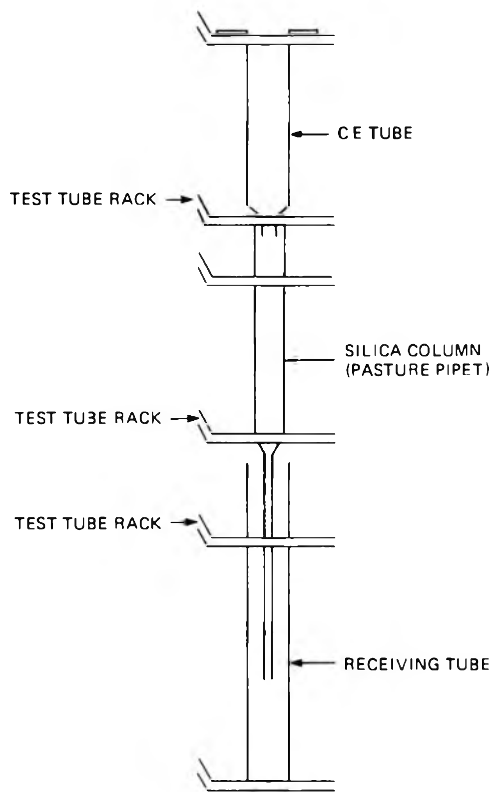

Figure 1. Schematic showing CE tube and silica column packed in Pasteur pipet with receiver, all supported by 3 test tube racks.

Table 1. Comparison of analysis for NDMA in herbicide formulations by NP and TEA detection systems (ppm NDMA found)

Sample type	Test method TSD	Test method TEA	TEA minus TSD	Ref. 9 method TEA	Ref. 9 method minus TSD
A	0.24	0.20	−0.04	0.25	0.01
A	0.16	0.18	0.02	0.19	0.04
A	n/d[a]	0.03	0.03	n/a[b]	
A	0.41	0.42	0.01	n/a	
A	n/d	0.05	0.05	n/d	
A	0.11	0.11	0.00	n/a	
A	0.11	0.10	−0.01	n/a	
A	0.76	0.85	0.10	n/a	
A	0.81	0.63	−0.18	n/a	
A	0.06	0.08	0.02	n/d	
A	0.08	0.14	0.06	n/d	
A	n/d	0.10	0.10	n/a	
A	0.12	0.12	0.00	0.08	−0.04
B	n/d	n/d	0.00	n/d	
B	0.08	0.06	−0.02	n/a	
C	n/d	0.04	0.04	n/d	
D	n/d	n/d	0.00	n/a	
E	2.01	2.15	0.14	1.99	−0.02
F	0.49	0.45	−0.04	0.40	−0.09
G[c]	28.4[d]	29.4[d]	1.0	n/a	
Mean	1.69	1.75	0.064		−0.020
SD	6.3	6.5	0.229		0.049

Concentration of 2,4-D amine salt active ingredient as stated below: A = 470–500 g/L; B = 250 g/L; C = 200 g/L; D = 140 g/L; E = 125 g/L + mecoprop; F = 95 g/L + mecoprop and dicamba; G = 720 g/L.

[a] Not detected above 0.05 ppm by TSD, 0.03 ppm by TEA.

[b] Not analyzed by reference method (9).

[c] NDMA confirmed by mass spectrometry; quantitation by GC/MS = 32 ppm.

[d] Mean of 5 replicate analyses: CV for TSD = 3.10; CV for TEA = 3.69.

Table 2. Recovery of NDMA from samples by standard addition

Sample type	Net NDMA found, ppm	Rec., %
A	0.47	94
A	0.47	94
A	0.50	100
A	1.46[a]	146
A	0.52	104
A	0.48	96
A	0.44	88
A	1.12[a]	112
A	0.99[a]	99
A	0.97[a]	97
A	0.50	100
A	0.55	110
A	0.46	92
B	0.52	104
B	0.51	102
C	0.55	110
D	0.46	92
E	0.97[a]	97
F	0.43	86
Mean rec., %		101.2
SD		13.00

Concentration of active ingredient of 2,4-D amine salt as stated below: A = 470–500 g/L; B = 250 g/L; C = 200 g/L; D = 140 g/L; E = 125 g/L + mecoprop; F = 95 g/L + mecoprop and dicamba.

[a] Spiked by addition of 1 μg/mL of sample. All remaining samples spiked by addition of 0.5 μg/mL.

FRG). Prepare as described for sodium sulfate. May be stored in plastic container on bench, under tight-sealing screw-cap lid. Verify elution time by running spikes.

(e) *Chem Elut (CE) disposable extraction tubes.*—Use as received from manufacturer (Analytichem International, Harbor City, CA, 1 mL CE1001, 3 mL CE1003).

(f) *Molecular sieve.*—10-16 mesh beads (grade 518, 40 nm, Davidson Chemical, Baltimore, MD). Re-activate by heating to 250°C. Cool in desiccator cabinet before placing in 2 L glass storage bottle.

(g) *Miscellaneous apparatus.*—Disposable glass test tubes, 16 × 125 mm (Kimble); Pasteur pipets, 9 in. long; 1 mL pipets; Vortex mixer; test tube racks, 12 × 12 mm mesh (Canlab Laboratory Supplies); wooden clothes pins or suitable clamp to retain packed columns.

Extraction and Cleanup

Add 1 mL formulation by pipet to CE1001 disposable extraction tube. Let tube stand until sample is absorbed and tube appears dry (20–30 min). Prepare silica gel column using 9-in. disposable Pasteur pipet. Pack column by tapping gently as materials are added. Insert small piece of glass wool, using second pipet to push glass wool to taper. Insert piece of fused silica column, 0.53 mm id, or other suitable object from bottom of pipet to loosen glass wool plug from below. This ensures free flow when column elutes. Then add 3 mm granular Na_2SO_4, 55–60 mm silica gel Keiselgel 60, and 3 mm granular Na_2SO_4.

Stack 3 test tube racks in fume hood (Figure 1). Insert CE tubes into upper rack so that tips protrude through bottom of rack into mouths of packed columns placed in middle rack. Retain columns in position by using clamps or clothes pins. Place bottom tip of columns into the test tubes held in bottom rack.

Elute CE tubes with 8 mL dichloromethane, adding one 4

Table 3. Recovery study conducted over 4 days with 5 levels of NDMA spikes

Detector:	TSD				TEA			
Injected:	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Control:[a]	0.13	0.12	0.13	0.12	0.14	0.13	0.14	0.12
Spike, μg[b]	Recovery, %				Recovery, %			
0.1	104	90	90	100	103	79	128	110
0.3	97	n/a[c]	93	100	101	n/a	110	100
0.5	n/a	95	90	96	n/a	95	102	92
0.7	99	83	96	n/a	n/a	103	94	91
1.0	102	94	96	98	101	97	104	94

Overall TSD recovery (n = 17) = 96%
Recovery at 0.1 μg (n = 4) = 96% CV = 7.4%
[a] Formulation containing 500 g/L of 2,4-D amine salt. An additional 20 replicate analyses of this formulation gave a mean of 0.14 ppm and a CV of 10.18%.
[b] Control sample with indicated amount of NDMA added.
[c] Recovery not performed on this day at this level.

mL portion and then 4 or more small volumes, taking care to avoid overflowing silica column. Remove top rack after CE tubes are drained, and elute silica gel columns with 5 mL hexane and 1 mL ethyl acetate; discard all eluates. Next, elute with 2 mL ethyl acetate, collect eluate, and dilute to known volume (2-3 mL) with ethyl acetate or add 1 mL (1 μg) internal standard to extract. Mix well, and transfer extract to auto-sampler (1.5 mL) vial. Cap with Teflon-faced septa and screw-cap tops. Store in refrigerator for later injection into gas chromatograph. No further concentration is required.

Determination of NDMA

Inject 1 μL aliquots of calibration solution into GC/TSD until successive injections yield variation of <5% in response factors as determined by integrator. Inject 1 μL aliquots of samples, and calculate concentration of NDMA by internal or external standard. When instability of the TSD occurs, whereby abnormal detector drift during analytical run causes change in response >5% from beginning to end of run, perform manual computation of response factor by injecting calibration solution until <5% variation is obtained between 2 successive injections. Inject set of 3-4 samples, followed by 2 additional calibration solutions. Use mean of 4 response factors bracketing set of samples to calculate concentration of NDMA by external standard.

Table 4. Results of analyses of MCPA formulations (results in ppm, based on 1 mL formulation)

Sample type	Test method TSD	Test method TEA	TEA minus TSD
A	0.06	n/d[a]	−0.06
A	0.42	0.42	0.00
A	0.44	0.42	−0.02
A	2.40	2.32	−0.08
A	0.14	0.13	−0.01
A	1.17	1.37	−0.20
A	0.49	0.46	−0.03
A	0.38	0.38	0.00
A	2.53	2.83	0.30
A	0.41	0.43	0.02
B	0.52	0.60	0.08
Mean, ppm			0.036
SD			0.115

A = 500 g/L MCPA amine; B = 275 g/L MCPA + mecoprop and dicamba amine.
[a] Not detected above 0.03 ppm by TEA.

Results and Discussion

The method was applied to 20 different 2,4-D products registered in Canada under the Pesticide Control Products Act. It was suitable for all formulations analyzed. The data in Tables 1, 2, and 3 show that good agreement was obtained between the nitrogen-selective TSD and the nitrosamine-specific TEA used for confirmation. Nineteen samples were spiked by addition of NDMA at 2 levels: 0.5 μg for samples with lower amounts of NDMA and 1.0 μg for those samples

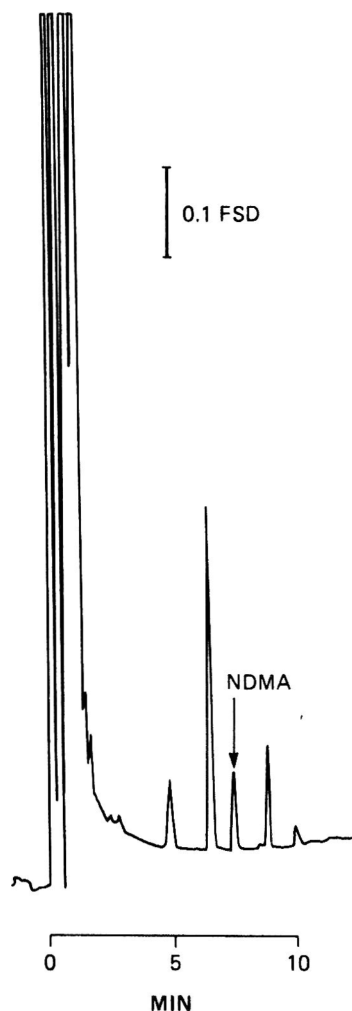

Figure 2. Typical NP chromatogram of 2,4-D extract: 0.11 ppm NDMA by NP, 0.10 ppm NDMA by TEA.

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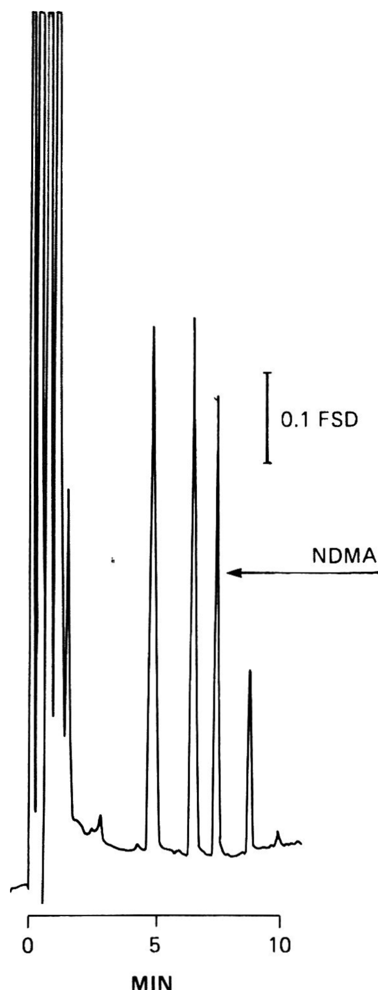

Figure 3. Typical NP chromatogram of MCPA extract: 0.44 ppm NDMA by NP, 0.42 ppm by TEA.

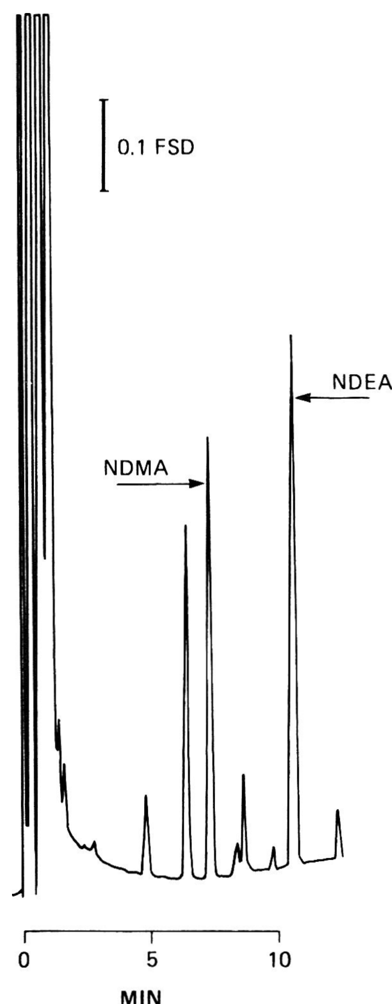

Figure 4. 2,4-D sample of Figure 2 spiked with 0.5 ppm NDMA plus 1 μg NDEA internal standard.

with higher levels of NDMA. Results are listed in Table 2, with the net NDMA listed. This is the amount found after spiking, less the amount found in the original analysis. Some of the samples were also analyzed by a similar method (9) with good agreement (Table 1, column 4). Results in Table 1 were compared by subtracting the TSD result from the TEA results obtained using the test method and the method of Hindle (9). Application of Student's *t*-test to these differences showed no difference at the 95% confidence level, even when the final result (sample type G), which is much greater than the others, is included. Similarly, the closeness of the average results for these unrelated samples shows that the 2 methods yield closely comparable results.

Table 3 lists results obtained when the control sample was spiked by adding 0.1, 0.3, 0.5, 0.7, and 1.0 μg NDMA to each of five 5 mL aliquots, respectively. On 4 separate days, 1 mL aliquots of the spiked samples were extracted. The extracts were injected on both the TSD and TEA systems and, in this case, the results were calculated by an internal standard method after the addition of 1 μg NDEA.

One formulation, containing the highest concentration of both 2,4-D and NDMA (final result in Table 1), could not be eluted through the silica gel column. This formulation was viscous and oily and the eluate from the CE1001 tube appeared to be a mixture of partly immisible liquids. To analyze this formulation, a 1 mL aliquot of sample was diluted with 2 mL purified water. The sample was mixed thoroughly in a Vortex mixer, and a 3 mL CE1003 tube was used in place of the 1 mL CE1001 tube. The sample was transferred to the CE1003 using a Pasteur pipet. A larger volume of dichloromethane was required to elute the sample. A total of 12 mL dichloromethane was used. The sample was then processed in the same manner as all other samples. Five replicate analyses were performed on this sample. The results are listed as the final sample (G) in Table 1. There is good agreement in the quantitation among all 3 modes of detection—GC-TSD, GC-TEA, and GC-MS—as well as confirmation of NDMA by mass spectrometry.

Table 4 shows results obtained when the method was employed to analyze 11 commercial formulations of MCPA. No changes were required to the method in this instance. Student's *t*-test shows no difference between the TSD and TEA results at the 95% confidence level. The chromatograms were similar to those of 2,4-D and contained, in varying amounts, the same extraneous peaks.

Most of the samples analyzed showed the pattern typically seen in Figures 2 and 3, so some extracts were analyzed by FID using the narrow-bore DB-225 column to confirm relative retention times of the unidentified peaks, which were run by GC-MS. These peaks were shown to be low molecular weight compounds containing 5 or 6 carbons, nitrogen, and oxygen. Divising a further cleanup step to remove these few remaining impurities did not seem warranted.

The quantitation of NDMA for the results in Table 2 was

based on an internal standard calculation by adding a known amount (1 μg) of NDEA to the final extract. The results for the control sample show good agreement between the TSD and TEA. This demonstrates that an internal standard of NDEA could be used successfully with some formulations; however, it is recommended that for untested formulations, preliminary extracts be analyzed to ensure no interferences in the zone of the chromatogram where NDEA elutes. (See Figure 4 for relative retention time of NDEA.) Linearity of the detector was verified from 0.1 to 2.0 μg/mL NDMA in this study; however, this should be confirmed as part of each laboratory's quality assurance.

A large number of samples can be extracted on a daily basis. The racks used can hold 48 extraction tubes, although using more than 24 spaces at one time could be difficult. Total time for preparation of a set of samples is approximately 1.5 h, but will vary, mainly in the time required to elute the CE tubes. Slow-eluting samples can be forced through the tubes by placing a clean 1 mL rubber pipet bulb over the top of the tube and gently forcing with small amounts of air. Care must be taken to avoid overflowing the silica column. The silica columns elute faster if they are not allowed to go dry, but results were not affected if this occurred. It is more productive to leave the rack (Figure 1) holding the CE tubes until all tubes are eluted and then simply lift off this rack as a unit. This permits the elution of all silica columns simultaneously. Slow-eluting silica columns were forced as above on occasion.

This method of sample preparation could be used for either TSD or TEA analysis of the extracts. The 5 mL hexane wash is not required if TEA is used. This wash is incorporated only to flush the chlorinated solvent from the silica column prior to elution of NDMA when the TSD detector is used, since injection of chlorinated solvents is known to affect the response of the TSD. Traditional methods use liquid-liquid partitioning of samples which often leads to emulsions and requires extra steps to separate and dry the organic layer containing the nitrosamine. The described method provides a more rapid method of extraction.

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Detection and Separation of Fenpropathrin, Flucythrinate, Fluvalinate, and PP 321 by Thin-Layer Chromatography

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Four synthetic pyrethroids having α-cyano ester groups, i.e., fenpropathrin, flucythrinate, fluvalinate, and PP 321, are separated by thin-layer chromatography and detected by a new set of chromogenic reagents. Synthetic pyrethroids containing the α-cyano group react with sodium hydroxide to liberate cyanide which forms pink spots with o-dinitrobenzene and p-nitrobenzaldehyde. The detection limit is 0.1 μg and the method can be applied for identification and confirmation of these synthetic pyrethroids in vegetables.

Fenpropathrin [I, (*R,S*)-α-cyano-3-phenoxybenzyl 2,2,3,3-tetramethylcyclopropanecarboxylate], fluvalinate [II, (*R,S*)-α-cyano-3-phenoxybenzyl (*R*)-2-(2-chloro-4-(trifluoromethyl)anilino) 3-methyl-butanoate], flucythrinate [III, (±)-cyano-(3-phenoxyphenyl)methyl (+)-4-(difluoromethoxy)-α-(1-methylethyl)benzeneacetate], and PP 321 [IV, α-cyano-3-phenoxybenzyl-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropane carboxylate] are comparatively recent synthetic pyrethroids having insecticidal activity against a wide range of pests. Flucythrinate especially has been found effective against those insects which have developed resistance to other classes of insecticides. Fluvalinate and fenpropathrin have the added advantage of also being effective acaricides. All these compounds have a cyanide group attached at the α-position to the carboxylate group.

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At the present, no thin-layer chromatographic method is available for detecting these compounds. TLC detection has been reported for some other synthetic pyrethroids (1–7). These methods use either UV light or chromogenic reagents such as phosphomolybdic acid and palladium chloride. Another sensitive method has been reported for halogenated synthetic pyrethroids (8), using silver nitrate as chromogenic reagent. Since this latter method is based on the presence of halogen atoms, it is obviously not applicable to the α-cyano ester compounds. We report here the use of *p*-nitrobenzaldehyde and *o*-dinitrobenzene, a sensitive and hitherto unreported set of chromogenic reagents, for the detection of synthetic pyrethroids having the α-cyano group. Comparative resolution in different solvent systems is reported for 4 such compounds: fenpropathrin, fluvalinate, flucythrinate, and PP 321.

METHOD

Apparatus and Reagents

(a) *TLC equipment.*—Variable thickness chromatograph spreader No. 20011 (Warner Chilott Laboratories, Instruments Division).

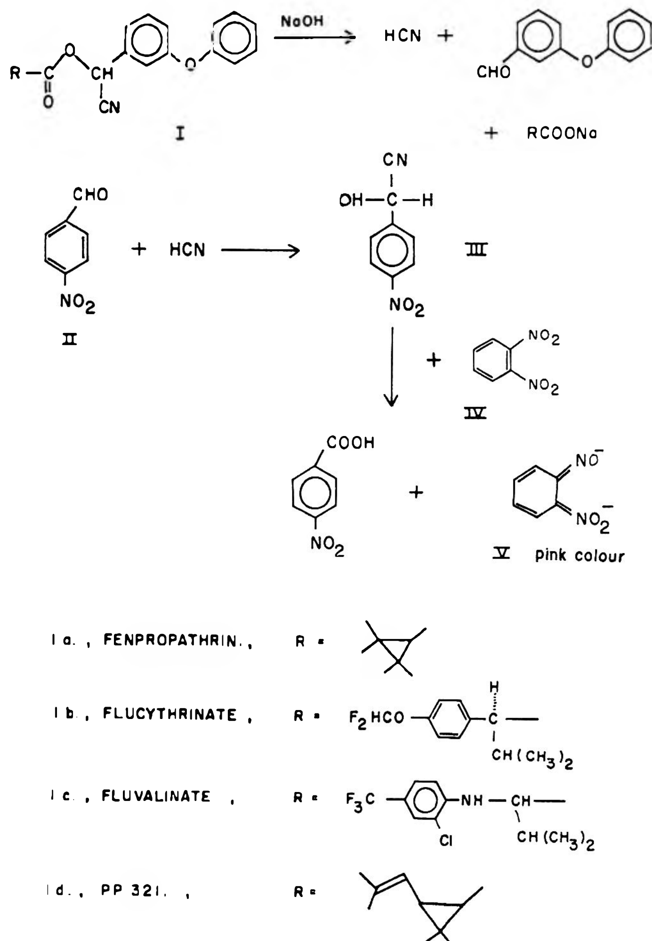

Figure 1. Color reaction of α-cyano ester pyrethroids with *p*-nitrobenzaldehyde and *o*-dinitrobenzene.

(b) *Silica gel.*—Silica gel G, 60 mesh, with 2% $CaSO_4$ as binder.

(c) *Solvents.*—Acetone, benzene, chloroform, ethyl acetate, methanol (all analytical grade).

(d) *Sodium hydroxide solution.*—Prepare 0.5N NaOH solution by dissolving 1 g NaOH in 5 mL water and diluting with methanol to 50 mL.

(e) *p-Nitrobenzaldehyde solution.*—Dissolve 0.30 g *p*-nitrobenzaldehyde in 10 mL methyl cellosolve.

(f) *o-Dinitrobenzene solution.*—Dissolve 0.25 g *o*-dinitrobenzene in 10 mL methyl cellosolve.

(g) *Pesticide reference standards.*—Fenpropathrin was supplied by M/s Rallis India Ltd, Bangalore. Fluvalinate was supplied by M/s Sandoz India, Bombay. Flucythrinate was supplied by Cyanamid India, Bombay. PP 321 was supplied by Imperial Chemical Industries, Calcutta.

Preparation of Plates

Mix 40 g silica gel with 80 mL water shaking vigorously to make slurry for 5 plates. Spread as 1 mm layer over 200 × 200 mm plates with help of applicator. Let plates air-dry 1 h and then activate for another 1 h in 100°C oven. Remove plates and store in desiccator over silica gel or calcium chloride until used.

Extraction of Samples

Transfer 25 g chopped sample to Waring blender. Add 100 mL acetone and churn for 2 min. Decant solvent with suction through Whatman filter paper No. 1 in Buchner funnel. Add additional 100 mL acetone to blender contents and re-extract for another 2 min. Filter mixture through Buchner funnel. Concentrate acetone layer; then dilute with 100 mL water. Extract with *n*-hexane (3 × 50 mL). Dry hexane extracts over sodium sulfate. Concentrate extract to small volume, using Kuderna-Danish evaporator.

Table 1. Mean R_f values of synthetic pyrethroids in different solvent systems

	R_f values			
Solvents	Fenpropathrin	Fluvalinate	Flucythrinate	PP 321
n-Hexane	0.22	0.19	0.16	0.13
Benzene	0.50	0.66	0.45	0.39
n-Hexane–benzene:				
80 + 20	0.30	0.2	0.19	0.15
50 + 50	0.34	0.35	0.28	0.19
40 + 60	0.38	0.48	0.37	0.25
20 + 80	0.44	0.61	0.42	0.32
n-Hexane–acetone:				
90 + 10	0.49	0.48	0.46	0.39
80 + 20	0.53	0.61	0.68	0.62
70 + 30	0.74	0.79	0.92	0.85
n-Hexane–chloroform:				
90 + 10	0.92	0.82	0.69	0.74
n-Hexane–ethyl acetate:				
95 + 5	0.85	0.79	0.60	0.71
90 + 10	0.92	0.85	0.66	0.83
85 + 15	0.85	0.94	0.79	0.87

Determination

Pre-saturate chromatographic tank with solvent for 1 h before use. Spot standard solution and extract 2 cm apart and 3 cm above base line. Place TLC plate in chromatographic chamber after air drying. Let solvent front develop to 15 cm. Remove plate and air dry. Spray entire plate with sodium hydroxide solution with help of sprayer. Let set for 3 min. Then spray entire plate with *p*-nitrobenzaldehyde solution, followed immediately by spraying with *o*-dinitrobenzene solution. Locate colored spots. Run each analysis in triplicate and record mean R_f value.

Results and Discussion

Alkaline hydrolysis of cyano ester pyrethroids has been shown to proceed by nucleophillic attack by a hydroxyl group, resulting in the formation of cyanohydrin derivative; this degrades to give HCN and corresponding benzaldehyde derivatives (9). The present 4 compounds would react in such a manner. The synthetic pyrethroids fluvalinate, fenpropathrin, flucythrinate, and PP 321 (I in Figure 1), on reaction with methanolic NaOH, would liberate HCN to react with *p*-nitrobenzaldehyde (II) to give *p*-nitrophenylcyanohydrin (III). *p*-Nitrophenylcyanohydrin reacts with *o*-dinitrobenzene (IV) to produce the colored compound, the dianion of *o*-nitrophenylhydroxylamine (V) and nitrobenzoic acid (10). A schematic representation of the reactions involved has been depicted in Figure 1.

All 4 insecticides appeared as pink spots on a white background. The intensity of color increased with increasing concentration. Because the spots were generally elliptical, the compounds did not overlap, even in systems producing close

R_f values. Mean R_f values of these compounds in single solvents of varying polarity and different solvent combinations are reported in Table 1.

By this technique, a minimum of 0.1 μg each of the 4 synthetic pyrethroid standards can be detected positively when spotted directly on the TLC plate. We detected residues of all 4 pesticides in fortified samples of cabbage and cauliflower without elaborate cleanup. The recoveries ranged from 85 to 90%, based on the minimum detection limit.

It should be emphasized that about 3 min should lapse after the methanolic sodium hydroxide is sprayed and before the other 2 reagents are sprayed, for optimum results. The latter 2 reagents, *p*-nitrobenzaldehyde and *o*-dinitrobenzene, can be sprayed in any order. They may even be pre-mixed and sprayed with no effect on the color of the spots.

The present method is simple and quick for qualitative detection of these pesticides in vegetables.

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TECHNICAL COMMUNICATION

Diazomethane Derivatization of Sulfamethazine: Formation of Isomeric Products

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Reaction of 4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl)benzenesulfonamide (sulfamethazine) with diazomethane yields not only 4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl)-*N*-methylbenzenesulfonamide but also 2-(4-aminobenzenesulfonimido)-1,4,6-trimethyl-1,2-dihydropyrimidine. Yields of the latter compound are highly variable and the compound does not show a response to gas chromatography. Thus, results of gas chromatographic determinations of residues of some sulfa drugs in edible meat tissues may be erroneous when diazomethane derivatization is used.

The widespread use of sulfonamide drugs in animal production has led tc concern about drug related residues in tissues used for human consumption. In response to this concern, analysts have developed methods to identify and quantify sulfonamide related residues in animal tissues (1-8). Most of the recently developed methods involve methylation with diazomethane to produce the N^1-methyl derivatives, which are more amenable to gas chromatography (GC) than are the parent compounds. We obtained approximately equal amounts of 2 isomeric methyl derivatives (*I* and *II*) from large scale (i.e., 5 g) reactions of sulfamethazine and diazomethane (Figure 1). Comparable products (N^1-methyl and ring-methyl) were reported when sulfathiazole (4-amino-*N*-2-thiazolylbenzenesulfonamide) was derivatized with diazomethane (9, 10). We also discovered that the ring-methyl derivatives of some sulfonamide drugs did not elute from the gas chromatographic (GC) columns used to assay the N^1-methyl derivatives. These findings suggested that use of diazomethane in these analyses should be reevaluated.

Experimental

Sulfamethazine (or ^{14}C labeled sulfamethazine) was reacted with diazomethane under a variety of conditions. Reactions were carried out in ether, methanol, acetone, and tetrahydrofuran as pure solvents and in combinations. Diazomethane solutions were added to solutions of sulfamethazine, and vice versa, in ratios of diazomethane to sulfamethazine that varied from 1:1 to approximately 100:1. The amount of sulfamethazine reacted varied from 1 μg to 5 g. Reaction temperatures were 60, 20, and −20°C. Reactions were run in new glassware, etched glassware, polypropylene vials, and Teflon vials. Porous glass adsorbent (120-140 mesh) was added in some reactions. Sulfamethazine was adsorbed on Chromosorb 102 or Porapak Q and reacted with diazomethane vapors by the method of Schwartz (11). Reaction mixtures were separated by liquid chromatography (LC) on C-18 columns using a 20-50% linear gradient of acetonitrile-water. A radioactivity flow monitor and/or UV detector operated at 254 nm were used to monitor the effluent. Nuclear magnetic resonance spectra were obtained in acetonitrile-d_3 with a JEOL FX 90Q spectrometer. Mass spectra were obtained with a Varian-MAT Model CH-5 DF mass spectrometer interfaced with an SS-200 data system and equipped with an AMD Intectra combination source and post accelerator. An Ion Tech saddle-field gun operated at 7 kV with xenon gas provided the beam of fast atoms to bombard glycerol solutions of the compounds. Gas chromatographic analyses were done on either methyl silicone or 5% phenyl methyl silicone capillary columns (Hewlett-Packard Ultra Performance columns, 12 m × 0.2 mm, 0.33 μm film thickness, 80-300°C at 10°/min; on-column injector; flame ionization detector).

Results and Discussion

4-Amino-*N*-(4,6-dimethyl-2-pyrimidinyl)-*N*-methylbenzenesulfonamide (*I*) and 2-(4-aminobenzenesulfonimido)-1,4,6-trimethyl-1,2-dihydropyrimidine (*II*) were prepared by reaction of sulfamethazine and diazomethane and were isolated by chromatography on a silica gel column. Ethyl acetate eluted *I*, and methanol eluted *II*. Recrystallization of *I* from ethyl acetate yielded a product that melted at 214-216°C. Mass spectrometric (MS) data were as follows: EIMS, m/z 228 (M − SO_2, 100), 227 (M − HSO_2, 87); FABMS in glycerol, m/z 293 (M + H, 100); NMR, δ 2.29 (s, 6H, pyrim. CH_3), 3.52 (s, 3H, N-CH_3), 6.64 (d J = 8.75 Hz 2H, 3,5 aryl H), 6.71 (s, 1H, pyrim. H), 7.74 (d, J = 8.75 Hz, 2H, 2,6 aryl H). Irradiation at δ 3.52 showed no nuclear Overhauser effect (nOe) at 2.29. Recrystallization of *II* from acetone yielded a product that did not melt but decomposed at 223°C. MS data were as follows: EIMS, m/z 292 (M^+, 35), 228 (M − SO_2, 63), 227 (M − HSO_2, 65), 106 (100); FABMS in glycerol, m/z 293 (M + H, 100); NMR δ 2.27 (s, 3H, 6-pyrim. CH_3), 2.35 (s, 3H, 4-pyrim. CH_3), 3.56 (s, 3H, 1-pyrim. CH_3), 6.51 (s, 1H, pyrim. H), 6.63 (d, J = 8.75 Hz, 2H, 3,5 aryl H), 7.67 (d, J = 8.75 Hz, 2H, 2,6 aryl H). Irradiation at δ 3.56 showed nuclear Overhauser effects of 15 and 9% for absorbances at δ 2.27 and 2.35, respectively.

When reaction of 5 g sulfamethazine with an excess of diazomethane yielded a 1:1 mixture of *I* and *II*, we concluded that both products may be formed under other conditions. We reexamined LC chromatograms obtained on extracts of swine tissues that contained from 0.1 ppb to 4.3 ppm sulfamethazine. The extracts of these tissues, which had been methylated with diazomethane (12, 13), showed the presence of *II* in some samples, but interfering peaks and large variations in *I*:*II* ratios obscured this isomer in many samples. Thus, we investigated the effect of different solvents and temperatures, the sequence in which solutions were mixed, the ratio of reactants, and the size of reactions and concentrations to determine which variables affected the relative yields of *I* and *II*. Variability in yields of these isomers was unusually great; however, the size of the reaction was a parameter that correlated with yields of *I* and *II*. The smallest amounts of sulfamethazine gave the lowest yields of *II*, which suggested the involvement of surface phenomena. Small amounts of porous glass adsorbent (120-140 mesh) added to increase the surface area for a given size reaction suppressed the formation of *II*. Large amounts of porous glass caused such a rapid decomposition of the diazomethane that much of the sulfamethazine was recovered; however, despite the recovery of unreacted sulfamethazine, di- and tri-methyl compounds

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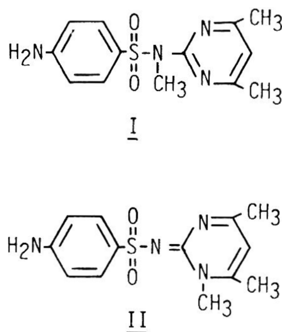

Figure 1. *N*[1]-Methyl and ring-methyl isomeric diazomethane derivatives of sulfamethazine. *I:* 4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl)-*N*-methylbenzenesulfonamide; *II:* 2-(4-aminobenzenesulfonimido)-1,4,6-trimethyl-1,2-dihydropyrimidine.

were also isolated. These polymethylated compounds were purified by liquid chromatography and were partially characterized by mass spectrometry, but a limited sample prevented rigorous identification. We found no conditions that yielded only *I*. The lowest yield of *II* was 8%, but some derivatizations yielded 30% of *II* when 200 μg sulfamethazine was reacted. The reaction of diazomethane vapors with sulfamethazine adsorbed on Chromosorb 102 or Porapak Q by the method of Schwartz (11) gave *I*:*II* ratios of 3 and 1.7, respectively.

Methylation products comparable to *I* and *II* resulted when 4-amino-*N*-(3,4-dimethyl-5-isoxazolyl)benzenesulfonamide (sulfisoxazole), *N*-(4,6-dimethyl-2-pyrimidinyl)benzenesulfonamide (desaminosulfamethazine), and 4-dimethylaminophenyl[4-(*N*-4,6-dimethyl-2-pyrimidinyl)sulfamidophenyl]-diazene were reacted with diazomethane. Other investigators (9, 10) also reported formation of the 2 products when sulfathiazole was reacted with diazomethane, suggesting that this is a general phenomenon with sulfonamide drugs that can tautomerize.

Gas chromatographic analysis of *I* on either methyl silicone or 5% phenyl methyl silicone capillary columns gave single peaks at 245° on both columns. When *II* was analyzed under the same conditions, no response was observed. Thus, unless appropriate corrective action is taken, the determination of tautomerizable sulfonamides by diazomethane derivatization and GC or (GC/MS) analysis may give erroneous results. The addition of deuterium or carbon-13 labeled sulfonamides before derivatization and the use of isotope ratio analysis for quantification should give reliable results.

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SYMPOSIUM ON PESTICIDES IN FOODS: COPING WITH THE ISSUE

102nd Annual International Meeting of AOAC, August 1988

The issue of pesticides continues to be highly visible for the scientific community and the general public. Concern appears to be growing based on coverage by the news media, studies by various government and consumer organizations, and oversight actions by Congress. Analytical chemists and AOAC are in the middle of the issue because methods of analysis for pesticide residues in foods are a fundamental consideration.

The symposium provided a unique opportunity for analytical chemists to deal with the facts of the task before us: analysis for residues in foods. The symposium papers that cover the "programs" portion of the symposium are presented here. The papers provide a balanced perspective of relevant activities in key federal, state, and international organizations as well as the food processing industry. Those activities strongly influence the challenges that residue analysts face now and in the future.

In addition to the papers presented here, which made up part I of the symposium, part II included 6 papers which dealt more directly with analytical methodology. Methods of immediate utility in pesticide residue monitoring in food were described along with emerging instrumental and biologically based analytical technology.

Those papers are not presented here; however, the authors may be contacted if further information is desired.

H. Anson Moye, University of Florida: *Overview of OTA Workshop on Technologies to Detect Pesticide Residues in Foods.*

Lamaat Shalaby, duPont: *Analysis of Pesticide Residues Using Themospray LC/MS.*

Leon Sawyer, FDA: *Efficient and Rapid Approach to Multiresidue Analysis: Sweep Co-Distillation.*

Harry Lento, Campbell Soup Co.: *Application of Robotics in Multiresidue Analysis.*

Philip L. Wylie, Hewlett-Packard: *Pesticide Residue Analysis Using Gas Chromatography with a Novel Microwave-Induced Helium Plasma Detector.*

Ralph Mumma, Pennsylvania State University: *Potential of Immunoassay in Monitoring Pesticide Residues.*

This symposium has shown that the subject of pesticide residues is one of high interest and concern and that government and private sectors are responding with increased attention. The residue analyst will be called upon to develop and employ a variety of methods in monitoring and regulating pesticide residues in food. The mix will no doubt include rapid and simplified methods as well as highly sophisticated instrumental methods. Individual researchers and AOAC face substantial challenges in meeting future demands on pesticide residue methodology.

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The FDA Pesticides Program: Goals and New Approaches

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The U.S. Food and Drug Administration (FDA) has carried out a large-scale monitoring program for pesticide residues in foods since the 1960s. The program has evolved continuously as evidenced by a number of recently incorporated modifications and initiatives. Included are greater emphasis on imports; increased and more specific targeting of pesticide/commodity combinations by geographic area or country; development of individual district sampling plans for domestic and imported foods; expanded use of single residue methods; linkage of information on foreign pesticide usage with food import volumes; development of an analytical methods research plan; and increased cooperative sampling and data exchange with the states. Initiatives to acquire and utilize private sector and other monitoring data are being explored, and aggressive steps are being taken to inform the public of FDA monitoring results in a timely and understandable manner.

Pesticides are required to produce a high quality, inexpensive food supply, and the use of pesticides in agriculture can be expected to result in residues in foods. This paper will describe the U.S. Food and Drug Administration (FDA) program to monitor for these residues, briefly summarize recent monitoring findings, and outline recent changes and future approaches.

Three federal agencies share the responsibility for regulating pesticides. The U.S. Environmental Protection Agency (EPA) registers or approves the use of pesticides, and establishes tolerances if use of the pesticide may lead to residues in foods. With the exception of meat and poultry, for which the U.S. Department of Agriculture (USDA) is responsible, FDA is charged with enforcing tolerances for foods shipped in interstate commerce.

FDA has carried out a large-scale monitoring program for pesticides since the early 1960s. The program has 2 principal approaches: (*1*) Regulatory or Commodity Monitoring, to measure *residue levels* in domestic and imported foods to enforce tolerances and other regulatory limits; and (*2*) the Total Diet Study, to determine *intakes* of pesticides in foods prepared for consumption. Each of these approaches will be described.

Regulatory Monitoring

The prime objective of the monitoring element is to prevent foods that contain illegal residues from entering interstate commerce. As a very important by-product, information is developed on the incidence and levels of pesticide residues. This provides FDA with a national overview of the pesticides residue situation in foods, and is a measure of the effectiveness of the U.S. regulatory system. These monitoring data are made available to EPA for their continuing reassessment of pesticide uses and tolerances. Finally, the monitoring results are summarized and made available to the general public in the scientific literature or by other means.

The focus of the monitoring is on the raw agricultural commodity. At present, about 15 000 samples (8000 import and 7000 domestic) are collected and analyzed by FDA field offices each year. Samples are collected as near as possible to the point of production or entry into the United States. There are currently about 300 pesticides with tolerances in or on foods, as well as a number of other pesticides and related chemicals that can exist as residues. Because of the tremendous number of possible pesticide/commodity combinations, the goal is to carry out *selective* monitoring to achieve an adequate level of consumer protection. The analytical approach is critical. Most determinations are made by 1 of 5 well tested and validated analytical methods: those which can determine a group of pesticides in a single analysis. In combination, these 5 methods can determine about half the pesticides with food tolerances and many of their metabolites, alteration products, and associated chemicals. Single residue methods are used when residues of interest are not amenable to determination by the multiresidue methods. The agency realized at an early stage that the use of a separate method for each pesticide in even a limited number of commodities would not provide adequate monitoring coverage.

The monitoring program is composed of 3 different, but complementary elements: Core Requirements, Individual District Programs, and Headquarters-Initiated Surveys.

Core Requirements

This element requires all 21 of the FDA field offices to collect and analyze specified minimum numbers of domestic and imported commodities that are susceptible to environmental contamination, or those likely to contain residues of lipid-soluble pesticides. The chief commodities sampled are those of animal origin, including eggs, milk and dairy products, and fish and/or shellfish.

Individual District Programs

Each district or field office is now required to develop formal annual sampling plans covering both domestic and imported foods. As part of the planning, increased coordination, cooperative sampling, and data exchange with the states are emphasized. Each district is also required to carry out a minimum of 2 domestic and 2 import special (selective) surveys for pesticide/commodity combinations not covered in its ongoing monitoring. The focus is on foods of dietary importance. Overall, the district programs are based on knowledge of historical pest problems, local and foreign pesticide use information, and import volumes. FDA has recently integrated data on import volumes from U.S. Customs with information on foreign pesticide use from an extensive database purchased from Battelle-Geneva. The integrated Battelle-Customs information is used by our field offices to help plan their import monitoring. The Surveillance Index (SI) also serves to guide the planning. The SI was initiated in 1979, and in this systematic approach, monitoring priorities are established for each pesticide based on how toxic it is, whether it has potential for dietary exposure, and whether it has been adequately covered by our ongoing monitoring. Historical problem areas also play a role in the development of the individual district plans.

Headquarters Surveys

The third element is the headquarters survey. These selective surveys are issued to monitor particular pesticide/commodity combinations. The surveys are initiated in response to a number of factors, including incidents involving misuse or contamination; requests from EPA for specific monitoring

Presented at the Symposium on Pesticides in Foods: Coping with the Issue—Programs and Analytical Methods, 102nd AOAC Annual International Meeting, Aug. 29–Sept. 1, 1988, Palm Beach, FL.

Table 1. Selective surveys conducted in 1987

Pesticide	Commodity
Aldicarb	potatoes
Benomyl	apples
	grapes
	peaches
Captafol	apples
	cherries
	rice
Captan	cherries
Daminozide	apples
Ethylenebisdithiocarbamates (EBDCs)[a] and ethylenethiourea (ETU)	various fruits & vegetables
Folpet	various fruits & vegetables
N-Methyl carbamates[b]	various fruits & vegetables
Propargite	peaches

[a] Includes amobam, mancozeb, maneb, metiram, nabam, and zineb.
[b] Includes such compounds as aldicarb, carbaryl, carbofuran, and methomyl.

data for a particular pesticide; new information on the toxicity of a pesticide or change in usage which may indicate a greater potential risk; lack of information on particular pesticides, commodities, geographic areas, or countries; or a high priority for monitoring as indicated by the SI. Table 1 lists the selective surveys carried out in fiscal year 1987.

The Total Diet Study

The second major element of the FDA pesticide program is the Total Diet Study. The prime objectives are to determine *dietary intakes* of pesticides, to compare these intakes with acceptable daily intakes (ADIs) as established by the World Health Organization (WHO), and to identify trends. Finally, the data are summarized and made public.

The Total Diet Study was begun in the 1960s, and the current version of the program has been in place since 1982. Selection of the diets was based on 2 nationwide surveys covering about 50 000 people, the 1977–1978 USDA survey and the 1976–1980 National Health and Nutrition Examination Survey. About 5000 different foods were identified in these 2 surveys. An aggregation scheme was used to select 234 foods to represent the 5000 foods. Most of the individual foods selected represent a group of foods similar in type and nutrient content; the analyzed food is the group member consumed in the greatest amount. For example, apple pie represents dozens of different fruit pies and pastries with fruit, and a number of pasta dishes are represented by spaghetti and meatballs in tomato sauce. Thus, the 234 foods can be said to represent all 5000 foods identified in the 2 nationwide dietary surveys.

Diets were constructed for 8 different age-sex groups: the 6- to 11-month-old, the 2-year-old, and males and females 14–16 years old, 25–30 years old, and 60–65 years old. Dietary intakes for each of these groups can be calculated because each of the individual 234 foods is analyzed separately. The individual foods and ingredients necessary for the preparation of recipe items are purchased at retail stores 4 times each year to give a total of 4 "Market Baskets." For each of these 4 Market Baskets, simultaneous sampling is carried out in 3 cities in 1 of 4 geographic areas of the country: Northeast, North Central, South, and West. For example, one Total Diet Market Basket collection in the South might take place in Baltimore, MD, Charleston, SC, and Tallahassee, FL.

The foods are then shipped to the FDA Total Diet Laboratory in Kansas City, MO, where the 3 samples of each recipe item are composited and used to prepare 234 cooked or otherwise table-ready foods. The degree of preparation varies from the very simple, such as peeling bananas, to preparing items such as lasagna and beef stew. The 234 foods are analyzed individually for residues of well over 100 pesticides, a host of industrial chemicals such as PCBs, 15 metals and essential minerals, and radionuclides. Since the Total Diet Study is conducted to determine levels of chemicals in foods *as normally eaten*, and since the levels of pesticide residues found are usually low, the analytical procedures have been modified to permit quantitation at levels 5–10 times lower than those achieved in the FDA regulatory monitoring program. Also, the identity of each pesticide residue found is confirmed by an alternative technique. The dietary intakes are then calculated and compared with ADIs (Acceptable Daily Intakes) established by WHO. Figure 1 is a summary of the overall pesticide program.

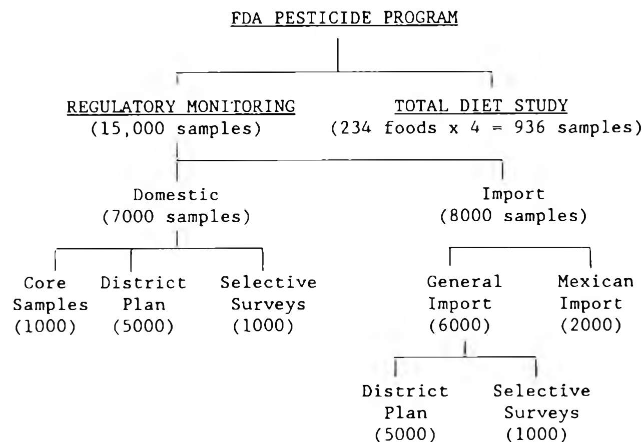

Figure 1. Summary of the FDA Pesticide Program.

To compare the 2 major components of the program, the Monitoring element is regulatory in nature, emphasizes the raw agricultural commodity, and is designed to deal with essentially all important pesticides, whereas the Total Diet Study is informational, examines foods as eaten, covers only selected pesticides, and allows the calculation of *dietary intakes*. Thus, the Monitoring and Total Diet elements of the program complement one another, and provide a picture of pesticide residues in foods "from the farm gate to the kitchen table."

Residue Findings

In fiscal year 1987, of the 253 different pesticides that would have been detected if their residues had been present, 113 were found. Tolerances were exceeded in less than 1% of the cases, and in over half the samples, *no* residues were found. About 12 000 surveillance samples were collected and analyzed in 1987. Surveillance samples are those collected without suspicion that illegal residues are present. Only 1.5% of the domestic and 3.4% of the imports were violative; most of the actionable samples were violative because there were no tolerances for those specific pesticide/commodity combinations. Compliance samples are collected to follow up findings of illegal residues in surveillance samples, or when other evidence suggests that a residue problem may exist in a particular shipment. As one might expect, a higher violation rate was observed on compliance samples, about 12% for both domestic and import samples. Only a small portion of these had residues that exceeded tolerances. Of all the samples collected in 1987, over 80% of the violations occurred because

Table 2. Frequency of occurrence of pesticides in Total Diet Study in 1987

Pesticide	Findings[a]		Pesticide	Findings[a]	
	Number	Percent		Number	Percent
Malathion	211	23	Carbaryl[b]	35	4
DDT, total	202	22	Quintozene, total	32	3
Diazinon	194	21	Chlorpropham	30	3
Chlorpyrifos	117	12	Acephate	24	3
Dieldrin	108	12	Dicofol, total	24	3
HCB	91	10	DCPA	20	2
Endosulfan, total	64	7	Ethion	20	2
BHC, *alpha* and *beta*	59	6	Dimethoate	19	2
Lindane	58	6	Phosalone	19	2
Heptachlor	57	6	Parathion	18	2
Chlordane, total	47	5	Toxaphene	17	2
Methamidophos	46	5	Permethrin	13	1
Chlorpyrifos-methyl	44	5	Omethoate	12	1
Dicloran	41	4			

[a] Based on 936 items.
[b] Reflects overall incidence; only 72 selected foods were analyzed for *N*-methyl carbamates.

there were no tolerances for those specific pesticide/commodity combinations, and of the total number of surveillance and compliance samples combined, less than 1% had residues that exceeded tolerances. The results of FDA's pesticide monitoring in fiscal year 1987 are summarized in a published report (*J. Assoc. Off. Anal. Chem.* (1988) **71,** 156A–174A).

With regard to the Total Diet Study, the dietary intakes calculated are well below ADIs established by WHO, in almost all cases by orders of magnitude. An ADI is the daily intake of a chemical which, if ingested over a lifetime, appears to be without appreciable risk. The continued declines in the intakes of the no-longer permitted organochlorine pesticides such as DDT and dieldrin are evident. Of the 253 pesticide chemicals that can be determined by the procedures used, 53 were found in the 1987 Market Baskets.

Malathion heads the list of the most frequently found pesticides. This chemical is used on many grains, fruits, and vegetables, and was found in about 23% of the 234 Total Diet foods. DDT, while no longer permitted for food use, continues to be found at very low levels in a great many foods, primarily those of animal origin. Table 2 lists some of the most frequently found pesticides, their frequency of occurrence, and their intakes expressed as a percentage of their ADIs.

Future Directions

The agency will continue to carry out an aggressive monitoring program with emphasis on imports and increasing coverage through the filling of gaps in information. The Total Diet Study will be continued and expanded to include a number of additional pesticides of interest. Ethylenethiourea (ETU) is slated for inclusion in 1989. The results of the new USDA nationwide food consumption survey will soon be available, and the composition of the Total Diet Study diets will be changed to reflect the new information. FDA will also consider increasing the number of age-sex groups, with a focus on infants and young children.

For some time, the expectations of the Congress and the public at large have clearly exceeded the level of monitoring coverage that FDA resources allow. The agency is currently emphasizing development of its "New Concepts" pesticide initiative. Key aspects include encouragement of the private sector to carry out additional monitoring of foods for pesticide residues, and the sharing of industry monitoring data and related information with FDA. The agency would then be able to augment its own monitoring data with information generated by others and allow FDA to better assess the overall public health significance of pesticide residues in foods. The FDA Commissioner recently circulated a draft document describing these new concepts to a number of organizations including the food industry, government agencies, the Congress, academia, and consumer groups. Once established, implementation will proceed in stages over about 5 years.

Finally, as far as the public is concerned, perception *is* reality. Pesticides continue to head the list of consumer fears about the food supply, yet FDA monitoring data show that the food supply is almost always free of excessive residues. This information would seem to indicate that the food supply is safe, and that the public's fears are unfounded. Selective surveys for many of the pesticides *not* covered as part of routine monitoring have continued to demonstrate the same overall picture: Over-tolerance residues are rarely, if ever, encountered. There is no compelling reason to believe that excessive or illegal residues of these pesticides will exist simply because they are not continuously monitored. The agency is presently taking a number of innovative steps to inform the public of its residue monitoring findings. Understandable and timely communication of the monitoring data and their significance is essential, if FDA is ever to change the public perception.

Changing Pesticide Technology in Meat and Poultry Products

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In response to consumer concerns about pesticide residues in meat and poultry, a National Residue Program was introduced in the 1960s. Rapidly developing analytical methods and instrument capability resulted in a laboratory-based program of sufficient size and technical capability to quantitatively determine an increasing number of pesticides. In 1985, at the request of the Food Safety and Inspection Service, the National Academy of Sciences evaluated the program and recommended that more emphasis be placed on preventing residues and providing a higher degree of safety to consumers. In response, FSIS made a commitment to increase and improve capability to test for more residues, including pesticides, and to develop or purchase rapid test systems to facilitate the planned expansion of the National Residue Program. Such systems are now being evaluated and integrated into the program. Factors important for acceptability of rapid tests are reviewed in detail.

Agricultural production today uses commercially available pesticides to combat a variety of weeds, insects, fungi, and other agricultural pests. As a result, consumers are exposed to pesticides, usually in minute quantities, in several food groups including meat, dairy products, fruits, vegetables, dried food goods, most processed foods, and many other household staples. Some of these pesticides are considered to be acutely or chronically toxic to humans and other segments of the environment and pose potentially serious health risks to nontarget organisms and species. This situation places a significant regulatory responsibility on public health-related agencies. Pesticides are a major health concern to consumers; they expect tc have pesticide-safe food.

The magnitude and complexity of the regulatory responsibility for pesticides is well developed by the 1986 Congressional Research Service report (1) and the 1987 National Academy of Sciences report (2). More than 8000 pesticide/commodity combinations have tolerances established under the U.S. Food, Drug, and Cosmetic Act, Sections 408 and 409. More than 300 individual pesticides (active ingredients) are registered for use on agricultural products. Based on data supplied by the U.S. Environmental Protection Agency, 53 of these pesticides have active ingredients identified by the National Academy of Sciences as oncogenic or potentially oncogenic (2). This does not include some of the chlorinated hydrocarbons considered to be oncogenic in animals or humans.

It is little consolation that the number of pesticides approved for use on meat and poultry products represents only a small percentage of all pesticides approved for use on agricultural crops and products. The Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture is clearly aware of and responsive to the need for pesticide residue analysis to ensure safe and wholesome meat and poultry products for consumers. Clearly, additional residue methodology capable of detecting many residues by the same analytical scheme would enhance the monitoring capabilities of FSIS.

Analytical methods play an important role in meat and poultry inspection systems designed to protect public health. We would like to have a universal analytical scheme that could simultaneously quantitate the presence of all compounds or classes of compounds of interest in animal tissue or fluid with acceptable accuracy and correctly identify the analyte or analytes. Yet at present very few of the available analytical procedures can simultaneously quantitate and confirm the identity of such residues. Since universal methods are not available, we must use methods with individual attributes of presumptive presence, quantitation, and positive identification. To accomplish our goal, methods will have to be integrated for a highly effective residue program.

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National Residue Program

As part of its inspection procedures, FSIS manages the National Residue Program to sample and test meat and meat products for residues of a variety of pesticides, environmental contaminants, and animal drugs. During the last 20 years, the NRP has evolved into a mature program with a focus that has changed from primarily residue detection to residue prevention.

Monitoring activities are designed to provide profile information on the occurrence of residue violations in specified animal populations on an annual national basis. The information is obtained through a statistically based selection of random samples from healthy-appearing animals under inspection. Although the monitoring program is not designed to provide precise estimates of the percent of violations in large populations, such estimates are available as supplemental information.

At present, monitoring information tells us with a 95% probability whether we have a residue problem in 1% or more of the national population of animals with a particular residue in a specific production class. It is not specifically designed to correct a problem or to stop the movement of potentially adulterated products into commerce. Residue violations revealed by the monitoring program initiate a series of subsequent activities.

Surveillance activities are designed to investigate and control the movement of potentially adulterated products. Surveillance sampling is biased and directed at particular carcasses or products in response to information about a potential problem from the monitoring segments of the NRP or from observations during ante-mortem or post-mortem inspection. Depending on the weight of evidence, the product may be retained at the plant while surveillance testing is carried out. When a producer source of a monitoring sample violation can be identified, follow-up surveillance testing is conducted on subsequent animals marketed from that source.

Established Methodology

Biotechnology is now a very popular topic in science, but it is not new to NRP or to regulatory analysis. One of the earliest analytical methods used for pesticides was a bioassay, in which the detector was the whole system, rather than a highly refined, specific entity: it was the fly. Meat samples were placed in an enclosed environment with a number of flies. If the flies died, pesticides were present. It was not sophisticated, highly sensitive, or definitive for chemical structure, but the analytical response was easy to read. Similarly, tests for antimicrobial and antibiotic residues have commonly been based on inhibition of microbial growth.

Twenty or more years ago, the development of the gas chromatograph was a breakthrough in pesticide analysis. It

was designed for materials that could be converted into a vapor or gaseous state without thermal degradation. Many pesticides were amenable to this type of analysis. Method sensitivity improved to the point at which submicrogram to nanogram quantities of analyte could be detected. These methods have served our pesticide residue program well; however, they require well equipped laboratories with competent, trained analysts.

This same time period saw the development of thin-layer chromatography and color-generating reagent systems to identify analytes. These color-generating systems evolved from relatively nonspecific to relatively specific capabilities providing color reactions dependent on functional groups. Specific reagent systems are now available for pesticides of the same chemical class.

A fairly recent major development has been the emergence of liquid chromatography. This technique broadened the range of analytes that could be separated and quantitated to include nonvolatile and thermally labile materials, including pesticides such as the carbamates. Sensitivity is now sufficient to detect nanogram and subnanogram quantities of analyte. Development of new chromogenic and fluorescent-generating materials also contributed to this dramatic improvement. Today an increasing number of our analytical methods depend on liquid chromatography. Again, these methods require well equipped laboratories to achieve the desired results.

Rapid Screening Methods

In the decade of the 1980s, heightened awareness about food safety demanded even more analytical capability and capacity. During this time, FSIS commissioned the National Academy of Sciences (NAS) to assess how effectively the agency was accomplishing its mission. In 1985, NAS issued its report on meat and poultry inspection (3). As the NAS study points out (and we concur), the most important responsibility of FSIS is the risk associated with bacteria and chemical residues, neither of which can be detected organoleptically or by eye. We believe that NAS has laid out the future of meat and poultry inspection, and we are prepared to implement their recommendations as fully as we can. To define our specific actions, FSIS prepared a report in 1986, titled "FSIS Future Agenda" (4). Actions for residue analysis are clearly stated in that report.

Higher levels of sampling were called for to detect residues in meat and poultry and to provide greater assurance of a safe food supply for consumers, as described by the proposed residue monitoring program through 1993. The need to rethink the program design was evident. Emphasis needed to be on developing and using biologically based rapid testing systems. Today, several immunoassay systems are being developed, such as the enzyme-linked immunosorbent assay (ELISA). For present and future needs, our regulatory programs will seek to employ methods with attributes of presumptive presence, quantitation, and positive identification.

Two important criteria for using screening methods are (a) their ability to analyze a relatively large number of samples in a given unit of time, and (b) their robust nature. This latter characteristic encourages the use of screening methods in nonlaboratory surroundings where tests may often be performed by individuals not experienced in analytical chemistry techniques. This characteristic places a constraint on certain types of methodology; it limits use of certain types of equipment, instruments, and reagents. Further, under these conditions methods must be written in simple, unambiguous test instructions that will enable a tester to correctly prepare the test material, conduct the analysis, and interpret and report the test findings. Process controls defining critical steps in the test procedure are highly relevant to the success of such a testing program.

We recognize that rapid screening methods generate useful but potentially imperfect information. They are designed primarily to detect the presence or absence of a compound or class of compounds at some designated level of interest and are often based on noninstrumental techniques of analyte determination. Consequently, results for a given sample are not as reliable as quantitative or confirmatory methods unless there is corroborating information. This point must be clearly understood. From our experience, these methods generally provide either reasonably good semiquantitative information but a lesser degree of identification of specific compounds or classes, or good compound/class identification but a lesser degree of quantitative information. Before they are used in our regulatory program, we develop data that define operating characteristics of reliable performance. Note that many of our microbiological assays and immunoassay test systems fall into this category of methods. They are used because of their convenience and potential suitability for field and in-plant environments, analytical speed, sample efficiency through batch analysis, portability to nonlaboratory environments, suitable sensitivity, and their ability to detect classes of compounds. One of the cautions that must be observed in using rapid screening methods is that proposed regulatory action based on individual positive results must be substantiated by quantitative or confirmatory methods as determined by the uncertainty of an individual result. However, epidemiological information may provide substantive data that reduce the uncertainty of individual results. We believe, therefore, that these methods offer several advantages to our residue control program.

The reliability of screening methods has to be judged in part by their performance characteristics, as well as their ability to handle relatively large numbers of samples within a given time. Two key characteristics requiring definition include the percentage of false positives (reporting a positive response when no analyte is present) and percentage of false negatives (reporting a negative response when the analyte is present) when measured against a validated quantitative assay in a statistically designed protocol. For public health reasons, the percentage of false negatives must be quite low at the levels of interest, whereas slightly more flexibility may be acceptable for false positives. A balance between these 2 parameters serves to establish a minimum value of residue detection and working range.

FSIS has set up procedures for approving rapid tests in response to the industry that is developing these test systems. The principal responsibility will be that of Technical Services, the program area responsible for developing and evaluating new inspection procedures. The agency will first determine whether a particular rapid test fits a program need. If it does, experts within FSIS will perform the needed evaluation. If these new tests are successful, they will be accepted by FSIS for use in its regulatory program. Further details are given in *Food Chemical News*, August 22, 1988 (5).

Criteria for Screening Tests

What are our criteria? To be suitable for regulatory purposes, methods must be reliable. To ensure analytical reliability, performance characteristics of a method must be determined by multilaboratory evaluation. In most cases, minimum standards should be designed to fit the needs of specific program requirements. By consensus with public

health agencies, standard setting organizations, and AOAC, the principal attributes considered for analytical methods are specificity, precision, systematic error, and sensitivity. These attributes have already been defined by the standard-setting organizations.

Regardless of what average recoveries are observed, we will look for low variability as a desirable feature. With rapid test systems, we will look for a degree of performance that routinely achieves parallel curves for standard solutions of the analyte and extracts of analyte added to a sample.

Performance criteria for analytical methods also provide a basis for good management decisions in future planning, evaluation, and product disposition. The sensitivity we seek in a method is a measure of its ability to discriminate small differences in analyte concentration. Accuracy requirements will vary with the objective of the test procedure: for screening methods, characteristics of false positives and false negatives will be a major factor in defining an operating range.

A number of other attributes are also desirable; the method should be

(a) Rugged or robust—relatively unaffected by small deviations from optimal parameter values.

(b) Cost effective—use relatively common reagents and instrumentation, and utilize resources efficiently.

(c) Relatively uncomplicated—use simple, straightforward mechanical or operational procedures.

(d) Portable—transferable from one location to another without loss of established performance characteristics.

(e) Capable of handling a set of samples simultaneously in a time-effective manner, thus reducing total analytical time. This is particularly important for screening methods, which are designed to analyze large numbers of samples in short or fixed time frames.

Safety considerations are essential. However, for screening tests it is especially important to keep in mind that end users of the tests may have limited analytical skills, may not have well equipped and ventilated work environments, and may have to run tests under adverse conditions, e.g., during July in Texas or January in Minnesota. These considerations place constraints on certain kinds of methodology, as discussed above.

Integrating rapid test methods into residue programs may depend on residue violation rates and public health/food safety issues. The following are 4 possible situations in which rapid tests may be used.

When the incidence of violations from statistically based random sampling programs is at a low level, screening methods are particularly attractive because they permit testing large numbers of food products. Residue monitoring data indicate that a large majority of samples contain nondetectable and below-tolerance concentrations of residues. A screening test provides a program with the opportunity to pass a product containing residues below a level of interest and to retain suspect positives for more definitive laboratory analysis. Screening tests permit more effective use of expensive laboratory resources and also reduce the significant cost involved in collection and shipment of all samples to a designated laboratory.

When the incidence of violation is known or is high, quantitative immunochemical-enzyme inhibition assays and thin-layer chromatographic systems designed for rapid testing in laboratory environments become very attractive. They provide data on which to take regulatory action on a violative product.

For detecting residues of unapproved pesticides or pesticides used in an unapproved manner, residue screening tests are very attractive. In these situations, detection of any amount of residue in meat and poultry is a violation. Screening test results normally require support by a confirmatory procedure, but in this situation, quantitation is not a specific requirement.

When sampling rates are increased, rapid tests fill a need. The FSIS response to provide greater consumer protection from pesticides includes sampling to detect a 0.1% incidence of residues with 95% confidence in the population of healthy animals presented for slaughter. This goal requires sampling 3000 animals in a single production class for a specific analyte. Sampling in these instances is an order of magnitude greater than generally provided by NRP protocols.

The great advantage of such test systems is their simplicity, which allows them to be performed by testers of limited experience in diagnostic or analytical procedures. An occasional disadvantage is that the tests are specifically designed for a single compound; separate test systems are required for a class of pesticides. In some instances, sufficient cross reactivity to a class of pesticides will allow other compounds to be detected in a sample matrix, usually at higher concentrations. Thus, there is some trade-off for development by laboratories and use in regulatory programs. Perceived constraints with screening tests are that they are not specific and that they consume too large a portion of valuable resources to identify the residue of interest. A possible resolution to this dilemma is to use emerging technology to develop a hierarchy of test methods based on simplicity in design and application, automation technology, and commercially available systems and equipment with potential for broad application. It is often possible to develop effective quantitative methods with the same technology. These assays require state-of-the-art instrumentation and must be performed by analysts in fully equipped laboratories.

Concerns with Immunochemical Assays

Some time will be required before confidence and recognized legal status is attained for qualitative or quantitative immunochemical assays. Regulatory agencies will probably need considerable experience and familiarization with the technology involved in the test systems containing unknown reagents ("black box" test systems) before they can develop procedures to assure themselves that public health protection is not being compromised.

There are other concerns with regard to immunochemical assays. First, in the development and design of ready-to-use products such as these tests, reliability and consistent performance of the assay can occasionally vary from lot-to-lot production. Quality control for production will likely improve with experience. Nevertheless, users of these systems must employ good quality control/quality assurance protocols to ensure method performance. Regulatory agencies planning to use such methods can facilitate their acceptance by developing standards for the manufacturers of the systems.

Second, some of these assays are more sensitive than the traditional quantitative and confirmatory assays, so that the qualitative results cannot be confirmed. This may limit further regulatory action. We hope that this apparent contradiction will force technology to develop new quantitative and confirmatory methodology to match the sensitivity levels. We need to be able to confirm what we have the capability to detect.

Third, the systems rely heavily on using aqueous media for performing the tests. For certain types of food, this may be of little consequence, but for other types such as meat it may be

a considerable deterrent. For example, most of our chemically based assays rely on organic solvents for releasing the analyte of interest from the test sample matrix. Thus, new solvent systems must be developed to provide sufficient transfer from the organic extraction solvent to the test system while not denaturing or deactivating the biological reagents. Progress is being made in this area. For example, ImmunoSystems has developed an assay for chlorinated triazines (Res-I-Mune®) that allows detection at low levels (ng/g, ppb) by using selected aqueous-organic solvent systems.

In using rapid test procedures in slaughter plants or farm environments where the test user is not likely to have analytical expertise, one of our chief considerations is quality assurance. We want to maintain a positive image with these rapid procedures because one measure of our success in meeting our commitments to improved inspection systems and analytical testing capability is the successful transfer of the new technology to our inspectors. In addition, if programs like pre-certification of animals or poultry are to be implemented, assistance to private practitioners and others will be needed. We have to develop controls and quality assurance plans to provide a high degree of reliability that the analytical test is being run properly, that necessary controls are being incorporated in the test system to monitor test performance, and that testers are interpreting and reporting results in a manner consistent with the test program. This is a tremendous task. We recently conducted a training and familiarization program for the on-site sulfa test for inspectors in swine slaughter plants, swine practitioners, and Extension Service veterinarians. The program required over 12 000 check samples for training and quality assurance. In addition, once inspectors began in-plant testing, procedures had to be developed to monitor for false negatives.

We are now evaluating rapid test immunochemical assays for the chlorinated triazines, paraquat, parathion, chlordane (heptachlor, dieldrin, endrin, aldrin, and endosulfan are detected via cross reactivity), and polychlorinated biphenyls (PCBs) at levels of interest for laboratory, field station, or slaughter plant use. We recognize the need to adapt the original design to fit our needs. Almost exclusively, these tests are designed to use aqueous systems, whereas we currently rely on organic extraction to release the compound(s) of interest from the sample matrix. These evaluations are under way. We have been able to demonstrate that extracting pesticides from fat and then using the extracts in the test system does not invalidate the test when the appropriate modifications are made. Our field laboratory method development units are currently working on optimizing these procedures for program use.

The immunoassay screening test for chlorinated triazines is now being studied in our laboratory at Athens, GA. The method test system is sensitive at the low parts per billion level for residues extracted with aqueous acetonitrile or aqueous methanol. Since we previously developed a quantitative chromatographic assay for triazines, we are encouraged that these methods will complement each other.

A commercial pesticide detection system based on cholinesterase enzyme inhibition has been developed by EnzyTech, Inc., and is being evaluated by FSIS. The enzyme ticket system detects common insecticides that account for about 85% of all insecticides used in the United States at concentrations in the low ng/g (ppb) range. Shelf stability for the test system is estimated to be several years. The design of the system allows for a 2-tier analytical scheme that will permit differentiation of organic sulfur-containing organophosphorus insecticides from their oxygen analogs. This advantage reduces some of the need for further analysis to quantitate and confirm these analytes.

Research is under way to enable analytes from an organic extract to be determined by the test system. Our approach is to prepare an organic extract of the pesticide from fat, and using the adsorbent "ticket" in the test kit on a filter funnel, slowly elute the extract through the ticket. After mild air or vacuum drying, the ticket can then be reinserted into the enzyme ticket system, the reagents added as originally designed, and the test result read visually. Results are encouraging for detection of compounds such as carbaryl, aldicarb, malathion, and methyl parathion at microgram to submicrogram quantities.

Our next concern will be adequate methods to quantitate and confirm specific analytes from multiresidue procedures to support these qualitative methods. Some of our recent method extension studies complement this screening test.

FSIS has method development contracts for developing immunochemical assays for heptachlor-related organochlorine pesticides, ivermectin, synthetic pyrethroids (permethrin, cypermethrin, and deltamethrin), and nitroimidazoles in meat and poultry tissue. These are expected to provide improved laboratory analytical capability for these analytes. The immunoassay for synthetic pyrethroids is currently being introduced into our field laboratories. We intend to pursue development of a swab-like test system for inspectors to detect surface contamination. With the reagents available and with no detailed extraction required, theoretically this should be a relatively easy adaptation.

The technology for rapid tests continues to develop. Additional analytes are being adapted to these chromogenic, enzymatic, and immunologic technologies. Newer technologies such as fluorescent immunoassays offer other possibilities for residue analysis, as do such systems when applied to forensic and clinical chemistry. These are exciting and challenging times for us. To be sure, we recognize that these new methods may raise new concerns, but given the options, we believe this is a prudent course of action to follow.

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State Programs for Pesticide Residues in Foods

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Two U.S. data collection and dissemination programs, FEEDCON and FOODCONTAM, are described. FEEDCON provides information on contamination levels in animal feeds of toxic chemical residues (pesticides, industrial chemicals, heavy metals, mycotoxins, natural plant toxins, salmonella, and therapeutic drug cross-contaminations). FEEDCON data are collected from approximately 40 state feed regulatory agencies, feed manufacturers, and related groups who subscribe ($100-$200 per year) to the program, which is sponsored by the Association of American Feed Control Officials. FOODCONTAM provides similar information, but is limited to pesticides, heavy metals and industrial chemicals (polychlorinated and polybrominated biphenyls, etc.) in human foods. Both programs have been developed and initiated under U.S. Food and Drug Administration contracts with the Mississippi State Chemical Laboratory. Program structures of both are outlined conceptually, and FOODCONTAM is described in detail. FOODCONTAM data-sharing program development is essentially complete, but expansion by incorporating FDA data with State Laboratory data is nearing reality.

Public concerns about adverse health effects of natural and synthetic chemicals in U.S. food supplies have increased greatly in the last 2 decades. Perceived, if not real, concerns and lack of quantitative data defining the extent, human health risk, and levels of agrichemicals and industrial chemicals in our nation's foods have increased public demands for banning or severely restricting use of such chemicals in food production and processing (1-3).

Monitoring of levels of chemical residue contaminants in animal feeds and human foods has been a programmatic element of both U.S. federal and state regulatory food quality assurance from the early 1950s, when a number of chlorinated hydrocarbon pesticides like DDT were introduced into commercial agriculture on a large scale. Accurate estimates of the extent of occurrence of pesticide residues in foods were relatively slow in developing, partly because of a lack of sophisticated methods to detect and quantify very low concentrations of pesticide residues in feeds and food crops. Introduction of gas chromatographs with sensitive electron-capture detectors made possible dramatic expansions of chemists' abilities to detect and quantify low levels of such residues in a wide variety of human foods, animal feeds, and feed ingredients (4-7). Analytical methods sensitive enough to detect and quantify pesticide residues in fractional parts per million were well developed by the early to mid-1960s.

Detection limits for many of these commonly used plant and animal protection chemicals were pushed lower and lower during the 1960s. Concurrently, public concern escalated greatly about the possible linkage of pesticide residue contaminations in foods to human cancer initiation. This concern was inversely proportional to amounts found, but directly proportional to the frequency of such findings.

By that time, the U.S. Food and Drug Administration (FDA) had begun systematic development and performance validations of "standard methods" (ultimately adopted by AOAC as official methods) for detection and quantification of these agrochemicals. FDA activity expanded as newer classes of organic herbicides, insecticides, fungicides, rodenticides, etc., were marketed. As chemical structures of such products became more complex, they became more polar and more rapidly degradable in the environment. These included a wide range of chlorinated hydrocarbons, organophosphates, carbamates, synthetic pyrethroids, and other classes of complex organic chemicals. Consumers in the United States and abroad became more aware and much more concerned about long-term health effects and residual toxicities of such chemicals and their decomposition residues in raw and processed foods. Analytical methods, oriented toward gas and liquid chromatographic instruments coupled to very sensitive and complex detectors, were introduced rapidly. Detection limits for many pesticides dropped to low parts per billion and even parts per trillion.

In 1963, as concerns about the health effects of such residues increased, FDA started the Total Diet Study, a program to estimate pesticide residues, industrial chemicals, toxic elements, radionuclides, and essential minerals consumed per day by a "typical" 18-year-old human male. Consumption was projected to lifetime intake to estimate maximum human exposures to each class of chemicals. The Total Diet Study has continued to the present, with major changes incorporated in 1982 (8).

In addition to FDA's nationwide programs for monitoring pesticide residues in raw food commodities, many states started comparable studies under their own state food regulatory program responsibilities. California, Florida, Michigan, Mississippi, New York, North Carolina, Virginia, Wisconsin, and many other states have extensive pesticide residue analytical capabilities and programs to assure quality and purity of foods produced and consumed in their state under state Pure Food and Drug Laws. Most of these laws were patterned after the landmark federal Food and Drug Act of 1906, which Harvey W. Wiley worked so hard to achieve. By the mid-1920s, many states had adopted their own food laws paralleling the 1906 federal act, and were conducting food regulatory programs under their state laws and regulations. Federal programs were conducted by FDA and the U.S. Department of Agriculture (USDA). State food protection agencies worked closely with USDA and later with FDA scientists and administrators to coordinate program findings, enforcement actions, and analytical methods development and performance validation under the auspices of AOAC. A long history thus began for federal-state cooperative programs to assure the quality of foods, drugs, and many consumer products to their own state citizens. Close cooperative relationships between most states with the U.S. Department of Agriculture, the U.S. Food and Drug Administration, and the U.S. Environmental Protection Agency have developed and flourished.

The need for more effective methods for sharing data among the 50 states conducting programs under their own laws and the various federal regulatory groups involved (FDA, USDA, and EPA) became even more critical in the mid-1970s. A number of localized incidents of food contaminations occurred, usually as accidents. They frequently resulted from poor management practices in production of livestock (beef, dairy cattle, hogs, and chickens), lack of observance of preharvest cessation of agrochemical applica-

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tions to food crops, and similar causes. Many derived from accidents in shipment, storage, or use of inappropriate feed ingredients, and by accidental contaminations (particularly in animal by-product ingredients). A few incidents occurred because of greed and deliberate sale of adulterated feed ingredients by unscrupulous individuals. Some of these persons have been arrested, tried, and sentenced to prison for criminal actions, but meanwhile thousands or even millions of dollars have been lost to feed and food industries, as well as to the citizens who have been thus harmed. Perhaps more important, such problems have created a general lack of public confidence in the safety of our food supply.

Because of the development of analytical methods of greatly increased sensitivity (lower detection limits) during the 1960s and 1970s, as well as increased state and FDA surveillance programs, a growing number and variety of residues of industrial chemicals and pesticides were detected in feed ingredients, feeds, and foods. These industrial chemicals included polychlorinated biphenyls, halogenated kerosenes, industrial solvents, phthalate esters, heavy metals (arsenic, copper, lead, etc.), and polybrominated biphenyls.

These and other contaminants, including cross-contamination of feeds with nonapproved animal drugs, mycotoxins in grain and other seeds, and toxic natural products such as plant alkaloids and gossypol, were being found with greater frequency in a wide variety of feeds, foods, and surface waters (although usually at trace levels) in the United States and in other industrialized nations as well.

A need was recognized in the late 1970s and early 1980s for some means of sharing *current* data on feed contaminants among all states and the federal regulatory agencies (FDA, USDA, EPA) to help limit the consequences of accidents and human errors. Discussions of these concerns among federal and state regulators involved in food quality assurance programs, together with a literature review, documented a number of past major national catastrophes that involved chemical contamination(s) in chickens, beef, grains, foods, cattle, hogs, and humans (9).

In addition to sporadic crises involving pesticide residues, new concerns were emerging in the early 1980s. These related to mycotoxins in feeds, newer industrial chemical containments like PCDDs, TCDDs, mercury in fish, seed treatment chemicals in meat and milk, *salmonella sp.* and *Listeria monocytogenes* in poultry and milk, dieldrin and heptachlor in broilers, and other problems. Such discussions caused state scientists and regulatory managers in the Association of American Feed Control Officials, Inc. (AAFCO) and in FDA to recommend initiation of an AAFCO-sponsored program for sharing data on contaminant residues.

In 1981, the AAFCO's Board of Directors established a Feed Contamination Prevention Task Force, chaired by G. A. Pearson, Feed Control Official from Virginia. J. P. Minyard and W. Y. Cobb were members, together with N. J. Neher, Madison, WI; B. Ginther, Olympia, WA; J. Helmer, Sacramento, CA; C. E. Jones, Denver, CO; and B. Paulson, Mesa, AZ. This Task Force (later Committee) recommended establishing a data-sharing program, along with educational programs and other means of containing such tragic and costly incidents. By 1982, Minyard, Pearson, Cobb, and Committee members had developed a conceptual outline, a list of desired data elements needed for input and output, a tentative format for data outputs, and an organizational procedure to provide a monthly report of shared data, to be provided to the program leader, J. P. Minyard, by cooperating state feed control officials.

From its initiation, the AAFCO-FEEDCON Data Gathering and Sharing Program was perceived to need certain key elements for its success. These included a broadly based information gathering network consisting of (*1*) state regulatory agencies, (*2*) U.S. and Canadian federal agencies like FDA, USDA, and Agriculture Canada, (*3*) feed industry members, (*4*) faculty of university Extension Services and Animal Science Departments, and (*5*) veterinary diagnostic laboratories, both federal and state university-related. A second major program element was to assure all data providers "anonymity" for data they supplied and nonpunitive regulatory actions by federal or state agencies who might have to make follow-up investigations.

Minyard and his staff offered to design a computerized data organization/dissemination format and microcomputer (APPLE /// SOS) programs which would convert hard copy (or computer-compatible input data submitted on 5½-in. diskettes) to standard outputs. Key information elements of each data line would include the feed matrix, the analyte, the geographic origin of the sample, and other needed data. All information collected on each sample would be reported, both significant (as judged by the agency/submitter) and nonsignificant values, including all zero findings (no residues found). Fast turnaround of information to groups contributing data was essential. It was expected that these findings could help states and industries sharing data to focus their own programs more effectively.

FEEDCON, the name given the computer program to input and print the data in an organized format, was initiated in the Mississippi State Chemical Laboratory (MSCL), located in the Hand Chemical Laboratories at Mississippi State University. All necessary microcomputer programs for data entry, file manipulations, and output were written by W. E. Roberts, computer programs manager in MSCL. FEEDCON was formally initiated September 1983 under the oversight and sponsorship of AAFCO, which provided an initial $10,000 for start-up costs. Developmental funding ($38,547 over 3 years) for this program was provided under contract from FDA. FDA's Executive Director for Regional Operations (EDRO), Federal-State Relations Office, provided this critical financial support, plus input on programmatic concepts during this 3-year initiation period. Organization of outputs and types of findings over the last 5 years will be described in a later paper.

FEEDCON was targeted to be self-supporting by September 1986, 3 years after start-up. It achieved that target within 1 year. Within 13 months (1983–84), 24 paid subscribers had joined the program, and had provided 11,873 lines of data code. Membership in the program has hovered around 40 paid members since the second year. A few individuals and laboratories receive reports at no charge because they support FEEDCON in other ways.

In 1983, after FEEDCON's initiation, W. Y. Cobb, then Director of Federal-State Relations, FDA, at Rockville, MD, visited MSCL to discuss the possibility of an FDA contract with MSCL to develop a parallel program to collect information generated by state food regulatory officials on pesticide contaminants found in food products in their state and organize it by computer. These FOODCONTAM programs, as they were immediately named, would be used by both state and federal agencies to provide current, complementary state food contaminant data to parallel FDA's federal program findings. At that time, such FDA national data were available only as historic summary reports published 1–2 years after they were available to FDA District personnel, and were of little use to state regulators to help focus their current programs. FDA awarded a fixed price, 3-year contract to

MSCL to build a FOODCONTAM database and manage its operation.

State Chemist Minyard was principal investigator, with W. E. Roberts, coinvestigator, as database manager and systems analyst. Computer programs were completed and debugged in 120 days, although debugging and modifications of output formats have continued since that time. Within 9 months FDA had advertised and contracted with 3 state food regulatory agencies (New York, California, and Massachusetts) to provide data on raw agricultural foods produced in their state. Massachusetts agreed to supply their limited food data without charge. Foods of interest were confined to raw agricultural vegetables, fruits, grains, and ocean and freshwater fish and shellfish. Cooked and prepared mixed foods were excluded, as were milk, meat and poultry, eggs, and related animal-derived foods, except fish and shellfish.

Program concepts and contract requirements covered many general expectations. FDA wanted as many state food laboratories as possible to contribute data (at least 10–12). They wanted to cover the entire United States with a reasonably uniform geographic distribution, and required analytes to be limited to pesticides, polychlorinated biphenyls, and similar toxic industrial chemicals in raw agricultural food products. FDA hoped that state food laboratories would contract with them to deliver data to the MSCL Management Center for $2.00 per dataline or less. Various input mechanisms for receiving data were expected by the MSCL contractor, including formatted data in hard copy or in ASCII code on tape or disk from the originators' computer systems. FDA required state data to be compatible with their District laboratories' files. This in turn required that product category and matrix codes, pesticide analyte codes, geographic sampling location codes, and analytical methodology codes be compatible, and that all analyte concentrations be reported as ppm of analyte.

FDA/EDRO managers of the contract expected MSCL database managers to build all computer data management programs to run on microcomputers (MS-DOS, PC-DOS, or APPLE /// SOS microcomputers), or on popular minicomputer operating systems. They anticipated that state data would be entered in the FOODCONTAM input database by supervised clerks and verified by contract managers, for all expected input modes. It was critical that all data be compatible with FDA codes and file layout to facilitate federal-state data exchanges and merging of files to produce integrated national data files.

FDA also expected that database managers at MSCL would provide overall quality assurance/quality control of outputs of data as well as the input data provided by contracting states. FDA and FOODCONTAM managers anticipated producing a variety of summary outputs, and expected flexibility from the contractor in providing a variety of appropriate profiles of findings for each state in various formats. These included a semilogarithmic display of concentration findings for each analyte in each matrix, to facilitate probability predictions. FDA required the submitter to furnish summary data on the range of positive (nonzero) findings by each chemical, with the average of the total range for each chemical/matrix combination, plus the number of findings judged "significant" (those marked with an asterisk) by the submitting state official on the basis of established tolerances for that pesticide/food combination. The contractor was to provide quarterly and annual hard copy summaries of all significant data, grouped state by state, and sorted into other useful arrangements (by analyte, food type, state, etc.).

During the first half-year of the contract, W. E. Roberts developed programs, wrote subroutines, and loaded FDA files into MSCL microcomputers. These were needed to facilitate customized data transfer from a diversity of contributors' computerized or hard-copy data systems. Originally, all programs ran on the MSCL APPLE /// microcomputers with SOS Operating Systems, 512K RAM linked to 40 MByte hard disk units. Programs now run on a UNISYS 5000/50 32 Bit minicomputer, UNIX based. This minicomputer has 4 MByte RAM, 220 MByte Disk, and is networked with the current MSCL microcomputers, both APPLE /// and MS-DOS IBM PC equivalents.

State food laboratory contractors were to provide data in proper format and examine FOODCONTAM outputs of states' formatted data. State food control officials could then use the findings to enhance their own state food quality regulation programs. State contractors were expected to keep informed on FDA regulations, pesticide/product tolerances, and related information so that federal and state programs would be as compatible as possible.

Tables 1–6 were built into the computer programs for FOODCONTAM. All sets of tables, sample data entry sheets, instructions, and program management elements were sent to each state's contracting officer/manager. A 75-page printout of FDA's Food Product Table was sent to state contractors. Table 1 shows FDA product codes, and Table 2 represents a small excerpt of FDA's extensive listing of FDA codes for various food product categories, using 5 characters (2 numeric, 1 alpha, 2 numeric) for each food type. Table 3 is a typical county code list, with 3-digit numbers for each of California's counties; such codes for each state are standard

Table 1. Product codes (from the table of contents, FDA Food Product Table)

Product code	Product name
02	Whole grains, milled grain products and starch
03	Bakery products, doughs, bakery mixes, and icings
04	Macaroni and noodle products
05	Cereal preparations, breakfast foods
07	Snack food items (flour, meal or vegetable base)
09	Milk, butter and dried milk products
12	Cheese and cheese products
13	Ice cream and related products
14	Filled milk and imitation milk products
15	Eggs and egg products
16	Fishery/seafood products
17	Meat, meat products, and poultry
18	Vegetable protein products (simulated meats)
20	Fruits and fruit products
21	Fruits and fruit products
22	Fruits and fruit products
23	Nuts and edible seeds
24	Vegetables and vegetable products
25	Vegetables and vegetable products
26	Vegetable oils (includes olive oil)
27	Dressings and condiments
28	Spices, flavors, and salts
29	Soft drinks and waters
30	Beverage base
31	Coffee and tea
33	Candy without chocolate, candy specialties, and gum
34	Chocolate and cocoa products
35	Gelatin, rennet, pudding mixes, and pie fillings
36	Food sweeteners (nutritive)
37	Multiple food dinners, gravies, sauces, and special
38	Soups
39	Prepared salad products
40	Baby (infant and junior) food products

Table 2. Food product categories—example segment

Product code	24 T Leaf and stem vegetables
01	Artichokes
02	Asparagus
03	Bamboo sprouts
04	Beet tops
05	Broccoli
06	Broccoli raab (raab)
07	Brussels sprouts
08	Cabbage
09	Carrot tops
10	Cauliflower
11	Celery
12	Chinese cabbage, celery cabbage
13	Collards
14	Dandelion greens
15	Endive, escarole, chicory root, or chicory leaf
16	Field cress
17	Hanover cress
18	Kale
19	Lettuce or romaine
20	Mustard greens
21	Parsley
22	Pole greens
23	Rape greens
24	Rhubarb
25	Spinach
26	Swiss chard
27	Turnip greens
28	Water cress
29	Seaweed
99	Other leaf and stem vegetables, N.E.C.

Table 3. County codes: California (example)

Code	State name: California State abbreviation: CA County name	Code	County name
001	Alameda	059	Orange
003	Alpine	061	Placer
005	Amador	063	Plumas
007	Butte	065	Riverside
009	Calaveras	067	Sacramento
011	Colusa	069	San Benito
013	Contra Costa	071	San Bernardino
015	Del Norte	073	San Diego
017	El Dorado	075	San Francisco
019	Fresno	077	San Joaquin
021	Glenn	079	San Luis Obispo
023	Humbolt	081	San Mateo
025	Imperial	083	Santa Barbara
027	Inyo	085	Santa Clara
029	Kern	087	San Cruz
031	Kings	089	Shasta
033	Lake	091	Sierra
035	Lassen	093	Siskiyou
037	Los Angeles	095	Solano
039	Madera	097	Sonoma
041	Marin	099	Stanislaus
043	Mariposa	101	Sutter
045	Mendocino	103	Tehama
047	Merced	105	Trinity
049	Modoc	107	Tulare
051	Mono	109	Tuolumme
053	Monterey	111	Ventura
055	Napa	113	Yolo
057	Nevada	115	Yuba

Table 4. Extraction and cleanup analytical method codes—example segment

Code	Extraction and cleanup method
002	PAM I, 211.1 with 6% + 15% EtO/PE eluants only
003	PAM I, 211.1 with 6 + 15 + 50% EtO/PE eluants only
004	PAM I, 211.1 with MeCl eluants 1 only
005	PAM I, 211.1 with MeCl eluants 1 + 2 only
006	PAM I, 211.1 with MeCl eluants 1 + 2 + 3
007	PAM I, 212.1 with 6% EtO/PE eluants only
008	PAM I, 212.1 with 6 + 15% EtO/PE eluants only
009	PAM I, 212.1 with 6 + 15 + 50% EtO/PE eluants
010	PAM I, 212.1 with MeCl eluants 1 only
011	PAM I, 212.1 with MeCl eluants 1 + 2 only
012	PAM I, 211.1 with MeCl eluants 1 + 2 + 3
013	PAM I, 232.3 charcoal column cleanup procedure
014	Holden, E. R., JAOAC 56, 713–717 (1973) modified by LIB 167 Carb
015	PAM I, 232.4, 424.1 Luke method without column chromatography
016	LIBA 1874 method for triazine analysis
017	PAM I, 212.2 Luke method with Florisil column cleanup
018	JAOAC 63, 539–545 (1980) Clower method for fumigants
019	Acid/base/alumina/Florisil method for (C16–C18) dioxins
020	Acid/base/alumina/Florisil/HPLC for tetrachlorodioxins
021	JAOAC 63, 1114–1124 (1980) Krause method for N-methyl carbamate
022	AOAC, 13th Ed., 29.123–29.126 Bong method for HCB and mirex
023	LIB 2306 Hopper GPC procedure for CPA residues
024	JAOAC 66, 534–536 (1983) Erney method for fish
025	LIB 2716 with 6% EtO/PE eluant (Rev. of LIBA 2120A superfat)
026	LIB 2716 with 6 + 15% EtO/PE eluants (Rev. of LIBA 2120A superfat)
028	LIB 2716 with MeCl eluant 1 (Rev. of LIB 2120A superfat)
029	LIB 2716 with MeCl eluant 1 + 2 (Rev. of LIB 2120A superfat)
030	LIB 2716 with MeCl eluant 1 + 2 + 3 (Rev. of LIB 2120A superfat)
031	JAOAC 64, 1252–1254 (1981) triple codistillation with hexane
032	LIB 2338C ethyl acetate codistillation for EDB in citrus fruits
101	PAM I, 211.1 with PE forerun + 6% mixed ether eluants (211.1)
102	PAM I, 211.1 with PE forerun + 6% + 15% mixed ether eluants
103	PAM I, 211.1 with PE forerun + 15% + 50% mixed ether eluants

U.S. Postal Codes for alphabetized county-parish names. Table 4 shows an example of FDA's codes for extraction and cleanup analytical methods used in their District laboratories. Each data line submitted for a specific analyte/matrix/pesticide finding is accomplished by such a code. Most of these are screening methods that cover a range of chemically related analytes. This information allows scientists to judge whether a specific analyte, if present, might have been found in the specific analyses run on that food by the state. Table 5 presents the standard FDA detection codes that define the type of instrumental detector used, and Table 6 is a portion of an alphabetic list of analytes that can be quantified by one or more of the various analytical methods. Approximately 700 analytes are covered, each coded by a 3-digit number.

Different kinds of hard-copy data input were accommodated by the FOODCONTAM management group. An FDA hard-copy laboratory management coding sheet is shown in Figure 1, for data submitted by the Virginia Division of Laboratory Services. The Commonwealth of Virginia was already using this form to share data with the FDA Baltimore District office. Photocopies of their submissions to Baltimore are now sent to program managers, and the data are rekeyed into FOODCONTAM format. Hard-copy data are handwritten by scientists or clerks under scientific super-

Table 5. Detection codes

Code	Detection procedure
01	GLC-electron capture detector
02	GLC-phosphorus selective detector (FPD)
03	GLC-sulfur selective detector (FPD)
04	GLC-nitrogen selective detector (Hall 700A)
05	GLC-halogen selective detector (Hall 700A or microcoulometric)
06	GLC-FID
07	GLC-MS
08	GLC electron capture detector
09	Thin layer chromatography (TLC)
10	Paper chromatography
11	GLC-UV detector
12	HPLC post-column derivative and fluorometric determination (Krause MTD carbamate)
13	GLC-phosphorus selective detector, LIB 2166 (aryl phosphates)
14	GLC-phosphorus selective detector, polar column-eg DEGS
15	GLC-nitrogen selective detector, polar column-eg DEGS
16	GLC-alkali flame detector (N/P or KC1TD)
17	GLC-halogen selective detector, LIB 1710/1710A early elute
50	Flame atomic absorption (AA)
51	Hydride generation (AA)
52	Cold vapor (AA)
53	Furnace (AA)
54	Flame emission (FE)
55	Inductively coupled plasma (ICP) FE
56	Direct current plasma (DCP) FE
57	Fluorescence spectroscopy
58	Gas-liquid chromatography (GLC)
59	Liquid chromatography (LC)
60	Spectrophotometric
61	Thin layer chromatography (TLC)
62	Differential pulse anodic stripping voltammetry
63	Differential pulse cathodic stripping voltammetry
64	Polarography (differential pulse)
65	Ion selective electrode
66	Other electrochemical method
67	Neutron activation
99	Other

Table 6. Analyte codes, alpha order (example segment)

Code	Analyte
515	1,1,1-Trichloroethane
557	1,1,2,3,4-Pentachloro-4-isopropoxybutadien, trans
643	1,1-Dichloro-2,2-bis(p-methoxyphenyl)ethane (methoxy)
577	1,2,3,4-Tetrachlorobenzene
575	1,2,3,4,6,7,8-Heptachlorobenzo-p-dioxin
574	1,2,3,4,6,7,9-Heptachlorobenzo-p-dioxin
531	1,2,3,5-Tetrachlorobenzene
572	1,2,3,6,7,8-Hexachlorobenzo-p-dioxin
571	1,2,3,6,7,9-Hexachlorobenzo-p-dioxin
573	1,2,3,7,8,9-Hexachlorobenzo-p-dioxin
570	1,2,4,6,7,9-Hexachlorodibenzo-p-dioxin
582	1,2,3-Trichlorobenzene
583	1,2,4-Trichlorobenzene
584	1,3,5-Trichlorobenzene
291	1,3-Pentadiene
559	1-Chloro-4-nitrobenzene
281	1-Hydroxychlordane
563	1-Methoxy-4-(1,2,2,2-tetrachloroethyl) benzene
152	2,3,4,6-Tetrachlorophenol
508	2,3,5,6-Tetrachloroaniline
535	2,3,5,6-Tetrachloroanisidine
533	2,3,5,6-Tetrachloroanisole
319	2,3,6-TBA
263	2,3,7,8-Tetrachlorodibenzo-p-dioxin
565	2,3-Dichloronitrobenzene
312	2,4,5-T
366	2,4,5-T N-butyl ester
619	2,4,5-T ethylhexyl ester
270	2,4,5-Trichlorophenol
269	2,4,6-Trichlorophenol
026	2,4-D
618	2,4-D ethylhexyl ester
630	2,4-D isopropyl ester
142	2,4-D, sodium salt
317	2,4-DB

vision. This tedious task is simplified by some states, who key data into commercial microcomputer database programs, organized in standard input format. After getting states oriented in program activities, Minyard and Roberts urged state officials to transfer data by tape or diskette, rearranged from state to FOODCONTAM input format. MSCL prefers to receive quarterly data in formatted ASCII code on $5^1/_2$ or $3^1/_2$-in. diskettes, or 9-track, 1600 BPI tape.

At the start of FOODCONTAM, FDA managers solicited data submission proposals by contract from all state food and drug officials. Only 3 states responded and contracted with FDA to supply data: New York Department of Agriculture and Markets, Massachusetts Department of Public Health, and California Department of Food and Agriculture. Because of the complexity required for responding to a proposal for a contract, several states who were interested did not initially contract with FDA (Virginia and Florida).

The 3 contracting states began sending data on April 1, 1986. Meanwhile, MSCL staff worked with Florida and Virginia to develop simpler data transfer mechanisms, and FDA simplified their contracting mechanism. Florida now sends 9-track 1600 BPI tapes of results, which are "translated" to FDA code equivalents. Virginia sends hard-copy information on FDA forms. Both Florida and Virginia participate on a simple cost reimbursement basis, at minimal costs to FDA. FDA's contract managers have now solicited more states to provide data via this simpler mechanism. As contract mechanisms have evolved and customized data transfer techniques have become easier, both Wisconsin and Michigan have joined. These states are paid at a negotiated rate per line of properly formatted and coded data.

A 2-year extension of the 3-year development contract was provided on September 25, 1987, to implement the program further, solicit increased participation by more states, and help merge state and FDA data outputs to generate a true national food contaminant database. Several more states plan to share their data, either free or under FDA contract. Four quarterly reports and one annual report of data have been published under this contract extension.

Program managers built several large "equivalency translator tables" for Florida to convert their internal coded management reports to FOODCONTAM equivalents, using tables to equate Florida's codes (matrixes, analytical methods, and instrumental detectors) to FOODCONTAM table equivalents. "Custom conversion" is available for any state willing to share data. Program managers have worked closely with each state to bridge the gap between that state's in-house report management systems and FOODCONTAM formats.

Figure 2 shows a portion of FOODCONTAM's annual report for fiscal year 1988 in tabular format. The FDA cooperative agreement with MSCL requires that data summaries be sorted in 3 possible sequences: product/analyte/state, analyte/product/state, and state/analyte/product. It also requires that data be printed in a semilogarithmic con-

LABORATORY MANAGEMENT CODING SHEET

(1) SAMPLE NUMBER: XVA47511
(2) PAC CODE: 04004A
(3) LID CODE: V
(5) PRODUCT DESCRIPTION: GREEN SWEET PEPPERS
(6) ANALYZING DISTRICT: 2
(7) SAMPLE FLAG: L
(8) SAMPLE TYPE: N
(9) CENTRAL FILE NUMBER: NOCE
(10) JD/TA: VA
(12) DATE COLLECTED (M M D D Y Y): 061785
(13) PRODUCT CODE: 24F--03
(14) HOME DISTRICT: 2
(15) SAMPLING DISTRICT: 2
(16) DATE SAMPLE RECEIVED (M M D D Y Y): 061985
(18) LAB ASSIGNED: X
(19) DATE ASSIGNED (M M D D Y Y): 061985
(20) ANALYST ASSIGNED: 00999
(21) PCLS: S
(23) NUMBER OF EXAMS: 0000
(24) HOURS WORKED: 000.0
(25) DATE ANALYSIS COMPLETED (M M D D Y Y): 071085
(26) LAB CLASS: 1
(27) DATE OUT OF LAB (M M D D Y Y): 071085

	RESI CODE	RESIDUE FOUND	EXT CODE	DETER CODE	VIOL RESI
1.	000		[illegible]	[illegible]	
2.	000		[illegible]	[illegible]	
3.	[illegible]		[illegible]	[illegible]	
4.					
5.					
6.					

PAC CODES

04004A - Pesticides and Industrial Chemicals

67001A - Chemical Contaminants in Animal Feeds

LAB CLASS

1 - Residue(s) < Tolerance or Acti[illegible] and No Residue Found Where No [illegible] Level or Tolerance Exists In T[illegible]
2 - Residue(s) at Trace Level Wher[illegible] Level or Tolerance Exists
3 - Violative

Figure 1. FDA hard-copy laboratory management coding sheet.

centration range format (similar to FEEDCON) including "N.D." (nondetected), "Trace," and 0.001-300 ppm in the ranges 0.001-0.003, 0.003-0.010, etc. An example of this product printout is shown in part in Figure 3 for each pesticide/food commodity combination, with a summary of the range of residue findings for each pesticide in that food. The portion of the report represented in Figure 3 also shows the total number of samples, total positive findings, and total significant findings for every pesticide/food combination examined. Codes for analytical methods used for each summary line are provided for FDA managers to use in subsequent scientific/regulatory evaluations of these detailed data. Six states (California, Florida, Massachusetts, New York, Virginia, Wisconsin) provided 34 181 lines of coded data to this program in federal FY88. Of this number of samples, 4454 (13%) were found to contain a measurable amount of a pesticide. Only 254 (0.7%) of these were listed as significant, i.e., exceeding federal tolerances for that food/pesticide combination.

As FOODCONTAM (and FEEDCON) have been publicized in presentations at regional and national meetings of food and feed regulatory officials and scientists, participation has grown. Many states have been solicited to join the program. North Carolina, Oregon, Wyoming, and Kentucky food control officials have been asked to join, either free or under negotiated FDA contract mechanisms, and some states are considering joining in the near future.

All FOODCONTAM programs will run on IBM equivalent machines with PC-DOS, MS-DOS, or C-PM operating systems. All programs, lookup tables of codes, and other codes are on diskettes, updated and backed up every week. Programs are documented (hard copy) and backed up on the University's UNIVAC 1100/70 mainframe located in a building adjacent to MSCL's location. Backup is on 9-track

01-06-1989

FDA Food Contaminant
Annual Report - FY88
Summary by Product/Analyte/State

BABY (INFANT AND JUNIOR) FOODS, N.E.C.: BABY (INFANTS AND JUNIOR) FOODS N.E.C.

	St	Ext	Det	PPM		
CARBARYL	FL	999	99	.02		40Y99
DDE; SEE 910,911	FL	999	99	.01	*	40Y99

BEANS-PEAS-CORN: BLACKEYED PEAS

	St	Ext	Det	PPM		
MALATHION	VA	012	01	.18		24A50

BEANS-PEAS-CORN: CORN

	St	Ext	Det	PPM		
CARBARYL	CA	999	59	.400		24A60
CARBARYL	CA	999	59	.440		24A60

BEANS-PEAS-CORN: FAVA BEANS

	St	Ext	Det	PPM		
CHLOROTHALONIL	CA	999	01	.060		24A05
DDE; SEE 910,911	CA	999	05	.070		24A05

BEANS-PEAS-CORN: FIELD PEAS (SMOOTH), CROWDER WHITE

	St	Ext	Det	PPM		
CARBARYL	FL	999	99	.26		24A54
DIAZINON	CA	999	02	.040		24A54

BEANS-PEAS-CORN: GREEN PEAS, GARDEN PEAS, SWEET PEAS

	St	Ext	Det	PPM		
CHLOROTHALONIL	CA	999	01	.030	*	24A51
CHLORPYRIFOS	CA	999	16	.340		24A51
DDE; SEE 910,911	CA	999	05	.100		24A51
DIAZINON	NY	015	16	0.02		24A51
DIMETHOATE	CA	999	02	.060		24A51
DIMETHOATE	CA	999	02	.060		24A51
DIMETHOATE	CA	999	02	.350		24A51
DIMETHOATE	CA	999	16	.080		24A51
DIMETHOATE	CA	999	16	.110		24A51
DIMETHOATE	CA	999	16	.130		24A51
DIMETHOATE	CA	999	16	.200		24A51
DIMETHOATE	CA	999	16	.200		24A51
DIMETHOATE	CA	999	16	.200		24A51
DIMETHOATE	CA	999	16	.280		24A51
DIMETHOATE	CA	999	16	.410		24A51
DIMETHOATE	CA	999	16	.660		24A51
DIMETHOATE	CA	999	16	.690		24A51
DIMETHOATE	NY	015	16	0.18		24A51
OMETHOATE	CA	999	02	.050		24A51
OMETHOATE	CA	999	16	.090		24A51

BEANS-PEAS-CORN: OTHER BEANS-PEAS-CORN, N.E.C.

	St	Ext	Det	PPM		
ACEPHATE	CA	999	16	.030		24A99
CARBARYL	CA	999	12	.240		24A99
DCPA	CA	999	05	.100		24A99
DCPA	FL	999	99	.05		24A99
ENDOSULFAN; SEE 900,901,902	CA	999	05	.300		24A99
ENDOSULFAN; SEE 900,901,902	CA	999	05	1.30		24A99
METHAMIDOPHOS	CA	999	02	.010	*	24A99
PARATHION	CA	999	02	.030		24A99
PARATHION	CA	999	02	.100		24A99
PARATHION	CA	999	02	.380		24A99

Figure 2. Example from the FOODCONTAM annual report for fiscal year 1988.

1600 BPI ASCII tapes and on MSCL removable 10 MByte cartridge tapes for an INFAX 201A tape unit in Hand Laboratories, where MSCL is located. Fiber-optics cables connect MSCL to the mainframe and thence to BITNET, a national high speed data network.

Roberts and Minyard have also developed a simplified data entry program to run on MS-DOS or PC-DOS equivalent microcomputers. The program disk contains all lookup tables necessary to provide easy access to the complex FDA product codes for food matrixes, analytes, and analytical methods, as well as county/state geographic location codes. These are selected by the operator from built-in tables as keyboard entry proceeds. This disk, plus user instructions, is available free to any state agency that will share its state food contaminant data with FOODCONTAM. The program, written in PASCAL, runs on microcomputers under MS/DOS Version 2.0 to 3.4. This data entry program has been described at several recent scientific meetings and is

FDA Food Contaminant
California (FY88)

01-07-1989

*****FRUITS AND FRUIT PRODUCTS Continued

Prod Code	Product Commodity	N.D.	Trace	0.001 0.003	0.003 0.010	0.010 0.030	0.030 0.100	0.100 0.300	0.300 1.000	1.000 3.000	3.000 10.00	10.00 30.00	30.00 100.0	100.0 300.0	300.0 >	Total Smp	Total Pos	Total Sig
	159 METHOMYL — Range 0.3 - 0.3																	
		.	.	.	.	.	.	.	1	.	.	.	.	.	.	1	1	0
	Methods -> 99912 - 1																	
	164 CHLOROTHALONIL — Range 0 - 0																	
		1	.	.	.	.	.	.	.	.	.	.	.	.	.	1	0	0
	Methods -> 99901 - 1																	
	166 PHOSALONE — Range 0 - 0.94																	
		1	.	.	.	.	.	.	1	.	.	.	.	.	.	2	1	0
	Methods -> 99905 - 1 99902 - 1																	
	171 DIMETHOATE — Range 0 - 0.9																	
		1	.	.	.	.	1	4	2	.	.	.	.	.	.	8	7	0
	Methods -> 99916 - 2 99902 - 6																	
	178 OMETHOATE — Range 0.4 - 0.4																	
		.	.	.	.	.	.	.	1	.	.	.	.	.	.	1	1	0
	Methods -> 99902 - 1																	
	593 PROCYMIDONE — Range 0 - 0																	
		22	.	.	.	.	.	.	.	.	.	.	.	.	.	22	0	0
	Methods -> 99905 - 22																	
	Commodity Summary: BERRIES: GRAPES															559	19	0
20A10	BERRIES: RAISINS (DRIED GRAPES)																	
	000 NO RESIDUE FOUND — Range 0 - 0																	
		3	.	.	.	.	.	.	.	.	.	.	.	.	.	3	0	0
	Methods -> 99902 - 1 99905 - 1 99960 - 1																	
	Commodity Summary: BERRIES: RAISINS (DRIED GRAPES)															3	0	0
20A12	BERRIES: RASBERRIES, BLACK																	
	000 NO RESIDUE FOUND — Range 0 - 0																	
		18	.	.	.	.	.	.	.	.	.	.	.	.	.	18	0	0
	Methods -> 99901 - 3 99916 - 3 99959 - 3 99902 - 3 99905 - 3 99912 - 1 99960 - 2																	
	Commodity Summary: BERRIES: RASBERRIES, BLACK															18	0	0
20A14	BERRIES: STRAWBERRIES																	
	47 DICOFOL; SEE 253, 254 — Range 2.7 - 2.7																	
		.	.	.	.	.	.	.	.	1	.	.	.	.	.	1	1	0
	Methods -> 99901 - 1																	
	52 MALATHION — Range 0.1 - 0.12																	
		.	.	.	.	.	.	2	.	.	.	.	.	.	.	2	2	0
	Methods -> 99902 - 2																	
	000 NO RESIDUE FOUND — Range 0 - 0.6																	
		262	.	.	.	.	.	.	1	.	.	.	.	.	.	263	1	0
	Methods -> 99902 - 36 99916 - 79 99905 - 14 99901 - 45 99912 - 11 99959 - 61 99960 - 17																	
	011 CAPTAN — Range 0 - 4.22																	
		1	.	.	.	.	.	3	9	6	1	.	.	.	.	20	19	0
	Methods -> 99905 - 11 99901 - 9																	

Figure 3. Example of semilogarithmic distribution of data; the example is taken from California data.

now used successfully by MSCL and several state laboratories. The program greatly simplifies data entry, and has stimulated states to cooperate more readily.

With the help of David Winters of the FDA Cincinnati District laboratory, Roberts and Minyard have shown how easily FDA quarterly national data from FDA District laboratories can be merged into FOODCONTAM. It is possible to report both state and FDA national data in a unified hardcopy output, or to communicate via BITNET at 54 KBaud to FDA or state laboratories that have computer access to this national network, on which Mississippi State University is a node. Data can also be transferred nationally by state and regional food regulators at minimum cost and difficulty through FDA's NRSTEN data network, which is available to state food regulatory program managers.

Plans are being developed by federal and state members of the Association of Food and Drug Officials (AFDO) for discussions on protocols for this expanded national data sharing, plus caveats and constraints on the appropriate use of such data. This will be done in cooperation with the staff of FDA's Office of Federal-State Relations (Heinz Wilms, Director).

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Effect of Commercial Processing on Pesticide Residues in Selected Fruits and Vegetables

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Commercial food processing operations such as washing, blanching, and cooking remove major portions of the pesticide residues that are currently permitted on the raw agricultural crop. These unit operations are reviewed for selected products, along with degree of residue removal at each step. For example, washing plus peeling removes 99% of carbaryl and malathion residues from tomatoes. Washing removes 83% of benomyl residue from tomatoes and further processing reduces the residue by 98% in tomato puree and catsup. Even in the most concentrated fraction from tomatoes (tomato paste), residues were below the initial level in the raw product.

The National Food Processors Association (NFPA) has long been interested in the effect of commercial processing on pesticide residues in foods. NFPA, a scientific, technically based trade association, represents nearly 600 companies including most of the major food processing companies in the United States. Approximately 450 of these companies are involved in processing fruits, vegetables, meats, fish, and specialty items, using canning, freezing, aseptic treatment, concentration, dehydration, and pickling as preservation methods. Simply put, the mission of NFPA is "To serve the food processing industry and consumers by helping to assure the safety, wholesomeness, and nutritional value of the nation's food supply."

A June 1987 report from the National Academy of Sciences on regulating pesticides in food suggests that many oncogenic pesticides concentrate in processed foods. Information is presented here to show that processing almost always considerably reduces residue levels.

However, residue control begins even before processing. We believe that the "focal point for controlling pesticide residues is at the point of application." Residue analysis of the final product requires significant analytical effort and the expenditure of large amounts of money. When the product is in the package and ready for shipment to the market, it is too late to exercise *preventive* residue control measures. For that reason, since 1960 the food processing industry has utilized the NFPA Protective Screen Program. The objective of this program is the prevention of illegal or unnecessary residues in processed foods. The program, a set of detailed recommendations that have evolved from more than 25 years of experience in the operation of active programs for the prevention of illegal and unnecessary residues, is published each year in *The Almanac of the Canning, Freezing and Preserving Industry* (James J. Judge Inc., Westminster, MD). The collection and analysis of samples is designed to verify that these preventive measures are working.

Presented at the Symposium on Pesticides in Foods: Coping with the Issue—Programs and Analytical Methods, 102nd AOAC Annual International Meeting, Aug. 29–Sept. 1, 1988, Palm Beach, FL.

Unit Operations that Remove Pesticide Residues

Recovery of the edible portion of a vegetable or fruit may involve husking, peeling, or shelling operations which effectively remove most of the pesticide with the discarded portions of the plant. Peas and corn are examples of products in which pesticides seldom if every come into contact with the edible portion.

On arrival at the canning plant, the product is subjected to several immersion or spray washing operations which sometimes involve use of a detergent to aid in cleaning. Blanching is a short treatment in hot water or steam applied to most vegetables such as peas, green beans, spinach, and broccoli. Washing and blanching may remove much of the pesticide residue. Most fruits are subjected to peeling or juice extraction. The peel and extracted plant material constitute a major portion of the solid waste resulting from procedures applied within the canning plant itself.

Filling and closing are of no significance in connection with the pesticide residue content, except for the diluting effect of the added syrup or brine. The heat process itself is ordinarily carried out on the closed and sealed container and could be expected to result in substantial destruction of compounds subject to hydrolysis and heat effects. Some pesticides, if present at the time of retorting or cooking, will leave trace amounts of their degradation products. For example, captan will degrade to THPI, daminozide to UDMH, and the ethylenebisdithiocarbamates to ethylenethiourea. The goal of the processor is to have no parent compound present at the time of heat processing so that degradation products of concern will not be formed.

Pesticide Removal by Washing

The extent to which pesticide residues are removed by commercial washing depends on a variety of factors, such as the chemical properties of the pesticide, the nature of the food commodity, the length of time the compound has been in contact with the food, and the formulation in which the pesticide was applied. Another factor that is often overlooked is whether it has rained since the last application. If it has rained, residues on the raw agricultural commodity may be quite low, but if it has not, residues could be quite high. Table 1 shows current tolerances for malathion, parathion, carbaryl, diazinon, benomyl, and maneb on tomatoes, spinach, and broccoli.

Figure 1 shows the effect of washing on carbaryl on tomatoes, spinach, and broccoli and on diazinon on tomatoes and spinach. Figure 2 shows the effect of washing on malathion on tomatoes and parathion on spinach and broccoli. These data were all obtained in field-treated products.

Table 1. Current U.S. tolerances for selected pesticides (parts per million)

Pesticide	Tomatoes	Spinach	Broccoli
Malathion	8	8	5
Parathion	1	1	1
Carbaryl	10	12	10
Diazinon	0.75	0.7	—[a]
Benomyl	5	0.2	0.2
Maneb	4	10	10

[a] Not registered for use on this crop.

Several detergents have been accepted for use as aids in washing products in preparation for canning (Figure 3). Detergents were significantly beneficial in increasing removal of parathion from spinach and broccoli.

Carbaryl was easily removed by washing from tomatoes (97%), from spinach (87%), and from broccoli (77%). Washing removed 88% of the diazinon residue from tomatoes, whereas an 11% increase was shown for spinach (Figure 1). The broad-leaf nature of the crop may be partly responsible for the difficulty of removing diazinon. However, these results are expressed on a dry weight basis, which in some cases can lead to an apparent increase in the residue and tends to lower the value for percent removed. Figure 2 shows that washing removed 95% of the malathion residue from tomatoes but only 9% of the parathion from spinach; the parathion residue on broccoli was increased by 11%.

The effect of washing on benomyl residues was as follows: for tomatoes (n = 5): unwashed, 1.76 ppm; washed, 0.31 ppm; removed, 82%. For oranges: unwashed, 3.28 ppm; washed, 0.75 ppm; removed, 77%. For apples (n = 7): unwashed, 1.06 ppm, washed, 0.89 ppm; removed, 16%.

The effect of washing on maneb residues was as follows: for leafy greens: unwashed, 23.0 ppm; washed, 6 ppm; removed, 73.9%. For spinach, first run: unwashed, 1.13 ppm; washed, 0.3 ppm; removed, 73.5%. For spinach, second run: unwashed, 23.1 ppm; washed, 2.5 ppm; removed, 94.3%.

The tolerance for maneb on leafy greens is 10 ppm before washing and, unlike any other commodity, 10 ppm on spinach after washing. The initial residue (23 ppm) is high but normal for this pesticide and this product, and may have been due to the absence of rain after the last application of maneb. Thorough washing is a very important step in processing spinach and leafy greens. Two types of washers are used. One is an immersion washer in which the greens are immersed and propelled through a tank of water by paddles, with considerable agitation. The other, a rotary-type washer, is a long reel equipped with even-speed, high-pressure water

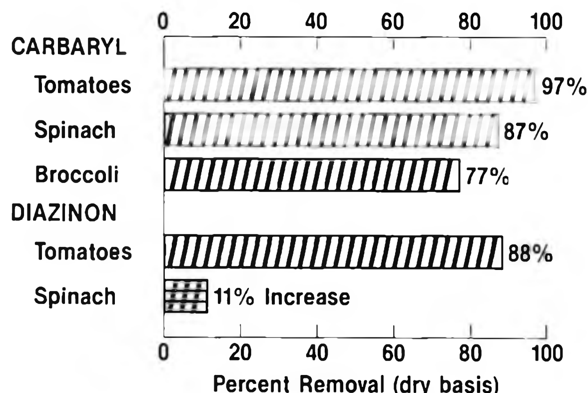

Figure 1. Pesticide removal by water washing: carbaryl and diazinon.

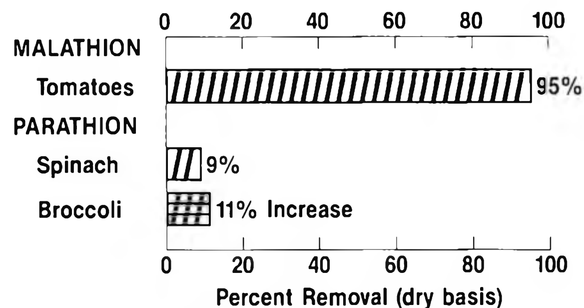

Figure 2. Pesticide removal by water washing: malathion and parathion.

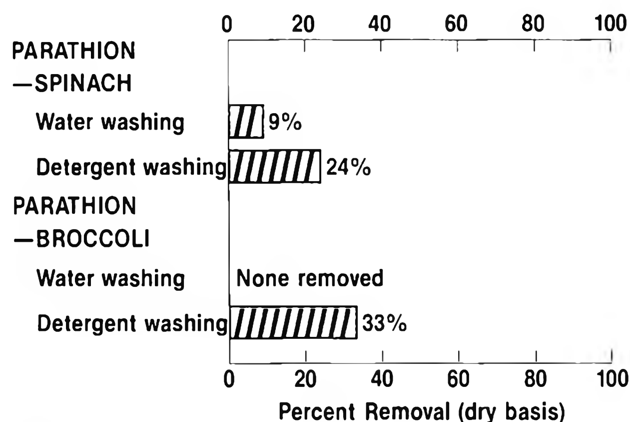

Figure 3. Effect of detergents as aids in pesticide residue removal.

sprays. The most efficient method of washing includes the use of both washers in series with the immersion washer first. At least 2 or 3 washings are necessary when processing greens.

Figure 4 shows pesticide removal by washing plus blanching for carbaryl and parathion. Removal of carbaryl from green beans by blanching only was 68%. Actually, green beans are washed and blanched at the same time. Total removal of carbaryl by washing and blanching was 97% from spinach and 98% from broccoli. Blanching alone removed 60% of the diazinon residue from spinach. It should be recalled that diazinon residues increased by 11% during washing.

Figure 5 shows 71% removal of malathion from green beans by blanching and parathion from spinach by washing and blanching. Blanching alone removed 10% of the parathion residue from broccoli.

Pesticide Removal by Washing, Blanching, and Processing

The effect of washing plus blanching on maneb residues on leafy greens was as follows: raw greens, 23 ppm; washed greens, 6 ppm, removed, 73.9%; blanched and frozen greens, 1.13 ppm, total removed, 95.1%.

Although turnip greens are canned, these experiments include freezing only. Commercial processing removed 95.1% of the original maneb residue.

The effect of washing, blanching, and canning on maneb residues on spinach was as follows: first run: unwashed, 1.13 ppm; washed, 0.3 ppm; blanched and canned, 0.05 ppm; removed, 100%. Second run: unwashed, 23.1 ppm; washed, 2.5 ppm; blanched and canned, 0.2 ppm; removed, 99.6%. Actually, virtually all the maneb residue is removed by canning. However, if any maneb residue is left after washing and blanching, the heat treatment will degrade it to ethylenethiourea (ETU).

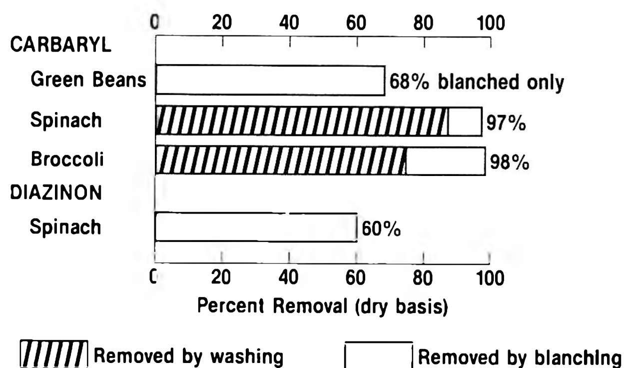

Figure 4. Pesticide removal by washing plus blanching: carbaryl and diazinon.

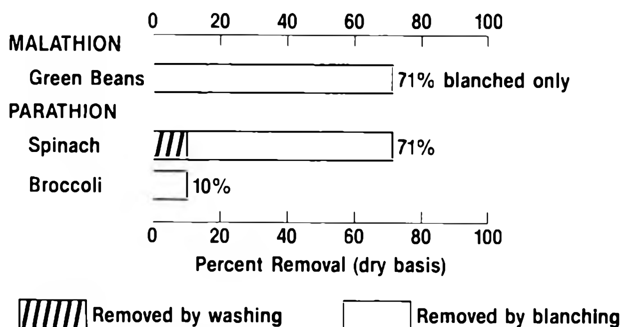

Figure 5. Pesticide removal by washing plus blanching: malathion and parathion.

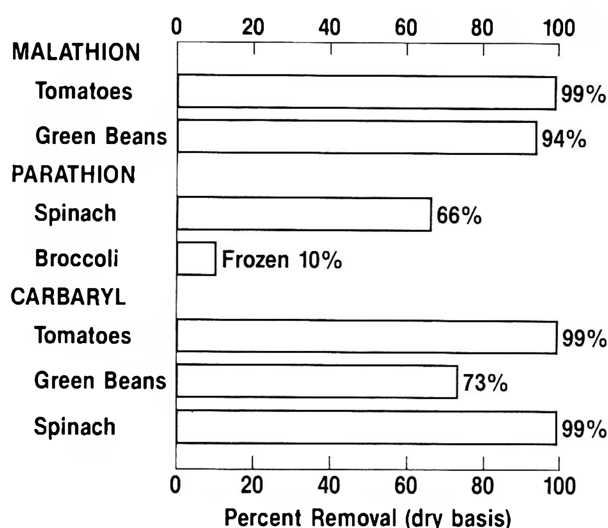

Figure 6. Pesticide removal by washing plus blanching plus canning.

Table 2. Residue content of tomatoes and tomato waste

Product	Malathion, ppm	Carbaryl, ppm
Unwashed	15.9	5.2
Washed	0.8	0.14
Peeled	0.1	trace
Waste	5.3	0.1

Effect on ETU residues: first run: unwashed, 0.06 ppm; washed, 0.09 ppm; blanched and canned, 1.1 ppm; increase, 94.5%. Second run: unwashed, 0.01 ppm; washed, 0.01 ppm; blanched and canned, 0.16 ppm; increase, 87.5%. Third run: unwashed, 0.7 ppm; washed, 0.04 ppm; blanched and canned, 0.54 ppm; removed, 23%. Note that some ETU is present on the raw agricultural commodity before washing. Run 1 was made on leafy greens that were not canned; the 1.1 ppm of ETU was the result of cooking the frozen turnip greens in a saucepan, which increased the ETU residue by 94.5%. The increase in ETU was due to degradation of the maneb present on the product at time of heat treatment. Run 3 shows a reduction in ETU, but this is due to the large amount that was present on the raw agricultural commodity before washing; 23.1 ppm of maneb was present on the raw spinach, and washing reduced the concentration to 2.5 ppm. Blanching and canning degraded the maneb to ETU, reducing the residue to 0.54 ppm.

Effect of processing on benomyl residues in tomato products was as follows: For tomatoes: unwashed, 1.76 ppm; washed, 0.31 ppm; removal, 82%. For juice: processed, 0.25 ppm; removed, 86%. For catrsup: processed, 0.03 ppm; total removed, 98%.

Benomyl is removed from oranges during processing into juice, molasses, and orange oil. Of the initial 3.28 ppm benomyl residue, 93% was removed by processing into juice, 90% by processing into molasses, and 23% by processing into oil.

Benomyl is removed from apples processed into juice, sauce, and slices. Washing removed 16% of the 1.06 ppm residue; 71% was removed in preparation of juice, 83% by processing into applesauce, and 91% by processing into slices.

Figure 6 shows pesticide removal by washing plus blanching plus canning. These operations removed 99% of the malathion from tomatoes and 94% from green beans. Of the initial parathion residue, 66% is removed from spinach but only 10% from broccoli; 99% of the carbaryl residue is removed from tomatoes and spinach and 73% from green beans.

The data on the residue content of tomatoes and tomato wastes (Table 2) indicate the kind of distribution that may occur in the waste from the processing and washing of fruits and vegetables. Malathion tends to concentrate in the peel or waste, while carbaryl, a fairly polar compound, is easily removed by washing. Carbaryl does not tend to concentrate in waste material.

Summary

As the information presented in this paper shows, canning plant operations remove most of the pesticide residues present on crops received for processing. The percentage removed varies; it depends on the nature of the crop, the pesticide and its formulation, and the weathering history. Many of the processing steps involved reduce residue levels by more than 90%. Steps such as concentration, dehydration, and extraction occur after the residue level from the raw product has already been significantly reduced. The net influence of processing almost always results in residue levels in processed foods well below the tolerance for the raw product this includes the systemic pesticides as well.

Finally, careful attention should be given to the concepts described in the NFPA Protective Screen Program to assure that desired residue levels are achieved in the food supply without the costs associated with excessive final product monitoring.

Tolerance Setting Process in the U.S. Environmental Protection Agency

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The U.S. Environmental Protection Agency (EPA) is responsible for setting tolerances for pesticide residues in food, under the authority of the Federal Food, Drug, and Cosmetic Act. The residue chemistry data required to set tolerances include metabolism in plants and animals, analytical methods, magnitude of the residue, and concentration in processed foods. A key aspect of tolerance-setting procedures is the identity of the residue in the matrix of concern; without knowledge of the chemical moieties that occur as residues, it is impossible to develop suitable methods or generate meaningful residue data. For new chemicals, EPA carries out a single-laboratory validation of the analytical method needed to generate residue data and to enforce tolerances. Tolerance enforcement methods need to be rapid and inexpensive and to use commercially available equipment and reagents. Methods are more complex for many newer pesticides, which are polar compounds that leave low levels of residue. EPA now requires that the registrants of older pesticides, for which methods are not acceptable by today's standards, must develop better methods.

The U.S. Environmental Protection Agency (EPA) is responsible for the registration of all pesticides sold or distributed in the United States. Before a pesticide can be registered for use on a food or feed crop, a tolerance for residues of that pesticide must be established. A tolerance is defined as the legal maximum residue concentration of pesticide chemical allowed in food or feed. Tolerances are set high enough to cover residues that result from registered use of the pesticide. Tolerances minimize uncertainty about food safety with regard to those pesticide residues, because if residues are below tolerance, EPA has determined that these residues are safe. If residues exceed the tolerance or if no tolerance is established, the crop may be considered adulterated and may be seized by the U.S. Food and Drug Administration (FDA), the U.S. Department of Agriculture (USDA), or a state enforcement agency. Although EPA establishes tolerances for pesticides, the agency has no responsibility for enforcing these tolerances. Enforcement is carried out by FDA, USDA, and the states.

Tolerances are set under the authority of the Federal Food, Drug, and Cosmetic Act. Section 408 of this Act applies to residues on raw agricultural commodities, and Section 409 applies to processed food or feed. Section 409 includes the Delaney Clause, which specifically prohibits the use of cancer-causing agents as food or feed additives. The EPA announced a new policy in October 1988 that adopts the position that the Delaney Clause does not prohibit a food or feed additive tolerance if the expected pesticide residues pose no more than a negligible risk of cancer.

The following is a description of the residue chemistry data required to establish a tolerance. In addition to residue chemistry data, data on toxicology, environmental fate, and ecological effects are also required. The data described below are not generated by EPA laboratories; rather, EPA requires the manufacturers of these pesticides to generate the data before a pesticide can be registered. For minor crops, the residue data are often generated by USDA as part of the Inter-Regional Project Number 4, (IR-4).

Presented at the Symposium on Pesticides in Foods: Coping with the Issue—Programs and Analytical Methods, 102nd AOAC Annual International Meeting, Aug. 29-Sept. 1, 1988, Palm Beach, FL.

Three general types of residue chemistry data are essential for establishing tolerances:

1. Qualitative data on metabolism degradation.
2. Quantitative data on magnitude of the residue.
3. Analytical methods.

The purpose of the residue data is to answer 2 basic questions. First, what is the chemical residue? Second, how much residue is there? Analytical methods are essential for providing answers to these 2 fundamental exposure questions. The "what" and "how much" information is used by EPA toxicologists to determine whether the dietary exposure is acceptable.

The starting point of any chemistry review is the composition of the test substance. EPA requires data on the composition of pesticide chemicals as part of its product chemistry data requirements. The product chemistry data are used to determine whether toxic impurities pose a risk to applicators of pesticides or whether impurities pose a risk as a residue in food. An analytical method capable of assaying the percent active ingredient in a pesticide product is required as part of the product chemistry data requirements. Collaborative studies on formulation methods are carried out under the auspices of the AOAC Committee on Pesticide Formulations and Disinfectants.

Metabolism Data

EPA requires metabolism data in order to answer the question: "What is the chemical residue in food?" Two types of metabolism data are required: data on plants and on animals.

Plant metabolism data characterize the nature of the residue that occurs in crops intended for consumption as a food or an animal feed. These data identify the alteration products the analyst should look for in the agricultural crops as the result of environmental transformation processes such as degradation and metabolism. These metabolism studies use radiolabeled pesticides, usually with ^{14}C. The radiolabeled pesticide is applied to the crop in a manner simulating actual use. The radiolabeled residue remaining in the harvested commodities is identified to the extent possible. It is very important to identify most of the residue; otherwise, unidentified residues may become a problem as more sensitive methods become available to detect these residues.

Plant metabolism studies are required in each of 3 dissimilar crops, such as a root crop, a leafy vegetable, and an oilseed. If the metabolism is similar in each of these crops, it is assumed to be similar in all crops, and no further metabolism studies are required.

The second type of metabolism data required is metabolism in animals. Whenever use of a pesticide results in residues in a livestock feed, or when a pesticide is applied directly to livestock, animal metabolism studies are required. The resulting data identify the pesticide residues to be looked for in the edible tissues of livestock, milk, and eggs that result from transformation processes in the animal. If the pesticide is not used on feed or applied directly to livestock, these data are not required. For livestock treatment uses, dermal application metabolism studies are generally required.

Animal metabolism studies are usually carried out on ruminants (cows or goats) and poultry (chickens). Swine metabolism studies may be required if a pesticide is applied directly to swine, or if the results from other animal metabolism studies show different metabolites in different species.

As with plant metabolism, the animal metabolism studies use radiolabeled pesticides, and the level of radioactivity resulting in muscle, liver, kidney, milk, and eggs is determined. If significant activity is found, the chemical identity of the activity is determined.

EPA chemists and toxicologists use the results of the metabolism studies to determine which metabolites are of concern and need to be included in the tolerance. Metabolites are included in the tolerance, depending on their toxicological significance, the percent and magnitude of the residue, and the availability of methodology to determine the metabolite. Methodology is essential for metabolites that are toxicologically significant and occur at significant levels. The determination of which metabolites need to be included in the tolerance is one of the most difficult decisions in reviewing tolerance data. If a metabolite is determined to be significant, an analytical method and residue data must be developed for the metabolite. This can significantly increase the cost of obtaining a tolerance and also delay the introduction of a new pesticide.

Analytical Methods

EPA requires analytical methods that are capable of determining all toxicologically significant components of the residue. In some cases, it is not possible to develop a single method that determines all components of the residue; rather, several methods are required.

Analytical methods are used for 2 purposes: The first is to generate residue data on which the dietary exposure assessment is based; the second is to enforce the tolerance after it is established. As stated previously, FDA and USDA are responsible for enforcing tolerances. Many states also have enforcement programs. To be sure that the method can be used to enforce a tolerance, EPA validates the analytical procedure in a single-laboratory review at laboratories in Beltsville, MD. In addition, EPA requires a second laboratory validation by the pesticide registrant before a residue method is submitted to EPA. No collaborative studies are required prior to publication of a tolerance.

EPA now sends copies of analytical methods to FDA and USDA before a tolerance is established in order to provide an opportunity early in the tolerance-setting process for the enforcement agencies to comment on the suitability of the methods to be used for enforcing tolerances. If a method is found to be totally inadequate for enforcing a tolerance, the tolerance will not be granted by EPA until a better method is developed. However, EPA does not require all tolerance enforcement methods to be as rapid and inexpensive as is desired for routine monitoring.

The method EPA approves for enforcing the tolerance must determine all components of the residue. The method should be specific and capable of determining residues at the tolerance level. EPA also requires that enforcement methods should not involve reagents or equipment that is not commercially available to enforcement agencies or take longer than 24 h to perform.

After a tolerance is established, the enforcement methods are published by FDA in the *Pesticide Analytical Manual*. EPA has recently initiated procedures to make analytical methods submitted by pesticide tolerance petitioners more readily available to FDA, USDA, the states, and other interested parties. In the *Federal Register* notice for every tolerance, EPA includes a specific statement on the availability of the analytical methodology. If the method has not yet been published in the *Pesticide Analytical Manual*, the *Federal Register* notice includes the address of the EPA Freedom of Information office from which the method can be obtained.

EPA has instituted a new requirement for analytical method data obtained by multiresidue methodology. Registrants must determine which pesticides and metabolites are determined by multiresidue methods used by FDA for routine monitoring. FDA uses the information on whether their multiresidue methods would detect a pesticide to compile information on the number of pesticides included in their monitoring program.

Field Trial Data

EPA requires field trial residue data to answer the question of how much residue remains on the crop at harvest. These are the studies in which the pesticide is applied to crops at known application rates, in a manner similar to the use directions that will eventually appear on the label. It is important to realize that the residue data must reflect the use directions that appear on the pesticide label. Pesticides can be applied either to the soil before the crop is planted, as foliar spray to the growing crop, or as a post-harvest treatment. Each of these modes of application can lead to widely different residue levels in the crop at harvest. The field trial must reflect the use of conditions that lead to the highest residue. This usually means the highest application rate, the maximum number of applications, and the shortest time between the last application and harvest. Data are normally required for each crop or crop group for which a tolerance and registration are requested. The residue field trial studies result in residue data for the raw agricultural commodity as it travels in interstate commerce. These data are used to determine the level at which the tolerance is set.

EPA also attempts to set U.S. tolerances at the same level as the Maximum Residue Limits (MRLs) set by the Codex Alimentarius Commission to facilitate international trade. (See the discussion of the Codex process by R. Maybury in this Symposium [*J. Assoc. Off. Anal. Chem.* (1989) **72,** 538–541].)

Processing Studies

Processing studies are required to determine whether residues in raw commodities can concentrate or degrade on processing. If residues concentrate on processing, food or feed additive tolerances are established. If residues do not concentrate on processing, the tolerance on the raw agricultural commodity applies to all processed food or feed derived from the raw agricultural commodity. It should be noted that the current EPA legal opinion is that the Delaney Clause applies to food and feed additive tolerances but not to raw agricultural commodity tolerances.

EPA also uses processing studies to determine whether residues dissipate on processing. If residues do dissipate, EPA can use this information to make a more realistic prediction of dietary risk. For example, residue levels in bread are always lower than the corresponding residue levels in wheat. In the past, EPA was criticized for assuming tolerance level residues in carrying out dietary exposure assessments. Now the Agency uses the results of processing studies to obtain a more realistic estimate of the residue levels in ready-to-eat food.

Livestock Feeding Studies

Livestock feeding studies are required to determine the level of residue in meat and milk resulting from livestock consuming residues on feeds. They are required whenever residues result in or on crops that are used as feed items. These studies provide data on the quantitative transfer of residues to meat, milk, poultry, and eggs. Similar studies are also required if a pesticide is applied directly to livestock.

In contrast to the animal metabolism studies, which are usually short-term, high-dosage studies, these feeding studies are longer-term, low-level studies designed to quantitate the transfer of residues to meat, milk, poultry, and eggs. Usually a control group and 3 dosage levels are used. Calculation of the 1× dosage level is based on the assumption of tolerance level residues in the feed and the percentage of the feed in the livestock diet.

In these feeding studies, animals are dosed for 30 days or until residues plateau in milk and eggs. The animals are sacrificed soon after the last dosing, and the muscle, kidney, liver, and fat are analyzed for the parent compound and all significant metabolites.

EPA also maintains a repository of analytical reference standards. Pesticide registrants are required to supply EPA with standards as a condition of registration. These standards are used to enforce tolerances and to assay for the percentage of active ingredient in formulations.

The availability of analytical reference standards is as important as the availability of good methods. In cases in which EPA is aware that analytical standards are not available, the Agency can act under its legal authority to call in the analytical standards. Failure of a registrant to provide or maintain analytical standards at the EPA repository can result in cancellation of the U.S. registration. EPA also provides analytical standards for those pesticides not having U.S. registrations if the producers of those pesticides are willing to supply the repository. FDA uses those standards to monitor imports.

In summary, tolerances are set by EPA to indicate the maximum level of residue that could result from use of a pesticide. This level is considered safe by EPA. Although the tolerance-setting process is complex, it is the best way to avoid uncertainty about the safety of pesticide residues in food. Most of the recent problems occurring with pesticide residues in food are due to the uncertainty about the safety of older pesticides for which the tolerance data are not available. Ethylene dibromide and Alar (daminozide) are the most recent examples of residues in foods causing problems due to a lack of complete information on the safety of the residue.

Codex Alimentarius Approach to Pesticide Residue Standards

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To protect consumers' health, most countries have maximum legal limits for pesticide residues in foods. Trade difficulties can arise when limits differ between countries. The Codex Alimentarius Commission was established in 1962 to implement the Joint FAO/WHO Food Standards Programme, the purpose of which is to protect consumer health and ensure fair practices in international food trade. The Codex Committee on Pesticide Residues (CCPR), an intergovernmental body which advises the Commission on matters related to pesticide residues, is responsible for establishing maximum residue limits (MRLs) for pesticides in foods and feeds that move in international trade. Codex MRLs are based on residue data obtained mainly from supervised trials that reflect approved pesticide use in accordance with "good agricultural practice." MRLs must be toxicologically acceptable in terms of estimated pesticide intake by consumers. CCPR Working Groups examine problems related to establishing and implementing MRLs, including sampling and methods of analysis. Despite time and effort expended, acceptance and application of Codex MRLs face many problems in international trade.

The use of pesticides for the production of food has provided numerous benefits in terms of increased production and quality of the product. Pesticides, on the other hand, are poisons, and to protect the health of the consumer, most countries have introduced laws governing not only the use of pesticides, but also setting limits for the levels of pesticide residues which may be tolerated in foods.

In general, these Maximum Residue Levels (MRLs) are based on the maximum residues that may be found when a pesticide is used at the minimum rate required to protect a crop against pests or diseases. The required rates of application may vary, however, under different agricultural and climatic conditions, from country to country, and between regions of a country. As a result, food with residue levels acceptable in one country may be rejected in another, a situation that leads to serious problems in international trade. Again, a food-exporting country may allow the use of a pesticide that is not registered in an importing country, with the result that shipments containing residues of that pesticide are rejected.

These conflicts would be resolved if agreement could be reached between all countries to apply the same residue limits for pesticides on foods in international trade. The harmonization of these and other food standards is the objective of the Codex Alimentarius system. This report will try to elucidate the system insofar as pesticide residues in foods are concerned.

Presented at the Symposium on Pesticides in Foods: Coping with the Issue—Programs and Analytical Methods, 102nd AOAC Annual International Meeting, Aug. 29–Sept. 1, 1988, Palm Beach, FL.

Joint FAO/WHO Food Standards Programme

The Codex Alimentarius Commission is an international body which was established in 1962 to implement the Joint FAO/WHO Food Standards Programme. Nations that are members of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) may become members of the Codex Alimentarius Commission. At last count, there were 129 member nations.

The Food Standards Programme has the following purposes:

(*1*) To protect the health of consumers and to ensure fair practices in the food trade.

(*2*) To promote coordination of all food standards work undertaken by international governmental and nongovernmental organizations.

(*3*) To determine priorities and to initiate and guide the preparation of draft standards through and with the aid of appropriate organizations.

(*4*) To finalize standards and, after acceptance by governments, publish them in a Codex Alimentarius.

To cover the different aspects of Food Standards, Codex is organized into a number of World-Wide General Subject Committees which report to the main body, the Codex Alimentarius Commission, and deal with the following concerns:

Committee	*Host Country*
Food Additives and Environmental Contaminants	Netherlands
Pesticide Residues	Netherlands
Analysis and Sampling	Hungary
General Principles	France
Food Labeling	Canada
Food Hygiene	United States
Drug Residues	United States

In addition, the Codex system includes 13 World-Wide Commodity Committees, dealing with products literally from soup to nuts, as well as regional and joint committees of various kinds. The Codex Committee on Pesticide Residues (CCPR) is, however, the one that will be described and related to the interests of AOAC in particular.

Codex Committee on Pesticide Residues

CCPR is an intergovernmental body which advises the Commission on all matters relating to pesticide residues. It meets every year in The Hague and submits its reports to the Commission. This meeting is attended by delegations from most member countries, as well as from the European Economic Community, the International Union of Pure and Applied Chemistry, and other international organizations, including AOAC.

CCPR has the following responsibilities:

(*1*) To establish maximum limits for pesticide residues in specific food items or in groups of foods.

(*2*) To establish maximum limits for pesticide residues in certain animal feedstuffs moving in international trade.

(*3*) To prepare priority lists of pesticides for evaluation by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR).

(*4*) To consider methods of sampling and analysis for the determination of pesticide residues in food and feed.

(*5*) To consider other matters in relation to the safety of foods and feeds containing pesticide residues.

(*6*) To establish maximum limits in specific food items or groups of food for environmental and industrial contaminants that show chemical or other similarity to pesticides.

To study these responsibilities in detail, CCPR establishes, on an ad hoc basis, a number of Working Groups that deal with questions of sampling, analysis, priorities, regulatory affairs, etc., as they arise.

Maximum Residue Limits

A list of Codex Maximum Residue Limits (MRLs) is published and updated on a regular basis (1), a typical page of which will indicate the following: (*1*) Pesticide number and name. (*2*) Classification number and name of the commodity. (*3*) MRL expressed as mg/kg. (*4*) Notes on the portion of the commodity to which the MRL applies. A number of considerations have to be agreed upon before the MRLs can be established and put into practice:

Classification of foods and feeds.—In dealing with such a variety of products on a worldwide basis, there is bound to be some uncertainty in the terminology for certain foods and crops. To overcome this, CCPR has produced a very useful document (2) which defines foods and some feeds. Each food is given a classification number which is included in the listing of MRLs. In this way, there is no doubt as to the commodity to which the MRL applies.

Portion of commodity to which Codex MRL applies.—Since residues are often localized on the outside of a commodity, it is important to define exactly what part is to be analyzed. CCPR and most countries apply their limits to the whole commodity which moves in commerce. Some countries, however, base their limits on the edible portion of the commodity. The outer leaves or skin, etc., which is not normally eaten, often contains the majority of the residues, so removal of this part before analysis can make a significant difference in the level of residues found. Agreement on such questions is obviously very critical for acceptance of Codex standards.

Methods of sampling.—Two possible sampling approaches for MRL enforcement are (a) Each individual unit of food in a consignment should conform with the MRL; then, if any unit is found to exceed the MRL, the whole consignment is considered to be not in compliance. (b) The MRL is applied to the average residue level in the sample taken from the lot. Under this approach, a number of units are sampled from the consignment, and if the sample composite is found to contain pesticide residues in excess of the MRL, the consignment is considered not in compliance.

CCPR has adopted the second of these approaches, i.e., the Codex limit applies to the average pesticide residue level of the sample taken from the lot under examination. Most countries have adopted the Codex approach, although some still insist that each unit of food must comply with the residue limit.

The Working Group on the Development of Residue Data and Sampling has drawn up detailed guidelines (3) for sampling all types of commodities.

Recommended methods of analysis.—The Working Group on Methods of Analysis deals with all questions that arise in CCPR with regard to the analysis of pesticide residues. This Group has published a book of recommended methods of analysis for residues of pesticides for which there are Codex MRLs (4). This book is reviewed annually by the Working Group but, because of the many possible combinations of commodity and pesticide, the list is not exhaustive.

A number of criteria are used in selecting the methods. The methods should be published in the open literature; collaboratively studied or known to have been validated in a number of laboratories with validation data having been published in the literature; capable of determining more than one residue, i.e., multiresidue methods; suitable for as many pesticide and commodity combinations as possible at or below the specified MRLs; applicable in a regulatory laboratory equipped with routine analytical instrumentation.

Good analytical practice.—The Working Group on Methods of Analysis has also elaborated Codex Guidelines on Good Analytical Practice in Pesticide Residue Analysis (5) which are intended to assist in improving the reliability of analytical results. This is particularly important in regulatory decision making, where the acceptability of a shipment of a commodity may be affected if it is decided that an MRL

is exceeded. In such cases the analyst must use scientific judgment with regard to analytical uncertainty, and it is recommended that the following are essential to ensure the reliability of results: regular assessment of the performance of the method at the limit of determination; confirmation of the identity of the pesticide residue; adequate replications so that results can be given with confidence.

Development of Maximum Residue Limits

The previous discussion covered some of the definitions and procedures that affect the establishment and use of MRLs. The next part will briefly describe the steps in the development of a Codex MRL.

First, the need for an MRL for a particular pesticide must be established. CCPR, through the Working Group on Priorities, develops a priority list of pesticides for evaluation. To be placed on the list, the candidate pesticide should meet certain criteria. It should:

(*1*) Result in residues on the food commodity;

(*2*) Be a matter of public health concern;

(*3*) Affect international trade to a significant degree;

(*4*) Be creating or have potential to create commercial problems;

(*5*) Not already be under review at some stage of the Codex procedure;

(*6*) Be available for use as a commercial product.

The priority list is submitted to the Joint FAO/WHO Meeting on Pesticide Residues (JMPR). This is a meeting of experts who serve on a personal basis, i.e., they do not represent any government or organization. They meet annually to evaluate pesticides in terms of their occurrence as residues in food and their potential effect on the health of consumers and on the environment.

Data are supplied by industry and governments on toxicology, chemistry, pest control practices, metabolism and fate, and other aspects of each pesticide. For each Joint Meeting, a report is published describing the general principles and conclusions reached and the recommended MRLs for the pesticides evaluated. A more detailed examination of the data is presented in the published evaluations.

Toxicology

Since safety is the primary concern, toxicological data requirements are extensive. Acute, subacute and chronic toxicity studies in several mammalian species are normally required, as well as various types of special studies, such as reproduction, teratogenicity, and neurotoxicity studies where appropriate. All of these studies include extensive tests on body functions and internal examination of sacrificed animals.

The total toxicological data base is evaluated in detail by the JMPR, and the "no observable effect levels" (NOELS) for the most sensitive toxicological parameters in the most sensitive species are determined. This maximum no-effect dose is then used as a basis for estimation of an acceptable daily intake (ADI) for humans by using a suitable safety factor. A margin of safety is necessary to allow for differences in sensitivity between the test species and humans, the wide variation in sensitivity among humans, and the small numbers of experimental animals in comparison with the human population that might be exposed. The margin of safety applied is generally 100; however, it may range from 10 to 5000, depending on a number of factors, including the range of studies available and the toxic effects observed at the maximum dosages. The Acceptable Daily Intake is the amount of chemical (mg/kg body weight/day) that may be ingested daily for a lifetime without appreciable risk to humans.

If there are deficiencies in the data base of a pesticide, or if there are concerns that the pesticide may be a carcinogen or teratogen for example, the Joint Meeting will not recommend an ADI, and a maximum residue level cannot then be considered. It should be noted that even though an ADI must be established first, it is not used in the development of the MRL.

Residue and Analytical Aspects

To recommend an MRL, JMPR requests that member governments, industry, and others provide residue data generated in supervised field trials. These should produce the residue levels to be expected under Good Agricultural Practice, the foundation on which the MRL is established. Good Agricultural Practice is defined by Codex as follows:

> Good Agricultural Practice in the use of pesticides is defined as the officially recommended or authorized usage of pesticides under practical conditions at any stage of production, storage, transport, distribution and processing of food and other agricultural commodities, bearing in mind the variations in requirements within and between regions, and which takes into account the minimum quantities necessary to achieve adequate control, applied in a manner so as to leave a residue which is the smallest amount practicable and which is toxicologically acceptable.

The recommended MRLs estimated by JMPR are then introduced into the CCPR system for elaboration as Codex MRLs.

The recommended MRL takes into consideration the maximum residues that might be expected to result when the pesticide is used according to approved instructions; the nature of the residues (parent compound + metabolites); the analytical methodology available for the residues; any other factors.

The views and recommendations of the FAO/WHO experts are taken as the basis on which the CCPR can judge whether residues of pesticides in foods are unavoidable when the pesticide is used in accordance with good agricultural practice.

The recommended MRL goes through a total of 8 steps before it is finally adopted by the Commission as a Codex Standard. During this process, opportunities are given for member countries and various international organizations to review and comment on the proposals. Once the Codex MRL has been established, it is submitted to member countries for acceptance, which may be full, limited, or target.

Predicted Dietary Intake of Pesticide Residues

Once MRLs for a pesticide have been established in a range of commodities, on the basis of results of globally generated residue data, it is important for authorities in a country to know whether the MRLs are acceptable from the public health point of view. In other words, will the total amount of the pesticide in the typical diet of that country exceed the acceptable daily intake?

To arrive at a realistic estimate of dietary intake the following factors have to be taken into account:

The kinds and quantities of foods consumed.—The kinds of food consumed can be obtained, on a global basis, from average intakes published in the FAO food balance sheets. More accurate "cultural" diets for specific regions or countries have also been prepared by FAO.

Pesticide residue levels in the foods.—If the pesticide residue level in a food is assumed to be at the MRL, a value

known as the Theoretical Maximum Daily Intake (TMDI) can be obtained by multiplying the MRL and the average daily per capita food consumption of each commodity for which an MRL has been established. The TMDI may be compared with the ADI, and if the ADI is not exceeded, any public health concern will be minimal. The TMDI is a gross overestimate because it does not take into account the following factors:

(*1*) The percentage of a crop treated with a pesticide is usually far less than 100%.

(*2*) Very few of the crops treated with a pesticide will contain residues at the maximum residue level.

(*3*) Residues normally dissipate during storage, transport, preparation, commercial processing, and cooking of the treated commodity.

(*4*) The MRL is set on the raw agricultural commodity, which frequently includes inedible portions that will be discarded, together with much of the residue.

Where these factors are taken into account, more realistic estimate of intake can be obtained. The most realistic estimate can be obtained if a typical diet is actually analyzed for its pesticide residue content, as in the case of Total Diet Studies in the United States and elsewhere.

If the ADI is exceeded in the more realistic intake estimates, public health may be of real concern, and the MRL in question may not be acceptable.

Conclusion

A great deal of information has been generated through the work of CCPR. MRLs have been established, or are being established, for some 153 pesticide chemicals in over 3000 pesticide and commodity combinations. Guidelines have been published for sampling, analysis, classification of foods, and other matters of significance in the regulation of trade at the international level.

For countries which do not have the necessary infrastructure to evaluate pesticides and develop their own system of MRLs, the Joint FAO/WHO Food Standards Programme as implemented by Codex provides the necessary information to protect the health of consumers and to facilitate exportation and importation of food commodities.

However, most of the major countries and trading blocks such as the European Economic Community have developed their own system of MRLs or tolerances which may or may not agree with the Codex values.

The Codex standards do help to facilitate international trade but a number of factors prevent universal adoption of Codex MRLs as national standards by countries:

(*1*) National legislation prevents some governments from accepting the Codex MRLs.

(*2*) Where Codex limits are lower than the national MRLs, there may be a problem for one country to accept the good agricultural practice of another. This is seen most clearly in the different requirements between countries with tropical and temperate climates.

(*3*) A national MRL may differ from the Codex MRL in terms of the definition of the residue, e.g., metabolites may be included in one case and not the other.

(*4*) There may be a difference in the definition of the portion of the food to which the MRL applies.

The Codex system has achieved a great deal by bringing together specialists from many disciplines worldwide to exchange information and resolve differences. Progress may be slow, but a great deal is being gained in the process of working toward the universal acceptance of Codex standards for pesticide residues.

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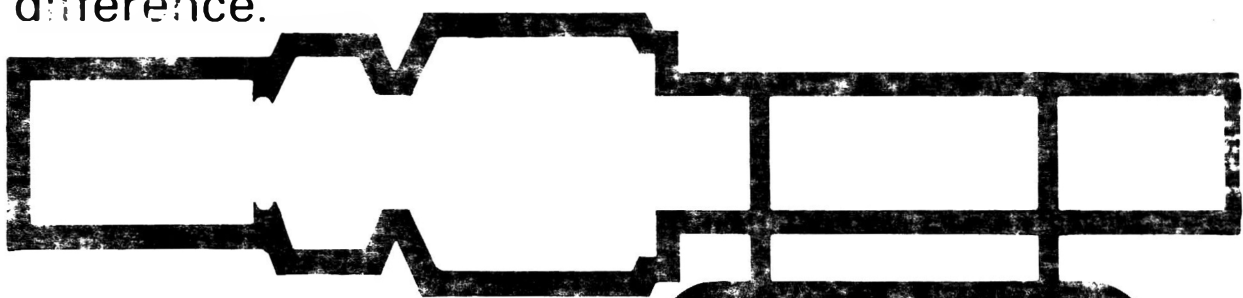

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