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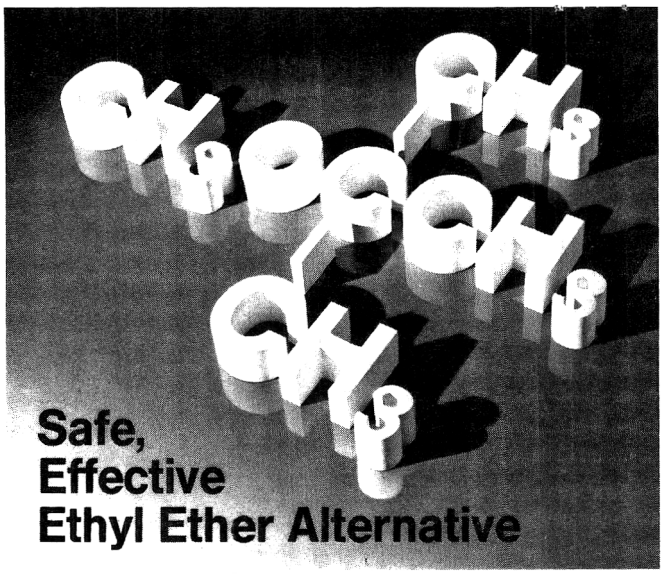

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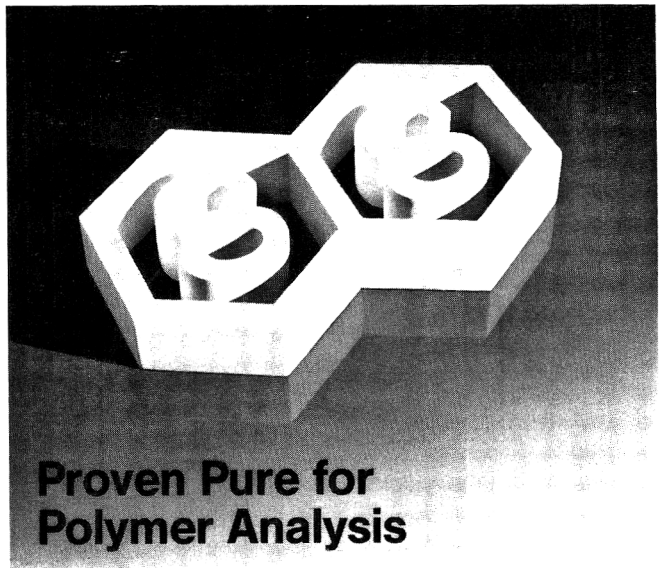

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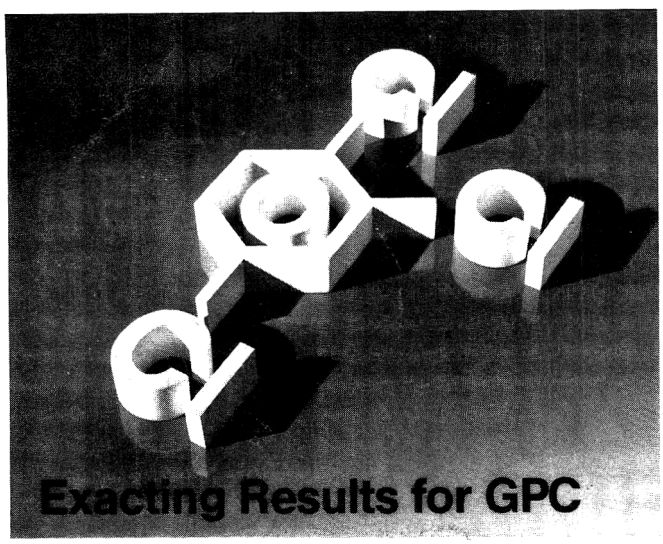

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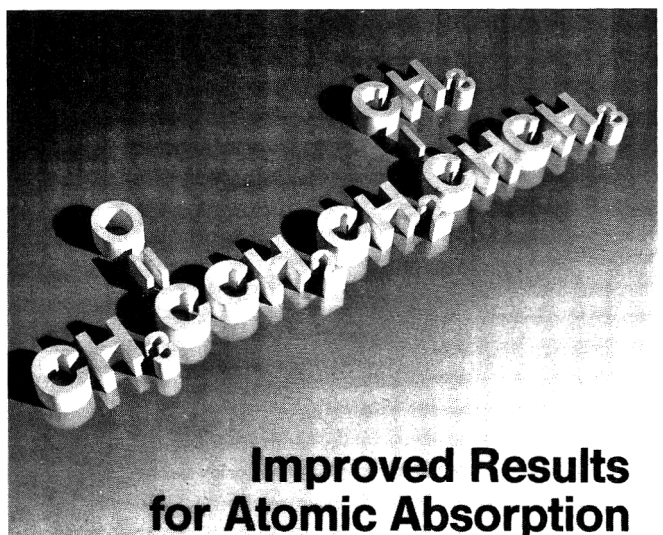

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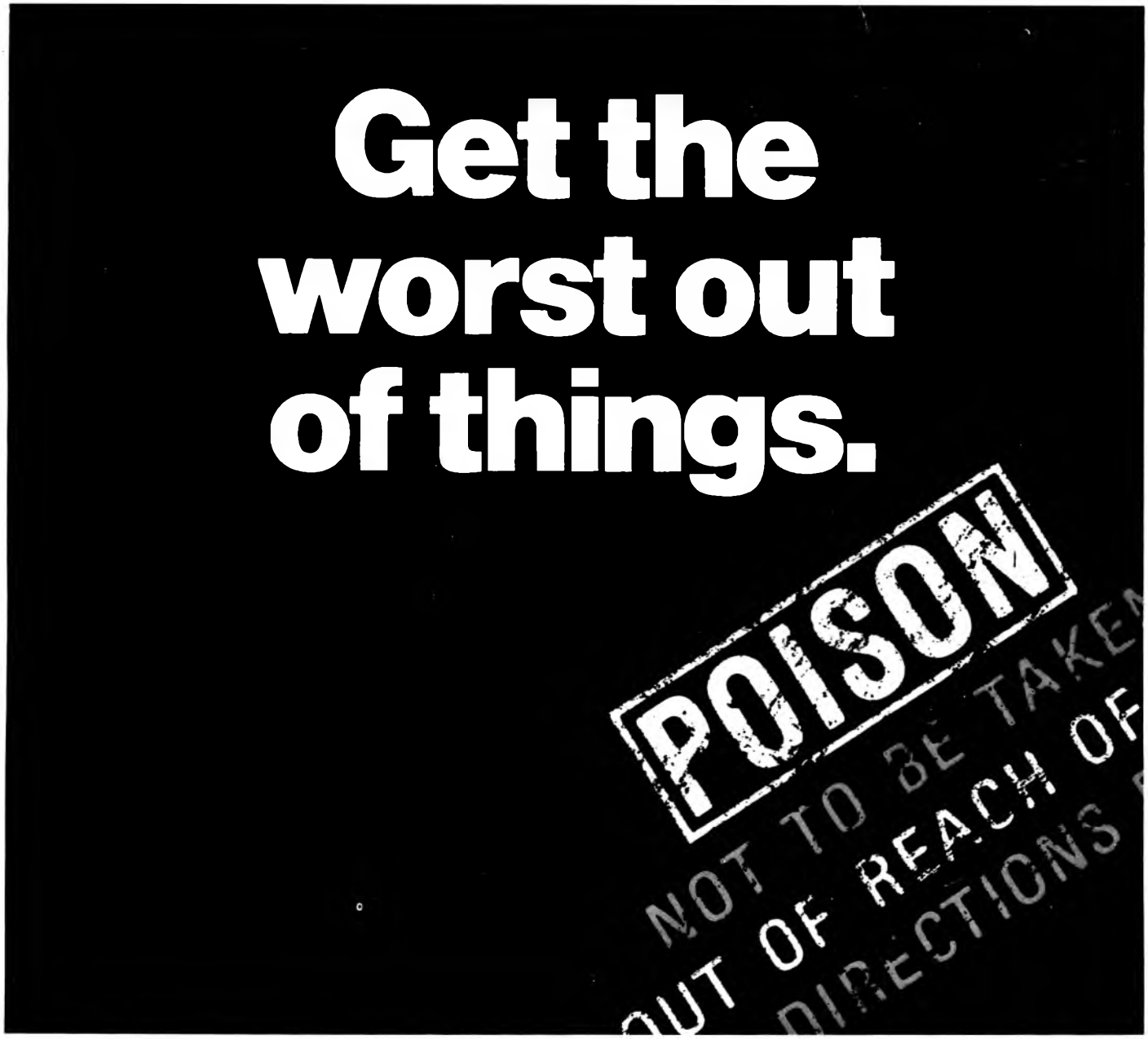

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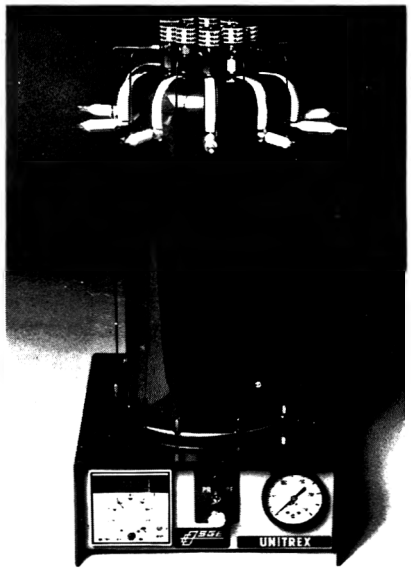

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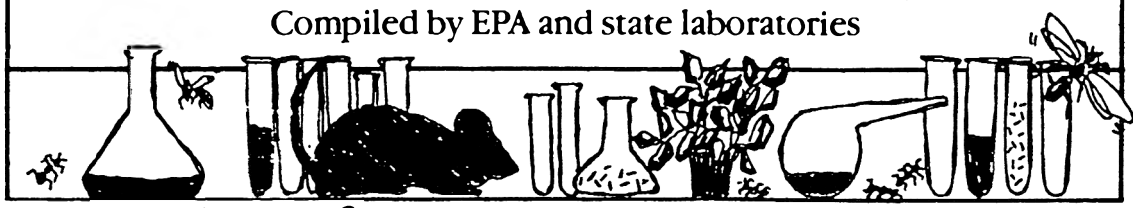

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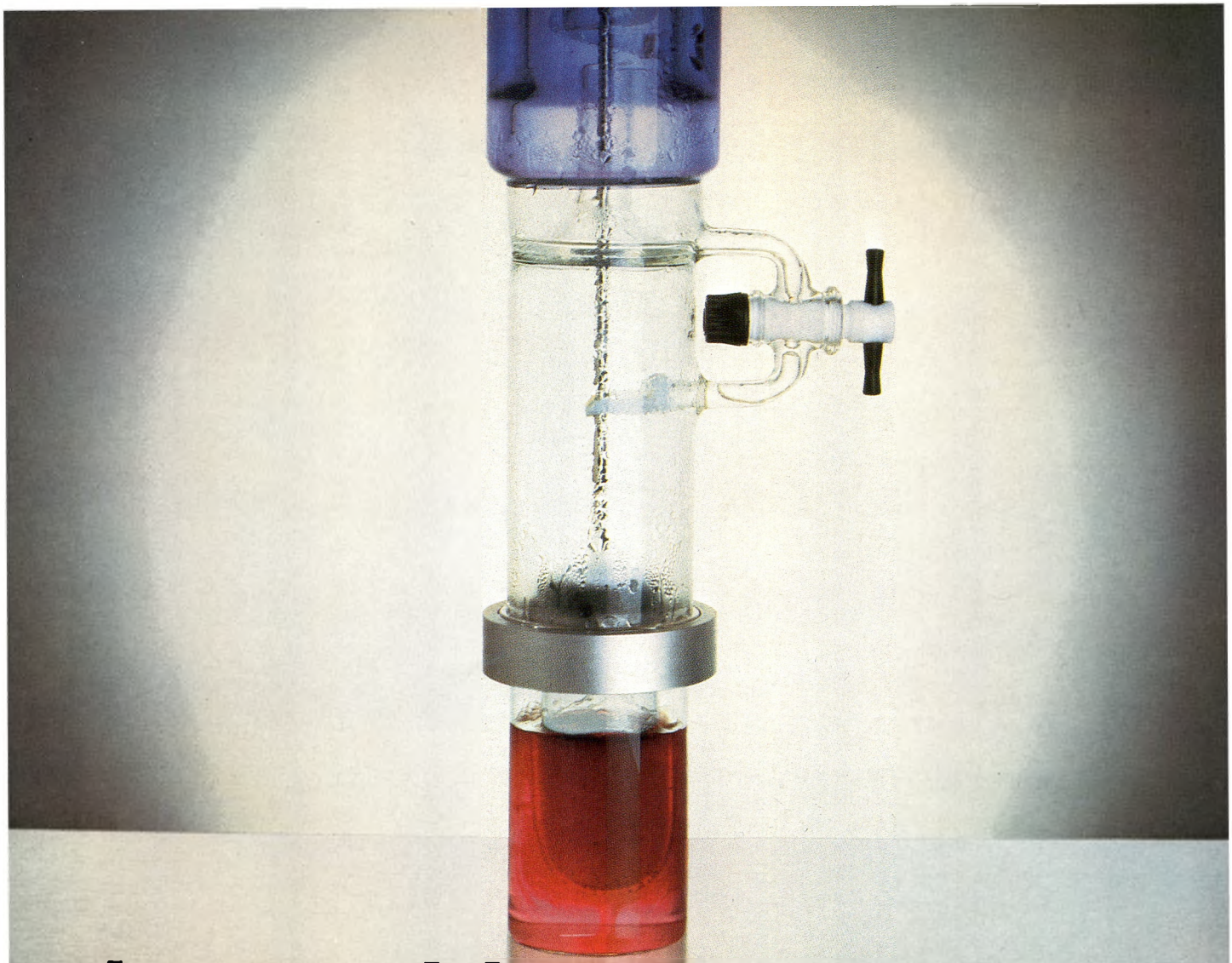

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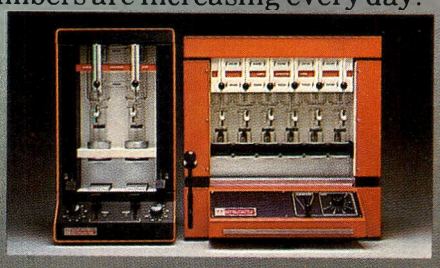

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Treatise on Analytical Chemistry, Second Edition. Edited by I. M. Kolthoff and P. J. Elving. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1983. 592 pp. Price: $70.00. ISBN 0471-49969-2.

This book is a complete source of information for analytical chemists, designed to stimulate research in pure and applied chemistry. Volume 3 of Part 1 on theory and practice, continues the discussion of solution equilibria and chemistry with such topics as oxidation-reduction equilibria and titration curves; surface chemistry: utilization in analysis; solubility; precipitates: formation, coprecipitation, and aging; precipitation equilibria and titrations in aqueous and nonaqueous media; reactive groups as reagents: introduction and inorganic applications; and reactive groups as reagents: organic applications.

Analytical Calorimetry, Volume 5. Edited by M. F. Johnson and P. S. Gill. Published by Plenum Publishing Corp., 233 Spring St, New York, NY 10013, 1984. 402 pp. Price: $69.50. ISBN 0-306-41507-0.

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Applied Complexometry. By R. Přibil. Edited by R. A. Chalmers. Published by Pergamon Press, Inc., Fairview Park, Elmsford, NY 10523, 1982. 410 pp.Price: $75.00. ISBN 0-08-026277-5.

Volume 5 in the Analytical Chemistry series deals with complexometry from the practical point of view in covering applied procedures that have been developed in the previous 3 decades. Topics covered include volumetric reagents, detection of the titration endpoint, masking reagents, separation methods, apparatus and solutions, classification of EDTA complexes, anions, analytical applications, analysis of alloys, analysis of rocks and minerals, analysis of silicates and rocks, analysis of slags, analysis of cements, and others.

Aquametry, Second Edition, Part II. Electrical and Electronic Methods: A Treatise on Methods for the Determination of Water. By D. M. Smith and J. M. Mitchell, Jr. Edited by P. J. Elving and J. D. Winefordner. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1984. 1352 pp. Price: $165.00. ISBN 0-471-02265-9.

Volume 5 of the Chemical Analysis series deals with the advances made in electrical and electronic methods for water determination based on dielectric, conductometric, and coulometric principles. The book presents major techniques in detail, covering significant historical aspects; assessments of important methods reported in the literature; proposals for improved techniques; and applications to many substances encountered in industrial and academic research and medical facilities.

Isolation, Separation, and Identification of Volatile Compounds in Aroma Research. By H. Maarse and R. Belz. Published by D. Reidel Publishing Co. Distributed by Kluwer Academic Publishers Group, PO Box 322, 3300 AH Dordrecht, Holland (outside U.S. and Canada); and by Kluwer Boston Inc., 190 Old Derby St, Higham, MA 02043 (U.S. and Canada). 290 pp. Price: $54.00 (U.S. and Canada)/Dfl. 130 (outside U.S. and Canada). ISBN 90-277-1432-0.

This monograph deals with modern analytical methods of aroma research, particularly, the isolation and concentration of aromatic substances, separation and assay techniques on the basis of chromatography, and the use of spectrometric techniques for identification. Topics include isolation and concentration of volatiles from foods, chromatographic separation, identification, and coordination of sensory and instrumental analysis.

Some Aromatic Amines, Anthroquinones and Nitroso Compounds, and Inorganic Fluorides Used in Drinking-Water and Dental Preparations. International Agency for Research on Cancer (IARC), Lyon, France. Distributed by the World Health Organization, 1211 Geneva 27, Switzerland. 1982. 341 pp. Price: $25.00 (U.S.)/SFr. 40.-. ISBN 92-8-3212274-4.

Volume 27 of the IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans presents monographs on aromatic amines, anthroquinones, nitroso compounds, and inorganic fluorides used in drinking water and dental preparations, as well as epidemiological evidence relating to the possible carcinogenic effects of hair dyes in hairdressers and in users of hair dyes.

Some Industrial Chemicals and Dyestuffs. International Agency for Research on Cancer (IARC), Lyon, France. Distributed by the World Health Organization, 1211 Geneva 27, Switzerland, 1982. 416 pp. Price: $30.00 (U.S.)/SFr. 60.-. ISBN 92-8-321229-0.

Volume 29 of the IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans contains 18 monographs on some industrial chemicals and dyestuffs. An annex discusses available methods for quantitative risk assessment and the limitations inherent in such assessments, using epidemiological data on benzene and benzidine as examples.

Miscellaneous Pesticides. International Agency for Research on Cancer (IARC), Lyon, France. Distributed by the World Health Organization, 1211 Geneva 27, Switzerland, 1983. 424 pp. Price: $30.00 (U.S.)/SFr. 60.-. ISBN 92-832-1230-3 (soft-bound); ISBN 92-832-1530-3 (hard-bound).

Volume 30 of the IARC Monographs of the Carcinogenic Risk of Chemicals to Humans contains monographs on miscellaneous pesticides, including some insecticides, herbicides, fungicides, and a rodenticide. The section, "Cancer Epidemiology of Pesticide Manufacturers, Formulators, and Users," reviews relevant epidemiological data on mixed exposures to pesticides. Also reviewed are the possible formation of *N*-nitroso compounds and references to studies on the carcinogenicity and/or mutagenicity of selected *N*-nitrosatable pesticides, tested either alone or in conjunction with sodium nitrate or on their *N*-nitrosated products.

Biological Magnetic Resonance, Volume 5. Edited by L. J. Berliner and J. Reuben. Published by Plenum Publishing Corp., 233 Spring St, New York, NY 10013, 1983. 303 pp. Price: $45.00 ISBN 0-306-41293-4.

Contributions by experts in the field include: a review of applications of C-13 NMR spectroscopy in investigations of metabolic pathways *in vivo*; a comprehensive survey of the use of N-15 NMR in studies of systems of biological interest; a description of P-31 NMR investigations of enzyme systems; an outline of the principles and a summary of the use of oxygen isotopes (0-17 and 0-18) in P-31 and 0-17 NMR studies of biophosphates; and a discussion of lipid–protein interactions as reflected in ESR and NMR data.

Short-Term Bioassays in the Analysis of Complex Environmental Mixtures III. Edited by M. D. Waters, S. S. Sandhu, J. Lewtas, L. Claxton, N. Chernoff, and S. Nesnow. Published by Plenum Publishing Corp., 233 Spring St, New York, NY 10013, 1983. 589 pp. Price: $69.50. ISBN 0-306-41191-1.

Volume 27 in the series, Environmental Science Research, is written by researchers in the field and describes techniques in the collection and preparation of environmental samples for bioassay. Researchers also report on advances in short-term in vitro and in vivo bioassays for mutagenicity, cytotoxicity, carcinogenicity, and teratogenicity, and review current efforts to integrate biological and chemical data to assess human health hazards.

Handbook of U.S. Colorants for Foods, Drugs, and Cosmetics, Second Edition. By D. M. Marmion. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1984. 466 pp. ISBN 0-471-09321-2.

This manual on color additives documents changes that have occurred in this field: The identity, status, and permitted areas of use of many colorants have been modernized, analytical technology has been improved, and specifications for a large number of color additives have been modernized. Part A provides a general background of color additives, Part B deals with colorant analysis, and Part C describes resolution of mixtures and analysis of commercial products.

Detergent Analysis: A Handbook for Cost-Effective Quality Control. By B. M. Milwidsky and D. M. Gabriel. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1982. 291 pp. Price: $57.95. ISBN 0-470-27257-0.

This handbook is intended for the industrial laboratory concerned with cost-effective quality control. Topics include laboratory apparatus; standard solutions and reagents; routine control analysis—rationale and background theory; scheme for routine control analysis; plant control procedures; new materials—development, evaluation, and performance tests; raw materials; ecological and environmental considerations; and analysis of unknowns.

Controlled-Release Technology: Bioengineering Aspects. Edited by K. G. Das. Published by John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1983. 225 pp. Price: $50.00 ISBN 0-471-08680-0.

This comprehensive, up-to-date analysis of the development of controlled-release systems presents an overview of the field and deals with design parameters; chemical and physical methods of controlled release; microencapsulation; determination of release rates; and chronic vs acute toxicity. Controlled-release technology, aimed at stimulating nature's processes by directing bioactive ingredients to specific target sites in required amounts, without significantly affecting nontarget materials, has applications in medicine, agriculture, public health, forestry, and the veterinary sciences.

Risk Management of Existing Chemicals. Published by Government Institutes, Inc., 966 Hungerford Dr, No. 24, Rockville, MD 20850, 1984. 184 pp. ISBN 0-86587-065-9.

This publication of the proceedings of a seminar conducted on December 8–9, 1983, at Washington, DC, treats the following topics: science and policy in risk control; industry's perspective on how risk management is working; Toxic Substances Contol Act: new attitudes; quantitative risk assessment: state-of-the-art for carcinogenesis; government data requirements for risk assessment; utilization of risk assessment in corporate risk management decisions; some case histories, and others.

Drug Metabolite Isolation and Determination. Edited by E. Reid and J. P. Leppard. Published by Plenum Publishing Corp., 233 Spring St, New York, NY 10013, 1983. 289 pp. Price: $42.50. ISBN 0-306-41265-9.

Volume 12 of the series: Methodological Surveys in Biochemistry and Analysis, deals with techniques applicable to metabolite investigation; investigation of metabolites, especially phase I; investigation of conjugates; determination of particular drugs and metabolites; and metabolite methodology. This volume also includes notes and comments following each section and a cumulative index of all previous volumes.

Modern Aspects of Electrochemistry, No. 15. Edited by R. E. White, J. O.'M. Bockris, and B. E. Conway. Published by Plenum Publishing Corp., 233 Spring St, New York, NY 10013, 1983. 361 pp. Price: $49.50. ISBN 0-306-41287-X.

Volume 15 of the series focuses on splitting water by electrolysis; splitting water by visible light; the recent development of lithium batteries; theoretical approaches to intercalation; and fundamental concepts of electrode kinetics, particularly, as applied to semiconductors.

Liquid Chromatography in Environmental Analysis. Edited by J. F. Lawrence. Published by The Humana Press, Inc., Crescent Manor, PO Box 2148, Clifton, NJ 07015, 1984. 374 pp. Price: $55.00 (U.S.), $65.00 (export). ISBN 0-89603-045-8.

This volume, written by international contributors, reviews a variety of HPLC techniques and applications used in the analysis of environmental substances and contaminants. Topics include HPLC as a cleanup technique, quality assurance in trace analysis, sample preparation and concentration, HPLC detector systems, sample injection, column switching, surfactant analysis, trace metal analysis, anion analysis, radioisotope fractionation, and acid rain analysis.

AOAC to Hold Tenth Annual Spring Training Workshop and Exposition, April 8–11, 1985

The Association of Official Analytical Chemists (AOAC) will sponsor the 10th Annual Spring Training Workshop and Exposition, April 8–11, 1985, at the Sheraton Dallas Plaza of the Americas Hotel in Dallas, TX.

This meeting will be the final event of our Centennial Celebration, and the technical sessions will provide the latest information on various topics including LC, recent advances and optimization of techniques; drugs and metabolites; thin layer chromatography; immunoligand techniques; food chemistry; atomic absorption spectrophotometry; elemental analysis; electroanalytical techniques; drug residues in animal tissues; forensics, drugs of abuse and physical evidence; fertilizers; laboratory data management; robotics; seafoods, microbiological testing; vitamins; microbiological testing of drugs and cosmetics; natural toxins; and pesticides and industrial chemical residues in the environment.

An exhibition of the most recent advances in laboratory instrumentation, equipment, and supplies will be held Monday, April 8th, 5:30–8:30 pm; Tuesday, April 9th, 8:00 am–5:00 pm; and Wednesday, April 10th, 8:00 am–5:00 pm.

For more information, contact: M. Virginia Gibson, FDA, 3032 Bryan St, Dallas, TX 75204; 214/767-0312 and Molly Ready, Alcon Labs, 6201 S Freeway, Fort Worth, TX 76134; 817/293-0450.

John Bremner 1984 Wiley Award Winner

John Bremner, Professor of agronomy and biochemistry and Curtiss Distinguished Professor of agriculture at Iowa State University, is the 1984 winner of the AOAC Harvey W. Wiley Award for his contributions to analytical methodology for soil, plant, fertilizer, water, and air analyses. Charles W. Gehrke, 1984 AOAC president, will present the award to Dr. Bremner on Tuesday evening, October 31, at the AOAC Centennial International Meeting.

The $2500 award is given annually to a scientist who has made outstanding contributions to the development and validation of methods of analysis for foods, drugs, cosmetics, pesticides, feeds, fertilizers. environmental contaminants, or other related areas. The award was established in 1956 in honor of Harvey W. Wiley, "Father of the 1906 Pure Food and Drug Act and a founder of AOAC. The award's primary purpose is to emphasize the role of the scientist in protecting the consumer and the quality of the environment.

Dr. Bremner has been a pioneer in using new techniques for soil research. He was the first to utilize chromatographic techniques for the identification of nitrogenous compounds in soils, and the first to use selective ion electrodes for determination of nitrate, ammonium, and Kjeldahl digests in soils.

As major contributor to the 1965 monograph of the American Society of Agronomy, *Methods of Soil Analysis*, Dr. Bremner's methods have been used extensively around the world for the past 18 years. The Science Citation Index shows that he has been among the most extensively cited scientist in the world during the past 20 years.

Born in Scotland in 1922, he received his B.S. from Glasgow University in 1944; his Ph.D. in chemistry in 1948 and D.Sc. in soil chemistry in 1959, were both from London University. He was a Carnegie Research Scholar at Glasgow, in 1944–1945, and served as a scientific officer with the Rothamsted Experimental Station in Harpenden, England from 1945 to 1949. Named a Rockefeller Fellow 1957, he toured agricultural research centers in the United States and worked at Iowa State University for 5 months, where he became a professor in 1961, and was named to his present position in 1975. During that time, he also served as a technical expert with the International Atomic Energy Agency, in Vienna, Austria, in 1964–1965, and was a Guggenheim Fellow in 1968–1969.

He has been named a Fellow of the American Society of Agronomy, the American Association for Advancement of Science, the Soil Science Society of America, and the Iowa Academy of Science. He has won the Outstanding Research Award of the First Mississippi Corporation (1979), the Wilton Park International Service Award (1982), the Bouyoucos Soil Science Distinguished Career Award (1982), the Governor's Science Achievement Medal, Governor of Iowa (1983), and is the 1982 recipient of the prestigious Alexander Von Humboldt Award "for the most significant contribution to American agriculture during the past five years."

1984 Fellows of the AOAC

Six scientists have been chosen to receive the 1984 Fellow of the AOAC awards. Charles Gehrke, AOAC president, will make the presentations at the opening session of the Annual International Centennial Meeting of the AOAC, October 29, 1984.

The Fellow of the AOAC award was established in 1961 to recognize those persons giving meritorious service to the Association. Winners of the award have performed notably for 10 years or more, usually as officers, referees, or committee members. Nominations are made by AOAC members, reviewed by the Committee on Fellows, and finally approved by the Board of Directors. The following are award recipients.

Richard S. Wayne, American Cyanamid Co., Princeton, NJ, has served as Associate Referee (AR) for Malathion and Dimethoate and was chairman of the Committee on Gas-Liquid Chromatography and Liquid Chromatography of Pesticide Formulations, from 1972 to 1981. He is currently chairman of the Subcommittee on Gas and Liquid Chromatographic Column Specifications.

Milan Ihnat, Agriculture Canada, Ottawa, has served as AR for Maleic Hydrazide, Arsenic, and Selenium. He has been a member of the Editorial Board since 1974 and a member of the Interlaboratory Study Committee since 1980. He has also served as chairman of the Subcommittee on Survey of *Official Methods of Analysis*, and chairman of the Subcommittee on the Permanent Numbering System for *Official Methods of Analysis*.

Clyde E. Jones, Colorado Department of Agriculture, Denver, CO, has served as General Referee (GR) for Feeds since 1980, and served as AR for Zinc and for Soil and Plant Amendment Ingredients. He was a member of the Committee on the Wiley Award, the Committee on State Participation, and chairman of the Denver Spring Training Workshop.

Keith McCully, Health and Welfare Canada, Ottawa, has served as GR for Organophosphate Pesticides since 1967, is chairman of the Committee on Laboratory Quality Assurance, and is a member of the Interlaboratory Study Committee.

Harry B.S. Conacher, Health and Welfare Canada, Ottawa, has served as chairman of Committee C since 1983, and has served as AR for Brominated

Oils and for Fatty Acids by Gas Chromatography. He has served as a member of Committee on the Harvey W. Wiley Award and the Organizing Committee for the 1981 Spring Workshop.

Stanley M. Harmon, Food and Drug Administration, is AR for *Clostridium perfringens,* for *Bacillus cereus*—Enterotoxin, and for *Bacillus cereus*—Isolation and Enumeration.

1984 AOAC Scholarship Awarded to Christine Cherney of Hazelton, PA

Christine Cherney, a sophomore at Immaculata College, Immaculata, PA, is the winner of a 2-year, $1000 scholarship sponsored by AOAC.

As a chemistry major with a strong component of mathematics, Christine has maintained a 4.0 grade point average her first 2 years of college. In addition, she has worked in the chemistry department as a participant in the college's work-study program, and has participated in a hospital internship. Christine plans to do research in chemistry in graduate school

Each year AOAC awards a 2-year scholarship to a college sophomore who is studying a subject important to public health and agriculture. To qualify, the student must be in need of financial aid, maintain at least a B average during the first 2 years of undergraduate study, and plan to do research, regulatory work, quality control work, or teach in an area of interest to AOAC.

Nominations for the 1985 award must be received before May 1, 1985. Send 6 copies of a nomination letter and 2 supporting reference letters to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209.

AOAC Infant Formula Conference

Contributed papers are now being solicited for an AOAC scientific conference on the production, regulation, and analysis of infant formula, to be held May 14–16, 1985, at the Sheraton Beach Inn & Conference Center, Virginia Beach, VA.

The conference will consist of a series of invited lectures and presentations of contributed papers on topics such as analysis of infant formula; accuracy, precision, and standards in analysis; regulatory aspects of infant formula; medical aspects of infant formula; American Academy of Pediatrics Committee on Nutrition nutrient guidelines; labeling of infant formula; quality assurance; and special formulations.

Summaries of contributed papers of 700–900 words, with figures or tables counting as 150 words each, should be submitted no later than February 1, 1985. Authors of papers which are accepted will be expected to submit camera-ready manuscripts at the conference. Summaries should be submitted to: James Tanner, Food and Drug Administration, HFF-266, 200 C St, Washington, DC 20204; 202/472-5364.

EPA Project Summary

Major aspects of the report, "Analysis of the NBS Sediment by the MRI Sludge Protocol," are described in a project summary developed by V. Lopez-Avila, R.V. Northcutt, J. Onstot, and M. Wickham of the U.S. Environmental Protection Agency's (EPA) Environmental Monitoring and Support Laboratory.

The project studied isolation of 51 organic priority pollutants, spiked into an NBS (National Bureau of Standards) standard reference sediment, by applying the EPA Interim Protocol for the Analysis of Extractable Organic Priority Pollutants in Industrial and Municipal Wastewater Treatment Sludge. The extraction technique involved homogenization of a sediment/water slurry with dichloromethane at dual pH, followed by centrifugation, a silica gel fractionation of the base/neutral extract, and a gel permeation chromatographic fractionation of the acidic extract.

The project summary highlights the *Experimental* and *Results and Discussion* sections of the report, and includes tables. A brief summary section describes that the extraction method can be applied, with minor modifications, to sediment analysis, on the basis of the precision and accuracy results. Other nonpolar or weakly polar compounds can also be isolated, although polar compounds or reactive compounds containing nitro or epoxy groups cannot.

The complete report (Order No. PB 84-133 750) can be obtained for $16.00 (subject to change) from the National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161; 703/487-4650. For information, contact James E. Longbottom, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

NVLAP Annual Report and Laboratory Directory Published

The National Voluntary Laboratory Accreditation Programs (NVLAP) seventh annual report provides information on about 100 laboratories nationwide that are accredited in one or more areas of testing by the National Bureau of Standards. The report also describes NVLAP activities, such as the International Laboratory Accreditation Conference and the bilateral agreements that provide mutual recognition of testing laboratories between NBS and foreign laboratory accreditation systems.

The directory lists testing laboratories with proven competence, the test methods for which each laboratory is accredited, identifies the field of testing, and gives the locations of the laboratories.

For copies of the report or information on the NVLAP program, contact: Manager, Laboratory Accreditation, B141 Technology Building, National Bureau of Standards, Gaithersburg, MD 20899; 301/921-3431.

NSF Announces Nominations for Alan T. Waterman Award

The National Science Foundation (NSF) Alan T. Waterman Award Committee calls for nominations for the tenth annual award.

Intended to give recognition to an outstanding young researcher in any field of science, mathematics, or engineering, and to encourage further high quality research, the award was established by Congress in 1975 to honor the first Director of the Foundation, Dr Waterman.

The recipient shall receive up to $50 000 per year for up to 3 years of research or advanced study in the mathematical, physical, medical, biological, engineering, social, or other sciences at the institution of the recipient's choice.

Nominations for the award may be submitted by the scientific and educational communities, individuals, professional societies, industry, and other appropriate organizations. Six copies of each nomination should be submitted to the Alan T. Waterman Award Committee at NSF in Washington, DC, by December 31, 1984.

For information on candidate qualifications and any additional information, contact the Executive Secretary for the Award Committee, Lois J. Hamaty, National Science Foundation, Washington, DC 20550; 202/357-7512.

CMA Study Finds DEHP Safe

A study done by Joseph Rodericks and commissioned by the Chemical Manufacturers Association (CMA) con-

cludes that DEHP [di-(2-ethylhexyl) phthalate] is safe for use in toys and other products for children.

The study reviews experimental data on the health effects of DEHP and concludes that even when the most conservative risk assessment procedures are used, the risk of cancer from products containing DEHP is below the ranges considered to be worthy of regulatory action by the federal government.

DEHP is used to "plasticize," or soften, polyvinyl chloride plastic, which is used to make pacifiers, teething rings, squeeze toys, and vinyl plastics for infant furniture.

Single copies of the study are available from the Chemical Manufacturers Association, 2501 M St, NW, Washington, DC 20037; 202/887-1224.

Government Publications Listing

The bimonthly listing, "New Books," provides entries of all new and revised government books and periodicals for sale by the U.S. Government Printing Office. Entries are listed alphabetically and include government research reports, census data, laws and regulations, economic and labor statistics, reference works, grant information, and energy production and consumption compilations.

To be placed on the mailing list to receive "New Books" without charge, write to: New Books, U.S. Government Printing Office, Stop: MK, Washington, DC 20401.

1984 Catalog on Information Materials for the Food and Cosmetic Industries Published

The 1984 edition of the Food and Drug Administration's catalog, "Information for the Food and Cosmetic Industries," has just been published. The catalog is useful in locating information materials involving FDA food and cosmetic regulations, policies, and procedures. Materials listed in the catalog tell firms how to stay in compliance with these requirements and suggest methods for solving compliance problems, should they occur. The catalog lists publications, audiovisuals, manuals, and other information developed by FDA and other sources.

Copies are available by contacting the Center for Food Safety and Applied Nutrition Industry Programs Branch at 202/485-0251.

Standard Reference Materials Published

The National Bureau of Standards (NBS) Office of Standard Reference Materials announces the availability of SRM 1549, Non-Fat Milk Powder, developed for use in validating methods for the analysis of milk, milk products, and other biological materials.

The Certificate of Analysis provides certified concentrations and their uncertainties for 15 elements: cadmium, calcium, chlorine, chromium, copper, iodine, lead, magnesium, manganese, mercury, potassium, selenium, sodium, sulfur, and zinc. Lactose, ascorbic acid, and other element concentrations are given for information only.

Also available from NBS is SRM 2670, a powdered, freeze-dried form of urine intended for studies on exposure to toxic elements, and short- and long-term exposure to toxins. SRM 2670 comes in 4 bottles, 2 each at low and elevated toxin levels, and includes the following elements at both levels: arsenic, cadmium, chloride, chromium, copper, lead, magnesium, manganese, mercury, nickel, potassium, selenium, sodium, and sulfate.

SRM 1549, Non-Fat Milk Powder, may be purchased for $135/100 g sample. SRM 1549 and SRM 2670 may be obtained from Office of Standard Reference Materials, Room B311, Chemistry Building, National Bureau of Standards, Gaithersburg, MD 20899; 301/921-3181.

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 prices indicated, from American National Standards Institute, Inc., 1430 Broadway, New York, NY 10018; 212/354-3300

ISO 6465-1984 Whole cumin *(Cuminum cyminum Linnaeus)*—Specification—$12.00

ISO 6479-1984 Shelled sweet kernels of apricots—Specification—$16.00.

Courses

Fundamentals of Chromatography, December 3–7, 1984, presented at Kent State University, is co-sponsored by the Kent State University Chemistry Department, IBM Instruments, and the University Conference Bureau. Directed toward beginners and intermediates, the course will provide an overview of chemical separations, using chromatographic methods including gas, liquid, and thin layer methods, and will stress the 3 techniques as complementary processes. Information on theory and instrumentation with applications, lecture sessions, and accompanying hands-on laboratory sessions will also be presented.

For more information, contact: Carl J. Knauss, Chemistry Dept. Kent State University, Kent, OH 44242; 216/672-2327.

Milling for Cereal Chemists, January 3–5, 1985, presented at Kansas State University, Manhattan, KS, is sponsored by the American Association of Cereal Chemists (AACC). Highlights of the course include tours of a flour mill, bakery, and flour treatment lab; discussions on raw materials, experimental milling, and flour mill operation; and hands-on experience with roll stands and sifters.

Registration fee is $525.00 for AACC members, $625.00 for nonmembers. A $50.00 deposit is required to reserve space in the short course. Deadline for registration is December 12, 1984. To obtain a registration form, contact: Dotty Ginsburg, AACC Short Course Coordinator, 3340 Pilot Knob Rd, St. Paul, MN 55121; 612/454-7250.

Practical Process Measurement: Techniques and Applications, March 11–12, 1985, at the Warwick Post Oak, Houston, TX, and May 6–7, 1985, at the Philadelphia Hilton, Philadelphia, PA. Sponsored by *Chemical Engineering* of McGraw-Hill, Inc., this seminar offers field-tested procedures for: improving process measurement; improving process efficiency; reducing operating costs; and reducing energy usage. The comprehensive program encompasses such topics as temperature, pressure, flow, level, physical property, and chemical composition measurements; process chromatography; mass spectrometry; and case studies in process measurement. Each seminar is conducted by an expert in the field, and qualifies for CEUs. Contact: Cathy Edwards, McGraw-Hill Seminar Center, Suite 603, 331 Madison Ave, New York, NY 10017; 212/687-0243.

Practical Distillation Technology, February 11–12, 1985, at the Warwick Post Oak, Houston, TX, and May 6–7, 1985, at the Ambassador West, Chicago, IL. Sponsored by *Chemical Engi-*

neering of McGraw-Hill, Inc., this seminar offers field-tested techniques for effectively designing, troubleshooting, and operating distillation equipment. Program topics include key fractionation concepts, column process design, energy savings in new and operating column tray design and operation, packed columns, column auxiliaries, column revamp and debottlenecking techniques, column hydraulic design, and column startup. Each seminar is conducted by an expert in the field, and qualifies for CEUs. Contact: Cathy Edwards, McGraw-Hill Seminar Center, Suite 603, 331 Madison Ave, New York, NY 10017; 212/687-0243.

Meetings

April 8–11, 1985: AOAC 10th Annual Spring Training Workshop and Exposition, Sheraton Dallas Plaza of the Americas Hotel, Dallas, TX, USA.

May 1–3, 1985: Scientific Computing and Automation Conference and Exposition, Atlantic City Convention Center, Atlantic City, NJ, USA, is sponsored by Gordon Publications, Inc. Designed for laboratory practitioners and managers in analytical chemistry, biotechnology/biomedical research, clinical chemistry, and engineering, the conference topics have been grouped into 7 areas: automation, data acquisition and handling, information processing, proteins and nucleic acids, computer technology, theoretical calculations, and laboratory information management systems. Each topic will be covered in both a symposium session and a poster session. Also offered will be workshops, short courses, and exhibitions of the latest computer hardware and software technology for the laboratory. For information on attendance and submitting abstracts for paper presentations and/or poster sessions, contact Expocon Management Associates, Inc., 3695 Post Rd, Southport, CT 06490, USA.

May 14–16, 1985: AOAC Infant Formula Conference, Sheraton Beach Inn & Conference Center, Virginia Beach, VA, USA. (See *AOAC Infant Formula Conference* above.)

June 2–5, 1985: 68th Canadian Chemical Conference and Exhibition, Queen's University, Kingston, Ontario, Canada. Contributed papers are now being solicited from all areas of chemistry and chemical engineering for presentations or poster sessions. Symposia and general sessions are being planned. Titles and abstracts must be submitted on special forms to R.J.C. Brown, MCIC, Department of Chemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6, by December 15, 1984. Abstract forms and instructions to authors are available from The Chemical Institute of Canada, 151 Slater St, Suite 906, Ottawa, Ontario, Canada KIP 5H3; 613/233-5623 (Telex AIC 053-4306).

June 17–19, 1985: AOAC Northeast Regional Section Meeting, University of Massachusetts, Amherst, MA, USA. Contact: Audrey Gardner, NY Agricultural Experiment Station, Food Research Laboratory, Geneva, NY 14456, USA, 315/787-2282.

AOAC Midwest Regional Section Meeting, Holiday Inn, Hillside, IL, USA. Contact: Devendra Trivedi, Illinois Law Enforcement Dept, Bureau of Scientific Services, 515 E Woodruff Rd, Joliet, IL 60432, USA, 815/727-5301.

June 20–21, 1985: AOAC Northwest Regional Section Meeting, Evergreen College, Olympia, WA, USA. Contact: Mike Wehr, Oregon Dept of Agriculture, 635 Capitol St, NE, Salem, OR 97310, USA, 503/378-3793.

September 3–7, 1985: Second International Symposium on the Synthesis and Applications of Isotopically Labeled Compounds, Vista Motel, Kansas City, MO, USA. Topics will encompass synthesis, analysis, purification, and storage of isotopically labeled compounds and their applications in biomedical, clinical and environmental studies, metabolism, pharmacokinetics, and toxicology. Contact: Donald Wilk, Symposium Coordinator, School of Pharmacy, 5100 Rockhill Rd, Kansas City, MO 64110, USA, 816/276-1616.

October 12–16, 1985: AOAC 99th Annual International Meeting, Shoreham Hotel, Washington, DC, USA. Contact: AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, USA, 703/522-3032.

August 25–29, 1986: 10th International Symposium on Microchemical Techniques, Antwerp, Belgium. Topics include pure and applied aspects of analytical chemistry related to micro and trace analysis, and application of modern techniques in the field of trace analysis, such as microbeam analysis techniques. A workshop on laser microprobe mass analysis is planned. Contact: R. Dewolfs, University of Antwerp (UIA), Department of Chemistry, Universiteitsplein 1, B-2610 Wilrijk, Belgium; Telephone 03/828.25.28 (ext. 204), Telex 33646.

New Private Sustaining Members

AOAC welcomes the following 7 new private sustaining members to the growing list of organizations aware of the need to support an independent methods validation association: American Council of Independent Labs, Inc., Washington, DC; Corn Refiners Association, Inc., Washington, DC; Dickey-john Corp., USA, Auburn, IL; McCormick & Company, Inc., Hunt Valley, MD; Avon Products, Inc., Suffern, NY; Golden State Foods Corp., City of Industry, CA; and Zoecon Corp., Palo Alto, CA.

In Memoriam: Harold Egan

The news of the death of Dr Harold Egan on June 28, 1984, while attending a Chemrawn Conference in The Netherlands, was received with great sadness by his many colleagues in the UK (United Kingdom) and around the world.

Born on December 23, 1922, he was educated at Chiswick County School, Acton Technical College, and Imperial College, London, where he obtained BSc, PhD and DIC degrees. After 3 years in the Biochemistry Department of St. George's Hospital, he joined, in 1943, the Department (later Laboratory) of the Government Chemist. Between 1943 and 1981 when he retired, he rose rapidly through the Laboratory to the post of Government Chemist in 1970. He was the only Government Chemist to have risen through the ranks and, in so doing, contributed to a wide range of investigations, particularly in food, drug, and pesticide analyses, with special reference to trace determinations. As Government Chemist, his advice on many aspects of chemistry was frequently sought by government departments to assist in formulating or implementing policies. He published more than 100 papers on many aspects of analytical chemistry and was a frequent lecturer at conferences.

He had many scientific interests outside the laboratory. He was a visiting professor in the chemical sciences faculty of the University of East Anglia, and previously had held a similar appointment in food science at Queen Elizabeth College, London. He had served on several committees of the Royal Society of Chemistry and, as an enthusiastic member of the Analytical Methods Committee, was editing a new edition of Official, Standardized, and Recommended Methods of Analysis. An

active member of IUPAC (International Union of Pure and Applied Chemistry), he had served as secretary and president of the Applied Chemistry and Food Chemistry Divisions.

Harold Egan's connection with AOAC has been long and fruitful. As UK Government Chemist, his responsibility for official methods of analysis naturally led him to collaborate with AOAC, and to regularly attend meetings. He organized the first International Conference on Harmonization of Collaborative Studies in Helsinki, sponsored by IUPAC, and made most of the arrangements for the follow-up conference in Washington, jointly sponsored by IUPAC and AOAC, running consecutively with the Centennial Meeting. He was the AOAC representative in the UK and Ireland and actively encouraged individuals and companies to participate in the development of new analytical methods through collaborative studies.

Harold Egan will always be remembered by his friends and colleagues for his great energy which he applied in all aspects of his professional and personal life. Widely known and respected internationally for his contribution to analytical chemistry, his presence will be sorely missed following his untimely death.

He is survived by his wife Daphne and son Geoffrey.

RONALD F. COLEMAN

INSTRUCTIONS TO AUTHORS

Scope of Articles

The Journal of the AOAC will publish articles that present, within the fields of interest of the Association *(a)* unpublished original research; *(b)* new methods; *(c)* further studies of previously published methods; *(d)* background work leading to development of methods; *(e)* compilations of authentic data; *(f)* technical communications, cautionary notes, and comments on techniques, apparatus, and reagents; *(g)* invited reviews of methodology in special fields. The scope broadly encompasses the development and validation of analytical procedures pertaining to both the physical and biological sciences related to agriculture, public health and safety, consumer protection, and quality of the environment. Emphasis is focused on research and development to test and adopt precise, accurate, and sensitive methods for the analysis of foods, food additives, supplements and contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment. Compilations of authentic data include monitoring data of pesticide, metal, and industrial chemical residues in food, tissues, and the environment. All articles are reviewed for scientific content and appropriateness.

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Spectrophotometric, gas chromatographic, and liquid chromatographic nomenclature should follow the practice recommended by the American Society for Testing and Materials.

Rev. 1/84

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A CENTURY OF ANALYTICAL EXCELLENCE

Food Adulteration Detection: 100 Years of Progress in AOAC Methodology

EUGENE H. HOLEMAN[1]
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The Indiana farm boy, Harvey W. Wiley, ignored and overcame numerous hardships to educate himself in chemistry and medicine and then in the art and science of imparting his knowledge to college students at Purdue University; he later practiced the difficult tasks of performing chemical tests and experiments as chief chemist of the Bureau of Chemistry, USDA (U.S. Department of Agriculture), and making a name for himself in the political arena, which culminated in the passage of the first U.S. federal pure food and drugs act—the 1906 Pure Food and Drugs Law. Dr Wiley and other associates and supporters saw the need for the development of analytical methods of analysis for *(1)* orderly manufacturing practices in the food, feed, and fertilizer industries to produce uniform lots of these commodities; *(2)* use by federal, state, and municipal laboratories to determine adulteration of commodities and to check label and adulteration claims; and *(3)* protection of the public from hazards which they could not detect by elementary means.

Over the years—particularly in the 1880s and 1890s—accurate, reproducible methods were developed through collaborative studies promoted by the Association of Official Agricultural Chemists (now Association of Official Analytical Chemists). For a number of years, the AOAC-adopted methods were published by the U.S. Department of Agriculture, Division of Chemistry, as the "Proceedings of the Annual Convention of the Association of Official Agricultural Chemists." When the methods of analysis for "Foods, Commercial Fertilizers, Feeding Stuffs, Dairy Products, Fermented Liquors, and Sugars," edited by Dr Wiley, were adopted and published, they became the instruments for law enforcement by the federal government and state and municipal control officials. Naturally, these methods also became the standards by which industries developed and checked their products, which would then be offered for sale to the public.

These brief opening paragraphs do not touch on the tremendous courage, perseverance, and scientific intelligence required by the early chemists and administrators to develop uniform, reproducible methods of analysis and to move the proceedings from the workbench in the laboratory to official status and, finally, to become the basic tools for law enforcement. All of these efforts, by the dedicated few, were fought every step of the way. Individuals and laboratories had their own methods with which they felt comfortable and did not want to give them up to a collaborative committee for study and possible changes; industry methods were "secret" and industry analysts did not want to release their methods to public scrutiny or to their competitors; and the politicians did not want to "rock the boat" for a new approach to scientific methods.

During this scientific inquiry into methodology, Wiley threw himself into the political arena in the interest of the consumer and against fraudulent advertising, labeling, and the introduction of poisonous and deleterious substances into the food supply. He used the Chautauqua circuit and women's clubs of the day to plead for passage of a pure food and drug law. All of this furor is reflected in the presidential campaigns of Presidents Roosevelt, Taft, and Wilson. (A concise review by Oscar E. Anderson, Jr, appears in *American Historical Review*, Vol. 61, No. 3, April 1956.) Dr Wiley and his supporters were successful, and President Theodore Roosevelt signed into law the Pure Food and Drugs Act on June 30, 1906. Conditions later led to the 1938 Food, Drug and Cosmetic Act which, with amendments and regulations, is in force today.

"Harvey W. Wiley taught Latin and Greek at what is now Butler College, Indianapolis, Indiana, while a student at the Indiana Medical College. In his fourth year, he taught science at the Indianapolis High School and in 1872 received his diploma as a Doctor of Medicine. In 1893, at the suggestion of his friend Dr. Charles Edward Munroe, he applied for and was granted a petition to try for a degree from Harvard. (Author note: Here is one of the most remarkable achievements in academic history.) To secure the degree he went through a 17-day examination. First, he took the entrance examinations of the freshman class, then the sophomore class examination, then the junior, and finally the senior. He passed them all and on June 25, 1893, he was given the degree of Bachelor of Science. He had actually been a student at Harvard a little over five months.

In August 1874 Dr Wiley was asked to become Professor of Chemistry at Purdue University. He then became a member of its first faculty. In 1878 he went to Berlin to complete his studies in chemistry. *There he was attached to the Imperial Board of Health under Dr Sell and began his life work in the study of food adulteration* and lost all desire of ever becoming a physician." (Quoted from an article by Wiley's widow, Anna Kelton Wiley, written by her for the Section on Food, Drug and Cosmetic Law of the New York State Bar Association on the commemoration of the fortieth year of the passage of the original Pure Food and Drug Act, June 25, 1946.)

So we come now to the subject of this paper, food adulteration—progress in methods for detection. It has probably appeared that I was following an outline of a famous preacher I knew in Buffalo Valley, Tennessee. When asked his formula for such excellent sermons, he said, "I takes my text—I departs from it—and I never comes back."

Dairy Products

Among the earliest products to be subjected to control of adulteration were such dairy products as milk, butter, and cheese. By their physical nature they are subject to decomposition, contamination, and adulteration. In the past, the most vital component of milk has been milk fat, although

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[2]Burnside, who succeeded McClellan, *moved* and was badly beaten by one-half his force at Fredericksburg.

from a health standpoint today, solids are now considered more important. Because of the economic value of milk fat, this ingredient was skimmed off and the product was sold below the 4.0% standard, with other oils or fats substituted for the milk fat. So Babcock and others before him developed test methods for fat.

Fat determinations by gravimetric methods are reported in the early proceedings of the AOAC meetings, principally from French, German, and Scandinavian sources. Methods by Babcock (gravimetric) are also reported; however, the official AOAC method for determining fat in milk, in both 1889 and 1890, was the Evans method—modified by Wiley (USDA Bulletin No 28). The method could be performed in slightly over 2 hours.

Now, move to the 3rd edition (1930), *Official and Tentative Methods of Analysis of the AOAC*, page 217, and find 2 official methods for fat determination: The Roese-Gottlieb and Babcock methods. The latter method, published in the *AOAC Journal*, **8**, 4 (1924), is the classic centrifugal, 18 g, graduated bottle method. (In my high school days in a one-room school house plus a small house outdoors, we would run 12 samples, hand-whirling, on the machine for 5, 2, and then 1 minute.)

Sometime after 1889, the Evans method and then the Babcock method, which used a coil of filter paper plus 5 mL milk in a beaker-watchglass combination, disappeared as official methods. Interesting, however, is the durability of the techniques: The Roese-Gottlieb method still contains the admonition, "weigh cooled flask or dish *without wiping* . . .," and the Babcock method still says, "centrifuge for 5, 2, and 1 minute." And why not? When an acceptable accuracy has been reached, change for the sake of change can be disastrous (especially for the schoolboy looking out the one-room window instead of turning the handle of the centrifuge). But we begin to see already the necessity for decreasing the time for analysis and increasing the number of samples tested. The rapid detergent method, automated methods, and infrared methods went through the rugged collaborative test demanded by the Association, and are meeting the needs of modern analysts.

The early analysts were confronted with a variety of unsophisticated yet hazardous and fraudulent adulterants. A mixture widely used to fool the lactometer consisted of 1 gal. water, 2 oz sugar, 1 1/2 oz salt, and a little caramel—added to each 4 gallons of milk. Other old standbys were chalk, salt, annatto, gums, dextrin, saltpeter, saleratus, caustic soda, soda, formaldehyde, and, of course, the universal adulterant, water. A packing plant operator once told me, "I can't help making a profit as long as water can be added to my products." This practice came to a momentary halt when a superpumped ham exploded and almost drowned the butcher.

Butter was subjected to much adulteration, particularly with lard, as well as oils, cocoa butter, and various natural and synthetic colors. Cheese was extended and cheapened with other oils, fats, gums, and colors.

Many of the tests for adulterants in dairy products in the early years of the Association have subsequently been deleted from *Official Methods of Analysis*. However, the basic tests for compliance with standards remain, with some modification. The great advancements in detecting adulterants in dairy products are in the areas of automation and instrumentation and in the addition of methods for detecting potential health hazards. The *3rd edition of Official and Tentative Methods of Analysis*, 1930, lists 24 selected references to the *AOAC Journal* and others, while the 13th edition, 1980, lists 72 selected references. This jump from 24 to 72 selected references between the first and second half century of AOAC history illustrates the increased interest of scientists in bringing their methods to the Association for study and approval. Some interesting advancements in detecting adulterants in dairy products are in the areas of extraneous materials and microbiological methods; somatic cell count is a good example.

The following example of adulterated ice cream might be headed "Homogenized milk, cream, and ice cream—may not be sold before its time" (Food Inspection Decision, March 28, 1911): The use of Homogenized Butter and Skimmed Milk in the Manufacture of Ice Cream, opinion of Solicitor General Frances G. Caffey to D. F. Houston, Secretary of Agriculture, under Food and Drugs Act of 1906, published 1914, page 147. "Investigations have shown that there has lately come into use in the trade an apparatus known as a 'homogenizer,' which has the faculty of so disrupting the globules of fat that a whole milk homogenized does not permit the separation of the cream through ordinary gravity methods. . . . Investigations have further shown that butter and skimmed milk are passed through the homogenizer to form a so-called 'cream' which is used in the place of real cream in the manufacture of ice cream. . . . The Board is further of the opinion that the product made from a homogenized butter or skimmed milk cannot be properly called 'ice cream' . . . ."

This adulteration was practiced before the advent of the famous "gas-air-water,"and the "secret ingredient" stabilizer: If a flour base were made up from the gas-air-water and secret ingredient mixture, and baked, it was sold as *bread;* if frozen, it became *ice cream.*

The stabilizer was composed of gum mixtures, alginates, gelatin, and casein. Bread standards, pan size laws, and AOAC methods took care of the bread problem, but the "secret ingredient" formula became a favorite with some frozen dessert manufacturers of soft and hard ice cream. It was shown, in the author's experience, that a judicious use of the secret ingredient could produce an ice cream which, after initial freezing, could be kept in the home refrigerator without ice. Dip out a generous serving and sit in the sun (you may get a headache from cold food or drink), the molded shape would remain. However, the synthetic taste was not very appetizing.

The AOAC official methods, **16.290** for gum, **16.297** for alginates, and **16.298** for gelatin, gave accurate methodology for their detection, and a simpler method for frozen desserts evolved from earlier attempts. Air is a great expander, when held in place by the stabilizer: The stabilizer base mix expands from the 2 quarts to 4 quarts to achieve a 100% over-run. Final action sections **16.280** and **16.281** describe the method and apparatus for weight per unit volume of packaged ice cream.

Fruit Juice Adulteration

An intriguing and challenging subject for analysts in industry, university, and regulatory laboratories has been detection of juice adulteration. Chemists Lee McClaran, Travis Smotherman, and the author became interested in orange juice and lemonade adulteration in the 1950s (through the 1970s) when field samples obtained from a local processor did not compare favorably with authentic products made in the laboratory. Tennessee did not have a "citrus belt" although there were several processors, and there was little evidence of juice concentrate being imported. To help us determine the authenticity of the suspect samples, we searched the AOAC Book of Methods and appealed to our "Dean" in AOAC, Dr Harry Fisher, chief of the Department of Analytical Chemistry, Connecticut Experiment Station, New Haven,

Connecticut. The 8th and 9th editions, *Official Methods of Analysis*, were our chief references, and *General Methods of Analysis*, was taken from the Uniformity of Interpretation of Food and Drugs Laws report in the *Proceedings of the Association of Food and Drug Officials*, Vol. 27, June 1963.

The AOAC methods and others were used for a large number of fruit juice *constants*—which are not constant. Variations occur because of varietal differences, the season, and such climatic conditions as sun, soil, and rain; therefore, evaluation is made on a sliding scale. For example, ash of authentic orange juice varies from 0.27 to 0.71% and averages 0.41%. Gross adulteration was not too difficult to detect, however, and the "citrus belt" in Tennessee and Georgia—some "100% orange juice" producers in business without benefit of orange groves—was put to the test. In Georgia, State Chemist Harry Johnson and Raymond Summerlin came to our aid. The Tennessee chemists were congratulated on their analytical work by Food and Drug Administration Director Les McMillin, Region IV, Atlanta. At the time he didn't know that the analysts were helped by Billy Riddle's raid on a Georgia plant, where drums of potassium citrate and calcium chloride, sugar, pulp, orange oil, citric acid, ammonium citrate, concentrated tangerine juice, and artificial color (turmeric and annatto) were found and destroyed. It helps to know the unknown before starting the analysis.

Progress is being made. The old methods would detect gross adulteration but are not sensitive or specific enough for today. Fruit juices and their concentrates are shipped worldwide, and more sophisticated types of adulteration are being attempted. Likewise, the literature reflects the attempts of federal and state regulators and industry to detect these newer forms of adulteration, for example, by the use of *Lactobacillus plantarum* for detection of orange juice adulteration.

An older parameter which has been used effectively is the total amino acid index. The system has worked well for the past 10 years in detecting citrus juice adulteration. Alas, individual amino acids can be purchased and the adulterated juice can be fortified to the natural amino acid levels.

Sugar Products

In a letter to the solicitor general, reprinted in *Federal Food and Drugs Act and Decisions*, FID 75 (July 1907), the director of the Agricultural Experiment Station at Orono, Maine, states: "There are in Maine many syrups which are labeled something like this: 'A Fancy Quality Syrup Made from Pure Maple and White Sugar.' Many of these syrups carry but little maple, one company saying that in a syrup analogous to this they put 90% cane sugar and 10% maple." Honey and other sweeteners including "New Orleans" molasses and sorghum, the favorite syrup of the South, have long been tinkered with, adulterated, or mislabeled. How far does the honey bee fly to bring in specific nectars for the beekeeper to label his honey Sourwood or Clover, Raspberry or Wildflower, Tupelo or Honeysuckle? Which is the cheaper today—corn syrup, commercial glucose, fructose, lactose, or partially cooked cane juice or even Blackstrap molasses? Many scientists have made exhaustive studies of the many phases of sugar, honey, and sorghum adulteration.

After stopping 2 truck loads of "Pure New Crop Sorghum Molasses," the owner in DeKalb County, Mississippi, called the author and inquired, "What can I do?" I replied, "Plant some sorghum next year!" As in the "prior knowledge" illustration on imitation "100% Pure Orange Juice," I had discussed the matter with the county agent; he inferred that no sorghum had been planted by the defendant. Dr James P. Minyard, state chemist and professor at Mississippi State College, told me the following about some particular sorghum farmers: "I know they are crooks, they know they are crooks, and they know that I know they are crooks, so we understand each other." Nevertheless, Minyard went on and perfected his method for the determination of adulterated sorghum (and other syrups) where high fructose sweeteners are used.

Published liquid chromatographic (LC) methods on the determination of fructose, glucose, and sucrose provide a direct determination of sugar composition in honey and adulterated honey. Other supporting methods include carbon isotope ratio analysis. These methods supersede previous methods using older, less specific techniques, e.g., polarimetric methods.

A major contributor to methods of analysis for the detection of food adulteration is William Horwitz of the Food and Drug Administration, a frequent contributor to the *AOAC Journal* and other scientific journals. He comments about the theory and practice of recent methods of sugar analysis, particularly honey, maple sugar, sorghum, syrup, etc. "The advent of gas and liquid chromatography made it possible to isolate, identify, and quantitate each of the individual sugars with a high degree of reliability as well as to characterize individual, characteristic, nonsugar components of each of these food commodities. Therefore, we now have at our disposal the theoretical possibility of controlling these classical adulteration problems.

"However, there is a word of caution necessary before automatically taking the information in the literature and applying it to the current situation. We must remember that many of the methods for the determination of chemical constituents were performed by empirical methods and relied on either consistent systematic errors or compensation of errors to achieve reproducible results. Although the results may be reported in terms of sucrose, fructose, glucose, maltose, and other specific sugars, they did not really represent these sugars. But modern chromatography does permit us to determine quantitatively each of these sugars." Horwitz points out further: "The sucrose, fructose, glucose ratio of honey, as obtained by the classical reducing and nonreducing sugar methods, does not correspond to values obtained for these specific sugars by modern chromatography. They correspond in name only."

With respect to the new chromatographic and similar techniques that are being developed and used today, Horwitz has this to add: "The advent of these new techniques also permits us to go into problems involving safety, health, and nutrition of consumers, which have been deemed much more important by modern regulators than chasing adulterators."

Another interesting example of changes and advancement in methodology is the analysis of laetrile and amygdalin. "The Analytical Chemistry of Amygdalin" by Cairns et al. (*Anal. Chem.*, Vol. 50, pp. 317–322) provides a multidisciplinary approach using LC, GC (gas chromatography), NMR (nuclear magnetic resonance), and GC/MS (mass spectrometry). These techniques supersede the AOAC official method, an acid and alkaline titration of cyanide.

Alcoholic Beverages

The adulteration of malt liquors, wine, cider, and whiskey is as old as the art of production itself.

C. A. Crampton, assistant chemist at the USDA Division of Chemistry, reporting on malt liquors, wine, and cider in the justly famous *USDA Bulletin No. 13*, 1887, had this to say: "The production of malt liquors in the country as an industry is second only in importance to the production of breadstuffs. Their consumption is steadily on the increase,

as is also the amount consumed in proportion to other kinds of alcoholic beverages." Quoting further from *USDA Bulletin No. 13*, 1887, on the process of brewing: "It was practiced by the Egyptians, and the Greeks and Romans learned the art from them. . . . The Romans are also supposed to have derived a knowledge of the art from the Egyptians, and Pliny and Tacitus both speak of its use among the Gauls and Germans of Spain and France."

I would guess that the Celts and Danes had their mead and ale before this time, and if I had time to read all the sayings of Confucius (550–478 BC), he would probably have warned against excessive consumption of alcoholic beverages. Even before that, there is the story of the native Hawaiian who was knocked out by a falling coconut. Upon waking up, he broke open the coconut, drank the juice, and danced back to the compound in high spirits.

Analyses made in 1873 in New York City by Professor Doremus, reported in *American Analyst*, April 1, 1887, were made for specific gravity, alcohol, extractive matter, water, albuminoids, ash, phosphoric acid, and sugar. All of these tests are early AOAC methods. Additional tests made during this period were reported for glycerine and carbonic acid. Methods for finding adulterants generally came from London, Bavaria, Paris, Belgium. These adulterants were substitutes for malt (glucose) or hops (quassia and gentian roots) and occasionally included strychnine and preservatives (salicylic acid, boric acid, and phosphoric acid).

Wines and whiskeys follow the same pattern. AOAC Reporter W. B. Rising of Berkeley, CA, reporting to the 6th and 7th annual meetings of AOAC, listed the most common adulterants for wine to be salicylic acid, sulfurous acid, sulfites, boracic acid, carbonate of potash, salt, alum, plaster, water, alcohol, glucose, glycerine, colorings, antiseptics, brandy, tartaric acid, ether, magnesia, and phosphoric acid.

Current official methods for wine analysis continue emphasis on accuracy, mainly by instrumentation. Esters, aldehydes, and methanol are determined by spectrophotometric and gas chromatographic methods.

C. S. Ough, chairman of Viticulture and Enology at University of California, Davis, states "Modern-day methods of analysis of SO_2 in the wine industry have not changed appreciably in practice. They still use the Ripper method (direct iodine titration) or variations of it, using redox end point detection. New methods using equipment being developed by Dionex will eventually come into use but not for some time. We feel the Monier-Williams method is the one of choice at the present time." Many of the older methods for the determination of alcohol in whiskey will be superseded by the oscillating U-tube density meter procedure.

The American people were remarkably indifferent to the composition of their food or drink in the years of the Republic. The "messing-up" of whiskey, wine, and beer had as much to do with methods' development and passage of state and federal laws prohibiting adulteration and misbranding as did food problems. With the founding of AOAC in 1884, and the assurance of reliable methods for detection as well as the enactment of the Federal Food and Drugs Act of 1906, we began to see both consumer concern and the desire for regulation accelerate. This acceleration has also contributed to the disappearance of the Republic and the emergence of the one-person/one-vote syndrome, democracy.

Vitamins

The identification or discovery of a new vitamin has been hailed with enthusiasm in the past and undoubtedly will be in the future. Vitamins and other food components may be adulterated because of a deficiency as well as an excess or by an added substance (foreign matter). Mike J. Deutsch, Food and Drug Administration, AOAC General Referee for vitamins, outlined the progress made in vital assays for vitamin C potency by way of example:

The indophenol assay approach to vitamin C (reduced form) determination was introduced in 1920. The method was later improved (1933) and adopted by AOAC in 1945. The manual microfluorometric assay for total vitamin C using *o*-phenylenediamine, was adopted in 1967. Subsequent work resulted in the granting of official status for the semiautomated version of this assay in 1983.

Chicken and rat assays have been used for both water-soluble and fat-soluble methods development work, but current AOAC animal assays exist only for vitamins D_2 and D_3. (Rat gives total vitamin activity; chicken gives D_3 and D_2 by difference.) Considerable effort is being expended to replace these methods with automated instrumental assays, primarily liquid chromatography. Microbiological assays are still needed, however, and have been semiautomated, but investigators are trying to replace them with liquid chromatography, radioimmunoassay, and other similar techniques.

As pointed out in previous situations involving dairy products and wine, malt beverages, and spirits, the early "test tube" methods are giving way to faster, automated, multiple analysis methods. This does not imply that the earlier methods did not serve the scientists, regulatory officials, and industry. They certainly did and will continue to identify and therefore limit adulteration, while newer methods are being developed to meet AOAC standards.

Bacteria and Mold Contamination

The law enforcement official frequently becomes frustrated when there is clear-cut evidence of food adulteration but no clear-cut definition of the amount of adulteration that must be present to render the product unwholesome or subject to seizure, condemnation, or court action. On rare occasions, the courts will define limits or standards to solve the problem. In one example, the Tennessee Food and Drug Administration and resident federal inspectors cooperated in the initial phase of an investigation on breaking out, freezing, and shipping of hatchery-rejected eggs to Chicago. The seizure, testing, and court action in the FDA Chicago district resulted in the establishment of a limit for frozen egg adulteration of five million viable microorganisms per gram of egg material (*AOAC Journal*, **36**, 912, 316 (1953)). Such a standard represented a significant improvement over detecting decomposition by sniffing 3 to 4 holes bored into the frozen eggs.

The following mold count incident illustrates another type of food adulteration problem. In the early 1900s, there were 100 tomato canneries and 50 strawberry processors in Tennessee. They were seasonal and mobile—coming and going—frequently in the middle of the night. When FDA had a big spring meeting in West Tennessee, having gone through 2 days of high level advice on the do's and don'ts of sanitary practices, we came to the question and answer period for industry:

An itinerant canner in the back of the room asked the question: "Now, Doctor," (he was always in trouble so this was his usual salutation), "We are up on everything now, and I have only one question—*What is the tolerance for mold count?*"

Having been elected to respond, I said, "Sir, you know more about canning tomatoes or strawberries than I will ever know; however, there is one thing I know that you do not,

and that is the tolerance for mold count—*and I am not about to tell you!*" That broke up the meeting.

Later that summer, however, the Chief Inspector and I were in Gibson, Tennessee, where one of the conference participants was canning tomatoes. Because it was the end of the season, the tomatoes were mushy and infested. We observed the operation for a few minutes, and I asked the canner, "Why are the screens so dark?" "They are partially covered with tar paper," he replied, and then stated, "Boss, that's not tar paper; that's flies."

So I called the owner over and asked him to have all the workers stand back from the processing line. When the line was clear, I pulled the power switch and stopped the operation. The owner then shouted, "What's going on here?" The Chief Inspector replied, "It looks like the tomato canning season is over." And so it was. This is some of the stuff that food technicians are made of.

A vast field of possible food adulterants has opened up since the 1960s. L. Stoloff's General Referee report shows the increasing importance of mycotoxins because of their contamination of an increasing number of foods. The chick embryo bioassay for aflatoxin B_1 toxicity was one of the first techniques developed. Many innovative methods for determining mycotoxins followed; Stoloff lists 56 references in his 1980 report. The Harvey W. Wiley Award address by Odette L. Shotwell tells the interesting story of the successful cleanup and disposition of 400 000 bushels of white corn contaminated with aflatoxin at an elevator in Diehlstadt, Missouri. The Northern Regional Research Center, ARS, USDA, used AOAC- and AACC-approved methods of analysis to establish tolerances for aflatoxin in grain.

Peter M. Scott (Health and Welfare Canada), chairman of the Joint AOAC–AOCS–AACC Mycotoxin Committee and AOAC General Referee for Mycotoxins, evaluated current methods of analysis for the Fusarium molds and concluded: "The best technique to date for quantitating trichothecenes is gas chromatography with electron capture or mass spectrometric detection, subject to available instrumentation."

Technology of the Future

Several examples of food adulteration and the progress made by AOAC members to develop methods of analysis for detection over the past 100 years have been cited. I realize, however, that very little has been said about meat, seafoods, fruits and vegetables, spices, and condiments, which become adulterated in production, processing, shipping, and the place of sale. Extraneous matter, products of decomposition, rodent and insect contamination, artificial colors, preservatives, pesticides, hormones and drugs, radiation, or microbacteria or their toxins may be the culprits.

A Historical Perspective of Intentional Adulteration of Foods is the subject of a paper prepared and delivered by Robert M. Reeves, director of the Food and Dairy Division, Tennessee Department of Agriculture, to the 96th Annual International Meeting of AOAC, October 25, 1982. There probably were insidious inspectors in those early trading times, or perhaps the raiders from Athens and Rome had the tax-gatherer use a straight stick to strike off a unit of measure of grain. Mr. Reeves states: "In 1202, King John of England proclaimed the first English Food Law, the Assize of Bread. This made it illegal for bakers or others to adulterate bread by mixing into the flour such ingredients as ground peas or beans. Violators were put into the pillory with a certain quantity of such dough hung from their necks." But Mr. Reeves could mete out some severe punishment himself. He became suspicious of a 2000 lb shipment of "hamburgers" to a Tennessee state institution and had the lot sampled and tested. The findings were positive for horsemeat. As Mr. Reeves proceeded with notices, hearings, and retesting, a lonesome farmer came in and said, "That's my pony in that hamburger, and I want my $250 which I was promised." That's a good way to confirm your laboratory reports, and it also teaches the fraudulent operator: "Pay on receipt of the merchandise." The courts took a dim view of the matter and closed the plant.

There are other and more severe ways to deal with adulterators. A clipping from the *Sydney Australia Sunday Telegraph* (June 5, 1983) reads as follows:

"Russian Racketeer Executed"

"The boss of a band that marketed bogus fruit juice for personal profit has been executed by firing squad in Soviet Georgia. A local newspaper said the case put an end to the dealings of 'rascally schemers' who had operated for years." That was a deterrent for rascally schemers.

The following comments on trace chemical contaminants were submitted by Stephen Walters, Food and Drug Administration, Detroit, Michigan:

"One can probably speculate with some certainty that there was little concern a hundred years ago about adulteration of foods with synthetic chemicals. The proliferation of agricultural chemicals, many of which were chlorinated organics, in the mid-20th century prompted a major concern for the safety of our food supply in this regard.

"The advent about 25 years ago of gas chromatography with microcoulometric and electron capture detectors was the beginning of a new era in the analysis of pesticides and related industrial chemicals as food adulterants. Among the earliest applications of this technique to pesticide residue analysis were those reported in the *AOAC Journal* by Watts and Klein and Burke and Johnson in 1962. At that time, microcoulometric detection permitted determinations in the microgram range, while electron capture detection permitted nanogram level analysis of organochlorine compounds. The use of Florisil columns for purification of sample extracts, which was previously described in the *Journal* by Mills in 1959, permitted gas chromatographic quantification of many chemical residues at 1 ppm and lower. These techniques formed the basis for a surge of activity in the development of analytical applications and numerous collaborative studies culminating in the official AOAC multiresidue methodology in use today.

"In recent years, major advances have occurred in the development of high resolution capillary gas chromatography (HRCGC) and high performance or high pressure liquid chromatography (HPLC). These terms were virtually nonexistent 25 years ago but are in common usage today. Investigations of HRCGC for application to multiresidue analysis of pesticides and industrial chemicals were recently reported in the *AOAC Journal* by Ripley and Braun (1983) and Fehringer and Walters (1984). With the use of HPLC for sample purification and analyte isolation, state-of-the-art HRCGC with ^{63}Ni electron capture detection was shown by Niemann et al. (*Anal. Chem.* (1983) **55**, 1497) in 1983 to be capable of resolving and accurately quantifying 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in fish extracts at less than 20 parts per trillion. TCDD, which entered the food chain as a trace contaminant of certain chlorinated phenolic compounds, is the most toxic of a family of 75 related isomers of chlorinated dibenzodioxins and has often been described as "the most toxic chemical ever made." The interfacing of HRCGC to a modern mass spectrometer permits the identification of picogram quantities

of these isomers in food sample extracts with a high degree of certainty, and numerous applications of GC-MS to this analysis have been reported in recent years. Though not yet developed to "official" status, these techniques and methods are being increasingly applied in laboratories throughout the world.

"Thus, the past 25 years of development in quantitative methods for chemical contamination of foods has progressed through 6 orders of magnitude, from the parts-per-million level to the parts-per-trillion level. One has to wonder what the next 25 years will bring in analytical capability for trace contaminants in food. While our increased capabilities sometimes lead to excessive public concerns about food safety, we must press forward with the conviction that an informed approach is the best way to solve problems and assure that the safety of our food supply is maintained."

Discussions with Food and Drug Administration scientists on the technology of the future resulted in the following comments:

"The current revolutions in computer applications and biotechnology undoubtedly will make significant contributions to food adulteration detection. Studies of applications of computer multivariate analyses (such as pattern recognition methods) for determination of adulteration have been reported, and expanded studies are underway. Professor Bruce R. Kowalski of the University of Washington, a pioneer analytical chemist in this area, has reported successful studies on wine and Scotch whiskeys. FDA is currently studying applications of these methods for determination of orange and apple juice adulteration. Methods for determination of adulteration on the basis of analyses for a limited number of inexpensive constituents or adulterants usually have been circumvented by the addition of or substitution for the appropriate constituent or adulterant. In addition, these analyses are complicated by the natural variations of the fruit juices. Computer pattern recognition methods offer a means of data analysis using large numbers of measurements. In addition to such common analytical measurements as GC, LC, and elemental compositions, complex analytical data including infrared, fluorescence, and nuclear magnetic resonance spectra can be used in these analyses. The computer calculations with these data employ classical multivariate statistical methods. From these analyses, patterns—analogous to 'fingerprints'—for authentic and adulterated juices can be developed. As Kowalski explains, the use of computer multivariate data analysis 'can have a profound effect on a study by relieving the limitations of collecting only a few variables on a system under study and ensuring that all the useful chemical information can be squeezed from the data.'

"Under the broad new horizon of biotechnology, immunoassays are being developed for determination of adulteration. Such methods have been developed for determination of adulteration of beef products with kangaroo and horse (and probably pony and raccoon!) meat. Monoclonal technology is being used to develop very specific and sensitive assays. Several immunoassay methods for determination of mycotoxins are also being used. There are numerous potential other applications for these methods, such as determination of mold contamination.

"A major challenge for AOAC will be to carry these new technology methods through the validation process to official action."

Conclusions

As we look now to the beginning of the second century of the Association of Official Analytical Chemists, we can see what is ahead, if we have studied the past. Dedication, hard work, and an occasional flash of genius will enable the scientists of the future to meet and overcome the challenges ahead. The organization and programs of the Association are strong. True, there is some criticism, even some bitterness, particularly about the slow, laborious work required to bring a method to final action. This problem is being studied, and something good will come out of the efforts. A complex method of analysis, speedily adopted, which gives inaccurate results, is worse than bad—it would be terrible. George McClellan, General of the Army of the Potomac, is as good an example to use here as he was in the fall of 1862. He was practically within sight of General Lee across the river and had been for several months with a well equipped army of nearly 200 000 men while General Lee had about 75 000. Old Abe made several efforts to get McClellan to move, but he wouldn't. When the President's friend, Frank Blair, asked him what the trouble was, Lincoln replied: "He has got the slows, Mr Blair."[2]

AOAC has more to gain by having "the slows" and being accurate. You might say, "All deliberate speed," and be about right.

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EXTRANEOUS MATERIALS

Fecal Pellets from a Commensal Shrew *(Suncus murinus)* and a House Gecko (Gekkonidae)

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Examination of excreta pellets from commensal shrews *(Suncus murinus)* and tropical house geckos (Gekkonidae) showed several characteristics that were useful in distinguishing these pellets from those of commensal rodents. Commensal shrew pellets contained a large number of embedded insect fragments but no embedded hairs. Each shrew pellet was associated with a distinctive circular stain on the floor or container surface where it was found. Gecko pellets were composed entirely of tightly packed insect fragments with no intervening matrix. In contrast to both shrew and rodent pellets, there was no surface mucous coating. A small white body containing uric acid adhered to one end of each gecko pellet.

The U.S. Food and Drug Administration (FDA) regularly examines samples of a variety of imported food products to determine if they are adulterated with filth. Among the possible adulterants in imported food samples are excreta (fecal) pellets from commensal vertebrates, many of which are cosmopolitan pests such as the murine rats and the house mouse, whose excreta pellets have been studied previously (1, 2). Some food products entering the United States from tropical regions, however, may have been exposed to tropical-cosmopolitan or regional commensal pests whose excreta pellets have not been completely described in the literature. This report describes excreta pellets from the commensal shrew *(Suncus murinus)* and a house gecko (Gekkonidae), 2 commensals that are common in the Far Eastern tropics but not in the temperate regions of the United States.

Experimental

Authentic specimens of excreta pellets from commensal shrews and house geckos were collected in August 1982, on the island of Guam, a U.S. territory in the equatorial Western Pacific Ocean (Mariana Islands). Guam is a moderately active military and commercial port along the air and maritime trade routes connecting the Far East with the United States. The specimens were collected inside an inhabited Western-style building, confirming the commensal habits of these pests and their ability to enter and infest buildings constructed with modern materials and techniques. Pellet specimens were examined microscopically and dissected to compare their characteristics with those of excreta pellets from rats and mice. Portions of house gecko excreta pellets were also tested for the presence of uric acid (3). Examinations and chemical tests were performed at the FDA Los Angeles District Laboratory.

Results and Discussion

Excreta pellets (Figure 1) of the commensal shrew, also known as the Asiatic musk shrew, were collected from a closed storeroom which the shrews had entered through a gap under the entrance door. Twenty-five excreta pellets were found in the storeroom, which had a floorspace of about 20 sq. ft. The pellets were distributed singly and in pairs on the storeroom floor, on stacked multi-ply paper sacks of gardening supplies, and between the stacked bags. Each pellet was located in the center of a nearly circular stain of dried liquid with a maximum diameter 2–3 times the length of the pellet. Although specimens of this characteristic circular stain associated with each pellet could not be obtained, the stains had the general appearance of urine stains.

Microscopic examination showed that commensal shrew excreta pellets are cylindrical with moderately to strongly tapered ends. Larger pellets may be bent or twisted. Pellet length ranges from 4 to 12 mm, and color varies from brown to black. The surface is covered with a mucous coating which turns grayish and viscous when wet. Embedded insect fragments are usually visible.

Dissected pellets contained a large proportion of insect fragments, including roach fragments, which were embedded in a dark, amorphous matrix. Occasional bits of plant material, bagging paper, and textile fibers were found in some pellets. In contrast to rodent excreta pellets where embedded hairs are commonplace, none of the pellets dissected contained embedded hairs or hair fragments.

Commensal shrews feed on processed seafoods or other meat products and, in some regions, compete with commensal rats for living space (4). Because the commensal shrew is spread by commerce (5), its importance as a pest of stored products is likely to increase, creating a greater need for food analysts to become familiar with commensal shrew excreta pellets and other shrew adulterants.

Excreta pellets (Figure 2) of the house gecko (Sauria: Gekkonidae) were collected from a sink and adjacent countertops in a kitchen. The 12 pellets collected represented the overnight activity of 6–8 geckos, which were nesting in the blinds of a window above the sink. These 7–10 cm (3–4 in.) long nocturnal lizards prefer nesting sites in dark, undisturbed recesses (6), where dirt and static material also accumulate. The geckos probably entered the building through an outside door which was briefly opened for people to enter or leave. This is a frequently used entry route, since geckos are attracted to building entrances by the insect activity around adjacent porchlights. Once inside, geckos will nest and breed within a building; in this case, immature geckos were seen around the sink in the kitchen area and in the restroom.

Microscopic examination showed that house gecko excreta pellets are cylindrical with tapered ends. They are light to medium brown and range in length from 8 to 10 mm. The surface is uniformly smooth and featureless, with no surface mucous coating. A small, spherical, white body, 1–2 mm in diameter, adheres to each pellet near one end. The white body, which is easily detached from the pellet by probing or other handling, consists of white, chalky amorphous material which gives a positive reaction when tested for uric acid.

Dissected pellets consist entirely of tightly compressed insect fragments with no discernible connective matrix. The insect fragments in the pellets dissected for this study were from adult moths and included large and small body fragments, appendages, and scales. A few fragments of other nocturnal flying insects, such as mosquitos, were also present. Although the diet of these lizards may include roaches

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Figure 1. Typical excreta pellets of the commensal shrew, *Suncus murinus*.

Figure 2. Excreta pellets of a house gecko (Gekkonidae). The small white body associated with each pellet contains uric acid.

(7), no roach fragments were found in the dissected pellets. The dissected pellets were from geckos tentatively identified as *Hemidactylus frenatus*, the Philippine house gecko.

House geckos are tropical-cosmopolitan in distribution (7). There is a species of house gecko established along the U.S. Gulf Coast, where it is almost entirely confined to human habitations (8). The distinguishing characteristics are described here to aid food analysts in recognizing gecko excreta pellets.

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FRUITS AND FRUIT PRODUCTS

Liquid Chromatographic Determination of Hydroxymethylfurfural in Fruit Juices and Concentrates After Separation on Two Columns

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A liquid chromatographic (LC) method is described for the detection and quantitation of 5-hydroxymethyl-2-furaldehyde (HMF) in fruit juices and juice concentrates. HMF is separated by chromatography in 2 dimensions with different reverse phase columns. The first column is eluted with aqueous solvents, 5% methanol–2.5% acetonitrile–1.5% tetrahydrofuran (1 + 1 + 1). By using an electrically driven, 6-port valve, the collected impure HMF fraction is rechromatographed on the second column with 5% aqueous methanol as mobile phase. The compound was detected at 284 nm under conditions selected to separate HMF in 26 min with very little or no sample preparation. On a series of samples, this method gave lower values than did the colorimetric toluidine–barbituric acid method.

Hydroxymethylfurfural, or 5-hydroxymethyl-2-furaldehyde (HMF), is a cyclic aldehyde formed by dehydration when hexoses are heated (1); this reaction is acid-catalyzed (2, 3). A high concentration of HMF indicates excessive heat treatment (4) during concentration or pasteurization. Packaging, storage, and reactions with other compounds could also influence HMF concentration (5–7).

Several methods can be used to determine HMF. Some of these methods, however, cannot be applied to the analysis of food and fruit juices. Spectrophotometry (8) cannot be used because many other UV-absorbing substances present in fruit juices interfere. HMF has been detected by thin layer chromatography (TLC) (9) and quantitated by HPTLC (10). The colorimetric determination of HMF with *p*-toluidine–barbituric acid is presently recommended by the International Federation of Fruit Juice Producers (IFFJP) (1) in Europe. When applying this method, it is necessary to depectinize or dilute certain fruit juice concentrates to prevent precipitation in, or clouding of, the solution which is to be measured at 550 nm. The method is sensitive but has some disadvantages, such as toxicity of *p*-toluidine, instability of the color complex formed, and interference of sulfurous acid and possibly other compounds present in the fruit juice or concentrate.

LC (liquid chromatography) has been used for HMF determinations in spirits, honey, and caramel (11–13). The presence of interfering peaks complicates separation by LC of HMF in fruit juice concentrates. In many commercial fruit juices, separation could be achieved on a single RP-18 column. However, adequate separation could not be obtained by using only 1 column for pineapple and guava fruit juice concentrates because at low concentrations the HMF peak was masked by another peak. Separation on 2 columns (with column switching) eliminated these problems.

Experimental

Apparatus

The LC equipment consisted of a Varian 5000 pump and Valco inlet valve fitted with a 50 μL sample loop, a Vari-Chrom detector (284 nm) maintained at 35°C by a circulating water bath, and a Hewlett-Packard 3390A integrator/plotter. Two stainless steel columns (25 × 0.46 cm id) were slurry-packed, one with 5 μm C-1 Spherisorb and the other with 5 μm RP-18 (Merck). These columns were connected via a Valco electrically operated, 6-port valve. Figure 1 is a schematic diagram of the column configuration. All other columns tested were prepared in similar column blanks and packed in the same way.

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Chemicals

HMF [5-hydroxymethyl-2-furaldehyde] from Merck was used as a standard; 100 mg was diluted to 100 mL with water. The exact concentration of HMF was determined at 279 nm with a Gilford 2400-S spectrophotometer (14). RP-18, RP-8, and RP-2 (Merck), Nucleosil C-18 (Macherey and Nagel), and C-1 (Spherisorb) were used as packing materials. *p*-Toluidine was from Fluka; barbituric acid and AR grade acetonitrile, methanol, and tetrahydrofuran (THF) were from Merck.

Sample Preparation

Clear normal strength fruit juices were analyzed as such. Fruit juice concentrates were diluted by weighing 50 g concentrate and diluting it to 100 mL with water. After proper mixing, cloudy samples were centrifuged (50 000 × g) at 5°C for ≥20 min, depending on viscosity. The supernate was directly injected (50 μL). The same procedure was followed for turbid, normal strength fruit juices. When sulfurous acid was present, the sample had to be treated with acetaldehyde (1) to liberate the HMF.

Determinations

Mobile phase (A) of equal volumes of acetonitrile (2.5% in water, v/v), methanol (5% in water, v/v), and THF (1.5% in water, v/v) at 1 0 mL/min was used for HMF separation on column 1 (C-1). The impure HMF peak eluting from column 1 (in mobile phase A) between 4.0 and 5.5 min was transferred to column 2 (RP-18) by the 6-port valve. The first column was then cleaned with 100% methanol (B) and equilibrated with an aqueous mobile phase (C) of 5% methanol (or 5% acetonitrile for guava) before the valve was automatically activated to divert this mobile phase onto the second column. The valve and the switching of mobile phases were controlled by the pump microprocessor. The integrator was programmed to reset the baseline when the elution of compound from column 2 was started. The second column was then cleaned with mobile phase (C). The first column was equilibrated with mobile phase (A) for 15 min more. HMF separation can be done in 26 min; a sample can be injected every 50 min. Table 1 summarizes this procedure. An autosampler may be used to automate sample injection.

The colorimetric determination of HMF by the toluidine–barbituric acid method was carried out as described by IFFJP (1).

Calibration and Recovery

A calibration curve, area vs mg HMF/L, was obtained for LC determinations by making duplicate 50 μL injections of solutions containing 0.02, 0.18, 1.98, 9.88, and 19.76 mg

Table 1. Timing for mobile phase changes and column switching

Time, min	Mobile phase[a]	Flow rate, mL/mir	Columns[b]	Comment
0	A	1	1	sample on column 1
4.0	A	1	1 and 2	place HMF fraction on column 2
5.5	A	1	1	stop flow through column 2, elute column 1
5.6	B	2	1	clean column 1 with mobile phase B
9.4	C	2	1	equilibrate column 1 with mobile phase C
17.0	C	1	1	change flow rate
17.5	C	1	1 and 2	start eluting compound from column 2 with mobile phase C; reset baseline
40.0	A	2	1	equilibrate column 1 with mobile phase A
50.0	A	1	1	ready for next sample

[a]A: 2.5% acetonitrile–5% methanol–1.5% THF (1 + 1 + 1, v/v, aqueous)
B: 100% methanol
C: 5% methanol or 5% acetonitrile
[b]1: C-1 column; 2: RP-1E column.

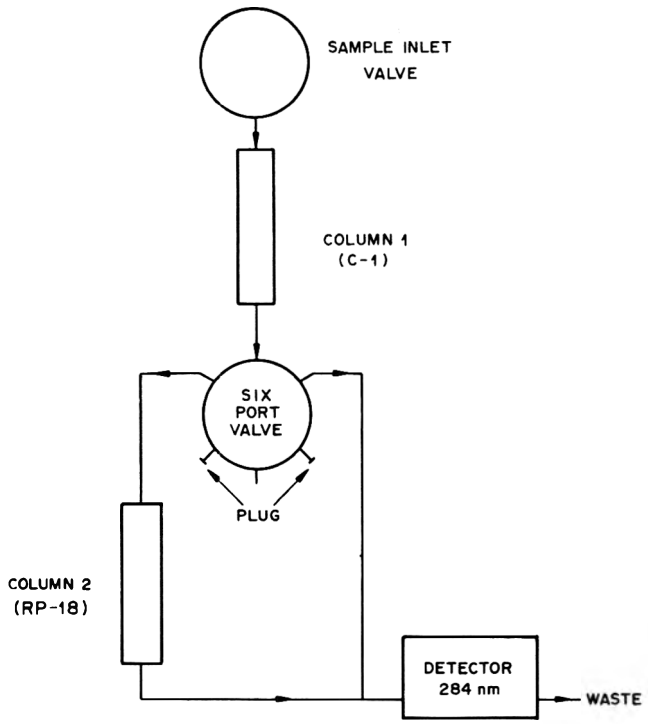

Figure 1. Schematic presentation of 2 reverse phase columns connected via a 6-port valve.

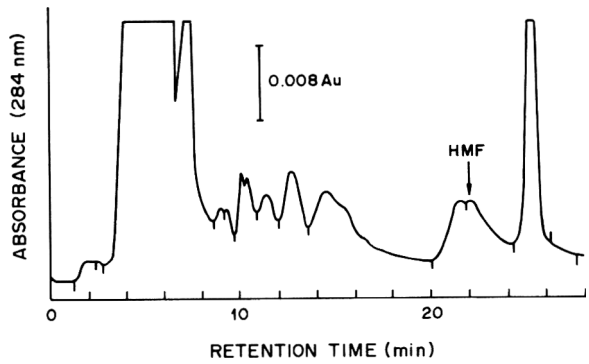

Figure 2. Pineapple concentrate (50 μL) chromatographed on two C-18 columns connected in series. Acetonitrile (5%) at 1.5 mL/min was used as mobile phase.

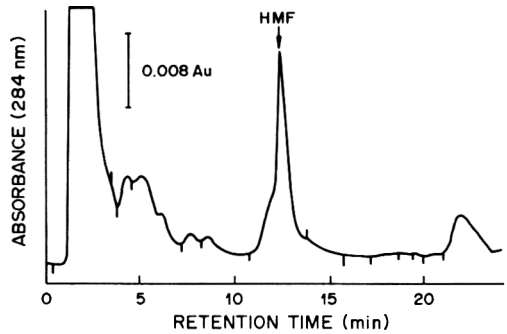

Figure 3. Pineapple concentrate (10 μL) chromatographed on RP-18 column with 5% methanol as mobile phase at 1.5 mL/min. Standard HMF was added.

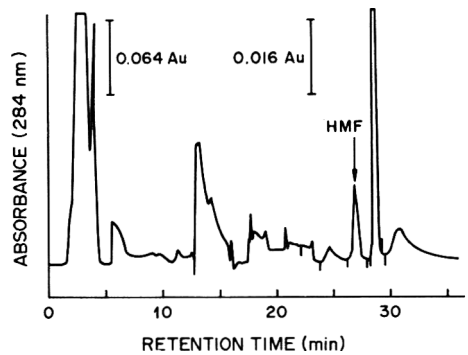

Figure 4. Pineapple concentrate (50 μL) chromatographed with column switching. Column 1 (C-1) was eluted with 2.5% acetonitrile–5% methanol–1.5% THF (1 + 1 + 1) at 1 mL/min. Column 2 (RP-18) was eluted with 5% methanol. After 23 min, attenuation was changed from 0.256 to 0.064 AUFS.

HMF/L. Samples containing between 0 and 22 mgHMF/L were used to determine the coefficient of variation (CV). The pooled CV was determined by squaring and summing the CV values for duplicate determinations, dividing by the total number of duplicates, and then taking the square root. Recovery determinations were done on solutions prepared by adding 8.8 and 21.9 mg/kg HMF standard to 50 g pineapple concentrate, which originally contained 7.9 mg HMF/kg as determined by LC, and diluting to volume (100 mL) with water.

Recovery tests were also done for the colorimetric method by adding 10 and 20 mg/kg of HMF standard to a pineapple juice concentrate containing 8 mg HMF/kg (as determined by the colorimetric method). A calibration curve was obtained by plotting absorbance vs mg/L HMF standards. The pooled CV was determined for samples containing HMF concentrations between 0 and 22 mg/L.

Samples Analyzed

Commercial normal strength apple, pineapple, and guava juices were obtained from a retail store and analyzed by LC and by the colorimetric method. Pineapple juice concentrates obtained from a factory were analyzed in the same way. These samples were also used to compare the 2 methods. The influence of sulfurous acid on HMF values was investigated by adding sodium metabisulfite to the diluted fruit juice concentrate to give approximately 100 ppm SO_2.

Results and Discussion

Different 5 μm reverse phase column packing materials (RP-18, C-18, RP-8, RP-2, and C-1) and aqueous mobile phases (5% methanol, 5% acetonitrile, or 5% tetrahydrofuran) were investigated to obtain adequate separation of HMF on a single column LC system (15–17).

Table 2. HMF values for normal strength pineapple, apple, and guava juices determined by colorimetric and LC methods

Sample	HMF, mg/L	
	Colorim.	LC
Pineapple juice		
P-C	1.5	1.0
P-F1	0.4	0.2
P-L	1.2	0.3
P-F2	1.9	0.3
Apple juice		
A-F1	1.8	1.5
A-L1 (clear)	1.0	0.6
A-L2 (turbid)	1.0	0.4
A-F2	1.9	1.2
Guava juice		
G-C	10.5	9.3

HMF in some single strength juice such as apple juice could be separated and quantitated on a single RP-18 column with an aqueous 5% acetonitrile mobile phase. In samples low in HMF, such as fruit juice concentrates and more viscous tropical juices (pineapple, guava, mango, and marula), separation of HMF even with two C-18 columns in series was insufficient to allow quantitation (Figure 2). In these samples, separation was best achieved with the 2-dimensional system described under *Determinations*. Separations achieved with a single RP-18 column and with the 2-dimensional system are shown in Figures 3 and 4, respectively.

The elution time of HMF was established by spiking fruit juice samples with a standard solution of HMF. We also found that fruit juices that received no heat treatment had no HMF present and that peak areas (due to HMF) could be correlated to heat treatment in several experiments concerning the processing of fruit juices.

For the LC method, a linear calibration curve, $Y = (161X + 0.244) \times 10^5$, correlation coefficient $r = 0.9999$, $N = 5$, was obtained for peak area vs mg HMF/L. Recovery of HMF from pineapple juice concentrate was 98.9 ± 0.6% (SE) ($N = 6$) and 103.9 ± 2.9% (SE) ($N = 3$), respectively, for the 21.9 and 8.8 mg/kg HMF standards added. The pooled CV for 8 samples (different fruit juices) was 4%. Pooled recovery was 99.3 ± 0.8% (SE); recoveries for 8.8 and 21.9 mg/kg standards did not differ significantly ($P > 0.5$).

For the colorimetric determination, a linear calibration curve ($Y = 0.030X$, $r = 0.9998$, $N = 5$) was found for absorbance vs mg HMF/L. Recovery of HMF from pineapple juice concentrate was 105 ± 0.9% (SE) ($N = 4$) for 10 mg/kg and 103 ± 0.7% (SE) ($N = 4$) for 20 mg/kg added standards. The pooled CV for 8 samples (different fruit juices) was 7%. Pooled recovery was 103.4 ± 0.6% (SE); recoveries for 10 and 20 mg/kg did not differ significantly ($P > 0.4$).

The 2 methods differ significantly with respect to the above (pooled) recovery estimates ($P < 0.005$). The results obtained with the 2 methods differ statistically at $P \leq 0.01$ (mean diff. = 0.711, SE = 0.161, $t = 4.43$, $N = 8$); the colorimetric method gave higher values than the LC method. HMF values for normal strength pineapple, apple, and guava juices determined by the colorimetric method and by LC are shown in Table 2.

The presence of sulfurous acid adversely affects the HMF values obtained by both the colorimetric and LC methods. This was established by determining the HMF content after adding sulfurous acid to the samples. After treatment with acetaldehyde (1), the original HMF values were obtained by both methods. Samples that contain sulfurous acid must be treated with acetaldehyde, as described for the colorimetric method, before LC determination.

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DECOMPOSITION AND FILTH IN FOODS

Determination of Histamine in Fish by Liquid Chromatography with Post-Column Reaction and Fluorometric Detection

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A liquid chromatographic (LC) system, using a bonded cation-exchange column, resolves histamine from histidine and other related compounds. Detection sensitivity and specificity are enhanced by introduction of a post-column reaction with o-phthalaldehyde to form a fluorescent derivative. The limit of detection is 1.5 ng histamine, and the response is linear over a range of 7–750 ng. The average histamine recovery from fortified tuna and mahimahi samples was 98.4% with a relative standard deviation of 2.18%. The results for canned tuna and mahimahi were comparable to those obtained by using the official AOAC method. The LC method requires only simple extraction of the sample before introduction into the LC system, which resolves and detects cadaverine and putrescine in addition to histamine.

The presence of histamine has long been associated with the decomposition of fish and has been linked to scombroid poisoning (1–4). Although most reported illnesses involve tuna, other species of fish such as mahimahi have been implicated (3).

Histamine is present in fresh fish at levels <5 mg/100 g (1); larger amounts are formed during the decomposition process by the action of decarboxylating bacteria on histidine (4). Histamine is used as a chemical index of fish decomposition in regulatory guidelines (5). Other amines, e.g., cadaverine, putrescine, spermine, and spermidine, have also been associated with decomposition, and an index to include these compounds has been proposed (3).

Of the 2 official AOAC methods for histamine (6), the fluorometric procedure is most commonly used. This time-consuming procedure involves extraction of the fish with methanol, separation of histamine from amino acids by passing the extract through an ion-exchange column, and reaction with *o*-phthalaldehyde (OPA) under carefully controlled conditions, followed by fluorometric measurement. The gas chromatographic (GC) method of Mietz (3) is more specific than the AOAC procedure, but also involves lengthy extraction and derivatization steps.

The purpose of this work was to use modern chromatographic techniques to develop a reasonably fast screening procedure as a possible alternative to the official AOAC methods. Specificity, speed, sensitivity, and ready availability of equipment are the basic reasons for choosing liquid chromatography (LC). The detection of histamine by LC presents a problem because histamine does not have enough ultraviolet absorbance or fluorescence, nor does the molecule appear to be amenable to electrochemical (EC) detection. Histamine detection can be enhanced by either pre- (7–9) or post-column chemical derivatization.

Post-column reaction, which has been effective for other compounds (9–11), was selected because it requires less sample pretreatment and saves analyst time by automatically performing the reaction steps. Another advantage of this approach is that critical reaction conditions, which can cause reproducibility problems in manual methods, are under precise automatic control.

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The choice of OPA from the reagents reported for primary amine detection enhancement (1–3, 7–13) is on the basis of speed and other reaction condition requirements. OPA forms highly fluorescent substituted isoindoles with primary amines in the presence of 2-mercaptoethanol (13).

This paper reports the development and validation of an LC system with post-column reaction for the determination of histamine in canned tuna and mahimahi samples. The primary concern was the determination of histamine, with limited investigation into the retention behavior of the other significant amines.

Experimental

Reagents

(a) *Methanol.*—LC grade, filtered for particulates (Omni-Solv, E. M. Industries, Inc., Gibbstown, NH 08027).

(b) *Potassium phosphate solution.*—0.1M. Dissolve 13.6 g KH_2PO_4 in purified water (Milli-Q System, Millipore Corp., Bedford, MA 01730), adjust to pH 6.1 with 1N NaOH, dilute to 1 L with purified water, and filter through nylon membrane using apparatus (c)*(1)*.

(c) *Sodium borate solution.*—0.05M. Dissolve 19.1 g $Na_2B_4O_7 \cdot 10H_2O$ in purified water, adjust to pH 10.0 with 1N NaOH, dilute to 1 L with water, and filter through nylon membrane using apparatus (c)*(1)*.

(d) *Mobile solvent.*—Mix 700 mL potassium phosphate solution (b) with 300 mL methanol (a).

(e) *Mercaptoethanol stock solution.*—Mix 10 mL 2-mercaptoethanol (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) with 90 mL methanol (a).

(f) *OPA solutions.—Stock solution.*—Dissolve 500 mg OPA (Eastman Kodak Co., Rochester, NY 14650) in 500 mL methanol (a) and filter through nylon membrane. Store in refrigerator in dark. *OPA reagent.*—Mix 25 mL OPA stock solution into 300 mL sodium borate solution (c), add 5 mL mercaptoethanol stock solution (e), and dilute to 500 mL with sodium borate (c). Prepare fresh daily and protect from light.

(g) *Extraction solvent.*—Mix 200 mL methanol (a) with 50 mL 0.1N HCl.

(h) *Histamine standard solutions.*—Dry histamine dihydrochloride (Aldrich Chemical Co., Milwaukee, WI 53233 or USP) over silica gel 2 h before using. *Stock solution.*—1.2 mg/mL (equivalent to 0.7 mg/mL free base). Accurately weigh ca 120 mg histamine standard, dissolve in methanol (a), and dilute to 100.0 mL with methanol. Store in refrigerator. *Working solution.*—Dilute 2.0 mL stock solution to 100.0 mL with mobile solvent (d). Prepare fresh daily.

Apparatus

(a) *Liquid chromatograph.*—Consisting of pump, sample introduction valve equipped with 25 μL loop, thermostated column oven set at 45°C, 25 cm × 4.6 mm id stainless steel column packed with 8 μm spherical silica particles having aromatic sulfonic acid groups chemically bonded to silica surface (Model 850 system with Zorbax SCX column, DuPont Co., Wilmington, DE 19898), variable wavelength fluoro-

metric detector set at 359 nm with 5 nm slit for excitation and 445 nm with 10 nm slit for emission (Model 650-10 LC, Perkin-Elmer Corp., Norwalk, CT 06856), and recording integrator compatible with detector output (Model 3380A, Hewlett-Packard Co., Palo Alto, CA 94304).

(b) *Post-column reaction equipment.*—Consisting of pump with pulse dampener (Model 6000A with high sensitivity pulse dampener, Waters Associates Inc., Milford, MA 01757) connected to zero dead volume mixing T (Valco ZDV 1/16 in. T, Supelco, Inc., Bellefonte, PA 16823), installed between column outlet and detector. Coils of 120 cm long 0.01 in. id stainless steel tubing are used for connecting T to pump and detector.

(c) *Filtering apparatus.—(1) For solvents.*—47 mm diameter glass filter holder with 0.45 μm porosity nylon membrane filter and 1 L vacuum flask. *(2) For samples.*—25 mm diameter stainless steel or polypropylene filter holder with 0.45 μm porosity nylon membrane and 10 mL Luer-Lok syringe (Rainin Instrument Co., Inc., Woburn, MA 01801).

Sample Preparation

Transfer 10 g uniformly ground fish sample (sec. **18.012,** 6) to semimicro high-speed blender cup, add 50 mL extraction solvent, and blend 2 min. Decant into coarse fritted-glass crucible inserted into bell jar. Apply vacuum and let liquid filter, then transfer remaining sample residue to crucible. Rinse blender cup with three 10 mL portions of methanol, transfer each portion to crucible, stir, and filter. Dilute combined filtrate to 100.0 mL with methanol. Dilute 25 mL aliquot to 50.0 mL with potassium phosphate solution and filter portion through nylon membrane, discarding first 1–2 mL filtrate.

First extract is stable in refrigerator at least 1 week, but analysis should be performed same day as final dilution.

Procedure

Degas mobile solvent and OPA reagent. Establish flow through LC system using mobile solvent at 1.5 mL/min. Introduce OPA reagent, using post-column reaction equipment at flow rate of 1.2 mL/min. (OPA reagent pump must not be run without mobile solvent flow, because this may damage column.) When system eluate is alkaline to pH paper and steady baseline is recorded, proceed with injections of working solution. Adjust detector/integrator attenuation to obtain at least 30% full scale deflection for 25 μL histamine working solution. System is suitable when histamine elutes in 4–8 min and relative standard deviation (RSD) is <2.0% based on peak responses of 6 standard injections. Introduce duplicate 25 μL portions of prepared sample solutions. If necessary, make further dilutions of sample or standard using mobile solvent.

After use, flush entire system with methanol–water (50 + 50) until eluate is not alkaline to pH paper.

Calculation

$$\text{Histamine, mg/100 g fish} = 100\,(R_x/R_s) \times (C \times D/W_x)$$

where R_x and R_s are peak responses for sample and standard, respectively; C is concentration of final standard solution, mg histamine/mL; D is sample dilution factor, mL; and W_x is sample weight, g.

Recovery Experiments

Two samples of canned tuna in water and 2 samples of canned tuna in oil were analyzed in duplicate. Eight 10 g portions were then each fortified with ca 2 mg (20 mg/100 g fish) aliquot of histamine stock solution. The spiking solution soaked into the fish for ca 5 min before analysis proceeded. The same procedure was repeated for 5 mahimahi samples with a total of 8 portions fortified with 2 mg histamine and 5 portions fortified at the 5 mg/10 g (50 mg/100 g) level.

Results and Discussion

Preliminary studies were conducted to optimize the chromatographic separation and post-column reaction parameters. Histamine retention on 4 different columns was examined using solutions of 0.1M KH_2PO_4 adjusted to pH values ranging from 2.5 to 7.5 and mixed with methanol or acetonitrile. The octadecyl and cyanopropyl bonded columns (Zorbax ODS, Zorbax CN, DuPont) resulted in poor chromatography under these test conditions. A bonded cation-exchange column (Zorbax SCX, DuPont) gave the most symmetrical peaks with reasonable retention, and a trimethylsilyl (TMS) column (Zorbax TMS, DuPont) was also adequate. Figure 1 shows the relationship of mobile phase pH to the retention of histamine on the latter 2 columns. Histamine is more strongly retained on the cation-exchange column at lower pH with a change in slope corresponding to the imidazole group pKa at pH = 6.0 (14). The amine side-chain pKa of 9.9 (14) is outside the operating range for silica-based LC columns. Mobile phase pH has the opposite effect on the TMS column with histamine retention increasing at higher pH; this indicates different retention mechanisms for the 2 columns. The ionized form is better retained on the SCX column by an ion-exchange interaction, while the unionized free base is more attracted to the TMS column in a reverse phase ion suppression mode. Increasing the methanol content of the mobile phase to 50% decreases histamine retention on both columns. Higher methanol concentrations present a problem of buffer precipitation, and acetonitrile interferes with the post-column reaction. On the basis of these experiments, the cation-exchange column with a 30% methanol, pH 6.1, 0.1M KH_2PO_4 mobile phase was chosen as the chromatographic system for histamine.

OPA, the first reagent investigated for the post-column reaction, was satisfactory for meeting the basic requirements of a fast reaction and compatibility with the mobile phase. The reducing agent 2-mercaptoethanol and pH 10 borate buffer were incorporated into the reagent solution to obtain suitable reaction conditions. Introduction of the post-column reagent was achieved by means of simple stainless steel fittings and a second pump. Figure 2 shows a schematic of the equipment used. In a satisfactory post-column reaction system, the reagent must be introduced at a uniform, smooth flow rate, and mixing of the 2 streams must be adequate. Fluctuations of the baseline associated with the piston strokes of pump B was overcome by using the pulse dampener provided with the Waters 6000A pump and by connecting an additional 120 cm 0.01 in. id stainless steel tubing between pump B and the mixer. Baseline fluctuations were more pronounced at lower OPA pump flow rates, and 1.2 mL/min was selected to minimize this effect as well as to provide the alkaline reaction conditions required with the chromatographic mobile solvent flow of 1.5 mL/min. A zero dead volume stainless steel T was used for mixing the column effluent and OPA streams; no difference in performance was noted between 3 common fitting brands. The mixer was connected to the detector with 120 cm 0.01 in. id stainless steel tubing, which serves as a delay coil, an additional pulse dampener, and a mixing aid.

The excitation and emission maxima for the OPA-histamine reaction product were 359 and 445 nm, respectively, using a recording spectrophotofluorometer. The LC system detection parameters were set accordingly.

One can expect some loss in the overall LC system efficiency when a post-column reaction is added. In this case the loss was not significant, approximately 10%, calculated as

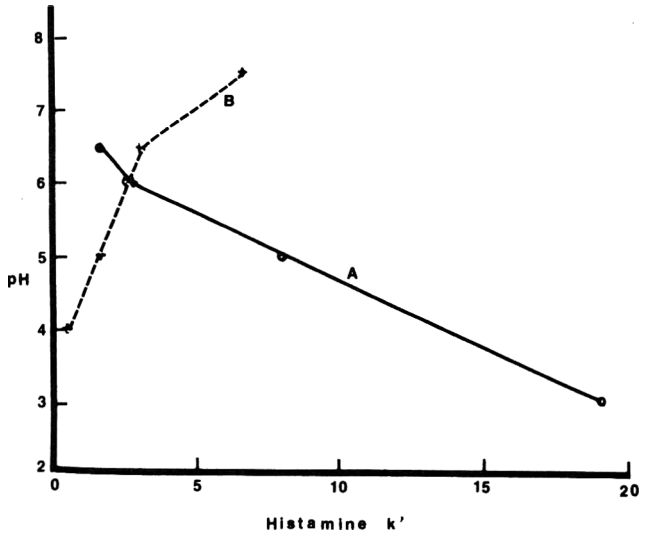

Figure 1. Effect of mobile phase pH on retention of histamine on A, Zorbax SCX column and B, Zorbax TMS column.

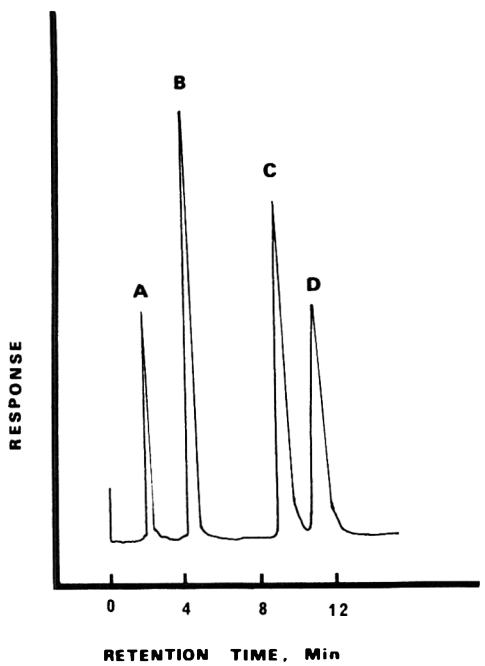

Figure 3. LC chromatogram showing separation of histamine (B) from histidine (A), putrescine (C), and cadaverine (D).

theoretical plate loss of the histamine peak compared to the same system without post-column equipment.

The limit of detection for histamine with this system was 1.5 ng, on the basis of a 2:1 signal-to-noise ratio. The response, measured as integrated peak area, was linear throughout the 7–750 ng range. The peak areas of 12 replicate injections of working standard showed an RSD of 1.6%, indicating satisfactory precision.

Figure 3 shows histamine separation from other compounds of interest. The amino acid histidine, which is naturally present in fish, is well resolved and does not interfere with the histamine peak. Putrescine and cadaverine are also well separated from histamine and from each other. Spermine and spermidine elute more than 30 min later than histamine and are not shown in Figure 3. A gradient solvent system may elute all of these compounds within a reasonable time; however, this has not yet been pursued.

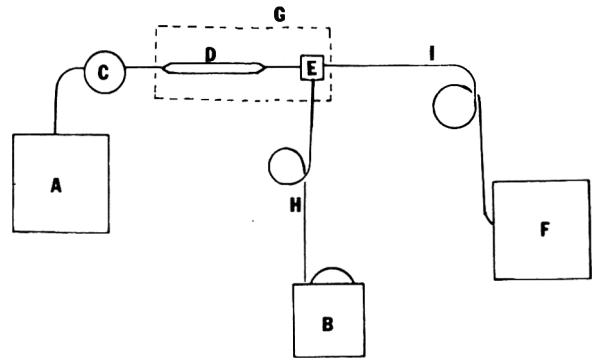

Figure 2. LC system with post-column reaction equipment. A, mobile solvent pump; B, OPA reagent pump with pulse dampener; C, sample injection valve; D, column; E, zero dead volume, stainless steel T; F, fluorometric detector; G, column oven; H and I, 120 cm, 0.01 in. id stainless steel tubing.

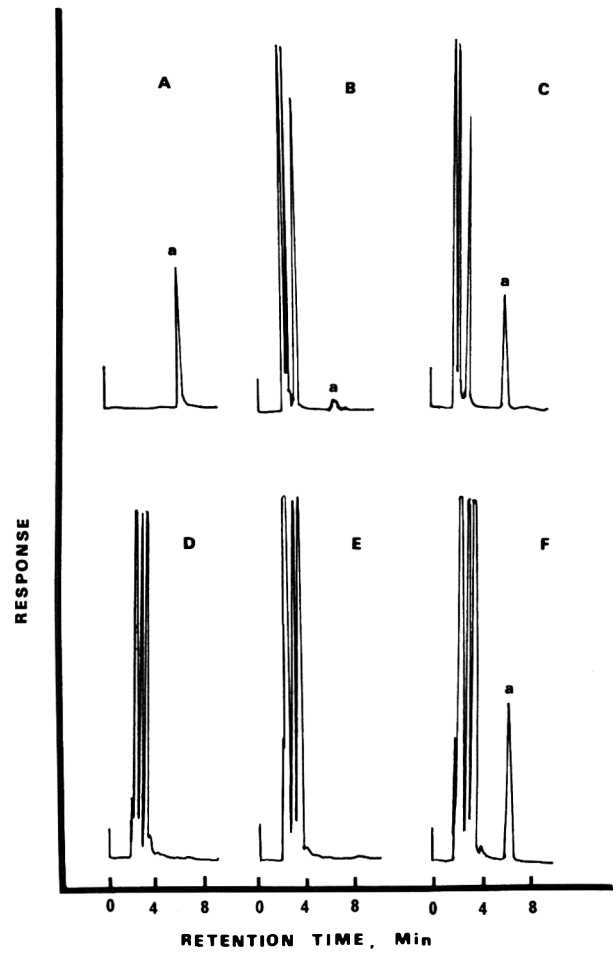

Figure 4. Typical LC chromatograms of fish extracts: A, standard histamine (a) (0.35 μg); B, mahimahi (2 mg histamine/100 g); C, mahimahi fortified with 20 mg histamine/100 g; D, canned tuna in water; E, canned tuna in oil; F, tuna fortified with 20 mg histamine/100 g.

Frozen mahimahi fillets and canned tuna in water as well as in oil were used in this study. Figure 4 shows typical chromatograms of sample extracts and fortified samples. No interfering background was noted in any of the sample chromatograms. Because the LC system effectively separates histamine from possible interferences, only a simple extraction and filtration of the fish sample is required before the determinative step.

Table 1 summarizes results of recovery experiments. The tuna samples were fortified with 20 mg histamine/100 g and the mahimahi with 20 and 50 mg histamine/100 g fish. Some

Table 1. Recovery of histamine (mg/100 g) from fortified fish samples, using LC method

Sample No.	Initially found[a]	Added	Total recd	Total histamine recd, %
		Tuna in Water		
1	0	20.8	20.6	99.1
1	0	20.8	21.1	101.4
2	0.3	20.4	19.5	94.2
2	0.3	20.4	20.0	96.6
		Tuna in Oil		
3	0	20.8	20.3	97.6
3	0	20.4	20.3	99.5
4	0	20.4	20.4	100.0
4	0	20.4	19.8	97.1
		Mahimahi		
5	0[b]	21.8	21.6	99.1
6	2.4	20.4	21.8	95.6
6	2.4	20.4	22.2	97.4
7	55.7	20.4	76.0	100.2
8	69.2	20.8	86.4	96.0
8	69.2	20.8	90.5	100.6
9	89.4	20.8	109.2	99.1
9	89.4	20.8	112.8	102.4
6	2.4	47.5	47.8	95.8
6	2.4	50.0	50.5	96.2
7	55.7	47.5	102.2	99.0
7	55.7	47.5	101.8	98.6
8	69.2	50.0	120.0	100.4
			Overall mean (n = 21) =	98.4
			SD =	2.14
			RSD =	2.18%

[a]Average of duplicate determinations.
[b]Single determination.

of the mahimahi samples contained considerable histamine before fortification; therefore, the total histamine found ranged up to 120 mg/100 g fish. The percent recovery values based on the total histamine recovered were used, because calculations based only on the added amount would multiply the experimental error and introduce a false bias into the results for samples 7–9, which had a high histamine content before fortification. Good recoveries were obtained with a 98.4% overall mean and 2.18% RSD. No obvious differences in the recoveries were noted between the tuna and mahimahi.

Table 2 shows a comparison of the results obtained by the LC procedure and by the AOAC fluorometric method (6). The data generally agree; the differences can be attributed to the time lapse between obtaining the 2 sets of data. Throughout this study, although the samples were kept frozen, their histamine content increases slightly with time; higher results were obtained regardless of the method. An increase of as much as 40% in 12 months was observed. This indicates some continued decomposition in the frozen state, but no explanation of the mechanisms involved can be offered at this time.

The results for canned tuna before fortification or forced decomposition were consistent with the 0–5 mg histamine/100 g range reported for acceptable tuna by Staruszkiewicz (2) and Mietz (3). As anticipated, the 2 tuna samples subjected to forced decomposition by exposure to room temperature for 6 days showed substantially higher histamine content by both methods.

Table 2. Amines (mg/100 g) found in tuna and mahimahi, using LC and AOAC methods

Sample	Histamine		Estimated by LC	
	AOAC (6)	LC[a]	Cadaverine	Putrescine
Tuna in water	0	0.3	0	0
Tuna in water[b]	29.3	30.6	0	0
Tuna in oil	0	0	0	0
Tuna in oil[b]	6.0	6.9	0	0
Mahimahi	3.0	2.4	1.5	0
Mahimahi	61.0	55.7	7.4	0
Mahimahi	73.5	69.2	14.6	0.5
Mahimahi	92.1	89.4	5.5	0

[a]Average of duplicate determinations.
[b]After forced decomposition.

The chromatograms were also examined for the presence of cadaverine and putrescine. Cadaverine was detected in 4 mahimahi samples and a trace of putrescine in one sample. The amounts of these compounds were estimated, and future work to validate the method for determining the other amines is planned. It is not known at present whether the differences between the AOAC and LC results are partly associated with the presence of cadaverine and putrescine, because data regarding their behavior in the AOAC method were not available.

The LC method is based on essentially the same principles as the AOAC fluorometric procedure; it involves an ion-exchange isolation of histamine, followed by OPA reaction, and fluorometric measurement of the derivatized histamine. These steps are accomplished automatically in the LC system, thus reducing the analyst's time and effort.

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VITAMINS AND OTHER NUTRIENTS

Determination of Total Dietary Fiber in Foods, Food Products, and Total Diets: Interlaboratory Study

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An interlaboratory study was conducted to determine the total dietary fiber (TDF) content of food, food products, and total diets, using a combination of enzymatic and gravimetric procedures. Thirteen unknown products including 2 mixed diets (one lacto-ovo vegetarian and the other nonvegetarian) were analyzed by 32 analysts. Duplicate samples of dried foods were gelatinized with Termamyl, a heat-stable alpha-amylase, and then enzymatically digested with protease and amyloglucosidase to remove the protein and starch present in the sample. Four volumes of 95% ethanol were added to precipitate the soluble dietary fiber. The total residue was filtered, and then washed with 74% ethanol, 95% ethanol, and acetone. After drying, the residue was weighed. One of the duplicates was analyzed for protein, and the other was ashed at 525°C and the ash was measured. TDF was calculated as the weight of the residue less the weight of protein and ash. Coefficients of variation for 10 of the samples ranged from 2.95 to 26.39%. For 3 of the samples high coefficients of variation were obtained. The results compared satisfactorily with those obtained previously by the best method available for the individual foods studied.

The development of methodology for the determination of total dietary fiber (TDF) has evolved over a long time and has been hampered by lack of a definition. Part of the complication has arisen because fiber is not a single entity but is a complex mixture of many substances.

Dietary fiber was first defined by Hipsley (1) to include lignin, cellulose, and hemicellulose, and has been broadened by some to include soluble substances such as pectins, gums, and mucilages (2). This current broad definition acknowledges the significance of fiber as a chemical and physiological component of the diet as compared with the static definition of crude fiber, "the residue of plant food left after extraction with solvent, dilute acid, and dilute alkali" (3).

In 1975, AOAC published an official first action method for acid detergent fiber (ADF) which measures cellulose and lignin (4). A neutral detergent fiber (NDF) method was officially adopted by the American Association of Cereal Chemists (AACC) as an alternative to the crude fiber method (5). The NDF chemical method is rapid and gives higher estimations of fiber than does the crude fiber method because of improved recoveries of cellulose, hemicellulose, and lignin. Complete removal of starch by conventional methods is difficult in some food samples, so the method was modified to include an alpha-amylase treatment to remove residual starch (6).

Neither the ADF nor the NDF method include all the components that have been encompassed by the term dietary fiber, nor have these methods been subjected to collaborative analyses in diverse foods and feeds. At the 93rd AOAC Annual Meeting (7), the authors announced their intention of seeking a definition and a method for the determination of TDF which would then be subjected to interlaboratory study.

At the 1981 AOAC Spring Workshop, Asp, Baker, Heckman, Southgate, and Van Soest (8) reported on their fiber methodology research. The scientists present concluded that 2 methods for the determination of TDF should be developed: (*1*) a rapid enzymatic gravimetric method based on procedures developed by Asp et al. (9), Furda et al. (10), and Schweizer and Würsch (11); and (*2*) a more comprehensive method, such as a modification of the methods of Southgate (12) or Theander and Åman (13) to determine the individual dietary fiber components. In the rapid gravimetric method, the sum of the soluble and insoluble polysaccharides and lignin would be defined and measured as a unit; in the second method, each of the specific components of TDF would be identified and measured separately (14). There was recognition that the needs of cereal chemists, who are mainly interested in the fiber content, are different from those of physiologists, who are primarily interested in identifying the fiber fractions that most consistently elicit physiologic response.

By 1981, at the 95th AOAC Annual Meeting, more than 100 responses had been received expressing interest in dietary fiber and suggesting definitions of dietary fiber and a preferred method of analysis. Most respondents preferred the definition of Trowell (15), "Dietary fiber consists of remnants of the plant cells resistant to hydrolysis by the alimentary enzymes of man." This definition was later modified by Trowell et al. (2) and Van Soest and McQueen (3) to include hemicelluloses, celluloses, lignins, nondigestible oligosaccharides, pectins, gums, and waxes.

At that time, the preferred methods for the determination of dietary fiber were modifications of Southgate's procedures (12). Because of the complexities and time involved with this procedure (acknowledging that the method gave the most complete results), an intermediate method was desired. Such a method would include determination of the insoluble fraction as well as the soluble fraction, which was lost in previous methods (ADF, NDF), and yet would be simpler than the Southgate method, so that it could be carried out in most general chemical laboratories. Thus, the interlaboratory study for the gravimetric method developed by Asp, DeVries, Furda, and Schweizer and described in this paper was submitted for study in February 1982.

Enzymatic methods were first used by Williams and Olmsted (16). Pancreatin was used to remove starch and protein followed by acid hydrolysis and subsequent identification and measurement of the sugar fractions. Hellendoorn et al. (17) used pepsin for the hydrolysis of protein and pancreatin for subsequent starch hydrolysis. Furda (18) assessed existing analytical methods and suggested new methodology that would be based on the use of appropriate enzymes and inclusion of the soluble fiber fraction. Asp et al. (9, 19) evaluated some of the more widely used methods for the determination of dietary fiber and proposed enzymatic modifications using pepsin, pancreatin, and Termamyl, a heat-stable alpha-amy-

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lase. Theander and Åman (20) introduced the use of the Termamyl enzyme for combined gelatinization and starch degradation in dietary fiber analysis. At the 93rd AOAC Annual Meeting, Furda et al. (10) reported that use of mammalian as well as bacterial enzymes gave similar values for TDF when these enzymes were free of cellulase, hemicellulase, and pectinase activity. In addition, they sought a measurement of the soluble fractions of dietary fiber.

Heckman and Lane (21) analyzed several foods by various dietary fiber methods: ADF method (22), NDF by the Schaller modification using hog pancreas amylase (23), Robertson and Van Soest's enzyme-modified NDF method using *Bacillus subtilis* amylase (24), the method of Furda et al. for soluble and insoluble dietary fiber using physiological enzymes (10), the Hellendoorn et al. method for "indigestible residue" (17), and Southgate's method for TDF (12). Results showed that enzyme-modified NDF was lower than NDF and depended principally on the starch content of the foods examined. The Furda et al. method, with its capability for measuring soluble dietary fiber, and its additional features of simplicity and rapidity, seemed the most promising. Later Englyst et al. (25) measured the nonstarch polysaccharides (the major components of "dietary fiber") from plant foods. They removed starch from samples by adding hog pancreatic alpha-amylase together with pullulanase and amyloglucosidase, and analyzed the starch-free material, after acid hydrolysis, by gas chromatography (GC).

An enzymatic gravimetric method apparently was the most practical and simplest way to meet the challenge, i.e., the practical approach with the major components identified as dietary fiber. A broad collaborative study testing various gravimetric, colorimetric, and GC methods on 9 food samples was conducted in 1978 and reported by James and Theander (26). There was a large variation in results among different methods, and Southgate's colorimetric method proved difficult to reproduce in different laboratories.

The method studied and reported here was based on several methods (Asp, Englyst, Furda, Schweizer, Southgate, Theander, Van Soest) and was developed at General Mills, Inc., Minneapolis, MN, by DeVries and Furda; at Kemicentrum, University of Lund, Sweden, by Asp; and at the Nestlé Research Department, La Tour-de-Peilz, Switzerland, by Schweizer.

Interlaboratory Study

Portions of the 13 foods described were sent to each of the 42 analysts for TDF analysis. The foods were unknown to the analysts.

(1) Corn bran (A. E. Staley Manufacturing Co., Decatur, IL, Lot CFJ 3101 A-G Regular) was donated by the company and used as provided.

(2) Iceberg lettuce. For every 350 g lettuce, 50 mL deionized water was added and the mixture was blended in a Waring blender for 2 min until homogeneous. The slurry was freeze-dried for 48 h and stored in tightly sealed plastic bags at room temperature until shipped.

(3) Oats, quick cooking (donated by The Quaker Oats Co., Barrington, IL), were blended in a Waring blender for 3 min and stored in plastic bags at room temperature until used.

(4) Potatoes, instant (Giant Foods Inc., Washington, DC), were used directly.

(5) Raisins, seedless (Giant Foods), were blended with enough water to make a thick paste, freeze-dried for 48 h, removed from the pan with a spatula, and placed in sample vials.

(6) Rice, enriched, long grain (Giant Foods), was ground 5 min in a Model 4E Straub electric mixer (Philadelphia, PA) to a uniform powder. There was visible separation of the outer coating, leading to lack of particle uniformity.

(7) Rye bread (Deli-Rye, Giant Foods), was broken into pieces ca 1 × 3 cm in diameter, placed in an aluminum loaf pan, and dried 4 h at 80°C. The dried pieces were blended in a Waring blender for 2 min.

(8) Soy isolate (donated by Ralston-Purina Co., St. Louis, MO) was used as received.

(9) Wheat Bran, AACC Certified Food Grade, was purchased from AACC, St. Paul, MN, and used as received.

(10) Whole wheat flour, high extraction, was donated by General Mills, Inc., Minneapolis, MN, and used as received.

(11) White wheat flour, low extraction, was donated by General Mills, and used as received.

(12) and (13) These materials consisted of a nonvegetarian mixed diet and a lacto-ovo vegetarian mixed diet. The diet comprised the foods reported by Oberleas and Harland (27). Casseroles and baked goods were either purchased ready-to-eat or prepared in the Food and Drug Administration laboratory. The 17 foods in the nonvegetarian diet and 20 foods in the vegetarian diet are shown in Tables 1 and 2, with the amounts of each food expressed as grams per ca 2200 g diet. After all foods listed on the diets were purchased or prepared, homogenates were prepared by adding foods one at a time and blending without adding water until the homogenate was uniform. One hour was needed to homogenize an entire diet. The homogenates were freeze-dried for 48 h, and the powder was again blended in a Waring blender for 2 min before being placed in sample vials.

Because the proposed method recommended fat extraction for samples containing >5% fat, we calculated the fat content of each diet (28, 29). Samples 1–11 did not require fat extraction. Calculations showed that diet composites 12 and 13 contained >5% fat, so fat extraction was recommended for those samples.

All samples were placed in 25 mL scintillation vials with screw caps, and the sample number was taped to each vial.

METHOD

Principle

Duplicate samples of dried foods, fat-extracted if containing >5% fat, are gelatinized with Termamyl (heat-stable alpha-amylase), and then enzymatically digested with protease and amyloglucosidase to remove the protein and starch. Four volumes of 95% ethanol are added to precipitate the soluble dietary fiber. The total residue is filtered and then washed with 74% ethanol, 95% ethanol, and then acetone. The residue is dried and then weighed. One of the duplicates is analyzed for protein, and the other is incinerated at 525°C and the ash is determined. TDF is the weight of the residue less the weight of the protein and ash.

Apparatus

(a) *Balance.*—Analytical, capable of weighing to 0.1 mg.

(b) *Fritted crucible.*—Porosity 2. Clean thoroughly, ash at 525°C, soak in water, and rinse in water. Add ca 0.5 g Celite 545 to crucibles before drying to obtain constant weight. Dry 1 h at 130°C, cool, and store in desiccator until used. A Pyrex

Table 1. Daily diet for a nonvegetarian adult

Food No.	Description of food	Wt of food added to composite, g
1	Wheat flakes, Grape Nuts Flakes, Post	30.0
2	Sugar, granulated	5.0
3	Milk, 2% fat	350.9
4	Egg, medium, raw	51.0
5	White bread, sliced	75.0
6	Margarine	15.0
7	Orange juice	240.0
8	Coffee, brewed, Maxwell House, Electra-Perk	360.0
9	Roast beef	90.0
10	Tomato soup, Campbell Soup Co.	120.0
11	Carrots, raw	119.5
12	Banana	110.8
13	Green bean casserole with cheddar cheese	236.0
14	Corn, whole kernel, cooked	100.0
15	Iceberg lettuce, wedge	155.0
16	Rolls, hard, french	50.0
17	Peach pie	131.0
	Total wt	2239.2

Table 2. Daily diet for a lacto-ovo vegetarian adult

Food No.	Description of food	Wt of food added to composite, g
1	Wheat germ pancakes, 1 tbsp. wheat germ/ pancake	90.0
2	Margarine	25.0
3	Sugar, granulated	10.0
4	Apple, fresh	181.6
5	Granola mix: almonds, coconut, sunflower seeds, and raisins in equal proportions	32.6
6	Cheddar cheese	15.0
7	Grain and spice beverage, Celestial Seasonings Roast Aroma	180.0
8	Peanut butter, Peter Pan, crunchy	30.0
9	Whole wheat bread, sliced	56.0
10	Sliced tomatoes	120.0
11	Five-bean salad: navy, kidney, lima, wax, and green beans; sugar and vinegar dressing	277.0
12	Peach, fresh	188.0
13	Milk, fluid, vitamin D, homogenized	232.0
14	Spinach greens with lemon butter	219.0
15	Potato salad with egg	194.1
16	Corn pudding	62.0
17	Deli-rye bread, sliced	18.0
18	White cake, lightly iced	73.0
19	Peanuts for cake, salted, roasted, crushed	30.8
20	Coffee, brewed, Maxwell House, Electra-Perk	180.0
	Total wt	2214.1

32940 coarse ASTM 40–60 μm fritted crucible was used (available from Scientific Products Co., C-8525-1; V.W.R. Scientific Co., 23863-040; Fisher Scientific Co., 08237-1A; Sargent Welch Co., F-243-90-B or F-243-90-C, depending on size needed).

Crucibles indicated in the procedure may not be available in Europe. Porosity 2 in Europe signifies pores of 40–90 μm, whereas it means 40–60 μm in the United States. Several analysts have reported breakage of crucibles when the temperature was increased to 525°C and have recommended Corning No. 36060 Buchner, fritted disk, Pyrex, 60 mL ASTM 40–60 μm, which seemed to break less frequently and gave the same results.

(c) *Vacuum source.*—Vacuum pump or aspirator equipped with an in-line double vacuum flask should be used to prevent contamination in case of water back-up.

(d) *Vacuum oven.*—70°C and desiccator. Alternatively, an air oven capable of operating at 105°C can be used.

(e) *Constant temperature water bath.*—Adjustable to 60°C, and equipped to provide constant agitation of digestion flasks during enzymatic hydrolysis. This can be accomplished with either a multistation shaker or multistation magnetic stirrer.

(f) *Beakers.*—Tall form, 400 mL.

Reagents

(a) *Ethanol.*—95%, technical grade.

(b) *Ethanol.*—74%. Place 250 mL water in 1 L volumetric flask. Dilute to volume with 95% ethanol. Mix and dilute to volume again with 95% ethanol if necessary. Mix.

(c) *Phosphate buffer pH 6.0.*—Dissolve 1.5 g sodium phosphate dibasic and 10.0 g sodium phosphate monobasic monohydrate in ca 700 mL water. Dilute to 1 L with water and adjust pH to 6.0 by adding drops of dilute monobasic or dibasic sodium phosphate, if necessary.

(d) *Termamyl (heat-stable alpha-amylase) solution.*—120 L (Novo Laboratories Inc., Wilton, CT). Store enzyme solution in refrigerator after each use.

(e) *Protease P-5380.*—Subtilopeptidase A, Type VIII (Sigma Chemical Co., St. Louis, MO 63178). One unit will hydrolyze casein to produce color equivalent to 100 μm tyrosine (181 μg)/min at pH 7.5 at 37°C. Refrigerate dry enzyme after each use.

(f) *Amyloglucosidase A-9268.*—E.C.3.2.1.3 (Sigma Chemical Co.). One unit will liberate 1.0 mg glucose from starch in 3 min at pH 4.5 and 55°C. Keep refrigerated when not in use.

(g) *NaOH solution.*—0.285N. Dissolve 11.4 g NaOH in ca 700 mL water in 1 L volumetric flask. Dilute to volume with water.

(h) *Phosphoric acid solution.*—0.329M. Dissolve 37.9 g phosphoric acid (85%) in water in 1 L volumetric flask. Dilute to volume with water.

(i) *Celite 545.*—Fisher Scientific Co., Fair Lawn, NJ.

Because the method requires enzymes for gelatinization and initial starch breakdown, and then for protein and further starch digestion, the activity of the enzymes was critical to the success of the proposed method. A sufficient quantity of the 3 enzymes (d–f) necessary for the study was pretested before shipment to the analysts. To ensure that undesirable enzymatic activity was not present in the enzymes used in this procedure, analysts were requested to check enzyme activity each time the lot of enzymes was changed, or at a maximal interval of 6 months.

Test substrate	*Activity tested*	*Substrate wt, g*	*Expected rec. of product, %*
Citrus pectin	pectinase	0.1	95–100
Stractan (larch gum)	hemicellulase	0.1	95–100
Wheat starch	amylase	1.0	0–1
Corn starch	amylase	1.0	0–2
Casein	protease	0.3	0–2
beta-Glucan (oat gum)	beta-glucanase	0.1	95–100

Sample Preparation

For this study, no sample preparation was required other than drying overnight in a 70°C vacuum oven and cooling in a desiccator before weighing. However, if fat content is >5%, defat with petroleum ether (decant with three 25 mL portions/g sample) before milling. Record loss of weight due to fat removal and make appropriate correction to final percent TDF. Store dry, milled sample in capped jar in desiccator until analysis. When sample preparation is necessary, homogenize sample and dry overnight as described; then dry-mill portion to pass through 0.3–0.5 mm mesh sieve. If sample cannot be heated, freeze-dry before milling.

When dealing with unknowns, fat-extract all samples. For fat extraction, place ca 10 g sample in 250 mL beaker and add 25 mL petroleum ether. Stir mixture 15 min, using magnetic stirrer. Let stand 1 min; then decant petroleum ether. Repeat this procedure with two 25 mL portions of petroleum ether. Place beaker in 70°C oven and dry overnight. If apparatus is unavailable for milling samples to pass through 0.3 or 0.5 mm mesh sieve, grind in mortar.

Determination

(1) Run blank through entire procedure along with samples to measure any contribution from reagents to residue.
(2) Weigh, in duplicate, 1 g sample accurate to 0.1 mg (sample weights should not differ by more than 20 mg) into 400 mL tall-form beakers. Add 50 mL pH 6.0 phosphate buffer to each beaker.
(3) Add 100 μL Termamyl solution.
(4) Cover beakers with aluminum foil and place in boiling water bath. Shake gently every 5 min. Continue this process for 15 min after beaker contents have stabilized at 95°C.
(5) Cool. Adjust to pH 7.5 ± 0.1 by adding 10 mL 0.285N NaOH solution.
(6) Add 5 mg protease. Protease adheres to spatula, so it may be preferable to prepare solution just before use by adding small amount (ca 100 μL) PO_4 buffer to enzyme, and to transfer required amount by pipet.
(7) Cover beakers with aluminum foil and place in 60°C constant temperature water bath with constant agitation. Maintain incubation for 30 min after internal temperature of beaker contents reaches 55°C.
(8) Cool. Add 10 mL 0.329M phosphoric acid solution to adjust pH to 4.5 ± 0.2.
(9) Add 0.3 mL amyloglucosidase solution.
(10) Same as *(7)*.
(11) Add 280 mL 95% ethanol, preheated to 60°C. Mix.
(12) Let precipitate form at room temperature for 60 min.
(13) Weigh crucible containing Celite to nearest 0.1 mg. After weighing, redistribute bed of Celite in crucible by using stream of 74% ethanol from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as even mat. When fiber is filtered, i.e., step *14*, Celite effectively separates fiber from fritted glass of crucible, and allows easy removal of crucible contents.
(14) Filter enzyme digest from step *12* through crucible.
(15) Wash residue successively with three 20 mL portions of 74% ethanol, two 10 mL portions of 95% ethanol, and two 10 mL portions of acetone. With some materials, gum is formed, which traps liquid. If surface film that develops after adding suspension to Celite is broken with spatula, filtration is improved. Time for filtration and washing will vary from 0.1 to 6 h, averaging 0.5 h/sample. Long filtration times can be avoided by careful intermittent suction throughout the filtration. Normal suction can be applied at washing. Back-bubbling air is another way of speeding up filtrations.
(16) Dry crucible containing residue overnight in 70°C vacuum oven or 105°C air oven.
(17) Cool in desiccator and weigh crucible, Celite, and residue to nearest 0.1 mg.
(18) Analyze residue from one sample of set of duplicates for protein. Protein is probably most easily analyzed by carefully scraping Celite and fiber mat onto filter paper, which can then be folded and digested. A similar piece of filter paper should be analyzed as a blank. Use Kjeldahl analysis as specified in *Official Methods of Analysis*, secs **47.021–47.023** (30).
(19) Incinerate second sample of duplicate set 5 h at 525°C.
(20) Cool in desiccator. Weigh crucible containing Celite and ash to nearest 0.1 mg. Analyses may be made in quadruplicate so that duplicate protein and ash values would be available if increased precision of results is desired.

Calculation

$$\text{TDF (\%)} = \text{mg residue} - \frac{[(\text{\% protein in residue} + \text{\% ash in residue}) \times \text{mg residue}] - \text{blank} \times 100}{\text{mg sample (wt)}}$$

Results and Discussion

The foods to be used to test the proposed method were chosen so that they differed not only in the amounts of dietary fiber but also in the distribution of the dietary fiber components in a variety of cellular matrices. Many dietary fiber methods are applicable to individual products, but fail when applied to a variety of products. The method used in this study, a modification of several methods, was designed to include the correct kinds and amounts of enzymes so that fiber could be determined successfully. Each laboratory took 2 separate aliquots from each sample. Starch and the bulk of the protein were digested by enzymes. Residual protein was determined for one sample and ash weight for the other. Ash weight was subtracted from the portion in which protein was determined, to arrive at a value for dietary fiber. Protein was subtracted from the portion on which ash weight was determined, to give a second value for dietary fiber.

Thirty-two of the 42 laboratories submitted results. The remaining 10 laboratories either used other procedures or did not send in analytical results. Some laboratories repeated the determination; all values are reported in Table 3. The data in Table 3 show that, for example, 30 laboratories reported results for Sample 1. The results ranged from 81.59 to 94.08% dietary fiber; however, the values of 81.59 and 86.74 found by Analyst 8 were not used in the final calculation because they were outliers by the Cochran test (31). (The range of replicates was wider than could be accounted for by chance alone.) Thirty-two laboratories reported results for Sample 2. These results ranged from 15.89 to 29.64% dietary fiber; however, the values of 26.63 and 19.20 from Analyst 31 were not used in the final calculation because they were Cochran outliers. Over all samples, 9 analyses were not used because they were Cochran outliers. After looking at the results in some detail, it became apparent that Analyst 31 had been unable to perform the procedure. That analyst had great difficulty with step *16;* most filtrations took as long as or

Table 3. Results of TDF (%) determination in foods

Analyst	Sample[a] 1	2	3	4	5	6	7	8	9	10	11	12	13
1	87.90	19.22	11.66	2.32	4.33	5.07	4.30	13.92	42.86	14.27	3.64	10.45	13.97
	89.66	19.13	11.64	2.30	4.26	5.16	4.32	13.88	42.86	14.27	3.64	10.45	13.94
3	91.08	22.45	9.99	7.06	3.94	1.32	5.98	0.42	40.81	12.31	3.26	6.21	6.38
	92.56	23.03	12.61	6.95	4.18	1.29	5.69	0.02	43.08	12.55	2.85	6.65	7.64
6	86.40	25.03	17.09	10.51	4.25	7.65	10.56	17.35	43.70	12.28	5.94	11.37	8.77
	87.16	22.37	15.89	8.25	4.79	5.75	7.58	15.80	43.05	13.81	3.42	7.57	9.15
7	92.36	24.91	11.33	7.92	6.37	1.91	5.89	11.15	44.31	12.82	2.75	6.98	7.82
	92.46	27.33	11.49	7.66	4.80	1.94	5.76	11.05	41.82	12.94	2.58	8.97	7.78
8	86.74[b]	22.50	9.74	7.87	3.30	7.19	5.35	2.16	41.41	11.49	3.21		7.25
	81.59[b]	24.41	6.09		8.40	1.13	4.62	3.32	44.22	16.17	0.83	8.00	
9	91.11	25.76	16.64	6.77	3.24	4.27	6.10	18.23	43.92	13.34	3.07	6.70	7.71
	91.11	25.20	15.30	7.61	4.53	4.42		18.23	44.32	14.64	3.15		
			14.19										
			15.10										
10	88.82	23.63	13.69	7.11	4.11	9.83	5.03	12.80	42.50	12.97	3.85	8.10	9.60
	88.64	23.61	13.69	7.10	3.71	9.82	5.05	12.79	42.66	12.98	3.84	8.10	9.63
	85.37	22.04											
		21.97											
11	88.79	19.54	20.43	8.93	4.65	5.52	7.20	12.80	39.93	11.42	3.09	5.46	7.75
	88.80	15.89	18.05	6.67	4.40	5.44	7.34	11.46	42.55	12.23	1.85	6.15	7.70
13	86.79	21.21	7.41	6.17	3.38	3.12	7.85	0.83	40.12	5.43	0.98	4.16	8.34
	87.17	20.61	8.89	6.30	2.86	3.16	6.90	0.57	40.59	5.33	1.61	5.23	6.49
14	87.10	19.60	9.00	6.10	3.20	1.20	5.70	0.90	40.40	7.90	2.10	11.70	14.50
	85.70	18.50	9.80	6.20	3.20	0.60	4.00	0.80	40.00	11.50	1.90	12.20	16.10
	84.90	18.30		5.90	3.00		5.00				1.60	12.30	18.80
16	85.91	23.10	17.60	5.57	4.20	2.98	7.42	0.48	37.27	10.61	0.12	4.74	6.61
	88.92	18.81	16.35		3.93	1.67	5.15	0.59	40.55	11.59	1.68		
											0.90		
17	93.70	24.16	13.08	7.19	4.96	3.10	7.45	1.79	45.68	12.26	3.86	6.64	7.51
	93.53	25.28	11.56	7.63	4.46	3.06	5.91	2.01	45.81	13.26	3.20		
19	92.10	29.60	17.14	7.79	5.08	4.34	9.06	21.99	43.28	13.61	2.47	9.72	9.48
	92.37	29.64	16.78	7.50	4.66	4.73	6.51	19.58	45.55	14.43	2.95		
	89.76												
20	—[c]	25.42	16.10	8.11	—	—	—	11.09	41.97	—	4.51	8.97	11.56
21	89.53	23.48	14.60	6.58	4.06	4.83	6.14	20.62	45.60	12.92	2.74	7.72	9.02
	89.28	24.38	14.60	7.02	4.49	4.82	6.27	21.06	44.56	12.98	2.75	7.91	9.02
22	—	26.51	10.33	7.72	9.86	—	—	1.76	—	—	2.20	3.39	7.81
		29.47	10.99	6.54	6.20			1.92			6.40	5.19	7.03
23	85.73	23.14	9.33	6.85	3.74	6.99	2.62	1.06	41.23	12.09	3.57	7.44	7.79
	87.33	23.80	10.06		2.84	6.18	2.66	1.00	39.73	12.09	3.48	7.54	7.50
25	87.58	22.53	16.58	8.03	4.22	9.32	5.90	19.71	45.07	14.20	4.76	8.93	11.36
26	85.58	22.21	10.90	6.97	1.67	2.36	2.06	1.90	42.85	11.74	3.70	4.28	9.41
	85.32	21.03	10.71	7.11	3.69	2.30	5.37	2.32	45.19	12.35	2.42	7.35	10.14
27	86.08	19.60	8.89	7.61	4.32	3.19	6.22	0.87	39.83	11.31	3.04	5.60	6.53
	86.30	21.70	9.87	7.40	3.73	2.92	5.59	0.92	40.57	11.81	2.75	6.11	6.44
29	86.25	24.51	9.92	7.37	4.31	3.96	6.19	0.23	40.96	11.18	2.52	6.35	7.22
	86.23	24.80	9.68	7.34	4.38	3.68	5.72	0.18	41.69	11.62	2.63	6.06	7.58
					4.01		6.01						
					4.17		5.33						
30	87.14	22.30	11.58	6.28	3.18	2.06	5.23	1.80	40.65	11.48	2.24	5.64	6.96
31	89.14	19.20[b]	12.37	4.93	1.97	3.34	3.12[b]	2.99	36.79	12.81	9.11	9.15[b]	8.27
	86.53	26.63[b]	11.84	4.00	4.52	1.74	8.47[b]	1.58	40.68	10.80	4.81	15.28[b]	7.54
				5.57	5.42	0.60	1.53[b]		44.74	8.70	0.00		
						0.26				16.98	0.07		
34	90.82	23.80	12.37	7.66	5.22	4.02	8.94	2.09	42.17	15.34	10.52	6.18	7.79
	91.59	24.27	11.80	7.09	5.96	5.14	7.87	−1.86	43.46	14.97	12.25	6.44	9.41
			11.15						40.39		9.58	6.98	
			13.10						42.00		3.37		
35	94.08	24.63	10.74	7.98	6.98	2.98	6.13	0.45	42.06	14.05	3.40	9.94	9.38
36	84.93	21.81	10.37	6.51	3.25	1.26	4.56	2.78	40.33	11.97	2.55	5.40	6.72
37	85.95	22.75	10.30	6.33	4.25	0.94	5.61	0.40	37.11	14.91	4.72	6.02	7.30
	87.20	22.01	10.72	7.75	4.53	0.89	3.65		43.51	10.94	3.26	5.97	8.20
38	92.00	25.12	19.00	6.00	4.75	4.92	8.00	20.50	47.22	15.98	3.68	4.73	8.19
39	92.13	26.11	13.08	7.04	5.98	4.37	5.35	12.09	41.18	12.15	2.83	8.48	9.79
	92.18	29.35	12.50	6.78	6.18	4.08	5.29	12.18	41.58	12.57	2.85	8.74	9.94
40	91.17	23.50	15.10	9.84	5.85	2.36	8.13	16.26	45.43	15.79	5.14	8.48	12.28
	90.60	21.53	16.08	9.40	6.95	4.25	7.42	18.93	43.23	15.97	4.67	8.07	14.99
41	87.88	18.93	7.58	7.63	3.66	0.44	4.58	5.02	36.54	11.37	1.30	5.93	7.37
42	90.38	22.98	9.85	7.48	5.29	1.56	5.09	5.00	41.26	13.26	2.95	5.50	7.56
	90.21	23.84	10.21	7.38	5.07	1.57	4.90	4.82	41.62	13.09	3.08	5.69	7.42

[a]Samples: 1, corn bran; 2, lettuce, freeze-dried; 3, oats, quick cooking; 4, potatoes, instant; 5, raisins, seedless; 6, rice, powdered; 7, rye bread, dried; 8, soya isolate; 9, wheat bran; 10, whole wheat flour; 11, white wheat flour; 12, nonvegetarian mixed diet; 13, lacto-ovo vegetarian mixed diet.
[b]Outlier, not used in statistical calculation.
[c]— = no values reported.

Table 4. Measures of precision for determining dietary fiber

Sample[a]	No. of labs[b]	Average % dietary fiber	Std dev.	Reproducibility (CV), %
1	30	89.02	2.63	2.95
2	32	23.31	2.75	11.79
3	32	12.47	3.20	25.64
4	32	7.22	0.96	13.24[c]
5	31	4.43	1.03	23.12[c]
6	30	3.67	2.35	64.15
7	30	5.90	1.45	24.41
8	32	7.51	7.58	100.93
9	31	42.25	2.23	5.29
10	30	12.92	1.43	11.04[c]
11	32	3.07	1.01	32.95[c]
12	32	7.19	1.90	26.39
13	32	8.59	1.90	22.07[c]

[a]See footnote a, Table 3, for sample descriptions.
[b]When fewer than 32 laboratories are shown, some laboratory(ies) did not submit any results for some sample(s).
[c]A Grubbs' outlier laboratory was omitted before computing these measures of precision. Outlier rejection level of 0.05 was used in the "one-tailed test" (31).

longer than the maximum. The time required at this stage has been identified as a major cause of problems with replication; blank, and by inference, sample precipitation increases extensively during prolonged filtration or standing. High blanks and ash residues were found in replicates that filtered slowly or were left overnight in an attempt to reduce the filtration time. To achieve good replication, this stage must be controlled. Analyst 31 reported poor replicate values for at least 7 samples. Analyst 8 also had poor replicates in 6 cases, but the mean results fell in a reasonable range. Because the majority of laboratories provided good replicates, we assume that the problem of Analysts 8 and 31 were not due to the method.

Table 4 gives a statistical evaluation of the results. Of the approximately 834 analyses performed, only 5 were omitted because they were Grubbs' outliers (31) (extreme variance). Although there were no apparent statistical correlations between the coefficients of variation (CV) and dietary fiber levels, the foods containing the highest levels of dietary fiber, 89.02, 42.25, 23.31, and 12.92%, had the lower CV values, i.e., 2.95, 5.29, 11.79, and 11.04%, respectively. Conversely, foods containing the lowest amounts of dietary fiber, 3.07, 3.67, 4.43, and 5.90%, had the higher CV values, i.e., 32.95, 64.15, 23.12, and 24.41%. These findings are of value because the physiological importance of dietary fiber decreases as the level in the diet decreases, making greater variations in low fiber values of little importance, particularly for label declaration and claims. On the other hand, foods with a low fiber content (potato, for example) are often eaten in large amounts and therefore contribute significantly to fiber intakes. This would require accurate analyses of low level fiber foods.

Player and Wood (32) carried out a collaborative study of methods of analysis for crude fiber in flours and found that the CV values were between 9 and 133%, but the largest proportion of values was between 10 and 20%. Although our CV values were within those reported by Player and Wood, the values of dietary fiber for Samples 6, rice (64% CV), and 8, soy isolate (101% CV), were not acceptable in this study. Both samples presented special problems to the analysts.

Rice had the highest amount of starch of any of the foods analyzed, 77%, as determined using method B (O. Theander, Swedish University of Agricultural Sciences, Uppsala, Sweden, personal communication, 1983). We suspected that the sample was not homogeneous because there was a noticeable separation of the outer coating after milling. However, the major concern was the ability of the enzyme to completely digest the starch from the food. This was accomplished by using an active amylase and amyloglucosidase enzyme preparation along with an incubation period sufficient to allow total hydrolysis of the starch. Unfortunately, shipping these enzymes by mail to various parts of the United States and to other countries may have caused partial reduction or inactivation of the enzyme activity.

Through the comments of several analysts, we determined that the desired temperature of the incubation mixture of food plus enzyme was not achieved. For example, when the directions called for incubation of Termamyl with the buffered food solution for 15 min in a boiling water bath, we assumed that the volume of the bath was great enough compared with the volume of the beaker containing the incubation mixture for the mixture to achieve a temperature of 100°C. This would not be the case if the volumes of the beakers containing the incubation mixture were large compared with the bath volume; in this instance, the internal temperature of the incubation mixture would reach a temperature of only about 70°C in the 15 min called for in the directions. To correct this, the instructions were revised to indicate that the internal temperature of the incubation solution should reach 100°C, and the time of incubation in the boiling water bath should be extended from 15 to 30 min.

A similar type of problem existed when the starch was hydrolyzed by using amyloglucosidase. The 30-min incubation period must begin when the internal temperature of the incubation mixture reaches 60°C. The large CV for dietary fiber in rice is mainly a reflection of high fiber values that result from incomplete removal of starch from the dietary fiber residue.

In rice, however, this was mainly due to insufficient milling, making amylase penetration into the hard rice particles difficult and incomplete. This is supported by the fact that Analyst 36, who pulverized the sample in a mortar before analysis, obtained a very low value (1.26%), and that the other samples with very high starch content (4, potato and 11, white wheat flour) gave no problems with starch removal. If the high starch content was the problem, Samples 4 and 11 also should have given large CV values for dietary fiber. Rice samples with low fiber values resulted from the complete removal of starch in those samples.

The other food that had a high CV was Sample 8, soy isolate. This food had the highest protein content, 90%, of any of the foods analyzed and therefore would have very little dietary fiber. All analysts were instructed to multiply the nitrogen content by 6.25 to convert to percent protein. While the average factor, 6.25, is applied to foods in general, specific factors may be used for products with known protein and nitrogen relationships. Wheat flour protein, for example, contains 17.5% nitrogen and the factor is 5.71. We found that the

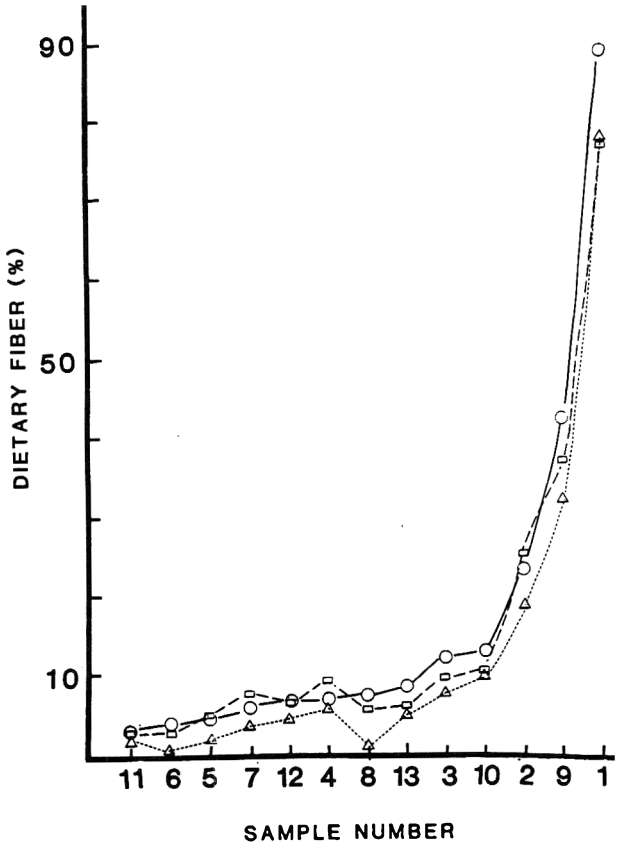

Figure 1. Comparison of percent dietary fiber by proposed method (○), method of Englyst et al. (25) (△), and method of Theander and Åman (20) (□).

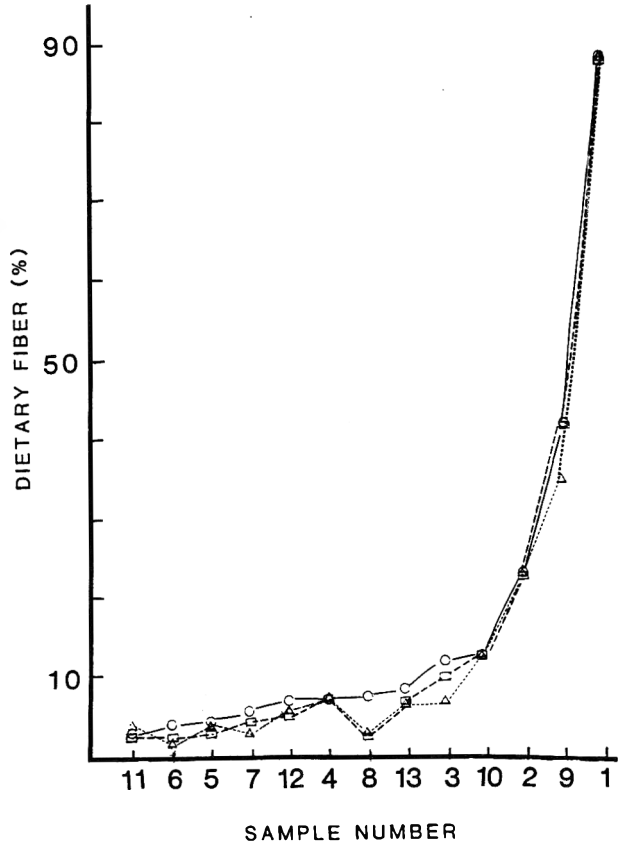

Figure 2. Comparison of percent dietary fiber by proposed method (○), method of Schweizer and Würsch (11) (△), and method of Asp et al. (9) (□).

analysts performed protein analyses by a variety of methods, which may lead to inaccurate protein values and, hence, to questionable dietary fiber results.

The instructions have been changed to state that the nitrogen determination be performed by the Kjeldahl method (30) and that 6.25 be used as the protein conversion factor. If the higher fiber values (>3%) for Sample 8 were discarded, the average TDF would be 1.38% ± 0.29 (17 laboratory results ranged from 0.12 to 2.78%). The CV_x was 88% but the quantity of fiber was so small that this large CV_x becomes almost meaningless under physiological conditions. Detailed analysis of the results for the other samples showed that analysts who reported high TDF for Sample 8 also reported high values for other samples more frequently than did analysts who found <3% TDF in Sample 8.

Products of the Maillard reaction may also present problems in the determination of TDF. The effect of various kinds of heat treatments on the determination of TDF appears to be important. When food is heated, the contribution of the noncarbohydrate dietary fiber residue increases due to the formation of modified proteins and new carbohydrate polymers that are different from those in the plant food material (26). Products of the Maillard reaction are present in significant amounts in many food products, but presumably only in Samples 12 and 13 in this study.

Because there are no known mixtures of dietary fiber nor known true percentages of dietary fiber in foods, it was impossible to do a recovery study. However, for some products we were able to obtain the following dietary fiber values from the company producing the food.

Sample 1—Staley refined corn bran, 88–92% dietary fiber by the method of Goering and Van Soest (22) and Hart and Fisher (33).

Sample 9—AACC Wheat Bran, 40.2% dietary fiber by the method of Van Soest and Wine (5), modified to use hog pancreatic amylase.

Sample 10—General Mills, Inc., whole wheat flour, 8–9% dietary fiber by the AOAC method for ADF (secs **7.069–7.071**, 30).

Sample 11—General Mills, Inc., white wheat flour, 0.6–1.0% dietary fiber by the NDF method (6).

The methods used were suitable for the products analyzed; however, they would not necessarily be suitable for determining fiber in other foods.

Two investigators used their own methods for dietary fiber (20, 25) to analyze the samples in this study; their values were not included in the statistical evaluation of results. Two other investigators provided results using both the AOAC method and their own published methods (9, 11). The TDF values they obtained by the proposed method were included in the final statistics; however, the TDF values that they obtained by their own methods were not included in the statistical evaluation of results. The results are compared in Figures 1 and 2. The data showed that the Englyst et al. method (25) was comparable to the AOAC procedure; however, the values for dietary fiber were lower, in all cases, than those obtained by the AOAC method. In the Englyst method, the

analysis of dietary fiber is limited to the determination of nonstarch polysaccharides and does not involve lignin. The other 3 methods were in very good agreement, with the exception of Sample 8, soy isolate, which was previously discussed. The fact that these more complicated methods agree with the present rapid and simple procedure demonstrates the suitability of the latter for routine measurements. Finally, determination of TDF performed at Kellogg Co. by using a modified NDF method (22) showed a correlation coefficient of 0.994 with the proposed method. Samples 4 and 8 were excluded from the correlation determination due to filtering problems. The Kellogg method yielded generally higher values but these were all within the statistical variation of the proposed method.

With the valuable data gathered in this effort, a collaborative study will be initiated shortly.

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METHOD PERFORMANCE

Reduction in Variability of Instrumental Methods

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The largest contribution to experimental error in most analyses is systematic, resulting from preparation of standards and calibration procedures. By their very nature, systematic errors are difficult to detect and measure, particularly without interlaboratory comparisons. It is suggested that systematic errors can be reduced by incorporating reference physical constants (such as molar absorptivity) and certified standards to verify the correctness of the calibration curve and the standard solutions from which they were constructed.

Modern analytical chemistry is conducted through the use of instruments. Probably the only major technique which can be said not to require instrumentation for its performance is thin layer chromatography, but even here, some analytical chemists like to place their plates in a densitometer for the final spot density reading, although the eye can estimate spot intensity in comparison to a standard as reliably as the instrument. One cannot visit a modern analytical laboratory now without noting the presence of gas and liquid chromatographs, as well as visible, ultraviolet (UV), infrared, and atomic absorption spectrophotometers.

Instruments have brought about a renaissance in analytical chemistry. It is largely through the application of instruments that analyses can be performed reliably in the parts per million region and, with slightly more difficulty, in the parts per billion region. With great care and expense, analyses can be done at the parts per trillion level. Because trace analyses are characterized by the nature of the instrument used to obtain the final measurements, there is a tendency in analytical chemistry to ignore the separation science that permits the removal of unwanted materials which may constitute 99.9999% of the mass accompanying the 0.0001% that is the compound of interest. Focusing on instrumentation causes the equally important separation science of the analytical system to be overlooked.

The total analytical system consists of several parts. We start with a lot of goods or a particle of interest to determine composition or properties. If the material is too large to be conveniently handled in the laboratory, it must be *sampled*. This sample, usually taken by personnel outside the laboratory, must be further reduced in size, still maintaining its original distribution of components, to match the scale of laboratory operations. Although, at times, the laboratory sample can be used directly, more often, all or part of it must be dissolved for further chemical manipulation. The *analysis* begins with the weighing or measuring of a test portion, followed by the application of chemical and physical principles, primarily solubility and adsorption relationships. Much of the systematic error of analytical chemistry occurs at these steps, usually through losses from nonoptimum or nonequilibrium conditions and gains through contamination from the laboratory environment. These steps are intended to isolate the constituent of interest (the analyte) from the bulk of the carrier (matrix) in a form that is acceptable to the measuring instrument. The instrument transforms a property of the analyte into a *measurable* response that can be converted into concentration or amount through calibration with a standard. Finally, the concentration reported must be *interpreted* in terms of the composition or properties of the original lot or particle.

Although the title implies primary consideration of the instrument measurement step, to concentrate on this one aspect of an analytical method would be misleading. The relative magnitude of the error involved in instrument measurements, compared with that of sampling and chemical manipulations, must always be considered for a proper perspective of the entire analytical system.

Sampling

The problem of sampling is usually handled on an arbitrary basis, depending on the expected degree of homogeneity of the commodity. Knowledge of the general extent of uniformity within a commodity is usually available on a historical basis. Some examples in the progression from fairly uniform to extremely heterogeneous include the following:

Many instructions for the assay of tablets for drugs start with a sample of 20 tablets, with an auxiliary tablet uniformity requirement that will permit extrapolation from the sample to the lot.

The regulation dealing with nutritional labeling specifies that the sample shall consist of a composite of 12 subsamples (consumer units), each taken from 12 different randomly chosen shipping cases.

The U.S. Department of Agriculture instructions for sampling raw, shelled peanuts for aflatoxin, where the sampling error may constitute as much as 90% of the total error, provide for collecting three 48 lb samples to be analyzed sequentially in duplicate until a decision to accept or reject can be made on the basis of predetermined statistical considerations.

The pattern of homogeneity within any commodity is in some degree characteristic of that commodity, and practical sampling takes advantage of this. It is assumed that if lots are sampled in the same way, the same sampling error exists from lot to lot. In theory, the sampling error is unique for every lot. If the variation about the true composition for a constituent within a lot is required, the sampling error for that lot must be estimated by replicate sampling and analysis of that lot.

Some important points about sampling and its relation to other parts of the analytical system are as follows: If the analytical error is less than one-third the sampling error, further lowering of the former is of little importance in reducing total error (1). Sampling error, however, can always be reduced by increasing the sample size or the number of sample units, within economic or practical limits.

Analysis

Analysts are usually preoccupied with the chemical aspects of analysis. They seldom attempt to determine the significant parts of the problem and concentrate on those parts. In their research on the development and improvement of methods of analysis, they separate the method into its various com-

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ponent steps, attempt to optimize the individual steps, and then try to optimize the method as a whole. Considerable effort is also devoted to problems of interference by other components of the sample matrix. In trace element analysis, for example, both considerable and contradictory literature has accumulated on the initial isolation step, the elimination of organic matter by wet oxidation or dry ashing. Although such studies are indispensable to minimize errors in the routine performance of analytical work, the properly optimized method may still fail to overcome the residual and variable baseline error which appears to be unique for each laboratory at low trace levels.

Many practical analysts fail to understand the fundamental error structure of analytical chemistry, and thereby concentrate on minimizing some of the least consequential sources of error. The 2 major components of analytical error are systematic error and random error. Random error is easiest to measure. Although individual measurement errors are unpredictable and uncontrollable, these fluctuations are predictable and reproducible as a group. When the limits of variation of a process can be predicted, the system is said to be in a state of statistical control (2). While random errors are characterized by a distribution function, systematic error is characterized by a constant. There are some easily understood systematic errors: A defective weight will introduce a constant bias into all weighings using that weight; adsorption and desorption of pesticides on a cleanup column almost invariably result in a consistent loss of a small fraction of the residue; and an incorrect standard used to prepare a calibration curve will subsequently influence all measurements made using that curve. Systematic errors are potentially correctable, if the correct answer is available from external sources.

Other types of errors are sometimes present which should be absent from a well run analysis. One is blunders—bad mistakes that chemists occasionally and unknowingly make, which will appear in the data as obvious outliers. Another error is a consistent influence of external variables with time, usually manifested as a monotonic drift. These errors will be ignored because they occur relatively infrequently.

The normal distribution function is the one best known to analytical chemists, and it is usually assumed that most analytical measurements (except counting techniques) follow this distribution. The important point about the presence of a distribution function is that, whenever it is present, there is some fuzziness or uncertainty associated with the accompanying value, as shown in Figure 1. The uncertainty interval can be predicted, but not the position of any given value. This uncertainty of the random error associated with the measurement also limits the ability to calculate the systematic error.

There are very few studies in the literature which have been conducted in such detail that they permit a separation of the components of error of the individual steps of the method to determine where to improve reliability. This is because the method originator has usually tested many factors to minimize variability in his own hands. In fact, optimization of the method variables produces a deceptive small within-laboratory repeatability and blinds the analyst to the fact that the major indicator of variability in the performance of a method is external to his operations.

Horwitz and Albert (3) have been investigating these external factors by reviewing the methods for drug dosage forms that have been approved by AOAC over the past 10 years. Most of the methods now being adopted by AOAC contain an instrumental measurement step. Boyer et al. (4) are doing the same for trace element analyses. To date, 41 collaborative

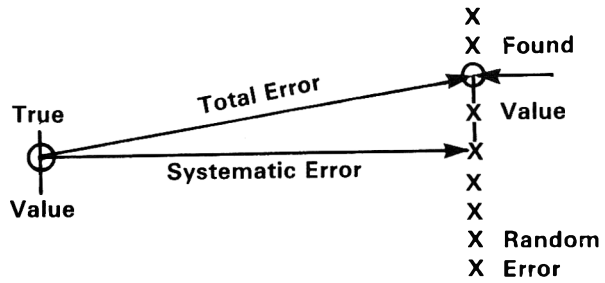

Figure 1. Representation of the relationship of the systematic and random errors. Although the reported value appears to be a specific point, it is confounded with the unpredictable random error (indicated by the spread of the X values) and the constant systematic error (indicated by the horizontal line measured from the true value). The total effect is a vector sum.

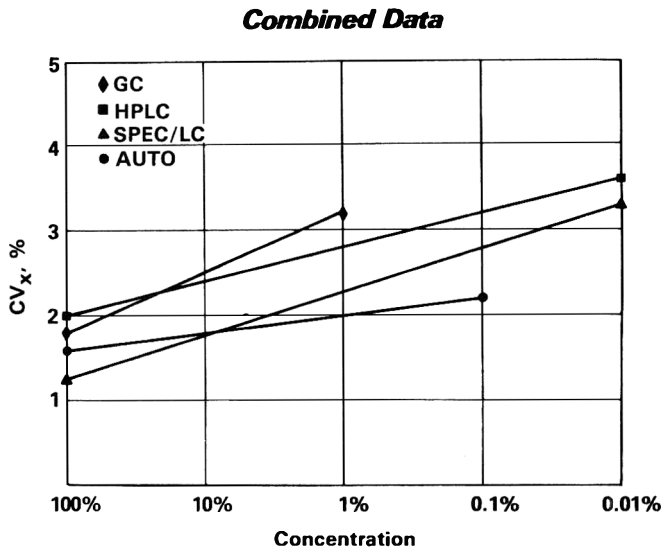

Figure 2. Reproducibility coefficients of variation (CV_x) of 4 types of instrumental methods applied to drug dosage forms as a function of concentration plotted logarithmically, but labeled linearly. Abbreviations: GC, gas chromatographic; HPLC, high pressure liquid chromatographic; SPEC/LC, liquid chromatographic separation, spectrophotometric or fluorometric measurement; AUTO, automated.

studies of drug dosage forms, using 4 basic measurement techniques, have been reviewed. These techniques are spectrophotometry and fluorometry preceded by a liquid chromatographic (LC) separation, gas chromatography (GC), automated methods, which usually include a spectrophotometric measuring step, and high pressure LC. Polarographic and other electrometric methods, atomic absorption, and miscellaneous methods are yet to be reviewed.

Figure 2 shows a summary of the coefficients of variation (CV, %) for these drug methods, plotted against concentration, marked off on a logarithmic scale but labeled linearly. Although each technique appears to give a characteristic line, the estimated 95% confidence band around each line is relatively large, on the order of at least 30%, so that the eventual statistical analysis will probably show that there is no statistically significant difference among the lines. Such a conclusion is further reinforced by the unexpected positions of some of the lines. For example, the simplest method, where the procedure is merely dilution and injection into the gas chromatograph, shows the highest CV, whereas the most complex method, chromatographic separation followed by spectrophotometric measurement, is in the lowest CV category. For 3 of the 4 methods, the ratio of the repeatability to reproducibility standard deviations is approximately 1:1, but for the

fourth (LC), it is approximately 1:2. In other words, even if the within-laboratory (repeatability) error, which is largely random in nature, could be entirely eliminated, there would still be a substantial residual among-laboratory variability. This residual variability for each laboratory is systematic in nature and is somewhat characteristic of each laboratory. Random error can be reduced to any desired extent by sufficient independent replication. On the other hand, systematic error can be reduced only by eliminating factors that produce the deviations or by correcting for them through use of a reference standard. However, it is known that one of the most important contributors to systematic error arises from the construction of a calibration curve.

Some chemists think that preparation of a calibration curve is one of the simplest and least demanding parts of an analysis. For stable systems, the curve can be used almost indefinitely, with occasional checks to ensure that the points are repeatable. Yet, when Brown and his colleagues (5) attempted to develop an atomic absorption method for the determination of calcium in blood serum, consisting of merely dilution and atomization into the instrument, about 3 years of work by 12 laboratories went into the establishment of the final method. The group started with a method that was stated to have been fully developed by the National Bureau of Standards (NBS) for general methodology and reliability. The objective was to establish the transferability of the NBS method to other laboratories so that any competent laboratory could establish the calcium content of blood serum to within 2% of the "true" value, with 95% confidence, over the concentration range 2.00–3.00 mmol/L (80–120 ppm). Weighing and pipeting were found to make negligible contributions to the inaccuracy of the calibrating solutions. The occasional occurrence of aberrant values (outliers) required the incorporation of reference accuracy controls into the protocol. Instrumental error was only a small part of the total variability and was "not a factor which limited the excellence of analytical performance." It was concluded that, "Perceptible sources of variability were associated with the preparation and dilution of the calibration solutions and the specimens." Also noted was the inability to guarantee the accuracy of performance of a reference method on the basis of a strictly defined protocol alone, i.e., following a method exactly does not ensure production of the correct result. The major source of variability was laboratory-associated effects, which is a systematic error for any given laboratory but which acts as a random error for the consensus value in different exercises. The authors significantly commented that it is unfortunate that values with large errors are the least precisely measured. It is relatively easy for the instrument to replicate readings from the final solution and from the matrix, both of which make only small contributions to the total error. But to replicate the entire study, which contributes only 1 result per laboratory to an estimation of laboratory-associated effects, requires a substantial allocation of resources.

The final reference method that was developed requires 4 working days to perform—the equivalent of 17 h of working time. To partially neutralize the random errors of preparation of the standard solutions, 5 calibrating solutions must be prepared independently from NBS-certified calcium carbonate. In addition, an aqueous and a serum reference material must be run in parallel to serve as an "accuracy control" as well as to provide benchmarks for the precision and linearity that must be met to establish values for the concurrently run unknowns. Each calibration sequence is run 4 times; each specimen and reference material is prepared in duplicate and is run 4 times. Obviously this method is not recommended for routine assays but for establishing reference values and as a standard against which other methods are tested.

The significance here is the necessity for attention to calibration and control to achieve an absolute overall error (or deviation from the accepted value) of 1%. This is almost a whole order of magnitude better than the expected reproducibility precision anticipated in interlaboratory studies of equivalent methods approved by AOAC, methods which often include little control over the systematic error. The tremendous amount of time and effort that would be required with the technique just described to render any meaningful improvement in the current acceptability standards suggests the need for exploring less costly alternatives for improvement of instrumental methods.

Potential Control of Systematic Error

The first general purpose instrument introduced into the laboratories of the Food and Drug Administration was the Beckman DU visible and UV spectrophotometer. The spectrophotometer is still an important analytical instrument in its own right and as the measuring part of other instruments such as liquid chromatographs, atomic absorption spectrophotometers, and in automated methods. Very soon after the first few methods were adopted, the AOAC referees on drugs and the reviewing committee decided that this instrument had to be calibrated with a standard solution prepared from the pure analyte. The calculations could not be based on preassigned absorptivities, except for secondary corrections. At that time, instruments were a novelty and interlaboratory studies revealed discrepancies in both wavelength and absorbance scales. The wavelength scale was easily calibrated against reference mercury, hydrogen, or sodium lines but the absolute absorbance scale was seldom calibrated. Now we have more stable and reproducible instruments, and even more important, primary certified reference standards exist for the absorbance measurement. Furthermore, the introduction of good laboratory practices into our operations, mandated for clinical chemistry laboratories engaged in interstate commerce and for laboratories requesting approval for marketing regulated commodities, has resulted in a greater sensitivity to periodic checking of instrument scales, preventive maintenance, and quality control. It would appear that a test of the original assumption that reference physical constants cannot be relied on as the basis for instrumental reference points should be instituted under modern conditions. Even if the use of reference values for modern analytical instruments would not provide sufficient reproducibility and security against the introduction of invisible systematic errors, there are advantages to the incorporation of reference constants as quality control points in the description of the method of analysis, particularly in the instrument specifications. This can easily be done by requesting all collaborating laboratories to report the response of their working standard solutions. The consensus value can be incorporated into the method as a guide to the analyst as to the approximate response to be expected at a certain concentration and the change in response that should be observed per unit change in concentration.

One official AOAC method already incorporates such a concept but only because there was no practical alternative. Extractable color is one of the important quality attributes of paprika. The chemical compounds responsible for the color are not available as a reference standard, so the American Spice Trade Association (ASTA) supplied a factor for converting absorbance at 460 nm to "ASTA color values." Initially the spectrophotometer was calibrated with a stable

Table 1. ASTA (American Spice Trade Association) color units for extractable color in oleoresin paprika

Parameter	Sample 1		Sample 2	
	NBS std glass	Chem. std	NBS std glass	Chem. std
Mean	996	1027	2012	2078
CV_o, % (within-labs)	0.89	0.88	0.50	0.57
CV_x, % (among-labs)	1.4	4.8	2.2	7.8

solution of potassium dichromate and ammonium cobaltous sulfate. The use of this chemical standard was compared in a collaborative study with the use of NBS certified glass filter (6). The repeatability CV_o was about the same with either standard, about 0.7%. The reproducibility CV_x was reduced by about one-third by the substitution of the certified glass filter for the chemical standard, as shown in Table 1.

One group of AOAC methods, those for mycotoxins (7), uses physical constants to establish the concentration of the standard solutions. For example, pure aflatoxins in crystalline form must have specified molar absorptivities in methanol within ± 95% confidence limits, which, for the 4 aflatoxins, average about 6.5% of the mean. This corresponds to a CV of about 3 to 4% (8, 9). Because of the minute quantities of standard analyte required, 10 μg/mL, aflatoxin standards are frequently purchased as films in a sealed vial. Solvent is added and the absorbance is determined in a spectrophotometer calibrated with reference standard potassium dichromate solution. The concentration of the standard compound is then calculated from the specified absorptivity at specified wavelengths. The among-laboratories CV_L for the absorbance measurement alone is about 3–4% (8, 9). This procedure for determining concentration of standards is also used for the other mycotoxins for which there are official AOAC methods: ochratoxins, patulin, sterigmatocystin, and zearalenone.

Recently adopted drug methods, LC methods for physostigmine, **38.C05** (10), and oxazepam, **40.D05** (11), have included sections on "Systems Suitability Check." These checks, however, only include tests for reproducibility (which should be repeatability) and resolution. We suggest that it would be helpful to the analyst to ensure that the detector is operating properly if the method indicated an approximate peak height (response) for a given amount of substance, as in the pesticide multiresidue method, **29.001–29.018** (12). This general method states that the system "should be capable of producing ca ½ scale deflection for 1 ng heptachlor epoxide by electron capture detection and for 2 ng parathion by KCl-thermionic detection and should resolve mixt. of heptachlor, aldrin, heptachlor epoxide, ethion, and carbophenothion into sep. peaks. Retention time for aldrin should be ca 4.5 min." These are excellent guidelines, and they provide the analyst with benchmarks for system performance.

Although the sensitivity factor is easily altered by the instrument controls, this can be handled for internal standard methods by specifying the acceptable range of the response ratio. With external standard methods, the use of 2 different concentrations of standard solutions would be required to fix the sensitivity (response per unit concentration), or response could always be related to a reference compound as in the case of heptachlor epoxide for the multiresidue pesticide method. Alternatively, built-in electronic components could handle this requirement.

Although the chemist may react adversely to suggestions of being replaced by an electrical component, some evidence already exists that the limiting factor of the reliability of instrumental methods for measuring milk constituents is the overall error induced by the variability of the assigned value of the calibrating solutions. This source of error is especially pronounced in those cases where the constituents are defined by the method of analysis, such as total solids, protein, and fat.

This situation provides an interesting example of the transformation of random error into systematic error. In a determination of fat in milk, there is no systematic error by definition because the result obtained by the specified method is the correct or "true" value. However, if the calibration is repeated, and the 2 results are not identical, which result should be used? The answer is undoubtedly the average, but then the "true" value depends on the number of determinations entering into the average. As a matter of fact, there are 3 such methods in the 1980 edition of *Official Methods of Analysis of the AOAC* (13). One requires calibrating with 20 samples, the second has no specification, and the third requires 8 values. Two of the methods contain performance specifications for the calibration; the third does not. Once a value is chosen, all other values, regardless of distribution, have a systematic error with reference to the chosen value.

It was stated several decades ago that the Babcock test for fat in milk was the most frequently performed chemical test in the United States. This is probably no longer true as instruments now perform this measurement. Knowing the variability of these chemical methods (of the order of 1–2%, relative) at the concentration range of interest, the use of any single or even duplicate values will introduce a variable systematic error into the analytical system with time as new calibration standards replace the old ones. But a stable, permanent, certifiable standard for milk constituents, such as the certified NBS glass absorbance standard, does not seem like a viable possibility. Restandardization with variable chemical calibration procedures introduces sudden shifts in output values, which may be small, but nevertheless significant, when millions of dollars in payments are involved. Perhaps this is a problem for instrument designers: How to maintain a constant relationship between input values, usually a difference in light intensity or in electrical potential, and an output reading of an undefinable mixture of chemical constituents which make up the protein, fat, carbohydrates, and minerals of milk. Even if this can be achieved, we can imagine how difficult it will be to get chemists to substitute a resistance box or oscilloscope settings for their cherished Kjeldahl protein or Roese-Gottlieb fat calibration determinations. But perhaps they would be willing to reduce within-laboratory variability by the routine analysis of stable "house standards."

At present, few investigations have been made to estimate the relative importance of the various sources of error that arise from sampling, preparation of sample, extraction, cleanup, and measurement. In milk analysis alone, sampling introduces a considerable error which, because of frequent deliveries, reduces to a systematic error of sampling which may be very difficult to define. The error of determination of the primary food constituents of milk is probably negligible, but is more significant for contaminants and drugs. The random measurement error of modern instruments is probably negligible compared with the sampling and chemical separation errors, and use of a surrogate physical standard such as a calibrated glass or electrical reference circuit can probably even eliminate much of the random error of calibration and at least stabilize the systematic error. Systems suitability constants would supply the chemical optimization parameters for such surrogate calibration functions and for the peri-

odic checking required. Then analytical research effort could probably most profitably be spent on reducing the chemical noise of the isolation or cleanup stages of chemical analysis.

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PESTICIDE FORMULATIONS

Liquid Chromatographic Determination of Sodium Fluoroacetate (Compound 1080) in Meat Baits and Formulations

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A liquid chromatographic (LC) method is described for the determination of sodium fluoroacetate in meat baits and formulations. Baits were extracted with water, ultrafiltered, partitioned into butanone, back-partitioned into dilute base, and diluted with acetonitrile. Aqueous formulations of 1080 were diluted with acetonitrile. The solutions were esterified with *p*-bromophenacyl bromide, using crown ether catalysis, and chromatographed on a 10 μm reverse phase column. Ultraviolet absorbance was monitored at 260 nm. Samples spiked to contain 1 mg and 10 mg 1080/100 g meat gave recoveries of 84.0–103.4%.

Sodium fluoroacetate (Compound 1080) is used in baits in Australia for the control of pests such as dingoes, feral pigs, and rabbits.

In our laboratory we have conducted a series of experiments to evaluate techniques for the preparation of fresh meat baits containing 1080. We required an analytical method for the determination of 1080 that was rapid, reproducible, and capable of handling large numbers of whole baits.

Published methods for the determination of 1080 use gas chromatography or gas chromatography/mass spectrometry (1–4), thin layer chromatography (5), fluoride ion-selective electrode (6), and liquid chromatography (LC) (7, 8). However, all these methods have one problem in common—they involve lengthy extraction procedures that make them unsuitable for the rapid analysis of a large number of samples. Some of these methods also lack specificity by detecting fluoride only, while some have low or erratic recoveries.

A method designed to overcome these deficiencies is described. Sodium fluoroacetate is determined as its *p*-bromophenacyl ester, using LC with a reverse phase column and ultraviolet detection.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Spectra Physics Model 8000B equipped with variable wavelength UV detector, oven, and printer/plotter.

(b) *Columns.*—4.6 mm id × 25 cm reverse phase RP-18 (10 μm) (Brownlee Laboratories). 4 mm id × 25 cm silica LiChrosorb (10 μm) (E. Merck).

(c) *Ultrafiltration system.*—47 mm stirred molecular filtration cell and 30 000 nominal molecular weight limit (NMWL) filters (Millipore Corp.).

(d) *Stomacher.*—Laboratory blender, Model 400 (Colworth Laboratory Equipment, UK).

Reagents

(a) *Solvents.*—LC grade acetonitrile, 2,2,4-trimethylpentane, and tetrahydrofuran (Waters Associates). Analytical reagent grade ethyl acetate. Laboratory reagent grade butanone (Ajax Chemicals). The latter solvent was fractionally distilled (bp 79.5–80.0°C). Water for LC use was purified by passing distilled water through a Gelman Sciences Water I purification unit.

(b) *Fluoroacetic acid.*—98% purity (Merck).

(c) *Sodium fluoroacetate.*—Technical grade (Rentokil Pty Ltd, UK). This chemical was decolorized with activated charcoal and recrystallized; purity was checked against fluoroacetic acid, using esterification procedure described below.

(d) *p-Bromophenacyl bromide, p-BPB.*—98% purity (Aldrich Chemical Co.). Decolorized with activated charcoal and recrystallized 3 times from ethanol.

(e) *Esterifying solution.*—150 mg *p*-bromophenacyl bromide and 25 mg 18-crown-6 (Aldrich Chemical Co.) dissolved in 5 mL LC grade acetonitrile.

Sample Preparation

(a) *Meat.*—Weigh the whole bait and dice it into ca 1 cm cubes in shallow tray. Transfer sample to blender bag and rinse tray with volume of water (V_A mL) equivalent to twice the weight of the bait. Transfer rinsings to bag and blend 1 min. Filter sample through glass wool. Determine approximate moisture content of bait (V_M mL) by recovering the solids and drying them overnight at 80°C. (It is important to note the total volume of water (V_{TOT}) involved in extraction of 1080. This volume consists of added water (V_A) plus moisture content of meat (V_M). V_{TOT} will be used to calculate amount of 1080 per bait.) Adjust pH of solution filtered through glass wool to 1.5–2.0, using 2N HCl and pH paper. Transfer solution to ultrafiltration cell and filter through 30 000 NMWL filter. Collect 5 mL ultrafiltrate and extract 3 times with 5 mL butanone. Centrifuge if layers do not rapidly separate. Add 1 mL water to combined organic solvent extracts. Add 3 drops of phenolphthalein indicator and add 0.5N KOH dropwise with shaking until permanent pink color is achieved. Remove aqueous layer with Pasteur pipet and estimate volume. If necessary, evaporate to ca 2.0–2.5 mL (water content of solution to be esterified should not exceed 10%). Transfer aqueous layer to 25 mL volumetric flask and dilute to volume with acetonitrile. Filter ca 5 mL solution through Millex-SR filter unit.

(b) *1080 Formulations.*—Prepare solution containing ca 2 mg/mL of 1080. Transfer 5 mL aliquot to 100 mL volumetric flask and adjust pH, using 0.5N KOH and phenolphthalein until permanent pink color is achieved. Dilute to 100 mL with acetonitrile. Filter ca 5 mL solution through Millex-SR filter unit.

Derivatization

Transfer 2 mL filtered solution to small screw-cap tube. Add 0.1 mL esterifying solution, cap, and shake tube. Heat at 80°C for 75 min.

Standards

Prepare 50 mg/L aqueous 1080 standard. Transfer 2, 4, and 6 mL standard to three 100 mL volumetric flasks; to each add 3 drops of phenolphthalein solution and add 0.5N KOH drop-

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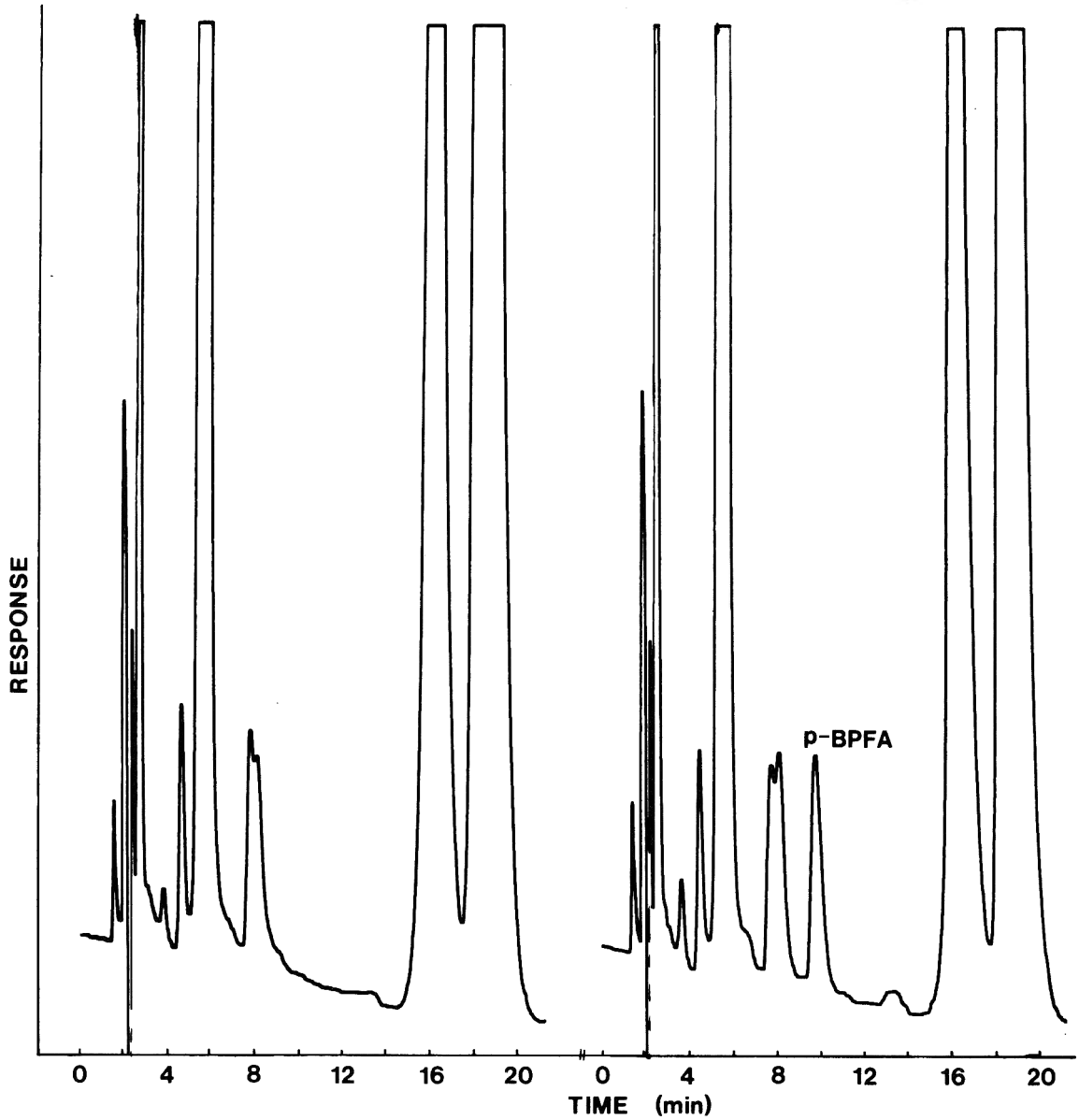

Figure 1. Typical chromatograms of 1080 as *p*-BPFA in fresh meat extracts (reverse phase column, 0.02 AUFS). Left = unspiked meat; right = meat spiked with 100 ppm 1080.

wise with swirling until permanent pink color is achieved. Dilute them to volume with acetonitrile. Esterify 2 mL each of dilute standards as described above.

Prepare standards for quantitation of formulations by diluting aliquots of 2.5 mg/mL 1080 standard in the same manner.

Chromatography

Analyze solutions on reverse phase column, using mobile phase containing 37% tetrahydrofuran in water. Operating conditions: temperature, 35°C; flow rate, 2 mL/min; chart speed, 0.5 cm/min; sensitivity, 0.01–0.02 AUFS; wavelength, 260 nm; loop size, 10 μL. Quantitate by measuring peak heights.

Confirm presence of 1080 by evaporating esterified solution to dryness on water bath. Add 1 mL acetonitrile and sonicate 5 min. Analyze solution by liquid chromatography, using silica column and mobile phase containing 8% ethyl acetate in 2,2,4-trimethylpentane. Use operating conditions previously described except reduce flow rate to 1.5 mL/min. Retention time of *p*-bromophenacyl fluoroacetate is 12.5 min.

Calculate quantity of 1080 in a bait as follows:

$$\text{mg 1080/bait} = P_M/P_S \times C_{1080} \times 2.7\ V_{TOT}$$

where P_M = peak height of meat sample; P_S = peak height of appropriate standard; C_{1080} = mg 1080 in esterified standard; and $V_{TOT} = V_A + V_M$. The factor 2.7 allows for aliquoting and recovery.

Calculate concentration of 1080 in formulations as follows:

$$\text{mg 1080/mL} = P_F/P_S \times 20C_{1080}$$

where P_F = peak height of formulation; P_S = peak height of appropriate standard; C_{1080} = mg 1080/mL in esterified standard; and 20 is dilution factor for formulation containing ca 2 mg 1080/mL.

Results

The retention time for *p*-bromophenacyl fluoroacetate (*p*-BPFA) on the reverse phase column was 10.5 min, with all compounds eluting within 25 min. Although no chromatographic interferences were observed from the formate, acetate, lactate, or propanoate esters, problems were originally encountered with chromatographic resolution due to impurities in the *p*-BPB and butanone. These reagents were purified as described in the *Reagents* section.

Table 1 shows the recoveries obtained when 100 g portions of meat were spiked with 1 mg and 10 mg 1080. Figures 1 and

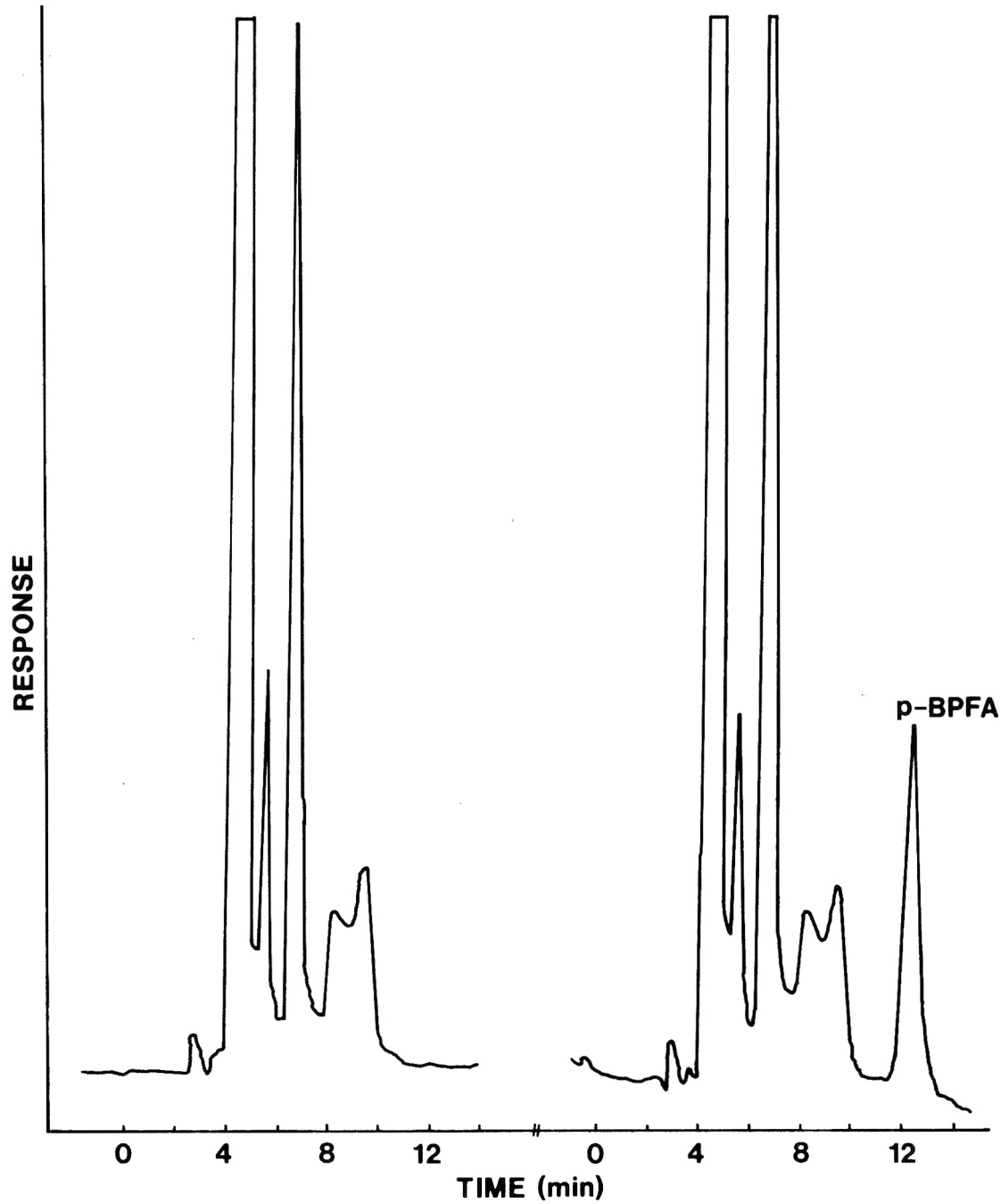

Figure 2. Typical chromatograms of 1080 as *p*-BPFA in fresh meat extracts (silica column, 0.02 AUFS). Left = unspiked meat; right = meat spiked with 100 ppm 1080.

2 show chromatograms of a blank meat extract and meat spiked with 1080. The standard graph for *p*-BPFA was linear from 2 to 100 mg/L of 1080 in the final extract.

The method has been used to detect 1080 in baits at concentrations as low as 2 mg/kg, which is adequately sensitive for the analysis of suspect bait materials. The minimum amount of derivative detectable in a 10 μL injection at 0.005 AUFS was equivalent to about 1.5 ng 1080. This detection limit was determined on the basis of the sensitivity of the method for standards (signal/noise = 2). The sensitivity of the method was increased by evaporating the esterified solution to dryness and redissolving in a minimum volume of 80% tetrahydrofuran in water.

Discussion

The analysis of whole baits is necessary because sub-sampling does not provide a representative sample. The standard weight of a fresh meat dingo bait in Queensland is 125 g, but baits varying in weight from 70 to 250 g have been analyzed. The blender specified cannot process samples weighing more than 150 g. Larger samples were processed in portions and recombined before ultrafiltration.

Most large protein molecules were removed by ultrafiltration. This technique was adopted because it was found that chemical deproteinizing agents interfered with the esterification. The flow rate through the ultrafiltration membrane was increased by adjusting the pH of the sample extract to 1.5–2.0 to increase protein solubility. This technique produced 6 mL colorless filtrate in less than 10 min, with no retention of fluoroacetic acid. Membranes were reusable at least 12 times and were soaked and stored after use in a solution of sodium hypochlorite (1% available chlorine).

Partition coefficients of fluoroacetic acid (pKa = 2.58) from acidified water (pH <2.5) into equal volumes of ethyl ether,

Table 1. Recovery of 1080 added to meat

1080 added, mg	Recovered, mg	Rec., % ± SD
1	0.93, 0.84, 0.92, 0.87, 0.88, 0.98	90.0 ± 5.0
10	9.12, 9.24, 9.15, 8.76, 9.72, 10.34	93.9 ± 5.6

butanone, 4-methyl pentan-2-one, and methylene chloride were evaluated, and butanone was selected as the most suitable solvent (partition coefficient = 50%) for extraction of fluoroacetic acid from the ultrafiltrate. Esterification did not proceed satisfactorily in water-saturated butanone. An esterification technique was adopted that was a modification of a method used by Patience and Thomas (9). By back-extracting fluoroacetic acid into dilute base and diluting with acetonitrile, the esterification can be made quantitative with the use of crown ethers as catalysts. The crown ether-catalyzed esterification proceeds in the presence of up to 10% water with negligible hydrolysis of *p*-BPB. The derivatization was rapidly driven to completion by the crown ether which strongly solvates the potassium ion and enhances the reactivity of the fluoroacetate anion (10). *p*-BPFA formed under these conditions was stable for at least 2 days at room temperature. High concentrations of chloride interfered in the esterification by converting *p*-BPB to *p*-bromophenacyl chloride, the latter being unreactive as an esterifying reagent. This interference was overcome by adding excess *p*-BPB.

Initial attempts to quantitate *p*-BPFA on the reverse phase column, using mobile phase combinations of methanol, acetonitrile, and water, gave unsatisfactory resolution of the ester from either the lactate or acetate ester. Resolution did not improve with the use of nonaqueous mobile phases or cyano, diol, RP-8, or RP-300 columns. Additional esters were prepared (*p*-phenyl and *p*-phenylphenacyl), but these did not improve resolution and sensitivity was lower.

A major reason for the simplicity of this method is that esterification is done in the presence of water. All other methods using derivatization for the determination of 1080 require anhydrous conditions, involving additional time-consuming solvent extraction or drying steps. Consequently, under these conditions only a few samples can be handled at a time. The ability to handle numerous samples was an important consideration in the development of a new method for the determination of 1080 in baits, because we envisaged at least 500–600 analyses during research on 1080 bait preparation techniques and the stability of 1080 in baits laid in the field. The method described in this paper permits the processing and analysis of 20 baits or 30 formulations of 1080 per day. No other published method is suitable for both the rapid analysis of a suspect 1080 bait and the batch analyses associated with experimentation with 1080 baits. This method has been used to analyze 250 samples and has proved to be rapid and reproducible. Application of the method in other areas is being investigated.

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DAIRY PRODUCTS

Detection of Adulteration in Grated Cheese by Using Calcium, Phosphorus, Magnesium, and Lactose Indices

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Calcium, phosphorus, and magnesium levels and calcium/phosphorus and calcium/magnesium ratios were determined for 74 authentic cheeses. Lactose was determined for 40 authentic grating cheeses. Phosphorus was measured by an AOAC method, calcium and magnesium by a modified AOAC atomic absorption method, and lactose by liquid chromatography. The average calcium/phosphorus and calcium/magnesium ratios were 1.55 and 23.3, respectively, and were used with lactose data as criteria for evaluating grated cheese adulteration.

Grated cheeses such as Parmesan and Romano are easily adulterated with other dairy products. We became aware of some of the problems when we discovered extensive use of added whey solids in grated cheese. Lactose (1) was then used as an index of whey solids adulteration.

Subsequent investigations indicated that partial substitution of hard grating cheese with processed cheeses and other dried natural cheeses was not uncommon. The calcium/phosphorus ratio was suggested as an index for detecting these substitutes, especially processed cheese. Magnesium is another mineral constituent of cheese, so the calcium/magnesium ratio might also be useful in detecting adulteration.

There are no AOAC official methods for calcium, phosphorus, magnesium, or lactose in hard cheese. AOAC method **2.109** (2) is an atomic absorption spectrophotometric (AAS) method for minor nutrients in fertilizers, which includes determination of calcium and magnesium. AAS has also been used to determine a number of elements in milk (3, 4), including calcium and magnesium. We chose AAS for our study because of its speed and reproducibility, and modified existing procedures to reduce matrix interferences by using standards whose mineral content closely approximates that of cheese. Phosphorus was determined by using AOAC molybdovanadate method **22.042** (2).

Lactose can be determined in processed cheese by using AOAC method **16.268** (2). The method detects reducing sugars, however, and is not specific for lactose. Liquid chromatographic (LC) methods (5, 6) are more specific. We used a modification of AOAC method **16.268** (2) for sample preparation, and determined lactose by LC as described in the following section.

METHOD

Apparatus

(a) *Atomic absorption spectrophotometer.*—Perkin Elmer Model 4000 equipped with time-averaged integration and automatic curvature correction for standards above linear range, or equivalent. Determine Ca at 422.7 nm, and Mg at 285.2 nm. Set up instrument according to manufacturer's instructions for optimum responses. Average sample responses for minimum of 9 s.

(b) *Liquid chromatograph.*—Waters ALC 201 with differential refractometer, M6000 pump, and U6K injector, or equivalent. Column, μBondapak carbohydrate 4 mm id × 30 cm; solvent, acetonitrile–water (75 + 25); flow, 1.5 mL/min; recorder-integrator, Spectro Physics 4100.

(c) *Spectrophotometer.*—Beckman DB, or equivalent with matched 1 cm cells. Determine P at 400 nm.

Reagents

(a) *Lanthanum stock solution.*—10 g La/L. Dissolve 11.73 g La_2O_3 in 25 mL HNO_3. Dilute to 1 L with water.

(b) *Calcium stock solution.*—500 mg/L. Dissolve 1.249 g $CaCO_3$ in minimum amount of 3N HCl. Dilute to 1 L with water.

(c) *Magnesium stock solution.*—1000 mg/L. Place 1.000 g Mg metal in 50 mL water and slowly add 10 mL HCl. Dilute to 1 L with water.

(d) *Sodium stock solution.*—1000 mg/L. Dissolve 2.542 g NaCl in water and dilute to 1 L.

(e) *Phosphate stock solution.*—500 mg/L as P_2O_5. Dissolve 0.959 g KH_2PO_4 dried 2 h at 105°C in water and dilute to 1 L.

(f) *Potassium stock solution.*—1000 mg/L as K_2O. Dissolve 2.297 g KCl in water and dilute to 1 L.

(g) *Atomic absorption standard solution.—(1) Stock solution.*—Add 20 mL Ca stock solution **(b)**, 1 mL Mg stock solution **(c)**, 10 mL Na stock solution **(d)**, 40 mL P_2O_5 stock solution **(e)**, 1 mL K_2O stock solution **(f)**, and 1 mL HCl to 100 mL volumetric flask. Dilute to volume with water. *(2) Working standards.*—Add 0, 1, 4, and 10 mL portions of stock solution *(1)* to 100 mL volumetric flasks. Add 20 mL La stock solution. Dilute to volume to obtain 0, 1, 4, and 10 ppm Ca and 0, 0.1, 0.4, and 1.0 ppm Mg, respectively, in 0.2% La.

(h) *Molybdovanadate reagent.*—**22.042(b).**

(i) *Phosphate working solutions.*—Proceed as in **22.042(c)** using P_2O_5 stock solution **(e)** as stock solution *(1)*.

(j) *Lactose standard solutions.*—Dissolve 0.2 g and 2 g lactose in water and dilute to 100 mL to obtain 0.2 and 2.0% lactose solutions, respectively.

(k) *Phosphotungstic acid solution.*—Dissolve 200 g phosphotungstic acid in 600 mL water. Add 20 mL H_2SO_4 and dilute to 1 L with water.

Sample Preparation for Calcium, Magnesium, and Phosphorus Determinations

Mix cheese thoroughly. Weigh approximately 1 g cheese into 100 mL Vycor dish and dry in forced air oven 1 h at 100°C. Char dried cheese on hot plate at high setting. Ash in furnace at 525°C, and cool.

Dissolve ash in 10 mL HCl (1 + 3) and evaporate to dryness on steam bath. Dissolve residue in 10 mL HCl (1 + 9) on steam bath and transfer to 100 mL volumetric flask (Solution A). Dilute to 100 mL with water.

Calcium and Magnesium Determination

Pipet 1 mL Solution A into 25 mL volumetric flask. Add 5 mL La solution **(a)** and dilute to volume to prepare assay solution. Determine ppm Ca and Mg in assay solution vs working standards **(g)***(2)*. Calculate mg Ca/100 g cheese and

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Table 1. Analytical values for authentic Parmesan and Romano cheeses

Statistic	Found, mg/100 g Ca	P	Mg	Ca/P	Ca/Mg	Fat, %	Moisture, %	Fat in solids, %	Apparent lactose,[a] %
					Parmesan (20 Samples)				
Av.	1089	695	48.5	1.58	22.1	25.72	32.15	37.65	
High	1206	767	57.7	1.66	24.6	32.58	39.19	47.41	1.0
Low	900	605	39.3	1.47	20.9	18.72	26.80	29.61	0
SD	87	44	4.7	0.06	1.1	3.63	2.82	4.01	
					Romano (11 Samples)				
Av.	989	640	44.8	1.54	22.4	28.54	30.98	41.32	
High	1191	721	54.1	1.66	26.5	34.75	34.52	48.85	0.6
Low	836	567	31.5	1.44	17.8	23.92	26.40	35.25	0
SD	112	55	7.3	0.08	2.5	2.94	2.47	3.63	
					Pecorino Romano (9 Samples)				
Av.	979	624	57.1	1.57	17.2	31.14	31.16	45.19	
High	1088	663	67.3	1.64	19.7	33.90	34.48	47.28	0.3
Low	912	591	49.8	1.50	15.0	27.58	27.68	40.76	0
SD	51	29	5.6	0.05	1.5	2.38	2.13	2.40	

[a]LC determination.

Table 2. Analytical values for authentic Gruyere, Provolone, and Mozzarella cheeses

Statistic	Found, mg/100 g Ca	P	Mg	Ca/P	Ca/Mg
			Gruyere (13 Samples)		
Av.	890	581	36.6	1.53	24.3
High	961	616	40.4	1.60	26.8
Low	814	524	33.6	1.48	22.2
SD	48	27	2.4	0.04	1.5
			Provolone (11 Samples)		
Av.	785	510	33.1	1.54	23.8
High	964	612	42.4	1.58	25.7
Low	666	449	28.2	1.48	21.5
SD	77	42	3.9	0.04	1.2
			Mozzarella (10 Samples)		
Av.	780	506	33.5	1.54	23.3
High	833	552	35.7	1.61	24.4
Low	733	473	31.3	1.46	21.8
SD	33	26	1.6	0.05	0.7

mg Mg/100 g cheese from concentrations in assay solution as follows:

$$M = Z \times 250/W$$

where M = mineral content of cheese in mg/100 g, Z = ppm mineral in assay solution, and W = weight of cheese taken.

Phosphorus Determination

Pipet 10 mL Solution A into 100 mL volumetric flask and dilute to volume to prepare P assay solution. Prepare P_2O_5 standard curve as in **22.043** (2). Determine P_2O_5 in 10 mL assay solution as in **22.045** (2). Calculate mg P/100 g cheese from mg P_2O_5/10 mL assay solution as follows:

$$P = (Y \times 10\,000)/W \times 2.29$$

where P = phosphorus content of cheese in mg/100 g, Y = mg P_2O_5/10 mL assay solution extrapolated from standard curve, and W = weight of cheese taken.

Determine Ca/P and Ca/Mg ratios for cheese.

Lactose Determination

(a) *Qualitative.*—Weigh ca 10 g cheese into 250 mL beaker. Add ca 20 mL 8% alcoholic KOH and mix. Place on steam bath 10–15 min. Observe cheese from top and bottom of beaker. Extensive charring of cheese particles indicates high levels of lactose present. Scattered charred particles of cheese indicate low levels of lactose present. No charring indicates negligible or no lactose present.

(b) *Quantitative.*—Weigh 10.00 g cheese into tall 200 mL beaker. Add 70 mL water and 1 mL NH_4OH. Stir on heater/stirrer at medium heat setting until cheese curd is emulsified, ca 15 min. Heat 1 min at high heat setting. Transfer hot mixture to 100 mL glass-stopper graduated cylinder. Add 25 mL phosphotungstic acid solution (k) and add water to bring volume to slightly in excess of 100 mL. Mix thoroughly, cool, and let precipitate settle. Remove portion of supernate and filter through 0.45 μm filter before LC determination.

Use acetonitrile:water ratio of LC solvent to elute lactose in 8–10 min at 1.5 mL/min flow rate. On basis of qualitative lactose test, determine high lactose levels vs 2.0% standard and low lactose levels vs 0.5% standard.

$$\%\ \text{lactose in cheese} = \%\ \text{lactose in assay} \times 10$$

Whey solids can be calculated from lactose values as follows:

$$\%\ \text{whey solids in cheese} = \%\ \text{lactose in cheese}/0.735$$

Results and Discussion

The study included the analysis of 74 domestic and imported authentic natural cheeses. They were purchased in bulk form and included 20 Parmesan, 20 Romano, 13 Gruyere, 11 Provolone, and 10 Mozzarella cheeses. Unless labeled otherwise, all were assumed to have been made from cow's milk. Nine samples of Romano, labeled Pecorino Romano, were assumed to have been made from sheep's milk. The cheeses were analyzed for Ca, Mg, and P. Ca/P and Ca/Mg ratios were calculated. In addition, the Parmesan and Romano cheeses were analyzed for lactose by LC and for moisture and fat, respectively, by AOAC **16.233** (2) and by modifications in **16.255** (2) as outlined in the AOAC General Referee's Report on Dairy Products (7).

In all cases, the Ca/P ratios (Tables 1 and 2) fall within a fairly narrow range. The average for all 74 authentic cheeses was 1.55 with a high of 1.66 and a low of 1.44. The standard deviation was 0.06.

There appears to be a significant difference between the Ca/Mg ratios in cheeses made from cow's milk and those made from sheep's milk. The 9 Pecorino Romano cheeses made from sheep's milk had an average Ca/Mg ratio of 17.2

Table 3. Comparison of LC and GC determinations of lactose (%) in cheeses

No. tested	Sample	Apparent lactose by LC	Confirmed lactose by GC
2	Authentic Romaro	0–0.2	0
6	Authentic Parmesan	0.5–1.0	0
1	Dried Ricotta	6.2	1.3
1	Grated cheese No. 1	0.5	0
1	Grated cheese No. 2	2.0	1.7
1	Grated cheese No. 3	2.0	1.4
1	Grated Parmesan No. 1	0.6	0.2
1	Grated Parmesan No. 2	10.6	8.6

with a high of 19.7 and a low of 15.0. The standard deviation was 1.5. The overall average for 65 authentic cheeses assumed to have been made from cow's milk was 23.1 with a high of 26.8 and a low of 17.8. The standard deviation was 1.6. Only one of these cheeses, a Romano, had a ratio below 20.0. Perhaps the assumption that it was made from cow's milk was incorrect. None of the Parmesan, Gruyere, Provolone, or Mozzarella cheeses, which are traditionally made from cow's milk, had a Ca/Mg ratio below 20.0. Excluding this one Romano, the average Ca/Mg ratio for cheese made from cow's milk was 23.3 with a high of 26.8, a low of 20.0, and a standard deviation of 1.5.

Table 1 shows lactose values obtained by LC that were determined for only the Parmesan and Romano cheeses. Literature sources (8) indicate that when a cheese is aged, lactose is fermented to lactic acid. Therefore, no lactose should be present in an aged cheese. The values in the tables are listed as apparent lactose and were quantitated from small LC peaks with retention times similar to lactose; they may have been artifacts. Peak heights ranged from 0 to 3% full scale deflection and actual quantitations were 1% lactose or less. No averages or standard deviations were determined for the apparent lactose levels in these cheeses.

To study whether these small LC peaks were actually lactose, we analyzed a number of the LC assay solutions by gas chromatography (GC) for lactose after forming the trimethylsilyl derivative (9). The results are presented in Table 3. Six authentic Parmesan and 2 authentic Romano cheeses were analyzed that had apparent LC lactose levels of from 0.0 to 1.0%. No lactose was found by GC in any of these samples at the sensitivity level of 0.01%. Six samples of grated cheese analyzed by LC and known to contain lactose or suspected of containing low levels of lactose were analyzed by GC. The presence of lactose was confirmed in all but one of these samples, and this sample had an apparent lactose content of 0.5%. These observations tend to confirm that authentic aged Parmesan and Romano cheeses do not contain lactose. However, these data would also tend to confirm that LC does not reliably quantitate lactose in grated cheese at levels $\leq 1\%$.

Moisture and fat values were also determined only on the Parmesan and Romano cheeses (Table 1). Because moisture and fat standards for these cheeses (10, 11) differ, we attempted to differentiate one variety from another on that basis. The results show that there is too much overlap in the data between the 2 cheese varieties. No conclusive identification could be made solely from moisture and fat data. It is interesting to note that 10 of 20 Parmesan and 3 of 20 Romano cheeses did not comply with moisture and/or fat standards. Many of these cheeses were retested and the original results were verified.

Over 300 samples of grated cheese, in addition to those reported in this study, have been analyzed for authenticity. The most common adulterants detected were processed cheese and whey solids. These adulterants affect the calcium, phosphorus, magnesium, and lactose levels of grated cheese. Table 4 presents analytical values for these adulterants as well as other products which could be used for adulteration. Except where noted, the values were obtained by actual analysis.

Most processed cheeses contain phosphate additives. Table 4 shows three of the many processed cheeses analyzed. They represent typical processed cheeses, and the analytical values show lower Ca/P ratios but similar Ca/Mg ratios compared with natural cheese. The partial substitution of processed cheese for hard grating cheese would therefore significantly lower the Ca/P ratio but would have no effect on the Ca/Mg ratio.

Whey solids contain high levels of lactose and magnesium. Calcium and phosphorus levels in whey depend on whether the liquid whey is a by-product of cheese made by acid or rennin coagulation (13). Higher levels of calcium and phosphorus, for example, are found in cottage cheese whey resulting primarily from acid coagulation. Table 4 shows typical values for whey from both acid and rennin coagulation. Literature values for whey solids are also given. Addition of whey solids to grated cheese would drastically increase the lactose content to levels $\geq 10\%$ and would lower the Ca/Mg ratio. The Ca/P ratio could also be lowered, depending on the source of the whey.

Other adulterants, such as unaged cheeses made in part from whey solids or nonfat dry milk, would have varying levels of calcium, phosphorus, magnesium, and lactose compared with other cheese. Typical analytical values for these adulterants are represented (Table 4) by 2 dried ricotta cheeses, 2 cottage cheeses, and bakers cheese. Addition of these adulterants to grated Romano or Parmesan would be detected by low but usually detectable levels of lactose, a lower than normal Ca/Mg ratio, and possibly a lower Ca/P ratio.

Calcium and magnesium levels can also be affected by the use of permitted food additives. The standards of identity for Parmesan (10), Romano (11), and hard grating cheese (14), as well as for most natural cheeses, allow the addition of certain calcium and magnesium salts. During manufacturing, $CaCl_2$ may be added to milk to aid in curd formation in amounts not to exceed 0.02%. $CaSO_4$ and/or $MgCO_3$ may be added to aid in bleaching in a combined amount not to exceed 0.012%. At the highest levels allowed for any or all of these salts, no significant differences would occur in the Ca/P ratio. However, the Ca/Mg ratio could be lowered by 1.5.

The standard of identity for grated cheese (15) allows the addition of anticaking agents not to exceed 2% by weight of the finished product. These agents could include calcium and magnesium silicates. Magnesium trisilicate and calcium metasilicate were experimentally added to authentic Romano cheese at 1 and 2% levels and samples were analyzed. Data presented in Table 5 show that these substances greatly affect the Ca/P and Ca/Mg ratios. Magnesium trisilicate substantially lowers the Ca/Mg ratio while calcium metasilicate raises both the Ca/P and Ca/Mg ratios.

Manipulation of Ca/P and Ca/Mg ratios is possible with the addition of any number of salts. Additions of calcium, phosphorus, and/or magnesium salts would affect the overall levels of these minerals in cheese. Essentially all the minerals in cheese, except for a small amount of phosphorus in fat-soluble phospholipids, are found in the solids not fat. By expressing the mineral content of a cheese on the basis of the solids not fat (SNF), a range of indices may be obtained with which to judge the presence of added salts. Using values from Table 1, the computed average Ca (SNF) for all 40 Parmesan and Romano cheese was 2547 mg/100 g with a high of 2929, a low of 2135, and a standard deviation of 186. The computed

Table 4. Analytical values for common substitutes of hard grating cheese

Product	Found, mg/100 g Ca	P	Mg	Ca/P	Ca/Mg	Apparent lactose,[a] %
Past. proc. American	645	691	28.9	0.93	22.3	ND[b]
Past. proc. American	567	459	27.0	1.24	21.0	ND
Past. proc. Gruyere	826	1216	37.8	0.68	21.9	ND
Liquid whey (acid coag.)	787	462	82.9	1.73	9.6	ND
Liquid whey (rennin coag.)	283	315	73.3	0.90	3.9	ND
Whey solids (ref. 12)	646	589	130	1.10	5.0	73.5
NFDM (ref. 12)	1308	1016	143	1.29	9.1	52.3
Dried Ricotta	985	660	56.6	1.49	17.4	9.5
Dried Ricotta	1048	773	56.5	1.36	18.5	7.0
2% Cottage	56	114	6.4	0.49	8.8	ND
4% Cottage	83	131	8.9	0.63	9.3	ND
Bakers cheese	84	233	27.5	0.36	3.1	2.0
Cheddar	729	477	31.7	1.53	23.0	ND
Swiss	963	625	47.0	1.54	20.5	ND

[a]LC determination.
[b]Not determined.

Table 5. Effect of anticaking agents on Ca/P and Ca/Mg ratios

Sample	Found, mg/100 g Ca	P	Mg	Ca/P	ΔCa/P	Ca/Mg	ΔCa/Mg
Authentic Romano cheese	1005	684	44.2	1.47	—	22.8	—
1% $Mg_2Si_3O_8$ added	1012	673	201	1.50	+0.03	5.0	−17.8
2% $Mg_2Si_3O_8$ added	999	673	336	1.48	+0.01	3.0	−19.8
1% Ca SiO_3 added	1332	672	44.6	1.98	+0.51	29.9	+ 7.1
2% Ca SiO_3 added	1586	672	45.3	2.36	+0.89	35.1	+12.3

Table 6. Indices of grated cheese authenticity

Interpretation	Ca/P	Ca/Mg	Apparent lactose,[a] % by LC
A. Authentic Parmesan or Romano	1.37–1.73	18.8–27.8	≤ 1.0
B. Authentic Pecorino Romano	1.37–1.73	12.7–21.7	≤ 1.0
C. Adulterated with processed cheese	< 1.37	18.8–27.8	usually ≤ 1.0
D. Adulterated with whey solids	variable usually <1.37	5–15, below norm	> 10.0
E. Adulterated with unaged or manufactured cheese	variable usually <1.37	1–5, below norm	1.0–10.0
F. Authentic greated cheese with calcium silicate anticaking agent	> 1.73	2–10, above norm	≤ 1.0
G. Authentic grated cheese with magnesium silicate anticaking agent	1.37–1.73	10–20, below norm	≤ 1.0

[a]LC determination.

average P (SNF) for all 40 cheeses was 1632 mg/100 g with a high of 1783, a low of 1480, and a standard deviation of 78. Magnesium levels differ between cheese made from cow's milk and cheese made from sheep's milk. The computed average Mg (SNF) for 31 Parmesan and Romano cheeses, assumed to have been made from cow's milk, was 113.8 mg/100 g with a high of 144.8, a low of 82.0, and a standard deviation of 14.2. The computed average Mg (SNF) for 9 Pecorino Romano cheeses assumed to have been made from sheep's milk was 154.6 mg/100 g with a high of 177.0, a low of 129.0, and a standard deviation of 16.6. Mineral levels of grated Parmesan and Romano cheeses above and below these levels could be considered adulterated.

On the basis of Ca/P and Ca/Mg ratios and lactose data for authentic cheeses, indices were established to judge the authenticity of grated cheese (Table 6). The indices for Ca/P and Ca/Mg ratios were calculated from data derived from cheese made from cow's and sheep's milk, respectively, and are within ± 3 standard deviations of means. The index for lactose was based on the fact that no authentic grating cheese analyzed by LC contained more than 1.0% apparent lactose. Table 7 presents data showing the application of these indices in testing routine compliance samples for regulatory control of some forms of grated cheese adulteration.

Summary

Natural aged cheeses have distinct Ca/P and Ca/Mg ratios which fall within a fairly narrow range. We have also found a significant difference in the Ca/Mg ratio of cheeses made from cow's milk and those made from sheep's milk. Lactose

Table 7. Analytical values and interpretation of data for grated cheese samples

Lab. no.	Label	Ca/P	Ca/Mg	Apparent lactose,[a] %	Interpretation[b]
3015	Pecorino Romano	1.58	17.9	<1.0	B
3066	Parmesan	1.58	21.4	<1.0	A
3170	Parmesan & Romano	0.92	9.4	20.3	D
3171	Parmesan & Romano	0.99	9.9	19.8	D
3172	Romano	1.24	21.2	<1.0	C
3173	Parmesan & Romano	1.57	21.4	<1.0	A
3174	Parmesan & Romano	1.16	20.2	<1.0	C
3320	Parmesan & Romano	1.55	22.6	<1.0	A
3525	Parmesan	1.09	20.2	<1.0	C
3837	Romano	0.95	23.4	<1.0	C
3838	Parmesan	1.33	25.5	<1.0	C
3839	Imitation grated cheese	0.90	7.0	41.0	—[c]
3840	Parmesan	1.35	25.0	<1.0	C
3841	Parmesan & Romano	1.58	21.7	<1.0	A
3842	Romano	0.96	23.9	<1.0	C
3930	Romano	1.11	17.9	5.6	E
4481	Parmesan & Romano	1.34	18.1	<1.0	E
4489	Pecorino Romano	1.37	24.6	2.1	E
5000	Parmesan	2.28	26.1	<1.0	F
5221	Parmesan	0.84	13.3	<1.0	C,E
5260	Parmesan	1.37	14.4	10.6	D
5545	Parmesan	2.01	24.8	<1.0	F

[a]LC determination.
[b]See Table 6.
[c]Whey solids declared.

levels in Parmesan and Romano cheeses as determined by LC appear to be negligible. Adulteration should be suspected in any grated cheese having a Ca/P ratio, Ca/Mg ratio, and/or lactose level that falls outside the norms.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Rapid Determination of Organochlorine Pesticides and Polychlorinated Biphenyls, Using Selected Ion Monitoring Mass Spectrometry

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Methane chemical ionization (CI)-selected ion monitoring (SIM) mass spectrometry was used to identify and conclusively distinguish 19 organochlorine pesticides from polychlorinated biphenyls (PCBs) at parts-per-trillion to parts-per-billion levels in environmental water sample extracts with minimal sample cleanup. Two CI-SIM screens were developed. One set of ions scanned specifically for the presence of 4 classes of pesticides: diphenylmethane derivatives, bridged polycyclic chlorinated hydrocarbons, chlorinated benzenes, and acetanilide pesticides. The second set of ions responded exclusively to PCBs with biphenyl moieties containing from 1 to 8 chlorine atoms. Eight commercial Aroclor mixtures were analyzed and distinguished from the pesticide groups. The detection limit for pesticides and PCBs by CI-SIM screening was 0.005 and 0.1 ppb, respectively. CI-SIM can be used as an alternative method for the analysis of biological or environmental samples containing interferences that complicate the detection of PCBs and chlorinated pesticides.

Chlorinated pesticides and polychlorinated biphenyls (PCBs) are frequently isolated from biological or environmental samples in complex multicomponent mixtures. Most qualitative or quantitative methods for the analysis of these residues involve the use of gas chromatography with an electron capture detector (ECD-GC). Although a number of extraction and purification procedures have been described (1–3), column chromatographic purification techniques that can successfully separate organochlorine pesticides from PCBs have not been developed. In sample extracts that contain both chlorinated pesticide residues and PCBs, a number of peaks may be detected that cannot be conclusively identified solely on the basis of their GC retention times. The wide variety of nonpesticide and non-PCB residues present in crude sample extracts causes further nonspecific ECD responses.

Tentatively assigned compound identifications must be confirmed by using a more specific yet comparably sensitive detection method. The mass spectrometer in electron impact ionization mode (EI-MS) is commonly used to verify the structure of organic compounds. However, Pellizzari et al. (4) reported that the limits of detection for total ion monitoring EI-MS and ECD-GC differ by as much as 2 or 3 orders of magnitude. EI-MS is, therefore, often not sensitive enough to confirm the identities of compounds preliminarily identified by ECD-GC. In selected ion monitoring (SIM) mass spectrometry, sensitivity is improved by monitoring a few characteristic ions of the compounds under investigation rather than scanning the total ion current. The methane chemical ionization (CI) mass spectra of many chlorinated pesticides and PCBs (5–7) are simple and contain abundant, highly diagnostic ions that are suitable for SIM. The purpose of this study was to determine whether methane CI-SIM *(1)* could be used to unequivocally identify trace pesticides and PCB residues at detection limits comparable to those for ECD, and *(2)* could distinguish between these groups of compounds in sample extracts with minimal cleanup.

Two CI-SIM screens were developed. One set of ions scans specifically for the presence of the chlorinated pesticides listed in Table 1; the second set of ions is responsive to PCBs with biphenyl moieties containing from 1 to 8 chlorine atoms. The CI-SIM pesticide screen detects compounds representative of 4 classes of pesticides: diphenyl methane derivatives, bridged polycyclic chlorinated hydrocarbons, chlorinated benzenes, and acetanilide pesticides. The wide variety of Aroclor mixtures listed in Table 2 were analyzed using the PCB CI-SIM screen to determine whether the CI-SIM pattern obtained for PCBs could be used as a fingerprint for identifying Aroclor mixtures. The developed CI-SIM screens were applied to the analysis of parts-per-trillion to low parts-per-billion levels of chlorinated pesticides and PCBs in river water samples.

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Experimental

Standards and Samples

(a) *Pesticide and Aroclor standard solutions.*—Analytical reference standards of the pesticides and PCBs listed in Tables 1 and 2, respectively, were supplied by the U.S. Environmental Protection Agency Reference Standards Repository (MD-8, Research Triangle Park, NC 27711). Store individual stock solutions (100 ppm in methanol) of each compound in glass-stopper bottles at 4°C.

(b) *Extraction of river water and standard water samples.*—Standard water samples containing 0.005–1.0 ppb of each pesticide listed in Table 1 were prepared in 1 L deionized water (Millipore Q system) by adding appropriate volumes of the 100 ppm methanol stock solution of each pesticide. Similarly, 1 L laboratory-purified water samples containing 0.05–2.0 ppb of an individual Aroclor were prepared for each commercial Aroclor listed in Table 2. The prepared standard water samples and river water samples (5 samples collected from the Elbow River between March and May 1983) were processed in an identical manner. In a 2 L separatory funnel, 1 L water samples were extracted at pH 8.0 with three 50 mL volumes of methylene chloride. The combined extracts were dried with 1–2 g anhydrous Na_2SO_4 and concentrated using a Kuderna-Danish evaporator to ca 3.0 mL. The solvent volume was further reduced to 100 μL by passing a gentle stream of argon gas over the solution. One μL volumes of the crude sample extracts were injected onto the capillary column for CI-SIM analysis.

Apparatus

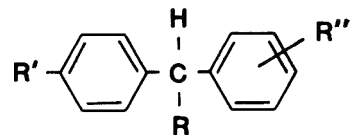
(a) *Gas chromatograph-mass spectrometer.*—Hewlett-Packard Model 5993 coupled to Model 1000 data system was used for both total ion scanning (120–500 amu) and selected ion monitoring. Methane chemical ionization (MS) operating conditions: temperatures (°C)—open split capillary interface 280, CI source 200, analyzer 180, injector 250; analyzer pressure (GC-MS valve open, methane gas present) 1×10^{-4} Torr; scan speed 216.7 amu/s; electron energy 70 eV; dwell time for SIM 50 ms.

(b) *Chromatographic conditions.*—12 m × 0.21 mm (id) OV-101 (methyl silicone, Carbowax deactivated) fused silica

Table 1. Names and structures of 19 compounds, representative of 4 pesticide classes shown, included in selected ion monitoring CI-MS pesticide screen

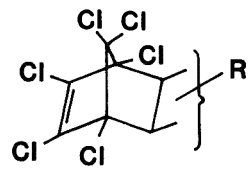
DIPHENYLMETHANE DERIVATIVES

o,p' - DDT / p,p' - DDT
o,p' - DDD / p,p' - DDD
o,p' - DDE / p,p' - DDE
Methoxychlor

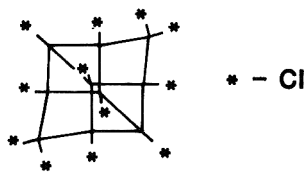


BRIDGED POLYCYCLIC CHLORINATED HYDROCARBONS

Aldrin
Dieldrin
∝- Chlordene / ϑ - Chlordene
∝- Chlordane / ϑ - Chlordene
Heptachlor epoxide

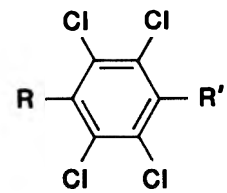


Mirex



CHLORINATED BENZENES

DCPA
PCNB
Pentachlorobenzene



ACETANILIDE PESTICIDES

Propachlor

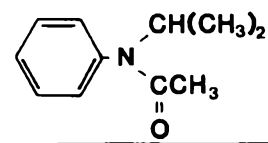
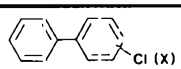


Table 2. Commercial Aroclor mixtures investigated by selected ion monitoring CI-MS



CAS No.[a]	Aroclor No.	Chlorination: Mean No. of Cl (X)	Chlorination: Cl, %
12674-11-2	1016	3.0	41.5
11104-28-2	1221	1.2	21.0
11141-16-5	1232	1.9	32.0
53469-21-9	1242	3.1	42.0
12672-29-6	1248	3.9	48.0
11097-69-1	1254	5.0	54.0
11096-82-5	1260	6.3	60.0
37324-23-5	1262	6.8	62.0

[a]Chemical Abstracts Services registry number.

capillary column, 50°C isothermal 1 min, then programmed from 50 to 240°C at 5°/min; methane carrier gas at 7 psi.

Methane Chemical Ionization Mass Spectrometry

(a) *Total ion mass spectral data.*—The abundances of the major fragment ions in the methane chemical ionization mass spectra of pesticides and PCBs included in this study are summarized in Tables 3 and 4, respectively. The MS data shown were used to select ions for the 2 CI-SIM screens. Ions for CI-SIM were chosen on the basis of maximum abundance, maximum selectivity, and lowest possible interference from other compounds.

(b) *Optimized selected ion monitoring.*—Ions chosen for CI-SIM scanning of pesticides and PCBs of interest in this study are listed in Tables 5 and 6, respectively. The Hewlett-Packard Model 1000 computer system is capable of monitoring 5 groups of selected ions with up to 20 ions monitored simultaneously per group. The maximum abundance mass to be dwelled on was determined by scanning at ion currents 0.1 amu apart for each mass to be included in a CI-SIM screen. Each ion shown in Tables 5 and 6 represents, to the nearest 0.1 amu, the determined maximum abundance peak. The ions chosen for pesticide CI-SIM were generally either the $(M + 1)^+$ ion or fragment ions generated by the loss of neutral molecules from the $(M + 1)^+$ ion. PCBs showed little fragmentation and, in all cases, the abundant $(M + 1)^+$ ions were chosen for PCB CI-SIM. Each group of PCB scanning ions overlaps the previous one because chlorobiphenyl isomers elute from the capillary column over a range of temperatures. The reliability of the SIM identification was increased by monitoring more than 1 ion characteristic of each com-

Table 3. Relative abundance of major fragment ions in methane CI mass spectra of chlorinated pesticides[a]

Compound	Species, % relative abundance			
	$(MH + H)^+$	$(MH - HCl)^+$	$(MH - Cl_2)^+$	$(MH - \bigcirc - R)^+$
Diphenylmethane derivatives:				
o,p' – DDD (53-19-0)[b]	—	23.2	100.0	21.9
p,p' – DDD (72-54-8)	—	21.8	100.0	20.7
o,p' – DDT (789-02-6)	—	6.8	63.5	100.0
p,p' – DDT (50-29-3)	—	8.8	50.7	100.0
Methoxychlor (72-43-5)	—	10.5	—	100.0
o,p' – DDE (3424-82-6)	100.0	89.0	50.7	—
p,p' – DDE (72-55-9)	100.0	45.6	18.7	—
Bridged polycyclic chlorinated hydrocarbons:				
Aldrin (309-00-2)	9.1	100.0	40.0	—
Dieldrin (60-57-1)	32.5	100.0	56.6	—
α-Chlordene (56534-02-2)	22.3	100.0	78.5	—
γ-Chlordene (56641-38-4)	3.0	100.0	12.1	—
α-Chlordane (5103-71-9)	—	100.0	37.9	—
γ-Chlordane (5103-74-2)	—	100.0	24.7	—
Heptachlor epoxide (1024-57-3)	64.1	100.0	40.6	—
Mirex (2385-85-5)	7.0	100.0	—	—
Chlorinated benzenes:				
DCPA (1861-32-1)	100.0	14.7	—	—
PCNB (82-68-8)	100.0	72.1	—	—
Pentachlorobenzene (608-93-5)	100.0	43.8	—	—

[a]Major fragment ions in CI spectrum of propachlor are obtained by more complex mechanisms as described in text.
[b]Chemical Abstracts Services registry number.

Table 4. Relative abundance of major fragment ions in methane CI mass spectra of PCB isomers containing from 1 to 8 chlorine atoms per biphenyl moiety

No. of Cl atoms	Species, % relative abundance		
	$(M + H)^+$	$(M + C_2H_5)^+$	$(MH - HCl)^+$
1	100.0	23.4	5.0
2	100.0	20.1	10.9
3	100.0	23.4	36.7
4	100.0	29.9	30.4
5	100.0	36.0	33.3
6	100.0	30.9	35.6
7	100.0	31.0	24.2
8	100.0	27.0	30.1

pound. As shown in Tables 5 and 6, respectively, from 2 to 5 diagnostic ions were scanned for each pesticide and PCB.

Discussion

The methane CI mass spectra of the compounds included in this study were comparatively simple and highly characteristic. Table 3 summarizes the major fragment ions present in the CI mass spectra of several groups of chlorinated pesticides. Because the compounds are all chlorinated, each of the fragments listed is composed of a cluster of ions due to the isotope distribution of Cl-35 and Cl-37. The EI spectra of diphenyl methane and polycyclic chlorinated insecticides (5) are generally dominated by fragments that arise directly or indirectly from retro-Diels Alder reactions. The contributions of such reactions to the ion currents of CI spectra are small. Major fragment ions in the CI spectra of DDD, DDE, DDT, and methoxychlor correspond to the loss of neutral molecules such as HCl, Cl_2, and substituted benzenes from the $(M + 1)^+$ ion (Table 3). The spectra of the *o,p'*- and *p,p'*-isomeric pairs of DDT, DDD, and DDE were very similar, and these compounds could not be distinguished on the basis of their CI mass spectral fragmentation patterns. The $(M + 1)^+$ ion was absent from the spectra of most diphenyl methane derivatives with the exception of *o,p'*- and *p,p'*-DDE.

In the mass spectra of the bridged polycyclic chlorinated hydrocarbon pesticides listed in Table 3, the base peak was produced by the loss of HCl from the $(M + 1)^+$ ion. The loss of a Cl_2 molecule from the $(M + 1)^+$ ion was also a predominant fragment in all of the spectra in this group and, with the exception of the chlordanes, the $(M + 1)^+$ ion was present. In general, the CI mass spectra of the bridged polycyclic chlorinated hydrocarbon pesticides included in this study are much more distinctive than are the corresponding EI mass spectra. For example, the EI spectra of mirex and kepone are very similar (5) while their CI spectra can be easily distinguished. Figure 1 shows the major fragments in the CI total ion spectra of mirex and kepone. The base peak in the CI spectrum of kepone is the $(M + 1)^+$ ion (m/z 490.8); mirex contains a distinctive base peak (m/z 510.7) generated by the loss of HCl from the $(M + 1)^+$ ion. The molecular ion, the only diagnostic feature in the EI mass spectra of mirex and kepone, is weak and often absent from the spectra of both compounds. In addition, the fragment ions produced in electron impact mode are common to many of the cyclodiene insecticides (5).

The chlorinated benzene compounds DCPA, PCNB, and pentachlorobenzene fragmented with the loss of an HCl molecule from the $(M + 1)^+$ ion; the hydrogenated molecular ion still remained the base peak in these spectra. The CI mass spectrum of propachlor, an *N*-isopropyl acetanilide herbicide, is unusual and cannot be described by the loss of simple fragment molecules from the $(M + 1)^+$ ion (m/z 212.0). The ion m/z 178.0 is suspected to be PhN $[CH(CH_3)_2]$ $[(C=OH)CH_3]^+$ which is formed from propachlor in the mass spectrometer (Hargesheimer et al., unpublished). The abundance of the ion m/z 178.0 is variable, because it is formed by chemical reaction in the mass spectrometer rather than by the typical rearrangement and fragmentation reactions. The ion m/z 178.0 is diagnostic of propachlor; however, it is not suitable for quantitative SIM analysis because its abundance is not consistent.

As shown in Table 4, the methane CI mass spectra of PCBs that contain from 1 to 8 chlorine atoms are all characterized by the presence of the $(M + 1)^+$ ion as the base peak. Ethyl- and propyl-addition ions were also observed. Story and Squires (6) reported that CI mass spectra of PCBs generated with methane reactant gas contained only $(M + 1)^+$, $(M + 29)^+$, and $(M + 41)^+$ species with no fragment ions observed. Under the methane CI-MS conditions used in this study, we

Table 5. Diagnostic ions used for CI-MS selected ion monitoring of chlorinated pesticides

Group No.	Group scan time, min	Peak No.	Pesticide	GC retention time, min	Scanning ions, m/z
I	14.2	1	pentachlorobenzene	18.92	248.8, 250.8
		2	propachlor	21.07	178.1, 212.0
		3	PCNB	24.48	263.9, 265.8
		4	α-chlordene	27.60	300.9, 302.9, 305.0, 338.9
		5	γ-chlordene	28.68	300.9, 302.9, 305.0, 338.9
		6	aldrin	28.85	292.9, 295.0, 326.9, 329.0
II	1.4	7	DCPA	29.42	331.0, 332.9, 334.9
		8	heptachlor epoxide	30.25	350.8, 352.8, 354.8, 388.9, 390.7
III	1.4	9	γ-chlordane	31.07	372.9, 374.9, 376.8
		10	*o,p* – DDE	31.50	283.0, 317.0, 319.0, 321.0
		11	α-chlordane	31.77	372.9, 374.9, 376.8
IV	4.2	12	dieldrin	32.55	309.0, 311.0, 344.9, 347.0
		13	*p,p'* – DDE	32.73	283.0, 285.0, 287.0, 317.0, 319.0
		14	*o,p'* – DDD	32.85	247.0, 249.0, 283.0, 285.0, 287.0
		15	*p,p'* – DDD	34.07	247.0, 249.0, 283.0, 285.0, 287.0
		16	*o,p'* – DDT	34.28	247.0, 249.0, 283.0, 285.0, 287.0
		17	*p,p'* – DDT	35.50	247.0, 249.0, 283.0, 285.0, 287.0
V	10.0	18	methoxychlor	37.62	239.0, 275.0
		19	mirex	39.00	509.0, 511.0, 513.0

Table 6. Diagnostic ions used for CI-MS selected ion monitoring of PCBs

Group No.	Run time, min	No. of Cl atoms per biphenyl moiety detected	Scanning ions, m/z
I	10.0–28.0	1	189.0, 191.0
		2	223.0, 225.0
		3	257.0, 259.0
II	28.0–32.0	3	257.0, 259.0
		4	291.0, 293.0, 295.0
		5	325.0, 327.0, 329.0
III	32.0–37.0	5	325.0, 327.0, 329.0
		6	359.0, 361.0, 363.0, 365.0
		7	393.0, 395.0
IV	37.0–47.0	6	361.0, 363.0
		7	393.0, 395.0, 397.0, 399.0
		8	429.0, 431.0, 433.0

observed fragmentation with the loss of HCl from the $(M + 1)^+$ ion. The abundance of this fragmentation was consistently less than 40% and, as shown in Table 4, the $(M + 1)^+$ ion remained the base peak in the CI mass spectra of PCBs containing from 1 to 8 chlorine atoms. Methane CI positive ion mass spectra are more diagnostic than are negative chemical ionization (NCI) mass spectra of PCBs. In NCI, the molecular ions of the chlorinated biphenyl compounds are absent or in low abundance (4). Although Pellizzari et al. (4) tried a variety of reagent/gas combinations, the predominant ions generated by NCI-MS were m/z 35 and m/z 37. These chlorine isotope ions do not represent diagnostic fragments and give little information concerning the identity of the compounds under investigation. Therefore, CI-MS unlike NCI-MS can be used to identify the degree of chlorination of a PCB moiety because $(M + 1)^+$ ions are present in the spectra.

The use of CI-SIM in combination with high resolution capillary GC allows the identification of compounds on the basis of their GC retention times as well as the presence of characteristic ions. Using a 12 m OV-101 capillary column, all 19 pesticides listed in Table 5 were resolved within less than 40 min. Figure 2 shows a portion (from 27 to 37 min) of the chemical ionization total ion chromatogram. Fourteen pesticides, with GC retention times differing by as little as 0.12 min, were resolved within 9 min. The use of methane as a GC carrier gas as well as CI-MS ion source reagent gas did not hamper GC resolution of the 19 pesticides included in this study.

Figure 3 shows a CI-SIM pesticide scan of a standard mixture of 19 pesticides. The ions scanned in each group correspond to the ions listed in Table 5; a total of 5 groups were scanned. Group I ions are diagnostic of pentachlorobenzene, aldrin, PCNB, propachlor, and the chlordenes. Aldrin, for example, is unequivocally identified only if a detector response is observed at all 4 selected ions diagnostic of aldrin: m/z 292.9, m/z 295.0, m/z 326.9, and m/z 329.0. Because the methane CI-MS spectra of the chlorinated pesticides included in this study contained few fragmentations, nonspecific response from interfering compounds and various background constituents was minimal. As shown in Figure 3, the peak heights obtained in selected ion monitoring are much larger than those in the total ion trace. The sensitivity of the mass spectrometer is increased in selected ion monitoring mode because data are being collected on the specific ions in each group for a longer period of time than is possible in the total ion scanning mode. In a 1 L water sample, the CI-SIM detection limit for the pesticides included in this study was 5 ppt. Pesticide residues were not detected in any of the extracts from 5 Elbow River water samples analyzed using the pesticide CI-SIM screen.

As shown in Table 4, PCBs undergo little fragmentation, and abundant $(M + 1)^+$ ions are available for CI-SIM. Figure 4 shows the CI-SIM screen of an extract prepared from a 1 L distilled water sample containing 0.5 ppb Aroclor 1254. The screening method can be used to determine the number of chlorines in each chromatographic peak of a complex mixture of PCBs. Each chlorinated biphenyl, from 1 to 8 chlorine atoms, is composed of a series of isomers. In Figure 4, the ions m/z 359.0, 361.0, 363.0, and 365.0 are all diagnostic of hexachlorobiphenyls. The pattern of isomers detected at all 4 ion currents is identical and identifies the presence of hexachlorobiphenyl. Figure 5a shows a portion of the CI-SIM screen obtained after injection of 1 μL crude extract of Elbow River water. Most of the scanning ions detected no peaks and simply showed a repetitive noise pattern. Although a number of small peaks were detected at m/z 291.0, tetrachlorobiphenyls were not present in the sample. To provide conclusive identification of the presence of tetrachlorobiphenyl, the other 2 ion currents, m/z 293.0 and 295.0, would have to be detected concurrently with the response at m/z 291.0. A second 1 L Elbow River water sample was extracted after the addition of 1.6 ppb Aroclor 1254, and the extract was analyzed with the PCB CI-SIM screen. As shown in Figure 5b,

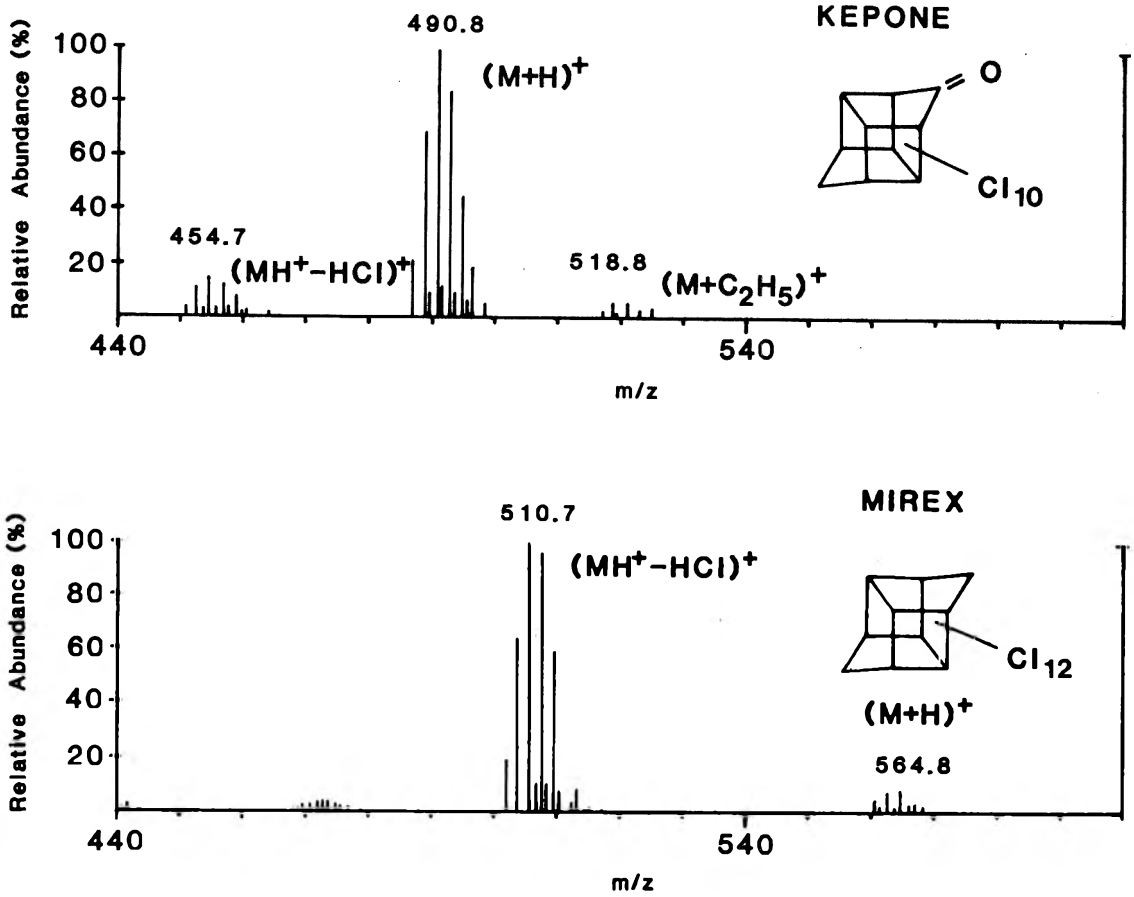


Figure 1. Methane CI mass spectra of kepone and mirex.

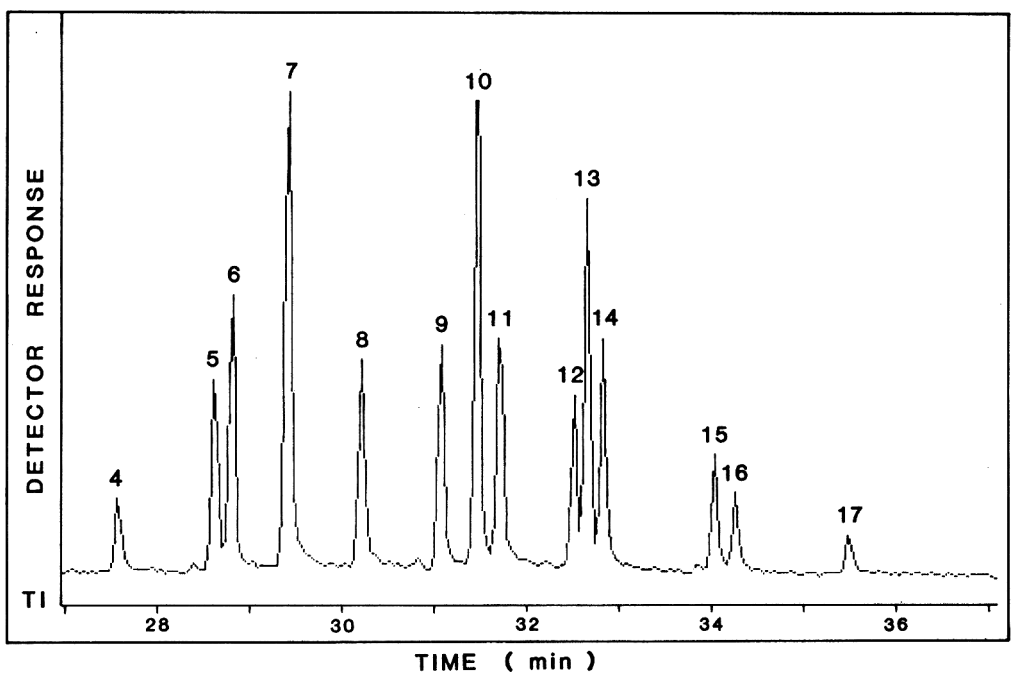


Figure 2. Computer-reconstructed MS total ion trace of portion of capillary gas chromatogram (27–37 min run) showing resolution of pesticides (1.0 ppb each) with GC retention times differing by as little as 0.12 min. See Table 5 for peak identification.

the very distinctive pattern of Aroclor 1254 was observed. In this case, all 3 ions diagnostic of tetrachlorobiphenyl (m/z 291.0, 293.0, and 295.0) detected the same pattern of isomers. Pentachlorobiphenyl as well as hexachlorobiphenyl isomers were detected. The detection limit for PCBs by CI-SIM screening was 0.1 ppb. PCBs were not detected in any of the 5 Elbow River water sample extracts examined.

Figure 6 shows the CI-SIM total ion chromatograms (a composite of all 31 PCB scanning ions) obtained for 5 different commercial Aroclor mixtures. The capillary GC pattern and CI-SIM scan of each commercial Aroclor mixture was distinctive. Further, as shown in Figure 4 for Aroclor 1254, CI-SIM indicated the number of chlorine atoms present in each chromatographic peak.

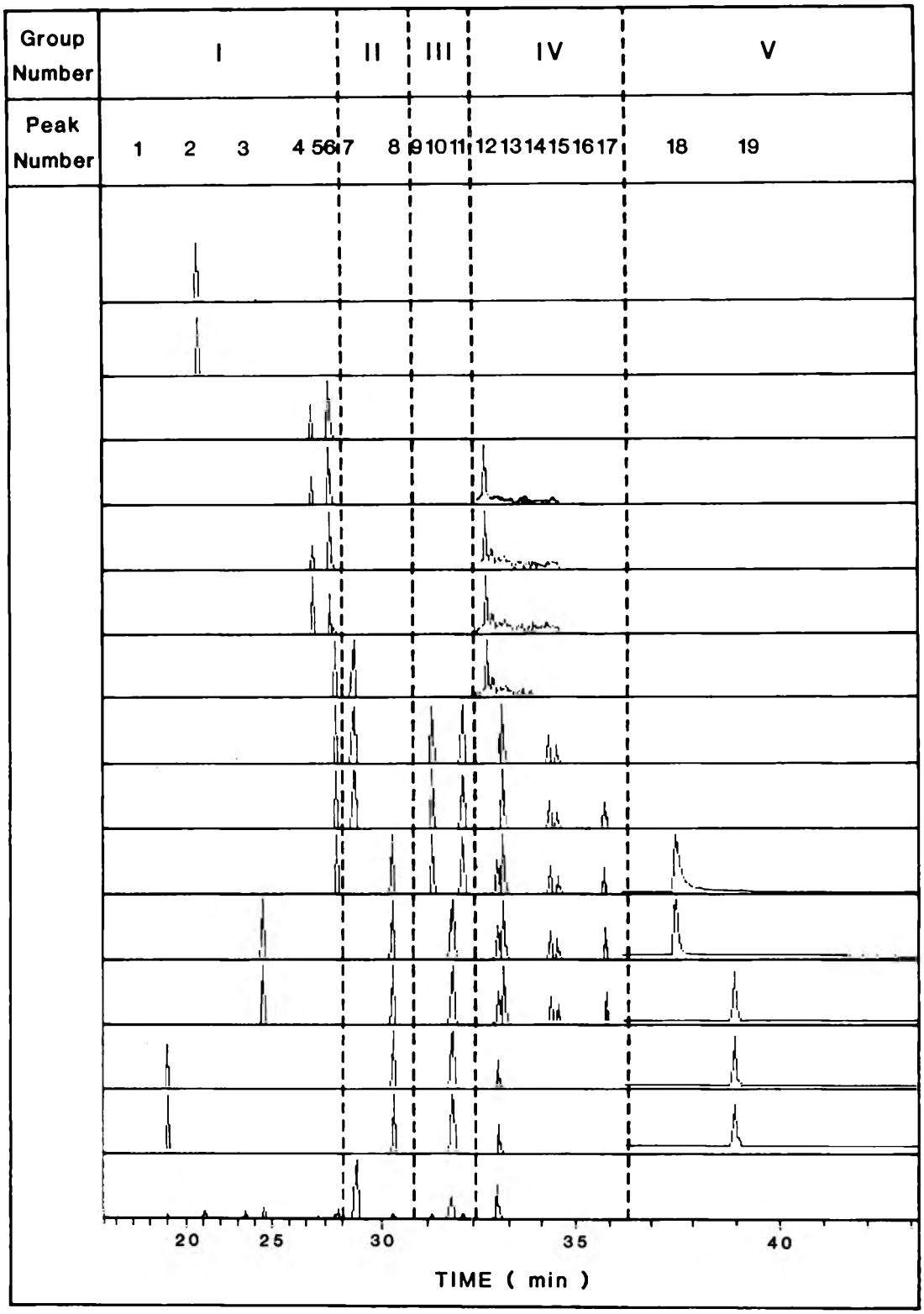


Figure 3. CI-MS selected ion monitoring scan of standard mixture of 19 pesticides (0.5 ppb of each). Group numbers, peak numbers, and scanning ions as listed in Table 5.

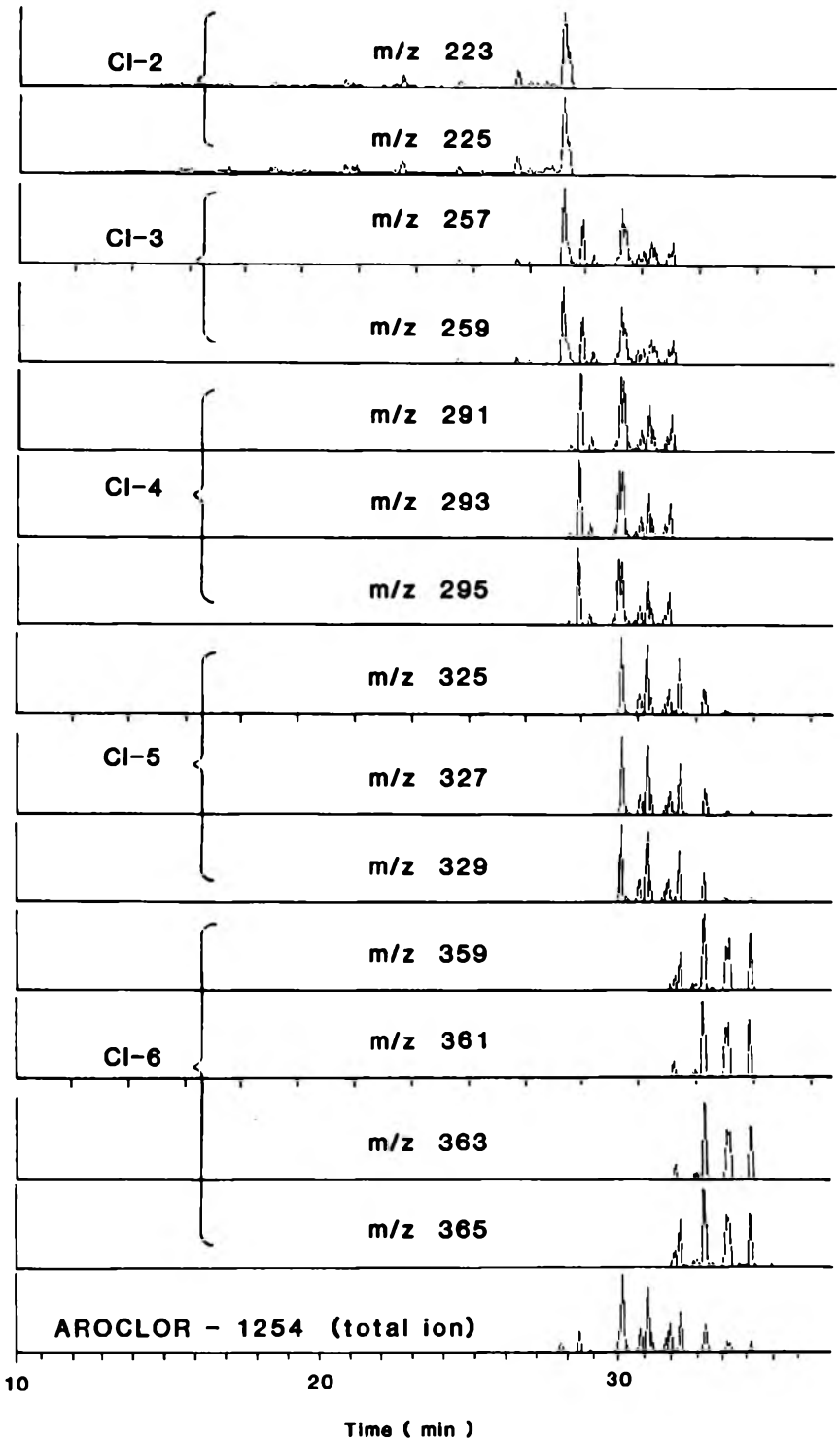


Figure 4. CI-MS selected ion monitoring scan of Aroclor 1254 (0.5 ppb). Scanning ions correspond to SIM program in Table 6.

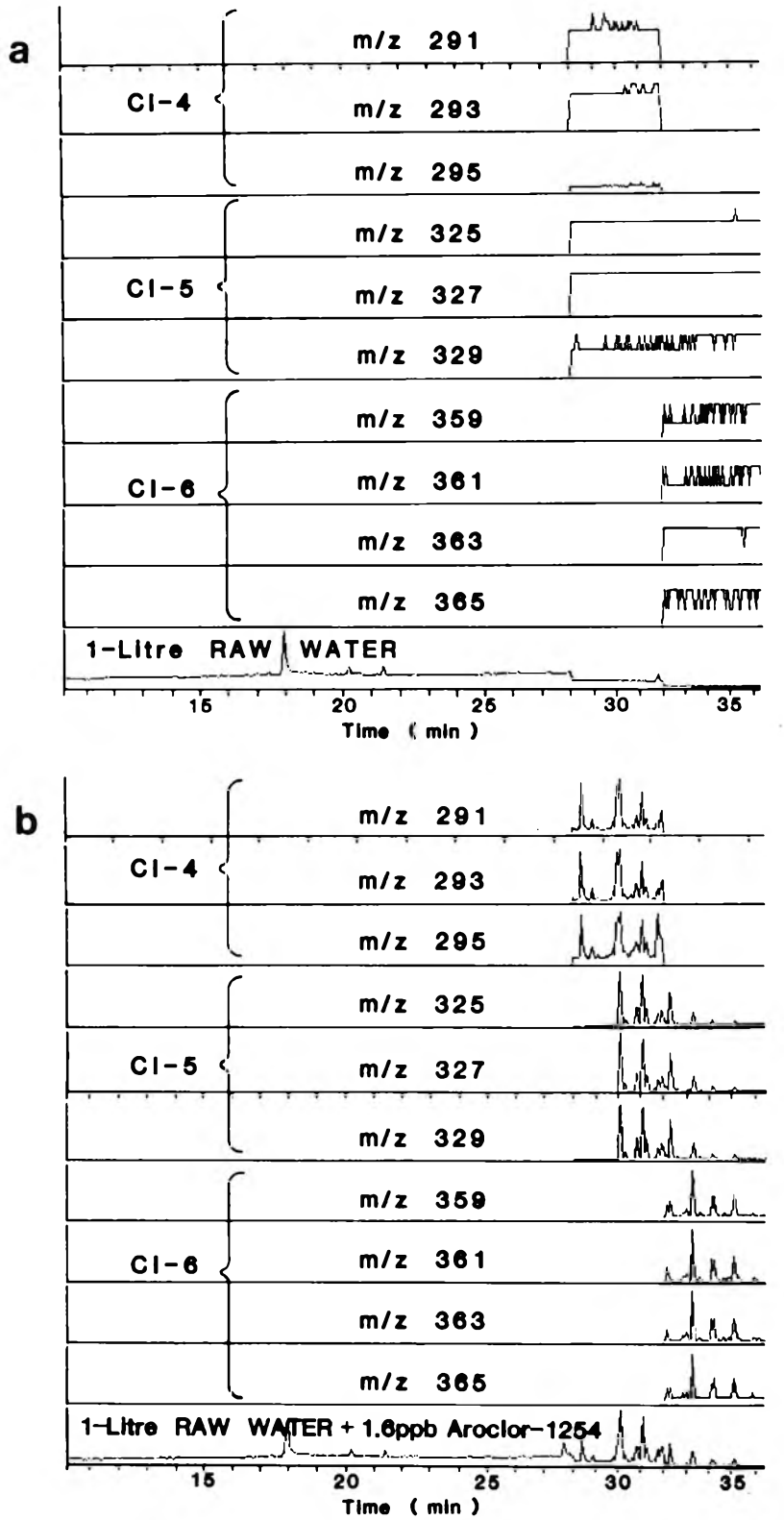


Figure 5. Selected ion monitoring PCB screen of (a) crude extract of Elbow River water; (b) extract of raw river water sample containing 1.6 ppb added Aroclor 1254.

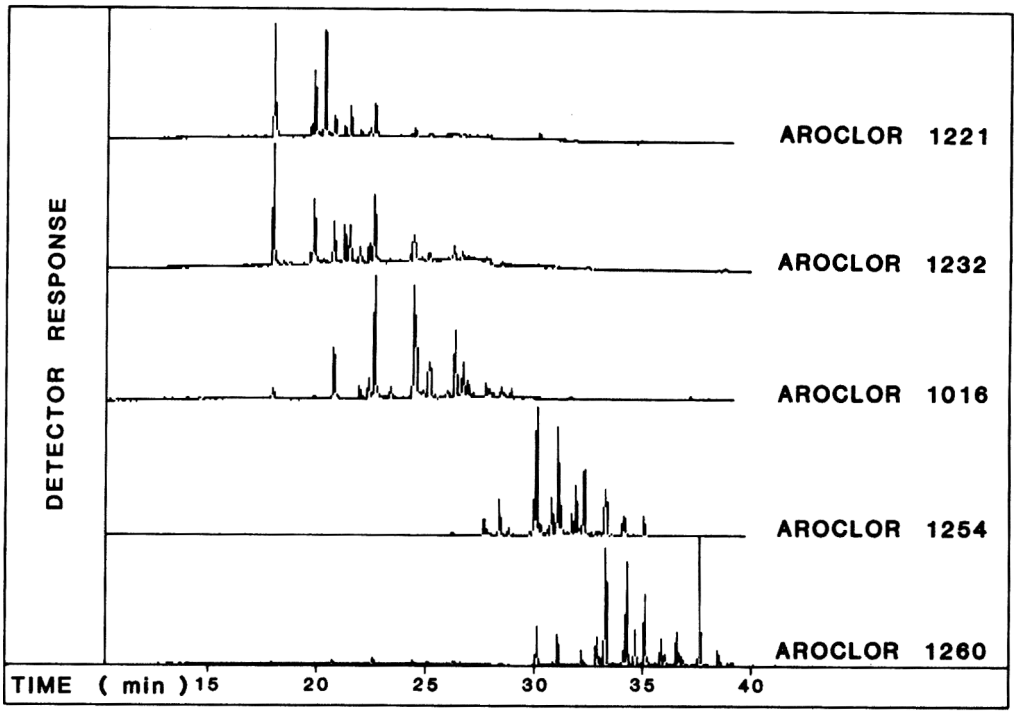


Figure 6. Computer-reconstructed MS total ion traces of 5 commercial Aroclor mixtures.

Various Aroclors and the 19 pesticides included in this study could be distinguished using CI-MS selected ion monitoring. A particular compound was considered conclusively identified only if peaks were observed at all of the specific monitoring ions at the expected GC retention time of the standard compound. When the extract containing Aroclor 1254 (Figure 5b) was analyzed using the pesticide CI-SIM screen, no response was obtained. The absence of interference between pesticide and PCB mixtures was further confirmed by first analyzing highly concentrated Aroclor extracts (100–200 ppb range) by using the pesticide CI-SIM screen and, second, by analyzing concentrated pesticide mixtures (100–200 ppb) by using PCB scanning ions.

Although no quantitative analysis was conducted using CI-SIM in this study, the method is potentially quantitative as well as qualitative with the addition of suitable internal standards. Individual reference standards with fragmentation and chemical characteristics similar to the compound of interest would be required for quantitative CI-SIM. Deuterated analogs are commonly used as internal standards (8).

In conclusion, CI-SIM is a reliable means of identification and confirmation of a wide variety of pesticides and PCB residues detected by ECD-GC. The selected ion monitoring technique can be expanded to include other classes of compounds of particular interest. Crude extracts of environmental water samples can be analyzed by SIM with minimal sample preparation.

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Liquid Chromatographic Determination of Trace Residues of Polynuclear Aromatic Hydrocarbons in Smoked Foods

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A liquid chromatographic (LC) method was developed and applied to the determination of polynuclear aromatic hydrocarbons (PAHs) in a variety of smoked, market basket commodities. The PAHs are extracted with 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113) from alcoholic KOH digests of the commodities. The extracts are purified by column chromatography through a deactivated silica gel/alumina column and by liquid-liquid partitioning between dimethyl sulfoxide and cyclohexane before separation of the PAHs by LC. Both fluorescence and UV detectors are used to monitor the LC column effluent to detect nanogram quantities of PAHs. Trace levels of carcinogenic and noncarcinogenic PAHs were found in all samples analyzed, although generally at <1 ppb levels.

There is continuing worldwide concern about the contamination of foods by polynuclear aromatic hydrocarbons (PAHs) with carcinogenic and mutagenic properties (1). Four sources considered to be the origin of PAHs in foods have been identified: natural causes, polluted environment, food additives and packaging, and curing smokes and other pyrolysis products (2). The last source probably contributes the bulk of PAHs found in foods because the PAHs become a part of the finished food as a result of the smoking process, the use of liquid smokes, and the heat treatment during the cooking process (1).

Recent investigations include those by researchers in many countries. Emerole (3) of Nigeria determined the likelihood of PAH contamination of smoked foods as the causative factor associated with the incidence of cancer in several Nigerian communities. Panalaks (4) of Canada analyzed charcoal-broiled and smoked foods to determine the demonstrated carcinogen benzo(a)pyrene (BaP). Grimmer and Böhnke (5) of Germany studied the occurrence of various carcinogenic PAHs in high protein foods, oils, and fats. Lintas and De Matthaeis (6) of Italy demonstrated that 73% of the commercially available smoked, cooked, and toasted food products contained BaP. Guerrero et al. (7) of the United States determined the extent of PAH contamination in shellfish exposed to oil-polluted waters.

A large number of procedures for separating and measuring various PAHs have been developed to assess the problem of PAH contamination of food. Generally, early investigators in the field used ultraviolet (UV) absorption and/or fluorescence spectroscopy for quantitative analysis of food extracts (8–11). Recently, the trend has been toward the use of capillary gas chromatography (GC) (12–20) and/or the use of liquid chromatography (LC) (4, 7, 21–27).

As indicated by Dunn and Armour (1), in a majority of the samples of interest. PAHs are accompanied by a complex array of other organic compounds which require extensive purification procedures to isolate the PAHs as a purified fraction before determination of individual PAHs. In their review of PAHs in foods, Lo and Sandi (2) indicated that approximately 37 PAHs had been identified.

In the present work, a rapid, sensitive, and reliable LC method is described for the determination of 12 representative PAHs in various smoked food samples. These PAHs include phenanthrene (Phen), fluoranthene (F), pyrene (Py), benz(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(e)pyrene (BeP), BaP, dibenz(a,h)anthracene (DBahA), indeno(1,2,3-c,d)pyrene (IcdPy), benzo(g,h,i)perylene (BghiP), dibenzo(a,i)pyrene (DBaiP), and coronene (Cor), with benzo(b)chrysene (BbCh) used as the internal standard. In this method, the samples are saponified in alcoholic KOH, extracted into trichlorotrifluoroethane (TCTFE) (Freon 113) or cyclohexane, cleaned up by column chromatography and liquid-liquid partitioning, and finally determined by reverse phase LC with UV and fluorescence detection. In several cases, fluorescence emission and excitation spectra were used to confirm the identity of some PAHs fractionated from various food samples.

METHOD

Apparatus

Clean all glassware with chromic acid/sulfuric acid solution, rinse thoroughly with distilled water, oven-dry at 120°C, and solvent-rinse before use to minimize interferences.

(a) *Meat chopper.*—Launders, Frary and Clark Co., New Britain, CT, or equivalent.

(b) *Digestion apparatus.*—*(1) Heating mantle.*—500 mL (Series O, hemispherical, K-721000, Kontes Glass Co., Vineland, NJ 08360, or equivalent). Use with variable auto transformer heat control (E 2101-2T, American Scientific Products Div., American Hospital Supply Corp., McGaw Park, IL 60085). *(2) Friedrich condenser.*—With inclined Ⓣ 24/40 outer joint and Ⓣ 24/40 inner, drip joint at bottom (Kontes K-437000, or equivalent). *(3) Boiling flask.*—Short-neck, round-bottom, 500 mL with Ⓣ 24/40 outer joint.

(c) *Separatory funnels.*—One 500 mL, one 125 mL, and two 250 mL, each equipped with Teflon stopcock.

(d) *Reservoir.*—250 mL with Ⓣ 24/40 upper and lower joints.

(e) *Chromatographic tube.*—14.5 × 250 mm with Ⓣ 24/40 joint at top.

(f) *Flasks.*—Pear-shape, 300 mL with Ⓣ 24/40 joint (Kontes K-294260, or equivalent).

(g) *Flash evaporator.*—Spiral condenser, with temperature-controlled water bath (Buchler Instruments Inc., Fort Lee, NJ 07024, or equivalent).

(h) *Filter funnel.*—Buchner, 60 mL with coarse porosity fritted disk.

(i) *Concentrator tubes.*—Kimax, 13 mL conical, graduated 0.1 mL subdivisions (American Scientific Products Div., American Hospital Supply Corp., or equivalent).

(j) *Sample clarification kit.*—Parts include 3 mL Luer-Lok syringe and 13 mm Swinny stainless steel disk filter holder (XX30-012-00, Millipore Corp., Bedford, MA 01730, or equivalent) with 0.2 μm filters (FGLP 01300, Millipore Corp.).

(k) *Liquid chromatograph.*—Model 332 programmable gradient system (Altex Scientific Inc., Berkeley, CA 94710). *Column.*—4.6 mm × 25 cm, Zorbax ODS, 5–6 μm particles (DuPont Co., Analytical Instruments Div., Wilmington, DE 19898), or equivalent C_{18} column. *Detectors.*—Model SF 770 UV-Vis detector (Schoeffel Instruments Div., Kratos, Inc., Westwood, NJ 07675) set at 289 nm; and Model FS 970 fluorometer (Schoeffel) set at 333 nm (excitation filter 7-54; emission filter KV-370) in series with UV detector. (Note: The 7-54 excitation filter has a broad transmission range with a maximum at 310 nm; the KV-370 emission filter has 10^{-3}% transmission at 340 nm and 99% transmission at 390 nm.) *Operating parameters.*—Mobile phase: Reservoir A contains

Table 1. Recovery (%) of PAHs from procedure blanks extracted by cyclohexane and TCTFE[a]

	Cyclohexane		TCTFE	
PAH[b]	Av.[c]	CV	Av.[c]	CV
Phen[d]	96	9.1	85	8.9
F	80	6.6	83	3.5
Py	78	9.2	81	6.2
BaA	80	6.6	94	1.8
BbF	85	7.2	98	2.0
BeP	91	3.4	94	4.3
BaP	83	7.8	91	4.6
DBahA	89	9.1	93	3.8
IcdPy	81	12.7	95	4.0
BghiP	82	17.1	93	5.9
DBaiP	65	18.6	85	4.2
Cor	85	8.3	91	4.0
Mean	83	9.6	90	4.4

[a]LC determination with fluorescence detection at a 333 nm activation wavelength (all PAHs except Phen).
[b]2.5 ppb spiking level for each PAH.
[c]Average of 3 determinations.
[d]LC determination with UV detection at 289 nm.

Table 2. Detection limits (ppb) of 12 PAHs used in this study

PAH	UV[a]	Fluorescence[b]
Phen	0.18	—
F	0.29	0.05
Py	0.39	0.07
BaA	0.09	0.03
BbF	0.09	0.03
BeP	0.12	0.21
BaP	0.06	0.07
DBahA	0.02	0.07
BbCh	0.06	0.07
IcdPy	0.11	0.15
BghiP	0.05	0.18
DBaiP	0.10	0.36
Cor	0.07	0.44

[a]With UV detection at 289 nm.
[b]With fluorescence detection at a 333 nm activation wavelength.

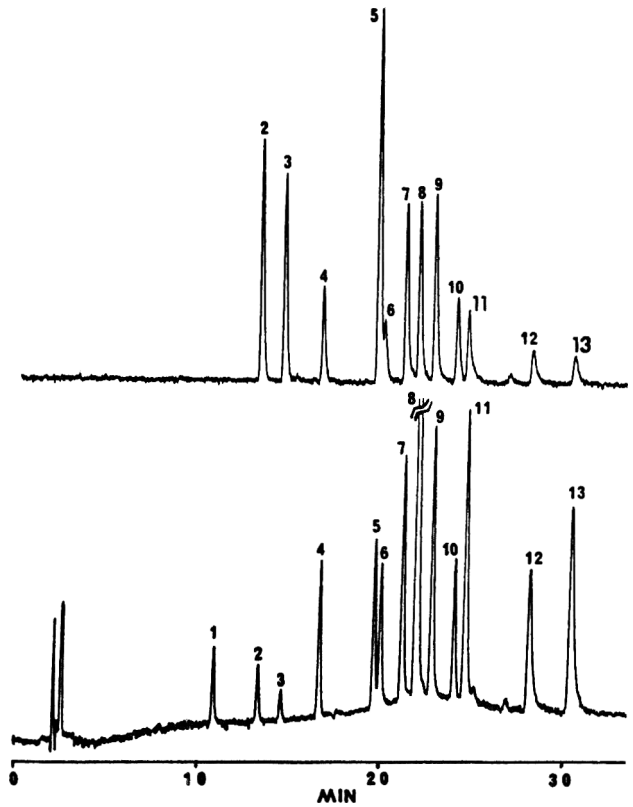

Figure 1. LC chromatograms for 5 ng of each PAH standard injected on column. Top chromatogram: fluorescence excitation 333 nm, range 0.2 μA. Bottom chromatogram: UV 289 nm, attenuation 0.02 absorbance unit full scale (AUFS). 1, Phen; 2, F; 3, Py; 4, BaA; 5, BbF; 6, BeP; 7, BaP; 8, DBahA; 9, BbCh (internal standard); 10, IcdPy; 11, BghiP; 12, DBaiP; 13, Cor.

water and reservoir B contains methanol–acetonitrile (1 + 1); flow rate 1 mL/min; injection volume 20 μL; gradient program Segment 1: 80 to 100% B in 20 min; Segment 2: hold at 100% B for 20 min; Segment 3 (equilibration of column): 100 to 80% B in 5 min, hold at 80% B for 20 min before next injection.

(l) *Recording data processor.*—Altex Model C-R1A (manufactured by Shimadzu Seisakusho Ltd, Kyoto, Japan), used with fluorescence detector; or equivalent instrumentation.

Reagents

(a) *1,1,2-Trichloro-1,2,2-trifluoroethane (TCTFE).*—Reagent grade (Fisher Scientific Co., Pittsburgh, PA 15219). Distill before use.

(b) *Solvents.*—Ethanol, 200 proof (Publicker Industries, Philadelphia, PA); methanol and acetonitrile, LC grade (Fisher Scientific); dimethyl sulfoxide (DMSO), spectrophotometric grade (J. T. Baker Chemical Co., Phillipsburg, NJ 08865); cyclohexane, distilled-in-glass (Burdick & Jackson Laboratories Inc., Muskegon, MI 49442).

(c) *Solvent B.*—Acetonitrile–methanol (1 + 1).

(d) *Solvent C.*—Solvent B–water (80 + 20).

(e) *Deionized water.*—Equilibrate one volume with TCTFE; equilibrate second volume with cyclohexane.

(f) *PAH standards.*—Phenanthrene (Phen), fluoranthene (F), pyrene (Py), benz(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(e)pyrene (BeP), benzo(a)pyrene (BaP), dibenz(a,h)anthracene (DBahA), benzo(b)chrysene (BbCh) (internal standard), indeno(1,2,3-c,d)pyrene (IcdPy), benzo(g,h,i)perylene (BghiP), dibenzo(a,i)pyrene (DBaiP), and coronene (Cor), obtained from Division of Chemistry and Physics, Food and Drug Administration, Washington, DC 20204.

(g) *Standard solutions.*—*(1) Stock solutions.*—0.1 mg/mL. Dissolve 1 mg of each PAH standard in 10 mL benzene. (DBaiP may require heating to dissolve.) *(2) Working standard solutions.*—0.25 μg/mL. Pipet 0.25 mL of each stock solution (except BbCh) into 100 mL volumetric flask and dilute to volume with solvent B. Use solvent C to prepare BbCh solution (0.25 μg/mL) separately. *(3) Internal standard reference solution.*—Pipet 1 mL working standard solution containing PAH mixture into concentrator tube and concentrate just to dryness under gentle stream of nitrogen, using 30°C water bath. Add 1 mL BbCh solution (0.25 μg/mL solvent C) and sonicate 1 min. Inject 20 μL for LC analysis.

(h) *Alumina.*—Aluminum oxide 90, active neutral (activity stage 1); particle size 0.063–0.200 mm (70–230 mesh ASTM) (E. Merck, Darmstadt, W. Germany; obtained from Brinkmann Instruments, Inc., Westbury, NY 11590, No. 1077).

(i) *Deactivated alumina.*—Prepare from alumina (h) above. Determine apparent weight loss by heating 10 g alumina in weighed platinum or porcelain dish to red heat for 15 min over Bunsen burner. Cover dish immediately and place in desiccator to cool before weighing. (Weighings should be made rapidly, because alumina readily adsorbs atmospheric water.) Calculate water content. Discard alumina that has been heated. Add enough distilled water to fresh alumina so that total added water accounts for 10% by weight of the final deactivated alumina. Vigorously shake alumina 15 min and store in brown glass bottle for at least 4 h before use.

Table 3. Recovery (%) of PAHs from various smoked foods[a]

PAH	Beef franks[b]	Turkey[b]	Chicken[c]	Ham[b]
Phen[d]	107	87	92	94
F	94	92	74	92
Py	87	84	76	106
BaA	104	93	104	92
BbF	97	102	80	80
BeP	96	101	80	80
BaP	91	96	76	94
DBahA	91	100	76	98
IcdPy	76	100	78	98
BghiP	98	92	76	84
DBaiP	75	91	76	82
Cor	65	96	70	84

[a]Values represent averages of duplicate analyses after correction by subtraction of control values; LC determination with fluorescence detection at a 333 nm activation wavelength (all PAHs except Phen).
[b]5 ppb spiking level for each PAH.
[c]2.5 ppb spiking level for each PAH.
[d]LC determination with UV detection at 289 nm.

(j) *Silica gel 60.*—Particle size 0.063–0.200 mm (70–230 mesh ASTM) (Brinkmann Instruments, Inc., No. 7734).

(k) *Deactivated silica gel.*—Prepare from silica gel (j). Heat portion of silica gel in tared Erlenmeyer flask in 160°C oven for 16 h (overnight). Heat tared glass stopper in separate beaker along with flask containing silica gel. Remove flask from oven, stopper immediately, and cool in desiccator. Weigh flask to determine weight of dried silica gel. Add enough distilled water to dried silica gel so that added water accounts for 15% by weight of the final deactivated silica gel. Immediately stopper flask and shake vigorously 15 min. Store ≥4 h before use.

Column Chromatography

Add 5 g deactivated silica gel (water content 15%), 5 g deactivated alumina (water content 10%), and 10 g Na_2SO_4, in that order, to 14.5 × 250 mm chromatographic tube containing glass wool plug by tapping gently during each addition. Fit column with 250 mL reservoir. Wash column with 50 mL TCTFE. Stop flow when liquid level just reaches top of Na_2SO_4 bed. Test before use as follows: Prepare 2 columns as described above (one column for blank and one for recovery). Add 40 mL TCTFE to one column and to the other column add 40 mL TCTFE spiked with 0.25 mL of a PAH mixture containing 0.25 μg/mL each of Phen, F, Py, BaA, BbF, BeP, BaP, DBahA, IcdPy, BghiP, DBaiP, and Cor (equivalent to 25 g sample spiked with each PAH at the 2.5 ppb level). Let solvent percolate through blank and spike columns. Collect eluates in separate 300 mL pear-shape flasks. Repeat column elution with two 25 mL portions of TCTFE. Let each eluant drain just to top of Na_2SO_4 bed before adding succeeding eluants. Evaporate combined TCTFE solutions to ca 5 mL, using flash evaporator with 30°C water bath. Transfer concentrates to 15 mL concentrator tubes with disposable Pasteur-type pipets. Rinse each flask with three 1 mL portions of TCTFE and transfer to the appropriate concentrator tube. Place tubes in 30°C water bath and concentrate solutions to dryness under gentle stream of nitrogen. Add 0.25 mL BbCh solution (0.25 μg/mL solvent C) and subject to ultrasonic vibration for ca 3 min. (If necessary, filter solutions with sample clarification kit into sample vial.) Subject to LC. Chromatograms of solvents must be free of interfering peaks, and recovery for each PAH should be >90%.

Extraction

Grind sample and place 25 g in 500 mL round-bottom boiling flask, which contains a few boiling chips. Add 100 mL ethanol and 4 g KOH. Place flask in heating mantle, set transformer to 50% output, insert Friedrich condenser, and digest 2 h. Let cool to room temperature. Fit glass filter funnel with ethanol-rinsed glass wool plug, and pour cool solution through glass wool into 500 mL separatory funnel. Sequentially rinse flask with 90 mL water pre-equilibrated with TCTFE, 40 mL TCTFE, and 50 mL ethanol, and combine with solution in separatory funnel. Stopper separatory funnel, then invert, and open stopcock while gently swirling contents of funnel a few seconds to vent. Close stopcock and extract by shaking funnel 2 min. Let layers separate, and then draw off lower TCTFE layer into chromatographic column (as prepared under *Column Chromatography*). Repeat extraction with two 40 mL portions of TCTFE. Let each extract percolate through column and collect eluate in 300 mL pear-shape flask. When third extract reaches top of Na_2SO_4, add 50 mL TCTFE to column as rinse and collect this eluate also in flask.

Add 10 mL DMSO to solution in flask. Evaporate the TCTFE, leaving the DMSO, using flash evaporator with water bath at 30°C. Quantitatively transfer DMSO concentrate to 125 mL separatory funnel, using 5 mL DMSO in small portions to aid transfer. Rinse flask with 50 mL cyclohexane, and add rinse to funnel. Extract by shaking funnel 2 min. After layers separate, draw off lower layer into 250 mL separatory funnel containing 25 mL cyclohexane and 90 mL water pre-equilibrated with cyclohexane. Repeat extraction of cyclohexane in 125 mL funnel with two 15 mL portions of DMSO. Add DMSO extracts to 250 mL funnel. Extract by vigorously shaking funnel 2 min. After layers separate, draw off lower layer into second 250 mL separatory funnel containing 25 mL cyclohexane. Extract by shaking funnel 2 min.

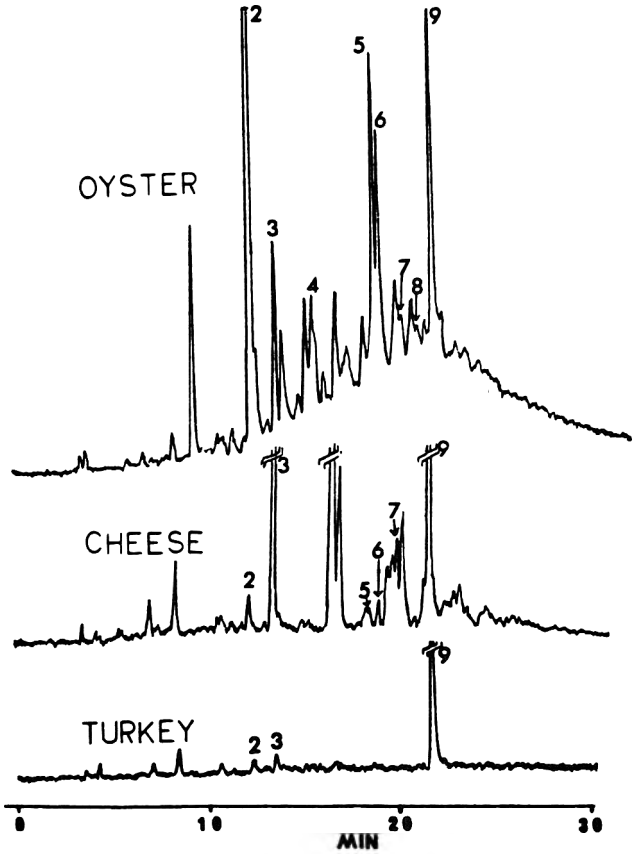

Figure 2. Representative LC chromatograms for extracts of unspiked, smoked oysters, cheese, and turkey (fluorescence excitation 333 nm, range 0.2 μA). 2, F; 3, Py; 4, BaA; 5, BbF; 6, BeP; 7, BaP; 8, DBahA; 9, BbCh (internal standard).

Table 4. PAHs (ppb) found in smoked marine samples[a]

PAH	Oysters[b]	Whitefish[c]	Whiting[c]	Salmon[c]	Labrador[c]
Phen[d]	14.5 (1)	5.9	—	—	—
F	15.4 (3)	1.0	3.2	1.3	3.7
Py	11.0 (3)	1.3	2.8	1.1	2.6
BaA	5.9 (3)	—	—	—	—
BbF	3.2 (3)	0.2	0.3	0.3	0.7
BeP	36.6 (3)	2.1	3.5	2.5	0.3
BaP	1.9 (3)	—	—	—	—
DBahA	0.7 (2)	—	0.5	—	—
IcdPy	1.3 (3)	—	0.7	—	1.1
BghIP	2.0 (2)	—	—	—	—
DBaIP	—	—	—	—	—
Cor	—	—	—	—	—

[a]LC determination with fluorescence detection at a 333 nm activation wavelength (all PAHs except Phen).
[b]Averages of duplicate analyses of 3 separate cans of the same brand; number of cans in which corresponding PAH was detected is given in parentheses.
[c]Averages of duplicate analyses of same sample.
[d]LC determination with UV detection at 289 nm.

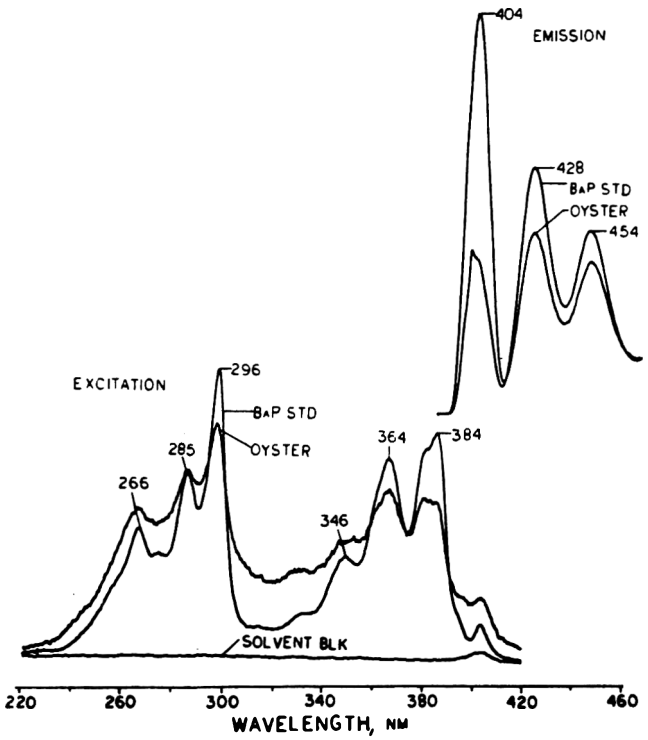

Figure 3. Excitation and emission spectra of standard BaP and a component (same retention time as BaP) isolated from the LC analysis of an oyster extract.

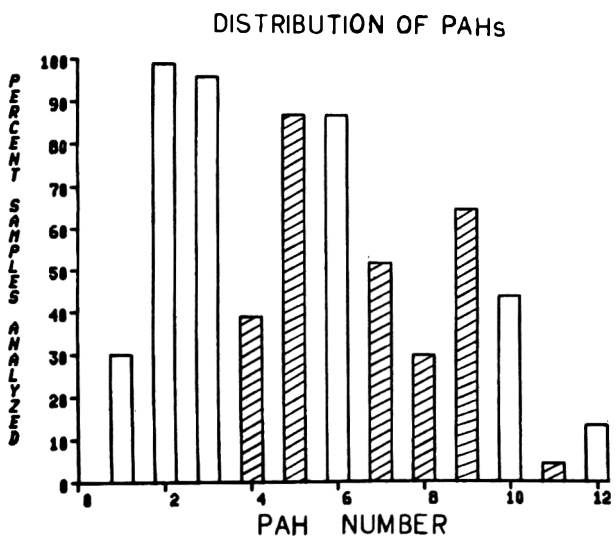

Figure 4. Distribution of PAHs in smoked foods analyzed. Striped bars show the percentage of samples analyzed in which a particular carcinogenic PAH was identified. Solid bars show the percentage of samples analyzed in which a particular noncarcinogenic PAH was identified. The PAH numbers correspond to elution order: 1, Phen; 2, F; 3, Py; 4, BaA; 5, BbF; 6, BeP; 7, BaP; 8, DBahA; 9, IcdPy; 10, BghIP; 11, DBaIP; 12, Cor.

After layers separate, discard lower, aqueous layer. Combine 2 cyclohexane extracts in first 250 mL funnel. Rinse second 250 mL funnel with two 10 mL portions of cyclohexane, and add rinses to first 250 mL funnel. Wash solution twice by gently shaking each time for 15 s with 100 mL water pre-equilibrated with cyclohexane. Discard aqueous layer after each wash.

Place 10 g deactivated alumina (water content 10%) followed by 50 g Na_2SO_4 in 60 mL Buchner funnel. Wash with 50 mL cyclohexane and discard wash. Pass combined cyclohexane extracts from separatory funnel through Buchner funnel into 300 mL pear-shape flask. Rinse separatory funnel with two 25 mL portions of cyclohexane, and pass each through Buchner funnel into flask. Use flash evaporator with water bath at 40°C to bring volume in flask to ca 2–5 mL. Transfer contents quantitatively to concentrator tube, using Pasteur-type, disposable pipet and ≤5 mL cyclohexane for rinsing. Bring contents in tube just to dryness in water bath at 30°C under gentle stream of nitrogen. Add 100 μL BbCh (0.25 μg/mL solvent C) and ultrasonicate for 3 min. Filter if necessary and analyze by LC.

Liquid Chromatography

Inject 20 μL sample extract or standard solution onto Zorbax ODS column and follow solvent program as described under *Apparatus* (k). Between injections, flush sample loop and port passages with ca 3 mL solvent B to prevent cross-contamination. Compare retention times of any peaks observed with those for known PAH standards chromatographed under same conditions. Inject standards after every third sample. Calculate quantity of PAH in sample extracts by internal standard procedure.

Calculation

Calculate level of each PAH found in sample extract as follows:

$$\text{PAH, ppb} = (F \times (Ru/Ris) \times IS \times (Vd/Vi))/W$$

where F = response of internal standard divided by response of PAH as obtained from analysis of standard mixture; Ru = response of given PAH in sample extract; Ris = response of

Table 5. PAHs (ppb) found in smoked cheese, poultry, pork, and beef products[a]

PAH	Edam cheese	Cheddar cheese	Sliced chicken	Sliced turkey	Sliced ham	Whole ham	Bacon[b]	Breakfast beef[c]	Beef franks
Phen[d]	—	10.1	1.3	—	1.4	—	—	—	1.0
F	0.2	3.5	1.0	1.1	0.3	1.2	1.1	1.3	0.3
Py	2.7	7.0	1.1	1.1	—	1.7	1.1	1.4	0.1
BaA	0.2	0.8	—	—	—	0.6	0.4	1.1	2.1
BbF	0.1	0.4	0.2	0.2	—	0.2	0.2	0.9	—
BeP	1.6	4.8	0.8	1.3	0.2	1.4	0.5	2.3	1.0
BaP	0.3	0.5	0.1	0.1	—	0.2	0.1	0.2	1.0
DBahA	0.1	0.1	0.1	0.4	0.1	—	—	—	—
IcdPy	0.7	0.3	—	0.4	0.1	0.2	—	—	0.3
BghiP	—	0.4	—	—	—	0.1	—	—	—
DBaiP	—	—	—	—	—	—	—	—	—
Cor	—	0.5	—	—	—	—	—	—	—

[a]Values represent averages of duplicate analyses of same sample; LC determination with fluorescence detection at a 333 nm activation wavelength (all PAHs except Phen).
[b]Averages of duplicate analyses of 3 separate samples (6 determinations).
[c]Averages of duplicate analyses of 2 separate samples (4 determinations).
[d]LC determination with UV detection at 289 nm.

internal standard added to same sample extract; *IS* = internal standard injected, ng; *Vd* = dilution volume, μL; *Vi* = volume of sample extract injected, μL; and *W* = weight, g, of sample taken for analysis.

Results and Discussion

In developing the analytical approach, PAH recoveries, background interference, and analysis time when an aliphatic solvent, such as cyclohexane, was used were compared to when TCTFE was used to extract the PAHs from an aqueous alcoholic KOH solution. Table 1 shows the results of the comparative study of recoveries obtained from procedure blanks that were fortified at levels of 2.5 ppb for each of the 12 PAH standards. Each recovery value shown is the average of 3 determinations. The range of the average recoveries is 65–96% with a mean CV of 9.6% for cyclohexane vs 81–98% with a mean CV of 4.4% for TCTFE. Similar background interferences were obtained for both solvents. The time studies showed that approximately 4 samples can be analyzed in 12 h using TCTFE, compared with 16 h using cyclohexane.

Because recoveries were higher, background interference was approximately equal, and analysis time was shorter using TCTFE, this solvent was chosen for the smoked food analyses reported in this investigation.

Figure 1 shows standard chromatograms for 5 ng quantities of 12 representative PAHs plus the internal standard BbCh. The top curve represents the fluorescence at a 333 nm activation wavelength; the lower curve represents the UV response at 289 nm. Peak identities are shown beneath the figures. Because Phen does not have an absorption band at or near 333 nm, it does not appear in the fluorescence chromatogram.

The detection limits for each PAH included in this study were determined on the basis of the chromatographic conditions used. Accordingly, each limit was based on the minimum amount of material that must be injected on the column in order to produce an observable peak. In this study, a peak was considered to be observable if it was twice the level of the background noise (28). The amounts calculated were then converted to ppb on the basis of a 25 g sample size. Table 2 shows these detection limits. The values obtained by UV detection at 289 nm and 0.02 AUFS range from 0.02 ppb for DBahA to 0.39 ppb for Py; the values obtained by fluorescence detection at an activation wavelength of 333 nm and range setting of 0.2 μA range from 0.03 ppb for BaA and BbF to 0.44 ppb for Cor.

PAHs extracted from samples were quantitated by using the stock solution internal standard procedure (29). In this technique, a stock solution of the internal standard BbCh and the components of interest was analyzed separately under the same conditions as the sample solution to which the internal standard was added to make the final BbCh concentration identical to that of the stock solution. The relative response factor, *F*, for each of the components of interest vs the internal standard was determined from the chromatogram of the stock solution. These response factors, which were reported previously (26), were then used to calculate the concentrations of the components of interest in the various smoked foods analyzed.

The PAHs separated by LC were tentatively identified by comparing the retention times of peaks in the sample chromatograms with those in the standard chromatograms. To enhance specificity, UV and fluorescence detectors were connected in series and set at 289 and 333 nm, respectively. Additional confirmatory qualitative information was obtained by isolating the components of interest and obtaining fluorescence spectra of the fractions collected. Repetitive fractions were collected for fluorescence confirmation whenever LC analysis indicated the presence of a particular PAH. To test for purity of isolated eluates, fluorescence peak ratios were calculated for the compounds of interest and compared with those for standards of the same compounds. These values were generally in very close agreement.

The smoked foods used in this investigation were purchased from local retail markets in the Washington, DC, area. A total of 23 samples were analyzed. The smoked foods analyzed include poultry, pork, beef, dairy, and marine products. Figure 2 shows fluorescence chromatograms of unspiked smoked oysters, cheese, and turkey samples. Peaks in all 3 chromatograms have retention times equivalent to those for F and Py; peaks in the top 2 chromatograms have retention times equivalent to those for BbF, BeP, and BaP.

The levels of PAHs in the samples analyzed range from approximately 0.1 ppb, found for a number of PAHs in various samples, to 37 ppb BeP found in oysters. Figure 3 shows typical emission and excitation spectra for the BaP fractions isolated from 2 oyster samples. These spectra support the identification of the components isolated from oyster samples as BaP, which appears in the LC chromatograms as a peak having the same retention time as the standard BaP peak. Other PAHs were identified in a similar manner.

To check the reliability of the analytical procedure, recovery studies were conducted with several smoked food samples. Table 3 shows the results of these investigations in which 12 PAHs were recovered from 4 different smoked food

Table 6. PAHs (ppb) found in miscellaneous smoked food products[a]

	Sausages[b]				Frankfurters[c]		Half-smokes[d]
PAH	1	2	3	4	1	2	1
Phen[e]	—	—	1.7	0.5	1.7	1.7	1.9
F	0.3	0.7	0.2	0.4	0.2	0.3	0.2
Py	0.9	0.9	0.3	0.3	0.3	0.4	0.3
BaA	0.3	—	—	0.5	—	—	—
BbF	0.1	0.2	0.1	0.1	0.1	—	0.2
BeP	0.3	0.7	—	0.6	—	0.3	0.2
BaP	0.1	0.1	—	0.1	—	—	—
DBahA	—	—	—	—	—	—	—
IcdPy	0.2	0.2	0.2	0.2	—	—	—
BghiP	0.2	0.2	0.2	—	0.2	—	0.3
DBaiP	—	—	0.4	—	—	—	—
Cor	—	—	0.4	—	0.4	—	—

[a]Values represent averages of duplicate analyses of same sample; LC determination with fluorescence detection at a 333 nm activation wavelength (all PAHs except Phen).
[b]4 different brands.
[c]2 different brands.
[d]1 brand
[e]LC determination with UV detection at 289 nm.

samples: beef franks, turkey, chicken, and ham. The chicken sample was fortified with 12 PAHs, each added at the 2.5 ppb level; the remaining samples were fortified with the PAHs added at the 5 ppb level. Recoveries corrected by subtraction of control values range from 65% for Cor to 107% for Phen. The results reported for Phen were obtained from the UV chromatographic data.

Table 4 summarizes the results for the marine samples analyzed: oysters, whitefish, whiting, salmon, and labradors. F, Py, BbF, and BeP were found in all these samples. In the case of the oysters, 3 cans of the same brand were analyzed separately, and the numbers in parentheses show the number of cans in which the corresponding PAHs were found. The ppb values shown for all the samples are the averages of duplicate analyses. These analyses showed agreement between duplicates to within a few tenths of a ppb. From these data, it is apparent that the oysters contained a much higher level of PAH contribution in comparison with the other marine samples.

Table 5 summarizes the data obtained for the analysis of some smoked cheese, poultry, pork, and beef products for their PAH content. The values shown are averages of duplicate analyses except in the case of bacon and breakfast beef. Three separate samples of bacon and 2 separate samples of breakfast beef were analyzed in duplicate. For the 9 products shown, the carcinogens BaA, BbF, BaP, and IcdPy appear to have been present in more than 50% of the samples analyzed. The cheddar cheese contained the greatest number of PAHs, 11, as well as the highest level of PAH contamination. In contrast, the sliced ham appears to have contained the fewest PAHs as well as the lowest level of PAH contamination among the samples represented in this table.

Table 6 shows the results obtained for 4 different brands of sausage, 2 brands of frankfurters, and 1 brand of half-smokes. Each result is the average of duplicate analyses. F and Py were present in all the samples analyzed. The carcinogen BaP was detected only in 3 brands of sausage at the 0.1 ppb level, whereas BbF was found in all but one of the samples shown.

It is apparent from the data in Tables 2–4 that trace levels of PAHs were present in all the smoked food samples analyzed. These data are summarized in the bar graph in Figure 4, which shows the percentage of total samples analyzed in which a particular PAH was found vs the PAH detected. The solid bars represent the noncarcinogenic PAHs and the striped bars represent the carcinogenic PAHs. From this graph it is apparent that F and Py were detected in at least 95% of the samples analyzed. More importantly, the carcinogens, BaA, BbF, BaP, DBahA, and IcdPy, were found in 30–87% of the samples analyzed, although generally at levels of <1.0 ppb. These data indicate that PAHs are widely dispersed in commercially available smoked foods.

Conclusion

A relatively rapid and reliable LC method has been developed for determining trace levels of PAHs in a variety of smoked foods. PAH contamination was found to be widespread in all the smoked foods analyzed. Carcinogenic PAHs, including BbF and BaP, were found in 30–87% of the samples analyzed at levels ranging from 0.1 to 6 ppb.

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Determination of Chlorinated Methylthiobenzenes and Their Sulfoxides and Sulfones in Fish

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A procedure was developed to determine chlorinated methylthiobenzenes and their respective sulfur oxidation products in fish. Perch samples fortified at the 0.1 ppm level with 2,4,5-trichloromethylthiobenzene, pentachloromethylthiobenzene, and their sulfoxides and sulfones were extracted and cleaned up using an adaptation of the official AOAC method for multiple residues of organochlorine pesticides. The Florisil column cleanup was modified; 200 mL 6% petroleum ether–ethyl ether eluted the methylthiobenzenes, 200 mL 50% PE-EE eluted the sulfones, and 200 mL EE eluted the sulfoxides. Recoveries determined by electron capture (ECD) gas chromatography (GC) were 75–101% for the methylthiobenzenes and their sulfones and 63–93% for the sulfoxides. Co-extracted materials in the Florisil eluates that interfered with the ECD/GC quantitation were removed by partitioning the sulfoxides and sulfones into sulfuric acid and by thin layer chromatography on silica gel, using methylene chloride–hexane (50 + 50) as the developing solvent. Seven fish samples containing residues of chlorinated benzenes or polychlorinated biphenyls (PCBs) were examined for chlorinated methylthiobenzenes, methylthio-PCBs, and their oxidation products by matching GC retention times obtained with the EC detector and a flame photometric detector operated in the sulfur mode. These analytes were not found in the fish samples above a detection level equivalent to 0.02 ppm 2,4,5-trichloromethylthiobenzene.

The chlorinated methylthiobenzenes, methylthiobiphenyls, and their sulfoxides and sulfones are fat-soluble substances that have been found as metabolites in the excreta of laboratory rodents given *p*-dichlorobenzene (1) or polychlorinated biphenyls (PCBs) (2). They have also been detected in the tissues of aquatic fauna containing residues of hexachlorobenzene (3) or PCBs (4). The sulfoxides and sulfones may go unobserved by analysts in the routine determination of the other fat-soluble residues because they have higher polarities than the chlorinated benzenes on adsorption cleanup columns and might not elute from the columns, and they have longer retention times (RTs) than the parent compounds on the gas chromatographic (GC) columns commonly used for residue determination (5). Although more specialized procedures have been developed to determine the methylthiobenzenes and their oxidation products in animal tissues, the recovery of these substances has not been adequately investigated.

This paper describes a procedure for determining some synthetic chlorinated methylthiobenzenes and their sulfoxides and sulfones at or below the 0.1 ppm level. The procedure can be used to determine these substances in edible fish tissue in which chlorinated benzenes or PCBs have been previously detected.

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Experimental

Apparatus

See sec. **29.012(e)** (5) for extraction apparatus.

See secs **29.014–29.015** (5) for cleanup apparatus.

(a) *Thin layer chromatographic (TLC) plates.*—20 × 20 cm × 0.5 mm silica gel, with fluorescent indicator (Analtech Inc., Newark, DE). Prewash plates with acetone (distilled in glass) and dry 30 min in hood. Store plates over Na_2SO_4 until needed.

(b) *TLC sandwich chamber.*—Chromaflex (Kontes Glass Co., Vineland, NJ).

(c) *TLC streak applicator.*—Camag Linomat III (Applied Analytical Industries, Wilmington, NC). Settings: nitrogen pressure 8 psig, 30 mm/μL, 5 s/μL.

(d) *Test tubes.*—Graduated 15.0 mL with $ 13 glass stoppers.

(e) *Spatula.*—Hayman stainless steel, 165 mm long with 3 × 29 mm curved end (A. H. Thomas, Philadephia, PA).

(f) *Gas chromatographs.*—Varian Model 3700 (Varian Associates, Palo Alto, CA) equipped with ^{63}Ni pulsed electron capture detector (ECD) and 6 ft × 2 mm id glass column packed with 3% OV-101 on Chromosorb W HP. Operating conditions: injection port temperature 230°C, detector temperature 310°C; argon–methane (95 + 5) carrier gas at flow rate of 30 mL/min; initial column temperature held for 1.0 min at 100°C, then programmed at 10°/min to final temperature of 260°C and held for 15 min; attenuation 256; range 10^{-11} amp/mV. A second Varian Model 3700 was equipped with flame photometric detector operating in sulfur mode (FPD(S)). Operating conditions: same as above with following exceptions: helium carrier gas 30–32 mL/min; detector temperature 240°C; air No. 1 flow rate to detector 80 mL/min, air No. 2 flow rate to detector 170 mL/min, hydrogen flow rate to detector 140 mL/min; range 10^{-10} amp/mV; attenuation 8.

(g) *Integrator.*—Shimadzu Chromatopac C-R1B (Shimadzu Corp., Columbia, MD). Settings: attenuation 4–6, slope 5000, minimum area 5000. Connect integrator to ECD/GC system with GC attenuation set at 1 to determine ECD linearity.

Reagents

(a) *Solvents.*—Distilled in glass petroleum ether (PE), acetonitrile, ethyl ether (EE), hexane, methylene chloride, acetone, benzene, and ethyl acetate (Burdick and Jackson Laboratories, Inc., Muskegon, MI). *Note:* Benzene is a possible carcinogen.

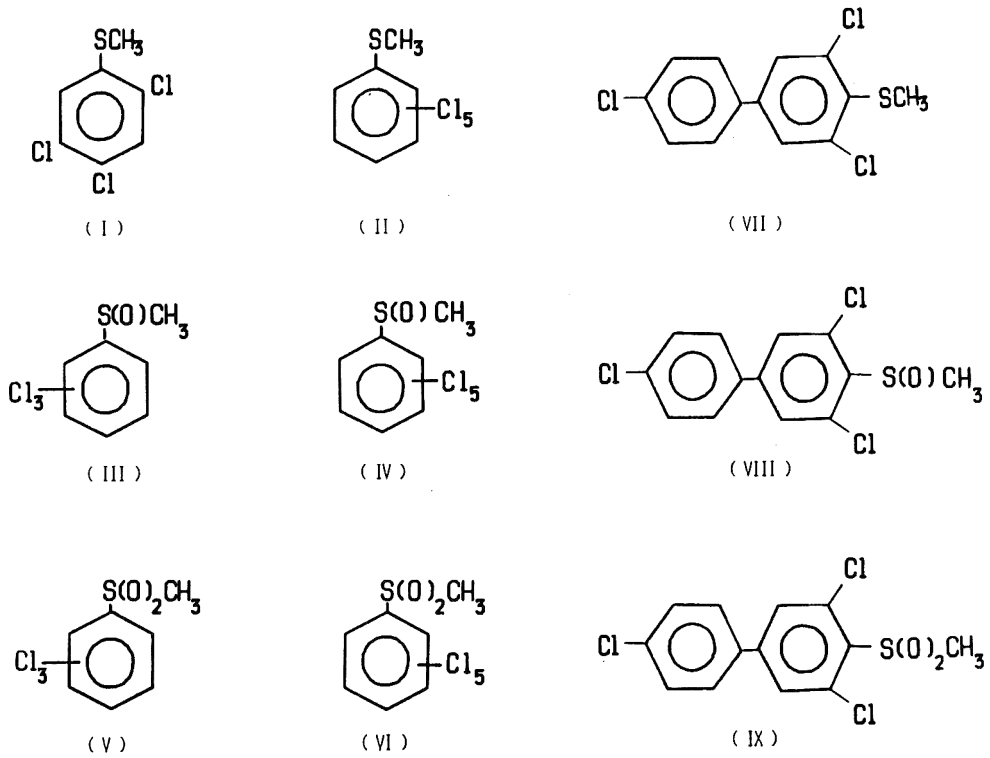

Figure 1. Chemical structures of synthetic test chemicals: I, 2,4,5-trichloromethylthiobenzene; II, pentachloromethylthiobenzene; III, 2,4,5-trichloromethylsulfinylbenzene; IV, pentachloromethylsulfinylbenzene; V, 2,4,5-trichloromethylsulfonylbenzene; VI, pentachloromethylsulfonylbenzene; VII, 3,4′,5-trichloro-4-methylthiobiphenyl; VIII, 3,4′,5-trichloro-4-methylsulfinylbiphenyl; and IX, 3,4′,5-trichloro-4-methylsulfonylbiphenyl.

(b) *Sodium sulfate.*—Anhydrous granular, free from interferences with ECD.

(c) *Florisil.*—PR grade, 60–100 mesh (Floridin Co., Berkeley Springs, WV), must meet sec. **29.002(i)** (5) specifications.

(d) *TLC solvent system.*—Methylene chloride–hexane (50 + 50).

(e) *m-Chloroperbenzoic acid.*—FMC Corp., Inorganic Chem. Div., New York, NY.

(f) *Synthetic test chemicals.*—Identification of the following synthesized chemicals was confirmed by mass spectrometry (MS) and, where noted, by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy. Prepare 2,4,5-trichloromethylthiobenzene (I) (Figure 1) by $NaBH_4$ reduction of 2,4,5-trichlorodisulfide (Aldrich Chemical Co., Milwaukee, WI) to trichlorothiophenol. Extract latter compound from acidic aqueous solution into methylene chloride and methylate with $(CH_3)_2SO_4$ in presence of OH^- and phase transfer catalyst, $C_6H_5CH_2N(Cl)(C_2H_5)_3$. ^{1}H-NMR ($CDCl_3$): δ 2.45 (3*H*), 7.18 (1*H*), and 7.45 (1*H*) ppm. ^{13}C-NMR ($CDCl_3$): δ 15.50, 126.71, 129.34, 130.50, 131.17, 131.73, and 132.56 ppm. Prepare pentachloromethylthiobenzene (II) by methylating pentachlorothiophenol (Pfaltz and Bauer Inc., Stamford, CT) with $(CH_3)_2SO_4$ as described above. Prepare 2,4,5-trichloromethylsulfinylbenzene (III) and pentachloromethylsulfinylbenzene (IV), the respective sulfoxides of I and II, by reacting small stoichiometric excess of *m*-chloroperbenzoic acid with I or II in benzene solution. Prepare 2,4,5-trichlorosulfonylbenzene (V) and pentachloromethylsulfonylbenzene (VI), the respective sulfones of I and II, by reacting large excess of *m*-chloroperbenzoic acid with I or II in benzene solution.

Prepare 3,4′,5-trichloro-4-methylthiobiphenyl (VII) by chlorinating 4-hydroxybiphenyl (Aldrich Chemical Co.) in EE, using excess sulfuryl chloride to yield 3,5-dichloro-4-hydroxybiphenyl. Reflux product in glacial acetic acid and excess iodine monochloride to yield 3,4′,5-trichloro-4-hydroxybiphenyl. Convert this product to 3,4′,5-trichloro-4-methylthiobiphenyl by method of Newman and Karnes (6). Methylate with $(CH_3)_2SO_4$ as described above. ^{1}H-NMR ($CDCl_3$): δ 2.44 (3*H*), 7.48 (4*H*), 7.58 (2*H*) ppm. Prepare 3,4′,5-trichloro-4-methylsulfinylbiphenyl (VIII) and 3,4′,5-trichloro-4-methylsulfonylbiphenyl (IX) by controlled oxidation with *m*-chloroperbenzoic acid as described above. Chemical structures of I–IX are shown in Figure 1. Chromatographic values for synthetic compounds are given in Table 1.

(g) *Test solutions.*—Use stock solutions of I–IX of 1–3 mg/mL ethyl acetate for TLC and 1 in 1000 dilutions of stock solutions for qualitative FPD(S)/GC. Prepare quantitative standards, 100, 200, and 300 pg/μL ethyl acetate, of I–IX for determining recoveries by ECD/GC from stock solutions. Prepare sulfoxide solutions separate from sulfone solutions because their retention times overlap.

Procedure

Contaminated fish was selected from samples at the Food and Drug Administration which had been previously analyzed for industrial chemicals and stored at <0°C. The procedure of Yurawecz and Puma (7) was used to determine contaminant levels.

Fortification for recovery determinations.—Add 1 mL hexane containing 1.9–2.5 μg of each synthetic test substance either to fat extracted from 25 g perch or to 25 g edible portion of perch meat mixed with 100 g Na_2SO_4 in blender jar.

Extraction and cleanup.—Extract fish as described in sec. **29.012(e)** (5). Clean up extracted oil as described in sec. **29.014** (5). Continue to clean up extract using Florisil column chromatography as described in sec. **29.015** (5) with the following modifications of eluting solvents:

(1) Elute chlorinated methylthiobenzenes with 200 mL 6% PE-EE.

(2) Elute chlorinated methylsulfonylbenzenes with 200 mL 50% PE-EE.

Table 1. GC retention times (RT) and TLC R_f values for chlorinated methylthio compounds and their oxidation products

Analyte	RT[a]	R_f[b]
2,4,5-Trichloromethylthiobenzene (I)[c]	0.60	0.83
2,4,5-Trichloromethylsulfinylbenzene (III)	0.76	0.04
2,4,5-Trichloromethylsulfonylbenzene (V)	0.76	0.18
Pentachloromethylthicbenzene (II)	0.81	0.86
Pentachloromethylsulfinylbenzene (IV)	1.00	0.05
Pentachloromethylsulfonylbenzene (VI)	1.00	0.24
3,4',5-Trichloro-4-methylthiobiphenyl (VII)	1.07	0.85
3,4',5-Trichloro-4-methylsulfinylbiphenyl (VIII)	1.35	0.01
3,4',5-Trichloro-4-methylsulfonylbiphenyl (IX)	1.35	0.10

[a]RT relative to pentachlcromethylsulfonylbenzene (RT 16.31 min). GC conditions given in text
[b]TLC conditions given in text.
[c]Roman numerals refer to structures in Figure 1.

(3) Elute chlorinated methylsulfinylbenzenes with 200 mL EE.

Test Florisil column by eluting mixture of synthetic test chemicals dissolved in <5 mL hexane. If standards elute from column in wrong eluting solvent, adjust amount of Florisil so that they elute as described above.

Concentrate eluates on steam bath to 10.0 mL for recovery studies. Use gentle stream of nitrogen to concentrate eluates with environmentally incurred contaminants to ≤2.0 mL.

H_2SO_4 extraction cleanup.—Clean up Florisil eluates 2 and 3 as follows: Carefully evaporate Florisil eluates to dryness under gentle stream of nitrogen. Transfer residue to 125 mL separatory funnel, using two 5.0 mL portions of hexane. Thoroughly mix hexane with 5.0 mL H_2SO_4 by vigorously shaking 1 min. Place separatory funnel on stand or in clamps. Slowly add 5.0 mL water to mixture and let heat gradually dissipate. (Caution: Rapid addition of water causes hexane to boil and to splash out of separatory funnel.) When mixture has cooled, add 40 mL water and thoroughly shake 1 min. Discard aqueous phase. Drain hexane layer into 15 mL graduated test tube and carefully evaporate hexane with gentle stream of nitrogen. Redissolve residue in 2.0 mL ethyl acetate.

Oxidation of chlorinated methylthiobenzenes or methylsulfinylbenzenes to sulfones.—Dissolve 20.0 mg *m*-chloroperbenzoic acid in 5.0 mL benzene. Mix 100 μL *m*-chloroperbenzoic acid solution with one-third of Florisil eluate 1 or 3 in 0.5 mL benzene in 15 mL graduated test tube. Let mixture react 1 h. Evaporate benzene with nitrogen stream and redissolve residue in 0.3 mL ethyl acetate.

TLC cleanup.—Cover area on acetone-prewashed TLC plate ca 25 mm above lower edge with clean 10 × 20 cm glass plate. Streak aliquots of Florisil eluates in band 11 cm long beginning at left edge of plate and on starting line 22 mm above and parallel to lower edge of plate. Use 10% Florisil eluate 2 and 50% oxidized Florisil eluates 1 or 3. Streak band 1 cm long of 10–20 μg V, VI, or IX on starting line at right side of plate. Let streaks dry 5 min. Develop streaks in sandwich chamber apparatus until solvent reaches 78 mm above starting line (or halfway up plate). Dry plate in hood 5 min. View plate under 254 nm ultraviolet light and mark upper and lower boundaries of test chemicals in space to left of band. Use spatula to scrape off area of fish extract zone having same R_f as sulfone test chemicals. Place scraped off adsorbent into glass wool-plugged disposable pipet and elute with 1–1.5 mL acetone. Collect eluate in 15 mL graduated test tube. Evaporate acetone with nitrogen stream and redissolve residue in volume of ethyl acetate equal to amount of extract originally streaked on TLC plate.

Determination

To determine recoveries by ECD/GC, inject 2 μL aliquot of 10.0 mL Florisil eluates. Plots of peak area vs amount (I–IX) go through 0 and are linear through 800 pg on ECD.

To determine sulfur-containing contaminants in fish with environmentally incurred residues, inject equivalents of 1 mg fish for ECD/GC and 100 mg fish for FPD(S)/GC. Use I to estimate amounts of chromatographable compounds in eluates. Detection limit (based on a signal-to-noise ratio of 2.0) of I is <4 pg by ECD and 2 ng by FPD(S). FPD(S) response appears to increase exponentially with increasing amounts of analytes.

Screening.—Screen GC responses for compounds containing both chlorine and sulfur as follows: Using FPD(S)/GC, determine RTs relative to VI of gas chromatographable components in Florisil eluates that have not been oxidized or cleaned up by TLC. Disregard responses with RTs shorter than parent chlorinated benzenes or PCBs. Using ECD/GC, identify eluates that have components with relative RTs within 3% of those obtained with FPD(S). These responses may represent compounds that contain both chlorine and sulfur in the same molecule. If selected eluate is from Florisil eluate 1 or 3, oxidize aliquot and clean up by TLC as described above. Rechromatograph this eluate by using FPD(S)/GC. If selected eluate is from Florisil eluate 2, clean up by TLC as described above. Using ECD/GC, rechromatograph TLC-cleaned up eluates. ECD responses remaining after TLC cleanup at similar relative RTs to those obtained on FPD(S) and equivalent to ≥0.02 ppm I are tentatively identified as chlorinated methylsulfonylbenzenes. Sulfones from Florisil eluates 1 or 3 represent chlorinated methylthiobenzenes or methylsulfinylbenzenes present before oxidation. If necessary, confirm all tentatively identified GC responses by GC/MS.

Results and Discussion

Recoveries of the test compounds are shown in Table 2. The recovery data for perch meat fortified after extraction (as perch oil) and before extraction indicated that the extraction and the modified Florisil column cleanup were adequate for determining these compounds at the 0.1 ppm level.

The perch sample used for the recovery studies was relatively free from ECD interferences. We used a sulfuric acid extraction cleanup described by Yoshida and Nakamura (8) and Richter et al. (9) for the more polar Florisil eluates (2 and 3) of samples of other fish to further reduce ECD interferences and thereby determine the compounds of interest at lower levels.

The TLC behavior of the test compounds indicated that all of the substances with a similar oxidation level (e.g., the methylsulfonylbenzenes) had similar R_f values (Table 1). The TLC cleanup, in conjunction with the *m*-chloroperbenzoic acid oxidation (for eluates 1 and 3), separated compounds with the sulfone moiety in samples showing matching GC RTs with the 2 GC detectors. Oxidation of eluates 1 and 3 converted any methylthio and methylsulfinyl compounds to the methylsulfonyls, which had R_f values of the methylsulfonyl compounds and GC relative RTs similar to or longer than their precursors. Longer GC RTs after TLC therefore corroborated the matched RT data for eluate 1. The RTs of methylsulfonyl and methylsulfinyl compounds in eluates 2 and 3 did not change after TLC. The sulfuric acid extraction, oxidation, and TLC steps in combination reduced the recoveries of compounds I–IX by an average of 50% of the recoveries obtained after Florisil column cleanup. The screening

Table 2. Recovery (%) of synthetic test chemicals from perch fortified at 0.1 ppm level[a]

Analyte	Perch oil[b]	Perch meat[b]
2,4,5-Trichloromethylthiobenzene (I)[c]	91,104 (98)	99,102 (101)
2,4,5-Trichloromethylsulfinylbenzene (III)	53,72 (63)	66,80 (73)
2,4,5-Trichloromethylsulfonylbenzene (V)	75,92 (84)	92,79 (86)
Pentachloromethylthiobenzene (II)	75,104 (90)	89,103 (96)
Pentachloromethylsulfinylbenzene (IV)	56,81 (69)	88,98 (93)
Pentachloromethylsulfonylbenzene (VI)	85,89 (87)	102,88 (95)
3,4',5-Trichloro-4-methylthiobiphenyl (VII)	72,81 (77)	
3,4',5-Trichloro-4-methylsulfinylbiphenyl (VIII)	79,75 (77)	
3,4',5-Trichloro-4-methylsulfonylbiphenyl (IX)	100,49 (75)	

[a]Extracted as described in sec. **29.012(e)** (5). Cleanup as described in secs **29.014–29.015** (5) as modified in text. Cleanup did not include H_2SO_4 extraction, oxidation, or TLC steps. Recoveries determined by ECD.
[b]Duplicate analyses, average in parentheses.
[c]Roman numerals refer to structures in Figure 1.

Table 3. Characteristics of fish samples analyzed for chlorinated methylthiobenzenes, methylthio-PCBs, and their oxidation products

Species	Residue[a]	Residue level, ppm[a]	Sample wt, g	No. of matching GC peaks[b]
Sucker	mono- and dichloro-benzenes	0.5–1.0	50.9	8
Striped bass	trichlorobenzene	0.5	3.9	1
Catfish	tetrachlorobenzene	0.4	38.7	8
Catfish	hexachlorobenzene	0.05	25.2	9
Goldf sh	PCB	50	22.5	11
Carp	PCB	335	20.5	4
Carp	PCB	52	25.0	1
Carp	—	—	34.5	4

[a]As determined by procedure of Yurawecz and Puma (7).
[b]Peaks found by FPD(S) within ±3% of relative RT of peaks obtained with ECD. GC conditions given in text. Oxidation and TLC followed by GC did not confirm any of the matching responses as representing methylthio compounds above a detection level of 0.02 ppm.

level, equivalent to 0.02 ppm I, was maintained by injecting a larger amount of fish extract into the gas chromatograph.

Detectable levels of chlorinated methylthiobenzenes and methylthio-PCBs, if present as metabolites, would be expected to be found in fish with high levels of the parent industrial chemical. Seven fish with the highest levels of environmentally incurred chlorinated benzenes and PCBs were selected from a repository of samples previously analyzed for industrial chemicals. The types of fish and the levels of contaminants found in them are shown in Table 3. On the basis of the chemical similarity between PCBs and chlorinated benzenes, we assumed that the methylthio-PCBs and their sulfoxides and sulfones would display cleanup characteristics similar to those of the respective chlorinated benzene substances. The limited recovery data for the methylthio-PCB test compounds in Table 2 sustantiated this assumption. Although many ECD/GC responses had RTs matching those of the FPD(S)/GC, no methylthio compounds or their oxidation products above a level equivalent to 0.02 ppm I were found after TLC cleanup. Sulfur-containing metabolites, such as the sulfates of naphthalene and phenols, have been found in fish (10–13). The absence of detectable levels of methylthio compounds in fish that contained residues of chlorinated benzenes or PCBs would indicate that fish may not form these metabolites as do some animals (1, 2) and other aquatic fauna (3, 4). Conversely, if methylthio metabolites are formed in fish, this work suggests that they are not stored in the edible portion as are the parent residues.

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Chemical Derivatization Analysis of Pesticide Residues. IX. Analysis of Phenol and 21 Chlorinated Phenols in Natural Waters by Formation of Pentafluorobenzyl Ether Derivatives[1]

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A sensitive, isomer-specific method is described for the simultaneous and quantitative analysis of 22 phenols (phenol, 18 chlorophenols, and 3 chloroalkylphenols) in natural waters. The sample was acidified to pH ≤2, extracted with dichloromethane, evaporated, and dissolved in acetone. The phenol extract was then reacted with pentafluorobenzyl bromide (PFBBr) to give the PFB ether derivatives. After silica gel column cleanup, the ethers were chromatographed on a 12 m OV-1 fused silica capillary column attached to an electron capture detector (ECD). The detection limit was 0.1 ppb for 1 L samples. Recoveries of phenols from pH 2 water samples fortified at 10, 1, and 0.1 ppb were ≥80% in most cases except for phenol which was only 30 to 35% recovered. Coefficients of variation were between 2 and 10% for all phenols. However, phenol recovery was quantitative when the sample volume was reduced to 100 mL. Because ECD sensitivities to the 22 phenol PFB ethers were similar, this method is most suitable for simultaneous screening of nonchlorinated and monochlorinated phenols as well as other higher chlorophenols at trace levels.

Because they are toxic compounds, analysis of chlorophenols in water samples has been the subject of many papers. However, published methods deal only with a few of the 19 chlorophenols, ranging from monochloro- to pentachlorophenols, or the methods lack the sensitivity required for analysis of environmental samples. Although free phenols can be analyzed by gas chromatography (GC) (1) and liquid chromatography (LC) (2), the generally less favorable chromatographic behavior and lack of sensitivity make this approach undesirable. Some workers prefer to prepare methyl ether derivatives of phenol extracts (3, 4) and analyze the anisoles by electron capture gas chromatography (EC-GC). This approach provides good sensitivity only for phenols with 2 or more chlorine substitutions. We reported the analysis of 15 chlorophenols in water by an in-situ aqueous acetylation technique (5); however, this method applies only to di-, tri-, tetra-, and pentachlorophenols. Analyses of nonchlorinated phenols by the formation of EC-sensitive derivatives with reagents such as heptafluorobutyrylimidazole (HFBI) (6), pentafluorobenzyl bromide (PFBBr) (7–10), 1-fluoro-2,4-dinitrobenzene (11), and bromine (12) have been reported. Previously, we demonstrated the formation and GC characteristics of pentafluorobenzyl ether derivatives of 32 substituted phenols (13). Because of the sensitivity and stability of these derivatives as well as the wide applicability (14–16) and reproducibility of this derivatization technique, PFB ethers are well suited for analysis of chlorophenols in water samples. We present here a comprehensive, quantitative, isomer-specific method for analysis of 22 phenols including phenol, 18 chlorophenols, and 3 chloroalkylphenols (Table 1) in natural waters at ppb levels by using a methylene chloride extraction and a PFB ether formation technique.

Experimental

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5880A equipped with ^{63}Ni electron capture detector, Model 7671A Autosampler, Level Four terminal, and split-splitless capillary column injection port. Operating temperatures (°C): injection port 250°; detector 300°; column initial 70°, hold for 0.5 min, programming rate 1, 25°/min (70–160°), rate 2, 2°/min (160–200°), final time 10 min. Splitless valve on for 30 s. Detector make-up gas, argon–methane (95 + 5), 25 mL/min. (Instrument available from Hewlett-Packard, Avondale, PA 19311.)

(b) *GC column.*—12 m × 0.2 mm id fused silica capillary column coated with crosslinked dimethyl silicone gum and surface deactivated by siloxane (Part No. 19091-60312, Hewlett-Packard). Carrier gas, helium. Linear velocity 25 cm/s.

(c) *Gas chromatograph—mass spectrometer (GC-MS).*—Finnigan Model 4000 GC-MS-DS operating in EI and CI modes. Column, 30 m × 0.25 mm id. DB-1 fused silica capillary column, 0.1 μm film thickness (J & W Scientific Inc., Rancho Cordova, CA 95670). Temperature program (°C): initial, 70°, hold for 2 min, programming rate 1, 10°/min (70–160°), rate 2, 2°/min (160–200°). Carrier gas, helium at 15 psi.

(d) *Vortex evaporator.*—Buchler Instruments Inc., Fort Lee, NJ.

Reagents

Use all pesticide grade solvents.

(a) *Phenols.*—Obtained from Aldrich Chemical Co. (PO Box 355, Milwaukee, WI 53201) or Supelco, Inc. (Phenol Kit 27, Bellefonte, PA 16823). 2,3,4,6-Tetrachlorophenol obtained from Eastman Organic Chemicals (Rochester, NY 14650). Prepare all stock solutions in toluene–isooctane (10 + 90) at 5 mg/mL. Keep in dark at 4°C.

(b) *PFBBr reagent.*—Dissolve 1 g PFBBr in 19 mL dry acetone (0.1% water). Keep in dark at 4°C. Prepare fresh reagent biweekly. Caution: Reagent is strong lachrymator.

(c) *K_2CO_3 solution.*—10%. Dissolve 10 g anhydrous K_2CO_3 in water and dilute to 100 mL.

(d) *Silica gel.*—GC Grade 950, 60–200 mesh (Fisher Scientific Co., Don Mills, Ontario, Canada M3A 1A9). Activate by heating overnight (14 h) at 130°C. Deactivate by adding 5 mL pure water to 95 g activated silica gel. Mix well by tumbling and keep in a tightly capped glass container overnight before use. Prepare fresh weekly.

Fortification of Water Sample

Add 100 μL phenol mixture in acetone at appropriate concentrations to 1 L water. Stir, and equilibrate 30 min before extraction.

Extraction

Stir water sample (1 L) collected in 1.15 L long-neck whiskey bottle or other suitable container, using Teflon-coated stirring bar so that vortex formed almost reaches bottom of bottle. Carefully add 1:1 (v/v) sulfuric acid until pH is ≤2 (pH paper).

[1]For Part VIII, see Lee, H.-B., Weng L.-D., & Chau, A. S. Y. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 789–794.

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Table 1. Retention times (min) and relative retention times (PCP PFB ether = 10.0) of 22 phenols on OV-101 crossedlink fused silica capillary column

Number	Parent phenol	RT	RRT
1	Phenol	4.90	2.40
2	2-Chloro-	6.33	3.10
3	3-Chloro-	6.64	3.25
4	4-Chloro-	6.82	3.34
5	2-Chloro-5-methyl-	7.44	3.64
6	2,6-Dichloro-	7.82	3.83
7	4-Chloro-3-methyl-	8.16	3.99
8	2,5-Dichloro-	8.41	4.11
9	2,4-Dichloro-	8.69	4.25
10	3,5-Dichloro-	8.83	4.32
11	2,3-Dichloro-	9.33	4.56
12	3,4-Dichloro-	9.76	4.77
13	2,4,6-Trichloro-	10.16	4.97
14	2,3,6-Trichloro-	10.96	5.36
15	2-Chloro-4-tert-butyl-	11.44	5.60
16	2,4,5-Trichloro-	11.87	5.81
17	2,3,4-Trichloro-	13.42	6.57
18	3,4,5-Trichloro-	13.57	6.64
19	2,3,5,6-Tetrachloro-	14.55	7.12
20	2,3,4,6-Tetrachloro-	14.81	7.25
21	2,3,4,5-Tetrachloro-	17.44	8.53
22	PCP	20.44	10.00

Add 50 mL dichloromethane, tightly cover bottle with piece of Teflon tape, and cap. After stirring 30 min, transfer contents to 1 L separatory funnel. Drain organic layer into 500 mL flask. Transfer aqueous layer back to original sample bottle. Rinse separatory funnel with 30 and 20 mL aliquots of dichloromethane and drain both fractions into sample bottle. Repeat above procedure twice. Discard water sample after last extraction. Filter combined organic extract through 5 cm anhydrous Na_2SO_4 in sintered glass filter column. Apply suction to dry column. Wash Na_2SO_4 column and original flask with 50 mL fresh dichloromethane in 2 portions and apply suction to collect filtrate.

Add 2 mL acetone and 250 μL 10% K_2CO_3 solution to organic extract in 500 mL flask and evaporate on rotary evaporator to ca 5 mL under reduced pressure (water bath temperature 35°C). Quantitatively transfer concentrated extract with Pasteur pipet to graduated centrifuge tube. Immediately add 2 mL acetone to original 500 mL flask, rinse container, and set aside. Evaporate extract in centrifuge tube to 0.8 mL in a 50°C bath under gentle stream of dry nitrogen. Transfer acetone rinse in 500 mL flask to centrifuge tube. Repeat rinsing with three 2 mL aliquots of acetone. Combine up to 8.0 mL acetone rinsings in the same centrifuge tube.

Derivatization of Phenols

Add 100 μL 10% K_2CO_3 solution and 100 μL 5% PFBBr reagent to phenol extract. Stopper tube tightly and mix contents on Vortex Genie. Heat tube 1 h in tube heater at 60°C, making sure stopper is tightly in place. After reaction, evaporate solution to 0.5 mL at 40°C with Buchler Vortex-Evaporator. Add 3 mL hexane and repeat evaporation to final volume of 0.5 mL.

Column Cleanup

Prepare mini cleanup column by plugging a long Pasteur pipet (23 × 0.5 cm id) with piece of silanized glass wool. Fill column with 5 cm 5% deactivated silica gel. Tap column gently and add 5 mm anhydrous Na_2SO_4 at top. Pre-wet column with 5 mL hexane and discard hexane eluate. With Pasteur pipet, quantitatively transfer concentrated derivatized extract in hexane and two 1 mL hexane rinsings from test tube to silica gel column. Discard hexane rinsings. Note: Total hexane rinsing should not exceed 3.0 mL. Elute column with toluene–hexane (25:75, v/v) and collect 8.0 mL. Dilute to 10.0 mL with same solvent. This fraction contains all 22 phenol PFB ether derivatives. Shake tube to mix contents and inject 2 μL into gas chromatograph in splitless mode.

Table 2. Mean % recovery and SD (in parenthesis) of phenols from 1 L fortified distilled water samples at pH 6

	No salt added	10 g anhyd. Na_2SO_4 added		
Phenol concn, ppb:	1	10	1	0.1
No. of replicates:	3	6	6	6
Parent phenol				
Phenol	31	33 (2.3)	36 (1.9)	—[a]
2-Chloro-	94	89 (3.3)	91 (3.7)	89 (7.3)
3-Chloro-	87	81 (1.6)	87 (3.3)	—[a]
4-Chloro-	72	80 (2.3)	85 (3.8)	100 (4.6)
2-Chloro-5-methyl-	97	94 (3.1)	96 (3.6)	94 (6.0)
2,6-Dichloro-	81	96 (2.4)	97 (3.9)	93 (5.9)
4-Chloro-3-methyl-	105	95 (2.2)	97 (3.6)	—[a]
2,5-Dichloro-	91	95 (2.0)	93 (5.9)	78 (8.1)
2,4-Dichloro-	91	94 (2.4)	95 (4.3)	81 (7.6)
3,5-Dichloro-	98	98 (3.5)	95 (5.2)	78 (11.1)
2,3-Dichloro-	94	96 (1.6)	99 (4.3)	84 (11.0)
3,4-Dichloro-	101	95 (2.0)	99 (4.5)	90 (6.5)
2,4,6-Trichloro-	90	97 (1.4)	105 (1.2)	101 (4.9)
2,3,6-Trichloro-	95	97 (2.1)	102 (3.6)	96 (5.8)
2-Chloro-4-tert-butyl-	86	93 (3.5)	92 (1.6)	89 (4.0)
2,4,5-Trichloro-	88	96 (1.7)	85 (4.7)	72 (5.9)
2,3,4-Trichloro-	88	92 (2.1)	88 (4.1)	81 (10.1)
3,4,5-Trichloro-	93	99 (2.2)	83 (6.0)	70 (7.1)
2,3,5,6-Tetrachloro-	100	95 (5.7)	103 (2.6)	78 (6.9)
2,3,4,6-Tetrachloro-	94	96 (3.9)	101 (1.5)	81 (8.3)
2,3,4,5-Tetrachloro-	97	90 (3.5)	88 (3.6)	74 (5.7)
PCP	99	92 (2.6)	97 (1.5)	75 (7.3)

[a]Interference.

Results and Discussion

In the development of this analytical method, 2,3,5-trichlorophenol was not included in the investigation, although the formation of its PFB ether has been shown (13) and the methylene chloride extraction procedure is likely to be applicable to its analysis. The compound was omitted because the PFB ethers of 2,3,5- and 2,4,5-trichlorophenols could not be resolved by all the GC columns investigated (13).

GC Separation of Chlorophenol PFB Ethers

A typical chromatogram (Figure 1) shows resolution of the 22 phenol PFB ethers on a 12 m OV-1 fused silica capillary column. Table 1 gives the retention times (RT) and relative retention times (RRT) of these derivatives on this column. In a previous paper (13), we reported the GC characteristics of these phenol PFB ethers on 6 different columns, and concluded that the nonpolar OV-101 fused silica capillary column with Carbowax deactivated surface was the most efficient. We recently tried a new column coated with crosslinked dimethyl silicone gum (OV-1) and which had a siloxane deactivated surface. We found that the order of elution for the phenol derivatives was identical on both columns; however, the OV-1 column was more efficient than the OV-101 column. For example, the number of theoretical plates calculated for PCP-PFB ether was 191 000 for the OV-1 column vs 120 000 for the OV-101 column. The new column was also more durable and there was a mere 10% drop in efficiency (without rinsing or cutting the column) over a period of 4 months during which over 650 injections of the PFB ether extracts were made. If a capillary column GC is not available, a 3% OV-1 packed column could be used. On this column, however, 3 pairs of derivatives could not be resolved, namely: 2,4- and 3,5-dichloro-; 2,3,4- and 3,4,5-trichloro-; and 2,3,5,6- and 2,3,4,6-tetrachlorophenols. For further discussions on the GC properties of phenol PFB ethers, see ref. 13.

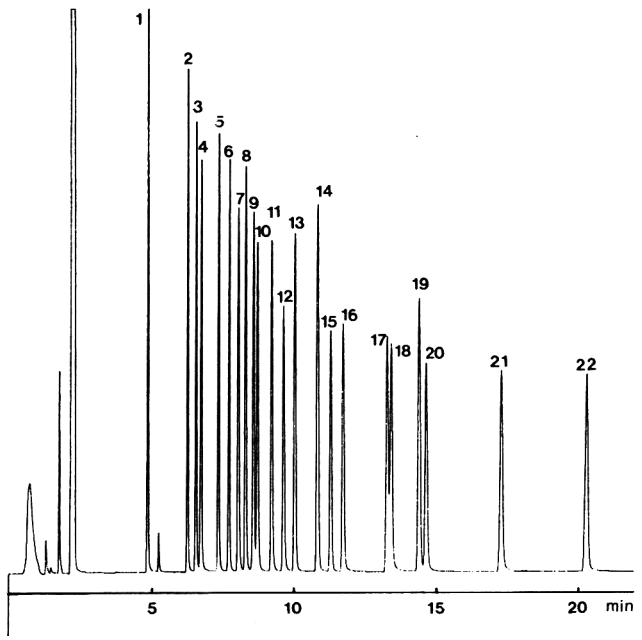

Figure 1. EC-GC chromatogram of PFB ether derivatives of 22 phenols resolved on a 12 m × 0.2 mm id OV-1 fused silica capillary column. See Table 1 for peak identification and *Experimental* for conditions.

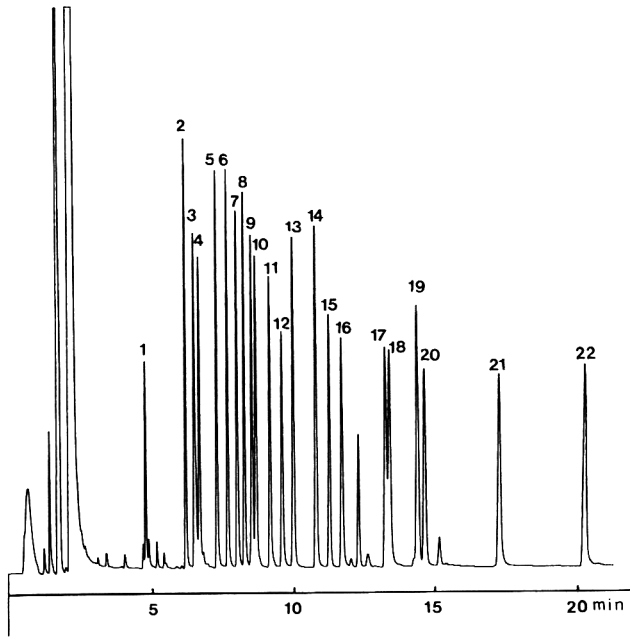

Figure 2. EC-GC chromatogram of 1 L natural water sample fortified at 1 ppb for each phenol after PFBBr derivatization.

GC-MS Analysis

Although under similar reaction conditions, PFB ethers of some phenols were synthesized and characterized by other workers (7–9), PFB ethers of the 22 chlorophenols were never prepared and identified on a macro scale in this study. However, a mixture of these derivatives was examined by capillary column GC-MS analysis. The mass spectrometer was operating in both electron impact (EI) and negative chemical ionization (NCI) modes. Close inspection of the individual mass spectrum indicated that *(1)* in the EI mode, the $C_6F_5CH_2^+$ fragment (m/z = 181) was always the base peak for all derivatives; *(2)* also in the EI mode, the intensities of the molecular ion (M^+) as well as the corresponding phenoxy ion of each component were much weaker, and under the conditions used in this experiment, the presence of the above species could only be observed for the dichloro- and lower chlorophenols; and *(3)* in the NCI mode, the predominant species in each mass spectrum was the corresponding phenoxy ion with the expected chlorine isotope distribution pattern. These data confirmed the formation of phenol PFB ethers.

Derivatization of Phenols

To produce the most reliable quantitative results, we studied various conditions to optimize the derivatization of phenols. Reaction was carried out at different volumes of acetone, such as 2, 3, 4, 6, 8, and 10 mL, with constant amounts of PFBBr and K_2CO_3. Results indicated that although yields of the ethers were basically identical for volumes between 4 and 10 mL, lower yields (55 to 80%) were experienced for all phenols if the amount of acetone was only 2 or 3 mL. Also, lower yields of the derivatives could be experienced if the test tube was not tightly capped during the reaction, even if the reaction volume was between 4 and 10 mL. Presumably, the PFB ethers formed or the unreacted phenols were partially distilled during the reaction.

We also studied the effect of residual CH_2Cl_2 (from extraction) in the reaction mixture on the yields of PFB ethers. We observed that although no effect on the yields was found for up to 10% residual CH_2Cl_2 in acetone, presence of higher (20 to 40%) amounts of CH_2Cl_2 in the reaction mixture caused lower yields for phenol (20–65%), 4-chlorophenol (65–75%), and 4-chloro-3-methylphenol (65–85%). Yields for other phenols were not affected by the presence of up to 40% CH_2Cl_2 in acetone. Therefore, for reliable results on these 3 phenols, the amount of residual CH_2Cl_2 must be kept to less than 10%.

Derivatization of phenols was carried out with different K_2CO_3 solutions. Results suggested that although the concentration of K_2CO_3 (1, 5, 10, or 30%) had no effect on the yield of the ethers, the volume of the aqueous base or water added was more critical to product yields. Highest yields of PFB ethers were obtained by using 75 or 100 μL of the base.

The yields for all phenols decreased to between 50 and 75% when the volume of K_2CO_3 solution used was 200 μL. On the other hand, although the yields of the higher chlorophenols were not affected, slightly lower yields (75–95%) were observed for phenol and monochlorophenols when the volume of base used was 25 or 50 μL.

The PFBBr reagent used throughout the experiments was a 5% solution in acetone. Higher PFBBr concentrations did not increase the yields of all phenol PFB ethers, while lower concentrations (e.g., 1% and 0.5%) tended to give lower yields of PCP, especially at high phenol concentrations. The PFBBr reagent should be prepared fresh biweekly; an old reagent could produce lower yields of the derivatives.

The reaction conditions cited in *Experimental* were tested and provided quantitative and reproducible results for all 22 phenols of concentrations between 0.1 and 20 μg each (or 2.2 and 440 μg of total phenols). However, when the amount of each individual phenol reached 50 μg in a mixture of 22 phenols, much lower yields were observed for phenol (10%), 4-chlorophenol (60%), and 4-chloro-3-methylphenol (40%), while the yields of the other 19 phenols were still quantitative. Therefore, for water samples with very high chlorophenol contents, a smaller volume should be extracted or a portion of the sample extract should be used for derivatization. The precision of the derivatization of these phenols was also estimated. For 5 replicate derivatization reactions done on the same day, the coefficient of variation was less than 6% for

Table 3. Effect of sample volume on mean % recovery of phenols from fortified water samples at pH ≤2 (n = 3)

	Distilled water				Lake Ontario
Sample volume, L:	1	0.5	0.25	0.1	0.1
Na_2SO_4 added, g:	10	5	2.5	1	1
Phenol concn, ppb:	1	2	4	10	10
Parent phenol					
Phenol	33	55	77	97	94
2-Chloro-	94	88	89	98	94
3-Chloro-	83	91	93	95	93
4-Chloro-	82	93	92	100	100
2-Chloro-5-methyl-	97	90	91	98	94
2,6-Dichloro-	92	92	91	99	97
4-Chloro-3-methyl-	101	89	101	96	97
2,5-Dichloro-	82	91	92	100	96
2,4-Dichloro-	86	91	93	102	97
3,5-Dichloro-	88	93	94	101	97
2,3-Dichloro-	91	87	93	98	93
3,4-Dichloro-	96	92	97	101	97
2,4,6-Trichloro-	92	90	93	99	97
2,3,6-Trichloro-	100	91	90	99	96
2-Chloro-4-tert-butyl-	86	88	90	96	92
2,4,5-Trichloro-	93	93	96	99	97
2,3,4-Trichloro-	88	99	100	103	102
3,4,5-Trichloro-	89	95	97	104	97
2,3,5,6-Tetrachloro-	101	94	93	94	96
2,3,4,6-Tetrachloro-	99	92	97	97	97
2,3,4,5-Tetrachloro-	97	102	95	105	105
PCP	100	95	93	95	97

all phenols. Hence, the PFBBr derivatization of phenol was sufficiently reproducible for quantitative purposes.

Evaporative Losses

Although it was not reported by other workers, we have experienced low recoveries of phenols due to losses during the evaporation of phenol solutions in CH_2Cl_2. In a typical experiment, a phenol solution consisting of 1 μg of each phenol in 200 mL CH_2Cl_2 was evaporated by using either a rotary evaporator (35°C bath) or a Kuderna-Danish (K-D) evaporator fitted with a macro 3-ball Snyder column (60–80°C bath). Mean (n = 6) evaporative recoveries for various phenols were only 30–70% (lower recovery for more volatile phenols) using the rotary evaporator. The K-D evaporator produced slightly better results and the mean (n = 6) recoveries for all phenols were between 53 and 73%. These results were clearly unsatisfactory for quantitative purposes.

In the presence of small amounts of acetone (2 mL) and 10% K_2CO_3 (250 μL), evaporative recoveries of all phenols in 200 mL CH_2Cl_2 could be improved to >90% by using a rotary evaporator. However, if the evaporation was done in a single stage from 200 mL down to about 2 mL, results might not be always quantitative. Low recoveries of phenol, the monochlorophenols, and the chloroalkylphenols were occasionally encountered. Consistent results were obtained, however, by a 2-stage evaporation. First, the phenol solution in the presence of keepers (acetone and K_2CO_3) was evaporated to about 5 mL in a rotary evaporator. The concentrated extract was then transferred to a centrifuge tube and evaporated to 0.5–1.0 mL under a gentle stream of dry nitrogen in a 50°C bath.

Extraction

Methylene chloride was chosen in this study because it provided satisfactory results for the recovery of chlorophenols in water. Because the same solvent has been shown, individually, to give quantitative recoveries of other classes of toxic organics such as PCBs (H. B. Lee and A. S. Y. Chau (1982) unpublished data), 18 organochlorine pesticides including mirex and photomirex, neutral (17) and acid (18) herbicides, several chlorobenzenes (19), and many organophosphorus pesticides (14), use of methylene chloride facilitates the generation of a multi-class/multi-residue method which will be initiated soon.

Table 2, column 1, shows the overall recoveries of the 22 chlorophenols at 1 ppb from 1 L, pH 6 distilled water. Recoveries of all phenols were ≥80% except for phenol (31%) and 4-chlorophenol (72%). Presumably, the high solubility of phenol in water caused its low recovery. Several approaches were attempted to improve the recoveries of these 2 phenols, such as addition of 10 g solvent-washed, oven-dried anhydrous sodium sulfate, which improved phenol recovery to 35% and 4-chlorophenol to 85%. The presence of sodium sulfate also provided slight increases in the recoveries of other phenols too. A further increase of sodium sulfate to 20 g did not produce any observable increase in phenol recoveries. Effects of the bromide, chloride, fluoroborate, and hydrogen sulfate of tetrabutylammonium salt as well as tetrapropylammonium bromide were also briefly examined. Of all the salts evaluated, bromides were the most effective in improving the recoveries of phenol and monochlorophenols. For instance, addition of 4 g of either bromide to a 1 L sample improved phenol recovery to about 70%, and recoveries of monochlorophenols and other chlorophenols were over 90%. However, quantitative recovery of phenol was still unattainable. Addition of these salts also caused various degrees of interference (depending on their purities) to the GC analysis of chlorophenols at lower levels.

A simple solution to improving phenol recovery without possible additional interference was decreasing sample size taken for extraction. Table 3 shows that phenol recovery increased markedly from 33% (1 L sample) to 55% (0.5 L sample), 77% (0.25 L sample), and finally to 97% (0.1 L sample). Percentage recoveries of 4-chlorophenol and some other chlorophenols also improved from lower eighties to upper nineties when the sample volume was reduced from 1 to 0.1 L.

Presumably because of their acidic nature, phenols are usually extracted from water samples acidified to < pH 2 (2, 6, 19). However, under such conditions other acidic organics in natural water samples are also extracted and the co-extractives could cause interferences. We examined extraction of chlorophenols in distilled water at pH 2, 4, and 6 and found that recoveries of phenols at these pH levels were essentially identical. Apart from the lower phenol recovery mentioned earlier, recoveries of the other 21 phenols at pH 6 in the presence of 10 g anhydrous sodium sulfate were >80% in all cases and >90% in most cases for phenol concentrations between 1 and 10 ppb (columns 2 and 3, Table 2). At 0.1 ppb, recoveries of phenols were generally lower (70–100%) (column 4, Table 2).

Extraction of natural water samples fortified with the 22 phenols at about pH 6 did not produce results similar to those derived from distilled water sample. In a Lake Erie sample, all six 2,6-disubstituted phenols, i.e., 2,6-dichloro-, 2,4,6-trichloro-, 2,3,6-trichloro-, 2,3,5,6-tetrachloro-, and pentachlorophenols were only 24–62% recovered at 1 ppb, although recoveries of other phenols were similar to the distilled water results (column 5, Table 4). Recoveries of all chlorophenols at 1 ppb were slightly lower for a Lake Ontario sample than for Lake Erie samples, at pH 6, and again the 2,6-disubstituted phenols had lower recoveries than the others (column 3, Table 4). However, when the extractions were performed at pH 2, all 2,6-disubstituted phenols were quantitatively recovered as were the other chlorinated phenols from fortified Lake Ontario and Lake Erie water samples (Table 4). A typical chromatogram of a natural water sample, fortified at

Table 4. Mean % recovery and SD (in parenthesis) of phenols from 1 L fortified natural water samples in presence of 10 g anhydrous Na_2SO_4

	Lake Ontario				Lake Erie		
pH:	6	2	2	2	6	2	2
Phenol concn, ppb:	10	10	1	0.1[a]	1	10	1
No. of replicates:	3	8	6	3	6	6	6
Parent phenol							
Phenol	28	28 (2.9)	34 (4.6)	—[b]	31 (3.5)	33 (2.4)	36 (4.8)
2-Chloro-	66	85 (4.6)	81 (9.3)	96	80 (6.3)	92 (2.5)	89 (5.8)
3-Chloro-	77	76 (3.9)	85 (7.8)	—[b]	76 (6.2)	79 (2.5)	81 (3.1)
4-Chloro-	69	74 (3.6)	76 (9.1)	—[b]	72 (6.2)	79 (2.2)	78 (5.4)
2-Chloro-5-methyl-	74	91 (4.5)	88 (9.0)	103	92 (7.0)	100 (2.9)	103 (4.7)
2,6-Dichloro-	60	94 (4.6)	93 (7.6)	100	61 (6.2)	93 (4.0)	93 (4.9)
4-Chloro-3-methyl-	86	95 (4.6)	93 (6.8)	—[b]	94 (6.9)	99 (2.9)	106 (5.8)
2,5-Dichloro-	75	93 (4.2)	89 (6.9)	105	84 (7.1)	93 (2.7)	86 (4.0)
2,4-Dichloro-	79	94 (4.8)	87 (7.4)	103	92 (7.9)	98 (2.2)	99 (4.1)
3,5-Dichloro-	88	95 (4.0)	92 (6.8)	103	91 (7.4)	97 (1.7)	94 (3.7)
2,3-Dichloro-	75	93 (3.8)	85 (6.9)	125	91 (8.4)	98 (1.7)	93 (3.7)
3,4-Dichloro-	86	95 (4.6)	85 (8.1)	130	93 (8.5)	98 (2.2)	99 (3.4)
2,4,6-Trichloro-	55	96 (3.4)	93 (5.3)	105	62 (5.8)	97 (4.4)	101 (4.2)
2,3,6-Trichloro-	37	97 (3.9)	94 (6.3)	102	41 (4.8)	98 (4.4)	90 (3.0)
2-Chloro-4-tert-butyl-	76	94 (3.8)	92 (7.7)	108	96 (6.2)	103 (2.1)	104 (3.1)
2,4,5-Trichloro-	76	98 (4.7)	89 (5.7)	101	89 (9.1)	98 (2.8)	89 (3.4)
2,3,4-Trichloro-	73	101 (6.0)	88 (7.5)	105	96 (9.9)	98 (2.8)	100 (4.0)
3,4,5-Trichloro-	88	101 (4.2)	92 (5.3)	104	98 (9.6)	105 (2.9)	101 (2.8)
2,3,5,6-Tetrachloro-	20	98 (3.4)	94 (6.7)	76	24 (3.8)	98 (2.0)	88 (4.1)
2,3,4,6-Tetrachloro-	32	100 (4.0)	91 (5.4)	96	40 (5.1)	99 (2.5)	89 (4.9)
2,3,4,5-Tetrachloro-	74	102 (6.7)	93 (4.7)	89	85 (8.8)	92 (2.5)	88 (4.3)
PCP	27	92 (4.2)	92 (5.9)	74	32 (4.2)	88 (2.3)	94 (5.6)

[a]Results not corrected for blanks.
[b]Interference.

1 ppb for each phenol, after extraction, derivatization, and cleanup is shown in Figure 2.

Interference

Some organochlorine insecticides co-extracted by CH_2Cl_2 and stable enough to survive the PFBBr derivatization would then interfere with the GC analysis of the phenol PFB ethers. However, such neutral organics could be effectively removed from the chlorophenols by base partitioning on the CH_2Cl_2 extract. Preliminary results indicated that chlorophenols were quantitatively removed by 0.01M KOH in 3 successive partitionings (40 + 30 + 30 mL) with the CH_2Cl_2 extract. After acidification to ≤2 pH, all chlorinated phenols together with phenol itself could then be recovered by 3 CH_2Cl_2 extractions without loss, because the volume of the aqueous sample was 100 mL.

In an earlier study (18), we reported the determination of 10 acid herbicides by forming their PFB esters. These acid herbicides, which would be co-extracted with the chlorophenols, would not interfere with chlorophenol analysis because the less polar chlorophenol PFB ethers are eluted first by toluene–hexane (25 + 75, v/v). Then more polar herbicide PFB esters are eluted with toluene–hexane (75 + 25) in the post-derivatization silica gel column cleanup.

PFBBr also forms PFB ethers with other types of phenols such as nitro-, thio-, alkyl-, cyano-, bromo-, and possibly phenols with combinations of the above functional groups as well as guaiacols and catechols. Although the nitro- and cyanophenols are separated from the chlorophenols by the post derivatization silica gel column cleanup, the PFB ethers of alkyl-, bromo-, and thiophenols, which are co-eluted with those of chlorophenols, could interfere in GC analysis (13). This will be investigated in the future.

At a concentration of 1 g/L, the presence of $CuSO_4$, which is often used to preserve water samples for phenol analysis, has no detrimental effect on the recovery of the 22 phenols.

Conclusion

Results of this study indicate that the extraction and PFBBr derivatization procedure presented are well suited to analysis of chlorophenols in natural waters. This method applies to the determination of phenol, monochlorophenols, and chloroalkylphenols as well as to the higher chlorophenols in water at low ppb levels, because the ECD is equally sensitive to PFB ethers of all 22 phenols. The PFBBr derivatization technique is the method of choice if simultaneous determination of non- or monochlorinated phenols together with polychlorinated phenols at trace levels is required in the same sample. If this method is applied to higher phenol concentrations, e.g., ≥1 ppb, a smaller sample, such as 100 mL, should be extracted so that quantitative recovery of all 22 phenols can be maintained. Because of reagent blanks and co-extractives, the detection limit of this method is 0.1 ppb for 1 L samples.

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Gas Chromatographic Determination of Chlorpyrifos Residues in Greenhouse Vegetables After Treatment of Potting Media with Dursban for Imported Fire Ant

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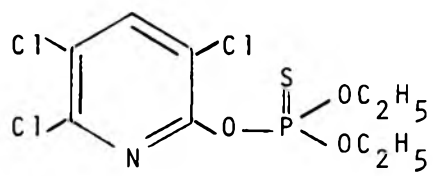
Residues of chlorpyrifos in vegetables were extracted in acetone, and then partitioned between 5% aqueous sodium sulfate solution and hexane to minimize the quantity of oily materials in the extract. Light green vegetable extracts were cleaned up on a silica cartridge, and dark green vegetable extracts were cleaned up with decolorizing carbon and silica gel column chromatography. Chlorpyrifos was determined by gas chromatography (GC) with electron capture detection. The vegetables analyzed were started as seedlings in potting soil treated with Dursban and were transferred to the field for maturation. Residue levels were determined in mature vegetables at the harvestable stage of growth, and ranged from < 0.01 ppm in vegetables, such as cabbage, squash, and tomato, to 0.06 ppm in parsley.

The imported fire ant (*Solenopsis richteri*) was probably brought into the Mobile, AL, area between 1933 and 1945 (1); soon thereafter it began to spread to other areas of Alabama and other southeastern states. By 1953, it was known to infest 102 counties in 9 states. Thirty years later, it had successfully spread across the southeast United States and extended its range to include over 230 million acres of land. This phenomenal rate of spread was because of 2 independent factors: First, natural spread through mating flights was inevitable and played an important role in ant dispersal; second, artificial or accidental spread was caused by humans (2).

The 1957 congressional appropriation of funds for a joint federal–state program recognized the need to slow or prevent artificial spread of the imported fire ant (IFA) through commercial shipments of infested items such as grass and nursery stock. A Federal Quarantine was invoked, and Environmental Protection Agency (EPA) regulations were issued governing the movement of certain articles (e.g., soil, sand, gravel, woody or herbaceous plants with soil attached, plants in pots or containers, grass sod, and unmanufactured forest products such as stump wood or timber with soil attached).

Initially, dieldrin and heptachlor at 3 lb actual insecticide (AI) per acre were used to treat grass sod and other plant material before certified movement was approved. The use of heptachlor and dieldrin was discontinued in preference for chlordane, which was applied to grass sod and field-grown ornamentals at the rate of 10 lb AI/acre. This treatment regimen was revised to 5 lb actual chlordane specified for treatment of nursery stock and nursery grass sod. In 1978, EPA cancelled the registration of chlordane for use on turf, although chlordane could still be used for treatment of nursery potting soil in which potted plants were grown, and on field-grown ornamental plants until December 1979, when the final EPA cancellation order concerning use of chlorinated hydrocarbon insecticides of all quarantine treatments became effective. With cancellation of chlordane use, Dursban® (a 5% formulation of chlorpyrifos) was introduced as a chlordane substitute for treatment of potting soil to prevent artificial spread of imported fire ants.



O,O-Diethyl *O*-(3,5,6-trichloro-2-pyridyl)-phosphorothioate

Chlorpyrifos is effective for control of a number of pests including flies, fleas, cockroaches, and ants (3–5). The acute oral LD_{50} (rat) is 97–276 mg/kg. Chlorpyrifos has very low solubility in water (0.0002 g/100 g), but is readily soluble in most organic solvents. Dursban FA-5 was, until recently, the leading candidate to replace chlordane in the nursery industry for the control of IFA. However, treatment of plants intended for food or feed was prohibited; therefore, vegetable transplants, such as peppers and tomatoes, could not be grown in Dursban-treated potting media.

The University of Florida Institute of Food and Agricultural Sciences, in cooperation with the National Monitoring and Residue Analysis Laboratory (NMRAL), conducted a project to determine if this restriction was necessary. This Interregional Research Project No. 4 used greenhouse vegetables raised as seedlings in potting media containing Dursban that was incorporated into the soil at the rate of 11.3 g AI per cubic yard (private correspondence, Herbert H. Bryan, Professor of Horticulture, Homestead, FL, 1981). The project was set up and funded by EPA to expedite registration of minor use pesticides.

The seedlings, which consisted of 20 different varieties of vegetables, were transferred for maturing to a field not treated with Dursban. Vegetables were analyzed for chlorpyrifos residues at the harvestable stage. Samples were bagged separately and quick-frozen for transport to the National Monitoring and Residue Analysis Laboratory for residue analysis.

It has since been determined that seed germination is delayed in Dursban-treated soil. Also, certain types of greenhouse nursery plants were abnormal after being grown in Dursban-treated media (unpublished data, Homer Collins, USDA, Gulfport, MS, 1981). Lawsuits were filed alleging phytotoxicity in succulents. All supplies of FA-5 have since been withdrawn from the market by the registrants.

Because of the removal of Dursban, the U.S. Department of Agriculture (USDA) currently does not have a certification treatment involving a soil-incorporated insecticide. The present study was undertaken to determine the residue levels of chlorpyrifos in vegetables grown from seedlings and harvested at maturity; this would be an indication of systemic activity.

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Experimental

Apparatus

(a) *Snyder column.*

(b) *Glass beads.*—4 mm.

(c) *Explosion-proof blender.*—General Electric, or equivalent, fitted with glass jar and lid.

(d) *Hot plate.*—GCA/Precision Scientific, Vari-Heat, or equivalent.

(e) *Gas chromatograph (GC).*—Tracor M.T. 220, with ^{63}Ni electron capture detector. Operating conditions: injector 250°C, column 175°C, detector 350°C; 40 psig inlet pressure; nitrogen carrier gas 100 mL/min; sensitivity $10^2 \times 32$; chart speed 0.25 in./min; glass column, 4 mm id × 6 ft, packed with 1.5% OV-17/1.95% QF-1 on 100–120 mesh Supelcoport and/or 3% XE-60 on 80–100 mesh Chromosorb WHP.

Reagents

(a) *Solvents.*—Acetone and hexane, Nanograde® (Mallinckrodt).

(b) *Silica gel.*—60–200 mesh (Baker Analyzed, Lot No. 26394), 20% deactivated.

(c) *Silica Sep-Pak® cartridge.*—Waters Associates, Inc., Lot No. P12601.

(d) *Carbon, decolorizing.*—Nuchar C-190N (Lot No. 736656, Fisher Scientific Co.)

Preparation of Sample

A subsample of mature plants at the harvestable stage, weighing 20 g, was blended with 100 mL acetone for 2–3 min at medium speed in an explosion-proof blender. The extract was filtered through glass wool, previously washed with acetone, into a clean 500 mL Erlenmeyer flask; the blender jar was rinsed once with acetone into the glass wool and the rinse was collected in the flask. Several glass beads were placed in the flask along with 1 mL 0.01% Nujol® in hexane, added to retard evaporation of chlorpyrifos.

The solution was concentrated to ca 50 mL, using a Snyder column, and transferred to a 500 mL separatory funnel containing 200 mL 5% aqueous (w/v) Na_2SO_4 solution; the flask was rinsed with acetone into the funnel. Chlorpyrifos was partitioned into hexane, using three 100 mL portions, shaking for 1 min each time, and allowing layers to separate. After each partition step, the top layer was drawn off and passed through anhydrous Na_2SO_4/glass wool, previously rinsed with hexane, into a 500 mL Erlenmeyer flask. The hexane extracts were concentrated in the flask to ca 10 mL, using a Snyder column and glass beads.

Cleanup

Dark green vegetables.—Vegetables containing the most chlorophyll were cleaned up with carbon and silica gel: Carbon (0.1 g) was added after concentration of the hexane extract. The flask was swirled ca 30 s and contents were filtered through Whatman No. 2V fluted paper, previously rinsed with acetone, into 250 mL Erlenmeyer flask. Glass beads were added, a Snyder column was attached, and the extract was concentrated to 10 mL. Ten mL hexane was added and the extract was again concentrated to 10 mL; this latter step was repeated twice more. The purpose of the hexane addition and concentration is to narrow the extract band on the silica gel column.

Five g deactivated silica gel, prepared by adding 20 mL water to 100 g 60–200 mesh silica gel, was placed in a 1 cm glass column containing a glass wool plug and 1 in. anhydrous Na_2SO_4. Glass wool and anhydrous Na_2SO_4 were also placed on top of the silica gel. Twenty mL hexane was added and allowed to drain to the top of the column; the hexane eluate was discarded. The 10 mL extract from the previous paragraph was added and allowed to drain to the top of the column; the flask containing the extract was rinsed with hexane and this rinse was also added to the top of the column. Ninety mL hexane was then added, and 90 mL eluate was collected. This solution was used for GC injection (A). Volume was adjusted either by concentration or dilution with hexane to achieve on-scale peaks.

Light green vegetables.—The Sep-Pak cartridge was prerinsed with hexane. The 10 mL sample extract was placed on the cartridge with a glass syringe and eluted with 10 mL 2% acetone in hexane. About 86% chlorpyrifos was recovered in the first elution. This solution was used for GC injection (B).

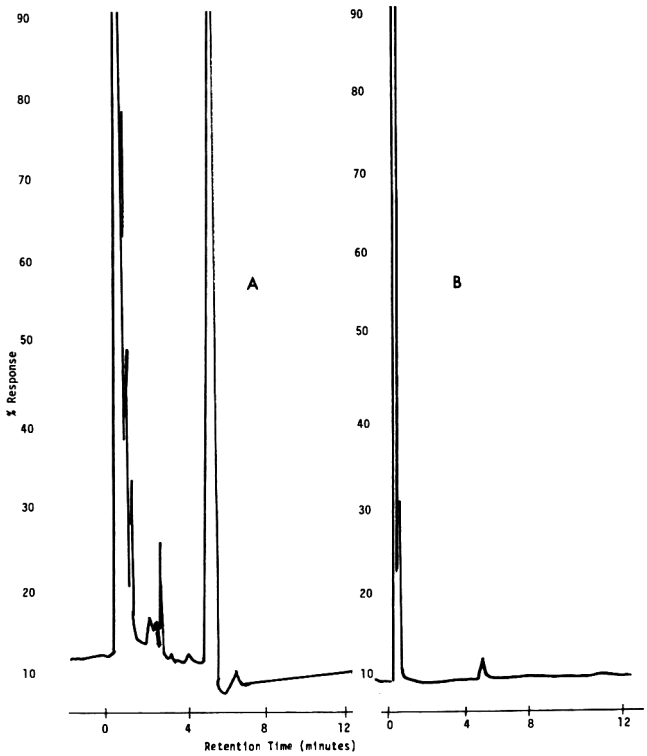

Figure 1. Sample chromatograms for cauliflower: A, cauliflower extract before cleanup, 1 μL, 20 g; B, after silica cartridge cleanup, 1 μL, 20 g

Table 1. Chlorpyrifos tolerances[a]

Vegetable	Tolerance, ppm
Brussel sprouts	2
Cabbage	2
Cauliflower	2
Cucumber	0.1
Onions (dry bulb)	0.5
Peppers	1
Tomatoes	0.5
Turnips (green)	1
Turnips (root)	3

[a]See 40 CFR 180.342–180.344, May 1, 1983.

Calculation

$$\text{ppm Chlorpyrifos} = (A/X) \times (B/Y) \times (1/Z)$$

where X = instrument sensitivity calculated from a standard in terms of peak height units/ng; A = measured peak height

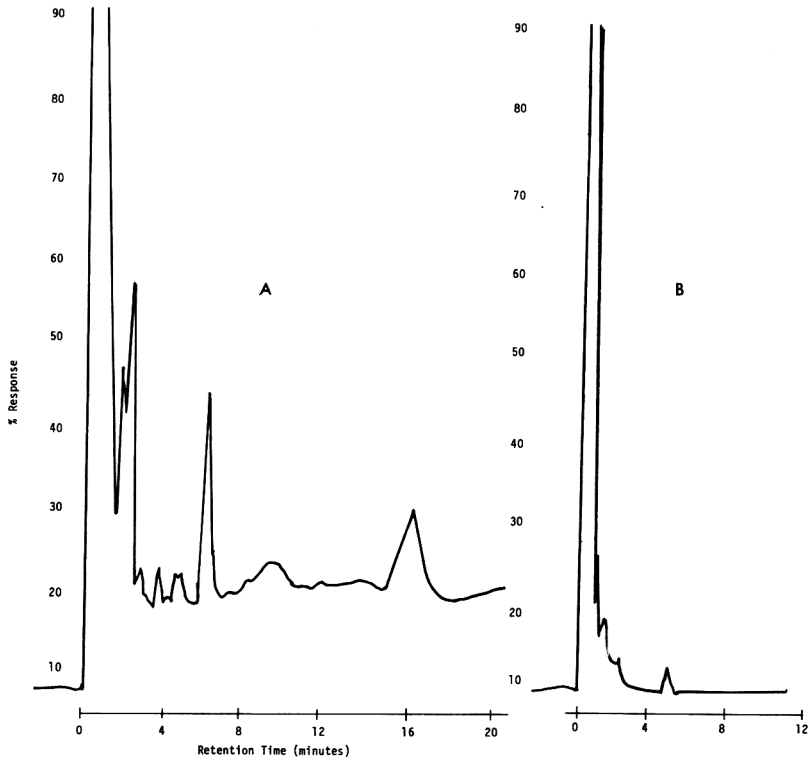

Figure 2. Sample chromatograms for collard greens: A, collard extract before cleanup, 1 μL, 20 g; B, after cleanup on carbon and silica gel column, 1 μL, 20 g.

Table 2. Chlorpyrifos found in vegetables

Vegetable	Replicate	Portion taken for analyses	Results, ppm
Brussel sprouts	1	whole plant	<0.010
	2		<0.010
	3		<0.010
Cabbage	1	whole plant	<0.010
	2		<0.010
	3		<0.010
Cauliflower	1	whole plant	<0.010
	2		0.011
	3		0.016
Celery	1	whole plant	<0.010
	2		<0.010
	3		<0.010
Collard greens	1	leaves	0.016
	2		0.013
	3		0.015
Cucumber	1	whole plant	0.011
	2		<0.010
	3		<0.010
Mustard greens	1	leaves; stems	0.013
	2		0.015
	3		0.013
Onions			
Granex top	1	green tops	0.010
Granex bulb	1	white bulb	<0.010
Liodon Dunchin top	1	green tops	0.025
Liodon Dunchin bulb	1	white bulbs	0.024
Parsley	1	whole plant	0.058
Pepper			
Bell	1	whole plant	<0.010
Hot	2		<0.010
	1		<0.010
	2		<0.010
Squash, yellow	1	whole plant	<0.010
	2		<0.010
	3		<0.010
Squash, zucchini	1	whole plant	0.067
	2		0.010
	3		0.010
Tomato	1	whole plant	<0.010
	2		<0.010
	3		<0.010

units of sample; B = volume of solution A or B; Y = weight of sample taken in g; Z = injection in μL.

Results and Discussion

A modified Dow Chemical Co. method of analysis was used that involves partitioning the acetone extract into hexane from a 5% aqueous Na_2SO_4 solution (6). This facilitates removal of plant matter that interferes with the chromatography. The combination of activated carbon and deactivated silica gel effectively removed chlorophyll from dark green vegetables. The silica cartridge sufficiently cleaned up light green vegetables.

The reliability of the analytical method was tested on locally purchased vegetables. Fortification levels were 0.1, 0.5, and 1.0 ppm for chlorpyrifos and 1.0 ppm for the oxygen analog. There was no attempt to determine if the metabolite 3,5,6-trichloro-2-pyridinol was present (it has been found in peppermint hay and oil) (7, 8). None of the oxygen analog was found in any vegetables. Recoveries from the fortified vegetables were 86 ± 16% for chlorpyrifos and 87 ± 10% for the oxygen analog. Sensitivity was 0.01 ppm for chlorpyrifos and 1.0 ppm for the oxygen analog.

Figure 1-A is a chromatogram of cauliflower extract before cleanup, while Figure 1-B is the same extract after cleanup on a silica cartridge. The difference in peak heights resulted from removal of interferences during the cleanup process. Retention time for chlorpyrifos was about 5 min.

Figure 2-A is a chromatogram of collard extract before cleanup, showing interference from plant components; Figure 2-B is the same extract after cleanup with silica gel and decolorizing carbon. Again, the difference in peak heights resulted from removal of interferences during the column cleanup process.

Table 1 shows established chlorpyrifos tolerances appearing in 40 CFR *(Code of Federal Regulations)*, ¶180.342–180.344, May 1, 1983. Analysis of mature vegetables, started as seedlings in Dursban-treated soil and collected at the harvestable stage of growth, gave residues ranging from < 0.01 ppm in most samples to 0.067 ppm in parsley. Results (Table 2) indicate that the tolerances were not exceeded (this study was undertaken in 1981). Further studies of this kind are needed to determine whether chlorpyrifos is phytotoxic in certain instances and, if so, to what degree, as well as its systemic activity.

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MYCOTOXINS

Determination of Xanthomegnin in Grains and Animal Feeds by Liquid Chromatography with Electrochemical Detection

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A sensitive, highly selective liquid chromatographic (LC) method is described which uses electrochemical (EC) reduction of the analyte in the determinative step. The method is capable of determining xanthomegnin in mixed animal feeds and grains at levels ranging from 15 to 1200 ng/g. The method can detect as little as 0.5 ng xanthomegnin injected on the LC column. Xanthomegnin is extracted with chloroform and 0.1M phosphoric acid. An aliquot of the crude extract is purified by silica gel column chromatography using a Sep-Pak silica gel cartridge. A novel feature of the method is that xanthomegnin is "backed off" the column by reversing the flow of the eluant through the column. LC is then used to separate xanthomegnin from other interfering substances. Xanthomegnin is detectd by EC reduction at −0.16 V. Recoveries of xanthomegnin added to samples at levels ranging from 15 to 1200 ng/g averaged 79% with a coefficient of variation of 7.9%. Results also demonstrate that this LC system can separate the related metabolites viomellein and rubrosulphin from each other and from xanthomegnin and that the same EC detection system can be used to detect these metabolites.

Xanthomegnin is a metabolite of a number of molds which have been shown to grow on grains that are used as foods and feeds (1). Among the more predominant of these molds are *Aspergillus ochraceus* (2) and *Penicillium viridicatum* (3). Xanthomegnin has also been shown to be toxic to swine (4) and laboratory mice (5). A more thorough discussion of the occurrence and toxicity of xanthomegnin is presented in ref. 6. Thus, methods for the determination of xanthomegnin in grains and animal feeds are needed to protect the public health.

We recently reported a liquid chromatographic (LC) method which relies on light absorption at 405 nm for the determination of xanthomegnin (6). The difficulties associated with absorption detection of xanthomegnin are a low molar absorptivity at 405 nm and interferences at other possible wavelengths. The absorption peaks for xanthomegnin in the LC mobile phase are located at 227, 280, and 390 nm. Although the molar absorptivities are sufficiently high at 227 and 280 nm to yield reasonable analytical sensitivity (approximately 26 000 and 19 000, respectively), many other co-extracted compounds absorb at these wavelengths and interfere with the detection of xanthomegnin. The molar absorptivity at 390 nm is only about 7900. Although it limits the sensitivity of the method, this wavelength is sufficiently selective for xanthomegnin to be unambiguously detected. The 405 nm wavelength was chosen in the previous work because it offered a slight increase in selectivity with almost no loss in sensitivity and because the absorption peak at 390 nm is very broad. The lowest amount of xanthomegnin detectable by the method is approximately 12 ng, which is equivalent to a concentration of 120 ng xanthomegnin/g sample.

In contrast, the method described here is slightly faster, less subject to sample interferences in the determinative step, and more sensitive by a factor of 10. The method can detect as little as 0.5 ng xanthomegnin injected on the LC column. The EC reaction on which this determinative step depends is illustrated in Figure 1 for quinones in general and for xanthomegnin in particular.

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Method

Apparatus

(a) *Sep-Pak rack.*—Waters Associates, Inc., Milford, MA 01757.

(b) *Silica gel cartridge.*—Sep-Pak (Waters Associates, Inc.).

(c) *Liquid chromatographic equipment.*—Beckman Model 100A pump with high pressure loop injection valve fitted with 20 μL sample injection loop; Beckman 5 μm octyl (C-8) column, 4.6 mm × 25 cm (Beckman Instruments, Inc., Berkeley, CA 94710).

(d) *EC detector.*—Bioanalytical Systems Model LC-4A amperometric detector fitted with Bioanalytical Systems Model TL-5A flow-through cell equipped with glassy carbon electrode (Bioanalytical Systems, Inc., West Lafayette, IN 47905).

(e) *Recorder.*—Kipp & Zonen Model BD-41 strip chart recorder with 10 mV input (Beckman Instruments, Inc.).

(f) *Metal Swinney filter holder.*—13 mm, No. 1xx30 012 00 (Millipore Corp., Bedford, MA 01730). Holder fitted with glass microfiber filter paper, 13 mm, Gelman type A/E, or equivalent (Gelman Sciences, Inc., Ann Arbor, MI 48106).

(g) *Wrist-action shaker.*—Burrell Model 75 (Burrell Corp., Pittsburgh, PA 15219).

(h) *Vacuum regulator assembly.*—Matheson Model Y3491 vacuum regulator and Model Y63-4105 vacuum gauge (Matheson Div., Searle Medical Products, Secaucus, NJ 07094).

(i) *TLC developing tank.*—Metal thin layer chromatographic (TLC) chamber with solvent trough and tight fitting lid.

(j) *Precoated silica gel plates.*—0.25 cm layer silica gel GHR (Brinkmann Instruments Co., Westbury, NY 11590).

Reagents

(a) *Solvents.*—Distilled-in-glass chloroform (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442); ACS reagant grade acetonitrile, benzene, anhydrous ethyl ether, glacial acetic acid, hexane, methanol, phosphoric acid, and ammonium hydroxide; LC grade water.

(b) *Mobile phase.*—Dissolve 1.4 g KH_2PO_4 in 450 mL LC grade water. Add 550 mL acetonitrile. Adjust pH to 3 with phosphoric acid.

(c) *Xanthomegnin.*—Obtained from Michael Stack, Food and Drug Administration, Washington, DC 20204. *(1) Stock solution.*—Dissolve crystalline xanthomegnin in chloroform–acetic acid (99 + 1) to obtain concentration of 30 μg/mL. When stored in amber glass in freezer, this solution is stable ca 2 months. *(2) LC working standard solutions.*—Transfer appropriate aliquots of stock solution into amber glass vials

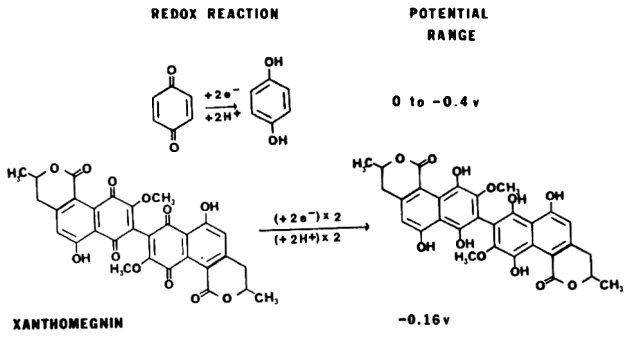

Figure 1. Electrochemical reduction of quinones and xanthomegnin.

to give following amounts of xanthomegnin per vial: 0.15, 1.5, 3, 6, and 12 μg. Evaporate working standards to dryness under stream of nitrogen at ≤40°C in dim light. These films are stable indefinitely when kept in freezer. Immediately before use, dissolve contents of each vial with 2.0 mL mobile phase. These solutions are stable ca 1 working day.

Sample Preparation and Extraction

Note: Because of the labile nature of xanthomegnin, it is desirable to carry the analysis through from start to finish without stopping. If extracts or cleaned-up solutions must be stored for even a short time, it is necessary to protect them from undue exposure to light. Keep extracts in the dark or store in amber glass containers.

Grind representative sample to pass 2 mm (U.S. No. 10) sieve. Weigh 25 g sample into 250 mL glass-stopper Erlenmeyer flask. Add 10 mL 0.1M phosphoric acid and 100 mL chloroform. Stopper and shake flask for 30 min, using wrist-action shaker set at moderate rate. Filter sample through fluted paper (S&S 588 or equivalent) into 100 mL Erlenmeyer flask.

Silica Gel Cartridge Chromatography

Transfer 20 mL aliquot of filtrate for each sample to be analyzed to 50 mL beaker and evaporate to 2–5 mL at ≤40°C under stream of nitrogen. Insert unused silica gel Sep-Pak cartridge into each position to be used (up to 8 samples may be run concurrently). Assemble cartridge rack according to manufacturer's instructions. Quantitatively transfer concentrated sample aliquots to column reservoirs with chloroform so that total volume for each sample is ≤8 mL. Turn on and regulate vacuum so that column flow is 1–2 drops/s. Collect these eluates in the first or "A" test tubes. Turn off vacuum and switch column reservoirs to the second or "B" test tubes. Add 8 mL hexane–ethyl ether (3 + 1) to each column reservoir, and turn on vacuum to obtain same flow rate as above until all reservoirs are empty. Turn off vacuum and invert each cartridge in rack. Switch column reservoirs to the third or "C" test tubes. Add 3 mL chloroform–acetic acid (98 + 2) to each column reservoir, and turn on vacuum to obtain same flow rate as above until all reservoirs are again empty. Turn off vacuum. Label two 7 mL amber glass vials for each sample and identify them as No. 1 and No. 2, respectively. Using chloroform, quantitatively transfer contents of "C" test tubes to 7 mL amber glass vials marked No. 1. Discard contents of first 2 test tubes for each sample. Evaporate these final eluates just to dryness (no odor of acetic acid) at ≤40°C under stream of nitrogen. Dissolve extracts in 1.0 mL chloroform. Transfer 500 μL of the solutions in vials marked No. 1 to vials marked No. 2 for LC determination. Retain extracts in No. 1 vials for confirmation. Evaporate contents of No. 2 vials just to dryness at ≤40°C under stream of nitrogen. These dry extracts may be stored, if necessary, for several days under refrigeration without loss of xanthomegnin. Just before LC determination, dissolve contents of these vials in 500 μL LC mobile phase. Clarify solutions, if necessary, by filtering through Swinney filter holder fitted with glass microfiber paper.

Table 1. Recovery of xanthomegnin from spiked commodities

	Rec., %		
Spike level, ng/g	Corn	Oats	Mixed feed
15	77	70	80
	77	79	85
	69	79	85
120	80	77	82
	80	87	81
	78	77	79
1200	64	80	87
	64	86	84
	80	87	82
Mean	74	80	83
CV, %	9.1	7.0	3.2

Overall mean = 79
Overall CV, % = 7.9

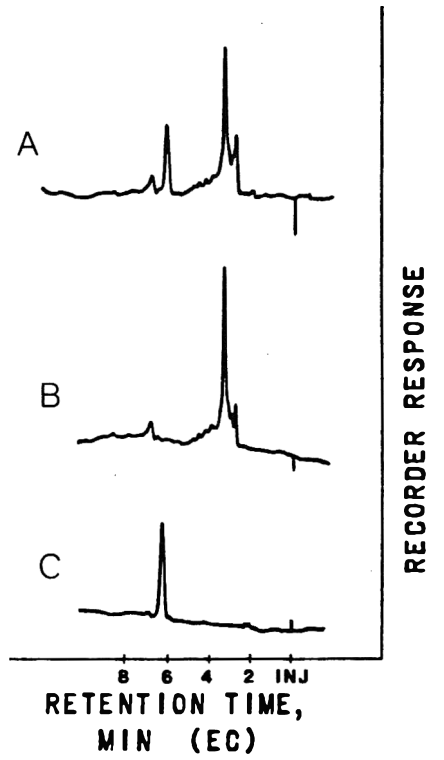

Figure 2. Chromatograms of sample and standard injections of xanthomegnin. (A) Spiked sample, 15 ng/g, corn; (B) blank sample, corn; (C) standard xanthomegnin, 1.5 ng injected. LC conditions as given in method.

Liquid Chromatography

Set LC flow rate to 1.0 mL/min. Turn on EC detector and set potential to −0.16 V in reduction mode vs Ag/AgCl reference electrode. Set range at 20 nA. Adjust this setting as required to keep sample and standard peaks on scale. Let system operate ca 30 min before use to permit LC column to equilibrate and detector to stabilize.

Inject full loop (20 μL) of each standard and construct standard curve from plot of peak height vs concentration to verify performance of system. This should be done daily. Retention time of xanthomegnin is 7–8 min. For purposes of

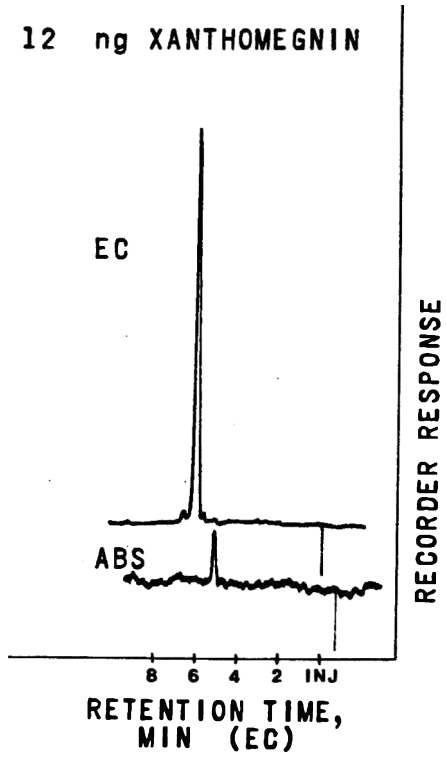

Figure 3. Chromatograms comparing relative sensitivities of electrochemical (EC) and UV-VIS absorption (ABS) detectors for detection of xanthomegnin. Instrument settings: (EC) −0.16 V vs Ag/AgCl, 20 nA FS; (ABS) 405 nm, 0.02 AUFS. Other LC conditions as given in method.

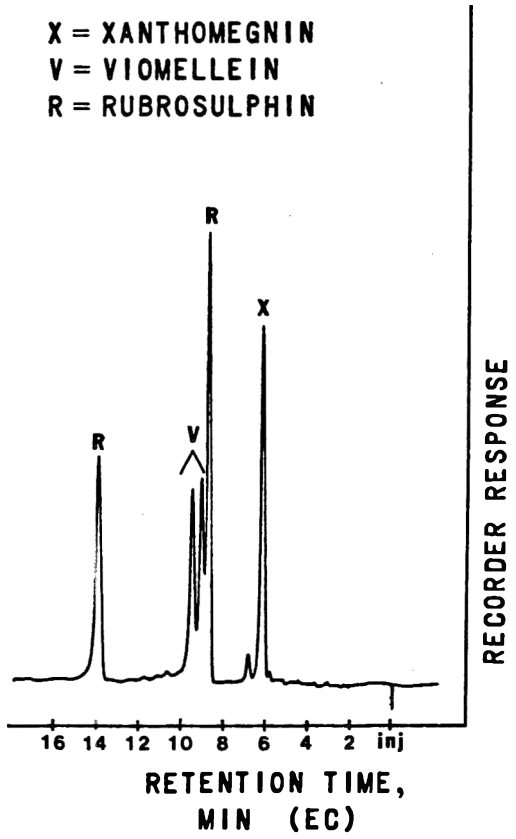

Figure 4. Chromatogram illustrating separation of xanthomegnin, rubrosulphin, and viomellein. Viomellein and rubrosulphin standards contain impurities. First peak in chromatogram of each of these standards is probably due to the pure compound.

screening, adjust detector sensitivity to give ca 10% full scale deflection for injection of 1.5 ng xanthomegnin.

Inject full loop (20 μL) of each sample solution. Determine concentration of xanthomegnin in samples by the following equation:

$$\text{Xanthomegnin, ng/g sample} = (A \times B/C) \times 1000$$

where A = concentration, μg/mL, of xanthomegnin in sample extract; B = volume, mL, of sample extract; C = weight of sample represented by extract (5 g).

TLC Confirmation of Identity

This TLC confirmation of identity is essentially the same as that described by Stack et al. for the LC determination of xanthomegnin in corn (7).

Evaporate contents of No. 1 vials just to dryness at ≤40°C under stream of nitrogen. Using calculated concentration of xanthomegnin found in sample as guide, dissolve contents of vials in chloroform so that concentration of xanthomegnin is ca 5 ng/μL. (Example: Vial contains extract from 2.5 g sample. Calculated result = 150 ng xanthomegnin/g sample. Vial therefore contains 375 ng xanthomegnin. Volume of chloroform required = 375 ng xanthomegnin ÷ 5 ng xanthomegnin/μL = 75 μL.) Spot 25 μL of this solution 4 cm from bottom of TLC plate. Spot 5 μL xanthomegnin stock solution 1 cm away on each side of sample spot. Develop TLC plate with benzene–acetic acid–methanol (90 + 5 + 5) in an unlined and unequilibrated tank until solvent front reaches height of 11–12 cm above spotting line. Let plate air-dry in fume hood ca 5 min. Expose plate to ammonia fumes 5 min. Red-to-violet spot in sample at same R_f as standard spot is positive confirmation of presence of xanthomegnin in sample.

Results and Discussion

To test the performance of the method, samples of corn, oats, and mixed animal feed were spiked with xanthomegnin at levels of 15, 120, and 1200 ppb. Recoveries of xanthomegnin added to these samples ranged from 64 to 87% with an average of 79% and a coefficient of variation of 7.9% (Table 1). Figure 2 shows chromatograms resulting from injections of 1.5 ng xanthomegnin standard, a sample spiked at the 15 ng/g level, and a blank sample.

The determinative step of this method is based on an EC reduction in an aqueous electrolyte solution at a glassy carbon electrode. A potential is applied to the working glassy carbon electrode, and the change in current caused by the EC reaction is measured. Figure 1 shows the EC reaction that occurs. It appears, from some preliminary polarographic work done in our laboratory, that this reaction is not reversible for xanthomegnin and that a static polarographic system is not suitable for xanthomegnin assay. The irreversible binding of xanthomegnin to wax-impregnated graphite and glassy carbon electrodes in a static cell made it impossible to obtain quantitative, reproducible results. No such problems were encountered in using a flow-through cell with a glassy carbon electrode. The same flow cell has been used in our laboratory for intervals of 6 months without cleaning and polishing the electrode.

To illustrate the relative sensitivities of EC and absorption detection for xanthomegnin, a standard solution equal to 12 ng xanthomegnin was injected into the liquid chromatograph under the conditions specified in the method. The detector responses for the absorption detector set at 405 nm and 0.02 AUFS and for the EC detector set at −0.16 V vs Ag/AgCl

and 20 nA FS were monitored simultaneously. Figure 3 shows the results of this experiment. The EC detector gives a usable signal-to-noise ratio even at sensitivity settings 2 and 4 times as large as that used in this experiment.

Three related metabolites, viomellein, viopurpurin, and rubrosulphin, are produced by the same molds that produce xanthomegnin (3, 8). Two of these compounds, rubrosulphin and viomellein, were available in sufficient quantities to explore the capabilities of this detection system for their separation and quantitation. Standard solutions of xanthomegnin, viomellein, and rubrosulphin were prepared in the LC mobile phase and injected into the liquid chromatograph under the conditions specified in this method. The concentrations used for viomellein and rubrosulphin were twice as large as the concentration used for xanthomegnin because each of these 2 compounds has only 1 reducible quinone group. Figure 4 shows the chromatogram obtained from this experiment. Although it appears that the rubrosulphin and viomellein contain impurities, it is also apparent that the separation and determination of these compounds are feasible with this system.

Acknowledgments

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Direct Enzyme-Linked Immunosorbent Assay for Determining Aflatoxin M_1 at Picogram Levels in Dairy Products

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Protocols for detecting picogram quantities of aflatoxin M_1 in dairy products were established. Milk samples were subjected to a reverse phase Sep-Pak C18 cartridge treatment before analysis by an enzyme-linked immunosorbent assay (ELISA) according to previously published procedures. M_1 in yogurt, brick cheddar, and ripened Brie cheese was extracted by a modified Pons method, subjected to a normal phase silica cartridge treatment, and analyzed by ELISA. The detection limits for M_1 in milk, yogurt, cheddar, and Brie were 10, 10, 50, and 25 ppt (ng/kg), respectively. Recovery for M_1 added to these products was in the range 70–110%. Good agreement was found for M_1 levels in several naturally contaminated milk samples analyzed by both ELISA and liquid chromatography.

Aflatoxin M_1 is one of the major metabolites of aflatoxin B_1. Lactating animals are able to excrete aflatoxin M_1 in milk after being fed aflatoxin B_1-contaminated feed (1, 2). Aflatoxin M_1 has been shown to cause hepatic carcinoma in rainbow trout (3) and other animal species, but the carcinogenicity is considerably weaker than that of B_1 (4). Aflatoxin M_1 has been reported to occur naturally in commercial dairy products in the United States and Europe (5–9) and is relatively stable during pasteurization, production, and storage of powdered milk and various cheeses (5, 6). Therefore, the M_1 problem is a potential health hazard to humans. Consequently, many countries have regulated the amount of M_1 in dairy products to levels as low as parts per trillion. For example, the U.S. Food and Drug Administration has an administrative guideline of 0.5 ppb M_1, whereas, in Switzerland, the permitted limit for M_1 in milk is 10 ppt.

A rapid and sensitive detection method is needed to control M_1 levels in dairy products. Currently, thin layer chromatography (TLC) (10, 11) or liquid chromatography (LC) (12, 13) is used for routine analyses. These methods exhibit high sensitivity but require extensive extraction and sophisticated cleanup procedures. Investigation in our laboratory has led to a sensitive and rapid enzyme-linked immunosorbent assay (ELISA) for M_1 in milk in which the milk sample can be used directly in the assay (14, 15). We recently found that with an additional cleanup on C18 reverse phase Sep-Pak cartridges, ELISA permitted detection of 25 ppt levels of M_1 in cow's milk and human urine (16). The present study describes the optimization of ELISA to detect parts per trillion levels of M_1 in various dairy products including liquid milk, powdered milk, yogurt, and cheese.

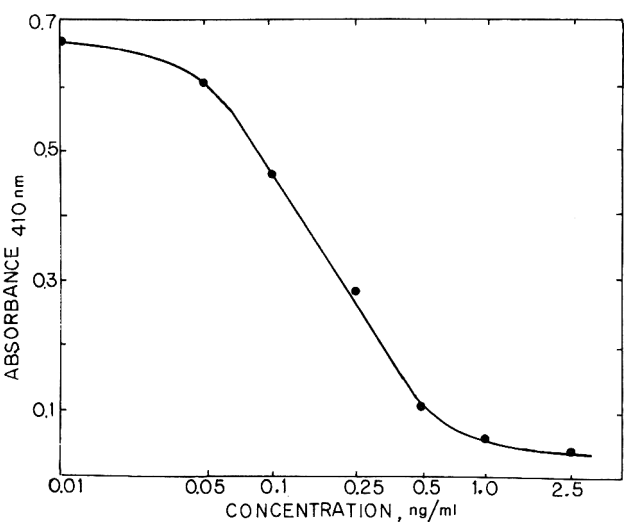

Figure 1. Aflatoxin M_1 ELISA standard curve. The x-axis indicates log of aflatoxin concentration (ng/mL); 25 μL sample was used in each analysis.

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Table 1. Recovery of M_1 by ELISA from artificially contaminated liquid and reconstituted powder milk

Added, ppt (ng/L)	Liquid raw milk		Reconstituted powder milk	
	ppt (ng/L)	Rec., %	ppt (ng/L)	Rec., %
5	NR[a] (19)[b]	—	5.8 ± 0.4 (12)	116 ± 8
10	10.8 ± 2.2 (12)	108.0 ± 22	10.71 ± 2.5 (14)	107.1 ± 24.4
14	15.6 ± 3.2 (14)	111.4 ± 22.8	ND	—
20	20.1 ± 3.5 (18)	100.5 ± 17.5	ND	—
25	ND	—	23.8 ± 7.3 (15)	95.2 ± 24.2
30	28.4 ± 1.2 (12)	94.7 ± 38.6	ND	—
50	49.3 ± 24 (18)	99.66 ± 48	43.46 ± 6.3 (13)	86.9 ± 12.6
100	77.0 ± 25 (9)	77.0 ± 25	85.7 ± 21.3 (13)	85.71 ± 21.3

[a]NR = not recovered; ND = not determined.
[b]Value in parenthesis indicates number of assays.

Table 2. Recovery of M_1 by ELISA from artificially contaminated yogurt

Added, ppt (ng/kg)	Recovered	
	ppt (ng/kg)	Rec., %
5	NR[a] (8)[b]	—
10	9.94 ± 1.4 (4)	99.4 ± 14.0
12.5	12.65 ± 1.81 (8)	101.2 ± 14.5
25	16.59 ± 5.9 (8)	66.4 ± 23.3
50	50.15 ± 19.0 (8)	100.3 ± 38.1
75	54.38 ± 15.7 (8)	72.5 ± 20.9

[a]NR = not recovered.
[b]Number of assays.

Table 3. Recovery of M_1 by ELISA from artificially contaminated cheddar cheese

Acded		Recovered	
ppt(ng/kg)	ng/2.5 g	ng/2.5 g	Rec., %
20	0.050	NR[a] (14)[b]	—
50	0.125	0.149 ± 0.027 (12)	119.3 ± 22.0
100	0.250	0.238 ± 0.040 (10)	114.2 ± 16.2
150	0.375	0.267 ± 0.062 (8)	71.2 ± 16.6
300	0.750	0.554 ± 0.138 (2)	73.9 ± 18.4

[a]NR = not recovered.
[b]Number of assays.

Experimental

Materials

(a) *Standards.*—Aflatoxin M_1 was prepared according to the procedure of Chu (17) and Stubblefield et al. (18). Concentrations of M_1 standards were determined spectrophotometrically according to official methods (19).

(b) *Cleanup columns.*—Normal silica gel and C18 reverse phase Sep-Pak cartridges (Millipore-Waters Associates, Inc., Milford, MA).

(c) *Chemicals.*—Horseradish peroxidase (Type VI), bovine serum albumin (BSA, RIA grade), Tween 20, water-soluble 1-ethyl-3,3-(dimethylaminopropyl)carbodiimide (EDPC), 2,2'-azino-diethyl-benzthiazoline-6-sulfonate (ABTS), and hydrogen peroxide (Sigma Chemical Co., St. Louis, MO). Aflatoxin M_1-carboxymethyl oxime was prepared according to Harder and Chu (14) and was conjugated to horseradish peroxidase as described previously (15).

(d) *Antiserum preparation.*—Specific antibodies against aflatoxin M_1 were produced in albino white rabbits, and were purified as described previously (14).

(e) *Solvents.*—All organic solvents were either chemically pure, reagent grade, or better.

(f) *Dairy products.*—Unpasteurized milk was obtained from the University of Wisconsin-Madison milk processing facility. Skim powdered milk, cheddar cheese, plain yogurt, and Brie cheese were purchased from local grocery stores. Naturally contaminated dry milk was obtained from Laboratoire Central des Services Veterinaires, Ministere de l'Agriculture (Paris, France).

Treatment of Samples

(a) *Preparation of samples.*—Uncontaminated and unspiked samples of liquid raw milk, dry milk, plain yogurt, cheddar cheese, and Brie cheese were analyzed for M_1 by a liquid chromatographic (LC) method as previously described (20) and were used as blanks. Liquid raw milk and dry milk (reconstituted at 10% in water) samples were spiked with aflatoxin M_1 solution at several levels from 0.005 to 0.250 μg/L. Cheddar cheese samples were also spiked with aflatoxin M_1 solution at several levels from 0.05 to 0.30 μg/kg.

Aflatoxin M_1 concentrations in the naturally contaminated milk samples were previously determined by LC procedure according to Fremy and Boursier (20). The M_1-contaminated yogurts were processed from the naturally contaminated milk at M_1 levels of 0.010, 0.025, and 0.050 μg/kg.

(b) *Purification procedure.*—For liquid milk and reconstituted powdered milk, all samples were subjected to C18 reverse phase cartridge treatment according to the procedure of Hu et al. (16). The cartridge was prewashed with 5 mL water, followed by 5 mL acetonitrile and again 25 mL water. In general, 10 mL milk sample, diluted with 15 mL water, was loaded on to the cartridge. After the cartridge was washed with 5 mL water followed by 15 mL 10% acetonitrile in water, aflatoxin M_1 was eluted with 10 mL 30% acetonitrile in water. The eluate containing aflatoxin M_1 was collected in a 30 mL centrifuge tube, vortex-mixed with 2 mL methylene chloride for 30 s, and centrifuged at 3000 rpm 10 min to separate the phases. The final volume of organic phase was measured and a 2 mL aliquot was transferred to a 5 mL vial and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 50 μL methanol, diluted with 450 μL 0.01M sodium phosphate buffer at pH 7.4, and then subjected to ELISA.

All samples of yogurt and various cheeses were subjected to an extraction step and then a silica cleanup treatment according to the procedure of Fremy and Boursier (20) with a slight modification. The normal phase Sep-Pak Si 60 cartridge was prewashed with 2 mL methanol and then with 20 mL chloroform before use. In general, 5 mL water with 10% NaCl, 60 mL methylene chloride, and 4 g Celite 545 were added to 10 g yogurt, 5 g Brie cheese, or 2.5 g cheddar cheese in a 100 mL Virtis vial. The mixture was stirred at low speed in a Virtis homogenizer (Model 45) 30 s to extract aflatoxin M_1. After filtration on Whatman No. 1 paper, the final volume

of organic filtrate was recorded (generally ca 40–50 mL) and then concentrated to 1–2 mL in a rotatory evaporator. The concentrate was quantitatively loaded on to a prewashed normal phase cartridge. After the cartridge was washed with 2 mL hexane and then 2 mL ethyl ether, aflatoxin M_1 was eluted with 5 mL 10% methanol in chloroform. The eluted fraction was evaporated to dryness under a gentle stream of nitrogen, dissolved in 50 μL methanol, then diluted with 450 μL 0.01M sodium phosphate buffer and finally subjected to ELISA.

(c) *LC analysis of naturally contaminated samples.*—Some naturally contaminated dry milk samples were prepared in the Laboratoire Central d'Hygiene Alimentaire (Paris, France). Four of these that had been previously analyzed for M_1 by LC were selected for the present study. Aflatoxin M_1 was separated on a 5 μm Spherisorb-5 ODS C18 reverse phase column, using 25% acetonitrile in water as the mobile phase. Details of this method were described previously by Fremy and Boursier (20).

Direct Competitive ELISA Procedure

The optimal dilutions of antiserum and carboxymethyl oxime-aflatoxin M_1-HRP conjugate were determined for each batch of antiserum and conjugate preparation, using the procedure described previously (15). The protocols for ELISA of M_1 were essentially the same as previously reported (15) except that the coating procedure was modified. Briefly, 50 μL antiserum diluted in PBS solution was coated directly to a 96-well polystyrene microwell Nunc plate prepared for protein binding (A/S Nunc Co., Roskilde, Denmark) and kept in a refrigerator overnight before use. Microplates prepared in this manner were stored in the refrigerator no more than one week. Plates were washed 4 times with PBS containing 0.05% Tween 20 before use Immediately after the washing, 325 μL PBS containing 1.0% BSA was added to each well. Plates were incubated 1 h at 37°C, and again washed 4 times in PBS-Tween. For competitive ELISA, 25 μL standard aflatoxin M_1 or dairy sample in PBS was added to each microplate well, followed by the addition of 25 μL of a 1/100 dilution of aflatoxin M_1-HRP conjugate in PBS containing 1.0% BSA. Plates were incubated 1 h at 37°C, washed 4 times in PBS-Tween, and reacted with 100 μL ABTS-hydrogen peroxide substrate (1 mL aqueous ABTS solution (22.9 mg/mL) mixed with 0.03 mL 30% H_2O_2 in 100 mL pH 4.0, 0.05M citrate buffer) for 15–20 min. The reaction was terminated by the addition of 100 μL hydrofluoric-ethylenediamine acetic acid stopping reagent. Absorbance at 410 nm was determined in an automatic microplate reader, MR 600 (Dynatech Lab. Inc., Alexandria, VA). Triplicate standards in the range 0.01–2.5 ng/mL and at least triplicate analyses of each sample were performed in each assay.

Results

Recovery of M_1 in spiked milk samples.—A typical standard curve for the quantitation of aflatoxin M_1 is shown in Figure 1. Table 1 shows results for recovery of aflatoxin M_1 added to raw milk and reconstituted milk. Aflatoxin M_1 at concentrations as low as 10 ppt for liquid raw milk and 5 ppt for skim dry milk can be determined by ELISA. Recovery in the range of 10–100 ppt was between 116 and 77%, with a linear response. For contamination higher than 100 ppt in the milk extract samples, a dilution is necessary.

Recovery of M_1 in spiked cheese samples.—Recovery of M_1 spiked in yogurts is shown in Table 2. Results indicate that aflatoxin M_1 at concentrations as low as 10 ng/kg (ppt) can be determined. Recovery of aflatoxin M_1 added in the range of 10–75 ppt is between 101.2 and 66.4%. At aflatoxin M_1 concentrations above 100 ppt, a dilution is necessary. Results for recovery of aflatoxin M_1 added to cheddar and Brie cheese samples are presented in Tables 3 and 4, respectively. Detection limits for M_1 in Brie and cheddar cheese are 25 and 50 ng/kg, respectively. A linear response for the recovery of aflatoxin M_1 added to cheddar cheese was observed between 50 to 300 ppt. The recovery of AFM1 from Brie cheese between 25 and 100 ppt was also linear with the yield of recovery between 115 and 95%.

Table 4. Determination of M_1 by ELISA from artificially contaminated Brie cheese

Added		Recovered	
ppt (ng/kg)	ng/5 g	ng/5 g	Rec., %
15	0.075	NR[a] (6)[b]	—
25	0.125	0.144 ± 0.025 (6)	115 ± 20
50	0.250	0.263 ± 0.062 (6)	105 ± 25
100	0.500	0.475 ± 0.350 (6)	95 ± 35

[a]NR = not recovered.
[b]Number of assays.

Table 5. Determination of M_1 in naturally contaminated products

Reconstituted dry milk, ppt (ng/L)		Yogurt, ppt (ng/kg)
LC values	ELISA values	ELISA values
NM[a] (≤30) (3)[b]	9.28 ± 0.75 (8)	9.71 ± 3.85 (6)
40.0 ± 5.0 (3)	26.84 ± 4.2 (12)	28.31 ± 5.10 (8)
70.0 ± 7.0 (3)	51.25 ± 2.3 (8)	63.75 ± 12.20 (8)
130.0 ± 15.0 (3)	81.60 ± 5.5 (10)	ND[c]

[a]NM = not measurable (under the detection limit).
[b]Number of assays.
[c]Not determined.

Analysis of naturally contaminated products.—Aflatoxin M_1 in the 4 selected dry milk samples was determined by both LC and ELISA procedures. As shown in Table 5, aflatoxin M_1 amounts determined by LC were about 30% higher than those found by ELISA. Smaller coefficients of variation are obtained by ELISA than by LC. Results for the M_1 concentration in yogurts made from these naturally contaminated milks are similar to those obtained in the initial milks. However, higher coefficients of variation are observed for the M_1 amount in yogurt than for the amounts in milk.

Discussion and Conclusions

In the present study, we confirm an earlier observation (16) that the sensitivity of ELISA for M_1 in milk samples increases almost 10-fold after treatment of samples with silica cartridges. This approach can also be used for determination of M_1 in other dairy products. For some dairy products, such as low-fat plain yogurt, cartridge treatment is not necessary at M_1 levels above 50 ppt. Nevertheless, the purification step is required for a quantitative and precise determination of low M_1 levels in various samples. Although all the dairy products treated by this manner can be analyzed either by LC or ELISA, the ELISA procedure seems to be more sensitive. For determining M_1 in naturally contaminated products, we found a higher variation in yogurt samples than in milk samples. This difference may be due to differences in sample treatments. Milk samples were directly subjected to the C18 reverse phase cartridge treatment, whereas yogurt samples were subjected to extraction and then cleanup with the normal phase cartridge. Because the latter samples required considerably more cleanup steps, each step may introduce some errors and subsequently have larger variations.

In the present study, diluted antiserum solution was directly coated to the Nunc microplates instead of using a glutaraldehyde method in which the antiserum is coated to the Falcon plate by cross-linking to BSA in the presence of glutaraldehyde. The ELISA test using this method appears to give very good results and requires less time. Aflatoxin M_1 can be determined in dairy samples which have been subjected to a cleanup step in less than 2 h.

Although treatment of samples with cleanup cartridges improved the sensitivity of ELISA for M_1 in dairy products, it should be emphasized that the main factor contributing to the successful application of ELISA is the high affinity of the antibody to M_1. A number of approaches have been used for eliciting antibodies against different aflatoxins (21). When aflatoxins B_1 (22, 23), B_{2a} (24, 25), and B_1-dichloride (26) were conjugated to protein carriers for antibody production, the elicited antibodies reacted weakly with M_1. Thus, these antibodies are not useful for determining M_1 in dairy products. Antibodies obtained from rabbits immunized with M_1-bovine serum albumin conjugate, however, are highly specific for M_1 and have some cross-reactivity with other aflatoxins (14).

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Improved Gas Chromatographic Method for Quantitation of Deoxynivalenol in Wheat, Corn, and Feed

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A gas chromatographic (GC) method is described to determine deoxynivalenol in wheat and corn at levels as low as 20 ppb. Ground samples are extracted with water, adsorbed onto a Clin Elut column, extracted with ethyl acetate, and passed through a silica gel Sep-Pak cartridge. The final extract is then derivatized with *N*-heptafluorobutyrylimidazole and quantitated by GC using an electron capture detector. Recoveries are greater than 85% for spiked samples at levels of 50–1000 ppb. Results for wheat, corn, and mixed feed samples are given as well as the results of an interlaboratory study on a naturally contaminated wheat sample.

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothecene-9-ene-8-one) (DON), also known as vomitoxin (1), is a trichothecene mycotoxin produced by certain species of *Fusarium* that infest grain crops in the field and storage. DON was originally isolated in Japan and in the United States from barley and corn infected in the field with *Fusarium graminearum* (1–3) and has been associated with feed refusal, vomiting, and general digestive disorders in animals ingesting moldy feeds (4–6).

Several analytical methods for DON have been reported that specify liquid chromatography (LC), gas chromatography (GC), and thin layer chromatography (TLC) (7–10). A recent evaluation of current methods for trichothecene analysis concluded that the method of Scott et al. (11), using GC with electron capture detection, was the best available for determining DON (10). The method is sensitive, accurate, and reproducible but requires substantial time and labor. Bennett et al. (12) recently reported modifications of the Scott method which result in a more rapid analysis but their method still requires a precipitation procedure, a partition w50h ethyl acetate, and chromatography on a silica gel column.

The present report describes substantial modifications to the Scott method in which water replaces aqueous methanol for extraction, the precipitation step is eliminated, a Clin Elut extraction column is used to avoid a lengthy partition procedure, and a silica gel Sep-Pak cartridge replaces the silica gel column. DON in the purified extract is derivatized with *N*-heptafluorobutyrylimidazole (HFBI) and quantitated by GC using an electron capture detector. DON determinations with the Research Foods Ltd method (RFL method) compared favorably with those of the Scott method in an interlaboratory study.

METHOD

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5830A equipped with ^{63}Ni electron capture detector and 3 m × 4 mm glass column packed with 3% OV-3 on 80–100 mesh Chromosorb W (HP).

(b) *Wrist-action shaker.*—Burrell Corp., Pittsburgh, PA.

(c) *Buchi flash evaporator.*—Brinkmann Instruments, Rexdale, Ontario, Canada.

(d) *Micro vials.*—2 mL, with Teflon screw cap (Chromatographic Specialties, Brockville, Ontario, Canada).

(e) *Centrifuge tubes.*—15 mL, with glass stoppers (Kimble 8064).

Reagents

(a) *Solvents.*—All solvents certified ACS grade.

(b) *DON standard.*—1 μg/mL toluene–acetonitrile (95 + 5). Standards were produced in-house and confirmed by comparison to authentic DON and by various spectrometric methods (infrared, ultraviolet, nuclear magnetic resonance, mass spectrometry, and gas liquid chromatography retention times).

(c) *Clin Elut columns.*—Nos. 1020 and 1005 (Analytichem Inc., Habor City, CA).

(d) *Silica gel Sep-Pak cartridges.*—No. 51900 (Waters Associates, Mississauga, Ontario, Canada).

(e) *Sodium bicarbonate solution.*—5% aqueous (w/v).

(f) *N-Heptafluorobutyrylimidazole (HFBI).*—1 g ampules (Pierce Chemical Co., Rockford, IL 61105). If reagent appears yellow, distill under reduced pressure. Store at 0°C.

Extraction and Cleanup

Grind 5 kg sample to pass 20 mesh sieve and mix well. Weigh 25.0 g ground sample into 500 mL round-bottom flask, add 125 mL water, and shake 1 h on wrist-action shaker. Transfer contents of flask to 250 mL centrifuge bottle and centrifuge 6 min at 3000 rpm. Pipet 20 mL supernate onto Clin Elut column No. 1020 and allow 3 min for adsorption. Elute with 140 mL ethyl acetate (dispensed to column in seven 20 mL aliquots) and evaporate eluate to dryness in rotary evaporator in 50°C water bath. Quantitatively transfer residue to 15 mL centrifuge tube with dichloromethane (2 + 1 + 1 mL). Examine flask carefully to assure that all residue has been transferred. Use additional dichloromethane if necessary. Evaporate to dryness under nitrogen in 50°C heating block. Quantitatively transfer residue to Sep-Pak cartridge, using 200 and 100 μL portions of dichloromethane. Wash cartridge with 5 mL toluene–acetone (95 + 5) and discard washings. Elute DON with 6 mL methanol–dichloromethane (1 + 9) into 15 mL test tube. Evaporate to dryness under nitrogen in 50°C heating block. Redissolve residue in 4 mL toluene–acetonitrile (95 + 5). This solution represents 1 g sample/mL and is reserved for derivatization.

Derivatization

Add 50 μL HFBI to 15 mL glass-stopper centrifuge tube, followed by 1 mL sample or DON standard solution. Stopper and seal with Parafilm. Mix 20 s, using vortex mixer, heat 1 h in 60°C sand bath, cool to room temperature, and vortex-mix 20 s. Add 1 mL 5% (w/v) sodium bicarbonate, vortex-mix 2 min, and let layers separate. Transfer 100 μL organic layer (equivalent to 0.1 g sample) to 2 mL vial containing 0.9 mL hexane. Prepare standard by diluting 25 μL derivatized DON standard with 975 μL hexane (0.025 μg DON/mL).

Gas Chromatography and Quantitation

If possible, analyze samples on the same day as derivatization; otherwise, store derivatized sample overnight at 0°C. Construct calibration curve on each day of analysis, using

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Table 1. Recovery of deoxynivalenol from spiked wheat samples by RFL method and Scott method (11)

DON added, ppb	Rec., %	Av., %
RFL Method[a]		
1000	97.0, 105, 69.3	90.4
500	82.6, 80.4, 92.8, 95.1, 76.5	85.5
200	91.3, 106, 87.8	95.0
100	76.8, 101	88.9
50	98.6, 97.0	97.8
500[b]	88.1, 84.7	86.4
Scott Method		
500	79.8, 81.1, 75.0, 70.5, 87.3, 88.4, 89.1	81.6

[a]20 mL Clin Elut column.
[b]5 mL Clin Elut column.

Table 2. Deoxynivalenol determinations in different commodities by RFL method and Scott method (11)

Commodity	Deoxynivalenol, ppm[a] Scott method	RFL method[b]	Alt. RFL method[c]
Wheat	0.40[d]	0.36[e]	—
Wheat	0.35	0.33[d]	0.32[d]
Wheat	0.91	0.85	0.92
Corn	0.24	0.27	0.24
Feed	0.23	0.29	0.25
Feed	0.36	0.32	0.39
Feed	0.64	0.59	0.64
Feed	0.21	0.22	0.20
Corn	0.42	0.40	0.34

[a]One determination.
[b]20 mL Clin Elut column.
[c]5 mL Clin Elut column.
[d]Two determinations.
[e]Four determinations.

the following GC conditions: column 185°C; injector and detector 255°C; carrier gas, argon–methane (95 + 5), 50 mL/min; attenuation 64. Peak height should vary linearly with amount injected over the range 20–200 pg. Inject 2–3 μL sample solution. If necessary, adjust injection volume of sample or dilute sample with hexane to obtain peak of about same height as standard. With described conditions, retention time for DON derivative is ca 16 min. Allow 35 min between injections.

Calculations

Deoxynivalenol, ppb = (250 × μL std soln injected × peak sample)/(peak ht std × μL sample injected)

If concentration of standard is not 0.025 μg/mL, then the following factor must be used instead of 250.

Factor = (ng/mL std)/(0.1 g/mL)

Alternative Cleanup Procedure

The cleanup procedure can be miniaturized by using 5 mL Clin Elut column instead of 20 mL size: Transfer 5 mL aqueous extract to 5 mL Clin Elut column and elute with 35 mL ethyl acetate (dispensed to column in seven 5 mL aliquots). Evaporate eluate to dryness in 50°C rotary evaporator. Using 200 and 100 μL portions of dichloromethane, transfer residue to silica gel Sep-Pak cartridge. Wash cartridge with 5 mL toluene–acetone (95 + 5) and then elute DON with 6 mL dichloromethane–methanol (9 + 1).

Evaporate this fraction to dryness as above. Dissolve in 1 mL toluene–acetonitrile (95 + 5) and derivatize entire extract, using derivatization method described previously. This 1 mL final extract represents 1 g sample.

Results and Discussion

We have found that most solvent systems for extraction of DON also extract extraneous compounds that necessitate further cleanup, usually in the form of a precipitation procedure followed by solvent partitioning and column chromatography. The high solubility of DON in water prompted us to test water as an extracting solvent. The resultant extract was sufficiently clean to permit elimination of a precipitation procedure. The aqueous extract was also adaptable to partitioning in a Clin Elut column and required only a silica gel cartridge to complete the cleanup. This extract also appears to be suitable for analysis by LC.

Extraction studies comparing a high-speed blender and a wrist-action shaker were performed on wheat samples that were either spiked or naturally contaminated with DON. No significant difference in the recovery of DON could be detected for a sample that was either blended for 5 min or put on the shaker for 60 min. Similar results were reported recently by Bennett et al. (12). For multiple analyses, the shaker proved to be more cost effective.

Table 1 compares DON recovery by our methods and the Scott method (11). In these recovery studies, a wheat sample free of DON was spiked with a stock solution of standard (50 μg DON/mL chloroform) which was allowed to evaporate before extraction. The average recoveries were 81.6, 91.5, and 86.4% for Scott's method, for RFL method using the 20 mL Clin Elut column, and for RFL method using the 5 mL Clin Elut column, respectively, over the range of 50–1000 ppb added DON. Our method using the 20 mL Clin Elut column for analysis of wheat spiked at 500 ppb DON had a coefficient of variation of 9.43%.

The sample was derivatized in toluene–acetonitrile (95 + 5) as described in ref. (11). We did not choose a higher column temperature to shorten the analysis time by GC because this resulted in a loss in resolution and, hence, reproducibility. Ten pg derivatized DON gave a peak height of 10% full scale. The GC analysis was performed on the same day as derivatization or, if this was impossible, the next morning after storage at 0°C. The derivatized DON standard does not appear to degrade even after 5 days at 0°C. The standard curve of DON peak height vs. amount injected is a straight line that passes through the origin and, thus, peak height can be used for quantitation. The electron capture detector sensitivity changes with time, particularly for the first few injections, so it is recommended that a calibration curve be run each day and a standard injected every 3–4 samples.

The concentration of the standard solution should also be checked every 2–3 months against a freshly weighed standard. We have observed a loss of response of approximately 10% in our standard solution over a 1 year period.

Table 2 contains the results of analyses of wheat, corn, and animal feed obtained by our method in comparison with that of Scott et al. (11). The methods show good agreement. We have found that our method can be used for animal feed without any modification even though animal feed often contains a variety of interfering compounds. Occasionally, a small shoulder peak elutes with DON but it does not affect quantitation. The detection limit for the above commodities is 20 ppb.

A small-scale interlaboratory study was conducted in which 3 other laboratories used our method to analyze a naturally

Table 3. Interlaboratory results of deoxynivalenol determinations in wheat, using RFL method and Scott method (11)

	Deoxynivalenol, ppm							
Method	Wheat 1	Wheat 1	Feed 2	Feed 3	Feed 4	Feed 5	Feed 6	Feed 7
Our Lab.								
RFL method	0.35	0.32[a]	—	—	3.22	2.63	1.46	0.94
Scott method	0.38	0.38	—	—	2.98	2.50	1.67	0.95
Lab. 1								
RFL method	0.33	—	0.08	1.69[b]	—	—	—	—
Scott method	0.39	—	0.03	1.66[b]	—	—	—	—
Lab. 2								
RFL method	0.32	—	—	—	—	—	—	—
Scott method	—	—	—	—	—	—	—	—
Lab. 3								
RFL method	0.29	—	—	—	—	—	—	—
Scott method	0.19	—	—	—	—	—	—	—

[a]Only determination to use 5 mL Clin Elut column.
[b]Sample spiked at 2.0 ppm DON.

contaminated wheat sample. Table 3 demonstrates that all the laboratories were in very close agreement.

Due to the unavailability of an authentic nivalenol standard, we could not include this toxin in our analyses, but we suspect that it could also be determined by our method.

In summary, our method can quickly and accurately determine DON in wheat, corn, and animal feed and has a sensitivity of 20 ppb. Because this method requires much less time and solvents, it represents a significant improvement over existing methods.

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Capillary Gas Chromatographic Determination of T-2 Toxin, HT-2 Toxin, and Diacetoxyscirpenol in Cereal Grains

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A capillary gas chromatographic (GC) method using an electron capture detector is described for determining T-2 and HT-2 toxins and diacetoxyscirpenol (DAS) in cereal grains at levels as low as 100 ppb for T-2 and DAS and 50 ppb for HT-2. Samples are extracted with methanol–water according to the Scott method, and further purified on a silica gel cartridge and a cyano column. Heptafluorobutyrylimidazole (HFBI) is added to form the esters of the analytes. Ester(s) of T-2, HT-2 and DAS are separated on a 30 m × 0.32 mm DB-5 fused silica column and measured with a ^{63}Ni electron capture detector. Samples were confimed by gas chromatography/mass spectrometry using electron impact ionization and single ion monitoring at the molecular mass of 501.11 m/z for T-2, 665.08 m/z for HT-2, and 502.12 m/z for DAS at 10 000 resolving power. The method was applied to wheat, oats, and barley. Average recoveries ranged from a low of 65% for T-2 in barley to a high of 99% for DAS in oats.

Trichothecene mycotoxins are mold metabolites produced by various strains of *Fusarium, Trichoderma, Myrothecium,* and other species of imperfect fungi. The group is characterized by the 12,13-epoxytrichothec-9-ene ring system. T-2 toxin, HT-2 toxin, and diacetoxyscirpenol (DAS) belong to the group that does not contain a carbonyl fraction at C_8 conjugated with a double bond in 9-10 (1). This feature prevents these compounds from being detected by UV absorption. T-2, HT-2, and DAS are capable of inducing skin necrotization, vomiting, and leucopenia in experimental animals (2, 3).

A general review on the analysis of trichothecenes has been published (4). T-2 and other mycotoxins have been analyzed by thin layer chromatography (5), radioimmunoassay (6, 7), and gas chromatography with electron capture detection (8) or gas chromatography combined with mass spectrometry for detection and confirmation (9). Before GC analysis, T-2 and DAS have been derivatized to trimethylsilyl ethers and heptafluorobutyrates (8).

Methanol–water, as described by Scott (10), is used in our method as the extraction solvent. After concentration, the analyte is cleaned up on a Sep-Pak silica gel cartridge by using dichloromethane and dichoromethane–methanol solvents, followed by a cyano column using chloroform–hexane and chloroform–ethanol solvents. The analytes are derivatized according to Scott's modification (10) and are subjected to capillary GC and electron capture detection.

METHOD

Apparatus

(a) *Gas chromatograph.*—Varian Model 6000 or equivalent equipped with direct capillary injector, 30 m × 0.32 mm id fused silica DB-5 capillary column, 0.25 μm film thickness, electron capture detector (^{63}Ni foil), and, preferably, autosampler.

(b) *High-speed blender.*—Waring, explosion-proof with 1 L jar.

(c) *Centrifuge bottles.*—250 mL, thick-wall glass bottles.

Reagents

(a) *Solvents.*—All distilled in glass (Caledon, Georgetown, Ontario).

(b) *Adsorbents.*—Sep-Pak silica cartridge (Waters Associates); cyano extraction column (J. T. Baker).

(c) *T-2, HT-2, DAS.*—Available from Myco Lab. Co., PO Box 321, Chesterfield, MO 63017.

(d) *N-Heptafluorobutyrylimidazole (HFBI).*—Pierce Chemical Co., Rockford, IL 61105.

Sample Preparation

Grind 1 kg sample to pass 2 mm screen. Mix thoroughly until 200 g representative sample is obtained. Further mix 200 g; then weigh 50 g into Waring blender.

Extraction and Cleanup

Blend 50 g sample with 250 mL methanol–water (1 + 1) for 5 min at high speed. Transfer mixture to 250 mL glass centrifuge bottle and centrifuge 5 min at 2000 rpm. Transfer 10 g equivalent (50 mL) to 250 mL beaker and add 100 mL 30% aqueous ammonium sulfate + 20 g Celite 545 and stir 2 min. Filter through Whatman No. 41 paper. Wash filtrate with 50 mL water. Extract combined filtrate with four 100 mL portions of ethyl acetate in 500 mL separatory funnel. Dry combined extracts with sodium sulfate and filter again through Whatman No. 41 paper, collecting filtrate in 500 mL round-bottom flask. Rinse filter paper with 25 mL ethyl acetate. Evaporate combined ethyl acetate extracts to dryness in Buchi Rota-vapor.

Dissolve residue in 2 mL dichloromethane and transfer to 10 mL glass syringe attached to silica gel Sep-Pak cartridge. Wash round-bottom flask with three 5 mL portions of dichloromethane and quantitatively transfer rinses to cartridge. Discard first 5 mL dichloromethane. Add 15 mL methanol–dichloromethane (5 + 95) and elute trichothecenes from cartridge into 50 mL centrifuge tube (A).

Evaporate fraction (A) from above to dryness. Dissolve residue in 2 mL chloroform–hexane (1 + 1) and transfer to cyano extraction column. Rinse tube again with 2 mL chloroform–hexane (1 + 1) and transfer rinse to cyano column. Add enough chloroform–hexane (1 + 1) to collect a total of 15 mL. This fraction (B) contains DAS and T-2. Add 5 mL chloroform to cyano column and discard eluate. Add enough chloroform–ethanol (98 + 2) to collect 5 mL. This fraction (C) contains HT-2.

Derivatization

Transfer 2 g equivalent of B and C into each of 2 separate tubes. Treat each similarly. Evaporate to dryness and add 100 μL HFBI to sample; mix well using tube mixer. Add 1 mL with toluene–acetonitrile (95 + 5). Heat tubes 1 h at 60°C. Cool and mix again. Add 1 mL 5% aqueous sodium bicarbonate solution and mix 2 min. Let stand to separate layers. Transfer 100 μL organic layer, using 100 μL syringe, to 2 mL autosampler vial and dilute to 1 mL with heptane.

Standard Solutions

(a) *Stock solution.*—Prepare solution containing 1.25 mg DAS and T-2, and 0.25 mg HT-2 in 100 mL toluene–acetonitrile (95 + 5).

(b) *Working standard solution.*—Transfer 2 mL stock solution to 15 mL centrifuge tube containing 100 μL HFBI.

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Table 1. Peak height and retention time reproducibility (30 replicates)

Std	Amount, pg	Pk ht	RSD	Retention (min)	RSD
DAS	250	20908	2.2	39.92	0.03
HT-2	50	19121	2.0	44.20	0.01
T-2	250	28355	1.9	49.47	0.02

Table 2. Linearity of response for different concentrations of DAS, HT-2, and T-2[a]

Compd	Range, pg	Index of determination
DAS	100–1000	0.993
HT-2	25– 500	0.991
T-2	100–1000	0.997

[a]Detector attenuation 64 × 10.

Table 3. Recovery of DAS, HT-2, and T-2 from wheat, barley, and oats[a]

Sample	Level, ppm	DAS, % Mean	DAS, % SD	HT-2, % Mean	HT-2, % SD	T-2, % Mean	T-2, % SD
Wheat	0.8	78.9	9.5			87.4	5.6
	0.4	83.4	12.9			85.5	13.2
	0.2	78.6	14.5	87.8	4.9	80.0	15.0
	0.1			90.0	9.3		
	0.05			85.0	14.6		
Barley	2.0	7	12.1			68	8.7
	0.8	84	11.1			65	9.1
	0.5			80	5.7		
	0.25			90.5	9.1		
Oats	4.0	99	1.7			81	3.4
	2.0	88.5	16.2			82	9.8
	1.0			91	10.8		
	0.5						

[a]All data are the results of 3 separate determinations.

Carry out derivatization. Transfer 100 μL derivatized mixture to 10 mL volumetric flask. Dilute to volume with heptane to give DAS and T-2 standards of 125 pg/μL, and HT-2 standard of 25 pg/μL.

Gas Chromatographic Analysis

If possible, carry out analysis on same day as derivatization, using the following oven temperature profile. Inject standards and samples and compare peak heights and retention times.

Initial value 75°C
Initial hold time 2.00 min

Step	*Final temp.*	*Rate*	*Hold time*
1	225	5.0	10.0
2	250	5.0	5.0
3	280	30.0	2.0

Additional conditions: injector 300°C; detector 350°C; helium carrier gas linear variety 35 cm/s; make-up gas (10% methane, 90% argon), flow rate 40 mL/min; chart speed 1 cm/min.

Results

Table 1 gives peak height reproducibility. Using an autosampler for all injections, the highest coefficient of variation (RSD) achieved for all trichocethene standards is 2.2%. Table 1 also gives retention times of derivatized trichothecenes; on the basis of 30 injections, retention time variation was ≤ 0.03%. Table 2 shows that within the range chosen for each trichothecene, detector response was linear on the basis of the index of determination approaching 1.0. Minimum detectable amount at attenuation 64 × 10 was 100 pg for DAS and T-2, and 25 pg for HT-2, with a signal-to-noise ratio better than 1%. All injections were done using an autosampler in the direct injection mode. Table 3 gives results from recovery studies.

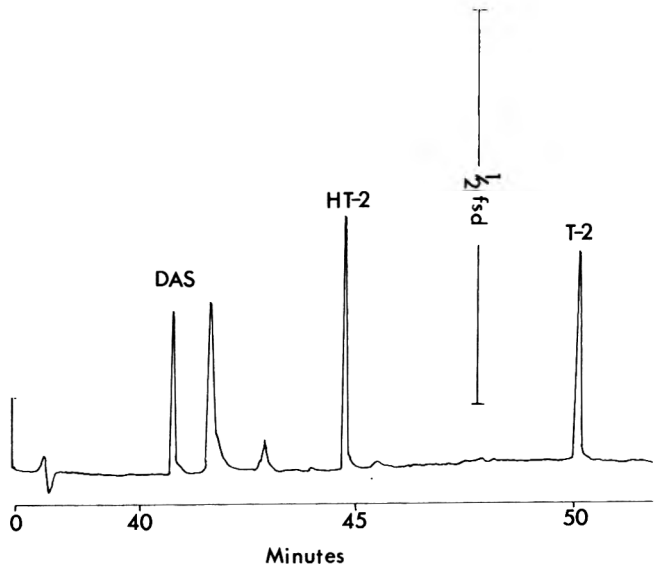

Figure 1. Chromatogram of 200 pg DAS, 50 pg HT-2, and 200 pg T-2 as heptafluorobutyrate derivatives.

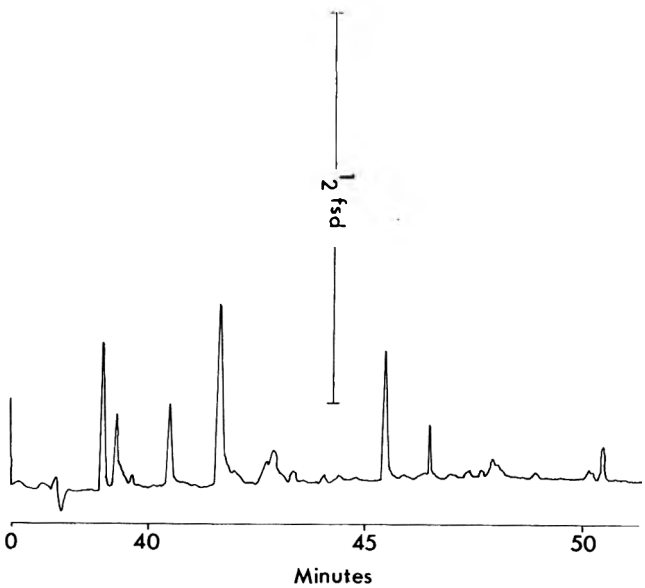

Figure 2. Chromatogram of barley blank sample equivalent to 0.5 g/mL.

Confirmation

T-2, HT-2, and DAS were confirmed by GC/MS with a Perkin-Elmer Sigma III chromatograph interfaced to a Kratos MS-50 mass spectrophotometer with a single stage glass jet separator. A 6 ft. × ¼ in. glass column packed with 3% SE-30 Ultraphase on 80–100 mesh Chrome 750 and operated isothermally at 220°C with a helium flow of 30 mL/min was used for chromatographic separations. High level and low level quantitation in samples was carried out by single ion monitoring at the molecular ion mass of 501.11 m/z for T-2, 655.08 m/z for HT-2, and 502.12 m/z for DAS at resolution of 10 000.

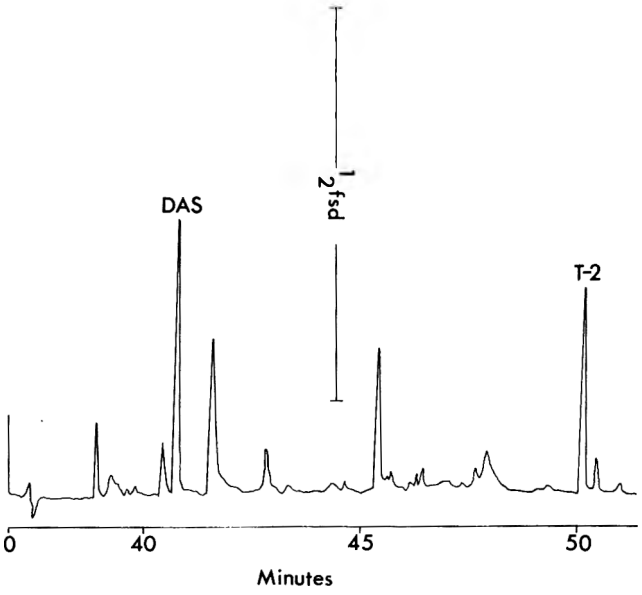

Figure 3. Chromatogram of barley sample spiked with 0.8 ppm DAS and T-2, equivalent to 0.5 g/mL.

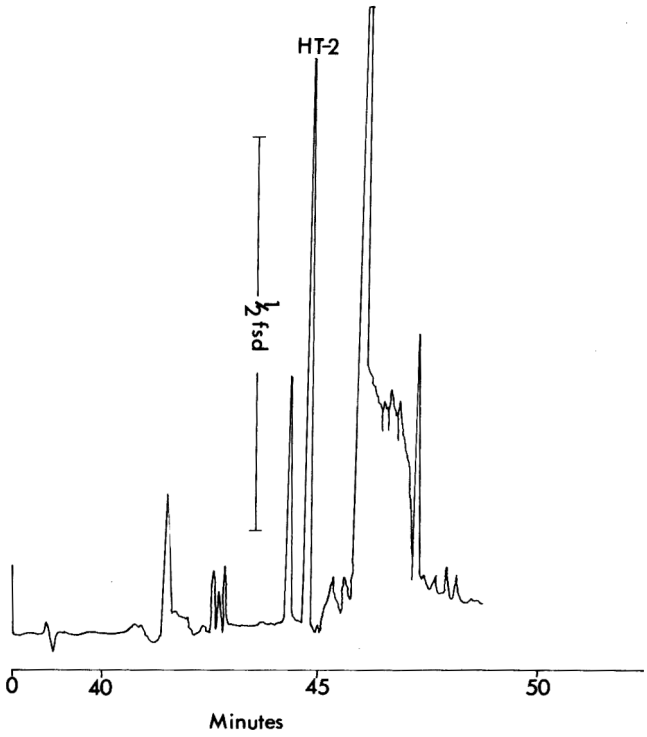

Figure 4. Chromatogram of barley blank sample spiked with 0.25 ppm HT-2, equivalent to 0.5 g/mL.

Discussion

The proposed method for determining DAS, HT-2, and T-2 was developed to detect residue levels in cereal grains. The methanol–water solvent quantitatively extracted the trichothecenes from the grains but with few co-extractives, thus permitting analysis free from interferences. The silica gel cartridge partially purified the sample by retaining polar compounds, but samples still were not suitable for capillary GC-EC analysis at this point.

Further purification was done on the cyano column; DAS and T-2 were eluted in one fraction and the more polar HT-2 was eluted in a separate fraction.

Table 1 demonstrates the good peak height and retention time reproducibility under the GC conditions described. Table 2 indicates the very good linearity achieved.

Table 3 is a compilation of recovery data from wheat, barley, and oats. Spiking levels varied from a high of 4 ppm to a low of 0.05 ppm. Recoveries averaged a low of 65% to a high of 99%. All samples were analyzed in triplicate along with a separate blank.

Figure 1 is a chromatogram of 200 pg DAS, 50 pg HT-2, and 200 pg T-2 as their respective HFBI derivatives. Figure 2 is a barley blank sample showing no interfering peaks in the areas of interest. Figure 3 is a blank sample of barley spiked at 0.8 ppm DAS and T-2. Figure 4 is a chromatogram of a barley sample spiked with 0.25 ppm HT-2.

The method offers the advantage of screening cereal grain for DAS, HT-2 and T-2 at residue levels. The GC capillary system and temperature programming adequately resolved all components. The HFB derivatives allow a very low limit of detection with the electron capture detector.

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Chemical Confirmatory Tests for Ochratoxin A, Citrinin, Penicillic Acid, Sterigmatocystin, and Zearalenone Performed Directly on Thin Layer Chromatographic Plates

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Published tests have been improved and a new procedure is described for chemical confirmation of mycotoxins directly on thin layer plates. After extraction and preliminary cleanup chromatography with *n*-hexane or chloroform, the mycotoxins ochratoxin A, citrinin, penicillic acid, sterigmatocystin, and zearalenone were easily separated by thin layer chromatography (TLC) using toluene–ethyl acetate–90% formic acid (6 + 3 + 1) developing solvent. In chemical confirmatory methods, the developed chromatogram was exposed to vapors of pyridine, acetic anhydride, or a mixture, or the mycotoxins were over-spotted. With this treatment, ochratoxin A, citrinin, penicillic acid, and zearalenone were converted to new fluorescent compounds, and observed under 365 nm light after re-chromatography with the same developing solvent. Sterigmatocystin was confirmed chemically using TLC plates impregnated with 0.6N H_2SO_4 or 10% oxalic acid in methanol. The described procedures are satisfactory for confirming mycotoxins present in standards, artificially contaminated grain samples (barley, corn, oat, rye, and wheat), and extracts from both fungal cultures and naturally contaminated grain samples.

The problem of mycotoxin contamination of food or animal feed has been widely recognized since the discovery of aflatoxins. Many laboratories are investigating the possible role of other toxic mold metabolites in human and animal health (1–5). Therefore, their unambiguous detection and analysis seems to be of prime concern.

Several procedures for detection of individual mycotoxins as well as simultaneous multimycotoxin analysis have been described (5–11). In our previous paper (12), we reported that screening analysis is often complicated by mycotoxin-like compounds that interfere with detection of ochratoxin A, citrinin, penicillic acid, sterigmatocystin, and zearalenone. These interfering compounds were present in extracts from both cereal grain and fungal cultures. To prevent false positive detections, chemical tests to confirm mycotoxin presence should be performed. The methods presented in this paper allow analysis and chemical confirmation for 5 mycotoxins (ochratoxin A, citrinin, penicillic acid, sterigmatocystin, and zearalenone).

Experimental

Apparatus

(a) *Thin layer chromatographic apparatus.*—See **26.001(k)** (13).

(b) *UV lamp.*—With 365 nm filter (Emita, Poland, or equivalent). Observe TLC plates on black background, 15 cm from lamp, in completely dark room. Protect eyes!

(c) *Chromato-vue cabinet.*—With 365 and 270 nm lamps (Hanau, West Germany, or equivalent).

(d) *Glass capillary micropipets.*—1, 5, and 10 μL (Drummond Scientific Co., or equivalent).

(e) *Chromatographic chambers.*— (Schott and Gen, Jena, DDR). 210 × 10 × 210 mm, or equivalent, adapted to generate ammonia, acetic anhydride, and pyridine vapors.

(f) *Rotary evaporator.*—Büchi, Switzerland, or equivalent.

(g) *Spectrofluorodensitometer.*—Vitatron TLD-100 Netherlands), or equivalent.

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Reagents

(a) *Mycotoxins standards.*—Analytical standards of ochratoxin A and sterigmatocystin (Calbiochem, Behring). Citrinin and penicillic acid (Hans P. van Egmond, National Institute of Public Health, Bilthoven, Netherlands). Zearalenone (Chester Mirocha, University of Minnesota, St. Paul, MN). Standards were checked by their spectrophotometric properties (2) and dissolved in benzene–acetonitrile (98 + 2) at the following concentrations (μg/mL): ochratoxin A 20, citrinin 100, penicillic acid 400, sterigmatocystin 200, and zearalenone 100. For sensitivity determinations, solutions were diluted to the following concentrations (μg/mL): ochratoxin A 0.1, citrinin 0.1, penicillic acid 15.0, sterigmatocystin 1.0, and zearalenone 5.0. Handle mycotoxins with care; may be carcinogenic and/or teratogenic.

(b) *Ground, mycotoxin-free cereal samples.*—Barley, corn, oat, rye, and wheat (supplied by Animal Feeding Station, Agricultural Academy in Poznań). Samples were spiked with standards at 2 different concentrations; minimum concentrations were ochratoxin A 0.2, citrinin 0.1, penicillic acid 5.0, sterigmatocystin 1.0, zearalenone 1.0 μg/g.

(c) *Naturally contaminated cereal samples.*—Obtained from 5 local factories producing mixed feeds for poultry.

(d) *Fungal isolate cultures.*—Incubated on sterilized corn (Institute of Food Technology, Agricultural Academy in Poznań).

(e) *Solvents.*—Analytical grade, glass-distilled before use (PPOCh Gliwice, Poland). Caution: Benzene and chloroform are possible carcinogens.

(f) *Pyridine and acetic anhydride.*—Analytical grade (International Enzymes Ltd, Windsor, Berkshire, England).

(g) *Acetic anhydride–pyridine (1 + 1).*—Caution: Pyridine and acetic anhydride are hazardous chemicals.

(h) *TLC plates.*—Aluminum sheets, 20 × 20 cm, precoated with 0.2 mm silica gel 60 (Merck No. 5553, GFR). Sheets were cut to 20 × 10 or 10 × 10 cm. When needed, plates were impregnated with 0.6N H_2SO_4 or 10% oxalic acid solution in methanol, and then dried at 50°C.

(i) *Developing solvent for TLC.*—Toluene–ethyl acetate–90% formic acid (6 + 3 + 1).

(j) *Ammonia vapors.*—Generated from 27% water solution of NH_3.

(k) *Ethanolic $AlCl_3$ spray solution.*—20%.

(l) *Filter papers.*—Medium wide pores, medium fast filtering for crystalline deposits (No. 389), and dense, narrow pores, slow filtering for finest deposits (No. 390) (Filtrak, Spezialpapierfabrik Niederschlag, DDR, or equivalent).

(m) *Extraction mixture.*—125 mL $CHCl_3$ and 12.5 mL 0.2M H_3PO_4. Caution: $CHCl_3$ is possible carcinogen.

(n) *Concentrated HCl.*—Analytical grade (PPOCh Gliwice).

Extraction

The Nesheim method (14) was adapted to extraction of ochratoxin A, citrinin, penicillic acid, sterigmatocystin, and zearalenone. Twenty g ground cereal samples, naturally and artificially contaminated, or 20 g fungal culture was extracted 30 min with 125 mL $CHCl_3$ and 12.5 mL 0.2M H_3PO_4. The mixture was acidified with HCl to pH 2–3 in a 250 mL glass-

stopper Erlenmeyer flask, shaken on a wrist-action shaker, and filtered through 10 g anhydrous Na_2SO_4 and 2 filter papers (Filtrak No. 389 and No. 390). The solids were washed with a small volume of chloroform and the combined filtrate was evaporated to dryness and then dissolved in 2 mL $CHCl_3$.

Thin Layer Chromatography

Mycotoxin extracts (10 μL) from naturally or artificially contaminated grain samples or from fungal cultures were spotted by using glass capillary micropipets on an imaginary line 1 cm from the lower edge of a TLC plate; in addition, 10 μL of each extract was overspotted with 5 μL internal standard and 5 μL reference standards on the same plate.

When large amounts of fatty impurities were present in extracts of cereals, interfering with developed chromatograms, preliminary cleanup chromatography was performed, either with *n*-hexane (preferred) or $CHCl_3$. Neither migration of mycotoxin spots nor loss of toxins, as determined fluorodensitometrically, was observed after this operation. After preliminary cleanup chromatography, the plate was developed with toluene–ethyl acetate–90% formic acid (6 + 3 + 1), up to 0.5 cm from upper edge of the TLC plate. For maximum sensitivity determination (minimum detected), different volumes of extract, 1, 5, or 10 μL, were spotted.

Chemical Confirmation of Mycotoxins

Chemical confirmation of mycotoxins was performed directly on the developed TLC plate. Pyridine, acetic anhydride, or a mixture (1 + 1) of the 2 was used as follows: (a) A developed chromatogram was exposed in a chromatographic chamber to vapors of the reacting agents; or (b) a mycotoxin spot was over-spotted with 0.05 mL droplet of these reagents. A gentle current of room temperature air (hair dryer) removed excess reacting mixture. This operation was performed in 15 min under a fume hood. Following either process, the plate was developed for a second time with the same toluene–ethyl acetate–90% formic acid (6 + 3 + 1) solvent. Procedure (a) is simpler and gives a better chromatogram after the second development. Fluorescent products of the chemical confirmation were observed under 365 and 270 nm UV lamps.

The presence of sterigmatocystin can be confirmed by TLC on aluminum sheets impregnated with either 0.6N H_2SO_4 or 10% oxalic acid in methanol. Ammonia vapors or 20% $AlCl_3$ spray was used for visualization and to increase the fluorescence intensity of the mycotoxins and/or chemical confirmation products.

Results

Recoveries of zearalenone and ochratoxin A extracted from artificially contaminated grain samples by the modified Nesheim method (14) and determined spectrofluorodensitometrically were 97% and 90%, respectively. Recoveries of sterigmatocystin, penicillic acid, and citrinin were determined visually to be about 75%.

The described chemical tests are satisfactory for confirming mycotoxins present in standards, extracts from artificially contaminated grain samples (barley, corn, oats, rye, and wheat), and extracts from fungal cultures and naturally contaminated grain samples. Application of the method is limited by quenching of mycotoxin fluorescence caused by impurities present in crude extracts. If preliminary cleanup procedures presented here are not satisfactory, additional purification steps (9, 10) will be necessary. The lowest amount of toxin permitting chemical confirmation was much higher for extracts from grain samples than for pure standards. Detection limits were comparable among extracts of all grains tested as follows: ochratoxin A 20, citrinin 15, penicillic acid 500, sterigmatocystin 75, and zearalenone 75 ng/spot. For standards and purified extracts, detection limits were ochratoxin A 1.25, citrinin 1.25, penicillic acid 150, sterigmatocystin 7.5, and zearalenone 50 ng/spot.

Techniques presented here prevent false positive reports for mycotoxin-like compounds (12). In all cases, spots with similar R_f values and fluorescence were definitively different from mycotoxin spots after chemical treatments. In addition, mycotoxin-free grain samples, after chemical confirmation, showed no mycotoxin-like spots on the TLC chromatogram.

The results of each chemical confirmatory test are presented in condensed form in Table 1; detailed observations are discussed below for each toxin individually.

Ochratoxin A

(a) A drop of acetic anhydride spotted on the toxin produced a new blue fluorescent, diffused spot with higher R_f value than a nontreated standard or the ester derivative obtained by the method of Paulsch et al. (15). We propose that esterification of the phenolic group in the ochratoxin A molecule occurs.

(b) Exposure to vapors of pyridine or pyridine–acetic anhydride gave a product exhibiting blue fluorescence and a lower R_f value than a nontreated standard. Probably, polar pyridium salts of the carboxylic group are formed in this reaction. Exposure to ammonia vapors or spraying with $AlCl_3$ increased fluorescence intensity to the toxin as well as confirmation products.

Citrinin

We modified and simplified the procedure of Hald and Krogh (8). The acetic acid ester of citrinin was formed directly on aluminum TLC sheets impregnated with 0.6N H_2SO_4 as follows:

(a) The developed chromatogram of citrinin was exposed 15 min to vapors of pyridine–acetic anhydride (1 + 1) or a drop of this mixture was spotted on the citrinin spot. During re-chromatography, the reaction product did not migrate, but exhibited yellow-green fluorescence.

(b) The developed TLC plate was exposed 15 min to vapors of acetic anhydride, or over-spotted with a droplet of acetic anhydride, and re-developed. Apart from the yellow-green spot (identical with the reaction product of (a)), an additional yellow-green spot was observed. The second spot exhibited the same fluorescence and R_f value as a citrinin standard re-developed on a TLC plate that had not been treated with either vapors or a droplet of acetic anhydride. Probably esterification during this treatment is not complete.

Penicillic Acid

A drop of acetic anhydride spotted at the origin on the sample extract, and re-developed, produced a new, diffused gray spot with a higher R_f value than a nontreated standard. Also, ammoniation (15 min) of the chromatogram can be used to visualize the toxin and products of confirmation.

Sterigmatocystin

(a) During chromatography of sterigmatocystin on aluminum sheets impregnated with 0.6N H_2SO_4, 2 orange fluorescent products were formed. The first (R_f = 0.69) exhibited a weaker fluorescent intensity than the second (R_f = 0.40). The R_f of sterigmatocystin on unimpregnated aluminum sheets was 0.66. Fluorescence changed to light yellow after spraying with 20% $AlCl_3$. We propose that during spotting and developing of the chromatogram, esterification occurs. This has

Table 1. Summary of results for new or modified chemical confirmatory tests for mycotoxins performed on TLC plates[a]

Test	Ochratoxin A	Citrinin	Penicillic acid	Sterigmatocystin	Zearalenone
Acetic anhyd. (drop)[b]	blue spot above std[c]	yellow-green spot does not migrate	grey spot above std after ammoniation	—	2 blue spots above std
Acetic anhyd.–pyridine (1 + 1) (vapor)	blue spot below std	yellow-green spot does not migrate	—	—	blue spot above std
Acetic anhyd.–pyridine (1 + 1) (drop)	—	yellow-green spot does not migrate	—	—	blue spot above std
Pyridine (vapor)	blue spot below std	—	—	—	—
0.6N H_2SO_4 on TLC plate	—	—	—	orange spots above and below std	—
10% oxalic acid on TLC plate	—	—	—	4–5 new red spots	—

[a]Toluene–ethyl acetate–formic acid developing solvent in all cases.
[b]Drop = ca 0.05 mL.
[c]Color of fluorescence observed under 360 nm UV light.

been supported by identical properties of spots obtained when the ester of sterigmatocystin was formed in vials according to the method described by Stack and Rodricks (6).

(b) During chromatography on aluminum sheets impregnated with oxalic acid, sterigmatocystin was converted to 4–5 new fluorescent spots; the most intense red fluorescent spot occurred at R_f = 0.68. Spraying with 20% $AlCl_3$ changed the fluorescence to yellow-orange.

Zearalenone

(a) Vapors of acetic anhydride–pyridine partially transformed zearalenone on the TLC plate to a new blue fluorescent compound with a higher R_f value than nontreated standard. The reaction was complete after 5 min vapor exposure, or with a drop of mixture spotted on the toxin spot.

(b) Vapors (30 min) or a drop of acetic anhydride esterified zearalenone. Two blue fluorescent spots with higher R_f values than nontreated standard appeared on the chromatogram. We explain this as 2 ester products of 2 different phenolic groups being formed. Fluorescence intensity of zearalenone, as well as its chemical confirmatory products, increased after the chromatoplate was sprayed with 20% $AlCl_3$.

Conclusions

The proposed methods allow 5 mycotoxins to be chemically confirmed and were successfully used for mycotoxin extracts from both fungal cultures and naturally contaminated grain samples. This procedure has several advantages: Interferences from mycotoxin-like compounds (12) exhibited definitely different properties than mycotoxins. The techniques are simple and can be performed by inexperienced operators. No additional operations like scraping mycotoxins and silica gel from the TLC plates for chemical confirmation are needed. Only one kind of aluminum sheet and one developing solvent mixture is used for chromatography before and after chemical confirmation. All operations can be performed directly on a TLC plate. All products of the chemical confirmatory tests are viewed on a chromatogram under 365 nm UV light. This is a rapid method which is relatively sensitive for grain contaminated with mycotoxins. Three or 4 mycotoxins can be confirmed in one operation, using acetic anhydride or vapors of pyridine–acetic anhydride, respectively.

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Improved Cleanup for Liquid Chromatographic Analysis and Fluorescence Detection of Aflatoxins M_1 and M_2 in Fluid Milk Products

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A rapid method is described for extraction and cleanup of raw and processed milk for determination of aflatoxins M_1 and M_2 by using a C_{18} Sep-Pak/silica gel cleanup column combination. Aflatoxins are separated by normal phase liquid chromatography and their concentrations are determined by fluorescence detection in a silica gel-packed flow cell. Recoveries ranged from 99 to 103% with coefficients of variation less than 2% for M_1 levels of 0.117–1.17 ng/mL added to raw milk. Similar recoveries were obtained for M_2. The coefficient of variation for analysis of 5 subsamples of naturally contaminated milk was less than 1%. Agreement with the official method is satisfactory. Each sample requires less than 25 mL solvent and 10 min actual handling time. Sample chromatograms show no interferences in the M_1-M_2 elution region and no late-eluting peaks, which permits spacing injections at 13–20 min intervals. Aflatoxin levels as low as 0.03 ppb may be determined by this procedure. Extracts have also been analyzed by thin layer chromatography.

Florida, a major milk-producing state, has over 380 dairy farms which supply 28 processing plants. In addition, milk is transported by tanker to these plants from neighboring states. Because of climatic conditions in this geographic region, dairy feeds are sometimes contaminated with aflatoxins, toxic metabolites of *Aspergillus* molds. To effectively and routinely monitor the aflatoxin level in the raw milk supply, a rapid and accurate method of analysis in the part-per-10 billion concentration range (0.1 ng/mL) is needed. Therefore, a cleanup procedure was developed that is rapid, rugged, and inexpensive and that gives reproducibly high recoveries with liquid chromatography (LC) and fluorescence detection. Extracts must also be free of late-eluting components so samples may be injected at short intervals without interference from earlier injections. Previously published methods (1–8) have not satisfactorily fulfilled all of these requirements. The cleanup and analytical procedure described here eliminates the drawbacks of methods currently available.

METHOD

Other suitable equipment or reagents may be substituted for those specified.

Apparatus

(a) *Liquid chromatograph.*—Waters Model ALC 204 with M6000 solvent delivery system with Model U6K injector (Waters Associates, Inc., Milford, MA 07157).

(b) *Detector.*—Varian Fluorichrom fluorescence detector with silica gel-packed flow cell and filters to provide 365 nm excitation and 431 nm emission wavelengths (Varian Instrument Division, Walnut Creek, CA 94303).

(c) *Recorder.*—Varian Aerograph A-25, 1 mV input, 10 in. chart, 0.1 in./min chart speed (Varian).

(d) *LC analytical column.*—μ-Porasil, 0.4 × 25 cm (Waters), or Micro-Pak SI-5, 0.4 × 30 cm (Varian), or Zorbax, 0.46 × 15 cm (DuPont Co., Wilmington, DE 19898).

(e) *Cleanup columns.*—Bio-Rad Econo column, 0.8 × 4.0 cm polypropylene column with Luer tip, 35 μm porous polypropylene bed support disk, and 10 mL reservoir (Bio-Rad Laboratories, Richmond, CA 94804). Alternatively use large volume Pasteur pipet, 0.8 × 14.5 cm (Fisher Scientific Co., Orlando, FL 32809), with tight glass wool plug to support silica bed.

(f) *Vacuum regulator.*—Spectrum Medical Industries Vacu/Trol (Fisher).

Reagents

(a) *Solvents.*—Distilled-in-glass anhydrous methanol, acetonitrile, chloroform, and methylene cloride (Burdick and Jackson, Muskegon, MI 49442). Reagent grade ethanol and anhydrous diethyl ether (0.01% ethanol preservative). Deionized water filtered through 0.45 μm filter.

(b) *Water–acetonitrile wash solution.*—(95 + 5).

(c) *Methylene chloride–ethanol elution solvent.*—(95 + 5).

(d) *LC mobile phase.*—Add 22.5 mL ethanol to 1 L 22.5% water-saturated chloroform (225 mL water-saturated chloroform + 775 mL chloroform). Stir to mix, and degas in ultrasonic bath.

(e) *Aflatoxin standards.*—Aflatoxin M_1 (Eureka Laboratories, Sacramento, CA 95816) and aflatoxin M_2 (Sigma Chemical Co., St. Louis, MO 63178). Prepare separate stock solutions in $CHCl_3$ at 1.0 μg/mL; freeze to store. Prepare LC standard daily by diluting aliquots of stock solution with fresh mobile phase tc obtain one solution that is 0.01 μg M_1/mL and 0.004 μg M_2/mL. Adjust detector attenuator so that 100 μL injection of standard (1.0 ng M_1, 0.4 ng M_2) gives 50–75% full scale deflection for aflatoxin M_1.

(f) *Sodium azide, purified.*—0.4% solution.

(g) *Cleanup column packing.*—E. Merck No. 9385 silica gel 60, particle size 0.040–0.063 mm (American Scientific Products, Ocala, FL 32670). Dry silica in 105°C oven for 1 h. Cool, and add 1% water by weight. Shake in sealed container and equilibrate overnight before use.

(h) *Extraction cartridges.*—C_{18} Sep-Pak sample preparation cartridges (Waters Associates).

Sample Preservation

If samples are to be stored more than ca 2 days before analysis, preserve in the following manner and refrigerate: Gently invert sample container ≥ 10 times to evenly distribute cream in nonhomogenized milk. Transfer known volume of sample to storage container. Add 5.0 mL sodium azide solution for each 100 mL milk, cap, and stir gently with magnetic stirrer. Preserved samples may be stored up to 2 weeks before analysis.

Extraction

Bring sample to room temperature. Gently invert sample container ≥ 10 times to evenly distribute cream in nonhomogenized samples. Transfer 20 mL milk (21 mL for azide-preserved samples) to 50 mL graduated cylinder containing 20 mL warm (ca 80°C) water.

Attach inlet (longer) stem of C_{18} Sep-Pak cartridge to Luer tip of 50 mL syringe. Assemble syringe, cartridge, and vacuum flask as shown in Figure 1. Adjust vacuum regulator to pull solvents through cartridge in dropwise manner (ca 5 mm Hg). Prime cartridge by adding 5 mL methanol followed by 5 mL water. Discontinue vacuum when flow of water stops, and release residual vacuum by lifting cartridge-syringe

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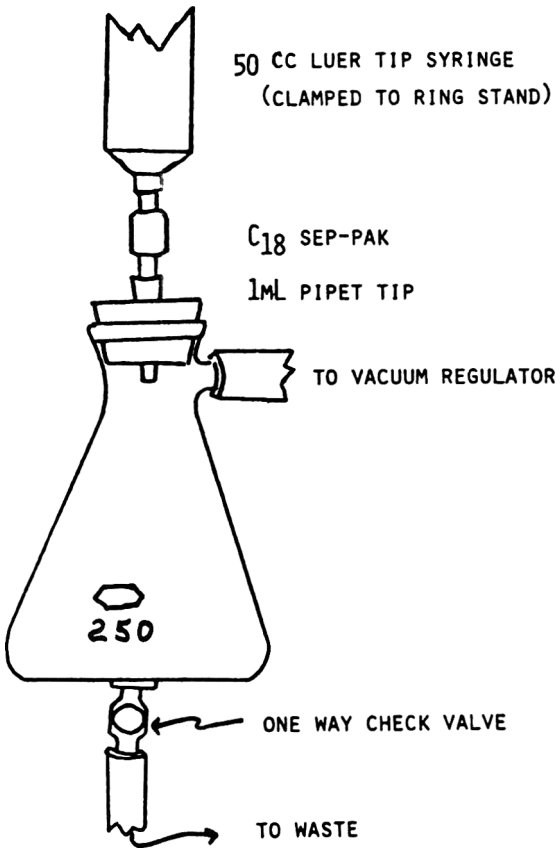

Figure 1. Assembly for extraction of aflatoxins M_1 and M_2 from raw milk.

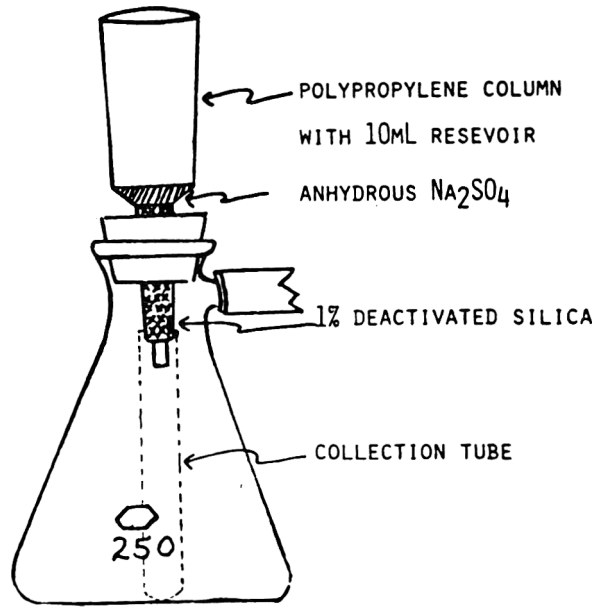

Figure 2. Cleanup column assembly for raw milk extracts. Dotted lines indicate position of collection tube.

assembly out of stopper to prevent loss of prime because of cartridge drying.

Replace cartridge-syringe assembly in stopper. Pour entire 40 mL of warm, diluted sample into syringe and gently pull sample through cartridge at ca 30 mL/min. (*Caution:* Too fast a flow will not allow sufficient time for aflatoxin to adsorb, and will result in low recoveries.) After milk has passed through cartridge, add 10 mL water–acetonitrile wash solution to syringe and pull through cartridge.

After wash has passed through cartridge, plug syringe barrel with rubber stopper and increase vacuum ca 30 s to remove retained wash solution from packing. Remove cartridge and dry both stems with cotton swab to eliminate remaining droplets of wash solution. Any excess water in cartridge that is subsequently transferred to silica gel column, will cause deactivation of silica and possible aflatoxin loss.

Cartridge prime may be partially destroyed while packing is dried. Reprime by adding 150 μL acetonitrile to inlet bed support disk and letting solvent soak into packing for several seconds. Attach cartridge to dry, glass 10 mL Luer-tip syringe, retaining same stem as inlet.

Silica Gel Column Chromatography

Assemble polypropylene column and 250 mL vacuum flask fitted with 1-hole stopper (Figure 2). Fill column to height of 4 cm with silica gel (ca 1 g). Use gentle vacuum to pack bed, and wash packing with 5 mL ether. Add ca 1 g anhydrous sodium sulfate to top of silica gel bed.

Add ca 7 mL ether to syringe-cartridge assembly positioned above silica gel column. With plunger, slowly force ether through cartridge, collecting eluate, with aflatoxin, in column reservoir. Pull ether slowly through silica column, using vacuum regulator to maintain ca 10 mL/min flow rate. Rinse silica column with 2 mL additional ether, still using vacuum. Aflatoxin is retained on silica column.

Remove column and stopper from flask and place in another 250 mL vacuum flask containing 16 × 125 mm collection tube (Figure 2). Add 7 mL methylene chloride–ethanol elution solvent to column reservoir. Pull elution solvent (methylene chloride–ethanol) through column at ca 10 mL/min flow rate, collecting eluate, with aflatoxin, in tube.

Discontinue vacuum, release slowly and remove collection tube from assembly. Evaporate eluate just to dryness under nitrogen stream, using heat to keep collection tube near room temperature. For liquid chromatography, dissolve residue in 1 mL fresh LC mobile phase and vortex-mix. Alternatively, residue may be dissolved in 100 μL $CHCl_3$ and analyzed by thin layer chromatography (TLC) as in AOAC method (9).

Liquid Chromatography

Stabilize instrument and detector for suitable period at 1.0 mL/min flow rate. Inject 100 μL LC standard several times until peak heights are constant. Inject sample extracts (typically 100 μL) with standard injections interspersed to ensure accurate quantitation.

Calculate aflatoxin concentration:

$$M_1 \text{ or } M_2, \text{ ppb} = (PH \times C' \times VI' \times V)/(PH' \times VI \times W)$$

where PH and PH' = peak height of sample and standard, respectively; C' = concentration of standard (ng/μL); VI and VI' = volume injected of standard and sample, respectively; V = volume in which dried sample eluate is dissolved (μL); and W = volume or weight of milk represented by final extract (typically 20 mL). Calculation is carried out separately for aflatoxins M_1 and M_2.

Results

Aflatoxin M_1 retention is about 10 min, using a 25 or 30 cm column (Figure 3-A), or about 8 min, using a 15 cm column. Aflatoxin M_2 is baseline-resolved, with a retention of 12 or 9 min for the 2 types of columns, respectively. The chromatograms of uncontaminated samples typically show 2 or 3 peaks, none of which has a retention time close to aflatoxins

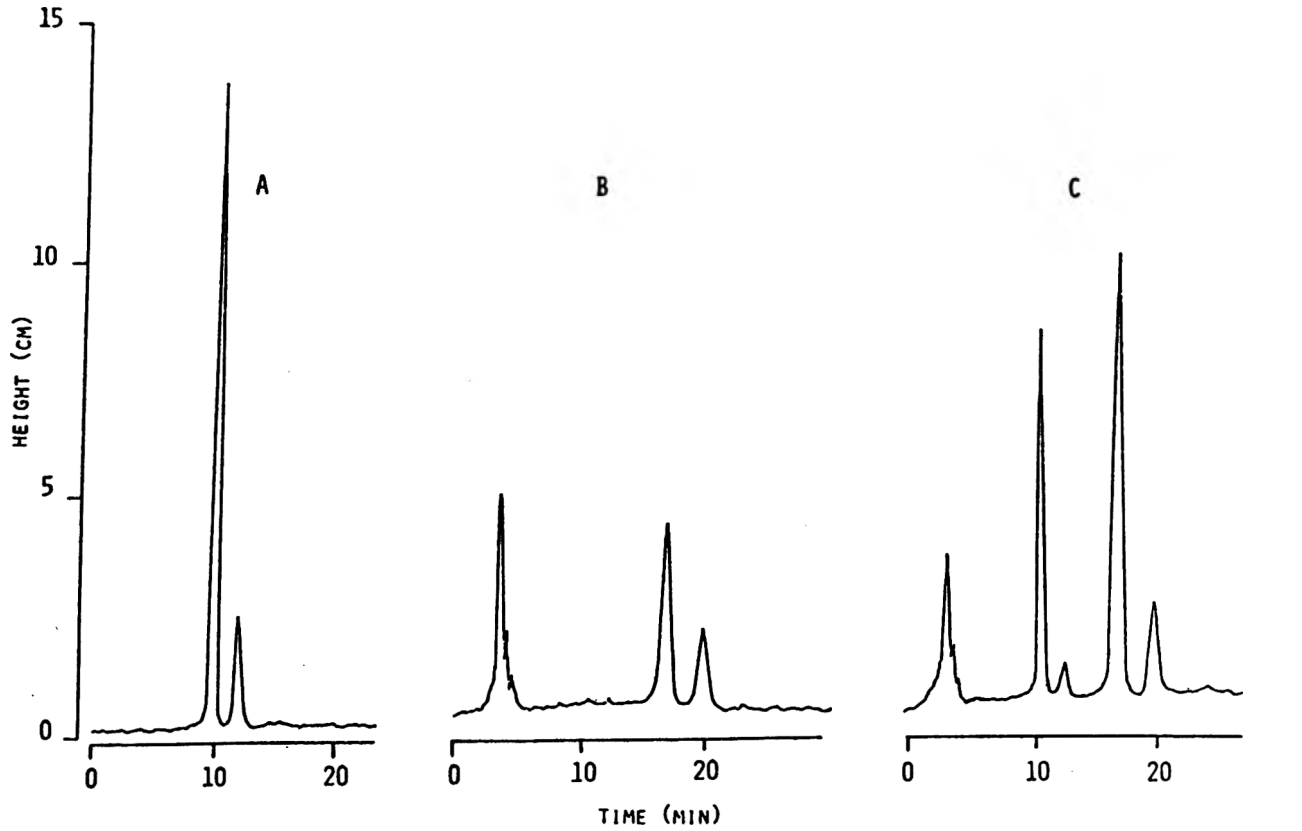

Figure 3. LC separation of 100 μL injections of A, standard solution containing 0.01 ng M_1/μL and 0.003 ng M_2/μL; B, 20 mL uncontaminated raw milk; and C, 20 mL naturally contaminated raw milk. Aflatoxin M_1 = 0.28 ppb; M_2 = 0.075 ppb.

(Figure 3-B). In contaminated samples, M_1 and M_2 are the second and third peaks eluted (Figure 3-C). A 0.03 ppb determination limit has been observed for this procedure.

Recovery studies were performed on aflatoxin-free, raw milk samples, spiked by adding M_1 + M_2 standard concentrate in ethanol to 1 L milk and mixing thoroughly but gently with magnetic stirrer. Several 20 mL subsamples were withdrawn for analysis; the sample was stirred before each aliquot was taken. Average recoveries were 103, 99, and 99% for added M_1 levels of 0.117, 0.497, and 1.17 ppb. Similar recoveries were obtained for M_2 (Table 1). For reconstituted nonfat dry milk, recoveries averaged 99% M_1 and 97% M_2 (Table 1). Recoveries are not shown for pasteurized milk because uncontaminated samples were not available during the development of the method.

Five 20 mL subsamples were drawn from 1 L of naturally contaminated raw milk to test the precision of the method on a sample that had not been artificially contaminated. The mean value for M_1 concentration was 0.951 ± 0.007 ng/mL (peak heights: 11.8, 11.9, 12.0, 11.8, 11.9 cm; M_1: 0.945, 0.953 0.961, 0.945, 0.953 ppb).

Twenty-six naturally contaminated raw milk samples were analyzed by the official cleanup with TLC analysis for M_1 in milk (10) and by the cleanup procedure described with LC analysis. A list of results appears in Table 2.

Several sample extracts prepared by this procedure were analyzed by thin layer chromatography, using the official procedure for milk (9). Aflatoxin M_1 is cleanly resolved with no streaking or interferences. A determination limit of 0.2 ppb was observed for visual analysis.

Discussion

In previously published methods, good recoveries and clean sample extracts are sometimes sacrificed for efficiency. Large amounts of solvents and analyst time are often required to obtain good extracts. The method described here produces good recoveries and high accuracy for sample extracts that are chromatographically clean and require short preparation time. Because extraction and cleanup take place on adsorption media, losses are virtually eliminated. The volume of solvents other than water used in the cleanup is less than 25 mL. Wash solutions and elution solvents are used directly from storage containers because the method is rugged enough for volumes to be measured with sufficient accuracy by milliliter graduations on a syringe barrel or cleanup column reservoir.

The method described is particularly suited for high sample throughput. Only about 10 min analyst time is required to process a sample to the eluate drying step. The directions and apparatus are especially suited to multiple sample handling. LC conditions permit injection to be made at 20 min intervals or alternatively, more often (about every 13 min) by spacing injections so that early (nonaflatoxin) peaks from one sample injection overlap later peaks from the previous sample. We have found that, realistically, a single analyst can prepare and analyze 25 samples in an 8 h workday. This can be increased if an automatic sampler is used with the LC column.

The method described in this paper has been used by the Food Laboratory of the Florida Department of Agriculture and Consumer Services on a routine basis since July 1980. Since that time, several thousand samples have been analyzed, and occasional problems have arisen which have been corrected.

After the development of the method and its presentation at the October 1980 AOAC meeting, we observed that the recoveries previously obtained could not be reproduced. Losses were found to result from failure to remove all of the aflatoxin from the C_{18} Sep-Pak cartridge. Cartridges marketed at that time behaved differently than those of earlier lots used

Table 1. Recovery of aflatoxin M_1 and M_2 from raw and nonfat dry milk

	M_1			M_2		
No. of detns	Spike, ppb	Av. rec., ppb (%)	SD	Spike, ppb	Av. rec., ppb (%)	SD
Raw milk:						
5	0.117	0.120 (103)	0.002	—	—	—
8	0.497	0.492 (99)	0.004	0.169	0.170 (100)	0.008
5	1.17	1.16 (99)	0.022	0.340	0.323 (95)	0.017
Nonfat dry milk:						
4	1.17	1.16 (99)	0.010	0.340	0.329 (97)	0.028

Table 2. Total (M_1 + M_2) aflatoxin (ppb) found in 26 raw milk samples by the official AOAC method and described LC method

Low level		High level	
AOAC	LC	AOAC	LC
0.21	0.20	0.84	0.66
0.21	0.16	0.84	1.05
0.42	0.46	1.26	1.18
NDA[a]	0.03	0.80	1.10
0.21	0.11	0.60	1.00
0.37	0.44	0.60	0.90
0.21	0.19	0.80	0.90
0.35	0.28	0.80	0.80
0.10	0.11	0.80	0.70
NDA[a]	0.04	0.60	0.60
NDA[a]	0.04	1.43	1.47
0.21	0.13	0.80	0.90
0.40	0.56	0.80	0.80

[a]No detectable amount.

for method development and sample analysis. Repriming the cartridges with acetonitrile (as described under *Method* in this paper) restored recoveries to the levels described. However, each lot of cartridges should be tested with a known sample for recovery.

On other occasions, losses have occurred from excess ethanol preservative or trace peroxide formation in the ether used for eluting the aflatoxin from the cartridge, or from deactivation of the silica gel column by excess water eluted from the cartridge.

As milk samples are extracted on the cartridge, the cartridge packing should take on a yellow color. When this is not observed, the analysis should be restarted. Adsorption of the sample has not occurred because the cartridge either was incorrectly primed or was faulty. (This is rare but has occurred.)

When extraneous peaks have appeared in chromatograms, they have been due to impurities in the solvents or silica gel. If silica is determined to be contaminated, it should be stirred in anhydrous methanol for 30 min, and excess liquid should be removed by filtration before drying.

Samples not preserved with sodium azide will begin to curdle after several days of storage. Partially coagulated samples are difficult to pull through the cartridge. Filtration through glass wool sometimes removes enough particulates to allow the sample to be extracted.

Although uncontaminated pasteurized milk could not be procured for recovery studies, several recoveries were carried out on samples contaminated with less than 0.1 ppb M_1. Corrected recoveries indicate that the described method will produce the same level of recovery for pasteurized milk as for raw milk. The trace levels of aflatoxin in these samples would not have been detected using most previously available methods.

It has been shown that this cleanup procedure can be used for TLC analysis with a slightly higher limit of determination. Preliminary investigations indicate that sample extracts prepared by the described method may also be analyzed by reverse phase LC.

Acknowledgments

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DRUGS IN FEEDS

Liquid Chromatographic Determination of Nicarbazin in Feeds and Premixes

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A liquid chromatographic (LC) method has been developed for the determination of nicarbazin in premixes and poultry feed. Liquid chromatography of the 4,4'-dinitrocarbanilide (DNC) portion of nicarbazin is performed isocratically with a reverse phase octadecylsilica column and a UV detector set at 340 nm. The 2-hydroxy-4,6-dimethylpyrimidine (HDP) portion of nicarbazin is chromatographed isocratically with a reverse phase octylsilica column and a UV detector set at 305 nm. Nicarbazin concentration can be calculated by assaying both DNC and HDP, or by assaying DNC or HDP and assuming that nicarbazin is a 1:1 molar ratio of the two. Average recoveries of DNC and HDP added to poultry feed were 101% and 87%, respectively. This procedure provides an alternative to existing colorimetric procedures for determining nicarbazin in premixes and poultry feeds.

Nicarbazin is the generic name for the complex of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) in a 1:1 molar ratio. Nicarbazin is used as a feed additive drug for the prevention of coccidiosis in poultry (1). Nicarbazin present in feeds and premixes can be analyzed by determining the DNC and/or HDP moiety (2–5). DNC is determined by developing a color with alcoholic sodium hydroxide and measuring the yellow absorbance at 430 nm in a spectrophotometer. HDP is reacted with diazotized sulfanilic acid in an acid medium to form a red complex, which absorbs at 540 nm. Although this method has undergone a number of collaborative studies by several laboratories, it is based on colorimetry and therefore subject to interferences.

Chromatographic techniques have been increasingly used to analyze for compounds in feeds and premixes. Methods based on liquid chromatography (LC) are sensitive and selective, and the instrumentation is becoming standard equipment in most analytical laboratories. This work describes the use of liquid chromatography to determine nicarbazin by assaying DNC and HDP in premixes and poultry feeds.

METHOD

Reagents and Apparatus

(a) *Solvents.*—Reagent grade dimethylformamide (DMF), glass-distilled water, methanol, acetonitrile, and *n*-hexane.

(b) *Chemicals.*—Nicarbazin standard, Alcoa alumina F-20, anhydrous sodium sulfate, silica gel 60 (35–70 mesh).

(c) *Apparatus.*—Hot plate; chromatography columns, 14 mm id × 250 mm long, equipped with stopcock; rotary vacuum evaporator (Rinco or equivalent) with a 45–50°C water bath; shaker table.

(d) *Liquid chromatograph.*—Waters Model 6000A pump; Waters Model 450 variable wavelength detector; reverse phase columns, 4 mm id × 30 cm, Hibar II RP18 and DuPont Zorbax C-8; Micromeretics Model 725 Autoinjector; and Fisher Recordall Series 5000.

(e) *Standard solutions.—Stock solution.*—Weigh 100 mg nicarbazin standard into 200 mL volumetric flask and dissolve in 75 mL DMF with the aid of gentle heat. Let flask cool, dilute contents to volume with DMF, and mix well. Use this standard for premix samples. *Analytical standards for feed samples.*—Using stock solution, prepare 50 μg/mL standard of nicarbazin in DMF and a series of standards between 1.0 and 6.0 μg/mL methanol.

Sample Preparation for 25% Premix Material

Weigh 400 mg premix sample into 200 mL volumetric flask. Add 175 mL DMF and heat, with intermittent stirring, just to boiling on hot plate in hood. Remove sample from hot plate, let cool to room temperature, and dilute to volume with DMF. No purification is needed and sample is ready for injection onto LC column for assay of DNC and HDP.

Sample Preparation for Poultry Feeds

(a) *DNC.*—Extract 10 g feed sample with 100 mL DMF and heat, with intermittent stirring, just to boiling on hot plate in hood. Remove sample from hot plate and let cool to room temperature. Filter sample and remove 25.0 mL aliquot for purification by column chromatography. Add 30 g alumina in 3 portions to glass chromatographic column containing glass wool pledget at bottom. Gently tap side of column with glass rod to let each portion settle. Add ca 2 g sodium sulfate on top of alumina. Open stopcock and add 25 mL DMF to top of column. Let solvent drain to top of sodium sulfate. Add 25.0 mL sample aliquot to column and let drain to top of sodium sulfate. Add five 5 mL portions of DMF, letting each portion drain to top of sodium sulfate. Add 15 mL methanol to column and let it drain to top of sodium sulfate. Discard all eluates to this point. Add 35 mL methanol to column and collect eluate in 50 mL volumetric flask, diluting to volume with methanol. Sample is ready for injection onto LC column for assay of DNC.

(b) *HDP.*—Extract 5 g feed sample with 20 mL water, swirl until large solid particles are dispersed, and then add 80 mL acetonitrile. Agitate on shaker table for 30 min. Pipet 10 mL aliquot of supernate and partition with 10 mL hexane. Discard top layer (hexane) and collect lower layer (acetonitrile). Evaporate acetonitrile to dryness in 50°C water bath. Sample is purified using silica gel column chromatography. Add 10 g silica gel and 15 mL acetonitrile to 50 mL beaker. Swirl and transfer to 14 mm id glass chromatographic column containing glass wool pledget at bottom. Rinse beaker and column with additional quantities of acetonitrile. After silica gel has settled, add ca 2 g sodium sulfate to top of silica gel. Open stopcock and let solvent drain to top of sodium sulfate at ca 2 mL/min. Quantitatively transfer sample to column, using three 5 mL portions of acetonitrile, letting each portion drain to top of sodium sulfate. Add 20 mL acetonitrile to flask that contained sample and add rinse to column, letting it drain to top of sodium sulfate. Using methanol–acetonitrile (25 + 75), add four 5 mL portions to flask that contained sample, and transfer rinses to column, letting each portion drain. Discard all eluate to this point. Add 75 mL methanol–acetonitrile (25 + 75) to column and collect eluate in 125 mL round-bottom flask. Evaporate sample to dryness in 50°C water bath. Redissolve sample in 10 mL methanol–acetonitrile (25 + 75). Sample is ready for injection onto LC column for assay of HDP.

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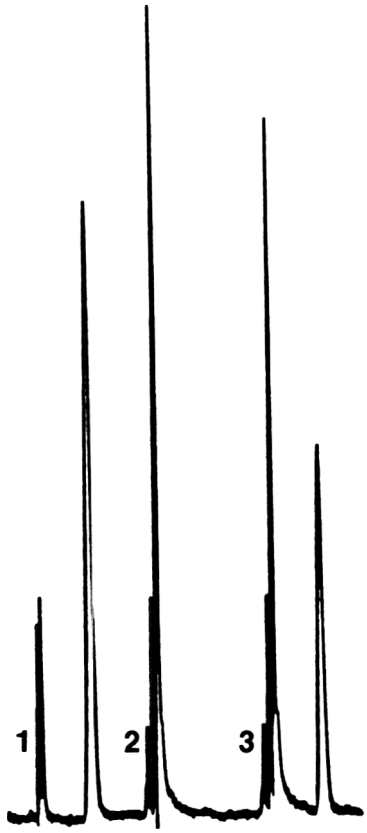

Figure 1. 1, Peak response for DNC equivalent to 1.2 μg (30 μL of 4.0 μg/mL) nicarbazin standard injected onto column; 2, control poultry feed sample; 3, control poultry feed sample fortified at 50 ppm with peak response for DNC equivalent to 0.72 μg (30 μL of 2.4 μg/mL) nicarbazin injected onto column, or 96% recovery.

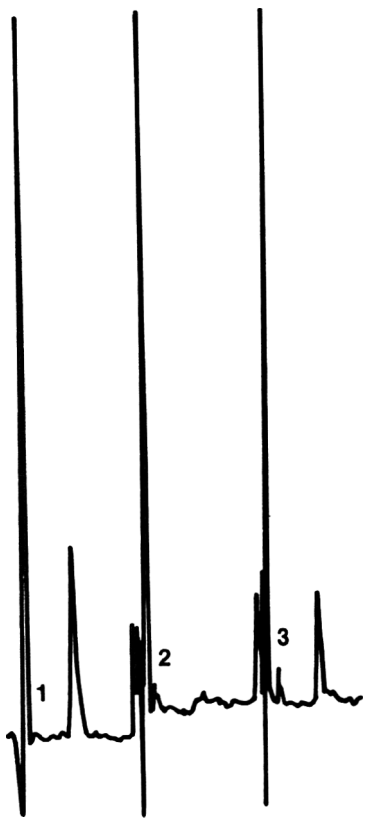

Figure 2. 1, Peak response for HDP equivalent to 1.2 μg (30 μL of 4.0 μg/mL) nicarbazin standard injected onto column; 2, control poultry feed sample; 3, control poultry feed sample fortified at 50 ppm with peak response for HDP equivalent to 0.69 μg (30 μL of 2.3 μg/mL) nicarbazin injected onto column, or 92% recovery.

Table 1. LC assays of 25% nicarbazin premix samples

% Based on DNC	% Based on HDP
24.2	25.8
24.6	26.7
24.5	25.5
24.0	24.9
24.3	27.1
24.5	26.1
24.5	24.1
24.9	24.7
24.3	24.4
24.7	23.7
25.0	24.1
24.6	24.4
AV. 24.5	25.1
SD 0.28	1.10
RSD, % 1.16	4.39

Table 2. Recovery of nicarbazin from poultry feed determined by assay of DNC

Fortification, ppm	Recovery ppm	Recovery %
25	27	108
25	27	108
25	27	108
25	28	112
25	24	96
25	24	96
25	25	98
25	24	94
25	24	96
25	24	96
50	56	112
50	54	108
50	50	100
50	51	102
50	52	104
50	51	102
50	57	114
50	53	109
50	48	96
50	49	97
50	48	96
50	47	94
50	47	94
50	48	96
100	109	109
100	103	103
100	96	96
100	95	95
	AV.	101.3
	SD	6.5
	RSD, %	6.4

Table 3. Recovery of nicarbazin from poultry feed determined by assay of HDP

Fortification, ppm	Recovery ppm	Recovery %
25	20	81
25	20	79
50	42	83
50	47	93
50	44	88
50	46	92
50	46	92
50	47	93
100	82	82
100	84	84
	AV.	87
	SD	5.3
	RSD, %	6.1

Chromatography

(a) *DNC.*—Quantitate DNC in purified extracts from premix samples by comparing detector responses 50 μg/mL standard of nicarbazin in DMF. Assay DNC in purified extracts from poultry feed samples by comparison with nicarbazin standard curve in methanol in concentration range between 1.0 and 6.0 μg/mL. LC operating conditions: temperature, ambient; mobile phase, water–methanol (25 + 75); column, Hibar II RP18; flow rate, 1.0–1.5 mL/min; detector wavelength, 340 nm; injection volume, 30 μL; retention volume of DNC peak, 6–12 mL. Measure peak heights or areas for samples and standards.

(b) *HDP.*—Quantitate HDP in purified extracts of premix samples by comparing detector responses 50 μg/mL standard of nicarbazin in DMF. Assay HDP in purified extracts of poultry feed samples by comparison with nicarbazin standard curve in methanol in concentration range between 1.0 and 6.0 μg/mL. LC operating conditions: temperature, ambient; mobile phase, water–methanol–acetic acid (90 + 10 + 0.01); column, DuPont Zorbax C-8; flow rate, 1.0–1.5 mL/min; detector wavelength, 305 nm; injection volume, 50 μL; retention volume of HDP peak, 6–12 mL. Measure peak heights or areas for samples and standards.

Results and Discussion

Feed and premix samples are analyzed for nicarbazin by quantitating DNC or HDP. The amount of nicarbazin is calculated from the experimentally determined concentration of DNC or HDP, assuming that nicarbazin is a 1:1 molar ratio of DNC and HDP. All assays of DNC and HDP are conducted using a nicarbazin reference standard.

Premix samples are readily assayed for DNC and HDP by simply extracting the sample with DMF and injecting the supernate into an appropriately prepared LC apparatus. No purification or color-forming reactions are necessary, simplifying the assay. To determine DNC and HDP in poultry feed, it is neccessary to purify and extract separate samples. The alumina column procedure (2) was used to purify the feed extracts before quantitation of DNC. The DMF solvent and alumina column can be used for feed samples containing a relatively high level of HDP (2). For samples containing a low concentration of HDP, an acetonitrile extraction followed by silica gel column chromatography is preferred. Figures 1 and 2 show typical chromatograms of a nicarbazin standard, control poultry feed, and control poultry feed fortified at 50 ppm with nicarbazin. Control poultry feed samples showed no background interferences at the retention times of the DNC or HDP peaks.

A comparative study on the colorimetric methods for DNC and HDP in poultry feeds resulted in relative standard deviations of 4 and 5%, respectively (2). The colorimetric methods were improved and shortened, and further comparative studies showed relative standard deviations of 3.5 and 4.6%, respectively (4, 5). These results compare well with the variability of the liquid chromatographic method shown in Tables 1–3. The advantages of the liquid chromatographic procedure are that the method is simpler because no color-forming reaction is necessary, and the assay is more specific with the high-resolution liquid chromatographic column.

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DRUGS

Liquid Chromatographic Determination of Methyldopa and Methyldopa-Thiazide Combinations in Tablets: Collaborative Study

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A reverse phase liquid chromatographic method for the determination of methyldopa, methyldopa-hydrochlorothiazide, and methyldopa-chlorothiazide in tablets was collaboratively studied by 8 laboratories. Each collaborator received 20 samples that included drug substance, synthetic and commercial tablet compositions. The overall repeatability and reproducibility standard deviations for commercial tablets were 1.11 and 1.75% for methyldopa, 0.96 and 1.62% for chlorothiazide, and 1.21 and 2.15% for hydrochlorothiazide, respectively. The overall recoveries of methyldopa, chlorothiazide, and hydrochlorothiazide added to synthetic tablets were 100.78, 100.70, and 101.34%, respectively. The method has been adopted official first action.

Methyldopa, 3-hydroxy-α-methyl-L-tyrosine, is an antihypertensive drug that is often used in combination with the diuretic thiazides. A rapid and specific isocratic liquid chromatographic (LC) method for the determination of methyldopa and of the components of methyldopa-hydrochlorothiazide and methyldopa-chlorothiazide combinations was previously reported by this laboratory (S. Ting (1983) *J. Assoc. Off. Anal. Chem.* **66**, 1436–1442). Analyses were carried out with a reverse phase C_{18} column, a mobile phase consisting of acetic acid–methanol–water, and photometric detection at 280 nm. The method was collaboratively studied by 8 laboratories.

Collaborative Study

Eight collaborators received 10 duplicate samples consisting of 1 methyldopa drug substance, 3 methyldopa tablet composites (1 synthetic and 2 commercial formulations), 3 methyldopa-hydrochlorothiazide tablet composites (1 synthetic and 2 commercial formulations), and 3 methyldopa-chlorothiazide tablet composites (1 synthetic and 2 commercial formulations). These samples were numbered 1 to 20. Samples were given false average tablet weights so that the presence of duplicates was not obvious to the collaborators. Each collaborator also received a set of instructions, a copy of the method, and vials of standards. The collaborators were directed to analyze each sample by the proposed method and to submit their results and worksheets to the Associate Referee.

Methyldopa, Methyldopa-Hydrochlorothiazide, or Methyldopa-Chlorothiazide in Tablets Liquid Chromatographic Method First Action

Principle

Methyldopa, chlorothiazide, and hydrochlorothiazide are detd by comparison with stds, using liq. chromatgy with UV detection and theobromine internal std.

Apparatus

(a) *Liquid chromatograph.*—Isocratic pump system, photometric detector capable of monitoring *A* at 280 nm, suitable recorder, and 20 μL injection loop.

(b) *Chromatographic column.*—300 × 3.9 mm id, contg μBondapak C_{18}, 10 μm particle size (Waters Associates, Milford, MA 01757).

(c) *Filter system.*—0.45 μm vac. filter app. (Millipore Corp., Bedford, MA 01730).

Reagents

(a) *Mobile phase.*—Mix 96 mL 3% HOAc with 4 mL MeOH, both either LC or reagent grade. Filter thru filter app., (c), before use.

(b) *Std soln.*—Transfer ca 50 mg anhyd. USP Methyldopa ref. std, accurately weighed, to 100 mL vol. flask. Add accurately weighed amt of USP Hydrochlorothiazide ref. std or USP Chlorothiazide ref. std, in same ratio with USP Methyldopa ref. std as that in sample tablets. Add 70 mL MeOH–H_2O (1 + 1) and mix by sonication with occasional swirling to dissolve. Let flask and contents cool to ambient temp. Dil. to vol. with MeOH–H_2O (1 + 1). Mix thoroly and filter thru 0.45 μm membrane filter, discarding first 5 mL filtrate.

(c) *Internal std soln.*—Weigh ca 22 mg theobromine into 100 mL vol. flask. Add ca 80 mL mobile phase. Warm gently on steam bath with occasional swirling to dissolve. Cool, dil. to vol. with mobile phase, and mix.

(d) *Methyldopa or methyldopa-chlorothiazide std soln.*—Pipet 4.0 mL std soln and 5.0 mL internal std soln into 25 mL vol. flask. Dil. to vol. with mobile phase, and mix.

(e) *Methyldopa-hydrochlorothiazide std soln.*—Pipet 10.0 mL std soln and 3.0 mL internal std soln into 25 mL vol. flask. Dil. to vol. with mobile phase, and mix. Do not use methyldopa-hydrochlorothiazide std soln >2 days old.

Preparation of Samples

(a) *Methyldopa and methyldopa-chlorothiazide tablets.*—Weigh and finely powder ≥20 tablets. Weigh portion of powder equiv. to 125 mg methyldopa and transfer to 250 mL vol. flask. Add 170 mL MeOH–H_2O (1 + 1) and mix by sonication with occasional swirling for at least the length of time needed to dissolve corresponding stds. Dil. to vol. with MeOH–H_2O (1 + 1) and mix thoroly. Filter thru 0.45 μm membrane filter, discarding first 5 mL filtrate. Transfer 4.0 mL filtrate to 25 mL vol. flask. Pipet 5.0 mL internal std soln into flask and mix. Dil. to vol. with mobile phase and mix.

(b) *Methyldopa-hydrochlorothiazide tablets.*—Follow procedure in (a), except transfer 10.0 mL sample filtrate and 3.0 mL internal std soln to 25 mL vol. flask.

(c) *Methyldopa drug substance.*—Accurately weigh portion of sample equiv. to ca 125 mg methyldopa, and transfer to 250 mL vol. flask. Follow procedure in (a), starting with "Add 170 mL MeOH-H_2O (1 + 1) . . . ."

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The recommendation of the Associate Referee was approved by the General Referee and Committee B and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.

Table 1. Collaborative results for methyldopa drug substance

Coll.	Found, %
1	99.9
	99.9
2	100.1
	100.4
3	99.8
	99.4
4	99.0
	100.7
5	100.8
	101.1
6	100.5
	101.9
7	99.1
	99.8
8	97.9
	101.0
Mean	100.1
CV, %	0.96
Repeatability CV, %	0.98
Reproducibility CV, %	0.98

Table 2. Collaborative results for methyldopa (MD) commercial and synthetic tablets

	Found, % of declared		
Coll.	500 mg tab.	125 mg tab.	Synthetic tab.[a] rec., %
1	98.04	98.64	100.22
	99.52	97.92	99.89
2	99.20	99.44	101.83
	99.60	97.36	101.62
3	97.38	97.84	99.65
	100.12	97.76	98.70
4	97.44	96.32	98.71
	97.00	97.44	99.71
5	100.30	99.78	101.85
	102.68	99.68	103.15
6	97.66	98.64	100.84
	97.44	101.04	102.66
7	97.88	97.60	102.10
	99.56	98.72	100.04
8	102.12	101.12	102.14
	101.34	105.92	98.63
Mean	99.21	99.08	100.73
CV, %	1.80	2.28	1.47
Repeatability CV, %	1.11	1.51	1.21
Reproducibility CV, %	1.85	2.32	1.49

[a]Prepared to contain 125 mg MD.

Suitability Test and Determination

Equilibrate LC column with mobile phase at 1.5 mL/min. Inject 20 μL std soln. Methyldopa, chlorothiazide, and hydrochlorothiazide peaks elute at ca 4, 6, and 7 min, resp. Retention time of theobromine internal std should be ≥9 min. Resolution factor between methyldopa and chlorothiazide and between hydrochlorothiazide and theobromine should be >3.5. Change flow rate or percentage of MeOH in mobile phase slightly if necessary. Adjust detector sensitivity so that peaks are ca 35–95% AUFS. Change detector sensitivity between methyldopa and hydrochlorothiazide peaks in combination samples. If necessary, adjust vol. of internal std soln added to sample soln and std soln to obtain satisfactory peak response for internal std. Make replicate injections of each std soln and compare peak responses between injections to det. reproducibility of system. In suitable system, coefficient of variation for 5 replicate injections is ≤2.0%. Proceed with sample analysis by injecting 20 μL each of sample soln and corresponding std soln.

Calculations

Using peak response ratios (R and R') relative to internal std, calc. mg drug per tablet from:

$$\text{mg/tablet} = (R/R') \times C \times (D/W) \times T$$

where R and R' = peak response ratios for sample soln and std soln relative to internal std, resp.; C = concn of std soln, mg/mL; W = wt of sample taken, mg; D = sample diln; and T = av. tablet wt, mg.

Results and Discussion

Tables 1–4 summarize the results received from the 8 collaborators on the analyses of methyldopa drug substance, methyldopa tablets, methyldopa-chlorothiazide tablets, and methyldopa-hydrochlorothiazide tablets. Statistical evaluation of these data showed the overall repeatability and reproducibility standard deviations to be 1.11 and 1.62% for methyldopa, 1.24 and 2.17% for chlorothiazide, and 1.04 and 1.94% for hydrochlorothiazide. The synthetic mixtures submitted to collaborative analyses were prepared by mixing the tablet excipients with concentrations of the corresponding drugs similar to those found in commercial formulations. Overall recoveries of methyldopa, chlorothiazide, and hydrochlorothiazide from these synthetic mixtures were 100.78, 100.70, and 101.34%, respectively.

Five collaborators used μBondapak C_{18} columns (Waters Associates, Milford, MA 01757); one collaborator used an Alltech C_{18} column (Alltech Associates, Inc., Deerfield, IL 60015); and one used a Partisil 10 ODS-3 column (Whatman Inc., Clifton, NJ 07014). For the calculations, 5 collaborators decided to measure peak heights, whereas the remaining 3 used peak areas.

None of the collaborators reported problems with the proposed method. Collaborator 4 suggested that allowance be made for the flow rate and for the mobile phase composition to permit attainment of the suitability requirements. These suggestions were incorporated into the proposed method. Collaborator 5 reported that a cloudy filtrate formed when the sample solution was filtered through the originally recommended Whatman No. 40 filter paper. For this reason, the proposed method was modified to direct that the sample solutions be filtered through a membrane filter rather than through a paper filter. Collaborator 7 suggested the use of a loop injector and of an electronic integrator that would eliminate the need for an internal standard and for attenuating the peak responses during a run, respectively. Although these suggestions will certainly improve the accuracy of the proposed method, the author decided to maintain the internal standard but to give the analyst the option to use either peak height or peak area measurements for the calculations. All chromatograms submitted by Collaborator 8 showed noisy baselines. Discussions of this problem with the collaborator revealed that it was caused by a faulty recorder.

In summary, the proposed LC method was shown to be simple, straightforward, accurate, and precise. It is generally applicable to tablets containing either methyldopa alone or combinations of this drug with hydrochlorothiazide or chlorothiazide.

Recommendation

The Associate Referee recommends that the LC method for methyldopa and methyldopa-thiazide combinations be adopted official first action.

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Table 3. Collaborative results for methyldopa (MD)-chlorothiazide (CH) commercial and synthetic tablets

	Found, % of declared					
	Commercial tab. 1[a]		Commercial tab. 2[b]		Synthetic tab.[c] rec., %	
Coll.	MD	CH	MD	CH	MD	CH
1	100.44	100.76	100.24	96.93	99.18	97.64
	99.76	100.08	98.04	99.86	102.45	99.56
2	100.36	102.08	101.40	101.20	103.67	103.11
	99.40	101.40	100.44	100.47	103.88	104.13
3	99.44	98.28	102.12	100.53	101.61	102.63
	97.08	98.24	101.36	101.53	102.76	101.50
4	99.92	101.24	101.20	102.93	99.63	105.09
	100.92	101.56	100.80	101.87	100.06	102.77
5	100.76	100.44	101.60	101.33	98.80	103.62
	99.60	102.48	101.20	100.19	98.67	104.13
6	101.64	99.36	100.72	100.33	100.65	97.37
	101.04	100.20	100.12	101.27	99.86	99.86
7	100.64	98.00	99.88	99.87	101.47	97.68
	99.56	98.40	100.12	99.60	101.96	100.44
8	99.76	98.44	100.60	103.73	97.14	98.43
	101.00	97.20	97.20	100.27	103.63	93.31
Mean	100.08	99.89	100.44	100.74	100.96	100.70
CV, %	1.048	1.64	1.269	1.52	2.01	3.21
Repeatability CV, %	0.88	0.69	1.07	1.26	1.84	1.79
Reproducibility CV, %	1.06	1.69	1.28	1.54	2.02	3.29

[a]Labeled to contain 250 mg MD and 250 mg CH.
[b]Labeled to contain 250 mg MD and 150 mg CH.
[c]Prepared to contain 250 mg MD and 150 mg CH.

Table 4. Collaborative results for methyldopa (MD)-hydrochlorothiazide (HC) commercial and synthetic tablets

	Found, % of declared					
	Commercial tab. 1[a]		Commercial tab. 2[b]		Synthetic tab.[c] rec., %	
Coll.	MD	HC	MD	HC	MD	HC
1	98.44	100.56	100.44	101.12	101.18	101.47
	98.50	101.44	99.32	100.96	101.96	100.55
2	99.92	102.12	99.76	100.80	101.37	100.74
	99.42	99.68	101.12	101.32	101.37	102.58
3	99.10	105.28	99.36	104.40	100.33	103.86
	99.60	105.46	99.08	104.28	100.54	104.78
4	99.10	101.60	99.48	99.88	100.37	100.18
	99.44	101.96	100.96	100.96	99.80	98.71
5	103.74	105.62	102.76	103.24	102.64	102.57
	103.38	104.22	102.32	102.36	102.66	101.84
6	99.86	100.74	99.48	100.24	100.07	100.37
	99.28	100.38	100.00	100.08	100.83	101.00
7	102.36	104.40	100.16	101.32	100.06	100.92
	100.18	103.00	100.52	102.00	100.04	100.37
8	99.20	100.38	93.00	97.12	98.04	101.65
	98.22	99.86	98.00	102.00	99.13	100.92
Mean	99.98	102.29	99.74	101.38	100.65	101.34
CV, %	1.68	2.04	2.16	1.77	1.21	1.50
Repeatability CV, %	0.65	0.82	1.45	1.59	0.37	0.73
Reproducibility CV, %	1.73	2.10	2.24	2.20	1.20	1.54

[a]Labeled to contain 500 mg MD and 50 mg HC.
[b]Labeled to contain 250 mg MD and 25 mg HC.
[c]Prepared to contain 250 mg MD and 25 mg HC.

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Liquid Chromatographic Determination of Allopurinol in Tablets: Collaborative Study

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A liquid chromatographic method for the determination of allopurinol in tablets was collaboratively studied by 7 laboratories. The method uses a C_{18} reverse phase column, a 0.05M ammonium phosphate mobile phase, hypoxanthine as the internal standard, and photometric detection at 254 nm. Collaborators were supplied with samples of 2 commercial tablets and 1 synthetic tablet powder. The mean recovery value of allopurinol from the synthetic tablet powder was 100.0%. The combined mean coefficient of variation for all 3 types of sample analyzed was less than 2%. The method has been adopted official first action.

Allopurinol, 1*H*-pyrazolo[3,4-d]pyrimidin-4-ol, is used in the treatment of gout and similar conditions. It acts by reducing the production of uric acid.

A liquid chromatographic (LC) method for the determination of allopurinol and its metabolites in biological fluids (1) was found suitable for the assay of allopurinol drug substance and for the detection of 3-amino-4-carboxamidopyrazole, its main degradation product (2). Because of its simplicity and good agreement between results by this method and those obtained by the spectrophotometric method of USP XX (3), it was decided to conduct a collaborative study of the LC method.

Collaborative Study

Collaborators from 7 laboratories received blind duplicate samples of 2 commercial tablets and 1 synthetic tablet powder. Each collaborator was also furnished an instruction sheet and a copy of the method. No restriction was placed on the source of the chromatographic column needed in the study.

Allopurinol in Tablets
Liquid Chromatographic Method
First Action

Principle

Allopurinol content is detd by liq. chromatgy on reverse phase column with aq. ammonium phosphate mobile phase, UV detection, and hypoxanthine internal std.

Apparatus

(a) *Liquid chromatograph.*—Model 950 solv. pump with Model 970A variable wavelength detector capable of monitoring *A* at 254 nm (Tracor Instruments, Inc., Austin, TX 78721), 20 μL injection valve (Valco Instruments, Inc., Houston, TX 77055), and suitable strip chart recorder, or equiv. LC system.

(b) *Chromatographic column.*—Stainless steel, 300 × 3.9 mm id, packed with μBondapak 10 μm (Waters Associates, Inc., Milford, MA 01757) or equiv. column meeting LC system suitability requirements.

Reagents

(a) *Ammonium phosphate.*—Monobasic, FW 115.031 (Fisher Scientific Co., Fair Lawn, NJ 07410), or equiv.

(b) *Hypoxanthine.*—Reagent grade (Fisher Scientific Co.), or equiv.

(c) *Mobile phase.*—Prep. 0.05M ammonium phosphate (monobasic) soln 5.75 g $(NH_4)H_2PO_4$/L H_2O. Degas under vac. or by ultrasonic treatment 5–10 min. (Do not leave mobile phase in column overnight; after draining mobile phase, flush entire system ≥20 min with H_2O, followed by MeOH for 20 min.)

(d) *Internal std prepn.*—Accurately weigh ca 50 mg hypoxanthine and transfer to 50 mL vol. flask. Add 10 mL 0.1N NaOH and shake mech. 10 min, or until completely dissolved. Dil. with H_2O to vol., and mix. Prep. this soln fresh daily.

(e) *Std prepn.*—Accurately weigh ca 50 mg USP Allopurinol Ref. Std and transfer to 50 mL vol. flask. Add 10 mL 0.1N NaOH and shake mech. 10 min. Dil. with H_2O to vol., and mix. Prep. this soln fresh daily. Transfer 4.0 mL of this soln to 200 mL vol. flask, add 2.0 mL internal std prepn, dil. with mobile phase to vol., and mix.

Sample Preparation

Weigh and finely powder ≥20 tablets. Accurately weigh portion of powder equiv. to ca 50 mg allopurinol, and transfer to 50 mL vol. flask. Add 10 mL 0.1N NaOH, shake mech. 10 min, dil. with H_2O to vol., and mix. From this point, proceed with detn without delay. Filter portion of soln thru suitable paper or 0.45 μm membrane filter, discarding first 10 mL filtrate. Transfer 4.0 mL filtrate to 200 mL vol. flask, add 2.0 internal std prepn, dil. with mobile phase to vol., and mix.

Suitability Test and Determination

Inject equal vols (10–20 μL) of sample prepn and std prepn into liq. chromatograph by means of sampling valve or high pressure microsyringe. Operate chromatograph at ambient temp. while pumping mobile phase at 1.5 mL/min. Adjust detector sensitivity so that peak response for allopurinol and hypoxanthine is 40–60% AUFS. In suitable chromatogram, lowest and highest peak response ratios of 3 successive injections of std prepn do not differ >3.0%, and resolution factor, *R*, for sample peak and internal std peak is ≥5. Retention times are hypoxanthine 7 min, allopurinol 12 min.

Calculation

Calc. amt of allopurinol in dosage form, using response ratios based on either peak hts or peak areas, according to following equation:

$$\text{mg/tablet} = (R/R') \times (W'/W) \times T$$

where R and R' = response ratios of allopurinol peak to internal std peak for sample and std, resp.; W and W' = mg taken for sample and std prepns, resp.; and T = av. tablet wt, mg.

Results and Discussion

When the liquid chromatograph was operated isocratically with a mobile phase flow rate of 1.5 mL/min, hypoxanthine and allopurinol were eluted with approximate retention times of 7 and 12 min, respectively. As shown in Figure 1, baseline resolution between these 2 compounds was achieved under the described experimental conditions. During the develop-

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The recommendation of the Associate Referee was approved by the General Referee and Committee B and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.

Table 1. Collaborative results (%) for determination of allopurinol in commercial and synthetic tablets by liquid chromatography

Coll.	Found, % of declared 100 mg/tab.	300 mg/tab.	Synthetic rec., %[a]
1	98.0	98.0	99.6
	99.2	98.8	100.0
2	102.1	99.7	99.8
	101.2	99.1	100.8
3	101.6	100.3	99.1
	102.5	99.9	101.3
4	98.0	97.9	100.0
	99.1	98.2	99.2
5	99.8	100.2	98.6
	99.6	99.7	99.4
6	100.0	100.0	101.5
	99.5	99.7	100.9
7[b]	97.5	93.3	95.6
	96.1	93.3	95.3
Mean	100.1	99.3	100.0
SD	1.49	0.87	1.04
CV, %	1.49	0.88	1.04
Reproducibility SD	1.55	0.90	0.93
Reproducibility CV, %	1.55	0.91	0.93
Repeatability SD	0.62	0.36	0.80
Repeatability CV, %	0.62	0.37	0.80

[a]Prepared to contain 31.9 mg allopurinol per 100 mg formulation.
[b]Outlier by ranking. Not included in statistical summary.

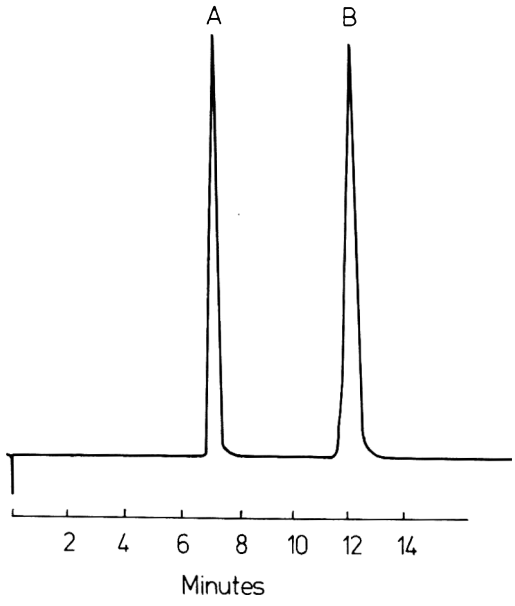

Figure 1. LC separation of A, hypoxanthine, the internal standard, and B, allopurinol.

mental stage of the method it was also possible to resolve allopurinol from its main degradation product, 3-amino-4-carboxamidopyrazole, which may be present in pharmaceutical dosage forms. This compound typically eluted with a retention time of approximately 4 min, and could be detected at a level of about 0.2%.

The collaborative results, together with the descriptive statistics, are presented in Table 1. All assays were conducted in duplicate. Results from Collaborator 7 showed systematic bias and were not included in the statistical calculations. This collaborator initially encountered difficulties in obtaining satisfactory sensitivity and minimal baseline noise, but noted some improvements when the detector and recorder were replaced. The remaining 6 laboratories reported no difficulties with the method. The combined mean reproducibility CV and the mean repeatability CV for the 2 commercial tablet samples were <1.3% and <0.50%, respectively; the respective values for the synthetic formulation were <1.0% in both cases.

Recommendation

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

Acknowledgments

The Associate Referee thanks Cesar A. Lau-Cam, Science Advisor, Food and Drug Administration, New York Regional Laboratory, Brooklyn, NY, and Professor of Pharmacognosy, St. John's University, College of Pharmacy and Allied Health Professions, Jamaica, NY, for his assistance in the preparation of this manuscript. Thanks are also extended to the following collaborators, all from the Food and Drug Administration, who participated in the study: M. P. Uribe, Dallas, TX; T. Poplawski, Brooklyn, NY; G. D. Reed, Kansas City, MO; F. Smith, Chicago, IL; D. Krieger, Brooklyn, NY; S. E. Roberts, Winchester, MA; T. S. Savage, Los Angeles, CA.

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Spectrophotometric Determination of Pentaerythritol Tetranitrate in Tablets

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A sensitive spectrophotometric method is reported for the quantitative determination of pentaerythritol tetranitrate in tablets. The method is based on reduction of pentaerythritol tetranitrate with zinc and calcium chloride, and reaction of the nitroso compound thus formed with 1-naphthylamine in acidic medium. The reaction gives a purple product having an absorbance maximum at 545 nm. Beer's law is obeyed in the concentration range of 1–10 μg/mL of reaction mixture. The presence of meprobamate in tablets does not interfere with the proposed analytical determination.

Pentaerythritol tetranitrate, 1,3-propanediol-2,2-bis[(nitrooxy)methyl]dinitrate, is used as a coronary vasodilator in the prophylactic management of patients with angina pectoris. Methods proposed for estimating pentaerythritol tetranitrate involve volumetric (1), spectrophotometric (2–5), titrimetric (6, 7), polarographic (8, 9), potentiometric (10, 11), and chromatographic (12, 13) techniques. The official compendia (14, 15) describe a spectrophotometric method for the determination of pentaerythritol tetranitrate in tablets.

We developed a simple, rapid, sensitive, and accurate method for estimating pentaerythritol tetranitrate. Various conditions such as volume and concentration of reagent, reduction times, and color development have been standardized.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Double beam, UV 240 nm (Shimadzu, Japan).

(b) *Dilute pentaerythritol tetranitrate BPC (14, 16).*—20% in lactose.

(c) *Zinc dust.*

(d) *Calcium chloride solution.*—10% in water.

(e) *1-Naphthylamine solution.*—0.2% in glacial acetic acid.

Preparation of Standard

Accurately weigh 125 mg dilute pentaerythritol tetranitrate BPC (equivalent to 25 mg pentaerythritol tetranitrate). Add 80 mL methanol, shake, and filter through Whatman No. 1 paper into 100 mL volumetric flask. Dilute solution to volume with same solvent.

Preparation of Standard Curve

Pipet 0.1, 0.2, etc., up to 1.0 mL pentaerythritol tetranitrate standard solution into series of 100 mL conical flasks, each containing 1.5 g zinc dust and 5 mL calcium chloride solution. Let stand 10 min at room temperature (27°C) with occasional shaking. Filter through Whatman No. 1 paper into separate 25 mL volumetric flasks. Wash each residue thoroughly with three 2 mL portions of methanol and add washings to flasks. Add 5 mL 1-naphthylamine solution to each flask, mix well, and let stand 20 min at room temperature (27°C). Adjust to volume with water and measure absorbance at 545 nm against blank prepared concomitantly.

Plot of absorbance vs drug concentration should be linear within range of 1–10 μg/mL.

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Preparation of Samples

Weigh and powder ≥ 20 tablets. Transfer amount of powder equivalent to 25 mg pentaerythritol tetranitrate to 100 mL conical flask. Add 80 mL methanol, shake, and filter through Whatman No. 1 paper into 100 mL volumetric flask. Dilute solution to volume with same solvent.

Pipet 0.5 mL sample solution into 100 mL conical flask, and proceed as in *Preparation of Standard Curve*, starting with ". . . containing . . . ."

Results and Discussion

Pentaerythritol tetranitrate, when reduced with zinc and calcium chloride solution, yields a nitroso derivative that further condenses with 1-naphthylamine in glacial acetic acid to produce a purple chromophore having an absorbance maximum at 545 nm. The colored species exhibited stability for 6 h at room temperature; a gradual decrease in absorbance was observed thereafter.

Various conditions have been standardized for the proposed analytical determination.

Effect of zinc dust.—Maximum intensity of color was developed with 1.5 g zinc dust; further increase in amount of zinc dust did not show any measurable change.

Effect of calcium chloride solution.—5 mL calcium chloride solution (10%) along with 1.5 g zinc dust was required for complete reduction of the drug solution in the working concentration range of 1–10 μg/mL.

Effect of reduction time.—Maximum color intensity was exhibited after reduction up to 10 min at room temperature; further increase in time showed no change in the absorbance values.

Effect of volume of 1-naphthylamine reagent solution.—To determine the volume of reagent required for maximum color intensity, varying quantities of reagent solution (e) were added to a fixed volume (1.0 mL) of standard drug solution; 5 mL reagent solution was sufficient to develop the color to full intensity.

Effect of concentration of reagent.—A series of experiments showed that a reagent concentration of 0.2% 1-naphthylamine in glacial acetic acid was optimum for maximum intensity of color.

Effect of time for color development.—The intensity of color increased up to 20 min and remained stable thereafter for 6 h at room temperature. Absorbance decreased gradually after this period.

The presence of meprobamate in tablets does not interfere in the determination of pentaerythritol tetranitrate. Commonly encountered tablet excipients such as starch, talc, lactose, magnesium stearate, and coloring agents, viz., brilliant blue and tartrazine, also do not interfere in the determination. The results of the proposed method compare favorably with those of the official method (Table 1).

Recovery experiments were conducted to check suitability and accuracy of the method. Known quantities of dilute pentaerythritol tetranitrate BPC were added to the pre-analyzed ground samples of the tablet, extracted with sufficient quantity of methanol, and filtered. The flasks were thoroughly washed with small quantities of methanol and the washings were added to the drug solution. The solution was then ana-

Table 1. Analysis of pentaerythritol tetranitrate in tablets by the official and proposed methods

Tablet	Amount found		Dilute pentaerythritol tetranitrate BPC added, mg	Recovery, % ± SD
	Official method, % ± SD	Proposed method, % ± SD[a]		
T_1	98.56 ± 0.224	98.33 ± 0.046	250.0	102.22 ± 0.125
T_2	98.00 ± 0.461	98.55 ± 0.050	125.0	103.05 ± 0.045
T_3[b]	101.25 ± 0.070	102.58 ± 0.046	62.5	97.76 ± 0.090
T_4[c]	100.22 ± 0.342	100.96 ± 0.429	125.0	99.16 ± 0.105

[a]Average of 3 determinations.
[b]Pentaerythritol tetranitrate + meprobamate.
[c]Pentaerythritol tetranitrate, 80 mg tablet.

lyzed by the proposed method. Percentage recovery and standard deviations were calculated and were acceptable.

It can thus be concluded that the proposed analytical method is simple, accurate, sensitive, and reproducible and can be used in the analytical control of pentaerythritol tetranitrate in tablets.

Acknowledgments

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SUGARS AND SUGAR PRODUCTS

Gas Chromatographic Determination of Nonvolatile Organic Acids in Sap of Sugar Maple (*Acer saccharum* Marsh.)

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Qualitative analysis of organic acids has never been reported for sugar maple sap, but only for its products, "sugar sand" and maple syrup. A gas chromatographic (GC) method is described for the simultaneous determination of up to 13 nonvolatile organic acids in sugar maple sap. Sap is filtered through Celite, and acids are isolated via cation- and anion-exchange chromatography. Reaction of dried acids with BSA [*N,O*-bis(trimethylsilyl)acetamide] in the presence of pyridine and methoxyamine hydrochloride yields the more volatile TMS (trimethylsilyl) esters. Oxalic, succinic, fumaric, L-malic, tartaric, *cis*-aconitic, citric, and/or shikimic acids were found in maple sap at concentrations ranging from less than 50 ppb to more than 45 ppm, depending on the particular acid and the date of sap flow. Percent recoveries and coefficients of variation for the acids at the 500 ppm level were 46.0 (3.2), 92.0 (2.9), 73.0 (0.77), 94.0 (2.0), 95.0 (–), 72.0 (–), and 97.0 (0.38), respectively. Various amounts of nonvolatile organic acids are reported in the sap of one sugar maple tree throughout a sap season, and of 3 individual maples during an early sap flow. Quantitation limits were as low as 15 ppb for individual acids in the analysis of a 100 mL sap sample. Esters were separated on a mixed liquid phase column of 4% SE-52/2% SE-30 on Chromosorb W-HP. They were identified by relative retention time, using a dual flame ionization detector. Naphthalene was used as the internal standard. Concurrent identification of pyruvic, malonic, glutaric, α-ketoglutaric, *cis*-aconitic, and isocitric acids with those previously mentioned is also possible.

Organic acids are important components in the flavor profile of many foods (1–3). They have been considered to influence color (1, 2, 4) and rate of spoilage (1, 2), and to act as indices of ripeness and adulteration (3, 5, 6).

The differential presence of organic acids in sugar maple sap products has been known for many years, but we found no references to qualitative analysis of those acids in maple sap. An early examination of "sugar sand," which Von Lippmann (7) called "calcium salts of organic acids" that precipitate during the maple sap boiling process, revealed L-malic acid as the major acid, followed by tartaric acid. Later Nelson (8) isolated traces of other nonvolatile organic acids (citric, fumaric, and succinic) in maple syrup by precipitation as lead salts, but tartaric acid was not found. These salts were esterified and fractionated by distillation. Hydrazides prepared from the fractions were identified by melting point and optical crystallographic examination.

Discovery of these trace acids in maple syrup necessitated the development of a more sensitive, selective, and rapid method for their determination in maple products. In 1951, Porter et al. (9) analyzed, by an anion-exchange procedure, one sample of commercial maple syrup diluted with water to 15%, and found malic, citric, succinic, fumaric, and other unidentified, nonvolatile organic acids. This author was incorrectly cited by Willits in the *Maple Producers Manual*, first published in 1958 and revised a few times until 1976 (10), as a reference for values of organic acids in maple sap. In 1959, the ion-exchange procedure to determine malic acid value as a measure of maple syrup purity was adopted by AOAC, under the assumption that malic acid levels are constant in all maple syrups (11–13). The procedure was powerful in its ability to selectively isolate and concentrate small amounts of acids from maple syrup—a complex natural sap product, high (about 66%) in total solids content (10). Further improvements in methodology were still needed, however.

Methods combining ion-exchange chromatography and gas chromatography (GC) have been demonstrated to overcome many of the deficiencies of earlier methods. A wide range of biological materials including grape juices (1, 3), strawberries (6), and plant tissues (14–18), have been successfully analyzed for organic acids by ion-exchange/GC methods.

We have adapted ion-exchange/GC methods, and we have analyzed nonvolatile organic acids in maple sap of one sugar maple tree (*Acer saccharum* Marsh.) throughout a sap season, and of 3 individual maples during an early sap flow. All trees were aseptically tapped. Sugar maple sap is a low density nutrient carbohydrate solution (average 2.5% total solids content) with less than 1% other compounds (10, 19–21), some of which are still unknown. They are the biochemical precursors of the different colors, flavors, and off-flavors of maple syrup.

METHOD

Apparatus

(a) *Gas chromatograph.*—Model 800, equipped with dual flame ionization detector (Perkin-Elmer Corp., Norwalk, CT 06856), or equivalent. Columns: 6 ft × ⅛ in. od, stainless steel, containing 80–100 mesh Chromosorb W-HP, coated with mixed liquid phase of 4% SE-52 and 2% SE-30 (Applied Science Laboratories, Inc., State College, PA 16801), or equivalent. Operating conditions: carrier gas (nitrogen), 30 mL/min; temperature program: 90 to 215°C at 5°/min; inlet, 200°C; detector, 260°C.

(b) *Recorder.*—1 mV with 0.5 cm/min chart speed (Perkin-Elmer Model 023), or equivalent.

(c) *Syringes.*—Hamilton syringe, 10 μL capacity, and a gas-tight syringe, 5 mL capacity (Hamilton Co., Reno, NV 89510).

(d) *Rotary vacuum evaporator.*—Büchi Rotavapor M, with heating bath (Brinkmann Instruments, Westbury, NY 11590), or all-glass equivalent.

(e) *Magnetic stir plate.*—Dylastir® (VWR Scientific Inc., Boston, MA 02101), or equivalent.

(f) *Reacti-Vials.*—With Teflon-lined septa, 5 mL, and triangular magnetic stir bars (Pierce Chemical Co., Rockford, IL 61105).

(g) *Chromatographic tubes.*—1 × 50 mL, Kimax burets, with Teflon stopcocks (VWR Scientific Inc.), or equivalent.

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Table 1. Percent recovery of organic acids via ion-exchange procedure at 500 ppm level

Acid	Rec.,[a] %	SD	CV,[b] %
Oxalic	46.0	1.5	3.2
Succinic	92.0	2.7	2.9
Fumaric	73.0	0.57	0.77
L-Mallc	94.0	1.8	2.0
Tartaric	95.0[c]	—	—
cis-Aconitic	72.0[c]	—	—
Citric	97.0	0.37	0.38

[a]Each value is the average of 2 determinations.
[b]Coefficient of variation = relative standard deviation.
[c]One determination only.

(h) *Lyophilizer.*—Freezemobile® 24 (Virtis, Gardiner, NY 12525), or equivalent vacuum sublimator.

(i) *Freezer.*—Ultra-Low® (Revco Inc., West Columbia, SC 29169), or equivalent (minimum temperature −115°C).

Reagents

(a) *Solvents.*—Ethyl ether, anhydrous (J. T. Baker Chemical Co., Phillipsburg, NJ 08865); pyridine, silylation grade (Pierce Chemical Co.).

(b) *Hydrochloric acid.*—2N. Add 172 mL HCl to water in 1 L volumetric flask Dilute to volume.

(c) *Sodium formate.*—2N. Dissolve 136 g $CHNaO_2$ in water in 1 L volumetric flask. Dilute to volume.

(d) *Dilute formic acid.*—44%. CH_2O_2 (1 + 1).

(e) *Silylating reagent.*—BSA (*N,O*-bis(trimethylsilyl)-acetamide) (Pierce Chemical Co.).

(f) *Organic acid solutions.*—500 μg acid/mL. Dry acid crystals (Sigma Chemical Co., St. Louis, MO 63178) 24 h in vacuum desiccator over anhydrous $CaSO_4$. Accurately weigh 0.5 g of each acid. Dissolve in water in 1 L volumetric flask. Dilute to volume. Determine purity and exact concentration by titration with standardized NaOH (12), to phenolphthalein end point.

(g) *Sodium hydroxide standard.*—Dissolve 80 g pellets in water in 1 L volumetric flask. Dilute to volume. Determine exact concentration by titration against primary standard (12).

(h) *Phenolphthalein indicator.*—Prepare 1% $C_{20}H_{16}O_4$ (w/v) in 80 mL ethanol. Dilute to 100 mL with water.

(i) *Organic acid standard solutions.—Stock solutions.*—Accurately weigh 0.001–0.01 g dry acid (corrected for purity, as determined in (f)) into 5 mL Reacti-Vial® containing triangular magnetic stir bar. Cap tightly using Teflon-lined septum. Add 2 mL pyridine via syringe. Stir until dissolved. *Working standards.*—Transfer appropriate aliquots of stock solution, via syringe, to Reacti-Vials. Dilute with pyridine as needed.

(j) *Filter aid.*—Celite® (Johns-Manville Products Corp., Denver, CO 80217).

(k) *Naphthalene GC internal standard solution.*—1 mg/mL.

(l) *Methoxyamine hydrochloride.*—(Eastman Organic Chemicals, Rochester, NY 14650).

Column Preparation

Prepare anion-exchange column by adding slurry of Dowex 1-X8 (200–400 mesh, formate form (Bio-Rad Laboratories, Richmond, CA 94804)) in 2N $CHNaO_2$ to height of ca 10 to 40 cm in column containing glass wool plug. Wash column with three 10 mL portions of 2N $CHNaO_2$, then with three 10 mL portions of water.

Prepare cation-exchange column by adding slurry of Dowex AG5OW-X2 (50–100 mesh, H^+ form (Bio-Rad Laboratories)) in 2N HCl to height of ca 15 cm in column containing glass wool plug. Wash column with three 10 mL portions of 2N HCl, and then with water until neutral to pH indicator paper.

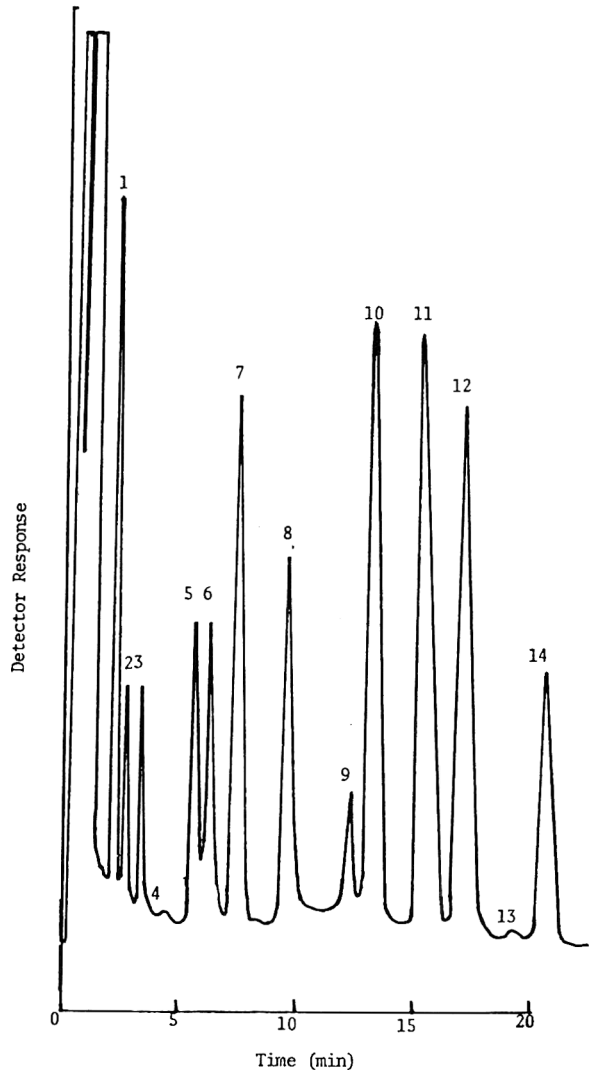

Figure 1. Gas chromatogram of standard organic acid esters. 1, pyruvic; 2, oxalic; 3, naphthalene (int. std); 4, malonic; 5, succinic; 6, fumaric; 7, glutaric; 8, L-malic; 9, α-ketoglutaric; 10, tartaric; 11, *cis*-aconitic; 12, citric/shikimic; 13, shikimic derivitive; 14, isocitric. Attenuation × 50.

Sample Collection

Five 50 mL maple sap samples were aseptically collected in sterile plastic bags during each sap flow throughout 1980 maple sap season from one sugar maple tree. On one date, sap was again aseptically collected at the same time from 2 other trees. Sap samples were immediately frozen at −20°C.

Sample Preparation

Place polyethylene bag containing frozen sap into beaker of warm water. Thaw to room temperature. Filter sap by suction through 1 cm layer of Celite in 5 cm diameter Buchner funnel.

Ion-Exchange Chromatography

Quantitatively transfer ca 100 mL filtered sap to cation-exchange column. Elute at 3 mL/min directly into anion-exchange column. When level of sap just touches top of cation resin, wash with 3 × 10 mL water. Add washes to anion-exchange column. Because of fine particle size of anion resin, fluid level will not go below top, and flow will be restricted to ca 1 mL/min. When cation eluate reaches top of anion column, wash with 3 × 10 mL water. Discard eluate and

Table 2. Organic acid esters in sugar maple sap (per 100 mL) throughout a maple season[a]

Flow date, 1980	Sample size, mL	Oxalic	Succinic	Fumaric	L-Malic	Tartaric	*cis*-Aconitic	Citric/Shikimic	Total
					Parts per Billion (ppb)				
3/20	156	760	2300	200	4300	95	100	140	7895
3/23	151	260	600	55	1400	46	—[b]	46	2407
3/27	101	130	740	1300	12 000	110	160	150	14 590
3/31	111	—[b]	340	30	45 000	36	—[b]	—[b]	45 406
4/07	148	—[b]	570	6800	12 000	67	320	110	19 867
					Individual Acids as % of Total Acids				
3/20		9.6	29.0	2.8	54.0	1.2	1.3	1.7	
3/23		11.0	25.0	2.3	58.0	1.9	—	1.9	
3/27		0.9	5.2	8.8	82.0	0.8	1.1	1.1	
3/31		—	0.7	<0.1	99.0	<0.1	—	—	
4/07		—	2.9	35.0	59.0	0.3	1.6	0.6	

[a]Five sap collecting dates, representing early-to-late sap flow season, from 1 sugar maple tree, one determination.
[b]Not detected using the above given sample size (quantitation limit = 15 ppb).

place 200 mL round-bottom flask beneath anion-exchange column. Elute organic acids with 10 mL formic acid solution. Wash with three 10 mL portions of water. Place flask containing ca 40 mL acidic eluate on rotary evaporator in 65°C water bath. Aspirate overnight or until the odor of formic acid is removed. Quantitatively transfer aqueous organic acid solution to 50 mL Erlenmeyer flask. Freeze at −70°C, and take to dryness in lyophilizer or vacuum sublimator.

Recharge cation column with 25 mL 2N HCl. Wash with three 25 mL portions of water, or until eluate is neutral to pH indicator paper. Recharge anion-exchange column with 25 mL 2N sodium formate. Wash with two 25 mL portions of water or until eluate is neutral. Column should be recharged after every sample to assure reproducible recoveries.

Derivitization/GC

Dissolve dry acids in 10 mL ether. Quantitatively transfer and evaporate solution in 5 mL Reacti-Vial containing magnetic stir bar. Add ca 5 mg methoxyamine hydrochloride and 1 mg naphthalene to vial. Dissolve with 1 mL pyridine and stir well. Add 2 mL BSA and stir 1 h to allow for complete reaction. Inject 3 μL sample via syringe into gas chromatograph. Attenuate as needed to keep peaks on scale. Final GC determinations are most reproducible after 18 h.

Calculations

Calculate response factor *(R)* for each acid just before determination. Linear response of detector over concentration range in question allows use of the following equation: std concn (ppm)/std peak ht (cm) = R (ppm/cm). The following formula was used to calculate individual organic acid concentrations in sap:

$$\text{Acid in sap, ppb} = (P \times R \times C)/(N \times I \times E)$$

where P = peak height (cm); R = response factor (ppm/cm peak height); N = number of 100 mL aliquots of sample used; I = injection size of sample/injection size of standard = 2; E = recovery factor (% recovery/100, Table 1); C = concentration factor (1000 (ppb/ppm)/33.33 = 30).

Recovery Studies

Recovery studies of individual organic acid esters via the ion-exchange procedure were done by running in triplicate 100 mL standardized aqueous solutions of individual organic acids (ca 500 ppm) through the lyophilization step. Recoveries were calculated by dissolving recovered acids in water and subsequently titrating them with standardized NaOH to phenolphthalein end point.

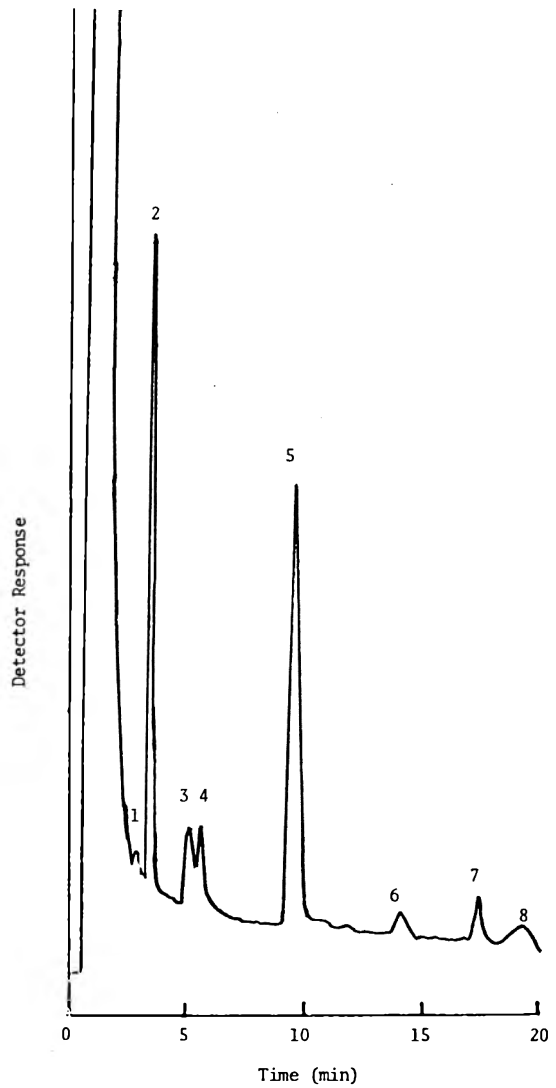

Figure 2. Gas chromatogram of organic acid esters in maple sap (one tree, early season sap, 3/23/80, 151 mL sap sample). 1, oxalic; 2, naphthalene (int. std); 3, succinic; 4, fumaric; 5, L-malic; 6, tartaric; 7, citric and/or shikimic; 8, shikimic derivative. Attenuation × 10.

Results and Discussion

In the method described, the ion-exchange procedure performed several tasks. In the cation-exchange column, interfering unknown colored compounds are removed from the sap. Also, organic acid salts are converted to free acids.

Table 3. Organic acid esters in sugar maple sap[a] (per 100 mL) in 3 individual sugar maples

Tree	Sample size, mL	Oxalic	Succinic	Fumaric	L-Malic	Tartaric	*cis*-Aconitic	Citric/Shikimic	Total
					Parts per Billion (ppb)				
1	151	260	600	55	1400	46	—[b]	46	2407
2	150	350	240	300	880	79	—[b]	190	2039
3	154	—[b]	710	1400	10 000	45	120	91	12 336
					Individual Acids as % of Total Acids				
1		11.0	25.0	2.3	58.0	1.9	—	1.9	
2		17.2	11.8	14.7	43.2	3.9	—	9.3	
3		—	5.7	11.3	80.9	0.4	1.0	0.7	

[a]One sap collection date (3/23/80), representing early sap flow, one determination per tree.
[b]Not detected using the above given sample size (quantitation limit = 15 ppb).

Recoveries are 100 ± 1% through this column for all acids. Direct elution into the anion-exchange column reduced analysis time significantly. In the anion-exchange column, free acids are retained while sugars and other interfering neutral components of the sap are removed. The acids are eluted as a group by mass action to a final volume of 40 mL and 2–4 times their concentration in sap. Recovery studies were performed because some acids are lost when the anion-exchange column is washed free of sugars. Although recovery levels (Table 1) were generally good, lower recoveries of oxalic, aconitic, and fumaric acids indicate that unsaturated acids are poorly adsorbed by Dowex AG50W-X2. Preliminary tests indicated that a 100 mL sap sample could contain 5 mg or more of acid. Because sap organic acids had not been previously determined, and to ensure that the columns would not be overloaded during a determination using ca 100 mL sap sample, they were tested for recovery with a 10-fold excess of acid (500 ppm). Tests on solutions containing a lower concentration of acid gave similar recovery results, as did sap samples that were spiked with standardized acids. Ideally, triplicate recovery determinations should be made; however, metals released by formic acid in one rotary evaporator which was not "all-glass," rendered some recovery data invalid. Only results from duplicate recovery determinations using an all-glass rotary evaporator are reported (Table 1) and used in the calculations.

To apply GC to the analysis of organic acid esters, it is first necessary to render the acids more volatile. Formation of TMS derivatives has been proven a rapid and convenient method to achieve this end (2, 15, 16). Derivitization increases detector response and the stability of the acids, while helping to decrease peak tailing. Detector response per unit weight of acid varied greatly among the different acids. In general, however, detector responses were higher for organic acids of higher molecular weight and/or greater number of derivitizable groups. We found that derivitization was essentially complete after 1 h, but results were most reproducible after about 18 h. This is especially true in the case of shikimic acid, which is silylated slowly and incompletely by BSA. Methoxyamine hydrochloride was added to the reaction mixtures before silylation to facilitate derivitization of α-keto acids, had they been present. Samples were injected after 1 h to check for traces of aconitic acid, because it has been found to desilylate with time (15), and to allow for modification of attenuation settings before the actual determination. Detection limits vary according to the sap sample size and the detector response for individual acids.

Silylation decomposition products accumulate rapidly on the detector anode. They were removed periodically with dichlorofluoromethane to maintain optimum detector sensitivity.

A preliminary investigation using a set of 6 ft × ⅛ in. columns packed with 3% OV-17 on Chromosorb W-HP was conducted, but broad peaks at later retention times were a problem for quantitation by peak height. Also, these columns could not resolve succinic and fumaric acids. The OV-17 column results do, however, confirm the presence of other acids, later identified with the mixed liquid phase columns. Figure 1 shows the separation of 13 standard organic acid esters on the mixed liquid phase columns used in the determination. Chromatographic conditions were selected to optimize the succinic/fumaric separation and to keep run times as short as possible. Reducing the temperature program rate to 2°C/min will permit further separation of succinic and fumaric acids and partial separation of even the *cis*- and *trans*-isomers of aconitic acid. No temperature program resolves citric and shikimic acids on these columns. Use of a 3% XE-60 column to completely separate these 2 acids in 7 min has been suggested (16).

Sap from a single tree was aseptically collected on 5 dates throughout a sap season. Oxalic, succinic, fumaric, malic, tartaric, aconitic, citric, and/or shikimic acids were found. Individual and total acid concentrations vary greatly over the season. Malic acid always predominates, although concentration varies from 0.8 to 45 ppm, comprising 43–99% of total acids (Tables 2 and 3). The so-called minor acids are also present in sufficiently large quantities in most samples. These acids should be included in the determination and interpretation of a full chemical profile of maple sap and its products. While Table 2 shows seasonal changes for each acid in the sap of one tree throughout a maple season, Table 3 compares the sap of 3 maple trees during an early season sap flow. Nelson (8) and Porter (9) identified citric but not shikimic acid in maple syrup, and we have identified shikimic acid in sap by the presence of its partially silylated derivative (Figure 2). We do not know, however, if both citric and shikimic acids are present in sap, or if citric is a product of sap boiling. The relative concentrations of the 2 acids are not known at this time. For ease of data handling, we give results in this paper without making the distinction.

The variety of similarities, differences, and trends evident in data from Tables 2 and 3 demonstrate different modes of metabolism in maple trees at different points during the sap season, which ends when tree growth resumes. An interesting point is that the tartaric acid concentration, although low, remains fairly constant in aseptically collected sap, both throughout the season and between trees, while the malic acid concentration varies considerably.

Although other previously described methods may be used to determine organic acid esters in maple sap and its products, we have demonstrated that gas chromatography provides greater resolution.

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Instrumental Color Classification of Honey: Collaborative Study

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A collaborative study was carried out to test the use of the Lovibond 2000 honey color comparator. Fourteen collaborators classified 6 honeys in the test. Results were generally favorable and the method has been adopted official first action.

The color of honey has been a factor in its grading and marketing for many years. Lighter color is associated with more acceptable flavor and higher price. Honey is classified by the U.S. Department of Agriculture (USDA) into 7 color categories by reference to the specifications of glass standards (defined in the U.S. Standards for Grades of Extracted Honey (1)). These were developed by Brice et al. (2, 3) to match the color of the previously used Pfund color grader at specified settings. The Pfund grader, used unofficially by the trade, provides continuous readings over the entire color range. USDA scientists use the commercially available USDA color classifier to describe honey and assign it to one of the 7 color classes.

A collaborative test of the latter instrument was completed in 1959 (4), and the USDA procedure was adopted by AOAC. In that test, 6 honey samples were classified in 5 USDA laboratories; in one of those laboratories, 4 individuals classified the samples. Of the 47 judgments (one sample was lost), 46 agreed with the consensus.

The present paper reports a collaborative test of a procedure using the Lovibond 2000 honey color comparator. This instrument consists of a viewing box containing an adjustable cell compartment with 33 mm cells and a detachable disc carrying 6 circular color glasses; the color of these glasses is intended to match that of the USDA color glasses, corrected by the difference in cell thickness (31.5 mm USDA, 33 mm Lovibond).[1] The disc can be rotated to bring any color glass into juxtaposition with the sample viewing port.

This report of the Associate Referee was presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

Submitted for approval November, 1984.

The recommendation of the Associate Referee was approved by the General Referee and Committee D and was adopted by the Association. See the General and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.

[1] A version of this instrument with 10 mm cells and corresponding color glasses has been commercially available.

Collaborative Study

Nine 2 lb samples of filtered honey ranging from 15.5 to 121 mm Pfund were obtained. These were slightly warmed and blended into 6 lots of honey for the test; each lot was thoroughly mixed. The master samples were classified by using the USDA color classifier, and aliquots were placed in 2 oz square wide-mouth, polyethylene screw-cap bottles for shipment by first class mail. A report sheet and instructions were included.

Samples were sent to 10 laboratories of the Processed Products Branch, Fruit and Vegetable Division, Agricultural Marketing Service, USDA, and to 6 laboratories possessing the necessary equipment, all of whom had agreed to collaborate. Because the number of instruments was limited, 5 were shared among 10 USDA laboratories. All samples supplied were clear, so the "cloudy suspensions" used to facilitate comparisons of turbid honeys were not furnished.

Color Classification of Honey
Instrumental Method II
First Action

Principle

Using com. comparator, honey is visually compared to std color glasses representing USDA color class limits.

Apparatus and Reagent

(a) *Color comparator.*—Lovibond 2000 (The Tintometer Co., 206 Packets Ct, Williamsburg, VA 23185).

(b) *Cloudy suspensions.*—Diat. earth (Hyflo Super-Cel, or equiv.), 100, 200, 400 mg/L H_2O. Stabilizer such as cellulose gum (0.5 g/L) or sorbic acid (0.1%) may be included.

Table 1. Collaborative results for instrumental classification of honey color

Coll.	Sample A	B	C	D	E	F	Diff. from consensus
1	W	A	LA	W	DA	ELA	1
2	EW	A	LA	W	DA	ELA	0
3	EW	A	LA	W	DA[a]	ELA	0
4	EW	A	LA	W	DA[a]	ELA	0
5	W	A	LA	W	DA	ELA	1
6	EW	A	LA	W	DA	ELA	0
7	EW[a]	A[a]	LA	W	A	ELA[a]	1
8	EW	A	LA	W	A	ELA	1
9	EW	A	LA	W	DA	ELA	0
10[b]	W	DA	LA	ELA	DA	LA	4
11	W	A	LA	ELA	DA	ELA	2
12A	EW	A	LA	W	DA	ELA	0
12B	EW	A	LA	W	A	ELA	1
13	EW	A	LA	W	DA[a]	ELA	0
14[c]	EW	A	LA	W	DA	ELA	0
USDA[c]	W	A	LA	W	DA	ELA	—

[a]Results are corrected interpretation of color, based on data provided in data sheet; see text.
[b]Data excluded from calculations; see text.
[c]Data of Associate Referee.

Table 2. Chi-square test of collaborative data

Coll.	Sample A	B	C	D	E	F	
1	0	1	1	1	1	1	
2	1	1	1	1	1	1	
3	1	1	1	1	1	1	
4	1	1	1	1	1	1	
5	0	1	1	1	1	1	
6	1	1	1	1	1	1	
7	1	1	1	1	0	1	
8	1	1	1	1	0	1	
9	1	1	1	1	1	1	
11	0	1	1	0	1	1	
12A	1	1	1	1	1	1	
12B	1	1	1	1	0	1	
13	1	1	1	1	1	1	
14	1	1	1	1	1	1	
N^1	11	14	14	13	11	14	77(B)
N^2	121	196	196	169	121	196	999(N^1)
X^1	10	13	13	12	10	13	71(A)

$$P_1 = \frac{71}{6\,(14 - 1)} = 0.9103$$

$$P_2 = \frac{77 - 71}{6} = 1$$

$$X_c = \frac{((13 \times 77) - (14 \times 71))^2}{14 \times 77 - 999} = \frac{49}{79} = 0.6203$$

Determination

Clear honey by minimal warming if necessary and let stand to clear bubbles as far as possible. Pour sample very carefully into 33 mm cell to avoid entrapping air.

Hold comparator in upright position. Insert honey disc into comparator, ensuring that printed standards are facing user. Adjust integrated large black cell attachment to fourth track and translucent spacer to second track. Count from closed end out. This will correctly set the cell holder to fit 33 mm test cell. Place cell contg honey sample in right hand compartment. Place 33 mm cell contg appropriate cloudy suspension soln in left hand compartment behind std if honey sample is cloudy.

Using good north daylight or white or daylight fluorescent source, rotate disc until sample appears to match or be lighter (i.e., less red) than glass. Glass represents the darkest color permitted in color class. Honey darker than "A" glass is classified as "Dark Amber."

Results and Discussion

Results are shown in Table 1. The 6 samples classified by the Associate Referee using the Lovibond and USDA procedures are included at the bottom of the table. Four of the collaborators, while recording their data for 6 readings as requested, did not assign the classification that was consistent with their data. In these cases, the correct classification resulting from their data was substituted by the Associate Referee. For example, collaborator 7 recorded that sample F was darker amber (redder) than W, and lighter amber (more yellow) than ELA, but recorded W as the classification. This was changed to the correct ELA by the Referee.

Two of the samples (A and E) were difficult to classify. Sample A was somewhat more yellow than many light honeys and presented a difficult choice between W and EW, as is evident in the Associate Referee classification. Sample E contained considerable buckwheat honey and, although clear, appeared to be darker than the glass reference instead of redder; it was close to the division between A and DA. Because these classifications are subjective comparisons, the spread of individual perceptions would be expected to overlap the class limits when sufficiently close. The Associate Referee believed that no real test of the instrument would have resulted had all samples been adjusted to the mid-points of the various color classifications.

Ten USDA collaborators and 3 other laboratories submitted reports. In one of the latter, two individuals classified the sample. Collaborator 10 was the last to report and made 4 classifications different from the consensus; each was darker. This collaborator agreed to classify the residual samples according to the USDA classifier; 5 of 6 samples verified his original reading. Four of these were darker than the values found by the Associate Referee before the test. It was concluded that improper exposure to excessive heat during storage or transit had probably darkened the honeys, and the results were not used in the calculations.

It is apparent by inspection of Table 1, after eliminating results from Collaborator 10, that of the 84 remaining classifications, 6 (7.1%) differed from the consensus: 5 laboratories show one difference each, and one laboratory showed 2. Of the 6 samples, 2 each showed 3 different of the 14 judgments, one showed one different, and unanimous judgments were made on 3.

This test is not a quantitative procedure giving a continuous gradation of values, but instead a qualitative test resulting in judgments; this limits the applicability of statistical evaluations normally applied to collaborative tests. Table 2 shows chi-square test results that indicate a chi-square value with one degree of freedom for 0.025 probability value of 0.6203 vs 5.024 for the null hypothesis, and support the recommendation of the Associate Referee.

On the basis of the collaborative results reported here, it is recommended that the color classification of honey by the Lovibond 2000 comparator be adopted official first action.

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FERTILIZERS

Liquid Chromatographic Determination of Ammoniacal and Urea Nitrogen in Fertilizer

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Ammoniacal nitrogen in fertilizer is converted to an ammonium-fluorophor derivative and determined by liquid chromatography (LC) on a LiChrosorb RP-18 column and subsequent measurement of fluorophotometric intensity. Urea is converted to ammonia with the aid of urease and then determined similarly. Detection limit of the method is 0.2 μg nitrogen in 2.2 mL. When 5.0 g fertilizer is diluted to 500 mL and then diluted 50-fold with deionized, distilled water, the detection limit equals 1% ammoniacal or urea nitrogen in commercial fertilizer.

Methods reported for the analysis of ammoniacal nitrogen in fertilizer include distillation (1), electrode (2, 3), and gas-phase molecular absorption (4). In the 2 official final action AOAC methods for determination of ammoniacal nitrogen in fertilizer (1), the conventional distillation step used to remove interfering materials is particularly time-consuming.

For analyzing urea nitrogen, the sample is diluted and interfering substances are removed by precipitation with $Ba(OH)_2$ and Na_2CO_3. After neutralization, urea is converted to ammoniacal nitrogen with the aid of urease. This official AOAC method (5) includes complicated acid-base titration steps; it is also difficult to reduce the interferences present in many kinds of commercial fertilizer. Because a large amount of fertilizer solution is used for the determination of urea-nitrogen, a similarly large amount of enzyme solution is needed to hydrolyze the urea.

Fluorescamine is a useful reagent for determining primary amines, including amino acids, peptides, and proteins (6–10). Fluorescamine reacts almost instantaneously with primary amines at a specific pH to form a stable fluorophor. Mizobuchi et al. reported the analysis of ammonium in human urine (11) and in water (12) by making an ammonium-fluorophor derivative and subjecting an aliquot to liquid chromatography (LC).

The authors applied this LC method to the analysis of ammoniacal nitrogen in fertilizer and of the ammoniacal nitrogen produced from urea. This paper describes a rapid, simple, and effective LC method to analyze these 2 kinds of nitrogen in fertilizers.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Shimadzu LC-3A liquid chromatograph (Shimadzu Seisakusho Ltd, Kyoto, Japan) with 250 × 4 mm id, stainless steel column prepacked with LiChrosorb RP-18 (5 μm, E. Merck, Darmstadt, Germany) and 50 × 3 mm id precolumn packed with Permaphase ODS (Du Pont, Wilmington, DE 19898) in our laboratory. Operating conditions: Flow rate 1.0 mL/min; column 40°C; range 4; response M; gain 10; sensitivity control 8.

(b) *Detector.*—Shimadzu Model RF-510LC spectrophotofluorometer set at 390 nm excitation and 470 nm emission wavelengths, respectively.

(c) *Mixer.*—Micro thermo-mixer (Thermonics, Tokyo, Japan).

(d) *Mobile phase.*—Mix 0.05M phosphate buffer solution (pH 2.0) and acetonitrile (60 + 40).

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(e) *Filter paper.*—Toyo No5C (Toyo Roshi Co., Ltd, Tokyo, Japan).

Reagents

(a) *Fluorescamine solution.*—Dissolve 75 mg fluorescamine (Hoffmann-La Roche Diagnostica, Basel, Switzerland) in 25 mL acetone and store at room temperature.

(b) *Borate buffer solution (pH 9.5).*—0.2M. Dissolve 12.366 g boric acid in 1 L water and adjust to pH 9.5 with 1M NaOH solution, using pH meter.

(c) *Phosphate buffer solution (pH 2.0).*—0.05M. Dissolve 6.8 g potassium dihydrogenphosphate in 1 L water and adjust to pH 2.0 with phosphoric acid, using pH meter.

(d) *Phosphate buffer solution (pH 7.0).*—Mix the following 2 solutions and adjust to pH 7.0: *(i)* 0.15M potassium dihydrogen phosphate—dissolve 9.07 g KH_2PO_4 in 1 L water; *(ii)* 0.15M sodium phosphate, dibasic, anhydrous—dissolve 9.46 g Na_2HPO_4 in 1 L water.

(e) *Standard solution of ammoniacal nitrogen.*—Dissolve 3.8188 g ammonium chloride (Wako Pure Chemical Industries, Ltd, Osaka, Japan), dried at 105°C, in deionized, distilled water and dilute to 1 L. 1.0 mL = 1.0 mg N.

(f) *Standard solution of urea nitrogen.*—Dissolve 2.1438 g urea (Wako Pure Chemical Industries, Ltd) in deionized, distilled water and dilute to 1 L. 1.0 mL = 1.0 mg N.

(g) *Urease solution (10 mg/mL).*—Dissolve 50 mg urease (Boehringer Mannheim, West Germany) in 5 mL phosphate buffer. Activity of this solution was 1.3 IU/mL or 0.13 IU/mg-enzyme.

Procedure

Ammoniacal nitrogen.—Place ca 30 g fertilizer in mortar and grind to make more homogenous. Dissolve 5.0 g ground fertilizer and dilute to 500 mL with deionized, distilled water. Filter an aliquot through paper and dilute 50-fold with water. Transfer 100 μL to 10 mL test tube and add 1.9 mL 0.2M borate buffer solution and 200 μL fluorescamine solution. Mix on mixer. Inject 10 μL into LC system.

Urea nitrogen.—Dissolve 5.0 g ground fertilizer and dilute to 500 mL with deionized, distilled water. Filter an aliquot through paper and dilute 10-fold with water (solution A). Transfer 1.0 mL solution A to 10 mL test tube and add 3.75 mL phosphate buffer; pre-incubate ≥ 30 min at 37°C. Dilute enzyme solution 3-fold and add 0.25 mL to sample. Let react 10 min. Transfer 100 μL reacted solution to 10 mL test tube and add 1.9 mL 0.2M borate buffer solution and 200 μL fluorescamine solution; mix on mixer (concentration B). Concomitantly, dilute an aliquot of solution A 5-fold with water and determine ammoniacal nitrogen according to procedure described above, beginning "Transfer 100 μL to 10 mL test tube . . ." (concentration C). Calculate urea nitrogen from difference of concentration (B − C).

Results

Figure 1 is a typical chromatogram of the ammonium-fluorophor derivative at 5.7 min retention time. A calibration

Figure 1. Liquid chromatogram of standard solution—abscissas: elution time (min).

curve of peak height vs concentration of ammoniacal nitrogen as nitrogen was linear from 0.5 to 10.0 μg N. The curve was, however, prepared up to 5.0 μg N for analysis of commercial fertilizers in this study.

For 5.0 g fertilizer dissolved and diluted to 500 mL, pH values were between 5.4 and 7.5, except one sample at pH 2.6. When the sample solution was mixed with borate buffer solution at 1:19, the value changed to 9.46; this showed that the components did not affect the formation of ammonium-fluorophor. The more diluted sample solution was used in the analytical procedure.

Effects of anionic species such as chloride, phosphate, and sulfate, the main anionic species of active ingredients in fertilizers, were measured on standard solutions (Table 1). No effects were noted below concentrations of 50 μg/mL on the fluorometric intensity of 2.0 μg ammoniacal nitrogen/mL.

Table 2 shows recoveries from 4 kinds of fertilizers fortified with 2.0 μg ammoniacal nitrogen after water dilution, and from 2 kinds of fertilizers fortified with 3.0 μg urea nitrogen. Average recoveries were 98.5% and 98.1%, respectively.

Table 3 gives the results of ammoniacal nitrogen determination in 12 different commercial fertilizers by the official magnesium oxide method and our proposed LC method.

Agreement between the 2 methods was good; difference did not exceed 5% except for one sample. Its ammoniacal nitrogen content was 13.0% and 14.0% by AOAC and our LC methods, respectively. All experimental results for content of ammoniacal nitrogen agreed with the manufacturers' information.

Table 4 shows the results of urea nitrogen by the official urease method and our LC method on 2 commercial fertilizers. One fertilizer, which contained only urea as the essential ingredient, showed good agreement among the label information and results by the AOAC and LC methods. The other one, however, gave different values compared with the label information.

Table 1. Effects of anionic species on determination of ammoniacal nitrogen

	Rel. fluor. intensity, %		
Anion, μg	Chloride	Phosphate	Sulfate
5	99	99	98
10	97	101	98
20	100	99	100
30	101	100	100
40	104	99	103
50	105	101	101

Table 2. Recoveries of added ammoniacal and urea nitrogen from commercial fertilizers by LC method

Sample (N-P-K)	Added, μg	Found, μg N[a]	Recovery, %[a]
Ammoniacal nitrogen:			
15-10-12	2.0	2.02 ± 0.08	100.9 ± 4.1
17-0-0	2.0	1.91 ± 0.05	95.5 ± 2.7
8-8-7	2.0	1.99 ± 0.04	99.6 ± 1.8
10-8-10	2.0	1.96 ± 0.08	98.2 ± 3.8
Urea nitrogen:			
urea	3.0	3.10 ± 0.06	100.3 ± 1.9
10-10-10	3.0	2.88 ± 0.12	96.0 ± 4.1

[a] $n = 5$; mean ± standard deviation.

Table 3. Comparison of results by official AOAC magnesium oxide and liquid chromatographic methods on 12 samples

Sample (N-P-K)	Ammoniacal N, %		
	Label	AOAC	LC
NH_4NO_3	17.2	17.4	18.0
$(NH_4)_2SO_4$	21.0	21.0	21.2
15-10-12	13.0	13.0	14.0
14-8-16	0	0.2	0
10-8-10	10.0	10.2	10.0
8-8-7	4.5	4.5	4.5
3-10-10	3.0	3.2	3.3
16-10-14	8.0	8.7	8.3
15-15-10	15.0	16.3	15.5
16-8-10	11.0	12.0	11.9
16-0-20	16.0	16.5	17.2
15-15-15	5.5	7.7	7.4

Table 4. Comparison of results by official AOAC urease and liquid chromatographic methods on 2 samples

Sample (urea-P-K)	Urea N, %		
	Label	AOAC	LC
urea	46.0	46.5	46.2
10-10-10	10.0	2.7[a]	2.6

[a] 3.0% by urease-distillation method.

Discussion

Ammoniacal nitrogen is one of the main ingredients in fertilizer and can be effectively determined by LC after simple 50-fold dilution. Other main components did not interfere with derivative formation or detection. The detection limit is 0.2 μg nitrogen in 2.2 mL, at a signal-to-noise ratio of 2. For 5.0 g fertilizer, the detection limit equals 1% ammoniacal or urea nitrogen.

The relation between fluorometric intensity of ammonium-fluorophor and pH value was shown in Figure 1 of ref. (11). We found that the intensity of the urea-fluorophor was less than 1% of that of ammonium-fluorophor, so the presence of urea does not affect the determination of ammoniacal nitrogen in fertilizer.

The AOAC method for analysis of urea is very complicated; it is difficult to remove interfering substances, to neu-

tralize, and to titrate with hydrochloric acid or sodium hydroxide, because each sample of complex fertilizer is different. We use a much more diluted sample solution so interferences on the enzymatic activity are minimized, and very little enzyme is needed to reduce urea to ammoniacal nitrogen.

Only one sample had urea nitrogen values different from the 10% urea content in the label information. Nitrogen accounts for 4.67% of this sample value, about 1.7 times the analytical results. We tested these data by another method: Urea in the sample was hydrolyzed with enzyme and the resulting ammonia was distilled. The content of urea nitrogen was 3.0%. We concluded that the analytical results by the AOAC and LC methods reflected the true content of urea nitrogen and have no explanation for the difference from the label.

The LC method is sensitive, requires no distillation step, is not affected by chloride, phosphate, or sulfate, and therefore is an effective method for analysis of ammoniacal and urea nitrogen in fertilizers.

Acknowledgments

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Determination of Tetracycline Residues in Meat and Fish

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A simple and rapid method was developed for quantitative determination of common tetracyclines, such as oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC), in meat and fish. Tetracyclines were extracted with aqueous HCl, and then centrifuged. The supernate was applied to an Amberlite XAD-2 column, which was washed with water and eluted with methanol. The eluate was concentrated to about 0.5 mL under vacuum at 35°C, and then measured by liquid chromatography and UV detection. Two analytical columns were used for confirmation. The average recoveries of OTC, TC, and CTC from meat and fish fortified at 1, 1, and 3 ppm were 82.6, 81.5, and 67.0% respectively.

Tetracycline antibiotics are widely used as a disease-preventative and food additive in cattle breeding and fishery culture. Residues may remain in meat or fish used for human food and lead to microbial substitution and development of bacterial resistance. Therefore, it is necessary to investigate the presence and amount of these antibiotics remaining in food.

Several microbiological methods (1, 2) are available to determine tetracycline antibiotics in food, but they are not specific. Chemical methods include spectrophotometry (3, 4), fluorophotometry (5–7), thin layer chromatography (8), gas chromatography (9), and liquid chromatography (LC) (10–14). These methods, however, are not sensitive enough to determine residues in food samples. We present a reliable method for determination of oxytetracycline, tetracycline, and chlortetracycline (OTC, TC, and CTC) in meat and fish, with pretreatment on an Amberlite XAD-2 column followed by liquid chromatography using 2 kinds of column.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Shimadzu Model LC-2 equipped with Model SPD-1 detector (Shimadzu Seisakusho Ltd, Kyoto, Japan). Operating conditions: temperature, ambient; flow rate, 1.5 mL/min for columns A and B; injection volume, 20 μL; sensitivity setting of detector, 0.04 AUFS for standard solutions ≤ 50 ppm and 0.08 AUFS for standard solutions > 50 ppm; wavelength, 370 nm.

(b) *Analytical column.*—Column A: stainless steel, 4 mm id × 25 cm, packed with Wako Gel DMS-10H (dimethyl silica, 10 μm) (Wako Pure Chemical Industry Ltd, Osaka, Japan). Column B: stainless steel, 4 mm id × 25 cm, packed with Shimadzu Gel PSG-100 (polystyrene gel, 10 μm) (Shimadzu Seisakusho Ltd). Guard column: stainless steel, 4 mm id × 5 cm, dry-packed (tap and fill method) with Bondapak C_{18} Corasil (Waters Associates, Milford, MA).

(c) *Centrifuge.*—Hitachi Model 18 PR-3 (Hitachi Ltd, Tokyo, Japan).

(d) *Homogenizer.*—Polytron® (Kinematica, Switzerland).

Reagents and Materials

(a) *Solvents.*—Methanol and acetonitrile (Wako Pure Chemical Industry Ltd).

(b) *β-Mercaptopropionic acid (β-MPA).*—Wako Pure Chemical Industry Ltd.

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(c) *Filter aid.*—Celite 545 (Wako Pure Chemical Industry Ltd).

(d) *Adsorbent.*—Amberlite XAD-2 resin (Rohm and Haas Ltd). Wash with acetone, methanol, and water, pack into 15 mm id × 25 cm glass column up to 10 cm, and then wash with 300 mL water before use.

(e) *Antibiotics.*—OTC dihydrate (OTC · $2H_2O$), TC (Sigma Chemical), and CTC hydrochloride (CTC · HCl) (Calbiochem).

(f) *Standard solution.*—Methanol solution containing 20 ppm OTC · $2H_2O$, 20 ppm TC, and 60 ppm CTC · HCl. Prepare weekly and store at 2–10°C in the dark (for recovery test).

(g) *Mobile phase for LC.*—Column A: 0.05M phosphate buffer (pH 2.5)–acetronitrile (90 + 10, v/v). Column B: 0.04M potassium phosphate, monobasic, and 0.08M EDTA–methanol (10 + 10 + 80, v/v).

Procedure

Place 20 g chopped sample in 500 mL beaker, add 100 mL 1N HCl, and then homogenize 5 min. Transfer mixture to 500 mL centrifuge tube, and centrifuge 15 min. Filter supernate through 5 g Celite 545. Re-extract residue with 50 mL 1N HCl. Pour combined filtrates onto Amberlite XAD-2 column, wash with 200 mL water, and then elute with 100 mL methanol. Purge bubbles if necessary. Collect 100 mL methanolic eluate in 200 mL Erlenmeyer flask. Add 1 mL 5% methanolic β-MPA solution, and concentrate to ca 0.5 mL under vacuum at 35°C. Transfer solution to test tube, using 1 mL methanol rinse, and then add methanol to give 2 mL (test solution). Filter test solution through micropore filter if necessary.

Determination

Inject 20 μL standard solution (OTC and TC 10 ppm, CTC 30 ppm) into liquid chromatograph (external standard method). Inject 20 μL test solution and compare peak height of standard *(S)* with peak height of test solution *(X)*.

$$\text{ppm Antibiotic} = (X/S) \times C \times (2/\text{sample wt})$$

where C = antibiotic concentration of standard.

Results and Discussion

Although OTC, TC, and CTC are stable in methanol for 2 weeks or more in the dark, they are slightly unstable in acid as shown in Table 1. Scales and Assinder (5) suggested that OTC is liable to photodecomposition, so β-MPA is added as a stabilizing agent. TC and CTC also showed results similar to that of OTC (Table 2), but loss of CTC was greater.

Figure 1 shows the elution patterns of OTC, TC, and CTC from Amberlite XAD-2 column. Elution was nearly complete with 80 mL methanol. Contrary to expectation, chromatographic separation was not observed because of formation of bubbles in the column. Ryan and Dupont (8) suggested that Amberlite XAD-2 resin was superior to other adsorbents, such as silica gel and Florisil, for cleanup of tetracycline residues. Sasaki et al. (7) used Amberlite XAD-8 resin, a more polar adsorbent, and eluted with aqueous alkali. This method, however, necessitates a time-consuming concentration of aqueous eluate, and recovery is slightly low.

Table 1. Degradation of OTC, TC, and CTC under various conditions

Condition	Hours[a]	Recovery,[b] % OTC	TC	CTC
Acidic (1N HCl)	16	90.6	68.8	101
	40	73.1	24.3	94.1
	88	60.6	7.4	94.0
Neutral (clear test tube)	1	100	100	79.3
	2	101	100	84.4
	6	100	100	81.0
Neutral (low-actinic test tube)	1	108	106	86.2
	2	104	102	86.2
	6	101	102	82.8

[a]Standing time at room temperature.
[b]Compared with initial concentration.

Table 2. Recoveries of OTC, TC, and CTC from concentration step[a]

Condition	Recovery,[b] % OTC	TC	CTC
Without β-MPA	70.0	69.6	57.3
With β-MPA[c]	101	101	80.4

[a]100 mL 95% methanol solution containing 40 μg OTC, 40 μg TC, and 120 μg CTC, concentrated to 0.5 mL under vacuum at 35°C, and adjusted to 4 mL with methanol.
[b]Average of 3 experiments.
[c]1 mL 5% methanolic β-MPA solution added to methanol solution before concentration procedure.

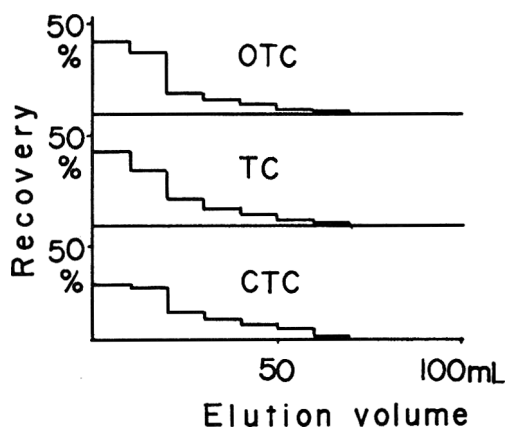

Figure 1. Elution patterns of OTC, TC, and CTC from Amberlite XAD-2 column with methanol.

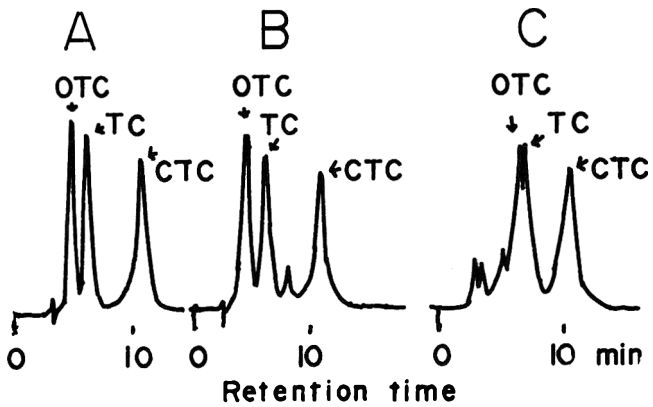

Figure 2. Typical LC chromatograms of OTC, TC, and CTC: A, standard solution (OTC and TC 10 ppm, CTC 30 ppm) and gel DMS-10H column; B, fish extract (OTC and TC 10 ppm, CTC 30 ppm) and gel DMS-10H column; C, fish extract (OTC and TC 10 ppm, CTC 30 ppm) and gel PSG-100 column.

Table 3. Recoveries of OTC, TC, and CTC from fortified meat and fish[a]

Sample	OTC Rec., %	OTC CV, %	TC Rec., %	TC CV, %	CTC Rec., %	CTC CV, %
Beef	84.3	6.4	83.3	4.0	65.4	7.0
Pork	87.4	5.3	78.9	5.3	60.7	4.8
Chicken	84.1	3.1	79.5	5.1	64.4	8.1
Yellow-tail	78.5	5.4	83.6	6.7	70.1	2.3
Porgy	78.9	4.6	82.3	2.1	74.3	4.0

[a]Sample fortified at 1 ppm (OTC, TC) and 3 ppm (CTC). Each value is the average for 3 samples.

Figure 2 shows the chromatograms of OTC, TC, and CTC on the 2 LC columns; good separation was obtained. Peak responses for OTC, TC, and CTC were the same on both columns. Two columns were used because, with a single column, the possibility of the presence of contaminants with the same retention time cannot be excluded.

These tetracyclines have strong absorption bands at 270 and 370 nm; we measured the peak heights at 370 nm. The response of CTC was lower than that of OTC or TC at this wavelength, so we used higher concentrations of the former. With the necessary changes in detector settings, a linear response was observed for standards from 1 to 100 ppm. The limits of detection for OTC, TC, and CTC were 20, 20, and 60 ng, respectively, for injection volumes of 2 μL and a signal-to-noise ratio of 3.

Recoveries of OTC, TC, and CTC from meat and fish were determined (Table 3) by fortifying samples with standard solutions at 1 ppm for OTC and TC, and 3 ppm for CTC. Average recoveries of OTC, TC, and CTC were 82.6, 81.5, and 67.0%, respectively. Recovery of CTC was lower than those of OTC and TC, corresponding to the decrease of recovery during concentration of residues under vacuum (Table 2).

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CHEMICAL CONTAMINANTS MONITORING

Development of a Quality Assurance Program for Determination of Ultratrace Background Levels of Lead and Cadmium in Raw Agricultural Crops by Differential Pulse Anodic Stripping Voltammetry

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Data on the background levels of lead and cadmium in the food supply are essential in order to establish a baseline from which to evaluate the extent of contamination in transport, processing, industrial atmospheric particulate fallout, and soil treatment (e.g., fertilizers, sewage sludge, etc.). This requires the establishment of site selection and sampling criteria as well as the development of a rigorous analytical method capable of performing routine analyses of Pb and Cd at ultratrace levels. The method used in this study, which was published previously, was designed to provide high sample throughput with minimal contamination. This involved control and measurement of blank levels and the establishment of quality control procedures to maintain confidence in the accuracy and precision of the method.

Methodology, quality control, and results are presented in the continuing study of background levels of lead and cadmium in selected raw agricultural crop samples as determined by differential pulse anodic stripping voltammetry at a static mercury drop electrode. Crop samples are dry-ashed at 500 ± 25°C with a 4% H_2SO_4 solution as an ashing aid. The low level nature of a background study necessitates the determination and reduction or elimination of possible sources of Pb and Cd contamination. Blank levels were reduced to 25 ± 24 pg Cd/mL and 64 ± 46 pg Pb/mL in solution in the cell. After dilutions are accounted for, these values correspond to procedural blank levels of 1.2 ± 1.2 ng Cd and 3.2 ± 2.3 ng Pb. Results for the analyses of NBS standard reference materials, replicate sample analyses, and recovery of standard spikes added to the samples demonstrate the accuracy and reproducibility of this method at ultratrace levels.

Data presented represent 1400 samples of carrots, field corn, onions, rice, spinach, and tomatoes collected during the second sampling phase. Blank data presented were acquired during the entire study (1, 2).

Experimental

Preparation of Sample Composite

Samples were collected according to the criteria previously reported (1, 2). Disposable polyethylene gloves were worn for all sample handling.

Spinach, onions, tomatoes, and carrots were rinsed with distilled deionized water (DDW), reduced to their edible portions, homogenized in a food processor, freeze-dried, blender-ground to ca 40 mesh, and stored in linear polyethylene bottles. Field corn was reduced to its edible portion, rinsed with DDW, soaked in DDW, and frozen to partially break down its cellular structure. The corn was then freeze-dried, blender-ground, and stored. Rice was reduced to its edible portion, rinsed with DDW, dried in a class 100 clean air environment as defined by Zief and Mitchell (3), blender-ground, and stored. Final sample composites represented 40 carrots, 5 ears field corn, 25 onions, 200 heads rice, 1000 spinach leaves, and 15 tomatoes.

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Sample Digestion

The dry ash procedure used was previously reported (1). However, with all Phase-II samples, only 3.0 mL 40% H_2SO_4 was used and DDW was added to ensure thorough wetting after agitation. All crop types except rice were heated 12 h at 500°C before HNO_3 oxidation. Because rice, when ashed at 500°C, required 4 or 5 additional HNO_3 treatments, depending on the sample size, higher initial ash temperatures were investigated. Oxidation of the rice matrix was found to be more complete at 625°C without additional analyte loss.

Contamination Control

Contamination is divided into 2 types of contributions to the analytical blank—systematic and random.

Reagents, instrumentation, and laboratory modifications were described earlier (1). The modifications and equipment were selected to provide a contamination control program which would reduce the level and the variability of systematic contamination in the analytical blank. All equipment and reagents used in preparation of the analytical sample were monitored for contribution to the analytical blank.

Random contributions to the analytical blank were caused by fluctuations in the Pb and Cd atmospheric levels and by the analyst. Ambient Pb levels were difficult to control, possibly because of the urban location and age of the laboratory building. Atmospheric contamination was reduced by keeping the samples in a clean air environment through as many stages of the determination as possible. The analyst's contributions to the blank can be reduced only by closely following quality control procedures.

Quality Control

Once the analytical method was developed, quality control procedures were established to maintain the validity of the analytical scheme when applied to a large number of samples over a 2 year period. The following procedures were designed to cover all aspects of the analytical determination.

All glassware was cleaned rigorously before digestion or analysis (1) and was stored in a clean air environment. These clean environments were periodically monitored for levels of contaminants by measuring contributions to a blank beaker after various intervals of its storage time in the clean area. Polarographic instrumentation was checked daily with elemental standards to verify adequate sensitivity, resolution, and reproducibility. Ag/AgCl reference electrode filling solution was replaced when the redox potentials shifted 0.05 V cathodically, due to diffusion of the saturated KCl-AgCl filling solution across the Vycor frit into the polarographic cell. Calibration of the microliter pipets used for standard additions was checked every 2 weeks by weighing the dispensed liquid.

A precision study was performed on each crop type to ensure reproducibility of the method. These studies consisted of 8–10 separate determinations on individual samples.

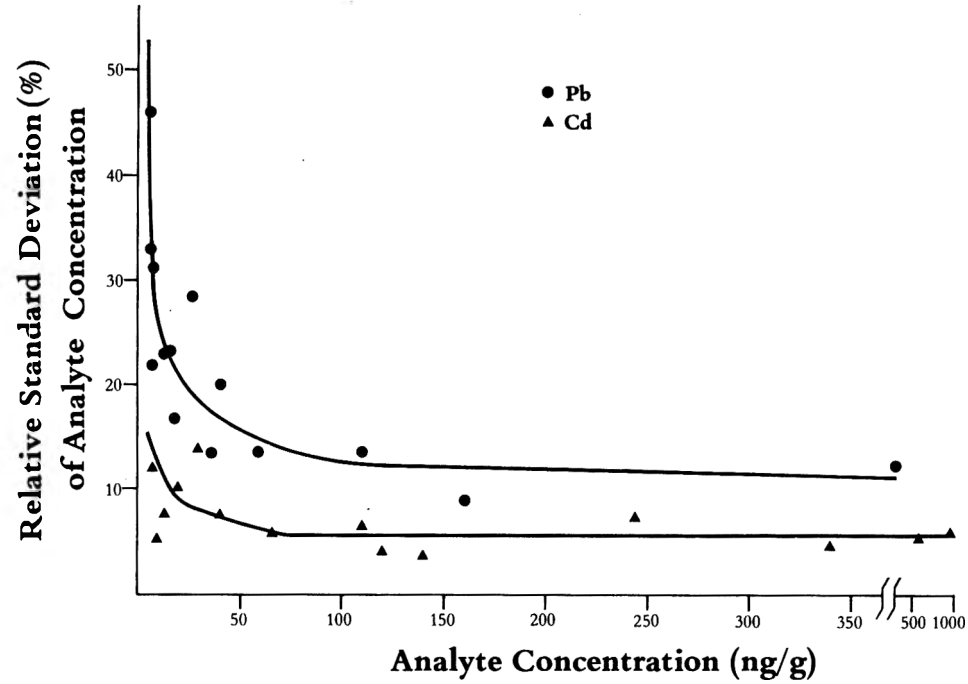

Figure 1. Effect of analyte concentration on analytical precision of determination of lead and cadmium in crops.

Table 1. Precision of Cd duplicates

Sample Cd content, ng/g	No. of duplicates[a]	Range,[b] %	Mean,[b] %
<10	28	0–74	29
10–50	35	0.7–47	12
51–100	29	1.6–19	7.1
101–500	88	0–32	8.2
>500	18	0.1–14	6.8

[a]Separate pairs of analyses.
[b]Percentages reported as relative standard deviation.

Table 2. Precision of Pb duplicates

Sample Pb content, ng/g	No. of duplicates[a]	Range,[b] %	Mean,[b] %
<10	34	3.4–78	30
10–50	65	0.2–82	15
51–100	14	0.7–25	6.9
101–500	46	0.4–30	9.7
>500	12	0.7–13	7.3

[a]Separate pairs of analyses.
[b]Percentages reported as relative standard deviation.

Figure 1 is based on 14 separate precision studies for Pb and Cd at different analyte levels in selected agricultural crops. The assumption is made that the precision is based solely on the analyte level, independent of sample homogeneity or sample matrix. As the concentration of analyte in a sample approaches the detection limit, the standard deviation associated with that determination increases. This was seen in the Pb analyses where random contamination at low levels was the major factor in a variation of about 46% at 4 times the detection limit. However, with Cd, the lowest level precision study was done at 10 times the detection limit, and the increase in relative standard deviation (RSD) at this concentration level was not as sharp. At 100 times the detection limit, RSD values fluctuated between 8 and 14% for Pb and between 4 and 8% for Cd. This is the best precision that can be expected for routine application of this analytical method in this laboratory.

Ten percent of the samples were analyzed in duplicate. RSD values calculated on each pair of duplicate measurements and the corresponding concentration ranges are tabulated in Tables 1 and 2. For example, Table 1 shows results for 28 pairs of duplicate samples ranging in concentration from the detection limit to less than 10 ng/g. For each pair of values, an RSD was calculated. The range reported represents the minimum and maximum of the 28 RSD values. RSD values ranging from 0 to 78% can be expected at this concentration level, since the RSD value is based on 2 determinations (4). The mean of these 28 values is also reported. At analyte levels less than 10 ng of either Pb or Cd, mean RSD values were approximately 30%. Mean RSD values reported in Tables 1 and 2 correlate with precision study values above. Another factor which may have contributed to these wide RSD ranges is that most of the values in this concentration range (<10 ng/g) were obtained on rice and field corn, both of which were difficult crops to homogenize.

An analyte-fortified sample was analyzed with each set of samples. In cases where recoveries were not within 80–120%, indicating possible sample matrix volatilization, temperature control problems, or contamination problems, the entire set of samples had to be re-analyzed.

Appropriate NBS biological standard reference materials were analyzed as well-characterized matrix models (e.g., Rice Flour), when available. A blank determination was performed with each set of samples (i.e., up to 20 samples per set) to monitor levels of contaminants. Samples were re-analyzed whenever an outlier in a particular crop series was obtained. A crop series consisted of approximately 15 samples of the same crop type from the same county. For example, on a particular series of onion samples, Pb values ranged from 20 to 40 ng/g plus a single value at 200 ng/g. This value was empirically determined to have resulted from random Pb contamination. If the Pb value decreased upon re-analysis, the high value was eliminated (Cd value remaining constant). If both Pb and Cd values were unchanged, mean values were reported. Random contamination by Cd was never encountered.

Table 3. Recoveries of Pb and Cd standards from crop samples

Crop type	Dry wt, g	Spike level, μg/g	Mean recovery, % Pb	Mean recovery, % Cd
Carrots	2.0	0.10	103 ± 13	108 ± 17
Field corn	3.0	0.03	100 ± 16	99 ± 14
Onions	2.0	0.10	104 ± 15	101 ± 14
Rice	2.0	0.05	100 ± 16	101 ± 13
Spinach	1.0	0.40	103 ± 13	111 ± 11
Tomatoes	3.0	0.13	103 ± 9	104 ± 15

Detection Limits

As the concentration of analyte in a sample approaches the concentration of analyte in the blank, the random error associated with the difference in those measurements becomes increasingly significant. The detection limit in this work was dependent on the standard deviation associated with the blank measurement and not on instrumental noise. The detection limit (DL) is defined as that level of analyte which produces a result significantly different from zero at the 99% confidence level. The analyte level is different from the reagent blank (5) if

$$\Delta \overline{X}_{s-b} > t_{\alpha,\phi}\, s_{xb} \left(\frac{1}{n_s} + \frac{1}{n_b}\right)^{1/2}$$

where $\overline{X}_{s-b}$ is the difference between the mean of the sample and blank populations; t, obtained from a table of significance limits of the Student's distribution, is a function of α (determined by the desired confidence level), and ϕ is the number of degrees of freedom after determination of x_b (the mean blank value). The standard deviation of the blank measurements is s_{xb}; n_s and n_t are the number of observations of the sample and blank, respectively. Because 140 blank experiments were conducted, $\phi = n - 1 = 139$. At the 99% confidence level, $\alpha = 0.005$ and the critical value of $t_{0.005,\,139} = 2.6117$ (6). If a single sample measurement is compared to 140 blank observations, the DL for Pb is 0.12 ng/mL, and the DL for Cd is 0.063 ng/mL. These correspond to minimum levels of analyte detectable above the blank in the cell. Based on a 3 g dry sample and a dilution factor of 50, the detection limits for Pb and Cd are 2 ng/g and 1 ng/g, respectively.

Results and Discussion

The greatest difficulty in this study involving the determination of Pb and Cd background levels in raw agricultural crops was to obtain a blank data set which demonstrates the capability to control the influx of contamination.

Even with improvements in the facilities and reduction of random error associated with the analyst, these were still control problems. For example, during one period of the study the Pb blank level increased by a factor of 5. The origin of this Pb influx was a bacterial growth in the water system, which resulted in the gradual release of Pb from the ion-exchange columns. The problem was eliminated by sanitizing the system with sodium hypochlorite and replacing the cartridges.

Ashing parameters were modified for rice samples because 4–5 additional HNO_3 oxidations were required to remove residual carbon when an initial ashing at 500°C was used. Investigation of elevated ash temperatures (up to 625°C) showed 100% recovery of Pb or Cd from rice samples fortified at 50 ng/g. Values for Cd in NBS Rice Flour, however, were below the certified NBS value by about 30%. A precision study of 11 separate determinations of Cd in rice ashed at 500°C produced a mean value of 20 ± 2 ng/g. Another precision study of 7 determinations of Cd in rice ashed at 625°C produced a mean value of 21 ± 5 ng/g. These values are compared tc 29 ± 4 ng/g reported by NBS. In both cases Pb recoveries agreed with the value reported by NBS. The Cd difference could be explained as a volatile organically bound Cd species in the reference material, which was lost before conversion to $CdSO_4$ during the initial ashing stages. Experiments using an HNO_3/$HClO_4$/H_2SO_4 wet ash to reduce the Cd volatilization possibility produced a mean value of 28 ± 2 ng/g. Further experiments are necessary to evaluate the extent to which volatile Cd species are lost from the rice samples.

Table 3 lists the number of recoveries performed on each crop type and the dry weight which produced a representative sampling, could be easily ashed, and produced enough analyte for quantitation. The level of fortification was commensurate with analyte concentration in the sample.

Samples were collected from "non-contaminated" fields in the major production areas of each crop throughout the United States with the intention of providing background Pb and Cd data. The lowest median Pb and Cd values acquired on a dry weight basis were less than 10 ng/g for field corn and rice. The highest median Pb and Cd values encountered on a dry weight basis were less than 1 μg/g in spinach. A thorough treatment of Pb, Cd, and multielement data is in preparation.

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Arsenic, Cadmium, Lead, Mercury, and Selenium in Sediments of Riverine and Pothole Wetlands of the North Central United States

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Surface sediments (0–10 cm) collected in 1980 and 1981 from 13 wetland areas in Iowa, Montana, Nebraska, North Dakota, and South Dakota were analyzed for total concentrations of arsenic, cadmium, lead, mercury, and selenium. Sediments from pothole-type wetlands had significantly higher concentrations of arsenic, cadmium, lead, and selenium than those from riverine wetlands. Mean (and range) of dry weight concentrations (mg/kg) for pothole and riverine locations, respectively, were arsenic, 4.4 (1.4–9.3) and 2.4 (0.7–6.1); cadmium, 0.52 (0.17–0.87) and 0.26 (0.01–0.55); lead, 13 (7.4–22) and 6.6 (1.1–14); selenium, 0.89 (0.13–2.1) and 0.52 (0.03–5.1). Mercury concentrations in sediment did not differ significantly between pothole and riverine type wetlands (mean, 0.03, range, 0.01–0.08). A comparison of the concentrations of elements found in this study with values reported in the literature indicated that, with the possible exception of one location, levels were within normal or background ranges.

The United States Fish and Wildlife Service (USFWS) has a responsibility to manage and preserve a large, publicly owned system of National Wildlife Refuges and Waterfowl Production Areas located throughout the Northern Prairie region of the United States. These areas can be broadly characterized as riverbottom marshland and pothole-type wetlands, maintained by surface runoff and groundwater, and are extremely important to migratory waterfowl. During summer they are used for breeding, nesting, and raising of young, and during spring and fall migrations they are used for resting and feeding.

Very little information is available on concentrations of certain elements in Northern Prairie wetland ecosystems. Resource managers have recently raised some concerns about possible contamination of these habitats with metals or other harmful elements from sources such as agricultural fertilizers (1, 2) and recently constructed or proposed coal-fired power plants (3, 4).

In 1980 we began a 2-year study of sediments in USFWS wetlands to obtain baseline information on specific element concentrations. Our objectives were to *(1)* determine the concentrations of arsenic, cadmium, lead, mercury, and selenium in the sediments of representative riverine and pothole wetlands in the North Central United States; *(2)* compare these elemental concentrations with values from the literature; and *(3)* make an initial assessment of the present state of these wetlands for possible contamination.

Description of Study Areas

Thirteen wetland areas in Iowa, Montana, Nebraska, North Dakota, and South Dakota were selected for sampling (Figure 1 and Table 1). Medicine Lake National Wildlife Refuge (NWR) is in Sheridan County, MT, and receives water primarily by diversion of Big Muddy Creek. The wetland consists of one pool with a surface area of about 34 sq. km and a maximum depth of about 4 m. Des Lacs NWR consists of 7 impoundments on the Des Lacs River in Burke and Ward Counties, ND. Total surface area of the wetlands is about 22 sq. km and maximum depth is about 4 m. J. Clark Salyer NWR is a series of 5 impoundments on the Souris River in Bottineau and McHenry Counties, ND. Total surface area of the wetlands is about 88 sq. km with a maximum depth of about 9 m. Audubon NWR in McLean County, ND, consists of a 43 sq. km portion of a large embayment on Garrison Reservoir, a 1472 sq. km impoundment of the Missouri River. Maximum depth is about 17 m.

The Coal Creek sampling area consists of 2, privately owned, pothole-type wetlands, less than 1 km from an 1100 megawatt, coal-fired power plant in McLean County, ND. The potholes have surface areas of less than 1 sq. km and maximum depths of 1–2 m. The Jamestown sampling area consists of 3 pothole-type Waterfowl Production Areas (WPA), located in Stutsman County, ND. These potholes were less than 1 sq. km surface area, with maximum depths of 1–2 m.

Arrowwood NWR is a series of 4 impoundments on the James River in Stutsman County, ND. Total surface area of the wetlands is about 32 sq. km and maximum depth is about 3 m. Sand Lake NWR is located on the James River in Brown County, SD. The wetland consists of 2 impoundments with a combined surface area of about 46 sq. km and a maximum depth of 2 m.

The Wike WPA is in Roberts County, SD, and consists of 3 pothole-type wetlands. The Madison WPAs included 4 pothole-type wetlands located in Brookings, Hamlin, and Lake Counties, SD. Each of these potholes had a surface area of less than 1 sq. km and a maximum depth of 1–2 m.

Lake Andes NWR, in Charles Mix County, SD, is a 19 sq. km natural lake with a maximum depth of about 2 m. Union Slough NWR is in Kossuth County, IA, and forms the headwaters of the East Fork Des Moines and Blue Earth Rivers. The wetland consists of 5 impoundments with a combined surface area of about 5 sq. km and a maximum depth of about 2 m.

The Hastings WPAs consist of 4 pothole wetlands in Clay and Fillmore Counties, NE, with surface areas of less than 1 sq. km and maximum depths of 1–2 m.

Overall, 5 locations can be characterized as typical pothole wetlands (Coal Creek, Jamestown, Wike, Madison, and Hastings), and 6 locations represent riverine marshland (Des Lacs, J. Clark Salyer, Audubon, Arrowwood, Sand Lake, and Union Slough). Two areas (Medicine Lake and Lake Andes) cannot be classified as either pothole or riverine, because of hydrological features regarding inflow and outflow. The size of the contributing watersheds for each of the locations varies greatly. The pothole wetlands typically drain relatively small watersheds, usually ranging from 4 to 20 sq. km. In contrast, the riverine wetlands drain more extensive watersheds often in excess of several thousand square kilometers. Land use within the watersheds is primarily agricultural. Vegetative cover ranges from 100% native prairie in at least one area to over 90% cultivated farm land in certain others. Most watersheds contain a mixture of small grain farming (wheat, barley, oats) and row crop farming (corn, soybeans, sunflowers) along with grazing of native grassland. With the exception of one site (Coal Creek), industrial development within the watersheds is limited or nonexistent.

Methods

Samples were collected in July and August of 1980 and 1981. Sampling sites within each of the 13 locations were

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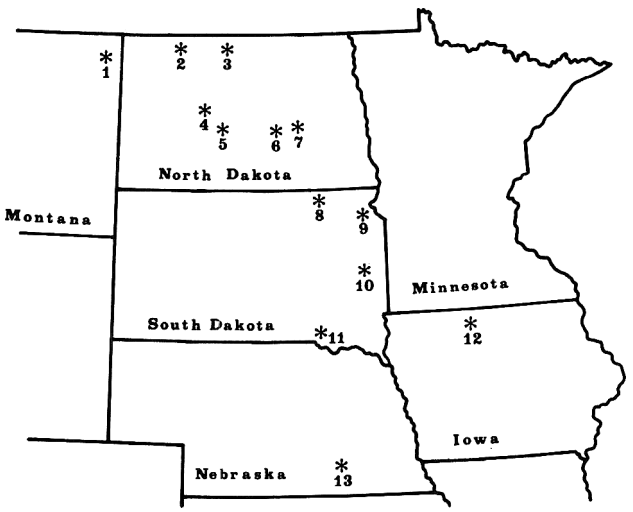

Figure 1. North Central United States showing locations of Waterfowl Production Areas (WPA) and National Wildlife Refuges (NWR) sampled: 1, Medicine Lake NWR; 2, Des Lacs NWR; 3, J. Clark Salyer NWR; 4, Audubon NWR; 5, Coal Creek WPA; 6, Jamestown WPA; 7, Arrowwood NWR; 8, Sand Lake NWR 9, Wike WPA; 10, Madison WPA; 11, Lake Andes NWR; 12, Un on Slough NWR; 13, Hastings WPA.

selected after consultation with refuge and wetland district managers. In riverine wetlands, samples were collected near the primary inlet and outlet of the refuge, as well as immediately downstream of selected major tributaries. In pothole wetlands, sampling sites were chosen on the basis of basin shape to represent as much of the wetland area as possible. Because there are no known or suspected point sources of contamination for any of the elements determined in this study, at any of the locations sampled, the samples are considered to be representative of these locations.

Sediment samples were collected with a hand-held polyethylene tube from the upper 10 cm substrate and were placed in linear polyethylene containers. Samples were frozen within 24 h of collection and shipped by air freight to Analytical Bio-Chemistry Laboratories, Inc., Columbia, MO, for analysis. There, samples were dried at 35–40°C, ground with a mortar and pestle, sieved through a 20 mesh screen, and mixed thoroughly. Two g subsamples of dried sediment were digested by one of 2 methods: Arsenic and selenium were analyzed on nitric–perchloric acid digestates, while cadmium, mercury, and lead were analyzed on nitric acid reflux digestates (5–8). All elements were determined by atomic absorption spectrophotometry (AAS). Instrumentation included a Perkin-Elmer 305-B spectrophotometer with background correction, an HGA-2100 graphite furnace, and an MHS-10 hydride generator. The limits for quantitation for this study were as follows:

Sediment	*Method*	*Detection limit, mg/kg*
Arsenic	hydride generation	0.05
Lead	flame	0.05
Mercury	cold vapor	0.005
Selenium	hydride generation	0.01
Cadmium	graphite furnace	0.01

Cadmium was also determined on some digestates by inductively coupled argon plasma emission to compare results

Table 1. Specific sediment sampling sites within each National Wildlife Refuge (NWR) and Waterfowl Production Area (WPA)

Map. No.	Location	Sampling sites
1	Medicine Lake (NWR)	A. West Edge of Rock Crossing B. East Tip of Gaffeney Pass Bay C. Above Dam #4, 1 mi. Below Sewage Lagoon
2	Des Lacs (NWR)	A. Upper Des Lacs Lake, 1 mi. South of U.S. Highway 52 Bridge B. Kenmare Lake, Above Dam C. East Edge of Lower Des Lacs Lake
3	J. Clark Salyer (NWR)	A. Below Dam #357, 1 mi. South of Canada Border B. Below Mouth of Boundary Creek C. Below Mouth of Deep River, Above Dam #332 D. Mouth of Willow Creek E. Souris River at Nelson Bridge
4	Audubon (NWR)	A. West End of Refuge, Section 2, R 83 W, T 147 N B. Southeast End of Refuge, Section 10, R 82 W, T 147 N C. North Edge of Refuge, Section 29, R 82 W, T 148 N
5	Coal Creek (WPA)	A. Wetland Immediately North and Adjacent to Ash and Sludge Disposal Pond B. Samuelson Slough
6	Jamestown (WPA)	A. NPWRC Study Pond P-1, Stutsman Co. B. NPWRC Study Pond P-10, Stutsman Co. C. Cottonwood Lake WPA, Stutsman Co.
7	Arrowwood (NWR)	A. Arrowwood Lake at State Highway 9 Bridge B. Arrowwood Lake below Dam #7 C. Arrowwood Lake near Picnic Area at Southeast End D. Lower Mud Lake at Bridge E. Lower Jim Lake above Jim Dam F. Above Depuy Dam
8	Sand Lake (NWR)	A. South Side of Hecla Grade at Bridge B. Above Houghton Dam at Spillway C. Columbia Road Reservoir at State Highway 10 Bridge D. Columbia Road Reservoir, 2 mi. North of Columbia Road Dam
9	Wike (WPA)	A. Wetland at South Boundary of WPA B. Wetland at East Edge of WPA C. Wetland near Farm Site at North Edge of WPA
10	Madison (WPA)	A. Ramsey WPA, Lake Co. B. Brush Lake WPA, Brookings Co. C. Juntunen WPA, Hamlin Co. D. Knapper WPA, Brookings Co.
11	Lake Andes (NWR)	A. Southeast Section of Owens Bay B. Northeast Section of Owens Bay C. North Unit
12	Union Slough (NWR)	A. North End of Pool D1, South of County Road B. South End of Pool B, North of Blacktop Road C. Pool C near Outlet of Ditch 99
13	Hastings (WPA)	A. Moger Lagoon WPA, Clay Co. B. Smith Lagoon WPA, Clay Co. C. Mallard Haven WPA, Fillmore Co. D. WPA, 4 mi. West of Harvard, Clay Co.

Table 2. Analytical precision and accuracy of elemental analyses

Analysis	Arsenic	Cadmium	Lead	Mercury	Selenium
			Recovery of Known Additions		
Number of samples	12	12	11	12	11
Concn added, mg/kg	10 & 2.0	2.0 & 1.0	20 & 5.0	1.2	10 & 2.0
Mean rec., (%)	94	97	98	100	101
95% Conf. int., %	74–114	79–115	84–112	88–112	79–123
			Duplicate Sample Analyses		
Number of samples	8	10	10	10	9
Mean Concn, mg/kg	3.3	0.50	8.6	0.03	0.50
Mean diff. of dupl. sets, mg/kg	0.2	0.03	0.6	0.005	0.06
			Analyses of Standard Reference Materials		
NBS No. 1645 River Sediment[a]					
Observed mean (SD)–*n*	—[b]	8.9(0.8)–6	670(22)–8	0.83(.05)–6	—
Certified range, mg/kg	—	8.7–11.7	686–742	0.6–1.6	—
DOE Sediment[a]					
Observed mean (SD)–*n*	5.0(0.7)–4	0.81(.05)–7	36(1.1)–3	0.34(.003)–2	0.55(.06)–5
Certified range, mg/kg	5–13	0.8–1.4	32–48	0.09–0.27	0.2–0.5
NBS No. 1566 Oyster Tissue					
Observed[c]	9.7	—	—	0.06	2.3
Certified, mg/kg	11.5–15.3	—	—	0.04–0.07	1.6–2.6
NBS No. 1571 Orchard Leaves					
Observed[c]	12	0.13	42	0.14	0.16
Certified, mg/kg	8–12	0.10–0.12	42–48	0.14–0.17	0.07–0.09

[a]NBS = U.S. National Bureau of Standards, DOE = U.S. Department of Energy.
[b]Not certified.
[c]Only one (1) analysis performed.

obtained with AAS. Instrumentation used was a Jarrell-Ash Model 800 series coupled to a PDP 8/a computer with dual floppy discs. Background correction on one side of the analytical line was done with a spectrum shifter. A forward power of 1 kw was used with the emission signal taken 16 mm above the load coil. Sample was introduced into the plasma with a fixed cross-flow nebulizer at 1.5 mL/min. Both methods of analysis gave similar results for cadmium, and all data reported in this paper were obtained by AAS.

Moisture in each sample was determined by drying a 2 g subsample in a vacuum oven at 100°C. All sediment concentrations are reported on a dry weight basis.

Analytical precision and accuracy for all 5 elements were estimated as follows: *(1)* Recovery of known additions to sediment samples was analyzed. Before digestion, a nitric acid solution containing As_2O_3, cadmium metal, lead metal, mercury metal, and selenium metal in the concentrations shown in Table 2 was added to the sediment sample and was carried through the entire digestion and analysis procedure. *(2)* Duplicate analyses were conducted on about 10% of the samples. Samples for duplicate analysis were selected at random from the sample set and carried through the entire digestion and analysis procedures. Duplicate samples were not collected in the field. *(3)* Certified standard reference materials were analyzed.

Results and Discussion

Average recoveries from spiked samples were within 10% of the added quantity for all elements (Table 2). There was no significant difference in recovery between the 2 fortification levels for any element, indicating a uniform recovery over the range of concentrations added. The average difference of duplicate sets ranged from 6 to 14% of the mean concentrations. Analyses of reference standards yielded average values that were within or near the specified certification ranges for all elements. No positive or negative bias was apparent in the analytical methodology. Therefore, all concentrations are reported as analyzed; no corrections were made to adjust for percentage recoveries.

Arsenic

Mean concentrations of arsenic at each location ranged from 1.9 mg/kg in Des Lacs to 12 mg/kg in Lake Andes (Table 3). The mean value for the 5 pothole locations (4.4 mg/kg) was significantly higher (t-test, $P < 0.01$) than the mean for the 6 riverine locations (2.4 mg/kg). The high mean for Lake Andes, which was nearly twice that of the next highest location, was biased by 3 samples from the same general area with unusually high concentrations of arsenic (21, 23, and 34 mg/kg). The remaining samples from Lake Andes (range 3.7–7.2 mg/kg) were comparable to values observed in the pothole locations.

Concentrations of arsenic in aquatic sediments from relatively unpolluted areas generally appear to be less than about 10 mg/kg. Huang and Liaw (1) found that arsenic in 10 Saskatchewan lakes ranged from 2.7 to 13 mg/kg, and they suggested that lakes at the upper end of this range showed evidence of contamination from runoff and erosion of agricultural land. Allan and Brunskill (9) reported that arsenic concentrations in Lake Winnipeg sediments ranged from 1 to 9 mg/kg, and they concluded that these levels did not indicate contamination from the surrounding watershed. Furr et al. (10) reported arsenic concentrations from the sediment of a farm pond receiving waste fly ash from a nearby coal-fired power plant and arsenic concentrations from a control pond receiving no waste fly ash. In the former, concentrations of arsenic in the sediment averaged 103 mg/kg, whereas in the latter the average was 9.8 mg/kg. The Environmental Protection Agency (11, 12) reported arsenic contamination in the sediments of streams below a large gold mining and processing facility in western South Dakota. Upstream of the mine, arsenic concentrations ranged from 3.6 to 21 mg/kg. Downstream of the mine, concentrations varied from 4.7 to 11 770 mg/kg. In Lake Texoma, where sediments are apparently

Table 3. Mean dry weight concentrations and ranges (in parentheses) of elements in sediment at 13 wetland locations (grouped according to habitat) in the Northern Prairie Region of the United States

Sample location and year collected	Number of samples	Concentration, mg/kg				
		Arsenic	Cadmium	Lead	Mercury	Selenium
			Potholes			
Coal Creek 1981	4	3.4 (2.4–5.0)	0.46 (0.17–0.87)	9.6 (7.5–12)	0.02 (0.01–0.03)	0.70 (0.27–1.0)
Jamestown 1981	6	6.2 (4.5–9.3)	0.46 (0.42–0.50)	12 (7.4–17)	0.04 (0.02–0.07)	0.99 (0.43–1.6)
Wike 1981	6	4.6 (3.7–6.3)	0.59 (0.46–0.77)	13 (8.6–15)	0.02 (0.01–0.04)	1.5 (1.2–2.1)
Madison 1980	12	4.8 (3.1–6.8)	0.62 (0.51–0.72)	12 (7.5–18)	0.04 (0.03–0.06)	0.98 (0.43–1.5)
Hastings 1981	12	3.2 (1.4–5.2)	0.44 (0.17–0.70)	14 (8.8–22)	0.03 (0.02–0.03)	0.50 (0.13–1.3)
All pothole samples	40	4.4** (1.4–9.3)	0.52** (0.17–0.87)	13** (7.4–22)	0.03 (0.01–0.07)	0.89* (0.13–2.1)
			Riverine			
Des Lacs 1980	9	1.9 (1.0–2.9)	0.25 (0.11–0.37)	6.8 (3.1–9.2)	0.04 (0.02–0.08)	0.35 (0.17–0.54)
J. Clark Salyer 1980	15	2.6 (1.0–6.0)	0.34 (0.10–0.55)	8.1 (2.1–12)	0.04 (0.02–0.06)	0.50 (0.12–1.1)
Audubon 1980	9	2.4 (1.1–5.0)	0.19 (0.09–0.30)	4.4 (1.5–6.9)	0.03 (0.01–0.05)	0.23 (0.08–0.53)
Arrowwood 1981	6	2.3 (0.7–4.0)	0.13 (0.01–0.26)	4.9 (1.1–8.7)	0.02 (0.01–0.05)	0.14 (0.03–0.39)
Sand Lake 1980	12	2.0 (1.1–3.2)	0.19 (0.08–0.34)	5.0 (1.6–14)	0.03 (0.03–0.04)	0.28 (0.06–0.43)
Union Slough 1980	9	3.0 (1.5–6.1)	0.40 (0.23–0.55)	9.1 (5.6–14)	0.03 (0.02–0.04)	1.6 (0.75–5.1)
All riverine samples	60	2.4 (0.7–6.1)	0.26 (0.01–0.55)	6.6 (1.1–14)	0.03 (0.01–0.08)	0.52 (0.03–5.1)
			Other			
Medicine Lake 1981	3	3.7 (1.6–6.2)	0.21 (0.04–0.37)	5.7 (1.7–9.7)	0.03 (0.01–0.04)	0.56 (0.10–1.1)
Lake Andes 1981	9	12 (3.7–34)	1.1 (1.0–1.3)	13 (7.5–17)	0.06 (0.04–0.11)	2.5 (0.34–6.8)

*Significant difference at $P < 0.05$ between grand mean concentrations in pothole and riverine samples.
**Significant difference at $P < 0.01$ between grand mean concentrations in pothole and riverine samples.

highly contaminated (from unspecified sources), Hunter et al. (13) found a mean arsenic concentration of 114 mg/kg with a range of 47–209 mg/kg. Cherry and Guthrie (14) reported arsenic in a coal-fired power plant ash disposal basin to range from 21 to 129 mg/kg. Sediments from a number of locations in a marsh system receiving effluent from this disposal basin averaged 20 and 27 mg/kg in 2 successive years. Greichus et al. (15) found that arsenic concentrations averaged 16 and 75 mg/kg in sediments of 2 South African reservoirs receiving mixed effluents from domestic, industrial, and agricultural sources.

With the exception of one location in Lake Andes, the range of arsenic concentrations in the sediment of the wetlands studied here (0.7–9.3 mg/kg) is comparable to background levels reported elsewhere. Arsenic contamination from unknown sources may have occurred in localized areas of Lake Andes; this is suggested both by the elevated levels of arsenic relative to other wetlands from the same geographic region and by comparison with values from the literature.

Cadmium

Mean concentrations of cadmium at each location ranged from 0.13 mg/kg in Arrowwood to 1.1 mg/kg in Lake Andes (Table 3). The overall mean for the pothole locations (0.52 mg/kg) was significantly higher ($P < 0.01$) than the mean for the riverine locations (0.26 mg/kg). The mean for Lake Andes was about twice that of the next highest location. However, unlike high arsenic which seemed to be more localized, the unusually high concentrations of cadmium were encountered in all of the samples from Lake Andes.

Background concentrations of cadmium in sediment, reported in the literature, vary by at least 2 orders of magnitude. Allan and Brunskill (9) found concentrations of 1–3 mg/kg in Lake Winnepeg. Jackson (16) considered concentrations ranging up to 13.7 mg/kg as background for Clay and Ball lakes in Ontario. Goldberg et al. (17) reported background levels in southern Lake Michigan as 0.25 mg/kg; recent contamination resulted in concentrations of 2.5 mg/kg in surficial sediments. Lund et al. (18) found that sediments of sewage effluent and sludge ponds contained from 0.25 to 1.4 and 0.38 to 3.6 mg/kg, respectively; control ponds in the same study ranged from 0.15 to 0.50 mg/kg. Cadmium concentrations in Derwent Reservoir (England), which received runoff from active fluorspar mining, averaged 13 mg/kg (19). Stephens Creek Reservoir (Australia), another area impacted by lead and zinc mining, had a mean sediment concentration of 4.3 mg/kg and a range of 2.2–6.9 mg/kg (20). Atchison et al. (21) reported sediment cadmium concentrations of 340–1300 mg/kg in Palestine Lake and 95–785 mg/kg in Little Center Lake (Indiana). These lakes receive contamination from an electroplating industry and urban runoff, respectively. In certain polluted lakes and streams near a smelter at Flin Flon, Manitoba, cadmium levels ranged from 2664 to 4260 mg/kg (22). Cherry and Guthrie (14) reported cadmium concentrations of 0.1–4.4 mg/kg in the sediment of a fly ash disposal basin. Sediment from various locations in the marsh receiving

effluent from the basin averaged 1.7–3.8 mg/kg in 2 successive years. Cadmium levels averaged 0.71 mg/kg in a nearby stream unaffected by the fly ash disposal. Greichus et al. (15) observed average cadmium concentrations of 0.87 and 0.19 mg/kg in the sediments of the 2 aforementioned South African reservoirs. Although not specifically reported as background concentrations by the authors, these cadmium levels do not appear to indicate contaminated conditions.

Cadmium concentrations in the sediments of the wetlands studied here (0.01–1.3 mg/kg) were comparable to the lower range of values reported in the literature and, therefore, do not suggest any contamination. All of the samples in this study, with the exception of those from Lake Andes, were below 1.0 mg/kg. Although the cadmium concentrations in Lake Andes are not particularly high relative to values reported elsewhere, they appear uncharacteristic for similar habitats in this geographic region.

Lead

Mean concentrations of lead at each location ranged from 4.4 mg/kg at Audubon to 14 mg/kg at Hastings (Table 3). The mean value for the pothole locations (13 mg/kg) was significantly higher ($P < 0.01$) than the mean for the riverine locations (6.6 mg/kg).

Background concentrations of lead in aquatic sediments vary considerably. Allan and Brunskill (9) found lead ranging from 2 to 278 mg/kg in Lake Winnipeg sediments. Harding and Whitton (19) reported a mean of 827 mg/kg for Derwent Reservoir, but the values ranged from below detection limits to more than 1500 mg/kg. In Little Center Lake (Indiana), which is contaminated by urban runoff, the mean lead concentration was 450 mg/kg with a range of 200–900 (21). Hamilton-Taylor (23) reported a mean lead concentration of 429 mg/kg (range 187–535 mg/kg) in the upper 11 cm of Windermere Lake sediment, and a mean background concentration of 56 mg/kg (range 51–62 mg/kg) in the same cores at depths below 11 cm. Coggins et al. (20) found a mean concentration of lead in the contaminated Stephens Creek Reservoir of 150 mg/kg (range 30–650 mg/kg). In 2 South African reservoirs, Greichus et al. (15) reported mean lead concentrations of 9 and 63 mg/kg. Goldberg et al. (17) found background lead concentrations of 10–30 mg/kg in Lake Michigan, with concentrations of up to 130 mg/kg in recent, contaminated sediments. In the Ottawa River, Oliver (24) considered 26 mg/kg as a background level. Williams et al. (25) reported recent accumulations of lead reaching 135 mg/kg in the Hudson River estuary, with a background concentration of 25 mg/kg.

Sediments from the wetlands studied here were not heavily contaminated with lead. All of the concentrations found (1.1–22 mg/kg) were near or considerably below the concentrations considered as background levels by other investigators. Furthermore, the range of values was rather uniform throughout the entire geographic region, with no evidence of abnormal localized enrichment.

Mercury

Mean concentrations of mercury at each location ranged from 0.02 mg/kg at Arrowwood, Wike, and Coal Creek to 0.06 mg/kg at Lake Andes (Table 3). No significant difference existed between the mean values for pothole and riverine locations. At Lake Andes, the samples having unusually high arsenic concentrations also had slightly elevated mercury concentrations (0.09–0.11 mg/kg).

Considerable data on concentrations of mercury in aquatic sediments are available; however, it is often difficult to distinguish those values representing natural or background levels from those affected by contamination. Allan and Brunskill (9) found mercury in Lake Winnipeg sediments ranging from 0.03 to 0.28 mg/kg. Walter et al. (26) reported mercury in Lake Oahe (a Missouri River reservoir in South Dakota) ranging from less than 0.03 to 0.62 mg/kg. Jackson (16) encountered mercury in a Canadian river-lake system ranging from 0.04 to 5.7 mg/kg. Sediments in Lake Ontario contained 0.03–2.1 mg/kg of mercury with a mean of 0.65 mg/kg (27). In Lake Paijanne (Finland) mercury ranged from 0.02 to 1.1 mg/kg with a mean of 0.36 mg/kg (28). In 2 South African reservoirs, Greichus et al. (15) reported mean concentrations of mercury of 0.06 and 0.60 mg/kg. Although not always stated, the above studies probably include data from both uncontaminated and mercury-contaminated areas. In a study of known mercury contamination, directly downstream of a gold processing plant in western South Dakota, the U.S. Environmental Protection Agency (11, 12) found mercury in stream sediments ranging from less than 0.1 to 4.1 mg/kg; in control sites in the same area mercury ranged from 0.02 to 0.10 mg/kg. In polluted lakes and streams near a smelter at Flin Flon, Manitoba, Jackson (22) reported mercury concentrations ranging from 0.89 to 1.8 mg/kg, whereas mercury in a control area averaged only 0.09 mg/kg. In the highly contaminated Gunneklewfjorden, Skei (29) found a range of sediment mercury concentrations of 90–350 mg/kg.

Mercury concentrations in the wetland sediments studied here (0.01–0.11 mg/kg) were comparable to the lowest concentrations reported in the literature. Therefore, discernable levels of mercury contamination at these sites are not evident. Slightly higher levels of mercury were found in the sediments from the previously noted area in Lake Andes, and some form of enrichment may have occurred there.

Selenium

Mean concentrations of selenium at each location ranged from 0.14 mg/kg at Arrowwood to 2.55 mg/kg at Lake Andes (Table 3). The mean value for the pothole locations (0.89 mg/kg) was significantly higher ($P < 0.05$) than the mean for the riverine locations (0.52 mg/kg). The 3 samples from Lake Andes with high arsenic concentrations were also high in selenium (4.8, 6.0, and 6.8 mg/kg). The remainder of the Lake Andes samples were comparable to other sites (0.34–1.3 mg/kg). One anomalously high selenium concentration (5.1 mg/kg) was encountered in Union Slough. The remainder of the values for Union Slough ranged from 0.75 to 1.5 mg/kg.

Background concentrations of selenium reported in the literature ranged from 0.9 to 2.2 mg/kg (10, 14). Concentrations of selenium in sediments from areas contaminated by ash from coal-fired power plants ranged from 2.4 to 17 mg/kg (3, 10).

Selenium concentrations in most of the wetland sediments studied here are well within the range of uncontaminated levels (0.03–2.1 mg/kg). However, the unusually high selenium concentrations from the same area, as previously noted for arsenic in Lake Andes, suggest that localized contamination may have occurred. The single, unusually high reading encountered in Union Slough is difficult to interpret.

Conclusion

A number of difficulties arise when comparing bulk element concentrations of sediment samples among locations. In most situations, bulk analyses alone are inadequate for recognition of all but the most polluted conditions. Element concentrations in sediments depend not only on input from external sources but also on a number of other factors, such as organic matter content, sulfide ion content, grain size, rate of sedi-

mentation, and solubility of the element under prevailing pH and redox conditions (16, 30, 31). Thus, one sediment sample might be richer in a particular element than another merely because it is finer grained or richer in organic matter and not because of increased input. Differences in sampling and analytical methods further complicate comparisons of various studies in the literature. For example, several different extraction techniques that are used report results as "total" concentrations of a given element in sediment.

In this study, sediments from pothole wetlands contained significantly more arsenic, cadmium, lead, and selenium than sediments from riverine wetlands, which may be due to differences in external loading or transport, rates of sedimentation, or to differences in sediment properties such as grain size and/or organic matter content. These questions focus on needs for additional studies of these elements in Northern Prairie wetland ecosystems. First, the primary sources of various elements in wetlands should be assessed. May and McKinney (2) summarized the primary sources of certain elements to aquatic environments in the United States. From their list, coal combustion, agricultural pesticides and fertilizers, and natural geologic sources are probably the most important in the geographic region we studied. Second, factors that regulate or influence the concentration of elements in wetland sediments should be determined. Knowledge of physical and chemical conditions in the sediment and the overlying waters is necessary (16, 22, 30). Hydrologic characteristics are also important in the transport and clearance of elements from aquatic systems (16, 31). Third, the ecological significance of specific elements in wetlands should be more clearly understood. Total quantities in any physical component such as sediment may be relatively unimportant. More important questions concern factors influencing availability to aquatic biota and effects of long-term, low level exposure on specific organisms.

The baseline data collected in the present survey indicate that, with minor exceptions, sediments from wetland habitats within the Northern Prairie region of the United States are relatively uncontaminated. The previously mentioned questions, regarding source and effect of specific elements in wetland environments need to be further addressed to ensure future protection of these resources.

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TECHNICAL COMMUNICATIONS

Evaluation of Laboratory Performance of the AOAC Method for PSP Toxin in Shellfish

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Laboratory performance of the official AOAC method for paralytic shellfish poison (PSP) toxin in shellfish was evaluated. Two series of naturally toxic shellfish split samples were distributed (15 in 1979 and 19 in 1982) to state shellfish-monitoring laboratories which participate in the National Shellfish Sanitation Program. The laboratories performed bioassays on duplicate 100 g portions of each 220 g split sample. Bioassays were consistent among the laboratories and compared favorably with those of previous studies.

The official AOAC method for the mouse bioassay (1–3) for paralytic shellfish poison (PSP) is performed routinely by many laboratories in the United States. To provide quality assurance among the state shellfish-monitoring laboratories which participate in the National Shellfish Sanitation Program (NSSP), Food and Drug Administration (FDA) personnel periodically visit the laboratories and evaluate routine procedures. In 1979 and 1982, FDA conducted studies in which a series of homogeneous shellfish meat split samples containing various levels of natural PSP toxin were analyzed by the state laboratories. The last such study (4), reported by McFarren in 1959, involved 11 laboratories with 8 spiked samples at 4 PSP levels. Naturally toxic clams and oysters comprised 7 other samples. The McFarren study led the way in developing and confirming statistical parameters associated with the mouse bioassay and in standardizing the test as it is known today.

Since 1959, however, laboratories, laboratory personnel, mouse colonies, and procedures have changed. To ensure that the participating state laboratories are uniformly providing equal public health protection, a continuing program of split samples has been established. This paper reports the results of the 1979 and 1982 split sample programs for 2 series of split shellfish samples. Results are compared with those obtained in surveys conducted in 1959 and 1979. The purpose of the study was to determine whether laboratories are generally conforming to standard methods and whether their individual results are within certain statistical limits.

Experimental

General

Bioassays were performed in duplicate 100 g portions by the participating laboratories on each 220 g split sample and results were expressed in μg/100 g. The split samples consisted of 2 levels of PSP toxin; one was targeted near the lower limit of the test, yet was still reasonably close to the recommended level of 80 μg/100 g, which is the level used for closure of harvesting areas. The higher value was selected to be significantly above the closure level but not so high as to present a problem in making dilutions. The low level samples were prepared by diluting shellfish meat containing naturally occurring toxin with nontoxic shellfish meat.

Both low and high levels were provided in duplicate so that repeatability could be evaluated. In the 1979 split sample no particular instructions were given to the laboratories except to follow the recommended AOAC method. The samples were treated completely as unknowns. In the 1982 split sample, however, instructions were given with respect to the dilution factor (DF); the DF was thus eliminated as a variable since it has been shown to affect the final outcome (4). With this controversial and significant variable eliminated, the potential was increased to compare variations among laboratories, such as personnel changes, mouse death time estimation, and mouse colony differences.

Preparation of Toxic Clam Samples

Approximately 40 lb of toxic butter clams *(Saxidomus giganteus)* were received frozen in dry ice from the FDA district laboratory in Seattle, Washington. The toxic clams originated from Barnaby Narrows, Vancouver, British Columbia, Canada, and ranged in weight from 15 to 50 g, with an estimated PSP concentration of 500–1000 μg/100 g. The external surface of the clams was scrubbed clean with tap water. Intact clam meats were shucked directly onto a No. 10 sieve without layering and were allowed to drain for 5 min. The drained meats were transferred into a blender and ground until homogeneous. The grinding time, which was dependent on the amount of clam sample, ranged from 1–3 min for 500 g of meats to 8–10 min for 4400 g of meats.

Split sample preparation involved development of 2 toxin levels. One homogenate contained a toxin concentration which required a dilution of the shellfish extract so that 3 injected mice died in 5–7 min. The second homogenate, the toxic extract, was injected undiluted, and the death time of 3 mice was between 5 and 60 min.

The split samples were prepared by mixing dilutions of toxic clams with nontoxic clam meats (50 and 10%, respectively) in a blender (1 gal. capacity). The blended sample was dispensed (or split) into plastic sample bottles in 220 g portions for duplicate bioassay. The samples were frozen at −20°C before packaging and shipping. Storage studies were done for up to 4 days to confirm the keeping quality of the sample and toxin during shipment.

Results and Discussion

The overall results of the 2 split samples showed that the quality assurance program provided some improvement in laboratory performance between 1979 and 1982. In 1982, the laboratories produced results within acceptable statistical limits, and despite the fact that more laboratories participated, the range of the limits to detect outliers narrowed. In 1979, the PSP level (μg/100 g) ranged from 28–101 (mean 61) to 100–336 (mean 218) (outer limits $x = 0.01$) (5). In comparison, the PSP level (μg/100 g) in 1982 ranged from 24–91 (mean 58) to 60–218 (mean 139). Of 15 and 19 participating laboratories in 1979 and 1982, respectively, 2 and 0 were outliers.

The split sample data in the McFarren study (4) clearly showed that the mouse bioassay test varied with the PSP concentration. The variance of repeated sampling at a single PSP level followed theoretical considerations (Figure 1). The line of best fit was calculated by regression analysis. Also plotted are the results of the 1979 and 1982 sample studies

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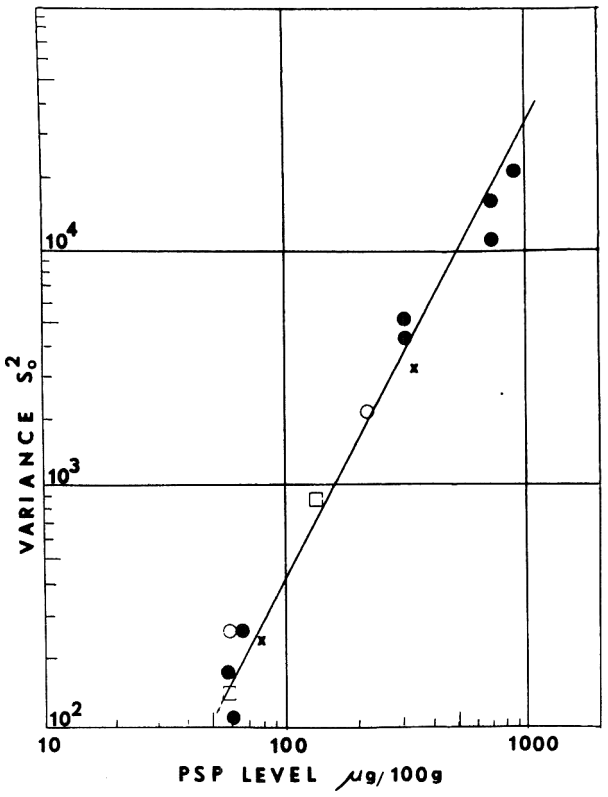

Figure 1. Within-laboratory variance of the mouse bioassay test for PSP toxin in shellfish, as a function of concentration. ○, FDA 1979; □, FDA 1982; x, Ares & Dymsza 1982; ●, McFarren 1959.

Table 1. Precision estimates by study and PSP level

Split sample	PSP level, µg/100 g	Standard deviation[a] S_x	S_o	S_L	CV_x
McFarren					
1959	61	14.5	7.0	12.7	24
1959	326	69.2	56.9	39.4	21
1959	740	151.2	71.6	133.2	20
1959	897	148.5	111.2	98.4	17
FDA					
1979	61	16.0	4.3	15.4	26
1979	215	43.6	15.7	40.7	20
FDA					
1982	58	12.4	3.4	11.9	21
1982	139	30.3	11.8	27.9	20
Ares and Dymsza[b]					
1982	78	14.8	5.9	13.6	19
1982	352	56.2	27.0	49.3	16

[a] S_o, repeatability (square root of variance of repeated measurements; S_L, square root of laboratory-to-laboratory variation; S_x, reproducibility (square root of the sum of S_o^2). CV_x, coefficient of variation of reproducibility (see ref. 5).
[b] See ref. 6.

and those of Ares and Dymszá (6), whose split samples involved mashed potatoes spiked with toxin. The graph demonstrates that the mouse bioassay has remained suitably consistent over the past 20 years and assures the NSSP that the participating laboratories have been performing satisfactorily. Overall variance (S_x^2) involves not only a component due to the laboratory-to-laboratory variation (S_L^2), but also a component due to the variation among repeated measurements (S_o^2). Although the McFarren study did not separate these components, we have done so (Table 1), using the methods of Youden and Steiner (7), and have expressed them as standard deviations (SD). The SD values (S_o and S_x) are, respectively, the repeatability and reproducibility of the assay.

In most cases, the SD reflecting the lab-to-lab variation (S_L) is higher than the SD reflecting the variation among repeated measurements (S_o). At the lower PSP levels, S_L is higher relative to S_o than at the higher PSP levels. Analysis of variance of the 2 FDA split samples showed significant lab-to-lab variation, even though the overall results indicated that nearly all laboratories fell within the outlier limits. The FDA split samples also showed that the results of 2 or 3 laboratories were consistently low or high for the duplicate samples. However, as shown in Table 1, the reproducibility coefficient of variation for all the studies approximates 20%.

Deviations from the AOAC recommended procedures for PSP bioassay included *(1)* omission of the conversion factor (CF) check value to calculate PSP concentrations; *(2)* application of weight correction factor to mice weighing less than 19 g or more than 20 g; *(3)* determination of the CF check from 3 mice rather than the recommended 5 mouse injections; *(4)* use of CF check value greater than ± 20% of the established CF; *(5)* use of clam samples less than 100 g; *(6)* use of shellfish extract dilutions which did not result in death of 3 mice in 5–7 min; *(7)* error in median mouse unit selection; *(8)* injection outside the peritoneal cavity.

In 1979, only 2 of 15 participating laboratories (13%) conformed with the standard operating procedures for the mouse bioassay; in 1982, that number increased to 9 of 19 laboratories (47%). This increase may be the result of the individual laboratory evaluations of mouse bioassay procedures, which are conducted once every 2 years as well as the FDA split sample program.

Overall, the mouse bioassay test for PSP toxin continues to provide assurance that shellfish may or may not be hazardous and that the various state laboratories can produce uniform results.

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Improved Gas Chromatographic Determination of Sorbitan Fatty Acid Esters in Confectionery Products

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A method is described for gas chromatographic (GC) determination of sorbitan fatty acid esters in confectionery products as sorbitan monostearate. A sample is homogenized with chloroform, filtered, dried, and saponified with 0.1N NaOH in ethanol 1 h at 80°C. The saponification mixture is acidified, washed with hexane, and dried. Isosorbide, 1,4-sorbitan, and D-sorbitol are each derivatized at 70°C with pyridine, hexamethyldisilazane, and trimethylchlorosilane. GC separation of the polyols as TMS (trimethylsilyl) derivatives was performed on a 2% Dexsil 300 column temperature-programmed from 120 to 250°C at 10°/min. The sorbitan monostearate content of the sample was calculated from the polyols, using the appropriate conversion factor. The procedure was applied to ice creams, cakes, and other confectionery products. Average recoveries from samples spiked with 1.0% Span 60 (sorbitan monostearate) were 91–96% for isosorbide, 83–99% for 1,4-sorbitan, and 82–98% for D-sorbitol. The detection limit was approximately 0.01%.

In Japan, sorbitan fatty acid esters are registered as a food additive. They are used as emulsifiers and stabilizers in such foods as cakes, ice creams, and other confectionery products at levels of 0.4–1%.

In 1964, Wetterau et al. (1) developed a gas chromatographic (GC) method for determination of sorbitan monostearate in cake mixes and baked cakes. A sample was extracted with ethanol, and interfering substances were removed by column chromatography. The sorbitan monostearate fraction was saponified with KOH, and the polyols were analyzed after purification on an ion-exchange column. The isosorbide was used for calculation of sorbitan monostearate.

Lundquist and Meloan (2) developed a rapid GC method for determination of sorbitan fatty acid esters in whipped cream without purification. Ethanol and water were added to the sample and the esters were extracted with diethyl ether and petroleum ether. The concentrated extract was directly injected onto a column of soda-lime beads that was connected to a column of 15% Carbowax 20M on silanized Chromosorb T. Samples were saponified on the reactive precolumn, and the resulting polyols were separated on the analytical column. This method is simple and rapid, but replacing the soda-lime beads after 10 injections is troublesome.

The Japan Ministry of Health and Welfare (3) proposed the following GC method: A freeze-dried sample is refluxed with chloroform and the filtrate is saponified with 0.5N KOH in ethanol. The evaporated reaction mixture is refluxed with 0.5N HCl, washed with hexane, and evaporated after neutralization. The dried residue is extracted with *n*-butanol and evaporated to dryness, and the polyols are analyzed as TMS derivatives by GC. The sorbitan monostearate content is calculated from the polyols using a conversion factor (0.27). However, this method is tedious and time-consuming.

We tried to simplify this GC method by substituting homogenized extraction for reflux extraction and omitting the desalting procedure with *n*-butanol. Our proposed method is rapid and can be applied to various confectionery products. We applied the procedure to commerical confectionery products and also surveyed sorbitan fatty acid ester contents.

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METHOD

Apparatus

(a) *High-speed homogenizer.*—Ultra-Turrax (Janke and Kunkel KG, GFR).

(b) *Hot dry bath.*—SHD III (Iuchi Seieido Co., Ltd, Osaka, Japan).

(c) *Gas chromatograph.*—Yanaco G 80 (Yanagimoto Mfg Co., Ltd, Kyoto, Japan) with flame ionization detector. Operating conditions: column temperature programmed from 120 to 250°C at 10°/min; detector and injection port temperature 275°C; glass GC column, 150 cm × 3 mm, packed with 2% Dexsil 300 GC on 60–80 mesh Chromosorb W (AW-DMCS).

Reagents

(a) *Sorbitan fatty acid esters.*—Span 60 (sorbitan monostearate) (Kao Atlas Co., Ltd, Japan).

(b) *Isosorbide and 1,4-sorbitan.*—Prepared in our laboratory by dehydration of D-sorbitol, analytical grade (Nakarai Chemicals Ltd, Kyoto, Japan) (3).

(c) *Polyols standard solutions.—Stock solutions.*—500 μg/mL. Dissolve 50 mg each of isosorbide, 1,4-sorbitan, and D-sorbitol in 100 mL pyridine. *Working solutions.*—Dilute aliquots of stock solution with pyridine to prepare solutions containing 5–50 μg polyols/mL.

(d) *TMS reagents.*—Trimethylchlorosilane (TMCS), hexamethyldisilazane (HMDS) (Nakarai Chemicals Ltd).

Procedure

Extraction of sorbitan fatty acid esters.—Weigh 5 g sample into 100 mL beaker and homogenize 3 min with 25 mL chloroform. Filter homogenate through paper No. 5A (Toyo Roshi Co., Ltd, Tokyo, Japan) into 50 mL volumetric flask. Repeat homogenization and filtration, and dilute to volume with chloroform.

Saponification of sorbitan fatty acid esters.—Pipet 5 mL filtrate into 10 mL stopper test tube, and evaporate to dryness under stream of nitrogen in 70°C hot dry bath. Add 2 mL 0.1N NaOH in ethanol, and carry out saponification in 80°C hot dry bath.

Removal of fatty acids.—Cool to room temperature, acidify with 2–2.5 mL 0.1N HCl, and shake vigorously 30 s with 2 mL hexane. Let layers separate, and discard upper layer. Repeat this operation, neutralize lower layer (pH 6–8) with 0.1N NaOH in ethanol (BTB indicator), and dilute to 5 mL with water.

Trimethylsilylation of polyols.—Pipet 1–2 mL neutralized solution into 5 mL stopper test tube, and evaporate to dryness (30 min) under stream of nitrogen in 70°C hot dry bath. Add 1 mL pyridine, 0.1 mL HMDS, and 0.1 mL TMCS. Shake vigorously, and heat 15 min at 70°C.

Preparation of standard curves.—Pipet 1 mL of each polyol standard solution, 5–50 μg/mL, into 5 mL stopper test tube, and derivatize in same manner as sample. Inject 5 μL derivative solution into gas chromatograph. Plot peak heights (cm) vs polyols (μg).

GC determination—Inject 5 μL sample solution into gas chromatograph. Measure peak heights and read concentra-

Table 1. Recovery of Span 60 as polyols from selected confectionery products

Sample	Added, %	Recovery, %[a]		
		Isosorbide	1,4-Sorbitan	D-Sorbitol
Cake	0.2	87 ± 5	103 ± 3	97 ± 7
	1.0	93 ± 4	99 ± 1	98 ± 2
Ice Cream	0.2	82 ± 8	83 ± 8	100 ± 7
	1.0	91 ± 9	83 ± 1	82 ± 5
Cream (bread)	0.2	80 ± 5	96 ± 3	90 ± 8
	1.0	96 ± 9	87 ± 6	82 ± 5
Biscuit	0.2	89 ± 8	83 ± 7	100 ± 7
	1.0	94 ± 5	87 ± 1	86 ± 3

[a]Each value is the average of 5 trials ± SD.

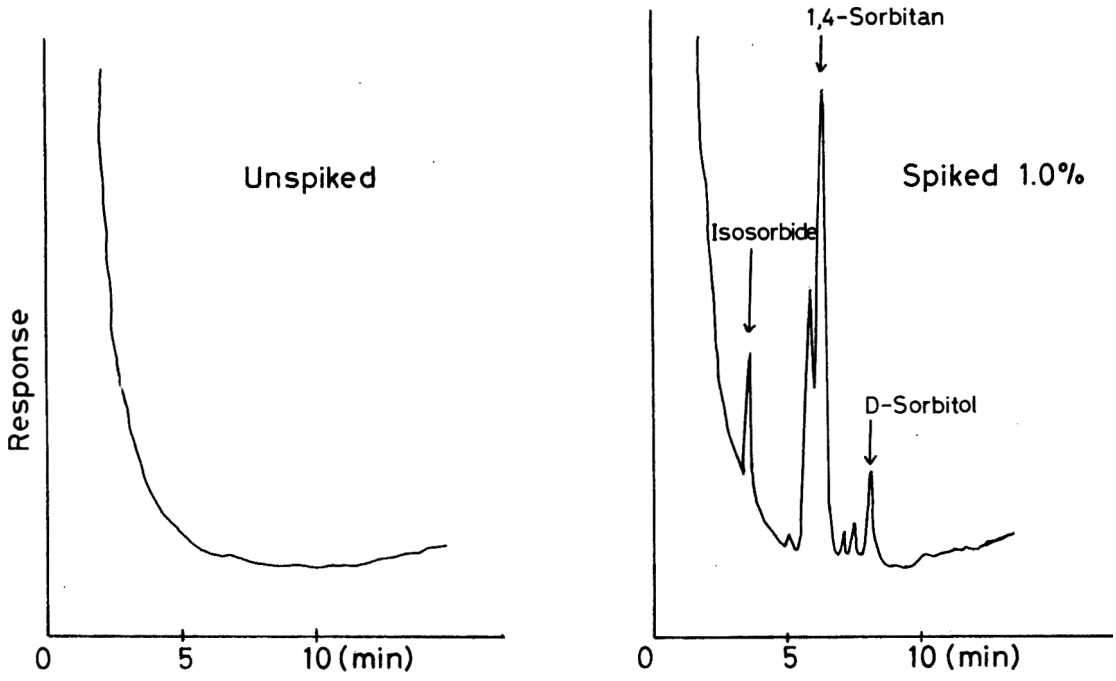

Figure 1. GC chromatograms obtained from recovery studies of cream in bread: left, unspiked; right, polyols as TMS derivatives derived from spiked Span 60.

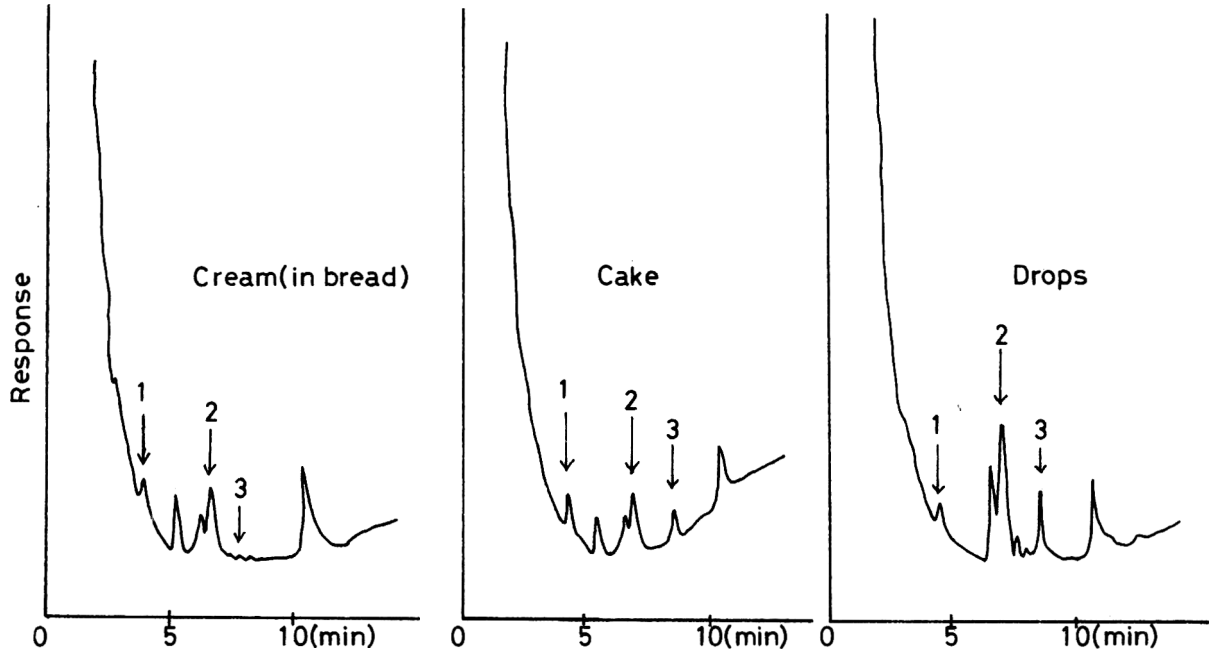

Figure 2. GC chromatograms of polyols as TMS derivatives derived from sorbitan fatty acid esters in commercial confectionery products: 1, isosorbide; 2, 1,4-sorbitan; 3, D-sorbitol.

Table 2. Contents of sorbitan fatty acid esters as sorbitan monostearate in commercial confectionery products

Sample	Contents, %
Ice cream I	ND[a]
Ice cream II	ND
Ice cream III	ND
Cream (bread) I	0.01
Cream (bread) II	ND
Cream (bread) III	ND
Cake I	0.04
Cake II	ND
Cake III	ND
Dry yeast	0.63
Drops	0.14

[a]None detected (detection limit 0.01%).

tion of polyols from standard curves. Calculate sorbitan monostearate content of sample as follows:

$$C = (W_1 + W_2 + W_3)/(10\,000 \times W \times f)$$

where C = sorbitan monostearate content (%); W_1, W_2, W_3 = isosorbide, 1,4-sorbitan, and D-sorbitol contents (μg) respectively; W = sample weight (g); and f = conversion factor (0.27, proposed by Japan Ministry of Health and Welfare (3)).

Results and Discussion

Saponification of Sorbitan Fatty Acid Esters

The saponification rate with 0.1, 0.2, and 0.5N NaOH in ethanol and for various intervals was investigated at 80°C. In all cases, reaction was complete after 1 h, and 0.1N was selected to minimize the salt generated by neutralization.

Interference Substances

Fructose, glucose, and D-sorbitol were tested. The monosaccharides (each 100 mg/2 mL water) were extracted 5 min with 50 mL chloroform, and 5 mL extract was analyzed as described above. No peaks were detected by GC (detection limit each 10 μg). Furthermore, fructose and glucose as TMS derivatives did not interfere in GC chromatograms of the polyol TMS derivatives of Span 60.

Recoveries from Selected Confectionery Products

Standard curves for polyols at 5–50 μg gave straight lines. In Japan, sorbitan monostearate is the most common of all sorbitan fatty acid esters used in food. Recovery studies were carried out by adding 0.2% and 1.0% Span 60 to 5 g samples and analyzing them by the method described above. Figure 1 and Table 1 show typical chromatograms and the results, respectively. Recoveries of Span 60 as polyols were 80–96% for isosorbide, 83–103% for 1,4-sorbitan, and 82–100% for D-sorbitol at 0.2% and 1.0% levels.

Contents of Sorbitan Fatty Acid Esters in Commercial Confectionery Products

Figure 2 shows typical GC chromatograms of polyols, as TMS derivatives, obtained from commercial confectionery products. Polyols were identified by comparison of GC retention times. Contents of sorbitan fatty acid esters were calculated as sorbitan monostearate, using the conversion factor 0.27. Table 2 shows the results obtained.

References

(1) Wetterau, F. P., Olsanski, V. L., & Smullin, C. F. (1964) *J. Am. Oil Chem. Soc.* **41**, 791–795
(2) Lundquist, G., & Meloan, C. E. (1971) *Anal. Chem.* **43**, 1122–1123
(3) *Analytical Methods for Food Additives in Foods* (1982) 1st Ed., Ministry of Health and Welfare, Tokyo, Japan, pp. 175–181

Inventory of IDF/ISO/AOAC Adopted Methods of Analysis for Milk and Milk Products

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This inventory is intended to provide a complete catalog of adopted methods of analysis prepared by IDF (International Dairy Federation), ISO (International Standardization Organization) (ISO/TC 34/SC 5), and AOAC for milk and milk products. At the request of the FAO/WHO Committee on the code of Principles for Milk and Milk Products, the 3 bodies have been cooperating for many years in the preparation of the methods of analysis needed to support the compositional standards associated with the Code of Principles. The present list includes not only these methods, but also indicates standard or adopted methods issued separately by the 3 organizations, and also methods under study by these organizations.

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The inventory is intended for use by the 3 organizations, by FAO/WHO (Code of Principles and the Codex Committees on Methods of Analysis and Sampling), and others (for example, the Inter-Agency meeting). Different users will have different requirements; accordingly, the inventory includes 2 lists, one classified on the basis of the products to which the methods apply (Table 1), and the other classified on the property or component being examined or analyzed (in alphabetical order) (Table 2).

The designations "reference" and "routine" in relation to the methods have not been included. Similarly, the classification scheme for methods devised by the Codex Committee on Methods of Analysis and Sampling has not been applied. On the other hand, it has been indicated whether a method has undergone an interlaboratory study to establish its precision.

Although work has not been completed, there are plans to compile an appendix, for use by IDF, ISO, and AOAC, listing those methods still required for the Code of Principles Standards.

Key to Symbols and Abbreviations
(Symbols for units and well known abbreviations, for example, TLC = thin layer chromatography, not included)

Column	Symbol/abbrev.	Explanation
Description/principle	RMP	Reichert-Meissl-Polenske value
	BDI	Bureau of Dairy Industries, USA
Collab. study	+	method has been subjected to an interlaboratory study (Youden and Steiner and/or ISO 5725 statistics)
	–	interlaboratory study not appropriate or method has not been subjectec to a fully rigorous interlaboratory study
FAO/WHO	B4:1967 (example)	Standard B4 associated with the Code of Principles concerning Milk and Milk Products, published 1967
	19th. App. IX-F (example)	Appendix IX-F to the report of the 19th session of the Committee of Government Experts on the Code of Principles concerning Milk and Milk Products
	CCEI	Codex Committee on Edible Ices
	submitted 1982	method in question was submitted to the Code of Principles Committee at the 1982 session (20th) but did not appear in an appendix to the report of the meeting
IDF	6A:1969 (example)	IDF Standard 6A, published 1969
	(rev.)	standard under revision by a joint IDF/ISO/AOAC group of experts
	Q 983/E (example)	Questionnaire 983/E latest draft
IDF, ISO, and AOAC	IDF/ISO/AOAC Group E 33 (example)	method is under consideration by joint IDF/ISO/AOAC Group E 33
ISO	1740:1980 (example)	ISO standard 1740, published 1980
	DIS	Draft International Standard
	DP	Draft Proposal
AOAC	OMA	Official Methods of Analysis, 13th edition (1980); 14th edition (1984)
	16.237 (example)	Chapter 16, paragraph 237 in OMA
	not	method referred to is not the same as those listed in the IDF and ISO columns

Table 1. IDF/ISO/AOAC inventory of standard and adopted methods of sampling and analysis for milk and milk products, arranged by product (published or in progress)

Product	Property/component	Description/principle	Collab. study	FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
Acid casein	ash	gravimetric, after incineration at 825°C	–	18th, App. IX-C				
Ammonium caseinate	ammonium	—				IDF/ISO/AOAC Group E 11		
Baby foods based on dried milk	colony count at 30°C	poured plates (yeast extract, tryptone glucose, skimmed milk)	–			IDF/ISO/AOAC Group E 22		
Butter	acid value; free fatty acid index	titrimetric	+	B4:1967	6A:1969	1740:1980	16.237–16.239	16.211–16.213
	aflatoxin M_1	TLC	+				26.090	26.090
	butter stirrer		–				44.002	44.002
	casein, ash, salt	oven <500°C, Cl/gravimetric	+				16.234	16.208
	chloride	titrimetric (Mohr)	+	B8:1967	12A:1969 (rev.)	1738:1980	16:235	16.209
	color additives	qualitative	+				16.244	16.218
	contaminating organisms	colony count at 30 and 20°C on carbohydrate-free medium	–		30:1964			
	critical temp. of dissolution	thermometric	+				16.241–16.243	16.215–16.217
	DDT residues	GC + identification	+				29.084	29.097
	fat	gravimetric (water, solids-not-fat, and fat content on one test portion)	+	B21:1978	80:1977	3727:1977	16.232	16.206
	fat	gravimetric, direct extraction	+				16.233	16.207
	filth	filtration	+				44.022–44.024	44.018–44.020
	lactic acid	spectrophotometric	+				16.245	16.219
		filtration	+				44.021	44.025
	organoleptic attributes	sensory evaluation	–		99:1981			
	pH value (of serum)	electrometric	–		104:1981	7238:1983		
	phosphatase, residual	photometric, 610 nm filter	+				16.256	16.230
	preservatives	various methods	+				16.254	16.228
	refractive index (butterfat)	refractometric	+	B5:1967	7A:1969	1739:1975	16.240	16.214
	sampling		–				16.012–16.014, 16.229	16.012–16.014, 16.203
	sediment	vacuum filtration	+				44.021	44.024
	solids-not-fat	gravimetric, on solvent extract	–		11:1960 (rev.)			
	solids-not-fat	gravimetric, on solvent extract (water, solids-not-fat, and fat on one test portion)	–	B21:1978	80:1977 (rev.)	3727:1977	16.205	16.231
	total solids (moisture)	gravimetric drying at 102°C	–		10:1960			
	total solids (water)	gravimetric, drying at 102°C (water, solids-not-fat, and fat on one test portion)	+	B21:1978	80:1977 (rev.)	3727:1977	16.231	16.205
	total solids (water)	gravimetric, heating (rapid method)				IDF/ISO/AOAC Group E 5		
	volatile acids	distillation, chromatographic separation	+				16.253	16.227
	volatile fatty acids	sterol acetate melting point (RMP value)	+				16.236	16.210
	water dispersion	indicator paper comparison	–		112:1982	DIS 7586		
	water-insoluble fatty acids	gravimetric	+				16.246	16.220
	water-insoluble fatty acids + butyric acid	chromatographic	+				16.247–16.251	16.221–16.225
Butter triers	sampling instrument	requirements	–			1193:1973		
Butter, cream, cheese, milk	pesticide residues (see *also organochlorine pesticides*)	GC + identification	+				29.001–29.028	29.001–29.028
Butterfat	iodine value	titrimetric (Wijs)	+				28.023	28.020
	iodine value	titrimetric with Na thiosulfate (Wijs)	–		8:1959			
Butteroil (see *also Milkfat products*)	water	titrimetric (Karl Fischer)	–		23:1964 (rev.)			
	fat	gravimetric (calculation from solids-not-fat and water)	–		24:1964			

Product	Property/component	Description/principle
Calf rennet and adult bovine pepsin	chymosine and bovine pepsin	clotting time, after chromatographic separation
Casein	filth	microscopic, after sieving
	phosphatase, residual	colorimetric and spectrophotometric
Caseinate *(see Ammonium caseinate)*		
Caseins *(see also Acid casein, Rennet casein)*	acidity (free acidity)	titrimetric, on aqueous extract
Caseins and caseinates	ash (fixed ash)	gravimetric, after incineration at 825°C
	colony count at 30°C	poured plates (yeast extract, tryptone glucose, skimmed milk)
	extraneous matter	
	fat	gravimetric (Schmid-Bondzynski-Ratzlaff)
	lactose	photometric, with phenol and H_2SO_4
	nitrate and nitrite	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction
	nitrogen (protein)	titrimetric, Kjeldahl
	pH value	electrometric measurement
	total solids (water)	gravimetric drying at 102°C
	whey protein	
Cheese		anhydride
	ash	furnace <550°C
	chloride	indicating-strip method
	chloride	titrimetric
	citric acid	gravimetric
	citric acid	qualitative test
	collection of sample	
	color additives	several methods
	DDT residues	TLC
	dehydroacetic acid	qualitative
	enzymes (residual)	
	examination of fat *(see also volatile fatty acids)*	RMP value, sterol acetate melting point
	extraneous materials	filtration
	fat	butyrometric (Van Gulik)
	fat butyrometers	requirements (Van Gulik)
	moisture *(see also total solids)*	distillation
	moisture	forced draft oven ± 130°C, screening
	moisture	microwave oven
	moisture	vacuum oven 100°C
	monofluoro acid residues	qualitative
	natamycin	photometric or LC, after extraction
	nisin	
	nitrate	(range 2–15 mg/kg)
	nitrate and nitrite	>1 ppm NO_3, Cd reduction method
	nitrate and nitrite	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction

Collab. study	FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
–		110:1982			
+				44.027	44.022
+				16.127–16.129	—
–	19th App. IX-F	91:1979	5547:1978		
–	19th App. IX-D	89:1979	5544:1978		
–		Q 883/E	DP 8198		
–			IDF/ISO/AOAC Group E 11		
+		Q 3183/E	DP 5543		
–	19th App. IX-A	106:1982	5548:1980		
+		120:1984	DP 8195		
–	19th App. IX-E	92:1979	5549:1978		
–	submitted 1982	115:1982	5546:1979		
–	19th App. IX-B	78B:1980 (rev.)	5550:1978		
–			IDF/ISO/AOAC Group E 11		
–	App. VI-D			differ	
+				16.267	16.241
+				16.273	16.244
+				16.272	—
+				16.291	16.262
+				16.290	16.261
–				16.257	16.231
+				16.277	16.248
+				IDF/ISO/AOAC Group E 12	
				29.109	29.097
+				20.069–20.070	20.052–20.053
–				IDF/ISO/AOAC Group E 32	
+				16.286	16.257
+				44.024–44.027	44.020–44.023
–			3433:1975		
–			3432:1975		
+				16.261–16.264	16.235–16.238
+				16.260	16.234
+				16.265–16.266	16.239–16.240
+				16.259	16.233
+				29.148–29.150	29.140–29.142
–				IDF/ISO/AOAC Group E 43	
–				IDF/ISO/AOAC Group E 43	
–				IDF/ISO/AOAC Group E 8	
+				16.278–16.283	16.249–16.254
+	B19:1978	84A:1984	4099:1978		

	nitrogen (protein)	titrimetric, Kjeldahl
	organoleptic attributes	sensory evaluation
	phosphatase, residual	photometric, 610 nm
	phosphatase, residual	qualitative, screening
	sampling	
	sediment	vacuum filtration
	sorbic acid	oxidation
	tartaric acid	qualitative
	tartaric acid	quantitative, titrimetric
	titanium	spectrophotometric
Cheese (pasteurized, stabilized)	phosphatase activity	photometric, with Gibbs reagent on phenol liberated
Cheese (processed)	chloride	potentiometric titration
	citric acid	photometric, with pyridine and acetic
	fat	gravimetric (Schmid-Bondzynski-Ratzlaff)
	phosphorus	photometric, molybdate-ascorbate
	total solids	gravimetric, drying at 102°C
Cheese products		
Cheese triers	sampling instrument	requirements
Chocolate frozen desserts	alginates	confirmation, qualitative
Cond. milk, sweetened	ash	furnace 525°C (2 methods)
	lactic acid	spectrophotometric
	lactose	gravimetric
	nitrogen (protein)	titrimetric, Kjeldahl
	sucrose	inversion and polarimetry
	total solids	gravimetric, drying at 102°C
Cond. and evap. milk	sampling	
Cottage cheese	gelatin	qualitative
Cream	ash	furnace < 550°C
	Babcock cream test bottle	
	collection of sample	
	color additives	qualitative
	DDT residues	TLC
	fat	Babcock
	fat	gravimetric (Roese-Gottlieb)
	filth	microscopic, after filtration
	gelatin	qualitative
	lactic acid	spectrophotometric
	lactose	gravimetric
	nitrogen (protein)	titrimetric, Kjeldahl
	phosphatase, reactivated and residual	differential test for phosphatase activity
	phosphatase, residual	photometric, 610 nm filter
	preservatives	various methods
	sediment	vacuum filtration
	volatile acids	chromatographic
	water-insoluble fatty acids	gravimetric
Dairy plant surfaces	sampling	microbiological
Dried milk	acidity (titratable acidity)	titrimetric, on dissolved product
	acidity (titratable acidity)	titrimetric, on dissolved product
	ammonium	
	ash	furnace < 550°C
	citric acid	gravimetric
	density	bulk density
	flecks	
	heat class (heat number)	calculation, from Kjeldahl N of precipitated casein plus some denatured serum protein

+			IDF/ISO/AOAC Group E 27		
				16.274	16.245
–		IDF group D6c			
+				16.304–16.306	16.275–16.277
+				16.307	16.278
–				16.015–16.018	16.015–16.018
+				44.021	44.024
+				16.303, 20.115	16.274, 20.098
+				16.287	16.258
+				16.288–16.289	16.259–16.260
+				16.275	16.246
–		53:1969			
+	B18:1978	88:1979	5943:1978	16.268–16.271	
+	B13:1972	34B:1971	2963:1974	16.292–16.296	16.321–16.325
+	B3:1967	5A:1969	1735:1975	16.284	16.313
–	B12:1972	33B:1982	2962:1984		
+	20th	4A:1982	DIS 5534	all methods differ	
–			1194:1973		
+				16.326	16.297
+				16.204	16.184
+				16.202	16.182
+				16.207	16.187
+				16.206	16.186
+	B14:1972	35:1966	2911:1976	16.208–16.209	16.188–16.189
–	20th App. VI-C	15A:1982	DIS 6734	*not* 16.203	*not* 16.183
–				16.008–16.009	16.008–16.009
+				16.302	16.273
+				16.173	16.153
–				16.177(a)	16.157(a)
–				16.164	16.144
+				16.181	16.161
+			IDF/ISO/AOAC Group E 12		
				29.109	29.097
+				16.177–16.178	16.157–16.158
+	B15:1973	16A:1971 (rev.)	DIS 245	16.176	16.156
+				44.022	44.019
+				16.179	16.159
+				16.167	16.147
+				16.175	16.155
+				16.174	16.154
+				16.185	16.165
+				16.183–16.184	16.163–16.164
+				16.180	16.160
+				44.021	44.024
+				16.170	16.150
+				16.168	16.148
–		121:1984	DP 8066		
–		81:1981	6092:1980		
–	19th App. IX-H	86:1981	6091:1980		
–			IDF/ISO/AOAC Group E 9		
+				16.216	16.196
+				16.221	16.201
–			IDF/ISO/AOAC Group E 2		
–			IDF/ISO/AOAC Group E 2		
–		114:1982	DIS 6735		

Table 1. (continued)

Product	Property/component	Description/principle	Collab. study
	insolubility index	dissolution and determination of insoluble residue	–
	lactic acid and lactates	colorimetric, with *p*-hydroxydiphenyl	–
	lactic acid and lactates	enzymatic	–
	neutralizers	calculation (from titratable acidity) and confirmation of Na, K, Ca content	+
	nitrate	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction (rapid method)	+
	nitrate and nitrite	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after reduction	+
	organoleptic attributes	sensory evaluation	–
	protein denaturation	(extra-low heating)	–
	protein denaturation	(heat classification)	–
	sampling		–
	sodium, potassium and calcium	flame photometric	+
	staphylococci, coagulase-positive	detection, after incubation at 37°C (Giolitti and Cantoni, Baird-Parker)	–
	Staphylococcus aureus	colony count at 37°C	–
	suitability for cheesemaking		–
	total solids (water)	gravimetric, drying at 102°C	–
Dried milk (enriched)	vitamin A	LC, colorimetry	+
Dried milk (instant)	dispersibility and wettability	dissolution and determination of solids dissolved, wetting time	–
Dried milk and dried milk products	sampling		–
Dried milk borers	sampling instrument	requirements	–
Dried milk, dried whey	coliforms	MPN at 30°C (brilliant green lactose bile)	–
Dried milk, dried whey, lactose	colony count at 30°C	poured plates (yeast extract, tryptone glucose, skimmed milk)	–
Dried milk, dried whey, dried buttermilk	fat	gravimetric (Roese-Gottlieb)	+
Dried milk, nonfat dry milk, malted milk	moisture (*see also total solids*)	ventilated oven 100°C	+
	nitrogen (protein)	dye-binding, spectrophotometric 480 nm	+
	nitrogen (protein)	titrimetric, Kjeldahl	+
	lactic acid	spectrophotometric	+
Dry skim milk	alkalinity of ash	titrimetric	+
Edible ices	density	immersion	+
	total solids	gravimetric, drying at 102°C	–
Edible ices and ice-mixes	fat	gravimetric (Roese-Gottlieb)	+
Evap. milk, unsweetened	preservatives	various methods	+
	albumin (*see also whey protein*)	titrimetric, Kjeldahl in filtrate of 16.194, 16.174	+
	ash	furnace < 550°C	+
	casein	titrimetric, Kjeldahl, ppt after acid-washing	+

FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
	Q1283/E	DIS 8156		
	69:1972			
	Q 1183/E	DIS 8069		
	102:1981			
	18:1984	DP 8151		
	95A:1984	6736:1982		
	99:1981			
		IDF/ISO/AOAC Group E 17		
		IDF/ISO/AOAC Group E 17		
			16.210	16.190
	Q 1183/E	DIS 8070		
	60A:1978			
		IDF/ISO/AOAC Group E 24		
		IDF/ISO/AOAC Group E 17		
	26:1964			
		IDF/ISO/AOAC Group E 46		
	87:1979			
			16.010–16.011	16.010–16.011
	71:1973 (rev.)	3003:1974		
	64:1971 (rev.)			
	109:1982	DP 7924		
B2:1967	9A:1969 (rev.)	DIS 1736	16.219–16.220	16.199–16.200
			16.212	16.192
			16.214	16.194
			16.213	16.193
			16.222	16.202
			16.217	16.197
CCEI	Q 1480/E	DP 6738	16.310	16.281
	70:1972	3728:1977		
submitted 1982	116:1983	DIS 7328	16.316	16.287
			16.198	16.178
			16.195	16.175
			16.190	16.170
			16.194	16.174

	color additives	qualitative
	gelatin	qualitative
	lactic acid	spectrophotometric
	lactose	polarimetric or gravimetric
	lead	AAS
	nitrogen (protein)	titrimetric, Kjeldahl
Evap. (unsweetened) and cond. (sweetened) milk	fat	gravimetric (Roese-Gottlieb)
Fermented milks	coliforms	MPN at 30°C (brilliant green lactose bile)
	contaminating organisms	colony count at 30 and 20°C on carbohydrate-free medium
Fluid milk products	beta-lactam (see also penicillin)	qualitative color reaction
Ice cream	separation of fat from ice cream	extraction + detection of fats
Ice cream and frozen desserts	color additives	qualitative
	gums	infrared
	lactic acid	spectrophotometric
	nitrogen (protein)	dye-binding
	nitrogen (protein)	titrimetric, Kjeldahl
	phosphatase, residual	photometric, 610 nm filter
	total solids	oven 100°C
Ice cream and milk ices	coliforms	colony count at 30°C and MPN (brilliant green lactose bile)
	colony count at 30°C	poured plates (yeast extract, tryptone, glucose, skimmed milk)
Ice-mixes *(see Edible ices)*		
Light cream, skim, whole milk	phosphatase, residual	qualitative, screening
Milk	acidity	titrimetric
	added water *(see also freezing point)*	refractometric (acetic serum)
	added water	refractometric (copper serum)
	added water, freezing point	osmometric, vapor pressure
	albumin *(see also whey protein)*	titrimetric, Kjeldahl in filtrate of 16.047
	antibiotics *(see also penicillin)*	many methods
	ash	furnace < 550°C
	ash (minerals)	automated methods
	automated methods *(see also individual components)*	statistics, overall accuracy and calibration
	calcium	titrimetric, after oxalate precipitation
	casein	titrimetric, Kjeldahl in filtrate and in milk
	casein	titrimetric, Kjeldahl N on acetic acid precipitate
	cesium 137	gamma-ray spectroscopy
	citric acid	gravimetric
	collection of sample	
	colony count at 30°C	plate loop inoculation
	colony count at 30°C	poured plates (yeast extract, tryptone, glucose, skimmed milk)
	color additives	qualitative
	correction table for specific gravity *(see also density)*	
	crude fat or extract	ether extraction
	DDT residues	TLC
	ethylenethiourea residues	GC
	extraneous matter	sediment
	fat	automated, Foss Electric, Mark III, and UFM 100

+				16.199	16.179
+				16.197	16.177
+				16.188	16.168
+				16.196	16.176
+				16.191	16.171
			IDF/ISO/AOAC Group E 15		
+				16.193	16.173
+	B7:1967	13A:1969 (rev.)	DIS 1737	16.205, 16.192	16.185, 16.172
–		65:1971 (rev.)			
–		66:1971			
+				16.152–16.157	—
+				16.317	16.288
+				16.328	16.299
+				16.319–16.325	16.290–16.296
+				16.318	16.289
+				16.315	16.286
+				16.314	16.285
+				16.329	16.300
+				16.313	16.284
–		62:1971 (rev.)			
–		61:1971 (rev.)			
+			IDF/ISO/AOAC Group E 51		
				16.123–16.124	16.127–16.128
+				16.023	16.023
+				16.093	16.097
+				16.094	16.098
+				16.101–16.104	16.105–16.108
+				16.050	—
–			IDF/ISO/AOAC Group E 47		
+				16.035	16.035
–			IDF/ISO/AOAC Group E 29		
–		Q 983/E	DP 8196		
–		36:1966			
+				16.048–16.049	16.048–16.049
+		29:1964		16.047	16.047
+				48.025	48.025
+				16.024–16.026	16.024–16.026
–				16.019–16.020	16.019–16.020
–		Q 984/E	DP 8553		
–		100:1981	DIS 6610		
+				16.109	16.113
–				52.023	52.023
+				7.064	7.059
+			IDF/ISO/AOAC Group E 12		
				29.109	29.097
+				29.119	29.112
+				16.110	16.114
+			IDF/ISO/AOAC Group E 29		
				16.068–16.072	16.063–16.067

Table 1. (continued)

Product	Property/component	Description/principle	Collab. study
	fat	Babcock	+
	fat	butyrometric (Gerber)	–
	fat	gravimetric (Roese-Gottlieb)	+
	fat	IR (automated method)	–
	fat butyrometers	requirements (Gerber)	–
	fat, protein, lactose, total solids	automated, Irma, Milko-Scan, Multispec	+
	freezing point *(see also added water)*	cryoscopic (thermistor)	+
	freezing point	cryoscopic, Hortvet	+
	hydrogen peroxide	qualitative	+
	hypochlorites, chloramines	qualitative	+
	iodide		–
	lactic acid	spectrophotometric	+
	lactose	enzymatic	+
	lactose	gravimetric	+
	lactose	infrared	+
	lactose	polarimetric	+
	lactose	titrimetric, with chloramine T and KI	–
	lead	anodic stripping voltammetry	+
	nitrogen (protein)	dye-binding (Orange 12)	+
	nitrogen (protein)	dye-binding (Amido Black)	+
	nitrogen (protein)	infrared	+
	nitrogen (protein)	titrimetric, Kjeldahl	+
	penicillin *(see also antibiotics)*	zones of inhibition of *Bacillus stearothermophilus* (disk assay)	+
	penicillins	affinity quantitative	+
	penicillins	qualitative disk II	+
	penicillins	quantitative disk *(Bacillus stearothermophilus)*	+
	penicillins	quantitative overnight method	+
	penicillins	zones of inhibition of *Bacillus subtilus* (field disk assay)	+
	phosphatase, reactivated and residual	differential test for phosphatase activity	+
	phosphatase, residual	colorimetric or spectrophotometric, 650 nm	+
	phosphatase, residual	dialysis, spectrophotometric, 550 nm	+
	phosphatase, residual (raw milk)	photometric, 610 nm	+
	phosphorus	photometric, NH_4 phosphomolybdate	–
	preservatives, formaldehyde, benzoic or salicylic acid	various methods	+
	protein-reducing substances	spectrophotometric	+
	psychotrophic organisms	colony count at 6, 5°C, 10 days	–
	quaternary ammonium compounds	qualitative and quantitative methods	+
	somatic cell count	automatic counter	+
	specific gravity *(see also density)*	pycnometer or std hydrometer	
	strontium-89 and strontium-90	ion exchange	+
	total solids	infrared	+
	total solids	lactometer	+
	total solids	microwave, IR (automated methods)	–

FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
			16.065–16.066	16.060–16.061
		2446:1976		
B6:1967	1B:1983	DIS 1211	16.064	16.059
		IDF/ISO/AOAC Group E 29		
	105:1981	488:1983		
		IDF/ISO/AOAC Group E 29		
			16.083–16.092	16.078–16.097
submitted 1982	108:1982	DIS 5764	16.096–16.100	16.100–16.104
			16.095	16.099
			20.083	20.066
			16.107	16.111
		IDF/ISO/AOAC Group E 15		
			16.027–16.031	16.027–16.031
			16.059–16.063	—
			16.057	16.057
		IDF/ISO/AOAC Group E 29		
			16.058	16.058
			16.055–16.056	16.055–16.056
	28:1974			
		IDF/ISO/AOAC Group E 15		
			25.101	25.080
			16.037	16.037
	98:1980	5542:1984	16.042–16.045	16.042–16.045
		IDF/ISO/AOAC Group E 29		
			16.046	16.046
	20:1962 (rev.)		16.036	16.036
	57:1970 (rev.)		16.140–16.145	16.131–16.136
			16.130–16.134	—
		IDF/ISO/AOAC Group E 47		
			16.146–16.151	—
			16.135–16.139	—
			16.163	16.142
			16.158–16.162	16.137–16.141
			16.125–16.126	16.129–16.130
			16.121–16.122	16.125–16.126
			16.116–16.120	16.120–16.124
			16.112–16.114	16.116–16.118
	42:1967 (rev.)			
			16.106	16.110
			16.051–16.054	16.051–16.054
	101:1981	DIS 6730		
			20.077–20.090	20.094–20.107
			16.022	16.022
			16.021	16.021
			48.016–48.024	48.016–48.024
			16.034	16.034
			16.033	16.033
		IDF/ISO/AOAC Group E 29		

Malted and choc. malted milk	casein	titrimetric, Kjeldahl
Malted milk	fat	gravimetric, Roese-Gottlieb
Milk and foodstuffs containing milk products	lactose (in the presence of other reducing substances)	photometric, after enzymic formation
Milk and liquid milk products (except evap. and sweet. condensed)	sampling	
Milk and milk powder, buttermilk and buttermilk powder, whey and whey. powder	phosphatase activity	color comparison of *p*-nitrophenol
Milk and milk powder, buttermilk and buttermilk powder	phosphatase activity	photometric, with Gibbs reagent on phenol liberated
Milk and milk products	aflatoxin M_1	
	coliforms	colony count at 30°C and MPN (brilliant green lactose bile)
	copper	AAS
	copper	photometric, diethyldithiocarbamate
	Enterobacteriaceae	
	Escherichia coli	
	iron	AAS
	iron	photometric with bathophenanthroline
	lactose	galactosidase
	lactose	glucose odixase
	lactose	LC
	lead (canned, liq.)	
	lead (canned, liq.)	AAS
	nitrogen/protein conversion	calculation
	organochlorine pesticides	many methods
	organoleptic attributes	sensory evaluation (general)
	polychlorinated biphenyls (PCBs)	many methods
	reproducibility and repeatability	statistics
	Salmonella	pre-enrichment, enrichment on selective media, recognition, confirmation
	sample preparation and dilutions (scorched particles, milk and milk products, caseins and caseinates)	preparation for microbiological examination, milk and milk products, visual comparison with standard disks, after filtering
	sampling	attributes sampling schemes
	sampling	general sampling techniques
	sampling	variables sampling schemes
	staphylococci, thermonuclease	color zones on toluidine blue 0-DNA medium
	tin (canned)	(quercetine)
	yeasts and molds	chloramphenicol agar, colony count at 25°C, 4 days
	zinc	
Milk chocolate	milk protein	titrimetric, Kjeldahl
	milkfat	calculation from RMP value
Milk products	vitamin E	TLC isolation, colorimetric
Milk, butter	lipolytic organisms	colony count at 30°C, sugar-free medium, Victoria blue indicator
Milk, cream and evaporated milk	total solids	gravimetric, drying at 102°C

+				16.215	16.195
+				16.218	16.198
–	19th	79:1977	DP 5765		
–				16.006–16.007, 16.019	16.006–16.007, 16.019
–		82:1978	DP 6090		
–		63:1971	3356:1975		
–			IDF/ISO/AOAC Group E 33		
–		73:1974 (rev.)	DP 5541		
–			IDF/ISO/AOAC Group E 15		
+	submitted 1982	76A:1980	5738:1980	25.066–25.071	25.038–25.043
–			IDF/ISO/AOAC Group E 32		
–			IDF/ISO/AOAC Group E 32		
–			IDF/ISO/AOAC Group E 15		
–	submitted 1982	103:1981	DIS 6732		
–			IDF/ISO/AOAC Group E 6		
–			IDF/ISO/AOAC Group E 6		
–			IDF/ISO/AOAC Group E 6		
–			IDF/ISO/AOAC Group E 15		
–			IDF/ISO/AOAC Group E 15		
–			IDF/ISO/AOAC Group E 27		
+		75B:1983	DIS 3890	29.001–29.028	29.001–29.028
–		99:1981	5495 (methodology)		
–		Q 2483/E	DP 8260		
–			IDF/ISO/AOAC Group E 30 5725		
–		93:1980	DIS 6785		
+	submitted 1982	E-Doc 1/3 107: 1982	DP 8261 5739: 1983		
–	20th App. VI-A	113: 1982	DP 5583		
–	B1:1966	50A: 1980 (rev.)	DIS 707	16.001–16.005	16.001–16.005
–		Q 1083/E	DP 8197		
–		83:1978			
–			IDF/ISO/AOAC Group E 15		
–		94:1980 (rev.)	DIS 6611		
–			IDF/ISO/AOAC Group E 15		
+			IDF/ISO/AOAC Group E 53		
				13.047	13.047
+				13.039–13.046	13.039–13.046
+				43.129	43.088
–		41:1966			
+	20th App. VI-B	21A:1982	DIS 6731	16.032, 16.171, 16.189	16.032, 16.151, 16.169

Table 1. (continued)

Product	Property/component	Description/principle	Collab. study
Milk, cream, buttermilk, evap. milk	gelatin	qualitative	+
Milk, dried milk, cheese	aflatoxin M_1	fluorodensitometric, after chromatographic separation	+
Milk-based foods, milk products (special cases)	fat	gravimetric	+
Milk-based infant foods with little starch	fat	gravimetric (Roese-Gottlieb)	–
Milkfat	foreign fat	GC of sterols	+
	foreign fat	phytosteryl acetate test	+
	foreign fat	TLC of steryl acetates	–
	volatile fatty acids	titrimetric, after saponification and distillation	–
Milkfat (anhydrous)	peroxide value	photometric, $FeCl_3$, NH_4CNS	+
Milkfat and other milk products	free fatty acids	BDI, auto-analyzer	–
	free fatty acids	titration, colorimetry, GC	–
Milkfat products	foreign fat	GC of sterols	+
Milkfat, milk products	BHA		–
	BHT		–
	TBHQ		–
Multicomponent food (milk products)	cholesterol	GC	+
Nonfat dry milk	*N*-nitrosodimethylamine	GC	+
Pasteurized milk	coliforms	MPN at 30°C (brilliant green lactose bile)	–
Pepsin *(see calf rennet)*			
Processed cheese	lactose	polarimetric	+
	nitrogen (protein)	titrimetric, Kjeldahl	–
Processed cheese products	ash	gravimetric, after incineration at 550°C	–
	phosphate emulsifying agents	calculation	–
	citrate emulsifying agents	calculation	–
Raw milk	coliforms	colony count at 30°C (violet red bile)	–
	psychotrophic organisms (rapid method)	colony count at 21°C, 25 h	–
Raw, whole, mixed herd milk	fat	automated	+
Rennet *(see calf rennet)*			
Rennet casein, and caseinates	ash	gravimetric, after incineration at 825°C	–

FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
			16.105	16.109
	111:1982	DP 7923	26.095, 26.090	26.090
	Q 3283/E	DP 8262		
	Q 384/E	DP 8381		
B17:1978	54:1979	3594:1976	28.100	28.089
B16:1978	32:1965 (rev.) 38:1966 (rev.) 37:1966	3595:1976	28.096	28.085
B20:1978	74:1974	3976:1977	*not* 28.025	*not* 28.022
		IDF/ISO/AOAC Group E 39		
		IDF/ISO/AOAC Group E 39		
			28.104	28.093
		IDF/ISO/AOAC Group E 43		
		IDF/ISO/AOAC Group E 43		
		IDF/ISO/AOAC Group E 43		
			43.283	43.229
			16.223–16.228	—
	40:1966			
			16.297	16.268
	25:1964			
	27:1964			
	51:1969			
	52:1969			
	39:1966			
	Q 1384/E	DP 8552		
		IDF/ISO/AOAC Group E 29		
			16.074–16.082	16.069–16.077
19th App. IX-C	90:1977	5545:1978		

Skimmed milk, whey, buttermilk	fat	gravimetric (Roese-Gottlieb)
Soft curd cheese	gums	qualitative
Sterilized milk	sampling and sample preparation	specific sampling technique
	stability (ethanol)	observation of precipitation on mixing with ethanol
Whey cheese	fat	gravimetric (Roese-Gottlieb)
	nitrate and nitrite	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction
	total solids (dry matter)	gravimetric, drying at 88°C
Whey powder	nitrate and nitrite	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction
Whole dry, nonfat dry milk	*Salmonella*	isolation in selective broth
Yogurt	benzoic acid	LC, capillary GC
	Lactobacillus bulgaricus	identification
	sorbic acid	LC, capillary GC
	Streptococcus thermophilus	
	sulfite	LC, capillary GC
	total solids	
	yogurt organisms	total count at 37°C (acidified MRS and M17 media)

+	submitted 1982	22A:1983	DIS 7208		
+				16.298–16.301	16.269–16.272
–		48:1969 (rev.)			
–		48:1969 (rev.)			
+	B10:1973	59:1970	1854:1972	16.285	16.256
+		96:1980	6739:1982		
–	B11:1970	58:1970	2920:1974		
+		97A:1984	DIS 6740		
				46.115–46.119	46.054–46.058
+			IDF/ISO/AOAC Group E 43		
–			IDF/ISO/AOAC Group E 44		
–			IDF/ISO/AOAC Group E 43		
			IDF/ISO/AOAC Group E 44		
–			IDF/ISO/AOAC Group E 43		
–			IDF/ISO/AOAC Group E 5		
–		117:1983	DP 7889		
–					

Table 2. IDF/ISO/AOAC inventory of standard and adopted methods of sampling and analysis for milk and milk products, arranged by component (published or in progress)

Property/component	Description/principle	Product	Collab. study	FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
Acid value and free fatty acid index	titrimetric	butter	+	B4:1967	6A:1969 (rev.)	1740:1980	16.237–16.239	16.211–16.213
Acidity	titrimetric	milk	+				16.023	16.023
	titrimetric	cheese	+				16.276	16.247
Acidity (free)	titrimetric, on aqueous extract	caseins	–	19th App. IX-F	91:1979	5547:1978		
Acidity (titratable)	titrimetric, on dissolved product	dried milk	–		81:1981	6092:1980		
	titrimetric, on dissolved product	dried milk	–	19th App. IX-H	86:1981	6091:1980		
Acids (citric, fatty) *(see under descriptor)*								
Added water *(see also Freezing point)*	refractometric (acetic serum)	milk	+				16.093	16.097
	refractometric (copper serum)	milk	+				16.094	16.098
Added water, freezing point	osmometric, vapor pressure	milk	+				16.101–16.104	16.105–16.108
Additives *(see individual additives)*								
Aerobic count *(see Colony count at 30°C)*								
Aflatoxin M_1		milk and milk products	–			IDF/ISO/AOAC Group E 33		
	fluorodensitometric, after chromatographic separation	milk, dried milk, cheese	+		111:1982	DP 7923	26.095, 26.090	26.090
	TLC	butter	+				26.090	26.090
Albumin *(see also whey protein)*	titrimetric, Kjeldahl	evap. milk, unsweetened	+				16.195	16.175
	titrimetric, Kjeldahl	milk	+				16.050	16.050
Alginates	confirmation, qualitative	chocolate frozen desserts	+				16.326	16.297
Alkalinity of ash	titrimetric	dry skim milk	+				16.217	16.197
Ammonium		ammonium caseinate	–			IDF/ISO/AOAC Group E 11		
		dried milk	–			IDF/ISO/AOAC Group E 9		
Antioxidants *(see BHA, BHT, TBHQ)*								
Antibiotics *(see also Penicillin)*	many methods	milk	–			IDF/ISO/AOAC Group E 47		
Ash	furnace < 550°C	milk	+				16.035	16.035
	furnace < 550°C	cheese	+				16.267	16.241
	furnace < 550°C	cream	+				16.173	16.153
	furnace < 550°C	dried milk	+				16.216	16.196
	furnace < 550°C	evap. milk, unsweetened	+				16.190	16.170
	furnace 525°C (2 methods)	cond. milk, sweetened	+				16.204	16.230
	gravimetric, after incineration at 550°C	processed cheese products	–		27:1964			
	gravimetric, after incineration at 825°C	acid casein	–	18th App. IX-C				
	gravimetric, after incineration at 825°C	rennet casein and caseinates	–	19th App. IX-C	90:1977	5545:1978		
Ash (fixed ash)	gravimetric, after incineration at 825°C	caseins and caseinates	–	19th App. IX-D	89:1979	5544:1978		
Ash (minerals)	automated methods	milk	–			IDF/ISO/AOAC Group E 29		
Automated methods *(see also individual components)*	statistics, overall accuracy and calibration	milk	–		Q 983/E	DP 8196		
Babcock cream test bottle		cream	+				16.177(a)	16.157(a)
Benzoic acid	LC, capillary GC	yogurt	–			IDF/ISO/AOAC Group E 43		
Beta-lactam *(see also Penicillin)*	qualitative color reaction	fluid milk products	+				16.152–16.157	—
BHA		milkfat, milk products	–			IDF/ISO/AOAC Group E 43		
BHT		milkfat, milk products	–			IDF/ISO/AOAC Group E 43		
Butter stirrer		butter	–				44.002	44.002
Calcium	titrimetric, after oxalate precipitation	milk	–		36:1966			

Calcium (dried milk) *(see Sodium)*		
Casein	titrimetric, Kjeldahl, ppt after acid-washing	evap. milk, unsweetened
	titrimetric, Kjeldahl in filtrate and in milk	milk
	titrimetric, Kjeldahl	malted and choc. malted milk
	titrimetric, Kjeldahl N on acetic acid precipitate	milk
Casein, ash, salt	oven < 500°C, Cl^- gravimetric	butter
Cesium-137	gamma-ray spectroscopy	milk
Chloride	indicating-strip method	cheese
	potentiometric titration	cheese and processed cheese
	titrimetric	cheese
	titrimetric (Mohr)	butter
Cholesterol	GC	multicomponent food (milk products)
Chymosine and bovine pepsin	clotting time, after chromatographic separation	calf rennet and adult bovine pepsin
Citrate emulsifying agents	calculation	processed cheese products
Citric acid	gravimetric	cheese
	gravimetric	dried milk
	gravimetric	milk
	photometric, with pyridine and acetic anhydride	cheese and processed cheese
	qualitative test	cheese
Coagulase-positive staphylococci *(See Staphylococci)*		
Coliforms	colony count at 30°C (violet red bile)	raw milk
	colony count at 30°C and MPN (brilliant green lactose bile)	ice cream and milk ices
	colony count at 30°C and MPN (brilliant green lactose bile)	milk and milk products
	MPN at 30°C (brilliant green lactose bile)	dried milk, dried whey
	MPN at 30°C (brilliant green lactose bile)	fermented milks
	MPN at 30°C (brilliant green lactose bile)	pasteurized milk
Collection of sample		cheese
		cream
		milk
Colony count at 30°C	poured plates (yeast extract, tryptone glucose, skimmed milk)	baby foods based on dried milk
	poured plates (yeast extract, tryptone glucose, skimmed milk)	caseins and caseinates
	poured plates (yeast extract, tryptone glucose, skimmed milk)	dried milk, dried whey, lactose
	poured plates (yeast extract, tryptone glucose, skimmed milk)	ice cream and milk ices
	poured plates (yeast extract, tryptone glucose, skimmed milk)	milk
	plate loop inoculation	milk
Color additives	qualitative	butter
	qualitative	ice cream and frozen desserts
	qualitative	milk
	qualitative	cream
	qualitative	evap. milk, unsweetened
	several methods	cheese
Contaminating organisms	colony count at 30 and 20°C on carbohydrate-free medium	butter

+				16.194	16.174
+				16.048–16.049	16.048–16.049
+				16.215	16.195
+		29:1964		16.047	16.047
+				16.234	16.208
+				48.025	48.025
+				16.273	16.244
+	B18:1978	88:1979	5943:1978	16.268–16.271	
+				16.272	—
+	B8:1967	12A:1969 (rev.)	1738:1980	16.235	16.209
+				43.283	43.229
–		110:1982			
–		52:1969			
+				16.291	16.292
+				16.221	16.201
+				16.024–16.026	16.024–16.026
+	B13:1972	34B:1971	2963:1974	16.292–16.296	16.321–16.325
+				16.290	16.261
–		39:1966			
–		62:1971 (rev.)			
–		73:1974 (rev.)	DP 5541		
–		64:1971 (rev.)			
–		65:1971 (rev.)			
–		40:1966			
–				16.257	16.231
–				16.164	16.144
–				16.019–16.020	16.019–16.020
–			IDF/ISO/AOAC Group E 22		
–		Q 883/E	DP 8198		
–		109:1982	DP 7924		
–		61:1971 (rev.)			
–		100:1981	DIS 6610		
–		Q 984/E	DP 8553		
+				16.244	16.218
+				16.328	16.299
+				16.109	16.113
+				16.181	16.161
+				16.199	16.179
+				16.277	16.248
–		30:1964			

Table 2. (continued)

Property/component	Description/principle	Product	Collab. study
	colony count at 30 and 20°C on carbohydrate-free medium	fermented milks	–
Copper	AAS	milk and milk products	–
	photometric, diethyldithiocarbamate	milk and milk products	+
Correction table for specific gravity *(see also Density)*		milk	–
Critical temp. of dissolution	thermometric	butter	+
Crude fat or extract	ether extraction	milk	+
DDT residues	GC + identification	butter	+
	TLC	cheese	+
	TLC	cream	+
	TLC	milk	+
Dehydroacetic acid	qualitative	cheese	+
Density	bulk density	dried milk	–
	immersion	edible ices	+
Dilutions *(see Sample preparation)*			
Dispersibility and wettability	dissolution and determination of solids dissolved, wetting time	dried milk (instant)	–
Dry matter *(see total solids)*			
Emulsifying agents *(see Citrate, phosphate)*			
Enterobacteriaceae		milk and milk products	–
Enzymes (residual)		cheese	–
Enzymic activity *(see Phosphatase)*			
Escherichia coli		milk and milk products	–
Ethylenethiourea residues	GC	milk	+
Examination of fat *(see also Volatile fatty acids)*	RMP value, sterol acetate melting point	cheese	+
Extraneous materials	filtration	cheese	+
		caseins and caseinates	–
	sediment	milk	+
Fat	automated, Foss Electric, Mark III and UFM 100	milk	+
	automated, Technicon Instr.	raw, whole, mixed herd milk	+
	Babcock	milk	+
	Babcock	cream	+
	gravimetric (Roese-Gottlieb)	ice cream and frozen desserts	+
	butyrometric (Gerber)	milk	–
	butyrometric (Van Gulik)	cheese	–
	gravimetric (calculation from solids-not-fat and water)	butteroil (milkfat products)	–
	gravimetric (Roese-Gottlieb)	cream	+
	gravimetric (Roese-Gottlieb)	dried milk, dried whey, dried buttermilk	+
	gravimetric (Roese-Gottlieb)	edible ices and ice-mixes	+
	gravimetric (Roese-Gottlieb)	evap. (unsweetened) and cond. (sweetened) milk	+

FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
	66:1971			
			IDF/ISO/AOAC Group E 15	
submitted 1982	76A:1980	5738:1980	25.066–25.071	25.038–25.043
			52.023	52.023
			16.241–16.243	16.215–16.217
			7.064	7.059
			29.109	29.097
			IDF/ISO/AOAC Group E 12	
			29.109	29.097
			IDF/ISO/AOAC Group E 12	
			29.109	29.097
			IDF/ISO/AOAC Group E 12	
			29.109	29.097
			20.069–20.070	20.052–20.053
			IDF/ISO/AOAC Group E 2	
CCEI	Q 1480/E	DP 6738	16.310	16.281
	87:1979			
			IDF/ISO/AOAC Group E 32	
			IDF/ISO/AOAC Group E 32	
			IDF/ISO/AOAC Group E 32	
			29.119	29.112
			16.286	16.257
			44.024–44.027	44.020–44.023
			IDF/ISO/AOAC Group E 11	
			16.110	16.114
			IDF/ISO/AOAC Group E 29	
			16.068–16.072	16.063–16.067
			IDF/ISO/AOAC Group E 29	
			16.074–16.082	16.069–16.087
			16.065–16.066	16.060–16.061
			16.177–16.178	16.157–16.158
			16.316	16.287
		2446:1976		
		3433:1975		
	24:1964			
B15:1973	16A:1971 (rev.)	DIS 2450	16.176	16.156
B2:1967	9A:1969 (rev.)	DIS 1736	16.219–16.220	16.199–16.200
submitted 1982	116:1983	DIS 7328	16.287	16.258
B7:1967	13A:1969 (rev.)	DIS 1737	16.205, 16.192	16.231, 16.172

	gravimetric (Roese-Gottlieb)	milk
	gravimetric (Roese-Gottlieb)	milk-based infant foods with little starch
	gravimetric (Roese-Gottlieb)	skimmed milk, whey, buttermilk
	gravimetric (Roese-Gottlieb)	whey cheese
	gravimetric (Schmid-Bondzynski-Ratzlaff)	caseins and caseinates
	gravimetric (Schmid-Bondzynski-Ratzlaff)	cheese and processed cheese
	gravimetric (water, solids-not-fat, and fat content on one test portion)	butter
	gravimetric (Weibull)	milk-based foods, milk products (special cases)
	gravimetric, direct extraction	butter
	gravimetric (Roese-Gottlieb)	malted milk
	IR (automated method)	milk
Fat butyrometers	requirements (Gerber)	milk
	requirements (Van Gulik)	cheese
Fat, foreign *(see Foreign fat)*		
Fat, protein, lactose, total solids	automated, Irma, Milko-Scan, Multispec	milk
Fatty acids *(see Free fatty acids, Volatile fatty acids)*		
Filth	filtration	butter
	microscopic, after filtration	cream
	microscopic, after sieving	casein
Fixed ash *(see Ash)*		
Flecks		dried milk
Foreign fat *(see also milkfat in mixtures)*	GC of sterols	milkfat
	GC of sterols	milkfat products
	phytosteryl acetate test	milkfat
	TLC of steryl acetates	milkfat
Free acidity *(see Acidity)*		
Free fatty acid index *(see Acid value)*		
Free fatty acids	BDI, auto-analyzer	milkfat and other products
	titration, colorimetry, GC	milkfat and other milk products
Freezing point *(see also Added water)*	cryoscopic (thermistor)	milk
Freezing point *(see also Added water)*	cryoscopic, Hortvet	milk
Gelatin	qualitative	cottage cheese
	qualitative	ice cream and frozen desserts
	qualitative	milk, cream, buttermilk, evap. milk
	qualitative	cream
	qualitative	evap. milk, unsweetened
Gums	infrared	ice cream and frozen desserts
	qualitative	soft curd cheese
Heat class (heat number)	calculation, from Kjeldahl N of precipitated casein plus some denatured serum protein	dried milk
Hydrogen peroxide	qualitative	milk
Hypochlorites, chloramines	qualitative	milk
Impurities *(see Extraneous matter, Flecks, Scorched particles)*		

+	B6:1967	1B:1983	DIS 1211	16.064	16.059
–		Q 384/E	DP 8381		
+	submitted 1982	22A:1983	DIS 7208		
+	B10:1973	59:1970	1854:1972	16.285	16.256
+		Q 3183/E	DP 5543		
+	B3:1967	5A:1969 (rev.)	1735:1975	16.284	16.313
+	B21:1978	80:1977	3727:1977	16.232	16.206
+		Q 3283/E	DP 8262		
+				16.233	16.207
+				16.218	16.198
–			IDF/ISO/AOAC Group E 29		
–		105:1981	488:1983		
–			3432:1975		
+			IDF/ISO/AOAC Group E 29		
				16.083–16.092	16.078–16.097
+				44.022–44.024	44.018–44.020
+				44.022	44.019
+				44.027	44.022
–			IDF/ISO/AOAC Group E 2		
+	B17:1978	54:1979	3594:1976	28.100	28.089
+				28.104	28.093
+	B16:1978	32:1965 (rev.)	3595:1976	28.096	28.085
–		38:1966 (rev.)			
–			IDF/ISO/AOAC Group E 39		
–			IDF/ISO/AOAC Group E 39		
+	submitted 1982	108:1982	DIS 5764	16.096–16.100	16.100–16.104
+				16.095	16.099
+				16.302	16.273
+				16.327	16.298
+				16.105	16.109
+				16.179	16.159
+				16.197	16.177
+				16.319–16.325	16.290–16.296
+				16.298–16.301	16.269–16.272
–		114:1982	DIS 6735		
+				20.083	20.066
+				16.107	16.111

Table 2. (continued)

Property/component	Description/principle	Product	Collab. study
Insolubility index	dissolution and determination of insoluble residue	dried milk	–
Iodide		milk	–
Iodine value	titrimetric (Wijs)	butterfat	+
	titrimetric with Na thiosulfate (Wijs)	butterfat	–
Iron	AAS	milk and milk products	–
	photometric with bathophenanthroline	milk and milk products	–
Lactic acid	spectrophotometric	butter	+
	spectrophotometric	cond. milk, sweetened	+
	spectrophotometric	cream	+
	spectrophotometric	dried milk, nonfat dry, malted milk	+
	spectrophotometric	evap. milk, unsweetened	+
	spectrophotometric	ice cream and frozen desserts	+
	spectrophotometric	milk	+
Lactic acid and lactates	colorimetric, with *p*-hydroxydiphenyl	dried milk	–
	enzymatic	dried milk	–
Lactobacillus bulgaricus (see also Yogurt)	identification	yogurt	–
Lactose	galactosidase	milk and milk products	–
	glucose oxidase	milk and milk products	–
	gravimetric	cond. milk, sweetened	+
	gravimetric	cream	+
	gravimetric	milk	+
	LC	milk and milk products	–
	infrared	milk	+
	photometric, with phenol and H_2SO_4	caseins and caseinates	–
	enzymatic	milk	+
	polarimetric	milk	+
	polarimetric	processed cheese	+
	polarimetric or gravimetric	evap. milk, unsweetened	+
	titrimetric, with chloramine T and KI	milk	–
Lactose (in the presence of reducing substances)	photometric, after enzymic formation of NADPH	milk and foodstuffs containing milk products	–
Lead		milk and milk products (canned, liquid)	–
	AAS	evap. milk, unsweetened	+
	AAS	milk and milk products (canned, liquid)	–
	anodic stripping voltammetry	milk	+
Lipolytic organisms	colony count at 30°C, sugar-free medium, Victoria blue indicator	milk, butter	–
Mass per unit volume *(see Density)*			
Milk protein	titrimetric, Kjeldahl	milk chocolate	+
Milkfat	calculation from RMP value	milk chocolate	+
Moisture *(see also Total solids)*	distillation	cheese	+
	forced draft oven ± 130°C, screening	cheese	+
	microwave oven	cheese	+
	vacuum oven 100°C	cheese	+
	ventilated oven 100°C	dried milk, nonfat dry milk,	+

FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
	Q 1283/E	DIS 8156		
			IDF/ISO/AOAC Group E 15	
			28.023	28.020
	8:1959			
			IDF/ISO/AOAC Group E 15	
submitted 1982	103:1981	DIS 6732		
			16.245	16.219
			16.202	16.182
			16.167	16.147
			16.222	16.202
			16.188	16.168
			16.318	16.289
			16.027–16.031	16.027–16.031
	69:1972			
	Q 1183/E	DIS 8069		
			IDF/ISO/AOAC Group E 44	
			IDF/ISO/AOAC Group E 6	
			IDF/ISO/AOAC Group E 6	
			16.207	16.187
			16.175	16.155
			16.057	16.057
			IDF/ISO/AOAC Group E 6	
			IDF/ISO/AOAC Group E 29	
			16.058	16.058
19th App. IX-A	106:1982	5548:1980		
			16.059–16.063	—
			16.055–16.056	16.055–16.056
			16.297	16.298
			16.196	16.176
	28:1974			
19th App. IX-G	79:1977 (rev.)	DP 5765		
			IDF/ISO/AOAC Group E 15	
			IDF/ISO/AOAC Group E 15	
			16.191	16.171
			IDF/ISO/AOAC Group E 15	
			IDF/ISO/AOAC Group E 15	
			25.101	25.080
	41:1966			
			IDF/ISO/AOAC Group E 53	
			13.047	13.047
			13.039–13.046	13.039–13.046
			16.261–16.264	16.235–16.238
			16.260	16.234
			16.265–16.266	16.239–16.240
			16.259	16.233
			16.212	16.192

		malted milk
Mold	filtration	butter
Monofluoro acid residues	qualitative	cheese
Mycotoxins *(see Aflatoxin M_1)*		
Natamycin	photometric or LC, after extraction	cheese
Neutralizers	calculation (from titratable acidity) and confirmation of Na, K, Ca content	dried milk
Nisin		cheese
Nitrate	(range 2–15 mg/kg)	cheese
	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction (rapid method)	dried milk
Nitrate and nitrite	> 1 ppm NO_3, cadmium reduction method	cheese
	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction	caseins and caseinates
	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction	cheese
	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction	dried milk
	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction	whey cheese
	photometric, with sulfanilamide and *N*-1-naphthylethylenediamine HCl after cadmium reduction	whey powder
Nitrogen (protein)	dye-binding (Amido Black)	milk
	dye-binding (Orange 12)	milk
	dye-binding	ice cream and frozen desserts
	dye-binding, spectrophotometric, 480 nm	dried milk, nonfat dry milk, malted milk
	infrared	milk
	titrimetric, Kjeldahl	caseins and caseinates
	titrimetric, Kjeldahl	cheese
	titrimetric, Kjeldahl	cond. milk, sweetened
	titrimetric, Kjeldahl	cream
	titrimetric, Kjeldahl	dried milk, nonfat dry milk, malted milk
	titrimetric, Kjeldahl	evap. milk, unsweetened
	titrimetric, Kjeldahl	ice cream and frozen desserts
	titrimetric, Kjeldahl	milk
	titrimetric, Kjeldahl	processed cheese
Nitrogen/protein conversion	calculation	milk and milk products
N-Nitrosodimethylamine	GC	nonfat dry milk
Organochlorine pesticides	many methods	milk and milk products
Organoleptic attributes	sensory evaluation	butter
	sensory evaluation	cheese
	sensory evaluation	dried milk
	sensory evaluation (general)	milk and milk products
Overrun *(see Density)*		
Oxidizing substances *(see Peroxide value)*		
PCBs *(see Polychlorinated biphenyls)*		

+				44.021	44.025
+				29.148–29.150	29.140–29.142
–			IDF/ISO/AOAC Group E 43		
+		102:1981			
–			IDF/ISO/AOAC Group E 43		
–			IDF/ISO/AOAC Group E 8		
+		118:1984	DP 8151		
+				16.278–16.283	16.249–16.254
+		120:1984	DP 8195		
+	B19:1978	84A:1984	4099:1978		
+		95A:1984	6736:1982		
+		96:1980	6739:1982		
+		97A:1984	DIS 6740		
+		98:1980	5542:1984	16.042–16.045	16.042–16.045
+				16.037	16.037
+				16.315	16.286
+				16.214	16.194
+			IDF/ISO/AOAC Group E 29		
–	19th App. IX-E	92:1979	5549:1978	16.046	16.046
+			IDF/ISO/AOAC Group E 27		
+				16.274	16.245
+				16.206	16.186
+				16.174	16.154
+				16.213	16.193
+				16.193	16.173
+				16.314	16.285
+		20:1962 (rev.)		16.036	16.036
–		25:1964			
–			IDF/ISO/AOAC Group E 27		
+				16.223–16.228	—
+		75B:1983	DIS 3890	29.001–29.028	29.001–29.028
–		99:1981			
–		IDF group D6c			
–		99:1981			
–		99:1981	5495 (methodology)		

Table 2.

Property/component	Description/principle	Product
Penicillin *(see also Antibiotics)*	zones of inhibition of *Bacillus stearothermophilus* (disk assay)	milk
	zones of inhibition of *Bacillus subtilus* (field disk assay)	milk
	affinity quantitative	milk
	qualitative disk II	milk
	quantitative disk *(Bacillus stearotherm.)*	milk
	quantitative overnight method	milk
Pepsin *(see Chymosin)*		
Peroxide value	photometric, $FeCl_3$, NH_4CNS	milkfat (anhydrous)
Pesticide residues *(see also Organochlorine pesticides)*	GC + identification	butter, cream, cheese, milk
pH value	electrometric measurement	caseins and caseinates
pH value (of serum)	electrometric	butter
Phosphatase activity	color comparison of *p*-nitrophenol	milk and milk powder, buttermilk and buttermilk powder, whey and whey powder
	photometric, with Gibbs reagent on phenol liberated	cheese (pasteurized, stabilized)
	photometric, with Gibbs reagent on phenol liberated	milk and milk powder, buttermilk and buttermilk powder, whey and whey powder
Phosphatase, reactivated and residual	differential test for phosphatase activity	cream
	differential test for phosphatase activity	milk
Phosphatase, residual	colorimetric and spectrophotometric	casein
	colorimetric or spectrophotometric 650 nm	milk
	dialysis, spectrophotometric 550 nm	milk
	photometric, 610 nm	butter
	photometric, 610 nm	raw milk
	photometric, 610 nm	cheese
	photometric, 610 nm	cream
	photometric, 610 nm	ice cream and frozen desserts
	qualitative, screening	cheese
	qualitative, screening	light cream, skim, whole milk
Phosphate emulsifying agents	calculation	processed cheese products
Phosphorus	photometric, molybdate-ascorbate	cheese and processed cheese products
	photometric, NH_4 phosphomolybdate	milk
Polychlorinated biphenyls (PCBs)	many methods	milk and milk products
Potassium *(see Sodium)*		
Preservatives	various methods	evap. milk, unsweetened
	various methods	butter
	various methods	cream
Preservatives, formaldehyde, benzoic, or salicylic acid	various methods	milk
Protein *(see Whey protein, Nitrogen)*		

(continued)

Collab. study	FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
+		57:1970 (rev.)		16.140–16.145	16.131–16.136
+				16.158–16.162	16.137–16.141
+				16.130–16.134	—
+				IDF/ISO/AOAC Group E 47	
				16.146–16.151	—
+				16.135–16.139	—
+				16.163	16.142
–	B20:1978	74:1974	3976:1977	*not* 28.025	*not* 28.022
+				29.001–29.028	29.001–29.028
–	submitted 1982	115:1982	5546:1979		
–		104:1981	DIS 7238		
–		82:1978	DP 6090		
–		53:1969			
–		63:1971	3356:1975		
+				16.185	16.165
+				16.125–16.126	16.137–16.141
+				16.127–16.129	—
+				16.121–16.122	16.125–16.126
+				16.116–16.120	16.120–16.124
+				16.256	16.230
+				16.112–16.114	16.116–16.118
+				16.304–16.306	16.275–16.277
+				16.183–16.184	16.163–16.164
+				16.329	16.300
+				16.307	16.278
+				IDF/ISO/AOAC Group E 51	
				16.123–16.124	16.127–16.128
–		51:1969			
–	B12:1972 (rev.)	33B:1982 (rev.)	2962:1984		
–		42:1967 (rev.)			
–		Q 2483/E	DP 8260		
+				16.198	16.178
+				16.254	16.228
+				16.180	16.160
+				16.106	16.110

Protein denaturation	(extra-low heating)	dried milk
	(heat classification)	dried milk
Protein reducing substances	spectrophotometric	milk
Proteins, functional properties		
Psychotrophic organisms	colony count at 6.5°C, 10 days	milk
	colony count at 21°C, 25 h (rapid method)	raw milk
Quaternary ammonium compounds	qualitative and quantitative methods	milk
Refractive index (butterfat)	refractometric	butter
Repeatability *(see Reproducibility)*		
Reproducibility and repeatability	statistics	milk and milk products
Salmonella	isolation in selective broth	whole dry, nonfat dry milk
Salmonella	pre-enrichment, enrichment on selective media, recognition, confirmation	milk and milk products
Salt *(see Chloride)*		
Sampling		butter
		cheese
		condensed and evap. milk
		dried milk
		dried milk and dried milk products
		milk and liquid milk products (except evap. and sweetened cond.)
	attributes sampling schemes	milk and milk products
	general sampling techniques	milk and milk products
	microbiological	dairy plant surfaces
	variables sampling schemes	milk and milk products
Sampling and sample preparation	specific sampling technique	sterilized milk
Sampling instrument	requirements	butter triers
	requirements	cheese triers
	requirements	dried milk borers
Sample preparation and dilutions (scorched particles)	preparation for microbiological examination visual comparison with standard disks, after filtering	milk and milk products caseins and caseinates
Sediment	vacuum filtration	butter
	vacuum filtration	cheese
	vacuum filtration	cream
Separation of fat from ice cream	extraction + detection of fats	ice cream
Sodium, potassium, and calcium	flame photometric	dried milk
Solids-not-fat	gravimetric, on solvent extract	butter
	gravimetric, on solvent extract (water, solids-not-fat, and fat on one test portion)	butter
Solubility (dried milk) *(see Insolubility)*		
Somatic cell count	automatic counter	milk
Sorbic acid	LC, capillary GC	yogurt
	oxidation	cheese
Specific gravity *(see also Density)*	pycnometer or std hydrometer	milk
Stability (ethanol)	observation of precipitation on mixing with ethanol	sterilized milk
Staphylococci, coagulase-positive	detection, after incubation at 37°C (Giolitti and Cantoni, Baird-Parker)	dried milk

–			IDF/ISO/AOAC Group E 17		
–			IDF/ISO/AOAC Group E 17		
+				16.051–16.054	16.051–16.054
–			IDF/ISO/AOAC Group E 53		
–		101:1981	DIS 6730		
–		Q 1384/E	DP 8552		
+				20.077–20.090	20.094–20.107
+	B5:1967	7A:1969	1739:1975	16.240	16.214
			IDF/ISO/AOAC Group E 30		
–			5725		
				46.115–46.119	46.054–46.058
+		93:1980	DIS 6785		
–					
–				16.012–16.014, 16.229	16.012–16.014, 16.203
–				16.015–16.018	16.015–16.018
–				16.008–16.009	16.008–16.009
–				16.210	16.190
–				16.010–16.011	16.010–16.011
–				16.006–16.007, 16.019	16.006–16.007, 16.019
–	20th App. VI-A	113:1982	DP 5583		
–	B1:1966	50A:1980 (rev.)	DIS 707	16.001–16.005	16.001–16.005
–			121:1984	DP 2066	
–		Q 1083/E	DP 8197		
–		48:1969 (rev.)			
–			1193:1973		
–			1194:1973		
–		71:1973 (rev.)	3003:1974		
+	submitted 1982	F-Doc 1973 107:1982	DP 8261 5739:1983		
+				44.021	44.024
+				44.021	44.024
+				44.021	44.024
+				16.317	16.288
+		119:1984	DIS 8070		
–		11:1960 (rev.)			
+	B21:1978	80:1977 (rev.)	3727:1977	16.205	16.185
+				16.022	16.022
–			IDF/ISO/AOAC Group E 43		
+				16.303, 20.115	16.274, 20.098
–				16.021	16.021
–		48:1969 (rev.)			
–		60A:1978			

Table 2.

Property/component	Description/principle	Product
Staphylococci, thermonuclease	color zones on toluidine blue O-DNA medium	milk and milk products
Staphylococcus aureus	colony count at 37°C	dried milk
Sterol, β sito-acetate (see foreign fat)		
Streptococcus thermophilus		yogurt
Strontium-89 and strontium-90	ion exchange	milk
Sucrose	inversion and polarimetry	cond. milk, sweetened
Suitability for cheesemaking		dried milk
Sulfite	LC, capillary GC	yogurt
Tartaric acid	qualitative	cheese
	quantitative, titrimetric	cheese
TBHQ		milkfat, milk products
Thermonuclease *(see Staphylococci)*		
Tin	(quercetine)	milk and milk products (canned)
Titanium	spectrophotometric	cheese
Titratable acidity *(see Acidity)*		
Total count *(see Colony count at 30°C)*		
Total solids		yogurt
	gravimetric, drying at 102°C	cheese and processed cheese
	gravimetric, drying at 102°C	cond. milk, sweetened
	gravimetric, drying at 102°C	edible ices
	gravimetric, drying at 102°C	milk, cream, and evaporated milk
	infrared	milk
	lactometer	milk
	microwave, IR (automated methods)	milk
	oven 100°C	ice cream and frozen desserts
Total solids (dry matter)	gravimetric, drying at 88°C	whey cheese
Total solids (moisture)	gravimetric, drying at 102°C	butter
Total solids (water)	gravimetric, heating (rapid method)	butter
	gravimetric, drying at 102°C	caseins and caseinates
	gravimetric, drying at 102°C	dried milk
	gravimetric, drying at 102°C (water, solids-not-fat, and fat on one test portion)	butter

(continued)

Collab. study	FAO/WHO	IDF	ISO	AOAC (14th Ed.)	AOAC (13th Ed.)
–		83:1978			
–			IDF/ISO/AOAC Group E 24		
–			IDF/ISO/AOAC Group E 44		
+				48.016–48.024	48.016–48.024
+	B14:1972	35:1966	2911:1976	16.208–16.209	16.188–16.189
–			IDF/ISO/AOAC Group E 17		
–			IDF/ISO/AOAC Group E 43		
+				16.287	16.258
+				16.288–16.289	16.259–16.260
–			IDF/ISO/AOAC Group E 43		
–			IDF/ISO/AOAC Group E 15		
+				16.275	16.246
–			IDF/ISO/AOAC Group E 5		
–	20th App. VI-D	4A:1982	DIS 5534	all methods differ	
–	20th App. VI-C	15A:1982	DIS 6734	*not* 16.203	*not* 16.183
–		70:1972 (rev.)	3728:1977		
+	20th App. VI-B	21A:1982	DIS 6731	16.032, 16.171, 16.189	16.032, 16.151, 16.169
+				16.034	16.034
+				16.033	16.033
–			IDF/ISO/AOAC Group E 29		
+				16.313	16.284
–	B11:1970	58:1970	2920:1974		
–		10:1960			
–			IDF/ISO/AOAC Group E 5		
–	19th App. IX-B	78B:1980 (rev.)	5550:1978		
–		26:1964			
+	B21:1978	80:1977 (rev.)	3727:1977	16.231	16.205

Vegetable fat *(see Foreign fat)*		
Vitamin A	LC, colorimetry	dried milk (enriched)
Vitamin E	TLC isolation, colorimetric	milk products
Volatile acids	chromatographic	cream
	distillation, chromatographic separation	butter
Volatile fatty acids	sterol acetate melting point	butter
	titrimetric, after saponification and distillation	milkfat
Water *(see also Total solids)*	titrimetric (Karl Fischer)	butteroil (milkfat products)
Water dispersion	indicator paper comparison	butter
Water-insoluble fatty acids	gravimetric	cream
	gravimetric	butter
Water-insoluble fatty acids + butyric acid	chromatographic	butter
Wettability *(see Dispersibility)*		
Whey protein		caseins and caseinates
Yeasts and molds	chloramphenicol agar, colony count at 25°C, 4 days	milk and milk products
Yogurt organisms	total count at 37°C (acidified MRS and M17 media)	yogurt
Zinc		milk and milk products

+		IDF/ISO/AOAC Group E 46		
+			43.129	43.088
+			16.170	16.150
+			16.253	16.227
+			16.236	16.210
–	37:1966			
–	23:1964 (rev.)			
–	112:1982	DIS 7586		
+			16.168	16.148
+			16.246	16.220
+			16.247–16.251	16.221–16.225
–		IDF/ISO/AOAC Group E 11		
–	94:1980 (rev.)	DIS 6611		
–	117:1983	DP 7889		
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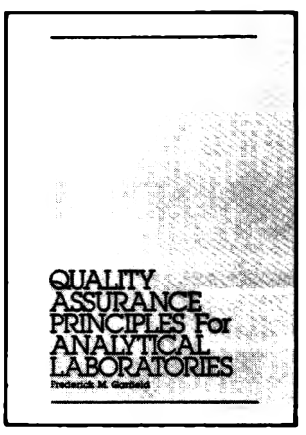

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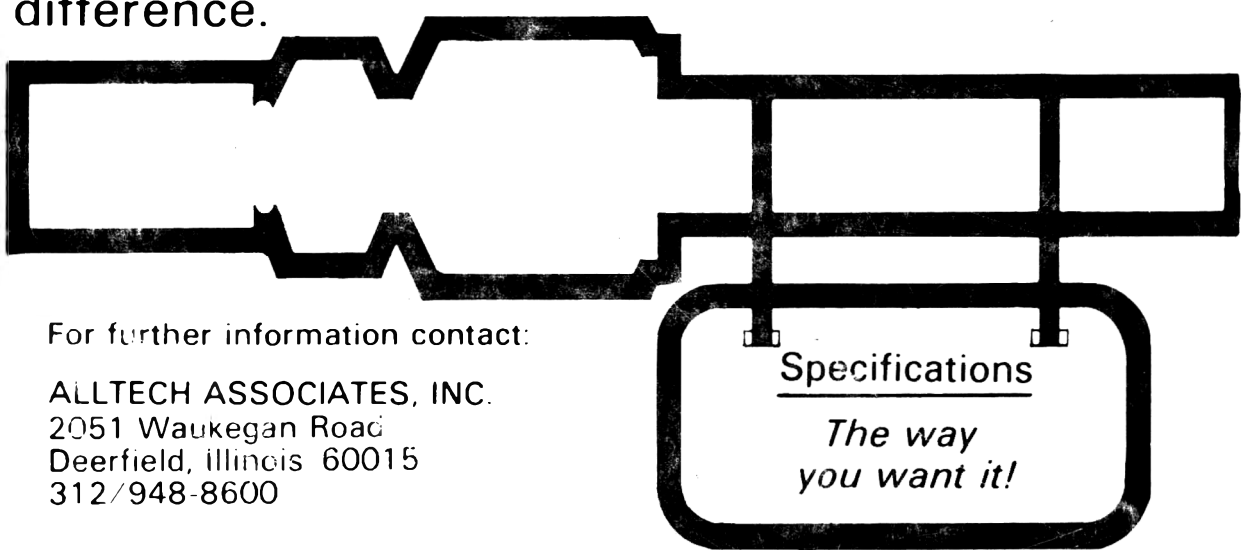

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