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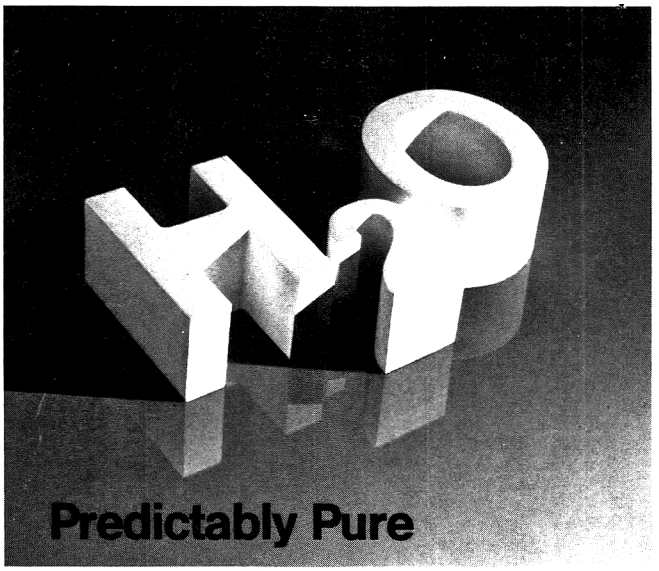

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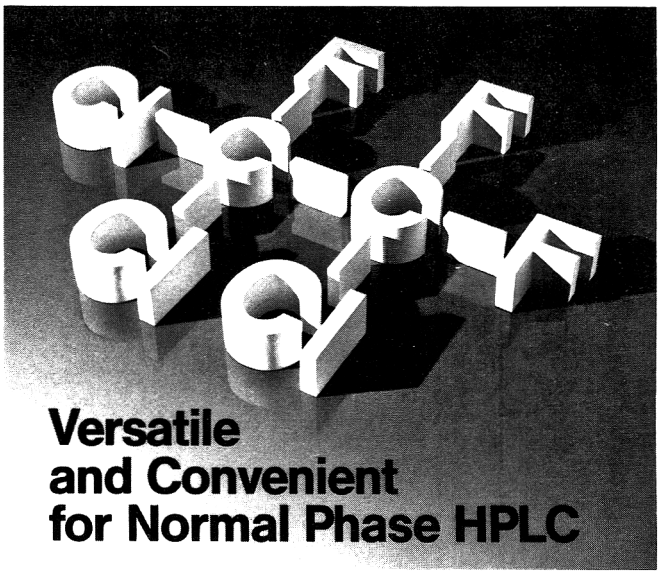

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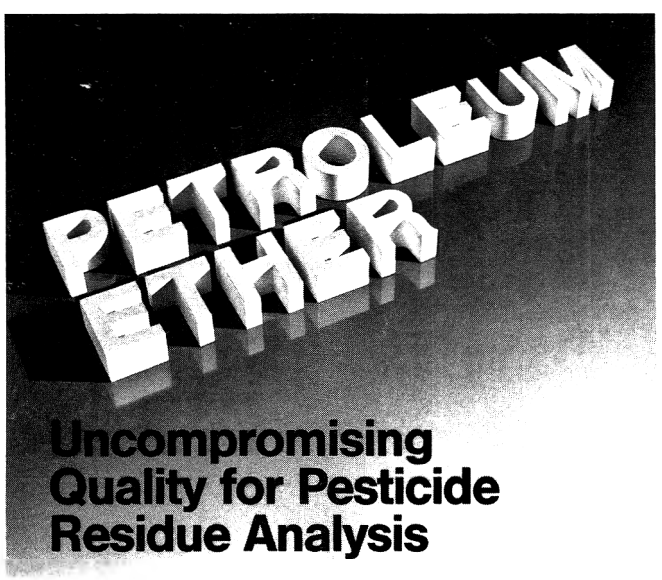

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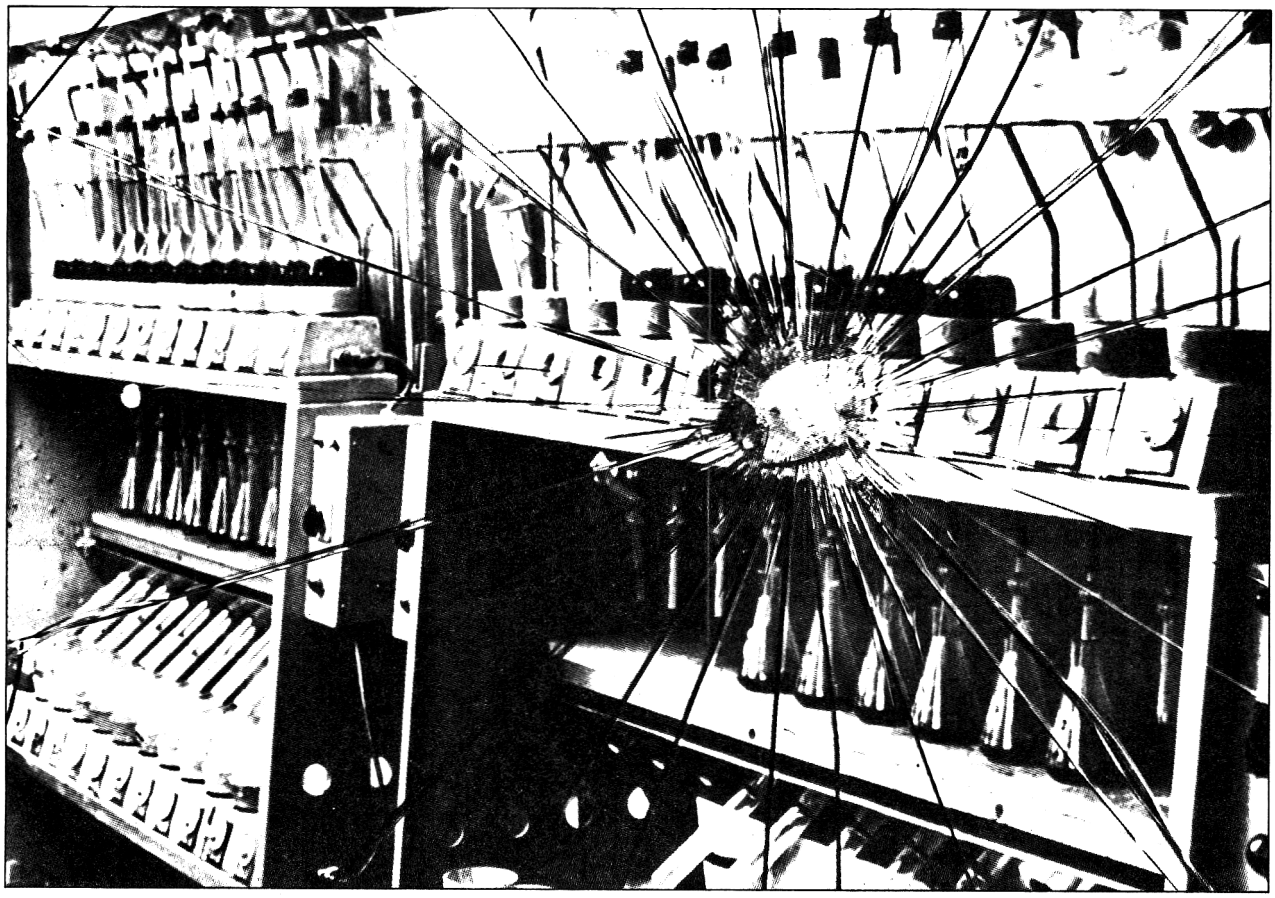

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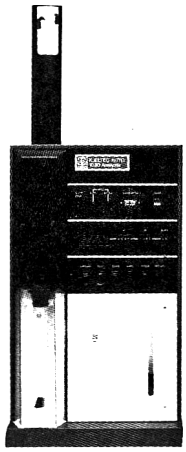

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ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

Basic & Applied Research in the Analytical Sciences Related to Agriculture and the Public Health

JULY/AUGUST 1984
VOL. 67, NO. 4

Technical Communications

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

The Journal The Journal of the Association of Official Analytical Chemists is published by AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. The Journal is issued six times a year in January, March, May, July, September, and November. Each volume will contain approximately 1400 pages. The scope of the Journal 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 the 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 and supplements, contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment.

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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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Authors must submit 3 copies of the complete manuscript, including tables and illustrations, to AOAC, 1111 N 19th St, Arlington, VA. The manuscript is to be typewritten on one side of white bond paper, 8½ × 11 inches, with page margins of 1 inch, and **double-spaced** throughout (i.e. title, authors' names and addresses, footnotes, tables, references, captions for illustrations, and the text itself). Tables are to be typed on separate sheets, *not* interspersed through the manuscript. Drawings and photographs should be mounted apart from the text or submitted as separate items.

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Methods, Results and/or Discussion, Acknowledgments, and Recommendations (applicable to reports of General and Associate Referees) should be placed in sections under appropriate headings.

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Methods: Methods should be written in imperative style, i.e., "Add 10 mL . . . Heat to boiling . . . Read in spectrophotometer." Special reagents and apparatus should be separated from the details of the procedure and placed in sections with appropriate headings; however, common reagents and apparatus or those which require no special preparation or assembly, need not be listed separately. Reagents and apparatus listed should be described in generic terms and in terms of performance; use of brand names should be avoided. Hazardous and/or carcinogenic chemicals should be noted. Any very long, detailed operation can be given in a separate section with an appropriate heading (e.g., Preparation of Sample: Extraction and Cleanup; Preparation of Standard Curve). Any necessary calculations should be included; number of significant figures must reflect the accuracy of the method. Wherever possible, metric units should be used for measurements or quantities.

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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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of steam boiler water, demineralized water, mine acids, mill waste, and where the determination of the ionic concentration of solution is required. Contact: Scientific Glass Engineering, Inc., 2007 Kramer Ln, Austin, TX 78758; 512/837-7190.
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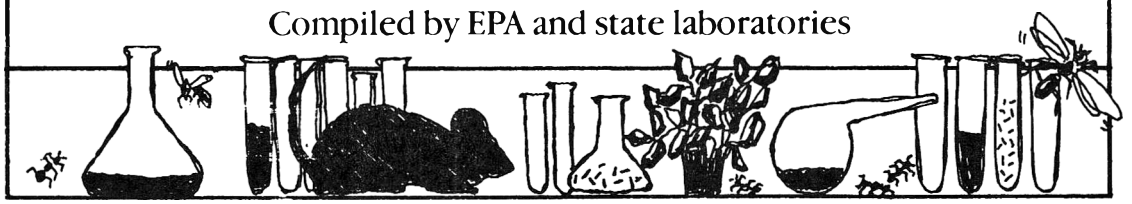
EPA MANUAL OF CHEMICAL METHODS FOR PESTICIDES AND DEVICES

Compiled by EPA and state laboratories



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BOOKS IN BRIEF

Methods of Enzymatic Analysis, Third Edition. Volume II: Samples, Reagents, Assessment of Results. H. U. Bergmeyer, Editor-in-Chief. Published by Verlag Chemie International, 1020 NW 6th St, Deerfield Beach, FL 33441, 1983. Approx. 530 pp.

This second of 10 volumes deals with preparation and processing of samples, cell and tissue disintegration, methods for protein determination, handling of reagents, biochemical reagents for general use, standard and reference materials, evaluation of experimental data, and the quality of experimental results.

Microcolumn High-Performance Liquid Chromatography. Journal of Chromatography Library Volume 28. Edited by P. Kucera. Published by Elsevier Science Publishers B.V., PO Box 330, 1000 AH Amsterdam, The Netherlands, 1984. Also available from Elsevier Science Publishing Co., Inc., 52 Vanderbilt Ave., New York, NY 10017. Approx. 318 pp. Price: U.S. $63.50/Dutch guiders 165.00. ISBN 0444-42290-0.

This book deals with the theoretical aspects of novel microcolumn techniques and discusses instrumentation, design, columns, detectors, injectors, connecting tubing, gradient elution and special analytical techniques, LC-MS, derivatizations, and other topics. Also, applications are described using various compounds, such as, drugs, substances of biological origin, proteins, nucleotides, and industrial extracts.

Meat Microbiology. Edited by M.H. Brown. Published by Applied Science Publishers, a division of Elsevier Science Publishing Co., Inc., PO Box 1663, Grand Central Station, New York, NY 10163, 1982. 529 pp. Price: $85.00. ISBN 0-85334-138-9.

This book presents a collection of chapters written by specialists on particular aspects of meat microbiology. The commodity chapters cover carcass meat, poultry meat, processed meat, cured meat, and by-products. The quality assurance chapters cover bacteria of public health significance, microbiological examination of meat, sampling, and limits.

Basic Gas Chromatography. By H. McNair and E. J. Bonnelli. Published by Varian Instrument Group, 220 Humboldt Ct, Sunnyvale, CA 94089, 1984. 306 pp. Price: $9.00 (Part No. 85-000007-00).

This book presents the basic aspects of modern high performance gas chromatography with emphasis on practical applications. Topics include: the chromatographic system, columns, detectors, temperature programming, applications, and servicing the instruments.

Handbook of Chemical Industry Labeling. Edited by C. J. O'Connor and S. I. Lirtzman. Published by Noyes Publications, Noyes Data Corp., Mill Rd at Grand Ave, Park Ridge, NJ 07656, 1984. 487 pp. Price: $64.00. ISBN 0-8155-0965-0.

This handbook has been designed to provide an in-depth review of, and to act as a source for, the major elements of a hazardous label communication program—to serve the needs of labor, industry, and the public.

Applications of Piezoelectric Quartz Crystal Microbalances. Edited by C. Lu and A. W. Czanderna. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1984. Also available from Elsevier Science Publishing Co., Inc., PO Box 1663, Grand Central Station, New York, NY 10163. Approx. 394 pp. Price: $100.00 U.S. & Canada/ 260.00 Dfl. (elsewhere). ISBN 0-444-42277-3.

This book provides information on the theory, design, sources of error, and applications of quartz crystal microbalances (QCMs) in vacuum and controlled environments. Application chapters deal with QCMs as thin film monitors and controls in surface science plasma-assisted etching, analytical chemistry, space system contamination, and for aerosol mass studies.

Toxic and Biomedical Effects of Fibers: Asbestos, Talc, Inorganic Fibers, Man-Made Vitreous Fibers, and Organic Fibers. By P. Gross and D. C. Braun. Published by Noyes Publications, Noyes Data Corp., Mill Rd at Grand Ave, Park Ridge, NJ 07656, 1984. 245 pp. Price: $36.00. ISBN 0-8155-0971-5.

This book reviews the toxic and biomedical effects of mineral and organic fibers, both natural and man-made. As part of the review of illnesses associated with exposure to various fibers, pathogenicity mechanisms are discussed as well as problems relating to determination of their cause.

Advances in Carbohydrate Chemistry and Biochemistry. Vol. 41. Edited by R. S. Tipson and D. Horton. Published by Academic Press, Inc., 111 5th Ave, New York, NY 10003, 1983. 416 pp. Price: $56.00. ISBN 0-12-007241-6.

This series is the only source of review articles in this field. Some topics in this volume include: C13 nuclear magnetic resonance spectroscopy of monosaccharides, structural chemistry of polysaccharides from fungi and lichens, biosynthesis of cellulose, and capsular polysaccharides as human vaccines.

Peanuts: Production, Processing, Products, Third Edition. Edited by J. G. Woodroof. Published by The AVI Publishing Co., Inc., 250 Post Rd E, PO Box 831, Westport, CT 06881, 1983. 414 pp. Price: $57.50. ISBN 0-87055-417-4.

This revised edition provides information on the status of peanut culture, production, and uses not only in the 4 peanut producing areas of the U.S., but also in Africa, Asia, Australia, and South America. Revised sections feature the peanut culture throughout the world including: perennial peanuts, harvesting peanuts for optimum quality, direct harvesting peanuts, the aflatoxin crisis, peanut oil extractions and specifications, integrated peanut processing, and sodium-free salted peanuts.

Clinical Liquid Chromatography. Volume I. Edited by P. M. Kabra and L. J. Marton. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1984. Approx. 224 pp. Price: $63.00, prepub., U.S./ $72.00, prepub. outside U.S. ISBN 0-8493-6637-2.

A reference for scientists involved in analyzing the exogenous and endogenous constituents in physiological fluids

through the use of liquid chromatography, this book contains a variety of liquid chromatographic methods and the basic concepts for development of each method. Some topics included in Volume I are: acetaminophen and phenacetin by ultraviolet detection, determination of acetaminophen in plasma by liquid chromatography, determination of ε-aminocaproic acid, analysis of aminoglycoside antibiotics by precolumn fluorescence derivatization, and many others.

CRC Handbook of Microbiology, 2nd Edition. Volume V: Microbial Products. Edited by A. I. Laskin and J. Lechevalier. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1984. Approx. 952 pp. Price: $99.50, prepub., U.S./$115.00, prepub., outside U.S. ISBN 0-8493-7205-4.

These volumes provide microbiologists and allied scientists with current, comprehensive information on the properties of microorganisms and their composition, products, and activities. Volume V includes the following topics: substances related to carbohydrates: simple aliphatic substances, hydrocarbons, esters, aldehydes, ketones, and alcohols; cyclitol antibiotics; disaccharides and trisaccharides; simple sulfur compounds; microbial sulfolipids; tetracyclines, including biosynthetic precursors; and others.

CRC Handbook of Chromatography. Volume I: Pesticides and Related Organic Chemicals. Edited by J. M. Follweiler and J. Sherma. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431. 1984. 368 pp. Price: $67.00, prepub., U.S./$77.00, prepub., outside U.S. ISBN 0-8493-3919-6.

Covering important published literature with data compiled into tables in sections for gas, liquid, and thin layer chromatography, this volume deals with chromatography of pesticides and related organic compounds, such as polychlorinated biphenyls. Information such as retention times, types of columns, temperatures, detectors used, sample preparation, and packing are also given and referenced.

Recent Vitamin Research. Edited by M. H. Briggs. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431, 1984. 256 pp. Price: $72.00, prepub., U.S./$83.00, prepub., outside U.S. ISBN 0-8493-5618-0.

This collection of papers by international experts discusses topics such as: biotin-responsive metabolic disorders of children, complex interactions between vitamin D and parathyroid hormone in bone calcification, and effects of vitamin supplements during pregnancy and intrauterine growth. Also included is a review of vitamin C and infectious diseases and possible protective effects of vitamin A against cancer.

Neutron Activation Analysis for Clinical Trace Element Research. Volume I. Edited by K. Heydorn. Published by CRC Press, Inc., 2000 Corporate Blvd, NW, Boca Raton, FL 33431. 288 pp. Price: $75.00, prepub., U.S./$86.00, prepub., outside U.S. ISBN 0-4893-5773-X.

Volume I discusses the role of quality control in obtaining statistical control of both trace and ultratrace element determinations. Researchers in laboratory medicine, analytical chemistry, and biochemistry will find this to be a valuable reference.

Herbicide Handbook, Fifth Edition. Edited by C. E. Beste. Published by the Weed Science Society of America, 309 W Clark St, Champaign, IL 61820, 1983. 515 pp. Price: $10.00. ISBN 0-911733-01-9.

With 12 new compounds added, this handbook contains technical information on 138 herbicides, desiccants, and growth regulators. Chemical and physical properties, physiological and biochemical behavior, toxicological properties, synthesis, and associated information on all compounds has been reviewed and revised. Also provided are sections on terminology, conversion factors, references, manufacturers, and tradenames.

NMR of Aromatic Compounds. By J. D. Memory and N. K. Wilson. Published by John Wiley & Sons, Inc., 605 Third Ave, New York, NY 10158, 1982. 252 pp. Price: $39.95. ISBN 0471-08899-4.

The text emphasizes high-resolution NMR done with ^{13}C nuclei and other nuclei, and attention is given to polycyclic aromatic compounds as prototypes of these compounds. Theories of NMR parameters and experimental techniques are provided. Geared to the information needs of organic, physical and analytical chemists at the graduate and professional levels, this book is a key to current research and a guide to laboratory techniques and information sources.

FOR YOUR INFORMATION

Information Service

The American Chemical Society (ACS) has begun a new public service program called the Information Service for Government to provide scientific and technical information about chemistry and chemical engineering to governmental bodies, especially Congress. The objectives of this program are to increase the scientific knowledge available to government and to enable congressional staff to gain a better understanding of the scientific and technical issues involved in policy debates.

Requests received so far have been for factual information and for names of experts on particular scientific issues. The Service does not answer questions requiring policy making or analysis, or value judgments of scientific and technological alternatives. Such questions are referred to the appropriate offices within ACS.

As well as answering questions, the Service staff plans to conduct occasional seminars for congressional staff on those emerging issues with many chemistry principles. The first seminar will be on the Society's revised "Guidelines for Data Acquisition and Data Quality Evaluation in Environmental Chemistry." Also, technical issue briefs will be prepared as appropriate.

The Service is located in the ACS Department of Public Affairs under Director R. G. Smerko. The program officer is H. S. Strauss, and there is a 10-member advisory committee made up of representatives from industry, public interest groups, academia, and government.

For a copy of the prospectus for the Service, contact the Dept of Public Affairs at ACS headquarters in Washington, DC.

Market Analysis Publication

U.S. suppliers of agricultural chemicals can benefit from a new market analysis publication offered by the U.S. Dept of Commerce's International Trade Administration (ITA). The Export Statistics Profile offers detailed statistics on industry exports for the last 5 years, plus an analysis of the industry's export potential.

Available in 2 forms, prepackaged and custom, the basic prepackaged profile summarizes essential market data, including: leading export items, fastest growing items, leading foreign markets, fastest growing markets, major foreign competitors, the industry's 10 best market prospects, and yearly statistical tables for the 1972–82 ranking of the industry's 75 top foreign markets. An expanded profile provides more detailed marketing information.

The custom profile enables exporters to tailor their own statistical information products. They can get data on products not covered by the general profile and can request import and export statistics.

For more information, including fees, plus order forms, contact: John Autin, U.S. Dept of Commerce, ITA/OTIS, PO Box 14207, Washington, DC 20044; 202/377-3253.

NSF Science Education Program

The National Science Foundation (NSF) is responsible for strengthening science education at the precollege level to prepare students for enhanced career options; for living in a high-technology, information society; and for pursuing higher education opportunities in mathematics, science, and technology.

NSF will consider proposals for support of precollege science and technology education projects that focus on research in teaching and learning and development of instructional materials for students and teachers; activities that provide incentives for teachers and develop their capabilities; and special activities for increasing extra-curricular science, mathematics, and technology education.

NSF evaluates proposals on the basis of 4 general criteria: intrinsic merit, performance competence, utility or relevance of the project, and effect on the infrastructure of science and engineering. NSF grants are administered in accord with NSF Form Letter 200, Grant General Conditions.

All institutions with a scientific or educational mission are eligible to submit proposals. Proposals may be submitted at any time from any field of science in which NSF supports research including: astronomy, atmospheric sciences, biological and behavioral sciences, chemistry, computer science, mathematical sciences, materials sciences, oceanography, physics, and the social sciences.

For information on preparation and submission of proposals and any other information, contact: Precollege Science and Mathematics Activities, Directorate for Science and Engineering Education, National Science Foundation, Washington, DC 20550.

New Publications from the American Chemical Society

The American Chemical Society's (ACS) publication, "Principles of Environmental Analysis," published in the December 1983 issue of *Analytical Chemistry*, is now available as a separate brochure. Developed by the ACS Subcommittee on Environmental Monitoring and Analysis, this publication identifies factors needed to obtain reliable data and those that produce unreliable data to aid in designing and conducting analysis of environmental samples. Types of analyses range from semiquantitative screening analyses to quality assurance programs. Discussion focuses on planning, quality assurance and quality control, verification and validation, precision and accuracy, sampling, measurements, and documentation and reporting.

While "Principles" is meant to aid those working in the field, ACS has also produced the "Ground Water Information Pamphlet," which is geared to the general public. This pamphlet focuses on what ground water is, where it occurs, its volume and quality; the scientific aspects of ground-water depletion and degradation; and the concepts about the chemistry of ground water. Written in clear, concise language, educators will find the pamphlet useful for high school and college students.

Single copies of the pamphlets are free, and multiple orders can also be obtained. Contact: J. Parr, Dept of Public Affairs, American Chemical Society, 1155 16th St, NW, Washington, DC 20036; 202/872-8725.

Meetings

October 28–November 2, 1984: AOAC Centennial International Meeting, Shoreham Hotel, Washington, DC. Contact: AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209; 703/522-3032.

September 10–12, 1984: 3rd International Conference of the Nature Publishing Co.: The Molecular Biology of Cancer, Boston Park Plaza Hotel, Boston, MA. Program topics are: oncogenes and growth factors; the cancer cell; and prospects and problems of drug resistance, monoclonal antibodies and drugs. For information on registration and fees, contact Ann Sobota, Nature Publishing Co., 15 E 26th St, New York, NY 10010. For more information, contact: Henry Dale or Gary Rekstad, 212/689-5900.

September 16–21, 1984: 11th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies, Philadelphia Marriott Hotel, Philadelphia, PA. Some program topics are: atomic spectroscopy, vibrational spectroscopy, chromatography, NMR spectroscopy, mass spectroscopy, flow injection analysis, computer aided technology, and many others. Workshops and short courses will be offered. Contact: FACSS XI Program Chairman Patricia B. Roush, Perkin-Elmer Corp., M/S 903, 901 Ethan Allen Highway, Ridgefield, CT 06877; 203/797-9481.

October 6–10, 1984: Project Management Institute Annual Seminar/Symposium: Innovation, Franklin Plaza Hotel, Philadelphia, PA. The theme, Innovation, will be enhanced by workshops, papers, presentations, and guest speakers, including Isaac Asimov. Contact: Project Management Institute, PO Box 43, Drexel Hill, PA 19026; 215/622-1796.

October 18, 1984: 11th Symposium of the Association of Analytical Chemists, Inc., Michigan State University Management Education Center, Troy, MI. Technical presentations include the following topics: laboratory automation, environmental and geological analysis, pharmaceutical analysis, separations, and spectroscopy. Contact: Paul M. Beckwith, The Detroit Edison Co., 6100 W Warren, Detroit, MI 48210; 313/897-1417.

April 9–11, 1985: AOAC 10th Annual Spring Workshop, Sheraton-Dallas Hotel, Dallas, TX. The program will contain sessions on a variety of topics including the environment, foods, feeds, drugs, and instrumentation. For information, contact: M. Virginia Gibson, FDA, 332 Bryan, Dallas, TX 75204; 214/767-0312 and Molly Ready, Alcon Labs, 6201 S Freeway, Fort Worth, TX 76134; 817/293-0450.

New Sustaining Members

AOAC welcomes new additions to the growing list of organizations aware of the need to support an independent methods validation association. New Private Sustaining Members are: Castleton Beverage Corp., Jacksonville, FL; DFA of California, Santa Clara, CA; McDonald's Corp., Westmont, IL; R.J. Reynolds Tobacco Co., Winston Salem, NC; and Mississippi Chemical Corp., Yazoo City, MI. A new Government Sustaining Member is the Alberta Dairymen's Association Research Unit of the University of Alberta, Edmonton, Alberta, Canada.

A CENTURY OF ANALYTICAL EXCELLENCE

A Perspective Review of the Development of AOAC Microbiological Methods

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Establishment of the Refereeship

The first General Referee Report on Microbiological Methods was submitted by A. C. Hunter (1) to Subcommittee C at the 49th annual meeting of the Association of Official Agricultural (Analytical) Chemists (AOAC) in 1933. The initial problem confronting the General Referee was to determine the most suitable approach for developing microbiological methods. Should the work be undertaken on a broad scale, covering a variety of foods, or should it be restricted to a particular type of food, at least at the beginning, until participating analysts became oriented? It was eventually decided that the latter alternative would be the more logical approach.

In choosing a particular type or class of food, a major concern was the risk of duplicating the work of organizations other than AOAC. Thus, certain foods or commodities were not considered because they had been studied or were currently under investigation by various organizations: milk (American Public Health Association (APHA), the American Dairy Science Association, and the International Association of Dairy and Milk Inspectors); oysters (APHA); water (APHA and the American Water Works Association); and, finally, foods in general, especially dairy products such as dry milk, ice cream, and butter (APHA).

Hunter believed that the class of food chosen should be based on 3 factors: *(1)* The food should be one that is normally examined by food microbiologists, *(2)* the bacteriological technique for examining such a food should be relatively simple, and *(3)* the bacteriological methods used in different laboratories for analyzing this food should be sufficiently diverse to justify study for the purpose of establishing a single, uniform, standard procedure. Based on these 3 factors, the general class of canned foods was chosen as the potential study area.

In an attempt to solicit comments on this approach, Hunter sent a questionnaire to 33 food microbiologists. Responses to the 11 questions demonstrated the extent of variability in the microbiological examination of canned foods: treatment of can exterior before opening (9 different methods); type of pipet used for sampling liquids in cans (ranging from 1 mL bacteriological pipet to a large, untapered length of glass tubing); culture media used (17 different formulations); time and temperature of incubation (31 different combinations); and quantity of sample taken from each can (20 varying amounts). Hunter came ". . . to the inescapable conclusion that the method of examining canned foods bacteriologically depends to a great extent on the ingenuity of the analyst and on the availability of certain apparatus and culture media." It was thus decided that the choice of canned products as a class of foods upon which to begin the development of standardized microbiological methods was the correct one. Accordingly, Hunter recommended the establishment of 5 Associate Refereeships to study the following topics: *(1)* Culture Media of Acid Products, *(2)* Culture Media of Non-acid Products, *(3)* Incubation Periods and Temperatures for Cultures, *(4)* Sampling Inoculum, and *(5)* Treatment of Unopened Container. Thus, these 5 Associate Referee topics were established to study techniques or fundamental procedures involved in the examination of canned food.

Orientation Period and Organization

The years 1933–35 can best be described as a period of orientation and organization. The 5 Associate Referees exchanged opinions, based on experience and actual experimentation, on the microbiological examination of canned foods, but no real progress was reported. In his report at the 1936 annual meeting, Hunter (2) recommended a change in direction. It was concluded that nothing more was to be gained by continuing the study of basic techniques or procedures involved in the examination of canned foods. Accordingly, Hunter recommended that the Associate Referee topics be reorganized according to specific foods: *(1)* Canned Fishery Products, *(2)* Canned Meats, *(3)* Canned Tomato Products, *(4)* Canned Vegetables, and *(5)* Sugar (included because it may become contaminated with microorganisms capable of causing spoilage in canned foods) (Table 1).

During the 4 years following the 1936 meeting, 3 new topics were established: Eggs and Egg Products (1937), Frozen Fruits and Vegetables (1940), and Nut Meats (1940). The topic, Canned Tomato Products, underwent 2 name changes: Canned Fruits (1939) and Canned Tomatoes and Other Acid Vegetable and Fruit Products (1940).

In his report at the 1941 meeting, Hunter (3) paralleled the courses being pursued by AOAC and APHA. He further mentioned that the then forthcoming edition of APHA's *Standard Methods for the Examination of Dairy Products* (4) contained methods for the bacteriological examination of sugar and frozen eggs as did AOAC's *Official Methods of Analysis* (5). It was fortunate, he continued, that the personnel involved in the preparation of these 2 methodologies were of a uniform viewpoint which prevented any conflict in the procedures recommended by the 2 organizations. Finally, he expressed the hope that a manual of methods would be prepared for the microbiological examination of foods which would contain contributions from both APHA and AOAC.

Because of war emergency conditions, no annual meeting was held in 1942, or in 1945 because of the post-war readjustment period. There were no major changes or recommendations for the intervening years, 1943–44.

Table 1. Microbiological methods Associate Refereeships, 1936–1963

Topic	Year of annual AOAC meeting										
	1936	1937–1938	1939	1940–1942	1943	1944	1946	1947	1948–1952	1953–1962	1963
Canned fishery products	X[a]	X	X	X	X	X	(X)[b]	(X)	X		
Canned meats	X	X	X	X	X	X	X	X	X		
Canned tomato products	X	X									
Canned vegetables	X	X	X	X	X	X	(X)	X	X		
Sugar	X	X	X	X	X	X	ND[c]	X	X		
Eggs and egg products		X	X	X	X	X	X	X	X	X	X
Canned fruits			X								
Canned tomatoes and other acid vegetable and fruit products				X	X	X	X				
Frozen fruits and vegetables				X	(X)	X	X	X	X		
Nut meats				X[d]	X[d]	X[d]	X[d]	X[d]	X[d]	ND[c]	X
Canned acid foods								X	X		
Crab meat											X

[a]X, officially listed as an Associate Refereeship in *J. Assoc. Off. Agric. Chem.* for the year indicated.
[b](X), not officially listed as an Associate Refereeship in *J. Assoc. Off. Agric. Chem.*, but existence of Associate Refereeship established by Committee's (Subcommittee's) and/or General Referee's Report(s).
[c]Could not be determined from available records.
[d]Associate Refereeship topic known as Nuts and Nut Products.

Tenure of G. G. Slocum

In 1946, G. G. Slocum became the new General Referee. In his first report at the 1947 meeting, Slocum (6) recommended a continuation of the general course established by his predecessor. Moreover, he emphasized the importance of continuing the close relationship of AOAC and APHA methods, not only to avoid any conflict in procedures, but also to minimize any duplication of effort by the 2 organizations.

In his report at the 1949 meeting, Slocum (7) again took the opportunity to emphasize that it was desirable, if not imperative, that AOAC methods be in essential agreement with other standard methods. AOAC methods for eggs and egg products, sugar, and nut meats were similar to those of APHA for the examination of these products but variations existed. He added that "these methods must and will be coordinated."

In his final report as General Referee, Slocum (8) asked Subcommittee C to reconsider AOAC's policy with respect to future development and adoption of microbiological methods, especially because APHA was developing its own methods manual (9) for the microbiological examination of foods. In responding to the General Referee's request, the subcommittee concluded (10) that AOAC ". . . should not work on microbiological methods which are not useful in regulatory work, and which are sponsored by other organizations, but only on methods for which there is definite need in regulatory work." Thus, the regulatory basis was established for the subsequent development of microbiological methods by AOAC.

Tenure of M. T. Bartram

M. T. Bartram became General Referee in 1953. Beginning that year and for the following 9 years, there was a single Associate Referee topic, Eggs and Egg Products (Table 1). It was during Bartram's term that AOAC adopted its first final action microbiological method for food analysis, the Examination of Eggs and Egg Products, **46.003–46.009** and **46.012** (11), about which more will be said later. In 1960, the General Refereeship for Microbiological Methods was placed under Subcommittee E.

Tenure of H. V. Leininger

It was discussed earlier how the Associate Referee topics were originally established according to fundamental techniques or procedures and then according to specific types or categories of foods. With the appointment of H. V. Leininger as General Referee in 1964 came a reorganization of the Associate Referee topics (Table 2). Although 5 of the 9 topics were still concerned with a particular food, for the first time topics were established to study a specific organism or group of organisms: anaerobes, *Salmonella* species, *Staphylococcus* species, and *Staphylococcus* species toxin.

At the 1965 meeting, the Associate Referee topic, Anaerobes, was subdivided into 2 topics: *(1) Clostridium botulinum* and Its Toxin, Detection, and *(2) Clostridium perfringens*, Isolation and Enumeration. This meeting also marked the beginning of the submission of the General Referee Reports to Subcommittee F (renamed Committee F in 1980), rather than to Subcommittee E. The remaining 3 years of Leininger's tenure resulted in the establishment of 7 additional topics: Canned Foods, Cereal Products, and Preservatives (1966); *Shigella* Species and *Vibrio parahaemolyticus* (1967); and Enterococci, and *Salmonella* Species, Fluorescent Antibody Technique (1968).

Tenure of R. Angelotti

R. Angelotti was appointed General Referee at the 1969 meeting, and in his first report (12) to Subcommittee F, he mentioned the proposed formation of a joint committee to act in an advisory capacity to the General Referee. Such a committee would be composed of members from industry, government, universities, AOAC, APHA, and the American Society for Microbiology. Other plans of the General Referee included reviewing the progress and expectations of the various Associate Referee topics and making the necessary consolidations and deletions to achieve a more efficient administration of the General Refereeship. Establishment of future Associate Referee topics, he continued, should be based on specific microorganisms or groups of organisms. Significantly, Angelotti noted that the International Commission on Microbiological Specifications for Foods (ICMSF) was conducting a collaborative study of a rapid direct plating method for enumeration of *Staphylococcus* species organisms, and that if the ICMSF collaborative study met the requirements for an AOAC collaborative study, it should be possible to adopt the ICMSF method as an official first action AOAC method. This concept offered the possibility of an extensive and accelerated expansion of microbiological methods officially approved by AOAC. Unfortunately, however, data from the ICMSF study could not be used. Nonetheless, Angelotti continued the trend established by his predecessors of

fostering cooperation between AOAC and other professional organizations. During his 2-year tenure the topic Sugar was reinstated, but no new topics were initiated.

Tenure of A. C. Sanders

In 1971, A. C. Sanders assumed the responsibilities of General Referee. At the very onset of his initial report to the Subcommittee in 1972, Sanders (13) indicated his intent to continue the realignment of Associate Referee topics from food types to specific organisms, as initiated by Leininger. Accordingly, 4 of the Associate Referee topics relating to foods or other commodities (Carbonated Beverages, Crab Meat, Eggs and Egg Products, and Frozen Food Analysis) were discontinued. In his report at the 1975 meeting, Sanders (14) anticipated a series of collaborative studies being conducted by FDA's Minneapolis Center for Microbiological Investigations (MCMI) in the interest of moving more methods into official AOAC status. Several co-Associate Referees were appointed for existing topics and new topics were established with Associate Referees and co-Associate Referees being appointed at MCMI and elsewhere. It was during Sanders' tenure that the General Refereeship experienced its greatest expansion. Topics were initiated to study established, well known pathogens (*Staphylococcus aureus* and *Vibrio cholerae*), emerging pathogens (Enteropathogenic *Escherichia coli, Yersinia enterocolitica,* and *Campylobacter* Species), toxin production (Endotoxins by *Limulus* Amebocyte Lysate), new techniques for detecting pathogens (Microbe Identification by Capillary Gas Chromatography), and automation (Automated Methods for Food and Cosmetics). During Sanders' 11-year tenure as General Referee, the number of Associate Referee topics increased from 11 in 1971 to 37 in 1981.

Subdivision of the General Refereeship

In 1982, the AOAC Executive Director and members of Committee F agreed that the General Refereeship for Microbiological Methods had expanded to the point where a subdivision was in order. Accordingly, that same year the General Refereeship for Microbiological Methods was subdivided into a General Refereeship for Drug and Device Related Microbiology (G. Oxborrow, General Referee) and a General Refereeship for Food Microbiology (W. Andrews, General Referee). Two topics were placed in the General Refereeship for Drug and Device Related Microbiology: Testing Biological Sterility Indicators, and Sterility Testing of Medical Devices. All other Associate Referee topics were placed in the General Refereeship for Food Microbiology. It was incumbent upon the recently appointed General Referee for Food Microbiology to review each of the topics in his area to determine past accomplishments, present performance, and potential for future activity. As a result, several topics were consolidated or discontinued. Thus, at the time of the 1983 meeting, there was a total of 27 topics in Food Microbiology.

Official Adoption of Early Methods for Foods

Table 3 shows the sequence of adoption of microbiological methods by the AOAC. It was not until 1941 that any microbiological methods were adopted official first action. Three methods received this status after having been adopted as tentative methods in 1939 (15). The designation "tentative," a term no longer used, focused attention on those methods which showed potential for eventually becoming an official method but had not yet been collaboratively studied.

Eggs and Egg Products

One of these early tentative methods, Examination of Eggs and Egg Products, **46.003–46.012,** was collaboratively studied in 1940, and the results were reported at the 1941 meeting (16). Frozen egg products used in the collaborative study included egg whites, egg yolks, whole egg, and sugar yolks. Five collaborators each received 15 samples of frozen egg products, which they analyzed for aerobic plate count values, coliforms (including *E. coli*), hemolytic staphylococci and streptococci, putrefactive anaerobes, fungi, and direct microscopic counts. Hemolytic staphylococci and streptococci were not detected in any of the samples by any of the collaborators; putrefactive anaerobes were not detected in 14 of the samples by any of the collaborators; and the determination of fungi counts was too variable to be useful. Nonetheless, the method for the microbiological examination of frozen eggs was adopted first action at the 1941 meeting (17).

Three years later, at the 1944 meeting (18), the first action method for frozen eggs was expanded to include tentative method status for the examination of liquid and dried eggs. It was not until the 1952 meeting (19) that 2 important revisions were made in the first action method. First, Butterfield's phosphate buffer was approved as an alternative to physiological (0.85%) saline as a diluent. Second, the incubation temperature was changed from 37°C to 35°C. Interestingly, in the collaborative study conducted in 1940, analysts were asked to inoculate triple sets of plate count agar and to incubate one set of plates at 20°C, one set at 32°C, and one set at 37°C, whereas coliforms were incubated only at 37°C (20). The method was further modified at the 1954 meeting by deleting the test for putrefactive anaerobes, because the General Referee (21) could find no indication that this group of organisms was of special significance in egg products.

In his report to the Subcommittee at the 1956 meeting, Bartram (22) presented the results of a second collaborative study on the microbiological examination of eggs and egg products. Based on that study, the first action method for the determination of aerobic plate count values, levels of coliforms (including *E. coli*), and direct microscopic count was adopted official final action that same year (23). Concomitantly, methods for sampling, sample preparation, and culture media preparation (as used in the examination of eggs and egg products) were adopted official final action. The first action methods for hemolytic staphylococci and for fungi were adopted as procedures. The Association defines procedures as ". . . generally sorting or screening methods, or well established types of examinations or auxiliary operations, such as sampling or sample preparation, which have not necessarily been subjected to a collaborative study" (24). Since 1956, the method for the examination of eggs and egg products has remained unchanged.

Frozen, Chilled, Precooked, or Prepared Foods

In 1965, the method for the microbiological examination of frozen, chilled, precooked, or prepared foods, **46.013–46.017,** was adopted official first action. This action followed completion of a collaborative study conducted by the Associate Referee on Frozen Food Analysis for the following analyses: aerobic plate count (APC), most probable numbers (MPNs) of coliforms (including *E. coli*), and enumeration of *Staphylococus aureus* on the basis of a single tube per dilution. This method was essentially that recommended by the Association of Food and Drug Officials of the United States (AFDOUS) (25) and had been used by the Food and Drug Administration (FDA) since 1958 (26). All the results from the collaborative

Table 2. Microbiological methods Associate Refereeships, 1964–1983

Topic	Year of annual AOAC meeting																		
	1964	1965	1966	1967	1968	1969	1970	1971	1972–1973	1974	1975	1976	1977	1978	1979	1980	1981	1982[a]	1983
Anaerobes in food	X[b]																		
Carbonated beverages	X	X	X																
Crab meat	X	X	X	X	X	X													
Eggs and egg products	X	X																	
Frozen food analysis	X	X	X	X	X	X													
Nut meats	X	X	X	X	X	X	X												
Salmonella species	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Staphylococcus species[c]	X	X	X	X	X	X	X	X	X	X	X	X	X	(X)[d]	X	X	X	X	X
Staphylococcus species toxin	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Clostridium botulinum and its toxin, detection		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Clostridium perfringens, isolation and enumeration		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Canned foods			X	X	X	X	X	X	X	X	X	X	X	X	X	X	(X)	X	X
Cereal products			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
Preservatives			X	X	X	X	X												
Shigella species				X	X	X	X	X	X	X	X	X	X	X	X	X			
Vibrio parahaemolyticus				X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Enterococci					X	X	X												
Salmonella species, fluorescent antibody technique					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Sugars						X[e]	X	X[e]	X[e]	D[f]	X[e]	X[e]	X[e]	(X)	(X)	(X)	(X)	X	X
Cosmetic microbiology									X	X	X	X	X	X	X	X	X		
Enteropathogenic *Escherichia coli*[g]									X	X	X	X	X	X	X	X	X		
Virology and animal oncology									X	X	X	X	X	X	X	X	X	X	X
Yeasts, molds, and actinomycetes[h]									X	X	X	X	X	X	X	X	(X)	X	X
Enteropathogenic *Escherichia coli*, direct fluorescent antibody technique											X	X	X	X	X	X	X	X	X
Automated methods for food and cosmetics												X	X	X	X	X	X	X	X
Coliform bacteriology[g]												X	X	X	X	X	X		
Parasitology												X	X	X	X	X	X	X	X
Yersinia enterocolitica												X	X	X	X	X	X	X	X
Bacillus cereus, enterotoxin[i]													X	X	X	X	X	X	X
Bacillus cereus, isolation and enumeration													X	X	X	X	X	X	X
Bacillus cereus, toxin[i]													X	X	X	X	X		
Escherichia coli and other coliforms[g]													X	X	X	X	X	X	X
Somatic cell, automated optical counting method													X	X	X	X	X	X	X
Somatic cell, Fossomatic counting method														X	X	X	X	X	X
Somatic cell, Millipore-DNA assay														X	X	X	X		
Staphylococcus aureus[c]														X	X	X	X		
Sterility testing of medical devices														X	X	X	X	X	X
Yeast and mold counts[h]														X	X	X	X		
Automated methods for fungi[h]															X	X	X		
Endotoxins by *Limulus* amebocyte lysate															X	X	X	X	X
Somatic cell, rolling ball viscometer procedure															X	X	X		
Testing biological sterility indicators															X	X	X	X	X
Campylobacter species																X	X	X	X
Vibrio cholerae and detection of its toxins																X	X	X	
Helium leaks, canned foods																	X	X	X
Microbe identification by capillary gas chromatography																	X		
Genetic methods for detection of bacterial pathogens																		X	X
Hydrophobic grid membrane filter methods																		X	X
Identification of microorganisms by biochemical kits																		X	X

Table 2. Microbiological methods Associate Refereeships, 1964–1983 (continued)

Topic	Year of annual AOAC meeting																		
	1964	1965	1966	1967	1968	1969	1970	1971	1972–1973	1974	1975	1976	1977	1978	1979	1980	1981	1982[a]	1983
Spore-formers and non-spore-formers in low acid foods																		X	X

[a]At the 1982 meeting, the General Refereeship for Microbiological Methods was subdivided into the General Refereeship for Food Microbiology and the General Refereeship for Drug and Device Related Microbiology. Two Associate Refereeships, Sterility Testing of Medical Devices and Testing Biological Sterility Indicators, were placed under the General Refereeship for Drug and Device Related Microbiology, whereas all other Associate Refereeships were placed under the General Refereeship for Food Microbiology.
[b]X, officially listed as an Associate Refereeship in *J. Assoc. Off. Anal. Chem.* for the year indicated. (*J. Assoc. Off. Agric. Chem.* before 1966.)
[c]Combined into the single topic, *Staphylococcus* Species, at the 1982 meeting.
[d](X), not officially listed as an Associate Refereeship in *J. Assoc. Off. Anal. Chem.*, but existence of Associate Refereeship established by Committee's (Subcommittee's) and/or General Referee's Report(s).
[e]Associate Refereeship topic known as Sugar and Sugar Products.
[f]Topic discontinued for 1 year.
[g]Combined into the single topic, *Escherichia coli* and Other Coliforms, at the 1982 meeting.
[h]Combined into the single topic, Yeasts, Molds, and Actinomycetes, at the 1982 meeting.
[i]Combined into the single topic, *Bacillus cereus* Enterotoxin, at the 1982 meeting.

study were valid except the *Staphylococcus* species data, which could not be analyzed statistically. Concomitant with this collaborative study, the Associate Referee on *Staphylococcus* Species conducted a collaborative study for the detection and enumeration of this organism (27). The method which was the subject of this latter collaborative study gave both reproducible results and better recovery of *Staphylococcus* species than the AFDOUS method. Accordingly, Subcommittee F accepted the General Referee's recommendation (28) that the method for *Staphylococcus* species as described by the Associate Referee on Frozen Food Analysis be set aside in favor of the *Staphylococcus* species method developed by the Associate Referee on that topic.

At the 1967 meeting, the method for frozen, chilled, precooked, or prepared foods was revised to include the enumeration of the coliform group and *E. coli* in nut meats (29). The plate count results from the collaborative study of tree nut meats, however, were too variable to be useful. In 1970, this method, **46.016,** was adopted official final action as applied exclusively to the examination of nut meats (30).

Later Methods

Staphylococcus aureus

Criticism of the *Staphylococcus* species method, **46.017,** developed. It focused on 2 points: *(1)* The method required too much time, and *(2)* the single-tube enrichment series was not as sensitive as a multiple-tube (MPN) series. In a review paper (31) on the development of the *Staphylococcus* species method, the Associate Referee noted that there were few options for reducing the analytical time. The most popoular alternative was a direct plating procedure which would shorten the time factor by about 48 h, but would be less sensitive than the liquid enrichment procedure. The theoretical detection limit of the liquid enrichment method (3 *Staphylococcus aureus* cells/g) was contrasted to that for a direct plate method (50 *Staphylococcus aureus* cells/g). The Associate Referee noted, however, that in actual experience, the sensitivity is less than theoretical for both methods. Further, it was concluded that most plating media probably would not detect fewer than 100 *Staphylococcus aureus* cells/g. It was the Associate Referee's opinion, however, that a method capable of detecting fewer than 100 *Staphylococcus aureus* cells is not always required, and in those instances a direct plating method would be applicable.

The second criticism of the *Staphylococcus* species method adopted in 1965 concerned the inadequacy of the precision of using a single tube per dilution of food sample compared to using the MPN technique. Indeed, in a comparison of a single-tube determination with a 3-tube MPN determination, the latter technique was superior (31). The Associate Referee also acknowledged the occurrence of false-positive coagulase reactions with the method adopted in 1965, but noted that these reactions could be eliminated by adding ethylenediaminetetraacetic acid (EDTA) or heparin to the coagulase plasma.

Thus, at the 1967 (29) and 1968 (32) meetings, the *Staphylococcus* species method was revised to substitute a 3-tube MPN determination for the single-tube method. In addition, supplementation of rehydrated coagulase with EDTA became part of the method. At the 1970 meeting (30), the recommended substitution of Baird-Parker agar for Vogel-Johnson agar was adopted first action. At the time of the recommended substitution there was no evidence of the superior efficiency of either of the 2 media. Rather, the Baird-Parker agar offered the advantages of being less inhibitory than Vogel-Johnson agar for selected strains of *Staphylococcus* species and also containing a diagnostic aid (egg yolk reaction) not contained in the Vogel-Johnson agar. It was at the 1974 meeting (33) that the 3-tube MPN enrichment method for *Staphylococcus aureus* was adopted official final action.

As mentioned previously, one of the objections to the tube enrichment procedure was the length of time required to complete the analysis. Another consideration noted by the Associate Referee was that thermally stressed *Staphylococcus aureus* cells are unable to grow in a medium with a high concentration of sodium chloride (34). The tube enrichment method, **46.017,** uses trypticase soy broth with 10% NaCl. Thus, injured *Staphylococcus aureus* cells that have been subjected to some sort of stress during processing may not be detected with such a medium. A practical alternative would be to use a direct plating medium which would also reduce analytical time. Based on a successful collaborative study conducted by the Associate Referee (34), a surface plating method for the isolation and enumeration of *Staphylococcus aureus* in food was adopted official first action at the 1974 meeting (33).

The Associate Referee's belief that most plating media, when used in a surface plating method, would not detect fewer than 100 *Staphylococcus aureus* cells/g has already been mentioned. In a collaborative study conducted by the Associate Referee, however, levels of 91, 34, and 20 *Staphylococcus aureus* cells/g were recovered with Baird-Parker

Table 3. Sequence of AOAC adoption of microbiological methods

	Official AOAC status	
Year(s)[a]	First action	Final action
1933–40	none	none
1941	examination of eggs and egg products, **46.003–46.012**[b] thermophilic bacterial spores in sugars, **46.026–46.030**[c] examination of canned vegetables (other than tomatoes)[d]	
1942–43	none	none
1944	examination of eggs and egg products, **46.003–46.012**[b]	none
1945–51	none	none
1952	examination of eggs and egg products, **46.003–46.012,** first action method modified	none
1953	none	none
1954	examination of eggs and egg products, **46.003–46.012,** first action method modified	none
1955	none	none
1956	examination of eggs and egg products, **46.010** and **46.011**[e]	examination of eggs and egg products, **46.003–46.009** and **46.012**[f]
1957–64	none	none
1965	examination of frozen, chilled, precooked, or prepared foods, **46.013–46.017**	none
1966	*Salmonella* species, **46.054–46.067**	none
1967	examination of frozen, chilled, precooked, or prepared foods, **46.013–46.017,** first action method expanded to include enumeration of coliform group and *Escherichia coli* in tree nut meats, **46.016,** and modified for detection and enumeration of *Staphylococcus aureus,* **46.017** *Salmonella* species, **46.054–46.067,** first action method modified to include lysine iron agar as supplemental test	none
1968	examination of frozen, chilled, precooked, or prepared foods, **46.013–46.017,** first action method modified for detection and enumeration of *Staphylococcus aureus,* **46.017** *Salmonella* species, **46.054–46.067,** first action method revised to include examination of nonfat dry milk and dry whole milk *Clostridium perfringens*[g]	none
1969	none	*Salmonella* species, **46.054–46.067,** for examination of dried whole egg, dried egg yolk, and dried egg white
1970	examination of frozen, chilled, precooked, or prepared foods, **46.013–46.017,** modified for detection and enumeration of *Staphylococcus aureus* by substitution of Baird-Parker medium for Vogel-Johnson medium, **46.017**	examination of frozen, chilled, precooked, or prepared foods, **46.013–46.017,** adopted final action for enumeration of coliform group or *Escherichia coli* in tree nut meats, **46.016**
1971	commercial sterility of low acid canned foods, **46.018–46.025** thermophilic bacterial spores in sugars, **46.026–46.030**[c]	none
1972	*Salmonella* species, **46.054–46.067,** final action method revised to include tandem inoculation of triple sugar iron agar and lysine iron agar optical somatic cell counting. Method I, **46.086–46.094**	none
1973	estimation of *Clostridium perfringens*, alpha-toxin method, **46.046–46.053**	*Salmonella* species, **46.054–46.067,** revised to include examination of nonfat dry milk and dry whole milk; method also revised to include tandem inoculation of triple sugar iron agar and lysine iron agar
1974	*Salmonella* species, fluorescent antibody method, **46.068–46.071** surface plating method for isolation and enumeration of *Staphylococcus aureus* in food, **46.075–46.076**[h] virus in ground beef, **46.120–46.122**	*Staphylococcus aureus,* **46.017**[i,j]
1975	*Clostridium perfringens,* **46.031–46.036**[k] *Salmonella* species, **46.054–46.067,** final action method revised to extend first action status to examination of dried active yeast staphylococcal enterotoxin, microslide gel double diffusion test, **46.077–46.085**	none
1976	*Clostridium botulinum* and its toxins, **46.037–46.045** spiral plate method for bacterial count, **46.110–46.116**	*Salmonella* species, **46.054–46.067,** revised to include examination of dried active yeast *Salmonella* species, fluorescent antibody method, **46.068–46.071** staphylococcal enterotoxin, microslide gel double diffusion test, **46.077–46.085**
1977	*Clostridium perfringens,* **46.031–46.036,** first action method revised to include procedures for maintaining vegetative cells in viable condition during storage and transport of food specimens to the laboratory *Salmonella* species, presumptive generic identification of *Salmonella* species using commercial biochemical kits, **46.072–46.074** optical somatic cell counting, Method II, **46.095–46.104** optical somatic cell counting, Method III, **46.105–46.109** fecal coliforms, **46.117–46.119**	commercial sterility of low acid canned foods, **46.018–46.025**
1978	*Salmonella* species, **46.054–46.067,** final action method revised to extend first action status to examination of onion and garlic powder	*Clostridium perfringens,* **46.031–46.036**[l] *Clostridium botulinum* and its toxins, **46.037–46.045** estimation of *Clostridium perfringens,* alpha-toxin method, **46.046–46.053** optical somatic cell counting, Method III, **46.105–46.109** fecal coliforms, **46.117–46.119**

Table 3. Sequence of AOAC adoption of microbiological methods (continued)

Year(s)[a]	Official AOAC status: First action	Official AOAC status: Final action
1979	somatic cell counting, membrane-filter deoxyribonucleic acid (MF-DNA) method, **46.A01–46.A04** extraction and separation of staphylococcal enterotoxins in foods, **46.A05–46.A09** enumeration and confirmation of *Bacillus cereus* in foods, **46.A10–46.A15**	*Salmonella* species, **46.054–46.067,** final action revised to include examination of onion and garlic powder optical somatic cell counting, Method I, **46.086–46.094** optical somatic cell counting, Method II, **46.095–46.104**
1980	*Salmonella* species, **46.054–46.067,** revised to extend first action status to examination of edible casein and milk chocolate; method also revised to delete the combination of brilliant green, *Salmonella-Shigella,* and bismuth sulfite agars, and replace them by a combination of bismuth sulfite, xylose lysine desoxycholate, and Hektoen enteric agars	*Clostridium perfringens,* **46.031–46.036,** procedures for maintaining vegetative cells in viable condition during storage and transport of food specimens to the laboratory *Salmonella* species, presumptive generic identification of *Salmonella* species using commercial biochemical kits, **46.072–46.074** spiral plate method for bacterial count, **46.110–46.116** somatic cell counting, membrane-filter deoxyribonucleic acid (MF-DNA) method, **46.A01–46.A04** extraction and separation of staphylococcal enterotoxin in foods, **46.A05–46.A09** enumeration and confirmation of *Bacillus cereus* in foods, **46.A10–46.A15**
1981	*Escherichia coli,* detection of invasiveness, **46.C01–46.C10**	*Salmonella* species, **46.054–46.067,** final action revised to include examination of edible casein and milk chocolate; method also revised to delete the combination of brilliant green, *Salmonella-Shigella,* and bismuth sulfite agars, and replace them by a combination of bismuth sulfite, xylose lysine desoxycholate, and Hektoen enteric agars
1982	differentiation of members of *Bacillus cereus* group, **46.D01–46.D03**[m] enumeration of coliforms in selected foods, hydrophobic grid membrane filter method, **46.D04–46.D08**	none
1983	*Salmonella* species, **46.054–46.067,** revised to incorporate a modification for the examination of nonfat dry milk bacterial contamination in low-acid canned foods, helium leak test, 1983 meeting genetic methods for detection of bacterial pathogens, detection of *Escherichia coli* producing heat-labile enterotoxin, DNA colony hybridization method, 1983 meeting total coliforms, fecal coliforms, and *Escherichia coli* in foods, hydrophobic grid membrane filter method, 1983 meeting *Escherichia coli* enterotoxins, mouse adrenal cell and suckling mouse assays, 1983 meeting	surface plating method for isolation and enumeration of *Staphylococcus aureus* in food, **46.075–46.076** differentiation of members of *Bacillus cereus* group, **46.D01–46.D03**

[a]Year of annual AOAC meeting.
[b]Microbiological method for frozen eggs, adopted official first action in 1941, was expanded to include tentative method status for liquid and dried eggs in 1944.
[c]First action method deleted in 1953 and reinstated in 1971.
[d]First action method deleted in 1953.
[e]Tests for incidence of hemolytic staphylococci and streptococci, **46.010,** and tests for fungi, **46.011,** adopted as procedures.
[f]Methods for sampling, sample preparation, culture media preparation, plate count determination, incidence of coliform group, and direct microscopic count adopted as final action.
[g]First action method subsequently repealed.
[h]Applicable for general purpose use in testing foods expected to contain ≥ 10 cells of *Staphylococcus aureus*/g.
[i]Applicable to detection of small numbers of *Staphylococcus aureus* cells in raw food ingredients and non-processed foods expected to contain large population of competing species.
[j]Adopted as first action method in 1965 and subsequently modified in 1967, 1968, and 1970 as part of the method for the examination of frozen, chilled, precooked, or prepared foods, **46.013–46.017.**
[k]Replaced existing first action method, adopted 1968, which was repealed.
[l]Exclusive of procedures for maintaining vegetative cells in viable condition during storage and transport of food specimens to the laboratory.
[m]Provides supplementary procedures for the identification of *Bacillus cereus* isolated from foods by method **46.A10–46.A15.**

agar used as a direct plating medium (34). Results from that study demonstrated that the surface plating method gave a more accurate estimate of the level of *Staphylococcus aureus* than did the tube enrichment method when the population level was ≥ 20 *Staphylococcus aureus* cells/g. Moreover, such a plating method was preferable to the tube enrichment method for examining processed foods containing stressed cells. This surface plating method for *Staphylococcus aureus,* **46.075–46.076,** was adopted official final action at the 1983 meeting.

Determination of APC and Coliform MPN Values by Two AOAC Methods

There is an official final action method for the determination of plate counts, coliforms, and *E. coli* in eggs and egg products, **46.003–46.009,** and yet a different method (official first action) for determining aerobic plate counts, coliforms, and *E. coli* in frozen, chilled, precooked, or prepared foods, **46.013–46.016.** These 2 methods differ with respect to 4 analytical operations. First, method **46.003–46.009** directs that frozen egg samples be thawed as rapidly as possible (≤ 45°C for ≤ 15 min), whereas method **46.013–46.016** specifies the use of unthawed (if frozen) samples. Although sample handling would be facilitated by thawing, there is a possibility that the population of nonthermally tolerant organisms would be reduced at the elevated temperature recommended for thawing. Moreover, there is no reason not to consider the liquid portion of a frozen food sample as being a valid part of that sample, provided that proportionate amounts of the liquid and solid portions are maintained in the actual sample to be analyzed.

A second difference between the 2 methods is the amount of sample analyzed. Because microorganisms are not uniformly distributed in a given sample, it follows that the larger the sample analyzed, the more representative that sample is

of the lot from which it was taken. The larger sample size specified in method **46.013–46.016** (50 g) probably gives a more accurate estimate of the true level of organisms in a sample than does method **46.003–46.009** (11 g).

The third and perhaps the most significant difference between the 2 methods is in the manner of sample preparation. According to method **46.003–46.009,** 11 g sample, 99 mL diluent, and 1 tablespoon of glass shot are shaken 25 times in a 30 cm arc within 7 s in a screw-cap bottle. Even though these directions appear precise, there is a potential for variability between laboratories and even among different analysts in the same laboratory. In method **46.013–46.016,** a 50 g sample is blended with 450 mL diluent in a "high-speed blender" jar. AOAC (11) defines a "high-speed blender" as a ". . . mixer with 4 canted, sharp-edge, stainless steel blades rotating at the bottom of 4-lobe jar at 10 000–12 000 rpm, or with equivalent shearing action. Suspended solids are reduced to fine pulp by action of blades and by lobular container, which swirls suspended solids into blades. Waring blender, or equivalent, meets these requirements." Even with this definition, the term "high-speed blender" and its definition have led to much confusion among food microbiologists. Although a speed of 10 000–12 000 rpm is specified, it is not clear if this specification applies to an empty jar or to a jar with sample and diluent. Moreover, the term "high-speed blender" implies that the sample should be blended at high speed, which may not be the intent of the method. The situation is further exacerbated when one considers that some blender models operate at "high speed" and "low speed" only, and either of these designations can vary considerably from model to model. Clearly this area needs further investigation to provide specific instructions for preparation of food samples for microbiological analysis.

The fourth difference between the 2 methods is the temperature at which the inoculated petri dishes of plate count agar are incubated. Method **46.003–46.009** recommends 3 days at 32°C, whereas method **46.013–46.016** recommends 48 ± 2 h at 35°C. The selection of 32°C for method **46.003–46.009** was probably based on the use of this temperature for the microbiological analysis of milk, because it has been shown that the greatest counts in milk were obtained when plates were incubated at 32°C (35, 36). However, a temperature between 30° and 32°C is considered the maximal growth range for many psychrotrophic microorganisms (37, 38). Mesophiles, however, can grow between 10° and 37°C (39). Thus, it follows that within the mesophilic range, the number of colonies will decrease rapidly as the temperature of incubation increases from 30° to 37°C.

Spiral Plate Count

Because the methods for determining APC values and MPNs of coliforms and *E. coli* have frequently been criticized as laborious and lengthy by some analysts. it was inevitable that labor- and time-saving methods for making these same determinations would be developed. One such method is the spiral plate method (40) for determining APC values. This method, **46.110–46.116,** uses an apparatus which progressively deposits a decreasing amount of sample in the form of an Archimedes spiral on the surface of a plate of agar as the stylus moves from the center to the edge of a plate. Thus, there is an exponential relationship between the radius of the agar and the volume deposited. The plate is incubated and colonies appear on the line of the spiral. The number of colonies is counted per unit length of line or per unit area of agar surface, and this number depends on the bacterial concentration in the deposited volume. This method was collaboratively studied (41) and received official first action status at the 1976 meeting (42) and official final action status at the 1980 meeting (43). The spiral plate method offers a number of advantages over the pour plate method. Much less time is needed for preparing a spiral plate (2 min) than for pour plates (10 min). Moreover, the growth rate of organisms of the same species is the same, resulting in more uniform, easily countable colonies. Another advantage is that the stylus stops before reaching the meniscus of the agar, resulting in the formation of colonies only on the level portion of the agar surface.

Rapid Methods for Coliforms

Rapid enumeration of total coliforms, fecal coliforms, and *E. coli* in foods by using a filtration method has been approved by AOAC. With the conventional MPN tube methods, **46.009** and **46.016,** determination of total coliforms (4 days), fecal coliforms (4 days), and *E. coli* (10 days) takes considerably longer than the hydrophobic grid membrane filter (HGMF) method (44), which provides these counts the day after analyses are started. The HGMF method, **46.D04–46.D08,** uses a membrane filter impregnated with hydrophobic material in a grid pattern. This hydrophobic material acts as a barrier, preventing the spread of "growth units" from one compartment to another. After filtration and incubation, the number of occupied squares is counted and converted to an MPN value by a formula specific for this method. It has been suggested (44) that this hydrophobic barrier is the reason why the HGMF method is more accurate than methods relying on the enumeration of colonies by plating, where a confluence or overlap of colonies could reduce the accuracy considerably. Further, the HGMF method avoids the poor precision and repeatability known to be inherent in the MPN procedure (45). Other advantages of the HGMF method include a resuscitation step for fecal coliforms and *E. coli,* and filtration to remove substances that may inhibit the organisms being enumerated. The use of this method for the enumeration of total coliforms, fecal coliforms, and *E. coli* in foods was adopted official first action at the 1983 meeting (46).

Another method, **46.117–46.119,** adopted official final action in 1978 (47) for the rapid enumeration of fecal coliforms, involves the inoculation of an MPN series of tubes containing a medium specific for this method. The inoculated tubes are resuscitated for 3 h at 35°C and then incubated at 44.5°C for 21 ± 2 h. Gas formation in the inner fermentation vial is indicative of fecal coliforms. The use of this method is restricted to the examination of shellfish-growing waters, however, because of some preliminary evidence that its efficiency for the analysis of shellfish meats is variable.

Enteropathogenic Escherichia coli

The role of *E. coli* in initiating clinical symptoms in humans has been reviewed by Mehlman et al. (48). The term enteropathogenic *E. coli* was applied to those cultures which had ". . . been identified as *E. coli* on the basis of adequate biochemical, physiological, and morphological criteria; possess somatic (O), capsular (K or B), and flagellar (H) antigens of biotypes frequently associated with human enteric illness. . .; and elicit a specific, consistently positive response in 2 or more standardized model pathogenicity systems of known sensitivity, physiological significance, and human equivalence." These 3 systems demonstrate *(1)* invasiveness, *(2)* production of heat labile toxin (LT), and *(3)* production of heat stable toxin (ST).

Invasiveness of Escherichia coli

At the 1981 meeting, a method to detect invasiveness of *E. coli*, **46.C01–46.C10,** was adopted official first action (49). A monolayer of HeLa cells is grown in tissue culture and is subsequently infected with the inoculum of bacteria. Nonpathogenic types are digested but pathogenic types grow intracellularly in the cytoplasm. The criterion for infection is ≥ 5 bacteria per cell, and invasiveness is defined as an infection of ≥ 1% of the HeLa cells.

Toxins of Escherichia coli

A method for detecting LT of *E. coli* was adopted official first action at the 1983 meeting (46). Rather than detecting the toxin itself, the method detects the genes encoding the toxin. These genes are specific fragments of deoxyribonucleic acid (DNA) produced by enzymatic cleavage of plasmid DNA. Such fragments are separated by gel electrophoresis, purified, radioactively labeled, and used as hybridization probes to detect homologous DNA sequences in *E. coli* colonies grown and lysed on nitrocellulose filters. This technique is simple, practical, and relatively inexpensive, with up to 50 colonies being assayed on a single 9 cm filter (50). A major disadvantage, however, is the necessity of using a radioactively labeled probe with a half-life of 14 days. Preparation of new probes is both inconvenient and time-consuming.

A second method for detection of LT of *E. coli* uses a Y1 mouse adrenal cell line. When this cell line is infected with LT-producing *E. coli*, the tissue culture cells react by a change in morphology from flat to round, a response mediated by the activation of adenyl cyclase. A score of more than 50% rounding is regarded as positive for LT. This method was adopted official first action at the 1983 meeting (46). This method and the bioassay method below will be printed with the collaborative study (*J. Assoc. Off. Anal. Chem.* (1984) **67,** September issue).

The suckling mouse is used in the bioassay model for determining the presence of ST. For each culture filtrate to be assayed, the milk-filled stomachs of 4 suckling mice are injected. After 3 h, the mice are sacrificed, and the ratio of the weight of pooled intestines to the weight of the pooled carcass remainders is determined. A ratio of ≥ 0.083 is considered positive and a ratio of ≤ 0.074 is reported as negative for ST. Intervening values indicate that samples should be re-examined. This method also was adopted official first action at the 1983 meeting (46).

The correlation between pathogenicity of an *E. coli* culture and demonstration of any one of the 3 pathogenicity factors above (invasiveness, LT production, and ST production) is not absolute. This phenomenon is the basis for the recommendation of Mehlman et al. (48), noted earlier, that 2, preferably 3, of the pathogenicity systems be used before making any final decision as to the pathogenicity of the particular culture under investigation.

Staphylococcal Enterotoxin

Even though the presence of relatively high numbers of staphylococci cells is cause for concern, demonstration of preformed staphylococcal enterotoxin signals a direct health hazard. The microslide gel double diffusion test, **46.077–46.085** (51), provides for specific identification of enterotoxin-producing cultures by observation of lines of coalescence between the unknown enterotoxin-antibody complex and the known reference enterotoxin-antibody complex. In addition to its high sensitivity (able to detect 0.01–0.1 μg enterotoxin per mL of culture fluid or concentrated food extract), this method is technically simple, requires small amounts of reagents, and is highly specific. It was adopted official final action at the 1976 meeting (42).

Because staphylococci cells may be killed during processing of foods, it may be necessary to determine the presence of staphylococcal enterotoxin in the food itself rather than merely identify the enterotoxin-producing culture as described above. Casman and Bennett (52) believed that food poisoning outbreaks are usually due to very small amounts of enterotoxin. Assuming a direct correlation between growth and enterotoxin production, they estimated that in separate food poisoning outbreaks involving 2 vehicles, each containing 50 million to 200 million staphylococci cells/g, there was about 0.01–0.04 μg toxin/g food. Three steps are required for extracting an adequate amount of enterotoxin to be tested in the microslide gel double diffusion test: *(1)* separation of enterotoxin from insoluble food constituents, *(2)* separation of enterotoxin from soluble extracting reagents, and *(3)* concentration of the extracted and separated enterotoxin. Such a method for the extraction and separation of staphylococcal enterotoxins, **46.A05–46.A09,** was collaboratively studied (53) and adopted official final action in 1980 (43).

Salmonella Species

A method for the detection of *Salmonella* species in dried whole egg, dried egg yolk, and dried egg white, **46.054–46.067,** was adopted official first action by the Association at the 1966 meeting (54). This method was essentially a modification of the method, proposed earlier by North (55), which has been used by FDA since 1961 to examine foods for *Salmonella* species. Since it was adopted official final action in 1969 (56), this method has been expanded to include modifications specific for the examination of other foods: nonfat dry milk and dry whole milk (adopted final action in 1973) (57); dried active yeast (1976) (42); onion and garlic powders (1979) (58); edible casein and milk chocolate (1981) (49). A major change in the final action method for nonfat dry milk, involving the manner of sample rehydration, was made at the 1983 meeting (46), and the method for the examination of this product has reverted to official first action status. In general, all these methods are capable of detecting *Salmonella* species when the contamination level is as low as 1 *Salmonella* species cell in a 25 g analytical unit. Other modifications in the *Salmonella* species method were the inclusion of lysine iron agar in the analytical protocol (57) and the substitution of a combination of bismuth sulfite, Hektoen enteric, and xylose lysine desoxycholate agars for a combination of bismuth sulfite, brilliant green, and *Salmonella-Shigella* agars (49).

One of the most frequent criticisms of the *Salmonella* species method has been the length of time needed to complete the analysis (5 days for a negative result and approximately 10–11 days to confirm a positive result). Specifically, the fifth day marks the observation of streaked BS plates after an incubation period of 48 h. Realistically and practically, however, an experienced analyst has a reliable indication that the sample is negative at the end of 4 days. Moreover, it has been this author's experience that it is highly unusual for a *Salmonella* species colony to appear on a plate of BS agar only after an incubation period of 48 h and not at 24 h (other selective agars are read after a 24 h incubation period only).

The fluorescent antibody (FA) technique, **46.068–46.071** (59), proposed as a rapid screening method for detecting *Salmonella* species, was adopted official final action by the Association in 1976 (42). With this method, a fluorescein dye is coupled with *Salmonella* species antiserum. When the coupled product is allowed to react with *Salmonella* species

antigen, a complex is formed which is visible when viewed through a fluorescent microscope. The appearance of short to medium rod-shaped cells with a fluorescence of ≥ 2 on a scale of 1 to 4 is a presumptive positive reaction. A negative result can be determined with the FA method 3 days after initiation of analysis, whereas a presumptive positive result must be confirmed by **46.057–46.067** (11). In addition to reducing analytical time, the FA method has the potential for being automated. Disadvantages of the method included the need to purchase expensive equipment, need for specialized training, and the occurrence of false positive reactions due to common antigens shared among closely related enteric bacteria.

Another scheme to reduce the analytical time for *Salmonella* species detection involves the use of commercial biochemical kits for the rapid presumptive generic identification of *Salmonella* species. Obtaining biochemical results by the conventional tube system, **46.060–46.063,** may require up to 4 days, but results with these kits are generally available 24 h after inoculation. The kits may consist of a series of microtubes containing test substrate affixed to a plastic strip (API) (Analytab Products, Inc., Plainview, New York); a self-contained compartmental plastic tube containing various substrates (Enterotube) (Roche Diagnostics, Nutley, NJ); or a series of paper disks impregnated with various chemical substrates (Minitek) (Bioquest, BBL, Cockeysville, MD). In addition to saving time, these kits have other advantages: easy to inoculate (Enterotube), easy to read (API and Minitek), versatile (Minitek), and substantial data base (API). Disadvantages include: difficult and time-consuming to inoculate (API and Minitek), limited shelf life (Enterotube), and expensive (Minitek). Use of these kits according to section **46.072–46.074** was approved official final action at the 1980 meeting (43).

Methods for Examination of Canned Foods

Early Methods

Sugars used in the canning industry can become contaminated with thermophilic bacteria. These bacteria are unusual in having an optimal growth temperature between 50 and 60°C. Both beet and cane sugar can become contaminated with spores of 3 types of thermophilic bacteria that may act as spoilage agents in low acid canned foods (finished equilibrium pH value > 4.6) which may have been understerilized: flat sour facultative anaerobes *(Bacillus stearothermophilus)*, anaerobes not producing hydrogen sulfide *(Clostridium thermosaccharolyticum)*, and sulfide spoilage bacteria or anaerobes producing hydrogen sulfide *(Clostridium nigrificans)*. In his Associate Referee Report at the 1935 meeting, Cameron (60) concluded that "no immediate significance is attached to the presence of 'non-spoilage' thermophiles, i.e., aerobic spore-formers, actinomycetes, etc." Subsequent to that report, however, the National Canners Association Laboratory investigated an outbreak of spoilage due to aerobic spore-formers of the *Bacillus subtilis* group (61). In reporting the results of a collaborative study of a method for the detection and enumeration of thermophilic bacteria in sugar at the 1939 meeting, Cameron (61) modified his earlier statement in reference to the significance of "non-spoilage" thermophiles. The method was adopted as tentative at the 1939 meeting (15) and as first action at the 1941 meeting (17). At the 1953 meeting, the General Referee noted that this method was used primarily to determine compliance with commercial specifications rather than for regulatory purposes (8). Accordingly, Subcommittee C accepted the General Referee's recommendation that the method be dropped (10). Seventeen years later, however, at the 1970 meeting, the General Referee proposed that the need for a microbiological method for sugars should be re-evaluated (12), and at the 1971 meeting Subcommittee F accepted the General Referee's recommendation to reinstate the method for sugars, **46.026–46.030,** which had been deleted earlier by Subcommittee C (62). This method has remained in first action status since that time with no major changes.

Table 3 lists the first action adoption of 3 methods at the 1941 meeting. The third of these methods, examination of canned vegetables, has not yet been discussed. Actually, this method was relatively short-lived since the General Referee's Report (8) at the 1953 meeting noted that the "microbiological examination of canned foods consists primarily of qualitative procedures designed to detect the presence of specific spoilage organisms, and thus has little forensic application." Accordingly, the official first action method for canned vegetables was discontinued that year (63).

Sterility Testing of Canned Foods

A microbiological method (64) was developed for analyzing low acid canned foods for sterility, in which potential for contamination by the analyst was minimized. Commercial sterility was defined ". . . as that condition achieved by application of heat which renders food free of viable forms of microorganisms having public health significance, as well as microorganisms not of health significance, capable of reproducing in the food under normal nonrefrigerated conditions of storage and distribution." Because hair on an individual greatly increases the surface area for harboring bacteria, analysts with beards, mustaches, or sideburns below the ear lobes should not perform such analyses unless these areas are completely covered with sterile caps and masks. According to method **46.018–46.025,** the analyst must initially wash the hands with soap and water, rewash hands with a detergent sanitizer solution, and finally wear a disposable operating room cap to cover the head completely. Such precautionary measures were adopted official final action at the 1977 meeting (65).

Helium Leak Test

Leakage after processing is the most prevalent cause of bacterial contamination of low acid canned food (J. E. Gilchrist, personal communication). Bacteria have little difficulty passing through holes with a diameter of 5 μm or greater. Of the 4 tests (vacuum leak, Mead jar, air pressure, and fluorescein dye) listed in the *Bacteriological Analytical Manual* (BAM) (66) to detect leaks in canned foods, the vacuum leak test is the one most frequently used in FDA laboratories. This test, however, has difficulty detecting holes smaller than 15 μm (J. E. Gilchrist, personal communication). Another method for detecting leaks in canned foods is the helium leak test. With this test the can is emptied, resealed, and placed in a pressurized tank. After exposure to helium gas, the can is pierced and a sample of the gas is collected for analysis by gas chromatography. Because of its very low molecular weight, helium gas can penetrate holes as small as 1 μm. In a collaborative study (67) comparing the efficiency of the vacuum leak test and the helium leak test, the latter test detected more leaks, and interpretation of results was less subjective. Another advantage is that the helium leak does not totally destroy the can so that it is possible to perform additional tests for can tightness. The helium leak test was adopted official first action at the 1983 meeting (46).

Other Methods

Clostridium botulinum

Because ingested *Clostridium botulinum* cells are incapable of multiplying and producing toxin *in vivo*, detection of their presence is not considered as evidence that the food caused a botulism outbreak. Detection of the toxin itself, however, is irrefutable proof of the role of the food in causing illness. Of the 7 antigenic types of *C. botulinum* (designated A, B, C, D, E, F, and G), most outbreaks in this country have involved types A, B, and E. The presence of toxin in food is determined by observing the death of mice after they have been injected intraperitoneally with a sample extract (68). The specific toxin(s) involved is identified by observing whether the mice are protected from death when injected with homologous antitoxin before injection with toxin. This mouse toxicity and protection method, **46.037–46.045,** adopted official final action at the 1978 meeting (47), is simple, specific, and widely used. Two precautions should be noted, however, in using this method. First, there is a slight degree of cross-neutralization between types E and F, so that it is possible for antitoxin E to neutralize toxin F and vice versa. Second, in observing death in mice, care must be taken to assure that death is caused by injection of botulism toxin and not by some other factor, e.g., high concentration of salt or presence of protein metabolic product indigenous to the food sample.

Clostridium perfringens

A method for the enumeration of *Clostridium perfringens* in food reported by Angelotti et al. (69) involved plating a series of dilutions onto sulfite-polymyxin-sulfadiazine agar. It was shown that the method was capable of recovering as few as 10 *C. perfringens* cells/g food. This agar, however, had the distinct disadvantage of supporting growth and formation of suspect colonies, not just of *C. perfringens*, but also of other sulfite-reducing clostridia. Nevertheless, this method appeared to be the best at the time for the quantitation of *C. perfringens* in food. Moreover, because of increasing evidence of the incrimination of *C. perfringens* in causing foodborne illness in this country, this method was adopted official first action at the 1968 meeting (32).

Seven years later the method was repealed in favor of the first action adoption of another method (70) at the 1975 meeting (71). This later method, **46.031–46.036,** uses tryptose–sulfite–cycloserine agar, which is regarded as the most useful of all the media for the quantitative recovery of *C. perfringens* (72). Although this agar adequately suppresses the growth of practically all facultative anaerobes, other anaerobic organisms may grow on this medium. Egg yolk is incorporated into the agar to measure the presence of lecithinase activity as evidenced by a halo or a zone of white precipitation around the suspect black *C. perfringens* colonies. It should be noted, however, that some strains do not necessarily produce this halo that is so characteristic of most other strains of *C. perfringens*. Moreover, some other sulfite-reducing clostridia, e.g., *C. bifermentans* and *C. sporogenes*, are capable of growing on this agar. It should be further noted that this method is applicable only for the examination of foods suspected of containing large numbers of vegetative cells.

It is often not possible to examine foods suspected of containing *C. perfringens* immediately after collection. It may be necessary to ship the samples over a long distance to the laboratory performing the actual analyses. Such shipment of samples under refrigeration or freezing conditions may result in a 3–4 log cycle reduction in the plate counts of this organism (73). In a collaborative study reported in 1977, however, Harmon and Placencia (74) demonstrated that samples treated with an equal amount by weight of buffered glycerol–sodium chloride solution and stored for 10 days with dry ice at −56°C gave plate counts of *C. perfringens* that were 2–4 log cycles higher than counts of untreated samples stored at −20°C. This method of maintaining viability of *C. perfringens* during shipment of samples was subsequently incorporated into the method for the enumeration of *C. perfringens*, **46.031–46.036,** which was adopted official final action in 1978 (47).

Even when the sampling and shipping method of Harmon and Placencia (74) is followed, there will be instances in which low plate count values of *C. perfringens* are expected. In those instances, the *alpha*-toxin method, **46.046–46.053** (75), for the estimation of the original level of *C. perfringens* in the food sample may be used. *Alpha*-toxin, produced by *C. perfringens*, can be detected when there are about 10^6 *C. perfringens* cells/g food, and the amount of toxin increases in proportion to an increase of the cell population. It should be emphasized, however, that this method provides an estimate, rather than an actual enumeration, of the *C. perfringens* cells present. This method was adopted official final action at the 1978 meeting (47).

Bacillus cereus

Detection of the foodborne pathogen, *Bacillus cereus*, by the AOAC method involves choosing 1 of 2 alternatives, depending on the expected level of *B. cereus* in the food sample. Direct plating on mannitol–egg yolk–polymyxin (MYP) agar is used when the food sample is suspected of containing more than 10^3 *B. cereus* cells/g. The MPN technique, which relies on enrichment of serial 10-fold dilutions of the sample in tubes of trypticase soy-polymyxin broth followed by streaking on MYP agar, is recommended when the sample is suspected of containing fewer than 10^3 *B. cereus* cells/g. This method, however, is capable of detecting even fewer than 10 *B. cereus* cells/g (76). With both techniques, suspect *B. cereus* colonies on MYP agar are confirmed biochemically.

The MYP agar contains egg yolk that is acted on by the enzyme lecithinase of *B. cereus*, resulting in a zone of lysis surrounding the *B. cereus* colony. Because these zones frequently coalesce, the countable range of *B. cereus* colonies is 3–30 colonies rather than the customary 30–300 colonies. It is not known to what extent accuracy is reduced by counting colonies in this range. Another problem is that *B. cereus* sporulates poorly on this medium, necessitating a subsequent transfer to a sporulation-promoting medium so that identification can be made.

The method for the enumeration and confirmation of *B. cereus*, **46.A10–46.A15,** was adopted official final action at the 1980 meeting (43). It was later deemed necessary to make a distinction between *B. cereus* and 3 closely related biotypes also enumerated by this method, because the role of these other 3 biotypes in causing foodborne illness in humans was not known at the time method **46.A10–46.A15** was adopted (77). Accordingly, a method to distinguish these 4 biotypes, **46.D01–46.D03,** was collaboratively studied (77) and adopted official first action in 1982 (78) and official final action in 1983 (46).

Viruses

Viruses in food are detected by determining the cytotoxic effects of a fluid extract of the sample on a tissue culture system. Separation of food particles from the viral particles is an essential part of obtaining this liquid inoculum. Of the various methods proposed to effect this separation, the glass wool filtration method has been reported as the most effective

(79). One advantageous consequence of this separation is the possible reduction in the level of bacteria which may have otherwise survived the concentration of antibiotics used in the tissue culture growth medium. This method, **46.120–46.122,** was collaboratively studied (80) and adopted official first action at the 1974 meeting (33). It should be noted, however, that not all viruses will grow in the particular cell culture system used in that collaborative study and that virus concentrations of less than 1 plaque forming unit/g may not be detected.

Somatic Cell Counting of Milk

The term "somatic cells" applies to those body cells in milk generally associated with inflammation of the cow's udder (81). The numerical relation between these somatic cells and the pathological condition of the animal is not indisputable. Presentation of such arguments, however, is beyond the scope of this paper and shall not be discussed here. The enumeration of these somatic cells by the direct microscopic somatic cell count (DMSCC) is based on spreading a measured drop (0.01 mL) of sample over a 1 sq. cm area on a glass slide. The film is fixed by drying, stained, and examined microscopically. The use of such very small volumes can be a source of considerable error. Other disadvantages include the tediousness of slide preparation and eye strain, both inherent to this technique.

The disadvantages of the DMSCC method led to the development of a number of fully automated electronic methods of somatic cell counting. Three of these methods have been subjected to an AOAC collaborative study: Method I, **46.086–46.094** (82); Method II, **46.095–46.104** (83); and Method III, **46.105–46.109** (84). Methods I and II both use an automated system (AutoAnalyzer, Technicon Corp., Tarrytown, NY) that automatically mixes milk samples with reagent matrix. This matrix dissolves fat particles, carbohydrates, and other extraneous materials, leaving somatic cells which are counted electronically as they pass through the stage of a dark field microscope. Method III uses an optical somatic cell counter (Fossomatic, Foss Electric Co., Hillerod, Denmark) that mixes milk samples with buffer and dye and transfers a portion of the mixture to a rotating disk which serves as an object plane for the microscope. When the somatic cell nuclei-dye complex is exposed to a xenon arc lamp, emitted fluorescent light is measured as electrical impulses. Methods I and II were adopted official final action at the 1979 meeting (58), whereas Method III was adopted official final action 1 year earlier at the 1978 meeting (85). In general, these electronic methods have been demonstrated to be as accurate as the DMSCC method, but the sophistication and expense of these machines may preclude their use in the smaller laboratories.

Somatic cell counting by the membrane-filter DNA method, **46.A01–46.A04** (58), may be especially suitable for the small laboratory because the equipment is simpler and less costly than that used in the electronic methods. After the milk sample is filtered, the membrane filter is heated in an acidic indole solution which lyses somatic cells. Indole reacts with cellular DNA to form a colored complex that is measured spectrophotometrically. Because the amount of cellular DNA is constant in individual somatic cells, the number of somatic cells in a particular milk sample can be determined on the basis of the amount of DNA that complexes with indole. One shortcoming of this method, however, is that 1 technician can analyze about 500 samples, whereas with Method II (80–120 samples/h) or Method III (180 samples/h), a larger sample load can be accommodated. This method was adopted official final action at the 1980 meeting (43).

Conclusion

After its establishment 50 years ago, we have seen the emergence of a General Refereeship marked by the productive validation of methods used for regulatory purposes. It would be an understatement of the full impact of these methods, however, to assume that AOAC methods are used exclusively for regulatory purposes, because these methods are internationally recognized and are used for such nonregulatory activities as research and quality control. What bestows such recognition on these methods is their submission to a collaborative study, the very essence of AOAC. It is only by subjecting a method to a collaborative study that the accuracy and reproducibility of that method can be substantiated.

The fact that the General Refereeship, Microbiological Methods, was subdivided at the 1982 meeting was a natural consequence of the rapid growth of scientific disciplines encompassed by that Refereeship. Such growth, however, is not without its problems, but this author is confident that these problems can be resolved and that the productivity of this Refereeship can be sustained.

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DRUGS

Liquid Chromatographic Determination of Prednisolone in Tablets and Bulk Drugs: Interlaboratory Study

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A normal phase liquid chromatographic (LC) method for the determination of prednisolone in tablets and bulk drugs was studied by 7 analysts. An LC system, consisting of a methanol–water–ethylene dichloride–acetic acid mobile phase and a silica column, was used to analyze bulk drugs, individual tablets, and composite samples. Analysts were supplied with 16 samples, including simulated formulations, composites of commercial tablets, intact tablets, and bulk drug substances. Results agreed with those obtained by the author. The coefficients of variation of the analysts' results ranged from 1.34% for bulk drugs to 2.14% for tablet composites. The LC method is suggested as an alternative to the official AOAC and USP XX blue tetrazolium colorimetric methods.

The official AOAC (1) and USP (2) methods for prednisolone use the blue tetrazolium reaction for both the assay and the content uniformity determination. This reaction, although sensitive and linear, is not specific and is subject to interferences by excipients (3–6).

The evaluation of existing methods and development of the liquid chromatographic (LC) method were part of the USP-Food and Drug Administration (FDA) Compendial Monograph Evaluation and Development Program.

The normal phase LC method described here is a modification of several published methods (7–12), and was selected for its ability to separate prednisolone from other closely related steroids, decomposition products, and precursors in the prednisolone synthesis.

Validation tests performed on the LC method showed that the system gave a linear response over a concentration range of 0.0125–0.20 mg prednisolone/mL and excellent reproducibility, as evidenced by a coefficient of variation (CV) of 0.15% for 10 replicate injections of prednisolone standard solution, using electronic integration of peak areas.

Recovery experiments on standard-spiked mixtures of excipients simulating 5 different manufacturers' prednisolone tablet formulations were performed by the LC method. A recovery of 100.0% and a CV of 1.10% were obtained for 25 determinations. The excellent recovery results obtained with the LC method indicated that there was little, if any, interference from common tablet excipients.

Three samples of prednisolone drug substances were analyzed by the LC and USP XX methods, which gave assay values (% by weight) of 99.2 and 99.5% and CV values of 0.79 and 1.52%, respectively, for 3 determinations by each method.

Comparable results were also obtained when representative samples of commercial prednisolone tablets were analyzed by the LC and USP XX methods (Table 1). This series of favorable results and comparisons justified the study described in this paper.

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METHOD

Principle

Prednisolone in bulk drugs and tablets is determined by normal phase LC, using a silica column and ultraviolet (UV) detection.

Apparatus

(a) *Liquid chromatograph.*—Equipped with Model 6000A solvent delivery system, Model 440 UV detector, and Model 730 data module (Waters Associates, Inc., Milford, MA 01757), or equivalent system. Operating conditions: flow rate 1.5 mL/min; 254 nm detector, 0.20 absorbance unit full scale; temperature ambient; 10–15 μL injection.

(b) *LC column.*—25 cm × 4.6 mm id column packed with 5–6 μm porous spherical particles (DuPont Instruments, Wilmington, DE 19898), or equivalent column that passes system suitability tests.

(c) *Filters.*—Polytetrafluoroethylene membrane filters Type FH and Type LS, pore sizes 0.5 and 5.0 μm, respectively (Millipore Corp., Bedford, MA 01730).

Reagents

(a) *Solvents.*—Distilled-in-glass (OmniSolv, EM Industries, Gibbstown, NJ 08027).

(b) *LC mobile phase.*—Mix 60 mL 95% methanol with 1.0 mL acetic acid and dilute to 1 L with water-washed ethylene dichloride. Filter through Type FH filter and degas 5 min.

(c) *Internal standard solution.*—1.0 mg/mL. Transfer 100 mg fluoxymesterone to 100 mL volumetric flask. Dissolve in 5 mL methanol and dilute to volume with methylene chloride.

(d) *Prednisolone standard solution.—(1) Stock solution.*—0.5 mg/mL. Accurately weigh ca 25 mg USP Reference Standard Prednisolone into 50 mL volumetric flask. Add 2 mL methanol and dilute to volume with methylene chloride. *(2) Working solution.*—0.05 mg/mL. Pipet 10.0 mL stock solution and 5.0 mL internal standard solution into 100 mL volumetric flask. Add 6 mL methanol and dilute to volume with methylene chloride.

Preparation of Samples

Bulk drugs.—Accurately weigh ca 50 mg sample (dried in vacuum at 105°C for 3 h) into 100 mL volumetric flask. Add 4 mL methanol and dilute to volume with methylene chloride. Pipet 10.0 mL sample solution and 5.0 mL internal standard solution into 100 mL volumetric flask. Add 6 mL methanol and dilute to volume with methylene chloride.

Tablets.—Determine average weight of ≥ 20 prednisolone tablets and grind to pass No. 60 sieve. Transfer accurately weighed portion of powder containing 5 mg prednisolone to 100 mL volumetric flask. Add 6 mL methanol and place flask in ultrasonic bath 2 min. Add ca 50 mL methylene chloride and return to ultrasonic bath 1 min. Add 5.0 mL internal standard solution and dilute to volume with methylene chlo-

Table 1. Assay of prednisolone tablet composites (% of declaration) by the LC and USP XX assay methods

	Composite strength, mg/tablet					
	5		1		5	
Statistic	LC	USP	LC	USP	LC	USP
Av., %[a]	93.6	94.2	94.0	95.4	97.6	99.0
SD	1.00	2.05	1.06	0.76	1.21	1.66
CV, %	1.07	2.18	1.13	0.80	1.24	1.68

[a]Four determinations for each method, % of declared.

Table 2. Interlaboratory results of determination of prednisolone (% by weight) in bulk drug substance by the LC method

	Sample					
Analyst	(1 and 6)		(2 and 5)		(3 and 4)	
1	98.3	98.3	99.6	99.6	99.7	99.7
2	100.8	99.7	100.4	101.5	100.3	101.0
3	101.3	98.8	103.8	102.3	98.1	97.2
4	98.4	97.8	97.9	99.1	98.6	98.3
5	99.3	98.6	99.6	100.5	99.4	99.5
6	99.4	100.7	99.5	102.0	100.7	102.1
7	100.6	100.9	99.2	99.0	99.2	99.1
Av.	99.5		100.3		99.5	
SD	1.17		1.59		1.27	
CV, %	1.18		1.58		1.28	

Table 3. Interlaboratory results of determination of prednisolone (% of label declaration) in tablet composites by the LC method

	Sample							
Analyst	(7 and 12)[a]		(8 and 11)[a]		(9 and 14)[b]		(10 and 13)[b]	
1	98.0	98.0	89.0	89.0	97.8	97.8	93.6	93.6
2	103.0	101.0	91.0	95.0	100.6	98.4	94.4	95.8
3	101.0	102.0	90.0	87.0	98.0	97.2	90.2	90.6
4	103.0	104.0	93.0	94.0	98.8	100.2	94.2	94.4
5	100.0	103.0	88.0	89.0	98.0	97.8	92.6	92.6
6	98.0	98.0	91.0	91.0	98.8	99.4	92.6	94.6
7	101.0	99.0	88.0	87.0	96.2	93.8	91.6	89.8
Av.	100.6		90.1		98.1		92.9	
SD	2.17		2.51		1.68		1.81	
CV, %	2.16		2.78		1.71		1.95	

[a]1 mg prednisolone/tablet.
[b]5 mg prednisolone/tablet.

ride. Shake flask vigorously and filter portion of solution through Type LS filter into 25 mL glass-stopper flask.

Individual tablet assay (content uniformity).—Place tablet in 125 mL glass-stopper flask and add 200 μL water. Let stand until tablet disintegrates and add 1 mL methanol for each mg prednisolone declared. Place flask in ultrasonic bath until tablet is dispersed. Add 1.0 mL internal standard solution for each mg prednisolone declared and dilute quantitatively with methylene chloride to ca 0.05 mg/mL. Shake flask vigorously and filter portion of solution through Type LS filter into 25 mL glass-stopper flask.

Determination

Let LC system equilibrate with 1.5 mL/min flow rate. Inject 10–15 μL prednisolone working standard solution. Retention times of fluoxymesterone and prednisolone should be ca 6 and 9 min, respectively, with an R value (13) ≥ 6. Inject 5 replicate aliquots of prednisolone working standard solution and calculate response ratios. CV will be $\leq 2.0\%$ in suitable systems. Proceed with sample analysis, injecting same amount of sample solution.

Calculations

Calculate results, using response ratios (A and A') relative to internal standard:

Bulk drugs

$$\% \text{ prednisolone} = (A/A') \times (C/S) \times 100$$

Tablets (composite)

$$\text{mg prednisolone/tablet} = (A/A') \times C \times (W/S) \times 100$$

Tablet (individual)

$$\text{mg prednisolone/tablet} = (A/A') \times C \times (T/D)$$

where A and A' are response ratios for sample and standard solutions, respectively; C is mg prednisolone/mL standard working solution; W is average tablet weight (g); S is sample weight (g); T is labeled quantity (mg) prednisolone in tablet; and D is concentration (mg/mL) of prednisolone in tablet solution, based on labeled quantity/tablet and extent of dilution.

Interlaboratory Study

Six analysts received 16 samples: 6 prednisolone bulk drugs as blind duplicates, 8 prednisolone tablet composites (2 synthetic and 6 commercial formulations) as blind duplicates, and 2 unground prednisolone tablet samples. Samples were chosen to be representative of commercial formulations.

Each analyst received instructions, the method, a vial of reference standard, and a reporting form. Analysts were given approximate sample weights for tablet composite assays and approximate dosages for individual tablet assays.

Results and Discussion

Analyst results (Tables 2–5) include means, percent recoveries or percent of label declarations, standard deviations (SD), and CV values for each sample. Dixon's test (14) was used to detect significant outliers; none were found in this study. The tabulated statistical summary is given in Table 6. The SD and CV for repeatability and reproducibility are acceptable for each of the products.

The author's results are listed as Analyst 1 in Tables 2–5.

Samples 7 and 12 (Table 3) were synthetic samples formulated to contain 1 mg prednisolone/tablet. The recovery of theoretical amount was 100.6%.

Sample 15 was from the same lot of tablets used to make ground composite Samples 9 and 14. The means of the results of the composite and individual tablet assays were 98.1 and 98.4%, respectively.

Sample 16 was from the same lot of tablets used to make ground composite Samples 8 and 11. The means of the results of the composite and individual tablet assays were 90.1 and 89.8%, respectively.

The analysts made the following comments:

One analyst reported that the resolution factor R (13) of ≥ 6 specified in the method could not be met with his column. The reported R values ranged from 5.32 to 10.0. Each analyst easily met the other system suitability test of $\leq 2\%$ reproducibility of 5 replicate injections. The reported results ranged from 0.12 to 1.21%.

Analysts used 3 manufacturers' columns in conducting the interlaboratory study: Spherisorb-Si (Altex), Zorbax-Sil (DuPont), and μ-Porasil (Waters Associates).

Analysts suggested some changes in the text of the method concerning sample preparation and calculation formulas.

The favorable results obtained by the interlaboratory study suggest that the LC method could be used as an alternative to the official AOAC and USP XX colorimetric methods.

Table 4. Interlaboratory results of content uniformity analysis (% of label declaration) of 5 mg prednisolone tablets (Sample 15)

	Tablet												
Analyst	1	2	3	4	5	6	7	8	9	10	Av.	SD	CV
1	95.4	94.8	98.6	97.4	97.4	96.8	100.0	98.6	97.4	98.8	97.5	1.584	1.62
2	95.8	97.4	99.0	98.8	96.2	97.6	97.0	95.6	96.6	95.2	97.0	1.296	1.34
3	101.6	100.6	102.8	93.8	96.8	101.2	97.6	102.4	97.4	120.6[a]	99.4	3.070	3.09
4	102.2	98.0	96.4	98.4	99.0	97.2	94.8	95.0	97.8	101.8	98.0	2.488	2.54
5	97.6	98.2	95.6	97.6	95.8	97.6	96.4	96.0	97.0	97.2	97.0	0.896	0.92
6	106.0	102.4	102.2	102.0	103.6	101.0	100.6	100.6	100.6	102.2	102.2	1.684	1.65
7	97.4	96.8	99.2	98.8	97.0	98.0	98.2	95.2	99.0	97.8	97.8	1.208	1.23
									Overall		98.4	2.484	2.53

[a]Not included in tabulation; internal standard was not added to sample.

Table 5. Interlaboratory results of content uniformity analysis (% of label declaration) of 1 mg prednisolone tablets (Sample 16)

	Tablet												
Analyst	1	2	3	4	5	6	7	8	9	10	Av.	SD	CV
1	87.0	89.3	90.4	87.4	90.2	92.0	90.1	91.5	91.2	90.5	90.0	1.646	1.83
2	93.4	91.3	89.2	87.4	83.6	86.0	89.0	85.2	88.6	88.6	88.2	2.872	3.25
3	91.5	97.3	91.6	90.8	90.2	91.0	95.7	94.2	92.9	91.5	92.7	2.340	2.52
4	89.5	90.2	92.9	89.3	90.6	94.7	91.8	90.3	87.8	91.7	90.9	1.971	2.17
5	89.0	90.0	95.0	95.0	96.0	89.0	91.0	92.0	90.0	90.0	91.7	2.669	2.91
6	80.8	81.6	81.6	84.8	85.6	87.2	98.4	84.8	86.4	90.4	86.2	5.199	6.03
7	90.3	87.6	89.9	90.2	90.7	88.0	87.6	88.5	89.5	87.8	89.0	1.233	1.38
									Overall		89.8	3.403	3.79

Table 6. Statistical analysis of the combined interlaboratory results

Statistic	Bulk drugs	Tablet composites	Indiv. tablets
Pooled mean, %[a]	99.8	95.4	94.1
S_1[b]	0.61	1.11	2.43
S_2[c]	1.34	2.04	3.07
CV1, %[b]	0.61	1.16	2.58
CV2, %[c]	1.34	2.14	3.26

[a]Percent of label declaration.
[b]S_1 and CV1 are the repeatability (within-laboratory error) and relative repeatability (100 S_1/mean) of the method.
[c]S_2 and CV2 are the reproducibility (between- and within-laboratory error) and relative reproducibility (100 S_2/mean) of the method.

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Semiautomated Determination of Tridihexethyl Chloride in Meprobamate Tablets

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A semiautomated method has been developed for the analysis of tablets containing tridihexethyl chloride in combination with meprobamate. The method is based on the USP assay for tridihexethyl chloride in tablets. The active ingredient is dissolved in water, the aqueous solution is mixed with buffer and dye solution, and the dye complex is extracted into chloroform. The absorbance of the chloroform solution is monitored at 408 nm. Results from the semiautomated method agree with results for the USP method. Recoveries from authentic formulations show no interference from the excipients tested.

A nationwide survey of meprobamate formulations was conducted during 1981 to ensure compliance with content uniformity, potency, and dissolution requirements. The survey included tridihexethyl chloride in combination with meprobamate. This combination can be used for treatment of peptic ulcer and irritable bowel syndrome (spastic colon, irritable colon, and mucous colitis).

Tridihexethyl chloride is one of a family of synthetic anticholinergics that include biperiden, cycrimine, trihexyphenidyl, and procyclidine. Gas chromatography (GC) has been used for analysis of formulations containing trihexyphenidyl hydrochloride (1–4), and for procyclidine, cycrimine, and biperiden formulations (4). However, a GC method has not been developed for the determination of tridihexethyl chloride.

The USP assay for tridihexethyl chloride in tablets (5) includes reaction of the drug with bromocresol purple to form a colored complex, extraction of the complex with chloroform, and colorimetric measurement at 408 nm. In the third supplement to USP XX, the assay for trihexyphenidyl HCl is a GC method (6). USP XX assays for biperiden, cycrimine, trihexyphenidyl, and procyclidine as the hydrochlorides use essentially the same dye complex-colorimetric procedure, which is time consuming and awkward when applied to content uniformity testing of tridihexethyl chloride in meprobamate tablets. Therefore, we automated the procedure, and present the method here.

This semiautomated method may be applicable to the other drugs in the same family as tridihexethyl chloride, i.e., the hydrochlorides of biperiden, cycrimine, procyclidine, and trihexyphenidyl in tablet form.

METHOD

Apparatus

(a) *Automated analyzer.*—AutoAnalyzer with following modules: Sampler II with cam or timing clock set at 30/h and sample-to-wash ratio of 2:1, proportioning pump 1, manifold (Technicon Instruments Corp., 511 Benedict Ave, Tarrytown, NY 10591) (Figure 1).

(b) *Spectrophotometer.*—Zeiss Model PM2DL (Carl Zeiss, Inc., 444 Fifth Ave, New York, NY 10018), or equivalent, fitted with Helma 10 mm, 180 μL flowcell (Beckman Instruments Inc., Fullerton, CA 92634).

(c) *Recorder.*—Servo/Riter II (Texas Instruments Inc., PO Box 1444, Houston, TX 77001), or equivalent.

(d) *Ultrasonic generator.*—Model II, 150 watt (Heat Systems-Ultrasonics, Inc., 38 E. Mall, Plainview, NY 11803), or equivalent.

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Reagents

(a) *Phosphate buffer (pH 5.3).*—Dissolve 38.0 g monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and 2.0 g anhydrous dibasic sodium phosphate (Na_2HPO_4) in water and dilute to 1 L.

(b) *Dye solution.*—Dissolve 400 mg bromocresol purple in 30 mL water and 6.4 mL 0.1N sodium hydroxide; dilute to 500 mL with water.

(c) *Chloroform.*—ACS reagent grade.

(d) *Tridihexethyl chloride standard solution.*—Accurately weigh ca 100 mg tridihexethyl chloride of known purity and transfer to 100 mL volumetric flask. Dissolve drug in water and dilute to volume. Transfer 25.0 mL aliquot to 250 mL volumetric flask and dilute to volume. Stock solution is stable at least 5 days.

Preparation of Sample

Place individual tablets or portion of ground composite in separate 250 mL volumetric flasks. Add ca 125 mL water to each flask and place in ultrasonic generator for 15–60 min or until tablet material is finely dispersed. Dilute contents of each flask to volume with water and mix. Let contents of each flask settle overnight or centrifuge portion of each. Use portion of clear liquid in each sample flask for analysis.

Determination

Arrange manifold according to Figure 1. Sample solutions from 3 mL polystyrene cups with Sampler II (sample rate 30/h; sample-to-wash ratio 2:1). Use sampling pattern of 3 standards, 5 samples, 1 standard, 5 samples, 1 standard, etc. Discard first 2 standard peaks. To start up system, pump alcohol through chloroform line for ca 3 min, followed by chloroform. After chloroform has filled all proper lines and flowcell, place all remaining lines in their respective reagents and let system establish steady baseline on recorder. To shut down system, remove all aqueous lines and pump until only chloroform is pumping through system. Remove chloroform line and pump alcohol through chloroform line for 5 min. Remove alcohol line and pump dry.

Draw tangent line to initial and final baselines. Subtract baseline to determine net absorbance values A and A' at 408 nm for each sample and standard peak, respectively. Discard values for first 2 and last standard peaks. Average all remaining standard peaks or use standards flanking sample pattern.

Calculate tridihexethyl chloride as follows:

$$\text{mg tridihexethyl chloride/tablet} = (A/A') \times C \times D \times (\text{atw/wt})$$

where C = concentration of standard, mg/mL, D = dilution factor for sample, atw = average tablet weight, and wt = sample weight.

Results and Discussion

The system gave a linear response with tridihexethyl chloride concentrations of 0 to 200% (0 to 200 μg/mL). The peaks obtained were 95% of steady state.

Composites were prepared from commercial tablet preparations and were analyzed by the USP assay and by the automated procedure. Two authentic tablet formulations rep-

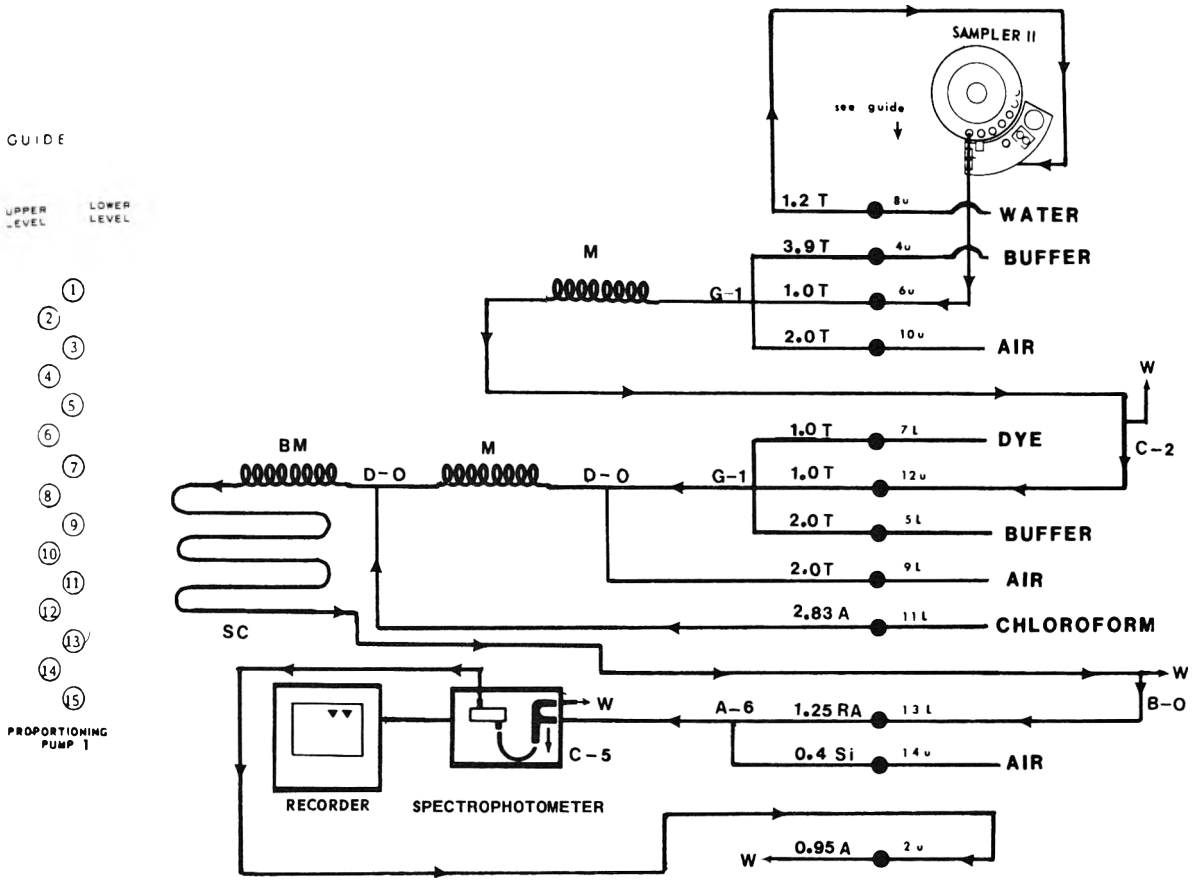

Figure 1. Flow diagram of automated system for tridihexethyl chloride. Key: T, Tygon pump tube; RA, red acicflex pump tube; A, acidflex pump tube; Si, silicone pump tube; M, 28-turn × 2.4 mm id mixing coil; BM, 28-turn beaded mixing coil; SC, 5-turn 2.4 mm id settling coil; C-2, B-0, D-0, G-1, C-5, A-6, Technicon nomenclature for glass fittings; W, waste. Pump tube sizes in milliliters per minute.

Table 1. Tridihexethyl chloride content (% of label claim) in meprobamate tablets by automated and USP methods

Mfr	Dosage, mg/tab.	Automated	USP
A	400/25[a]	100.7	100.0
B	400/25[a]	100.0	101.4
A	200/25[b]	107.4	107.1
B	200/25[b]	98.2	99.6
A	400/25[a]	102.3	104.9
B	400/25[a]	99.6(1.6)(30)[c]	99.8(1.0)(10)[c]
B	400/25[a,d]	100.0	—[e]
A	200/25[b,d]	100.0	—[e]

[a]Meprobamate/tridihexethyl chloride compressed tablets.
[b]Meprobamate/tridihexethyl chloride coated tablets.
[c]Coefficient of variation (%) and number of determinations in parentheses.
[d]Authentic formulation.
[e]Not analyzed.

resenting a 200 mg meprobamate/25 mg tridihexethyl chloride coated tablet and a 400 mg meprobamate/25 mg tridihexethyl chloride compressed tablet were also prepared and analyzed.

Table 1 shows the results of analysis with comparison to results from the USP XX method. Six commercial formulations representing 2 manufacturers, 2 different dosage levels of meprobamate, 2 different types of tablets, 6 different lots, and 2 authentic formulations were successfully analyzed by this method. There was no interference from meprobamate, excipients, or colors in the formulations in the analysis by the automated method. Recoveries of 100% were obtained by the automated method for analyses of the authentic formulations.

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Polarographic and Colorimetric Methods for Determination of Cyclophosphamide

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Polarographic and colorimetric methods for analysis of cyclophosphamide and its dosage forms were investigated. Both methods are based on the reaction of cyclophosphamide with nitrous acid. A single cathodic diffusion-controlled wave was used for dc polarographic determination of cyclophosphamide, with an accuracy of 99.98 ± 1.09%. The wave was well defined and irreversible. By differential pulse polarographic analysis, as little as 10 ppm cyclophosphamide was determined; overall accuracy at 10–60 ppm was 100.16 ± 0.99%. The linear relationship between absorbance for the lemon-yellow nitroso derivative and the concentration of cyclophosphamide was further used in colorimetric analysis; overall accuracy was 100.2 ± 0.99%.

Cyclophosphamide, 2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide, is the most effective alkylating agent against tumors (1, 2).

Several methods have been reported for the determination of cyclophosphamide. Some are based on determining nitrogen or phosphorus content (3). Cyclophosphamide can be identified by infrared spectroscopy and determined by gas chromatography (GC), as described in USP XX, NF XV (4). In the *British Pharmacopoeia* (5), cyclophosphamide is determined by treating its solution (after digestion) with citric–molybdic acid, then with quinoline. The precipitate is filtered, washed with water until it is free of acid, then a known excess of standard sodium hydroxide solution is added, and the excess is back-titrated with standard acid. Colorimetric (6, 7) and GC (8) methods have been developed for determining cyclophosphamide either in pure form or in pharmaceutical preparations.

Among the published methods, none has described a polarographic determination. The purpose of this work was to investigate the analytical behavior of cyclophosphamide in order to develop a new method. Colorimetric and dc and differential pulse polarographic techniques were investigated.

Experimental

Apparatus

Polarographic curves were recorded by using a Radelkis polarograph OH-102. A Model 384-1 polarographic analyzer system (EG&G Princeton Applied Research) was used for recording differential pulse polarographic (DPP) curves.

The rate of mercury flow through the capillary used for dc polarographic measurements, at a mercury height of 45 cm, was 1.8 mg/s; the drop time was 3.0 s (in distilled water). Except when the effect of mercury height was studied, a 45 cm mercury head was maintained. A dropping mercury electrode assembly, Model 303 SMDE (Princeton Applied Research) was used for DPP measurements.

A Unicam SP 600 Series 2 spectrophotometer with a matched pair of 1 cm quartz cells was used for colorimetric measurements.

Reagents

All reagents were analytical grade.

(a) *Stock standard solution.*—5 mg cyclophosphamide (authentic sample)/mL water.

(b) *Hydrochloric acid solution.*—20%.

(c) *Aqueous sodium nitrite solution.*—20%, freshly prepared.

(d) *Potassium hydroxide solution.*—20%.

Preparation of Samples

(a) *Tablets.*—Weigh 20 tablets and calculate average tablet weight. Accurately weigh an amount of tablet powder containing ca 300 mg cyclophosphamide. Extract with four 20 mL portions of ethyl alcohol. Filter the extract and wash the residue with ethyl alcohol. Evaporate the combined filtrate and washings to dryness on a water bath. Dissolve the dried residue in 20 mL water. Carry out the nitrosation process on an aliquot of the prepared solution as described below. By dilution with 20% potassium hydroxide, prepare final working solutions containing 1–2% active substance/10 mL for dc polarographic or colorimetric measurements.

(b) *Injections.*—Dissolve the contents of 10 vials in the appropriate amount of water to prepare a stock solution containing 5 mg cyclophosphamide/mL, and carry out the nitrosation process. By dilution with 20% potassium hydroxide, prepare final working solutions containing 1–2 mg active substance/10 mL for dc polarographic or colorimetric measurements.

Polarographic Method

Mix 25 mL stock standard solution with 25 mL 20% sodium nitrite solution and 25 mL 20% HCl in a 100 mL volumetric flask. Let the reaction proceed for 20 min on a water bath at 80°C with occasional shaking, then cool the solution to room temperature. Stop the reaction by diluting to volume with 20% potassium hydroxide solution. Prepare working standard solutions from the stock standard solution of the nitroso derivative by suitable dilution with Britton-Robinson buffer of different pH values, or with 20% potassium hydroxide solution for either dc (concentration range 0.5–5 mg/10 mL) or DP (concentration range 10–60 ppm) polarographic measurements.

Colorimetric Method

Prepare working standard solutions from the stock standard solution, prepared as for the polarographic method, by suitable dilution with 20% potassium hydroxide, to obtain solutions in the concentration range 1.5 to 4 mg/10 mL. Measure the absorbance of the yellow solution at 350 nm, against reagent blank. Calculate the concentration of cyclophosphamide from a calibration curve previously constructed.

Results and Discussion

The polarographic behavior of cyclophosphamide revealed that the compound and its brominated reaction product give an uncertain, ill-defined reduction wave. On the other hand, a well-defined cathodic wave was obtained from the prepared nitroso derivative. Similar polarograms were obtained by using different buffers and different unbuffered supporting electrolytes. Results showed that the $E_{1/2}$ value of the single cathodic wave of the nitroso derivative of cyclophosphamide obtained in all cases is about −0.9 V vs SCE, changing gradually to more negative potential values with an increase in concentration and pH of the solution. The most reproducible, well-defined cathodic wave was obtained, however, when

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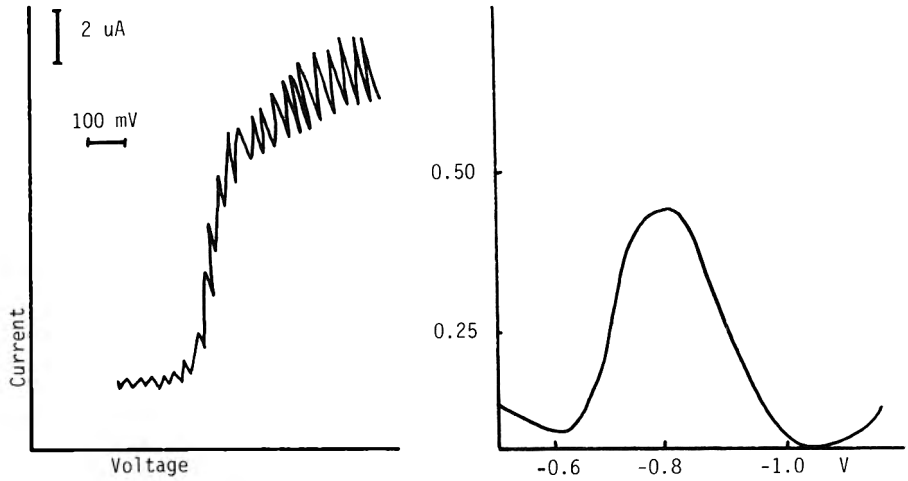

Figure 1. Studies of reaction of cyclophosphamide with nitrous acid: left, dc polarogram of reaction product (2.8 mg/10 mL); initial *E*, −0.6 V. Right, DP polarogram of reaction product (40 ppm). Current, × 10^3 NA; initial *E*, −0.6 V; final *E*, −1.1 V; peak, −0.834 V; 1.766E2 NA. Recorded in alkaline potassium hydroxide solution.

Table 1. dc Polarographic determination in alkaline medium of nitrosation product of authentic cyclophosphamide

Taken, mg/10 mL	Found,[a] mg/10 mL	Rec., %
0.5	0.50	100.0
1.2	1.19	99.2
2.8	2.78	99.3
3.6	3.65	101.4
5.0	5.00	100.0
Mean (P = 0.05)	99.98 ± 1.09	
SD	0.88	
Variance	0.78	

[a]Results are the average of at least 3 determinations each.

Table 2. DPP determination of nitrosation product of authentic cyclophosphamide

Taken, ppm	Found, ppm[a]	Rec., %
10.0	9.85	98.5
15.0	15.00	100.0
17.5	17.50	100.0
20.0	19.90	99.5
30.0	30.02	100.1
40.0	40.66	101.7
60.0	60.78	101.3
Mean (P = 0.05)	100.16 ± 0.99	
SD	1.073	
Variance	1.153	

[a]Results are the average of a least 5 determinations each.

the reaction between cyclophosphamide and nitrous acid was stopped by addition of 20% potassium hydroxide solution, and the polarogram was recorded in the alkaline potassium hydroxide solution as a supporting electrolyte (Figure 1).

The effect of mercury height, h, on the obtained current, i, in this alkaline potassium hydroxide solution was studied. A linear relationship was obtained between the limiting current and the square root of the mercury height. The log i–log h plot also is linear, with a slope very near 0.5, illustrating the diffusion-controlled character of the wave. The current was directly proportional to the concentration of the depolarizer over a wide concentration range. However, the range 0.05 to 0.5 mg/mL was used for constructing the dc polarographic calibration curve. It would be more difficult to measure the wave height at less than 0.05 mg/mL, although a concentration higher than 0.5 mg/mL can be used.

Thus, the reduction wave of cyclophosphamide, after its reaction with nitrous acid, is diffusion controlled and is suitable for quantitative polarographic determination.

The logarithmic analysis plot of the wave obtained under these experimental conditions shows a linear relationship with an exceptionally high slope that is significantly different from the theoretical values corresponding to a reversible system. The electrode reaction is, therefore, irreversible.

The derivatization procedure was applied to different concentrations of authentic cyclophosphamide. Results are shown in Table 1. The dc polarographic method can determine 0.5–5.0 mg cyclophosphamide/10 mL, after derivatization, with an accuracy of 99.98% and a confidence limit of ± 1.09%.

The DPP technique has also been used for the determination of cyclophosphamide through the obtained peak current (Figure 1), using the suggested derivatization method. The

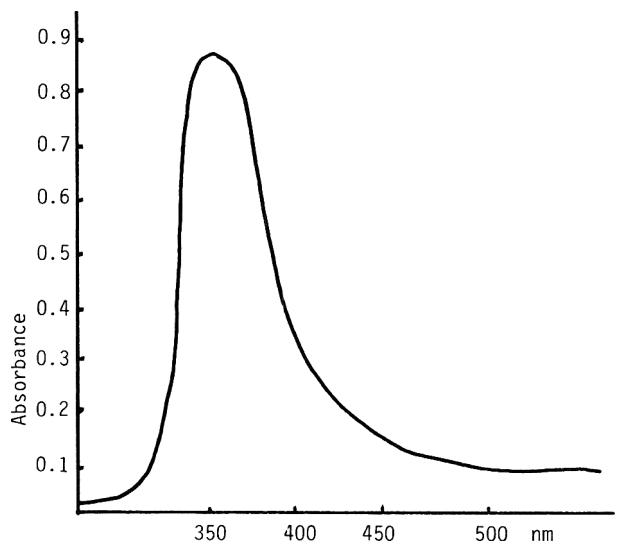

Figure 2. Study of reaction of cyclophosphamide and nitrous acid: spectrum of yellow solution obtained (4 mg/10 mL). Recorded in alkaline potassium hydroxide solution.

procedure was applied to authentic samples of cyclophosphamide after nitrosation. Results for quantities ranging between 10 and 60 ppm are shown in Table 2.

From the results shown in Table 2, it is evident that cyclophosphamide can be determined by using the proposed indirect DPP technique in the range 10–60 ppm, with a mean recovery of 100.16 ± 0.99%.

In the process of derivatization of cyclophosphamide a lemon-yellow reaction product was obtained. This reaction

Table 3. Colorimetric determination of authentic samples of cyclophosphamide by the proposed procedure

Taken, mg/10 mL	Found,[a] mg/10 mL	Rec., %
1.5	1.51	100.6
2.0	2.02	101.0
3.0	2.97	99.0
3.4	3.29	99.7
4.0	4.02	100.5
Mean (P = 0.05)	100.16 ± 0.99	
SD	0.80	
Variance	0.65	

[a]Results are the average of at least 3 determinations each.

Table 4. Analysis of some pharmaceutical preparations of cyclophosphamide[a] by the proposed procedures

Preparation and batch No.	Cyclophosphamide, % rec. dc Polarogr.	Colorim.
Tablets, 50 mg/tab. No. 5102	99.5	99.6
Tablets, 50 mg/tab. No. 5234	99.7	99.9
Injection, 100 mg/vial No. 5262	100.2	100.1
Injection, 200 mg/vial No. 6451	99.7	99.9

[a]Endoxan-Asta tablets and injections, Asta-Werke AG, Germany.

Table 5. Accuracy of the recovery of standard addition of cyclophosphamide to some market pharmaceutical preparations, using the proposed methods

dc Polarogr.			Colorim.		
Added, mg	Rec., % Tablets	Injections	Added, mg	Rec., % Tablets	Injections
0.8	100.0	99.6	0.5	99.8	99.8
1.6	98.8	98.8	1.0	100.1	99.9
2.8	98.2	101.2	1.5	100.2	100.1
3.2	102.3	100.9	2.0	100.2	100.1
4.0	101.1	99.2	2.5	99.8	99.9
Mean (P = 0.05)	100.1 ± 1.98	99.9 ± 1.29		100.0 ± 0.24	100.0 ± 0.17
SD	1.60	1.04		0.19	0.14
Variance	2.73	1.09		0.04	0.02

was, therefore, further used for colorimetric estimation of cyclophosphamide. Figure 2 shows the spectrum of the reaction product obtained by the proposed method. The color produced exhibited maximum absorbance at 350 nm.

Optimum conditions for maximum color development were studied. The use of equal volumes of 20% sodium nitrite, 20% hydrochloric acid, and cyclophosphamide solution gave the highest color intensity after heating 20 min on a water bath at 80°C. A 20% potassium hydroxide solution is used to stop the reaction and to adjust the pH to about 12. No detectable difference was found on using sodium hydroxide.

The proposed colorimetric method for the determination of cyclophosphamide through its nitrous acid reaction product was applied to determine separate authentic samples of cyclophosphamide, by measuring the absorbance at 350 nm. Results are shown in Table 3.

Endoxan-Asta tablets and injections produced by Asta-Werke AG, Germany, were analyzed by both the dc polarographic method and the colorimetric procedure. Results are shown in Table 4. No interference was observed from tablet excipients and coating materials.

The validity of both procedures was assured by applying the standard addition technique. The results obtained are presented in Table 5.

It is obvious from the results shown in Tables 1 and 2 that the suggested dc polarographic procedure can determine 0.5–5.0 mg cyclophosphamide/10 mL with an accuracy of 99.98 ± 1.09%, while the DPP procedure can determine 10–60 ppm cyclophosphamide with an accuracy of 100.16 ± 0.99%. The proposed colorimetric procedure, on the other hand, can determine 1.5–4.0 mg cyclophosphamide/10 mL with an accuracy of 100.16 ± 0.99%. Recoveries of added cyclophosphamide (Table 5) indicate the validity of the proposed procedures.

The reaction of cyclophosphamide with nitrous acid possibly proceeds in the same manner as the reaction of secondary amines (9, 10), with the formation of a nitroso derivative.

Acknowledgments

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Determination of Fluoride in Fluoride Tablets and Solutions by Ion-Selective Electrode: Collaborative Study

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A proposed method using the fluoride (F) ion-selective electrode has been developed for determining the fluoride ion concentration in tablets and solutions containing sodium fluoride. The method has been subjected to collaborative study. Eight samples consisting of 2 authentic fluoride solutions, 2 commercial fluoride solutions, and 4 commercial fluoride tablets were sent to each of 11 collaborators together with a copy of the method. Single assays on the authentic fluoride solutions known to contain 1 mg F/5 mL were performed with average recoveries of 99.5 and 99.6% and relative coefficients of variation (CV) of 2.11 and 1.91%, respectively. Single assays of 2 commercial fluoride solutions declared at 1 mg F/5 mL gave mean values of 0.994 and 0.990 mg and relative CV values of 1.88 and 2.36%, respectively. Single assays of 2 commercial fluoride tablet preparations declared at 0.5 mg F gave mean values of 0.485 and 0.478 mg and relative CV values of 3.12 and 3.71%, respectively. Single assays of 2 commercial fluoride tablet preparations declared at 1 mg F gave mean values of 0.991 and 0.981 mg and relative CV values of 2.99 and 2.85%, respectively. The method has been adopted official first action.

Sodium fluoride tablets and solutions are widely used as dental caries preventives in children. A rapid and accurate method employing the fluoride-selective electrode has been developed for the determination of fluoride (F) in tablets and solutions. Solutions containing F ion are buffered to pH 5.0–5.5 to eliminate formation of F complexes with moderate concentrations of polyvalent cations (1). The F content is then measured by direct potentiometry (2).

The determination of F by ion-selective electrode has the advantages of speed and accuracy of analyses, the ability to measure a large number of samples, and a wide range of sample concentrations. The method involves using a pH/mV meter equipped with an F-selective electrode and plotting a calibration curve on semilogarithmic graph paper. The electrode potentials of standard solutions are measured and plotted on the linear axis against their concentrations on the log axis. A pH/mV meter readable to 0.1 mV is required for good analytical precision.

Collaborative Study

Collaborators received 8 samples: 2 authentic F solutions of 1 mg F/5.0 mL, 2 commercial F solutions declared at 1 mg F/5.0 mL, 2 commercial F tablet preparations declared at 0.5 mg F/tablet, and 2 commercial F tablet preparations declared at 1 mg F/tablet. Practice F solution and practice F tablet samples with stated F contents were also included. The collaborators were asked to analyze the practice samples and to proceed with the analyses of the study samples only if the results were within ±2% of the stated value.

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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.* (1984) **67**, March issue.
This report of the Associate Referee was presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

Fluoride in Drug Tablets and Solutions
Fluoride-Selective Electrode Method
First Action

Principle

F in tablets and solns is detd by F-selective electrode, using total ionic strength adjustment buffer (TISAB) to eliminate complexation of F with polyvalent cations and direct potentiometry to measure concn of F ions in soln.

Reagents

(a) *Total ionic strength adjustment buffer (TISAB).*—TISAB II (Orion Research Inc., 380 Putnam Ave, Cambridge, MA 02139, Cat. No. 94-09-09); alternatively, in ca 500 mL H_2O in 1 L beaker, dissolve 57 mL HOAc, 58 g NaCl, and 4 g CDTA (cyclohexylene dinitrilo tetraacetic acid or 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid). Cool and adjust pH to 5.0–5.5 with 5M NaOH. Cool to room temp., transfer to 1 L vol. flask, and dil. to vol. with H_2O.

(b) *Fluoride std solns.—(1)* $10^{-2}M$.—Transfer 209.9 mg accurately weighed Ref. Std NaF, previously dried 4 h at 150°, to 500 mL vol. flask, dissolve, and dil. to vol. with H_2O. *(2)* $10^{-3}M$.—Pipet 25.0 mL of 10^{-2}M soln to 250 mL vol. flask. Dil. to vol. with H_2O. *(3)* $10^{-4}M$.—Pipet 25.0 mL 10^{-3}M soln to 250 mL vol. flask. Dil. to vol. with H_2O. (Store all std solns in plastic bottles.)

Apparatus

(Equiv. app. may be substituted)

(a) *Meter.*—Expanded-scale mV pH meter or selective ion meter (Fisher Accumet Selective Ion Analyzer, Model 750).

(b) *F-selective electrode.*—F-combination electrode (Orion Research Inc., Cat. No. 96-09-00), filled with internal filling soln (Orion Research, Cat. No. 90-00-01).

(c) *Plastic labware.*—Store all samples and stds in plastic containers because F reacts with glass.

(d) *Magnetic stirrer.*—Suitable mag. stirrer with asbestos pad placed on top to reduce heat transfer. Use Teflon-coated stir bar.

Preparation of Samples

(a) *Tablets.*—Det. no. of tablets equiv. to 10 mg F and completely disperse in 500 mL vol. flask contg ca 400 mL H_2O by heating on steam bath and shaking intermittently. Cool to room temp. and dil. to vol. with H_2O.

(b) *Solutions.*—Pipet aliquot of soln contg 1–2 mg F into 100 mL vol. flask, dil. to vol. with H_2O, and mix.

Do not let solns remain in glass >1 h. (Store sample solns in plastic bottles.)

Determination

Pipet 20.0 mL of each std and sample prepn into sep. plastic beakers. Pipet 20.0 mL TISAB into each beaker. Use potentiometer equipped with F-selective electrode. Immerse electrode in 10^{-3}M std soln and stir at const. rate with mag. stirrer. Take mV measurement when potential has stabilized

Table 1. Collaborative results for determination of fluoride in authentic fluoride solutions (1 mg F/5.0 mL) by ion selective electrode

Coll.	Found, mg F/5.0 mL Soln X	Soln Y
A	1.005	0.990
B	1.016	0.988
C	1.025	1.005
D	0.975	1.000
E	0.970	0.970
F	0.988	0.998
G	1.015	1.025
H	0.960	0.975
I	0.988	0.978
J	1.000	1.005
K	1.007	1.026
Added, mg F/5.0 mL	1.000	1.000
Av. found	0.995	0.996
Rec., %	99.5	99.6
SD	0.021	0.019
CV, %	2.11	1.91

within ±0.1 mV. Repeat with 10^{-2}M and 10^{-4}M std solns and with sample prepn. Using 2-cycle semi-log graph paper, prep. std curve plotting molar F std concn on log axis vs mV potential on linear axis. Det. molar F concn (C_i) in sample prepn from std curve. Prep. sep. mV measurements for each std soln and std curve for each sample detn.

Calculations

Tablets:

$$\text{mg F/tablet} = C_i \times (19 \text{ mg F/mL}) \times 500 \text{ mL/no. of tablets} = 9500\, C_i/\text{no. of tablets}$$

Solns:

$$\text{mg F/aliquot} = C_i \times (19 \text{ mg F/mL}) \times 100 \text{ mL} = 1900\, C_i$$

where C_i molar concn in sample prepn as detd from std curve.

Results and Discussion

Eight samples were sent to each of 11 collaborators during November 1982. All collaborators submitted their results to the Associate Referee by June 1983. Each collaborator indicated that the analyses were conducted during a 1–2 day period. None of the collaborators reported any difficulties with the proposed method.

Table 1 contains the results and a statistical evaluation of the data, with average F recoveries, standard deviations, and coefficients of variation for the 2 authentic fluoride solutions. The authentic fluoride solutions are "blind" duplicates prepared by the Associate Referee from the batch formulation of a commercial fluoride solution which contains all the manufacturer's excipients, with a concentration of 1 mg F/5.0 mL.

Table 2 contains the results of the study and a statistical evaluation of the data with the means, percent label declarations, ranges, standard deviations, and coefficients of variation for 6 different commercial fluoride preparations.

Direct potentiometry is a simple procedure for measuring a large number of samples. However, the ionic strength and the temperature of the solutions must remain constant throughout the determination.

TISAB II is added to each sample and standard solution and the resulting solutions are measured while being stirred at a constant rate; a constant temperature is maintained throughout. Continuous stirring, with a minimum of turbulence, causes the electrode to be exposed to a fresh supply of ions, and the addition of TISAB II maintains the ionic strength of the solution for measurement.

The pH of the solutions must be above pH 5 to avoid complexation by hydrogen ions and below pH 7 to avoid hydroxide interference. The addition of TISAB II adjusts solutions to pH 5.0–5.5. TISAB II also inhibits complexation of F with iron and aluminum ions by preferentially complexing with those ions. Up to 3–5 ppm iron or aluminum is complexed by TISAB II in the presence of 1 ppm F (1).

Electrode potentials are affected by changes in temperature. A 1° difference in temperature will cause about a 2% measurement error (1). Because a magnetic stirrer may generate sufficient heat to change the solution temperature, an asbestos pad is placed between the beaker and the magnetic stirrer to reduce heat transfer.

Changes in room temperature may also affect electrode potential measurements. Therefore, measurements of the standard solutions and subsequent preparation of the standard curve must be performed separately for each sample determination.

When the proper precautions are taken, the use of the F-selective electrode for the direct potentiometric determination of fluoride in tablets and solutions is a rapid, simple, and reliable procedure.

Table 2. Collaborative results for determination of fluoride tablets and solutions by ion-selective electrode[a]

Coll.	Sample 1 (1 mg F/ 5.0 mL)	Sample 2 (1 mg F/ 5.0 mL)	Sample 3 (0.5 mg F/ tab.)	Sample 4 (0.5 mg F/ tab.)	Sample 5 (1 mg F/ tab.)	Sample 6 (1 mg F/ tab.)
A	0.990	0.980	0.489	0.484	0.969	0.998
B	0.969	0.979	0.480	0.475	0.979	0.979
C	1.007	0.988	0.494	0.480	1.026	0.988
D	1.004	0.977	0.479	0.499	0.998	1.017
E	0.979	0.979	0.466	0.456	0.949	0.949
F	0.988	0.998	0.475	0.458	0.959	0.935
G	1.026	1.016	0.494	0.489	1.045	1.016
H	1.011	0.990	0.457	0.444	0.998	0.950
I	0.979	0.974	0.499	0.494	0.969	1.007
J	0.969	0.960	0.494	0.489	0.988	0.978
K	1.007	1.045	0.508	0.489	1.016	0.969
Mean	0.994	0.990	0.485	0.478	0.991	0.981
% Label decl.	99.4	99.0	97.0	95.6	99.1	98.1
Range: Low	0.969	0.960	0.457	0.444	0.949	0.935
High	1.026	1.045	0.508	0.499	1.045	1.017
SD	0.019	0.023	0.015	0.018	0.030	0.028
CV, %	1.88	2.36	3.12	3.71	2.99	2.85

[a]Six different commercial fluoride preparations; amount declared shown in parentheses.

Recommendation

It is recommended that the method for the determination of fluoride in tablets and solutions by F-selective electrode be adopted official first action.

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Rapid Differential Pulse Polarographic Determination of Tiaprofenic Acid

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Tiaprofenic acid (Surgam®) has been determined directly by using differential pulse polarography (DPP) and pH 5.5 acetate buffer as a supporting electrolyte. The differential pulse polarogram was obtained under constant amplitude pulses of 50 mV superimposed on a linearly increasing dc ramp. Peak current was measured at the peak potential of −0.990 V vs Ag/AgCl reference electrode. Under the experimental conditions used, a linear relationship between peak current and concentration was established over the range 0.5–5.0 μg/mL. Mean percentage recoveries for tiaprofenic acid in authentic and tablet forms were 100.27 ± 2.25 and 99.45 ± 1.44, respectively. The results obtained by the DPP method were compared with those of the spectrophotometric method used by the manufacturer for the analysis of tiaprofenic acid and its tablet form.

Tiaprofenic acid, α-(5-benzoyl-2-thienyl) propionic acid (Figure 1) is a new anti-inflammatory and analgesic drug with a marked therapeutic effect (1–3). A spectrophotometric method has been adopted by the manufacturer for the assay of authentic tiaprofenic acid and tablets (Roussel Laboratories, International Pharmaceutical Division, Paris, France, personal communication). Recently, Wahbi et al. (4) applied a differential spectrophotometic method to the determination of tiaprofenic acid in bulk and dosage forms.

The work in this report involves the application of differential pulse polarography (DPP) to the determination of tiaprofenic acid as an authentic sample and in tablet form. Because it is a carbonyl compound, tiaprofenic acid is expected to be readily reduced (5) at the dropping mercury electrode (DME) under the appropriate applied potential.

Experimental

Apparatus and Reagents

(a) *Polarographic systems.*—Metrohm Polarecord consisting of a Polarecord Unit 626 and a polarograph stand Model E505. Electrode assembly includes *(1)* silver-silver chloride reference electrode; *(2)* platinum auxiliary electrode; and *(3)* dropping mercury electrode, which is a fine capillary fitted with a drop controller that supplies a steady stream of mercury droplets at a frequency of 0.5/s and flow of ca 3.1 mg Hg/s.

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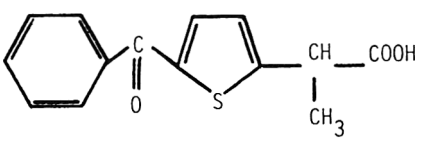

Figure 1. Tiaprofenic acid.

(b) *Spectrophotometer.*—Varian DMS 90 double beam with 1 cm quartz cuvet.

(c) *Supporting electrolyte.*—pH 5.5 acetate buffer. Dissolve 46.7 g sodium acetate in ca 500 mL water. Add 6 mL glacial acetic acid and dilute solution to 1 L.

(d) *Tiaprofenic acid.*—Supplied by Roussel Laboratories, Paris, France.

(e) *Tiaprofenic acid standard solution.*—Dissolve ca 50 mg authentic tiaprofenic acid, accurately weighed, in 100 mL volumetric flask, using 100 mL 0.01N HCl in methanol as solvent. Pipet 5 mL of this solution into 100 mL volumetric flask and dilute to volume with acetate buffer.

(f) *Benzophenone standard solution.*—Dissolve 20 mg benzophenone (BDH) in 100 mL methanol. Dilute 10 mL of this solution to 100 mL with the same solvent.

Preparation of Sample Solution

Weigh and finely grind 20 tablets and compute weight of one tablet. Accurately weigh quantity of powder equivalent to ca 50 mg tiaprofenic acid. Transfer powder into 100 mL volumetric flask, add 50 mL 0.01N HCl in methanol, shake, and dilute to volume with the same solvent.

Differential Pulse Polarography

Transfer exactly 30 mL supporting electrolyte containing 0.01% w/v gelatin (maximum suppressor) into polarographic vessel. Dilute 10 mL sample solution to 100 mL, using acetate buffer and maximum suppressor solutions. Add an aliquot (1.0–4.0 mL) of diluted sample solution to polarographic cell so that final concentration is maintained within linear range 0.5–5.0 μg/mL. De-aerate solution 10 min with stream of nitrogen [$W(N_2)$ = 0.9999]. Vary dc polarizing voltage from −0.75 to −1.2 V (convenient range) vs Ag/AgCl reference electrode at sweeping rate −5 mV/s. Adjust superimposed

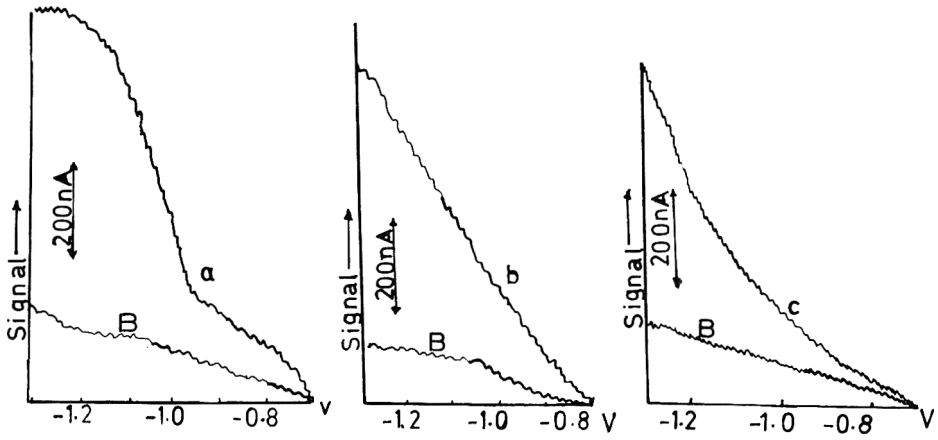

Figure 2. dc Polarograms of standard tiaprofenic acid solution (7.4 μg/mL): (a) pH 4.7 acetate buffer; (b) pH 7.4 phosphate buffer; (c) pH 9.3 borate buffer; (B) blank. Potential (E vs Ag/AgCl).

voltage pulse amplitude to 50 mV and record differential pulse polarogram. At the peak potential of −0.990 V vs Ag/AgCl reference electrode, measure peak current, *i*, from baseline and correct for dilution involved. Calculate concentration of tiaprofenic acid from calibration curve prepared by following same procedure, using 0.5, 1.5, 2.5, 3.5, and 4.0 mL standard tiaprofenic acid solution. Alternatively, standard addition method may be used.

Results and Discussion

Organic polarography is influenced by pH because hydrogen ions are involved in the electrode reaction. Consequently, pH 4.7 acetate buffer, pH 7.4 phosphate buffer, and pH 9.3 borate buffer were each tried as a supporting electrolyte for the development of dc polarograms of tiaprofenic acid at room temperature (Figure 2). Of these, the acetate buffer proved to be the most suitable. The single cathodic wave of tiaprofenic acid obtained in acetate buffer (Figure 3) shows a

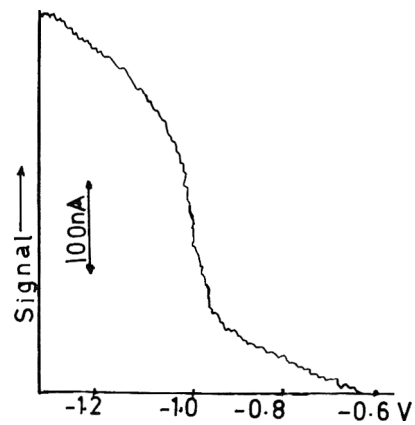

Figure 3. dc Polarogram of tiaprofenic acid standard solution (5 μg/mL) in pH 3.5 acetate buffer. Potential (E vs Ag/AgCl).

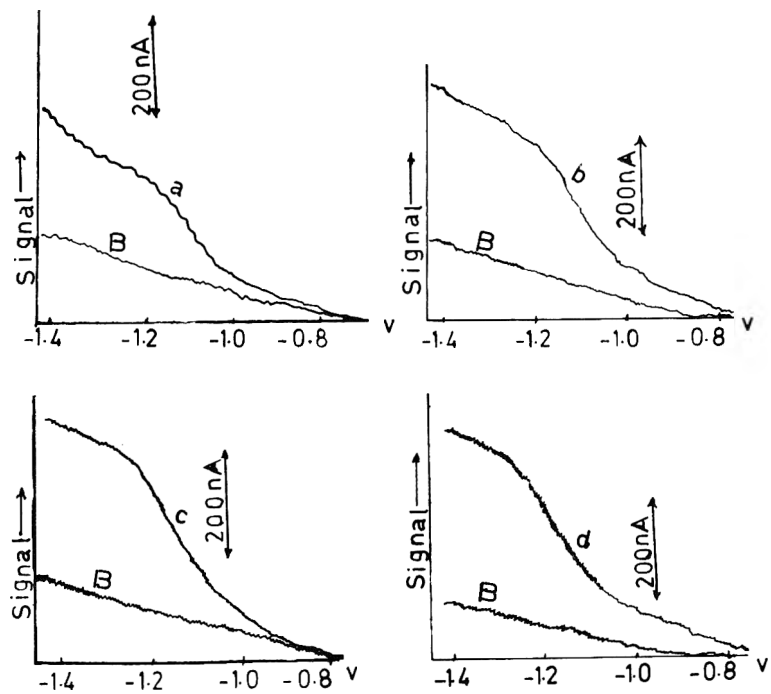

Figure 4. dc Polarograms of tiaprofenic acid standard solution (5 μg/mL) in acetate buffer pH (a) 4.0; (b) 4.5; (c) 5.5; (d) 6.0; (B) blank. Potential (E vs Ag/AgCl).

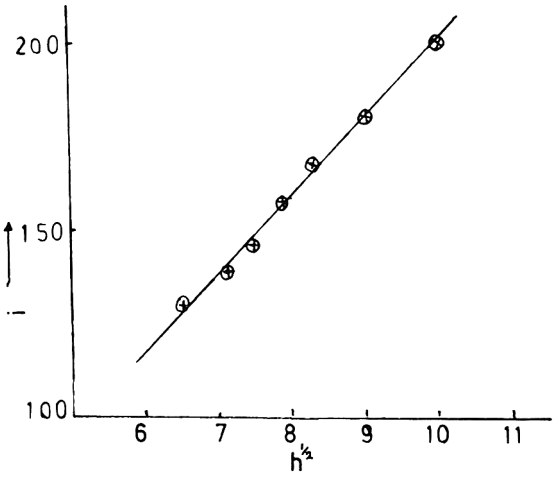

Figure 5. Variation of polarographic current (*i*) with square root of corrected mercury height (*h* cm).

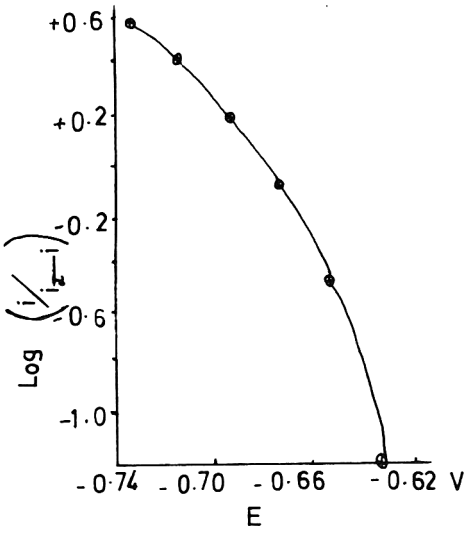

Figure 6. Relationship of log ($i/i_d - i$) to applied voltage E.

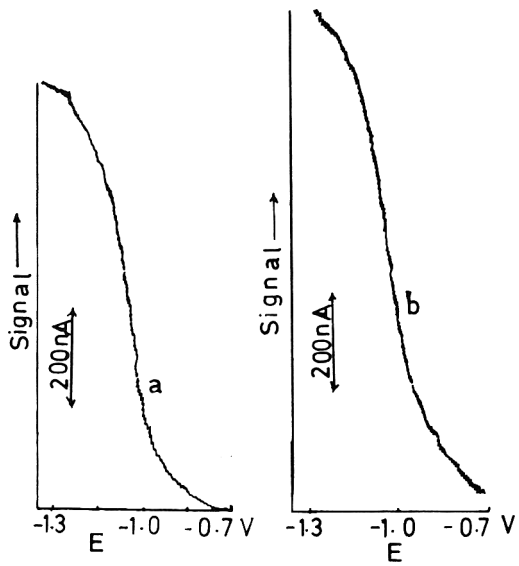

Figure 7. dc Polarograms in acetate buffer pH 5.5 of (a) benzophenone (10 μg/mL); (b) benzophenone (10 μg/mL) in presence of tiaprofenic acid (7.1 μg/mL). Potential (E vs Ag/AgCl).

half-wave potential of −0.95 V vs Ag/AgCl reference electrode.

The dependence of the half-wave potential on pH was studied using pH 3.5–6.5 acetate buffer solutions. The expression $E_{1/2} = -(0.74 + 0.06\ pH)$ was found to describe the variation of $E_{1/2}$ with pH up to pH 5.5 where a maximum height of the polarographic wave was obtained (Figure 4). Consequently, pH 5.5 acetate buffer solution was chosen as a supporting electrolyte for the quantitative measurements.

The effect of mercury height (*h*) on polarographic current (*i*) was investigated. A linear relationship (Figure 5) has been given between the recorded current and the square root of the corrected mercury height, suggesting that the recorded current is most probably diffusion-controlled.

The logarithmic analysis plot of the polarographic wave, obtained under the experimental conditions (Figure 6), showed a nonlinear relationship, indicating the irreversible character of the electrode reaction.

To verify that the keto group in tiaprofenic acid is the site of reduction, benzophenone solution was polarographed separately and in the presence of tiaprofenic acid using pH 5.5 acetate buffer. The polarographic waves obtained (Figure 7)

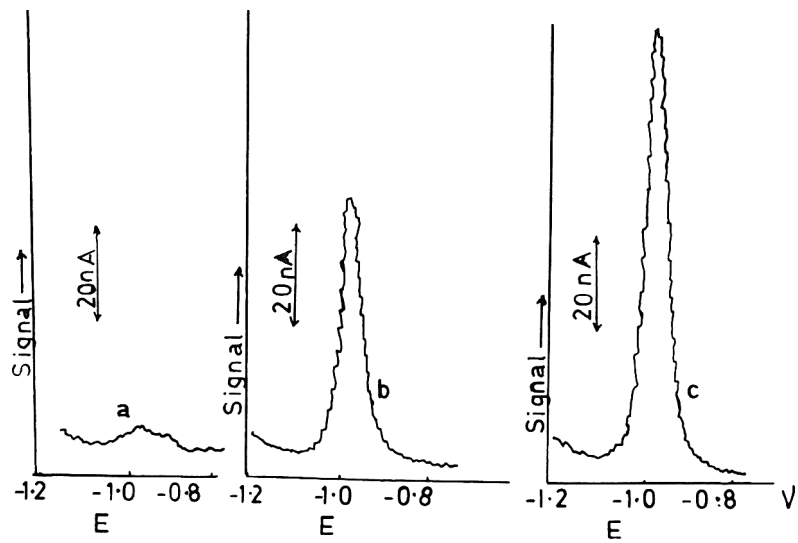

Figure 8. Typical differential pulse polarograms of (a) blank (pH 5.5 acetate buffer); (b) tiaprofenic acid standard solution (1.7 μg/mL); (c) tiaprofenic acid standard solution (3.4 μg/mL). Potential (E vs Ag/AgCl).

suggest that the keto group is most probably the site of reduction.

Typical differential pulse polarograms are shown in Figure 8. The peak current, measured from the baseline, is linearly related to concentration, C, over a range of 0.5–5 μg/mL. The relationship can be described by the expression $i = 17.03 + 45.35C$, where C is in μg/mL and (i) is the current in nanoamperes. The calculated correlation coefficient, r, is 0.999, indicating excellent linearity.

The mean percentage recovery of 5 determinations for an authentic sample, using the polarographic method, was 100.27 ± 2.25; 7 determinations on tablets (Surgram®, label claim 100 mg tiaprofenic acid/tablet) gave a mean recovery of 99.45 ± 1.44%. Mean percentage recoveries using the manufacturer's method, based on measuring absorbance at 306 nm, were 100.1 ± 0.12 (5 determinations) and 101.0 ± 0.34 (4 determinations) for authentic material and Surgram 100 mg tablets, respectively. The difference between the 2 means for the tablets is insignificant; the calculated t is 2.07 compared with a significant level of 2.26 at $P = 0.05$. Accordingly, the polarographic method can be used for the quantitative determination of tiaprofenic acid in tablets. Moreover, if the reduced form of tiaprofenic acid is present, which interferes spectrally with the keto form, the polarographic method will be advantageous because the measurements of peak current under the applied potential will not be affected. However, we have not substantiated this because we lack the reduced form of tiaprofenic acid.

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Liquid Chromatographic Determination of Primary and Secondary Amines as 8-Quinolinesulfonyl Chloride Derivatives

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The normal phase and reverse phase liquid chromatographic properties of seven 8-quinolinesulfonyl derivatives of primary and secondary amines are examined using dual wavelength ultraviolet detection. The amines are further identified by infrared spectrophotometry with emphasis on structural elucidation based on absorption bands at characteristic wavelengths.

The amine functional group is a component of many drugs such as amphetamine, ephedrine, methamphetamine, phenylpropanolamine, phentermine, and others. One of the major problems associated with the analysis of amines is the absence of absorbance or low absorbance in the ultraviolet (UV) region. In addition, the above problem is further compounded by the noncrystalline, hygroscopic, and volatile nature of many of these amines. Fluorescent derivatizing agents such as fluorescamine have been used to enhance the detectability of many amines (1). Jupille (2) reported the conversion of amines to strongly UV-absorbing *p*-nitrophenylacetamides by reaction with *N*-succinimidyl-*p*-nitrophenylacetate. A series of benzenesulfonamides and benzamides have also been reported for use in spectrophotometric detection of amines after liquid chromatography (3). The structural elucidation of low molecular weight primary and secondary amines via derivatization with phenylisothiocyanate (PIT) has been reported (4), as has the use of PIT to enhance detectability and chromatographic properties (5).

Primary and secondary amines readily react with acid chlorides to form substituted amides; this reaction is the basis for most derivatization procedures. The conversion of an amine to a sulfonamide may also be used to enhance detectability. Tertiary amines do not yield stable derivatives when subjected to reactions with acid chlorides, and covalent derivatization procedures are of little use for such compounds. This paper reports the qualitative aspects of using the acid chloride, 8-quinolinesulfonyl chloride, as derivatizing agent to enhance UV and liquid chromatography (LC) properties and to assist in the structural elucidation of primary and secondary amines by infrared spectrophotometry.

Experimental

Reagents and Chemicals

The hydrochloride salts of ephedrine, pseudoephedrine, methamphetamine, phenylpropanolamine, phentermine, and phenmetrazine, and the sulfate salt of amphetamine were received from their respective manufacturers and were used without further purification. 8-Quinolinesulfonyl chloride, 99%, was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. All chemicals were reagent grade or better (Fisher Scientific Co., Fair Lawn, NJ) and were used without further purification.

Instrumentation

Ultraviolet spectra were recorded on a Hitachi 100-80 spectrophotometer (Tokyo, Japan). Infrared spectra were recorded on a Perkin-Elmer Model 1330 infrared spectrophotometer (Norwalk, CT). The liquid chromatograph consisted of a Waters Associates (Milford, MA) Model 6000 A pump, Model U6K injector, Model 440 UV detector with dual wavelength accessory operated at 254 and 280 nm, and Houston Instrument (Austin, TX) OmniScribe dual pen recorder.

Synthesis of Amides

Ten mg each of the salts of ephedrine, pseudoephedrine, methamphetamine, phenylpropanolamine, phentermine, phenmetrazine, and amphetamine were dissolved in 10 mL 1.0N sodium carbonate. A 2.0 mL solution of 8-quinolinesulfonyl chloride (0.5 g dissolved in 100 mL LC grade acetone) was mixed with 1.0 mL amine solution in a 5.0 mL reaction vial. The vial was sealed and allowed to react 20 min

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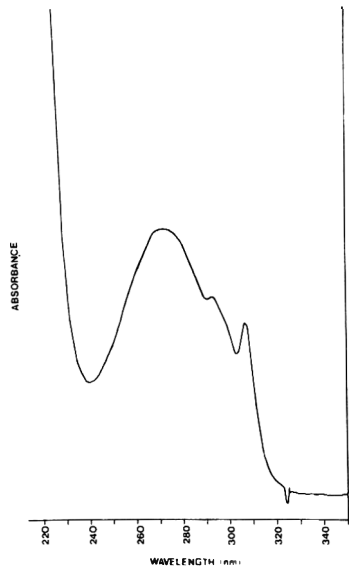

Figure 1. Ultraviolet absorption spectrum of the 8-quinolinesulfonamide of ephedrine.

at 65°C. The vial was cooled, and the mixture was added to a separatory funnel, extracted with chloroform (2 × 30 mL), dried with magnesium sulfate, and evaporated to dryness under a stream of air.

Chromatographic Procedures

Reverse phase separations were carried out on a 30 cm × 3.9 mm id μBondapak C_{18} column (Waters Associates) at ambient temperature. The analytical column was preceded by a 7 cm × 2.1 mm id guard column dry-packed with Co:Pell ODS (Whatman Inc., Clifton, NJ). The mobile phase consisted of water (double-distilled)–acetonitrile–acetic acid (59 + 40 + 1). The mobile phase flow rate was 1.5 mL/min.

Normal phase separations were carried out using a 30 cm × 3.9 mm id μPorasil column (Waters Associates) at ambient temperature. The analytical column was preceded by a 7 cm × 2.1 mm id guard column dry-packed with HC Pellosil (Whatman Inc.). The mobile phase consisted of *n*-hexane–methylene chloride–methanol–diethylamine (300 + 30 + 5 + 0.5). The mobile phase flow rate was 1.0 mL/min.

The ultraviolet absorbance detectors were connected in series and operated at 254 and 280 nm. Absorbance ratios were calculated from the average peak height measurements of a minimum of 3 injections for each drug tested.

Results and Discussion

The initial phase of this study was the synthesis of the 8-quinolinesulfonamides of representative amines followed by the determination of the UV absorbance properties of these derivatives. The amides were prepared by mixing an aqueous solution of the amine with an acetone solution of 8-quinolinesulfonylchloride in a 5 mL reaction vial. The reaction conditions included excess base (Na_2CO_3) and required heating at 65°C for 20 min. The product amides were isolated in pure form by solvent extraction. The infrared spectrum of the products confirmed the conversion of the amine to the sulfonamide. Further structural information concerning the degree of substitution of the amide-nitrogen can also be obtained from the infrared spectrum.

The UV absorption spectrum of a representative 8-quinolinesulfonamide is shown in Figure 1. The wavelength of maximum absorbance for ephedrine 8-quinolinesulfonamide

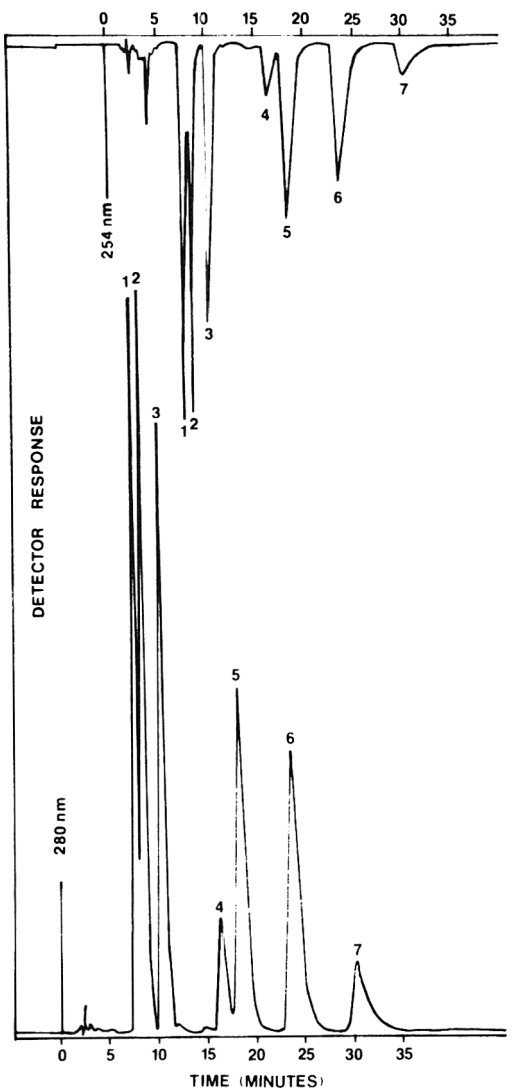

Figure 2. Reverse phase LC separation of 8-quinolinesulfonamides: 1, phenylpropanolamine; 2, ephedrine; 3, pseudoephedrine; 4, phenmetrazine; 5, amphetamine; 6, methamphetamine; 7, phentermine.

was observed to be 273 nm. The UV spectrum of the other amine-derivatives studied was essentially the same as that in Figure 1. This is to be expected since the derivatization reaction adds the same chromophore, the 8-quinolinesulfonyl group, to each of the drugs. Furthermore, the absorbance ratio (A_{254}/A_{280}) was the same for all derivatives studied. The A_{254}/A_{280} ratio calculated from the absorption spectrum was 0.68. Dual wavelength detection is an effective aid in the identification of chromatographic peaks. The strong absorbance properties of these sulfonamides at 254 and 280 nm would certainly allow dual wavelength analysis of LC peaks. These derivatives can be used to compliment other derivatization methods, particularly the PIT derivatives since PIT-amines exhibit greater UV absorbance at 254 nm ($A_{254}/A_{280} > 1$).

The chromatographic properties of the derivatives prepared in this study were examined in reverse phase and normal phase systems. The reverse phase separation of 8-quinolinesulfonamides is shown in Figure 2. The derivatives were separated on an octadecylsilane stationary phase, using acetonitrile–water–aqueous acetic acid as the mobile phase. Figure 2 shows the more hydrophilic amides eluting first in the reverse phase system. Phenylpropanolamine, ephedrine, and pseudoephedrine all contain a hydroxyl group which increases the polarity of these compounds relative to hydrogen. Thus,

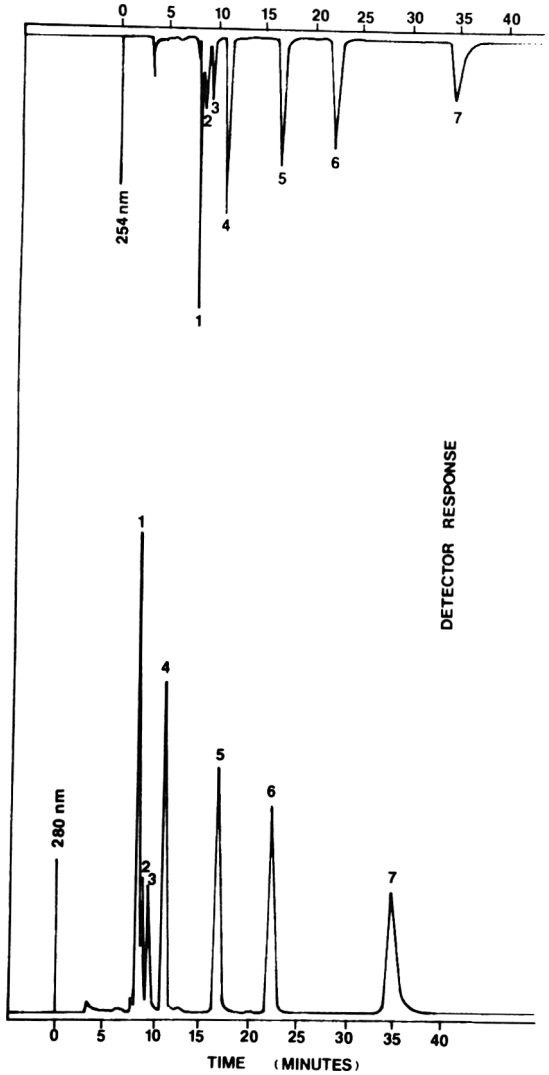

Figure 3. Normal phase LC separation of 8-quinolinesulfonamides: 1, methamphetamine; 2, phenmetrazine; 3, phentermine; 4, amphetamine; 5, pseudoephedrine; 6, ephedrine; 7, phenylpropanolamine.

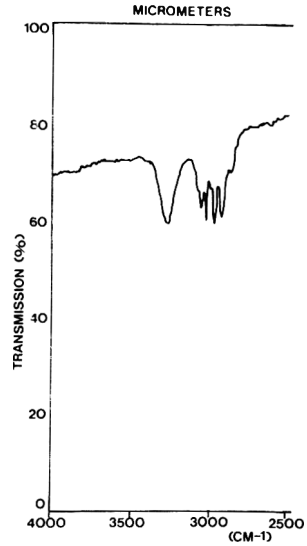

Figure 4. Portion of IR spectrum of 8-quinolinesulfonamide of amphetamine, illustrating absorption band at 3300 cm^{-1} due to hydrogen on primary amine.

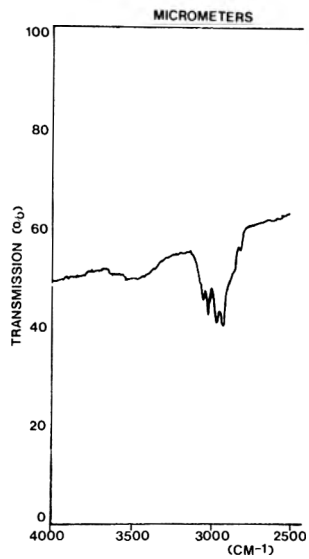

Figure 5. Portion of IR spectrum of 8-quinolinesulfonamide of methamphetamine, illustrating absence of absorption band at 3300 cm^{-1}.

these compounds associate more strongly with the solvent and are retained to a lesser degree. Current information suggests that the hydrocarbonaceous stationary phase plays only a passive role in solute retention, the mobile phase being primarily responsible for chromatography in reverse phase systems. The hydrophobic effect (6, 7) appears to play a major role in solute retention. Other factors collectively described as solvent selectivity also influence retention (8). Another advantage of using the 8-quinolinesulfonamide derivatives in conjunction with the LC separation of PIT derivatives is the identification of phenylpropanolamine, ephedrine, amphetamine and methamphetamine. The elution order is *(1)* phenylpropanolamine and *(2)* ephedrine, whereas the reverse phase separation of the PIT derivatives described previously (5) was the opposite. The same reverse relationship has been found to exist between amphetamine and methamphetamine.

The normal phase separation of the 8-quinolinesulfonamides examined in this study is shown in Figure 3. The elution order for the 3 hydroxyl group-containing amides is exactly opposite to that observed in the reverse phase system. In normal phase chromatography, the polar associations occur primarily between the solute and the stationary phase; therefore, the more polar solutes are more strongly retained. The derivatives were separated on a silica stationary phase with *n*-hexane–methylene chloride–methanol–diethylamine as the mobile phase. Although some time is required in changing solvents and columns from reverse phase to normal phase in LC systems, the use of both systems is important in establishing peak identity because elution orders in the 2 systems are changed.

The A_{254}/A_{280} ratio can be a useful aid in the identification of liquid chromatographic peaks. Baker et al. (9) used this ratio to determine the identity of drugs having similar elution characteristics in an LC system. However, the interlaboratory use of these ratios should be approached with caution. The A_{254}/A_{280} ratio for cocaine is reported as 0.86 (9) in one study and found to be 1.95 (10) in another. The ratio for diazepam has been reported as 6.04 (9) and 4.62 (11). These variations are not surprising because molar absorptivity and the wavelength of maximum absorbance vary with solvent composition, pH, and other factors (12). These ratios should be determined in an individual chromatographic system using reference standards. The A_{254}/A_{280} ratio was of little value in the identification of the 8-quinolinesulfonamides. The absorbance ratios of the compounds studied in reverse phase and

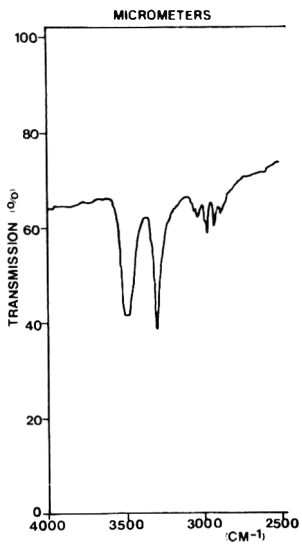

Figure 6. Portion of IR spectrum of 8-quinolinesulfonamide of phenylpropanolamine, illustrating absorption band at 3500 cm^{-1} due to –OH functional group and band at 3300 cm^{-1} due to hydrogen remaining on primary amine.

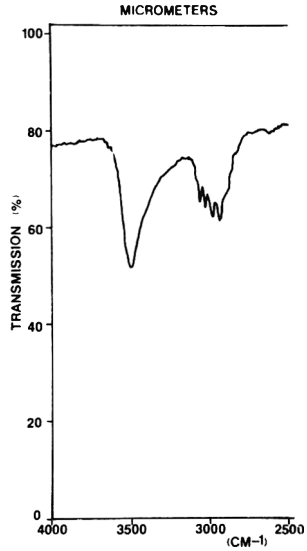

Figure 7. Portion of IR spectrum of 8-quinolinesulfonamide of ephedrine, illustrating absorption band at 3500 cm^{-1} due to –OH functional group and absence of the absorption band at 3300 cm^{-1}.

Table 1. Major infrared absorption frequencies of 8-quinolinesulfonamides (–SO_2N–)

Amine	Positions and intensities of absorption maxima (cm^{-1})[a]					
d-Amphetamine	3280	1600	1560	1490	1450	1420
	1380	1330 (B)	1220	1170 (A)	1150 (B)	1130 (C)
	1090	990	970	900	840	790
	750	700	680	640	610	580
	550	530	500			
Ephedrine	3500	1600	1560	1490	1450	1320 (A)
	1210	1160	1130 (B)	1030	950	880
	830	790	760	700	590	550
	530					
Methamphetamine	1600	1560	1490 (B)	1450	1380	1320 (A)
	1230	1210	1160	1230 (B)	1010	990
	950	890	860	830	790	750
	730	700	670	610	580	550
	530	510				
Phenmetrazine	1600	1550	1490	1440	1370	1330 (C)
	1320 (B)	1270	1200	1160	1140	1120
	1060	1040	990	970	930	920
	900	830	790	740	700	690
	580	570	500			
Phentermine	3260	1600	1560	1490	1450	1430
	1390	1370	1330 (B)	1220	1160 (C)	1140 (A)
	1000	960	830	790	740	700
	680	640	610	580	550	500
Phenylpropanolamine	3500	3300	1600	1560	1490	1450
	1420	1300 (A)	1230	1210	1160 (A)	1140 (B)
	1100	1060	1040 (C)	1030	900	830
	790	760	700	670	640	600
	580	570	550	510		
Pseudoephedrine	3500	3300	1600	1560	1490	1450
	1320 (A)	1210	1170 (C)	1130 (B)	1050	1000
	920	890	840	790	770	730
	700	590	550	510		

[a]A,B,C—major peaks in decreasing order of intensity

normal phase systems were approximately 0.50. The major advantage of absorbance ratios in the study is to show that derivatization has actually taken place and to possibly determine peak purity in cases of similarly eluting compounds.

The infrared spectra of the 8-quinolinesulfonamides studied exhibit absorption bands useful in determining whether the amine is primary or secondary. Typically, the hydrogen remaining on the primary amine, following amide formation, absorbs as a band around 3300 cm^{-1}. Secondary amines after derivatization have no hydrogen remaining on the nitrogen and would not be expected to show the band at 3300 cm^{-1}. Figure 4 illustrates the absorption band at 3300 cm^{-1} due to the remaining hydrogen on the primary amine of amphetamine. Figure 5 illustrates the absence of the 3300 cm^{-1} band of the secondary amine methamphetamine. Also of usefulness in determining structure was an absorption band occurring at 3500 cm^{-1}. This band is due to the –OH group on ephedrine, pseudoephedrine, and phenylpropanolamine. Figure 6 shows the absorption band at 3500 cm^{-1} due to the –OH functional group and the sharp band at 3300 cm^{-1} due

to the hydrogen remaining on the primary amine of phenylpropanolamine. Figure 7 is a portion of the infrared spectrum of ephedrine showing the absorption band at 3500 cm^{-1} but lacking the absorption band at 3300 cm^{-1} because no hydrogen remains on the secondary amine. The 8-quinolinesulfonamides studied were easily distinguishable by infrared spectrophotometry. Among the other characteristic absorption bands which were observed were sharp bands between 1350–1300 cm^{-1} and 1180–1100 cm^{-1} due to the $-SO_2N-$ functional portion of the 8-quinolinesulfonamides, which corresponds with the observations of other authors (13). Table 1 lists the major absorption frequencies of the compounds studied.

In conclusion, the primary and secondary amines of low natural detectability by UV can be derivatized with the chromophoric 8-quinolinesulfonyl group. The resulting amides display excellent chromatographic properties in reverse phase and normal phase LC systems and can be detected in trace amounts. Additional structural information can be obtained from the infrared absorption spectrum of these derivatives.

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MEAT AND MEAT PRODUCTS

Effect of Residual Ascorbate on Determination of Nitrite in Commercial Cured Meat Products

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Residual ascorbate in cured meat slurries results in different amounts of pigment being produced from different Griess reagent combinations. The phenomenon was used to study residual ascorbate in commercial cured meat products which had a variety of textures, acidities, moisture and meat content, fat, homogeneity, initial nitrite, and processing conditions. Diluting and heating the samples according to the AOAC procedure did not completely eliminate the ascorbate interference, but making the sample alkaline did. Determining nitrite separately in supernate and precipitate from the first dilution showed the effect of heating to be the elimination of interferences and solubilization or extraction of nitrite from the precipitate.

During a study of the kinetics of the Griess reaction, it was observed that the presence of ascorbate caused relatively more pigment to be produced from the reagent combination sulfanilic acid/*N*-(1-naphthyl)ethylenediamine (SAA/NED) than from the combination of sulfanilamide/1-naphthylamine (SAN/1-NA) (1). While 1-NA has been classified as a carcinogen (2), the combination of SAN/1-NA gave the best results in nitrite determination and gave the most disparate values when compared with the SAA/NED combination. In tests on heated meat slurries with and without added nitrite and various levels of ascorbate, it was found that the differential pigment production could be used as a test for residual ascorbate (3, 4). The meat slurries were uniform and the residual nitrite levels were relatively high. Commercial meat products present a more varied substrate, including variations in pH, moisture content, meat sources, tissue disruption, fat, homogeneity, initial nitrite content, and processing conditions. Because residual nitrite and ascorbate values vary greatly under these conditions, we determined nitrite by both colorimetric reagent combinations to see if the results from the slurry studies also applied to the more varied conditions of commercial meat products.

Experimental

Samples and Preparation

Commercial cured meat products were purchased at local markets. For the preliminary studies, 4 types of products were chosen: ham as an intact tissue product; bacon, because of its high fat content; frankfurters, representing emulsified products; and Lebanon bologna, a fermented (acid) comminuted meat product. The AOAC (5) procedure calls for finely mincing the sample and then dispersing it in hot water with a glass rod. We found that blending the sample gives more uniform results and is faster because it combines the comminution and dispersion steps. Therefore, the initial extracts were made by blending meat with an equal volume of water. A 2 mL portion of the resulting slurry was diluted to ca 80 mL with warm water and heated 2 h at 80°C, cooled, and centrifuged. Two departures from the AOAC procedure are noted, blending and centrifugation, but these do not appear to change the essence of the procedure.

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Nitrite Determination

Three colorimetric reagent combinations were used: *(1)* the AOAC procedure of adding sulfanilamide to the sample, waiting 5 min, then adding *N*-(1-naphthyl)ethylenediamine (SAN/5 min/NED); and the 2 combinations of *(2)* sulfanilamide with 1-naphthylamine (SAN/1-NA) (2), and *(3)* sulfanilic acid–*N*-(1-naphthyl)ethylenediamine (SAA/NED), with both pairs of reagents premixed. Ascorbate has been shown to interfere strongly in the AOAC colorimetric determination procedure (6), and the differential production of pigment from the latter 2 reagent combinations is a measure of the residual ascorbate in cooked meat slurries containing nitrite (3). For the sake of brevity, we shall refer to the comparison as differential colorimetric analysis. Chemiluminescent detection (CLD) of nitric oxide produced from nitrite is a sensitive (2.5 ppb) method for nitrite determination (7) and was used to confirm the results of colorimetric analysis.

Procedure Modification

Because nitrite is labile in meat, there can be no absolute measure of determinable nitrite. In a previous study, we used a consensus approach, using the results of methods that gave the maximal and most consistent results with a minimum of interference (4). These were CLD determination, making AOAC samples alkaline before heating, and charcoal addition. Therefore, portions of the AOAC samples were adjusted to pH 8.0 (alkaline AOAC) before heating. Other portions of the 1:1 meat:water slurries were diluted to 1:10 meat:water, 0.5 g charcoal was added, the samples were shaken for ½ h and then centrifuged according to the procedure of Adriaanse and Robbers (8). To determine nitrite partitioning between supernate and precipitate in the initial extracts, cold water-extracted samples were diluted 1:100 meat:water and a portion was centrifuged immediately to precipitate the water-insoluble proteins, mitochondria, connective tissue, etc. The precipitate was resuspended in a volume of water equal to the supernate, and the initial extract, supernate, and suspended precipitate were heated according to the AOAC procedure.

The charcoal, alkaline AOAC, and separated component samples were turbid after centrifugation or filtration. Turbidity is most commonly eliminated by heavy metal addition, usually iron, zinc, or mercury. Iron is commonly added as the ferricyanide (known as Carrez I) but it interferes in pigment production (4). Mercury is effective, but it also interferes (4) and is expensive and a pollutant. One molar zinc sulfate (Carrez II) added at a ratio of 1 to 20 parts sample before centrifugation gave clear solutions and had no demonstrable effect on pigment formation.

CLD is 20 times as sensitive as colorimetric determination, which allowed us to determine the precision and accuracy of the latter method by comparison with the former. To test recovery, nitrite solution was added to the samples to make them 5 and 80 ppm nitrite before nitrite determination. Five ppm $NaNO_2$ diluted 1:100 and determined with SAN/1-NA gives an absorbance at 525 nm of about 0.04 AU, about the threshold of colorimetric measurement. Comparisons were

Table 1. Percent recovery of nitrite added to commercial products immediately before sample preparation

Sample	Spike, ppm	Recovery, % CLD	AOAC	SAN	SAA
Bacon	5	86	86	93	108
	80	101	92	100	93
Frankfurters	5	95	103	117	129
	80	101	98	114	115
Ham	5	91	86	89	92
	80	99	98	101	95
Lebanon bologna	5	52	10	50	48
	80	90	83	97	104
Water	5	—	90	97	85
	80	—	95	100	83
$\bar{x}$		94.71	92.33	100.89	100.44
σ		5.91	6.69	9.13	14.98
CV, %		6.24	7.25	9.05	14.91

made between CLD (Antek Model 720 digital nitrogen detector) and Griess colorimetric determinations (GCD) (Cary 14 spectrophotometer) using techniques described previously (7). We prepared only 10 mL colored solution, but the reagent concentrations were those of the AOAC procedure. From the comparison, we were able to develop an estimate of the reliability of the colorimetric determinations, especially important because of the low nitrite levels encountered in cured meat products.

Results

Nitrite Recovery

The results of the nitrite recovery experiments are shown in Table 1. The low recovery value for the 5 ppm spiked Lebanon bologna samples was apparently due to sample preparation error because both nitrite determination methods gave the same low value. When samples were reanalyzed, recovery values were 113% and 132% by CLD and SAN, respectively. Statistical analyses of the data were made without the 5 ppm Lebanon bologna data, because the latter were found to be outliers (9). The analysis showed that the percent recovery values for GCD were not significantly different from 100% or CLD, except for the AOAC reagent combination which was low ($P \leq 0.05$). The coefficients of variation (CV) values are comparable to the pooled CV value of 4.4% (4) obtained for replicates in the slurry studies. Subgroup analyses were made of the data, for example, 5 vs 80 ppm nitrite, CLD vs colorimetric, but there were no significant differences. Since the precision of the CLD determination is 5.5% (CV) (6), from which the coefficients of variation of the colorimetric determinations do not differ significantly ($<4.56 \times CV_{CLD}$) (4), we conclude that the indicated values are close to the true precision and the accuracy of the colorimetric determinations. Since the residual nitrite values were subtracted from the total measured nitrite in the samples, the precision refers only to the spike and may not necessarily apply to residual nitrite in the products.

Supernate and Precipitate

Nitrite was determined in the supernate and precipitate before and after heating. Immediately after resuspension of the precipitate and before heating, there was no measurable nitrite in the precipitate. After heating, the average increases for 9 samples were 2 ppm nitrite in the supernate, 5.8 ppm in the precipitate, and 5.5 ppm in the whole extract. The sum of the increases in supernate and precipitate values (7.8 ppm) is larger than that for the whole sample, but not significantly. The values for measured nitrite in the supernates and precipitates of the 9 samples after heating are shown in Table 2. There was no significant difference between the sum of the parts (precipitate + supernate) and the whole extract. The

Table 2. Nitrite determination by CLD and SAN/1-NA in samples prepared by AOAC method

	Nitrite determination method							
	CLD				SAN/1-NA			
Sample	Spt + Ppt = Sum			Whole	Spt + Ppt = Sum			Whole
Bacon I	6.05	3.13	9.18	6.74	7.9	4.4	12.3	9.1
II	8.19	1.92	10.11	10.11	8.9	4.6	13.5	9.1
III	4.73	2.71	7.44	6.98	7.7	5.3	13.0	9.2
III (OH)	5.07	4.13	9.20	8.80	7.7	6.8	14.5	12.0
+ Carrez II		4.20		7.72		8.1		10.5
Frankfurters I	13.22	2.11	15.33	16.00	14.1	4.0	18.1	16.3
II	0.65	4.26	4.91	4.65	0.0	4.8	4.8	5.3
III	1.22	4.86	6.08	5.65	0.0	7.3	7.3	6.3
III (OH)	1.93	8.09	10.02	8.13	3.6	7.0	10.6	10.0
Ham I	2.20	1.62	3.82	4.99	4.4	2.2	6.6	4.6
II	0.63	3.16	3.79	4.46	1.6	4.0	5.6	6.1
III	0.90	3.46	4.36	3.56	1.8	6.1	7.9	8.1
III (OH)	1.35	5.77	7.12	7.00	2.8	8.3	11.1	9.2
+ Carrez II		5.00		5.68		11.1		9.6
Lebanon bologna I	3.24	4.90	8.14	8.58	4.8	6.1	10.9	10.5
II	1.03	5.59	6.62	8.60	2.6	4.8	7.4	8.1
III	1.57	5.83	7.40	8.55	1.0	10.0	11.0	10.6
III (OH)	2.52	11.81	14.33	12.84	1.7	14.8	16.5	14.9
Carrez II		11.23		12.76		13.2		14.9

Paired variate analysis

Pair	$\bar{d}$ (ppm)[a]	P	Pair	$\bar{d}$ (ppm)	P
Sum/whole: CLD	0.0	NS	0/+ alkali:spt	1.0	0.05
SAN	1.3	0.005	:ppt	2.7	<0.01
SAN/CLD: whole	1.6	<0.001	:sum	3.6	<0.001
0/+ Zinc: all	0.2	NS	:whole	3.0	<0.001

[a] $\bar{d}$ is average of differences of the first member of pair minus the second.

Table 3. Comparison of 3 methods of sample preparation with initial extract by CLD and 3 Griess reagent combinations

				Preparation methods									
	Initial			AOAC				AOAC-OH			Charcoal		
Sample	AOAC[a]	SAN	SAA	CLD	AOAC	SAN	SAA	AOAC	SAN	SAA	AOAC	SAN	SAA
Bacon	4.3	21.0	53.0	55.8	44.2	54.8	62.6	63.2	60.1	61.1	69.5	61.3	73.0
Corned beef	9.1	12.6	14.3	14.5	11.2	14.3	15.7	22.2	26.6	31.1	14.1	14.7	16.0
Frankfurter	2.9	19.4	55.0	60.5	49.2	57.4	66.4	61.0	65.0	69.8	69.0	60.7	72.3
Frankfurter, chicken	0.3	0.2	1.2	10.8	7.0	8.7	9.7	16.1	18 4	24.6	5.0	4.5	5.0
Genoa salami	0.6	1.2	1.9	5.2	3.4	5.1	4.7	10.2	12 1	12.2	5.8	6.1	6.7
Pepperoni	1.0	1.6	2.6	8.3	5.9	7.9	8.5	11.0	12.1	9.4	7.1	7.1	8.6
Pork butt	6.4	22.0	51.9	70.4	52.1	63.9	80.5	68.2	71.2	75.1	69.6	64.9	83.3
Smoky link	10.2	15.6	18.2	17.0	11.9	16.1	16.1	22.6	26.6	34.7	18.0	17.9	17.8

Prepn methods	Nitrite detn	$\bar{d}$ (%)	$S_{\bar{d}}$	P	Prepn methods	Nitrite detn	$\bar{d}$ (%)	$S_{\bar{d}}$	P
AOAC	CLD/AOAC	38	4.1	<0.001	AOAC	SAN/AOAC	29.1	3.6	<0.001
	CLD/SAN	4.5	1.2	<0.01		SAA/SAN	8.2	3.1	0.05
	CLD/SAA	−2.7	3.5	NS					
AOAC-OH/ AOAC	AOAC	88	10.6	<0.001	AOAC-OH	SAN/AOAC	10.8	3.0	<0.02
	SAN	61	17.2	<0.02		SAA/SAN	9.3	6.3	NS
	SAA	67	25.7	0.05					
AOAC-OH/ charcoal	AOAC	51.8	27.0	NS		AOAC/SAN	4.6	2.7	NS
	SAN	77.8	35.5	NS		SAA/SAN	14.7	3.1	<0.005
	SAA	80.4	47.6	NS	charcoal	SAA/AOAC	9.8	3.1	0.02

[a]Abbreviations used in this row are for the following methods of nitrite determination: AOAC, addition of sulfanilamide (SAN), wait 5 min, add *N*-(1-naphthyl)ethylenediamine (NED); SAN, combination of SAN and 1-naphthylamine; SAA, combination of sulfanilic acid and NED; CLD, chemiluminescent detection.

Griess reagent combination SAN/1-NA measured more nitrite than did CLD, but the difference was small (1.5 ppm). The addition of zinc (Carrez II) to the precipitate and whole samples removed the faint but visible turbidity that was present, but made no difference in the amount of nitrite determined. Finally, the adjustment of the extracts to pH 8.0 increased the measured nitrite 1.0 ppm in the supernates, 1.9 ppm in the precipitates, and 3.0 ppm in the whole samples.

Sample Preparation Procedures

Table 3 shows the results of nitrite determination by the 3 Griess reagent combinations in samples of the initial extract and after 3 sample preparation procedures. CLD was used to determine nitrite in the AOAC-prepared samples as a control. The nitrite concentrations in the initial extracts as measured by the AOAC (SAN/5 min/NED), SAN/1-NA, and SAA/NED reagent combinations show the effect of residual ascorbate in all of the cured meats, that is, the ordering of the determinations in terms of pigment formed was AOAC < SAN < SAA (4). A summary of the statistically significant differences between the preparation procedures and nitrite determination reagents is shown below the data. Because of the wide range of nitrite concentrations in the samples, the data were analyzed as percent changes between the 2 sets of data being compared. This treatment of the data is indicated because the differences between sample preparation procedures are the result of improving a given determination rather than adding a fixed amount to each nitrite concentration. The statistical analysis of the results of the 3 colorimetric preparation procedures and the CLD determination are shown in the first 3 sets of data, left side of Table 3. The $\bar{d}$ values are the average differences in percent increase or decrease of the first-indicated preparation method compared with the second. For example (first row), the CLD method gave 38% higher average nitrite values than did the AOAC colorimetric determination in the same AOAC preparation, indicating some residual ascorbate activity. The SAN/1-NA values averaged 4.5% lower than the CLD, and the SAA/NED 2.7% higher, again indicative of residual ascorbate. However, the differences between the SAA/NED and CLD values were more variable than the differences between the SAN/1-NA and CLD values, 3.5% and 1.2%. If we take CLD as the reference, the lower variation in the differences means SAN is giving more reliable values than SAA.

Referring to the second set, AOAC-OH/AOAC, making the samples alkaline before heating resulted in a significant increase in measured nitrite over the standard AOAC procedure (61–88%). Since one effect of alkalization is to reduce the ascorbate interference, the greatest increase was in the AOAC reagent determinations, although there was also a significant increase in the SAN/1-NA and SAA/NED values. The AOAC-OH procedure also gave higher measured nitrite values than did the charcoal treatment (third set), but the differences between the treatments were so variable that the increase (fourth column) was not significant (see next section).

The differences in nitrite measured by the 3 colorimetric reagent combinations on each of the 3 preparation methods are shown in the right hand sets of data (Table 3). The 29.1% difference between the AOAC and SAN determinations confirms that the AOAC preparation procedure is incompletely removing ascorbate. The difference of 8% between SAN/1-NA and SAA/NED in both the AOAC and AOAC-OH preparations is a minimum figure due to chloride in the samples (3). The order of increasing nitrite was AOAC/SAN/SAA for both heating procedures, but for the charcoal treatment it was SAN/AOAC/SAA (second set), which suggests that the 2 preparation procedures are not equivalent. The charcoal treatment eliminates the ascorbate effect since the AOAC and SAN/1-NA reagents are almost equivalent. The SAA/NED values are higher, but the combination is sensitive to Cl^- (3, 10), and ions from the charcoal may cause a false increase in the amount of pigment produced.

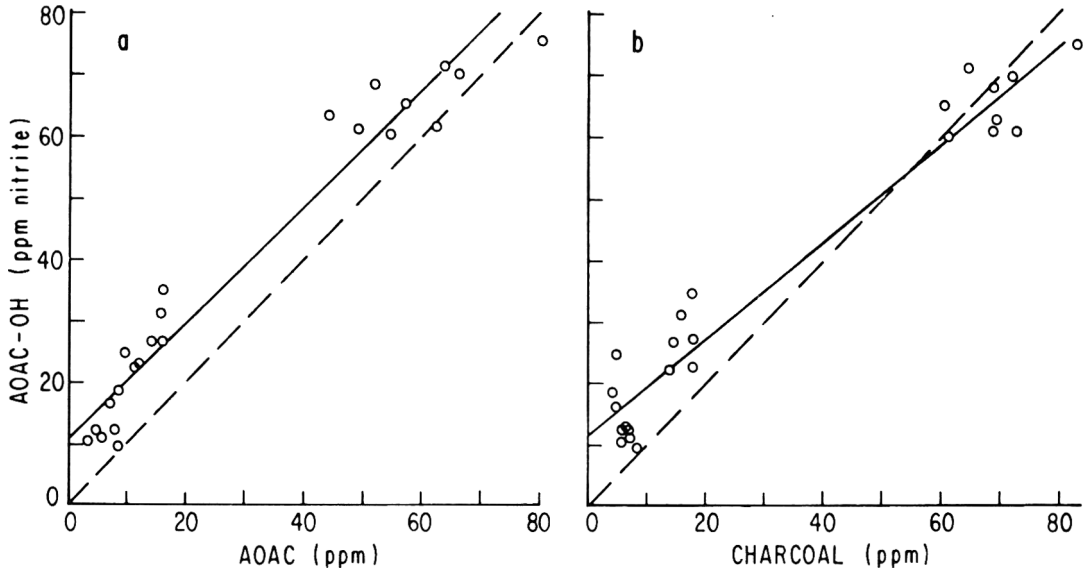

Figure 1. Nitrite as determined by 3 preparation methods. 1a—Covariant plot of nitrite concentrations as determined by sulfanilamide/1-naphthylamine in samples prepared by AOAC procedure, with and without alkalization. 1b—Same reagent, samples prepared by AOAC-OH and charcoal addition.

Comparison of AOAC-OH with AOAC and Charcoal Addition Preparation Methods

The plots for nitrite determined by SAN are shown in Figure 1, in which the AOAC-OH data are plotted as functions of the AOAC (1a) and charcoal addition (1b) methods. The dashed line is the expected regression if both sample preparation methods gave the same amount of measured nitrite for each sample. Similar to the slurry studies, making the samples alkaline improved recovery (Figure 1a) by about 10 ppm nitrite at all levels. In contrast, charcoal addition was as effective as AOAC-OH preparation at high nitrite concentrations, but yielded less nitrite by about 10 ppm at the lower nitrite levels. This explains the high average difference and standard deviation observed between the AOAC-OH and the other 2 preparation methods (second 2 sets of data, lower left side, Table 3).

Nitrite Determination in Different Products

As a final test of the different nitrite determination procedures, 16 different kinds of cured meat products were tested, including bacon (3 pork, 1 beef, and 1 Canadian), 2 corned beef, frankfurters (2 pork and beef, 2 chicken, and 1 turkey), 1 ham, 2 pepperoni, 1 pork butt, 2 Lebanon bologna, salami (1 hard and 2 Genoa), 1 alternative bacon product, 1 smoky link, and 1 beef strip. The results of the 3 colorimetric determinations are shown as a function of the reference method CLD in Figure 2. The regression equations are

$$\text{AOAC} = 0.803\ \text{CLD} - 1.37\ S_{yx} = 1.22$$
$$\text{SAN} = 0.938\ \text{CLD} + 0.77\ S_{yx} = 2.56$$
$$\text{SAA} = 0.996\ \text{CLD} + 4.98\ S_{yx} = 3.80$$

The intercepts of the AOAC and SAA plots are significantly different from zero ($P = 0.005$ and 0.002, respectively), but the SAN intercept is not. The solid line is the perfect correlation (slope = 1, intercept = 0), and it is apparent that over the entire range the SAN/1-NA combination is the closest to the ideal. The slope of the SAA/NED:CLD plot is closer to 1, but the offset (4.98 ppm) and the variability ($S_{yx} = 3.8$) are indicative of interference by residual ascorbate and chloride.

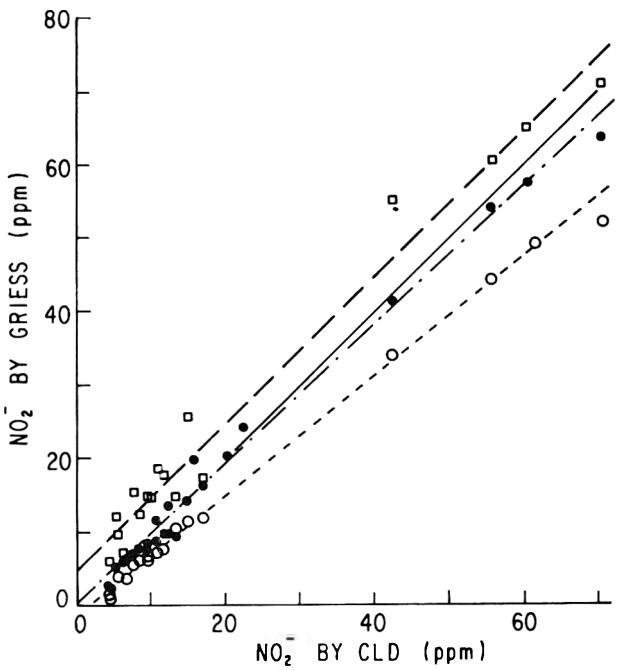

Figure 2. Nitrite in samples prepared by AOAC procedure as determined by chemiluminescent detection technique and 3 reagent combinations. 0, add sulfanilamide, wait 5 min, add *N*-(1-naphthyl)ethylenediamine (AOAC). •, Sulfanilamide/1-naphthylamine. □, Sulfanilic acid/*N*-(1-naphthyl)ethylenediamine.

Discussion

Magnitude of the Differences: Precision

The average differences between the various preparation techniques and reagent combinations tend to be quite small and in some cases negligible. For example, heating for 2 h resulted in an average increase of 5 ppm nitrite, partly by eliminating the ascorbate interference and partly by freeing nitrite from the precipitate; alkalization of the samples resulted in an average increase of 3 ppm in one set of values, in another set, 10 ppm. These differences do not at first appear to justify the use of one procedure or reagent over another, yet alkal-

ization is more effective in eliminating ascorbate interference, and because residual ascorbate, like nitrite, is variable, more uniform and accurate nitrite determinations result (4).

The same may be said for reagent combinations. By comparison with the reference method CLD, the reagent combination of SAN/1-NA, which is less sensitive to ascorbate, gave more precise and accurate values than did SAA/NED. Both the spike recovery figures and the covariance of the CLD and Griess nitrite measurements suggest an increasing variability in pigment formation in the order, AOAC < SAN < SAA. The CV of spike recovery increased in the same order, 7.25 < 9.05 < 14.91%, as did the S_{yx} of the CLD/Griess reagent covariant plots, 1.22 < 2.56 < 3.80. This coincidence led us to examine the data from previous studies and it was found indeed that the SAA/NED values showed a higher variation than did the SAN/1-NA values. It is safe, therefore, to conclude that the SAA/NED combination is inherently more variable (less precise) than the SAN/1-NA combination. The explanation is that the nitrosation rate of both the nitrosated species, SAA, and the coupling reagent, NED, are about equal (k_{1st} = 0.180 and 0.186 min^{-1}, respectively). Because of the molar excess of SAA over NED and the partial reversibility of the nitrosation of the latter, the reaction goes principally to the formation of pigment. Nevertheless, where the rates of nitrosation are approximately equal, any slight variation affecting the 2 rates differently will result in quite different amounts of pigment being formed. Slight variations in temperature, mixing rates, absolute reagent concentrations, other ions, etc., can cause the difference. For example, chloride affects the rate at which the pigment is formed (9). If the ratios of the reaction rates with SAA and NED are different for the 2 nitrosating species, nitrogen trioxide and nitrosyl chloride, the amount of pigment formed will be different with and without chloride. To counteract this effect, Sen and Donaldson (11) proposed adding excess chloride, which shifts the nitrosating species to nitrosyl chloride. However, the variability observed in the previous study was within isochloric sample groupings, and therefore could not be due to chloride variation. To decrease the variability, either faster reacting nitrosated species or slower reacting coupling reagents may be used, for example, SAN/1-NA, or the nitrosated species may be prereacted as in the AOAC reagent combination/procedure.

Spikes, Slurries, and Commercial Samples

The results obtained for the determination of nitrite in the spiked samples of this study and the slurries of the previous 2 studies (3, 4) illustrate the flaw in such procedures: It is too easy to get good results. Nitrite recovery from the spikes was very good, even for the AOAC colorimetric determination which is sensitive to residual ascorbate. Slurries, while useful for establishing mathematical parameters, are too uniform to serve as a test of the ruggedness of a determination procedure. The residual nitrite in the slurries was high and relatively uniform. Charcoal addition to the slurries resulted in nitrite concentration in measurements equivalent to the alkaline AOAC or CLD techniques (4), but in commercial samples, charcoal addition failed at low nitrite levels. Again, the necessity of testing procedures of both sample preparation and nitrite determination in a wide variety of products is confirmed. This does not imply that comparing techniques in model systems and then applying the technique of choice to commercial samples is sufficient. Rather, a formal testing of all techniques of interest in commercial products is required.

Nitrite Partitioning

The forms in which nitrite occurs in cured meat have not been fully defined. Part may occur as nitric oxide in the pigment (12), part perhaps as nitrosothiols (13), and part as some as yet unidentified small molecules (14). Nitrite, in the form of nitrosothiols or specifically bound to proteins, will be in the precipitate because most of the protein is the water-insoluble actomyosin, which contains the greater part of the free sulfhydryl groups of meat. Nitrite bound or reacted with the reducing compounds, primarily coenzymes or added ascorbate, will be in the supernate. The first step in analyzing sample preparation procedures is determining the partitioning of nitrite between the fractions. Mirna (15) and Olsman and van Leeuwen (13), in a study of the effect of mercuric chloride on nitrite in the precipitate, concluded that the nitrite was in the form of nitrosothiols, but they did not determine total nitrite nor did they study any other sample preparation procedure. We find that on average the nitrite partitions equally between the supernate and precipitate, and that the AOAC heating procedure is effective in releasing the protein-bound nitrite. In this regard, Rougie et al. (16) found that the nitrite could be freed from the precipitate simply by repeated extractions. The effect of heating is therefore twofold: to eliminate ascorbate and other reduced substances that interfere in the Griess reaction, and to free protein-bound nitrite.

Summary

Differential colorimetric analysis of a variety of commercial cured meats demonstrates that residual ascorbate is the major factor in reduced pigment formation in nitrite determination of these products. While the AOAC procedure of sample preparation is fairly effective in eliminating the interference, making the sample alkaline before heating improved yields and reduced ascorbate interference, regardless of the kind or initial acidity of the products. In the previous study on meat slurries, charcoal addition provided results equal to the alkaline-AOAC or CLD procedures, except that at lower nitrite concentrations in cured meats, it gave lower measured nitrite values. Nitrite determination by the AOAC reagent combination/procedure is highly sensitive to residual ascorbate and while the alkaline-AOAC sample preparation apparently did not completely eliminate the interference, the 3 reagent combinations gave values acceptably close to each other. As a result of these studies it is evident that the AOAC sample preparation procedure is acceptable but could be improved by making the samples alkaline before heating, which may necessitate addition of Zn^{++} ions to clarify the solutions. The nitrite determination procedure of the AOAC method is not a good technique because it gives low values for measured nitrite. It should be changed, preferably to the simultaneous addition of sulfanilamide and 1-naphthylamine, although the latter reagent may not be acceptable because it is a potential carcinogen.

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CACAO PRODUCTS

Nonaqueous Reverse Phase Liquid Chromatographic Analysis for Cholesterol in Milk Chocolate

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A method using liquid chromatography was developed for the analysis of cholesterol in milk chocolate products. The method involves saponification of the sample with methanolic KOH followed by extraction with ether. Potentially interfering components are eliminated through the use of a silica Sep-Pak cleanup step before injection. The nonaqueous reverse phase LC system consists of a C_{18} column and an isopropanol–hexane mobile phase with direct detection at 205 nm. Recoveries of 1, 3, and 5 mg cholesterol added to 1 g sample of milk chocolate were 88.6, 102.8, and 110.1%, respectively. Studies conducted with [4-^{14}C]-cholesterol were undertaken to further document the accuracy of the method.

The need to quantitate cholesterol content in foods has become increasingly important for 2 major reasons. The first is the health concern about the atherogenic role of excess plasma cholesterol and the influence of dietary intake on the level of plasma lipids (1, 2). It has recently become apparent that the development of atherosclerosis and coronary heart disease might be largely due to decreased levels of high-density lipoproteins (HDL). This appears to be due to the function of HDL in transporting cholesterol out of the arterial wall while inhibiting the uptake of low-density lipoprotein by vascular smooth muscle. Recent studies using cholesterol-fed animals have suggested that disturbed HDL metabolism might be a factor in the production of experimental atherosclerosis (3). The second reason deals with federal regulation regarding the nutritional labeling of foods (4).

Liquid chromatography (LC) has been used extensively for the analysis of lipids in foods (5–8). The analysis for steroids, especially cholesterol, has routinely been accomplished by gas chromatography. More recently, liquid chromatography has been applied to cholesterol determination through the use of UV-absorbing derivatives which usually involve lengthy sample preparation (9–11). Enzymatic methods have been proposed for steroid analysis, but problems result when other sterols are present because the enzyme attacks the common sterol site (12). Official AOAC methods for the determination of cholesterol in foods are lengthy and laborious. In fact, there is no officially recognized method for the determination of cholesterol specifically in milk chocolate.

Other modes of separation including reverse phase, ion-pairing, and normal phase were evaluated for this determination. None of these modes exhibited the selectivity for separation of stigmasterol and cholesterol that was available by nonaqueous reverse phase chromatography.

In this paper, a method is described for determining cholesterol in milk chocolate by using a nonaqueous reverse phase LC technique with detection at 205 nm. Samples are extracted and interferents are removed on a cleanup cartridge. The method is accurate and precise.

Experimental

Reagents

(a) *LC mobile phase.*—Isopropanol–*n*-hexane (UV grade) (0.1 + 99.9) (Fisher Scientific Co. and Burdick and Jackson Laboratories, Inc., respectively).

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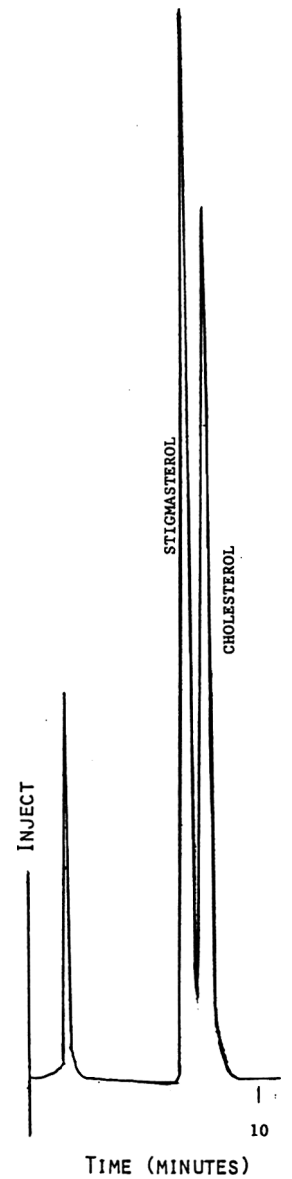

Figure 1. Chromatographic separation of stigmasterol–cholesterol standard, 1.00 μg/μL.

(b) *Extraction materials.*—Diethyl ether (preservative-free) (Burdick and Jackson). Petroleum ether (bp 30–60°C) (Fisher Scientific). Ten percent aqueous NaCl (w/v) from saturated stock solution of 31.7 g/100 mL. Saponification reagent, 0.5N KOH–methanol, prepared fresh daily.

(c) *Cocktail.*—Betacount liquid scintillation cocktail (J. T. Baker).

(d) *Standards.*—Cholesterol standard (Pfanstiehl Laboratories), at 0.1 mg/mL in LC mobile phase. [4-^{14}C]-cholesterol (0.02 mCi/mL) in benzene (New England Nuclear).

Apparatus

(a) *Liquid chromatograph.*—M6000A solvent delivery system (Waters Associates). Model 7125 injector with 50 μL

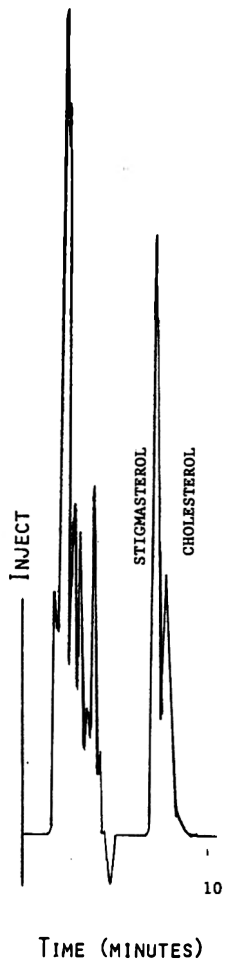

Figure 2. Chromatogram of milk chocolate sample.

loop (Rheodyne); C_{18} column (25 mm × 4.6 mm id), 10 μm particle size (Alltech Associates); Model 100-40 variable wavelength detector at 205 nm (Hitachi); Model-DKR 10 mV recorder (Sargent-Welch); ITG-4A digital integrator (Shimadzu Scientific, Inc.). The flow rate was 2.0 mL/min.

(b) *Liquid scintillation counter (LSC).*—Model 6881 Mark III liquid scintillation system (Tracor Analytical).

Procedure

Samples were placed in a freezer at < −15°C until completely frozen. A sample was then ground in a blender until the particle size was diminished and passed through a 710 μm sieve (Tyler Equivalent 24 mesh). Any portion of sample that did not pass through the sieve was refrozen and reground until all sample particles were reduced to <710 μm. This must be done to ensure a homogeneous sample, especially milk chocolate samples that contain nut meats.

About 1 g ground frozen sample was weighed (± 0.1 mg) and placed in a 250 mL round-bottom flask. Fifty mL freshly prepared 0.5N KOH–methanol was added and the sample was refluxed 30 min. The solution was transferred quantitatively while warm to a 250 mL separatory funnel. The round-bottom flask was washed with two 25 mL portions of water, which were added to the separatory funnel. This solution was cooled to room temperature and 10 mL 10% NaCl was added. The resulting solution was partitioned twice with two 100 mL portions of ethyl ether–petroleum ether (50 + 50). The ether phases were collected in a 250 mL round-bottom flask and flash-evaporated to dryness. Fifty mL petroleum ether was added to the sample and mixed by ultrasound until all sample

Table 1. Recovery of cholesterol added to 1 g milk chocolate

Added, mg	Rec., mg	Rec., %
0	0.23	—
1.0	1.09	88.6
3.0	3.32	102.8
5.0	5.76	110.1
Av.		100.5

Table 2. Survey of chocolate products for cholesterol

Product	Cholesterol, mg/g	CV, % (N = 5)
Pure milk chocolate	0.23	2.1
with almonds	0.14	3.4
with spanish peanuts	0.19	4.7
Dark chocolate	ND[a]	—
Baking chocolate	ND	—
Chocolate liquor	ND	—

[a]Not detected.

was redissolved. Any particulate matter was allowed to settle; 10.0 mL solution was withdrawn with a 10 mL glass syringe and passed through a silica Sep-Pak cartridge. The cartridge was washed with 10 mL ethyl ether–petroleum ether (7 + 93). The cholesterol-containing fraction was then eluted with 10 mL ethyl ether–petroleum ether (75 + 25). The cholesterol fraction was evaporated to dryness and diluted to volume in LC mobile phase. Samples and standards were injected onto the column in duplicate. Results were calculated by comparing peak areas obtained by injection of samples and standard. Figures 1 and 2 show sample chromatograms of cholesterol standard and milk chocolate. Figure 1 shows the separation between cholesterol and another plant sterol, stigmasterol, which is present in chocolate.

Results and Discussion

The analytical method described for cholesterol was evaluated for precision, accuracy, and linearity. The linearity study was conducted by repeated 20 μL injections of standard from a series of stock dilutions. The cholesterol standard was linear over the 400-fold range tested (0.052–20.72 μg/injection), with a correlation coefficient of 0.999. A precision study of cholesterol standard at 20.72 μg/injection (N = 12) and milk chocolate at 0.176 mg/injection (N = 4) resulted in coefficients of variation 2.0 and 5.7%, respectively. The precision for milk chocolate was less because of the small amount analyzed: only 3 times the lower limit of detection (3 × S/N). Recovery studies for milk chocolate are profiled in Table 1. The method exhibits a lower limit of detection of about 0.05 mg/g for the milk chocolate matrix, using 1 g sample.

In the method evaluation study, 0.2 μCi of [4-^{14}C]-cholesterol was added to the sample at the beginning of the procedure. Fractions (2 mL) were collected through the entire procedure, placed in LSC vials with 10.0 mL cocktail, and counted. The results, based on percentage of total decays, indicate that 98.8% of the cholesterol was extracted with the first 100 mL ether partition, with the remaining 1.2% collected with the second partition. These data show that the method is very effective in recovering cholesterol.

Before cartridge cleanup, the samples contained an excessive number of other components that resulted in an overloading of the column and detector at the wavelength of choice. The cleanup step removed these components and allowed interference-free analysis.

Table 2 shows a product survey of milk chocolate bars plus baking chocolate. Results indicate that the cholesterol content in milk chocolate is derived solely from the presence of

milk; samples without animal milk components had no trace of cholesterol.

In conclusion, an effective LC method has been developed for analysis for cholesterol in milk chocolate and a variety of milk chocolate products. Before this study, cholesterol content in milk chocolate products was based on literature values for cholesterol in milk, with adjustment to account for the percentage of milk in the final product. Using this method, actual cholesterol content is easily determined.

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DRUGS IN FEEDS

Liquid Chromatographic Determination of Sodium Salinomycin in Feeds, with Post-Column Reaction

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A method for the detection and quantitation of sodium salinomycin in feeds by a liquid chromatographic, post-column reaction system is presented. Sodium salinomycin is leached from feed with hexane, isolated as a dried residue, and dissolved in mobile phase for analysis. The drug is separated from interfering substances on a silica LC column, combined with vanillin reagent in a mixing tee, reacted with vanillin at elevated temperature in a coil, and detected in the effluent stream at 527 nm. Response, as peak area, is linear with concentration. Feed components, other common veterinary drugs, and closely related compounds do not interfere. Average recovery and coefficient of variation (CV) values for liquid spiked feeds and supplements were 101 ± 3.5% and 100 ± 0.3%, respectively. Results for laboratory blends and commercial scale feeds were within 6% of intent with CV values ranging from 0.4 to 5.1%.

Sodium salinomycin is a monocarboxylic polyether antibiotic having antimicrobial and anticoccidial activity (1, 2). The use of sodium salinomycin in swine and poultry feeds has created a need for a specific assay applicable to rations containing as little as 25 ppm drug. Liquid chromatography (LC) offers several advantages for determination of drugs in feeds, but its application to sodium salinomycin was hindered by the lack of a suitable means of detection. The drug has neither a strong chromophore nor electrochemical activity. The formation of a detectable derivative by an in-line, post-column reaction is a useful approach to this type of problem. Reviews of this technique have been made by Krull (3) and van der Wal (4).

The use of vanillin as a post-column reagent for sodium salinomycin was investigated. Vanillin has been used in conventional colorimetric analysis of certain other polyether antibiotics, i.e., lonomycin (5) and monensin (6), and for various other classes of compounds (7). Under anhydrous, acidic conditions, sodium salinomycin and vanillin form a product that absorbs strongly in the visible region. Heat is necessary to drive the reaction at a rate that is rapid enough for post-column use, but the temperature requirements are within practical limits of the system. Some natural components of feeds are also vanillin-reactive or absorb in the visible region, but these are separated from the drug on the LC column.

The feed sample is prepared for analysis as follows: Sodium salinomycin is leached from feed with hexane, the leachate is filtered and evaporated to dryness, and the residue is reconstituted with mobile phase and centrifuged. The final solution is injected into the LC-post-column reaction system where the silica column separates sodium salinomycin from interfering substances, the mixing tee combines the drug eluate with vanillin reagent, the reactor coil provides thermal energy for the reaction, the detector measures the absorbance of the sodium salinomycin vanillin reaction product at 527 nm, and the recorder/integrator measures the response of the peak in area units.

The proposed method was satisfactory with respect to accuracy and precision, linearity over a wide working range, applicability to a wide variety of feeds, and absence of interference from other drugs, closely related substances, and common feed additives.

Dimethylaminobenzaldehyde, which has been used in a similar procedure for sulfa drugs in feeds (8), was also evaluated for application to sodium salinomycin. The reagent formed a colored product with the drug, but was not compatible with the chromatographic system of choice. Recently, at the Eighth Annual Spring Workshop (1983) of AOAC, J. M. Rodewald et al. (Eli Lilly and Co., Greenfield, IN) described the use of vanillin as a post-column reagent for monensin in feed. This procedure, which had been developed independently, was similar in many respects to ours and was also capable of separating and responding to sodium salinomycin.

METHOD

Reagents

(a) *Mobile phase.*—Ethyl acetate, isooctane, acetic acid, triethylamine (750 + 250 + 4 + 2). Filter under vacuum through 5 μm Mitex® filter (Cat. No. LSWPO4700, Millipore Corp., Bedford, MA 01730). Degas by stirring 10 min under vacuum. Prepare fresh every 2 days.

(b) *Vanillin reagent.*—Dissolve 15.0 g vanillin (min. 99%), Cat. No. V110-4 (Aldrich Chemical Co., Milwaukee, WI 53201), in 480 mL ethanol. Filter under vacuum through 5 μm Mitex filter containing a prefilter pad (Cat. No. AP25 04700, Millipore Corp.). Degas by stirring 10 min under vacuum. While stirring gently, add 10 mL conc. H_2SO_4 dropwise and then wash down walls of container with 10 mL degassed ethanol. Protect solution from light. Prepare fresh daily.

(c) *Sodium salinomycin standard solution.*—Standard solution A—Dissolve 25.0 mg reference sample of sodium salinomycin in enough mobile phase to make 50.0 mL. Standard solution B—Volumetrically dilute 4.00 mL standard solution A to 100 mL with mobile phase. Prepare standard solutions fresh every 2 days.

Apparatus

(a) *Liquid chromatograph with post-column reactor.*—See schematic, Figure 1. Two reservoir filters (Part No. 25531, Waters Associates, Milford, MA 01757). Reservoir tubing, PTFE, 3.2 mm od, 3.1 mm id (Part No. 26809, Waters Associates), length as required. Two Model 98750 LC pumps (Waters Associates), at 0.54 mL/min for mobile phase and 0.27 mL/min for reagent. Two in-line filters, Nupro® 7 μm (Part No. SS-1F-7, Hartford Valve and Fitting, Avon, CT 06001). Two pulse dampeners (Part No. 79457, Waters Associates). Model CV-6-UHPa-N60 injection valve (Valco Instruments Co., Houston, TX 77055), with 50 μL loop and connectors to 1/16 in. od steel tubing. Mixing tee, 316 ss, 0.009 in. id, low dead volume (Part No. 75215, Waters Associates) with connectors to 1/16 in. od steel tubing. Tubing, 304 ss, 1/16 in. od, 0.010 in. id (Cat. No. HGC-001, Foxboro/Analabs, North Haven, CT 06473), 25 ft length for reactor coil, and

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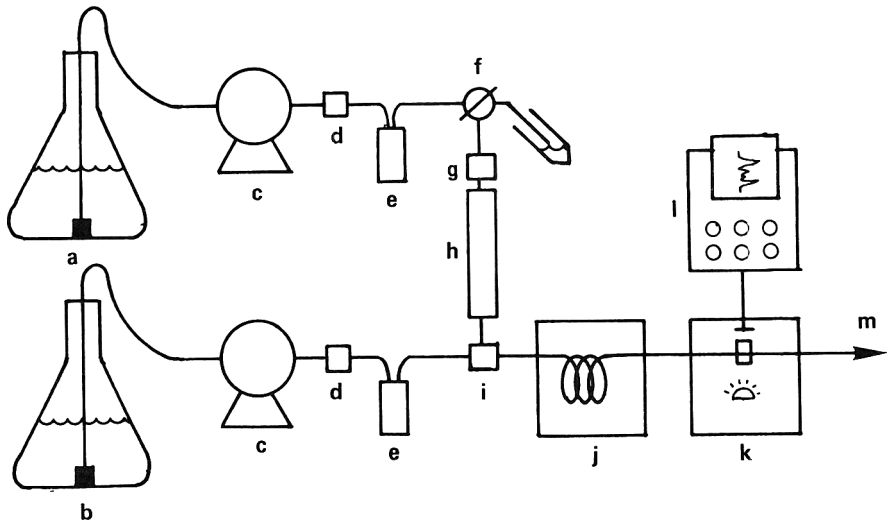

Figure 1. Schematic for LC-PCR system. a, mobile phase reservoir with filter and PTFE tubing; b, reagent reservoir with filter and PTFE tubing; c, pump; d, in-line filter; e, pulse dampener; f, injection valve; g, guard column; h, analytical column; i, mixing tee; j, reactor coil in controlled temperature bath; k, spectrophotometric detector; l, recording integrator; m, waste.

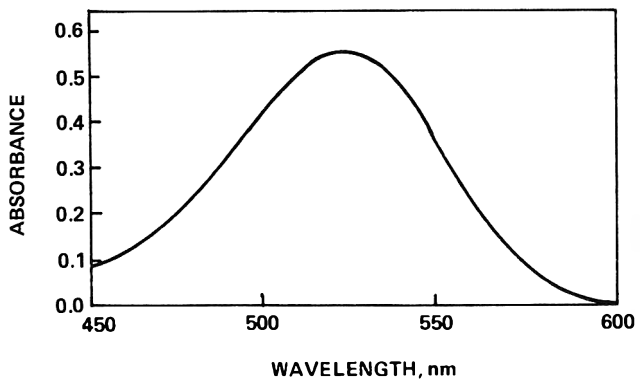

Figure 2. Visible region absorption spectrum of sodium salinomycin reaction product in mobile phase/reagent diluent system. Concn = 13.3 μg/mL, cell path = 1 cm.

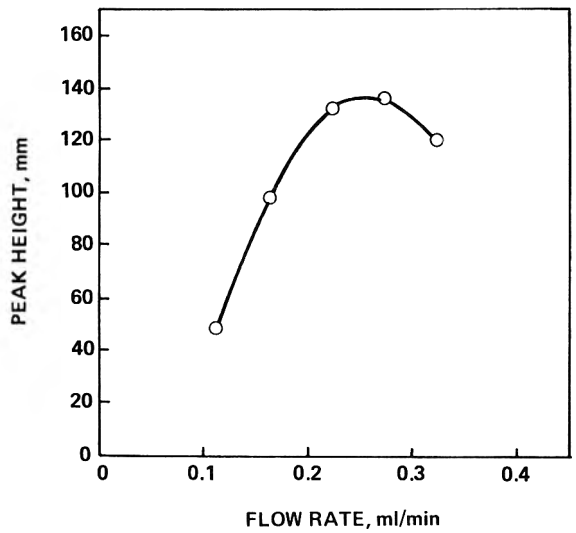

Figure 3. Response of LC-PCR system to sodium salinomycin as function of reagent flow rate.

lengths as required for all other connections. Insulated bath, 4 L capacity, filled with SF 81 (50) silicone fluid (General Electric Corp., Waterford, NY 12188). Immersion heater/circulator (Cat. No. 13-874-62, Fisher Scientific Co., Springfield, NJ 07081), at 95°C ± 0.2°C. Model 450 variable wavelength spectrophotometric detector (Waters Associates) at 527 nm, 0.1 AUFS. Model 3390A recording integrator (Hewlett Packard Co., Wallingford, CT 06492), at chart speed 0.2 cm/min.

(b) *Chromatographic column.*—4.6 mm id × 25 cm Zorbax® Sil (Part No. 850952701, DuPont Co., Wilmington, DE 19898), with Model 84550 guard column containing 400 mg Corasil II (Waters Associates).

(c) *Reciprocating shaker.*—Model 6005 (Eberbach Corp., Ann Arbor, MI 48106), at 180 cycles/min.

(d) *Rotary evaporator.*—Model PFE-1BN (Buchler Instruments, Inc., Fort Lee, NJ 07024) with vacuum pump, solvent trap, and 50°C bath.

Preparation of Samples

Weigh 20.0 g ground feed (25 ppm sodium salinomycin) or 4.00 g ground supplement (250 ppm sodium salinomycin) into 200 mL glass-stopper flask. Add 50 mL hexane (use 75 mL for very dry feeds), stopper tightly, and shake for minimum of 2 h, up to maximum of 18 h. Using three 25 mL hexane washings, quantitatively transfer mixture to Buchner funnel containing prefilter pad. Collect filtrate under vacuum in 500 mL round-bottom flask. Evaporate filtrate to dryness, or oily residue, with rotary evaporator. Using mobile phase solution, dissolve and quantitatively transfer residue to 50 mL volumetric flask for 25 ppm feeds or 100 mL volumetric flask for 250 ppm supplement. Adjust volume to mark with mobile phase and mix well. Clarify solution by centrifuging ca 35 mL at 1500 rpm for 20 min.

Determination

Condition column by successive 50 μL injections of standard B. When peak areas are reproducible to ±1%, inject standard and samples in the order B,S_1,S_2,B. Measure responses, R_B of standard and R_S of sample, as area. Calculate response factor F for standards as follows: $F = (p \times c_B) \div R_B$, where p = fractional purity of sodium salinomycin reference standard; c_B = concentration of standard B in mg/mL.

Using average response factor of 2 standards bracketing 2 samples, calculate sample potency as follows:

$$\text{ppm sodium salinomycin} = (F \times R_s \times \text{mL of } S \times 1000\ \mu\text{g/mg})/\text{g feed or supplement}$$

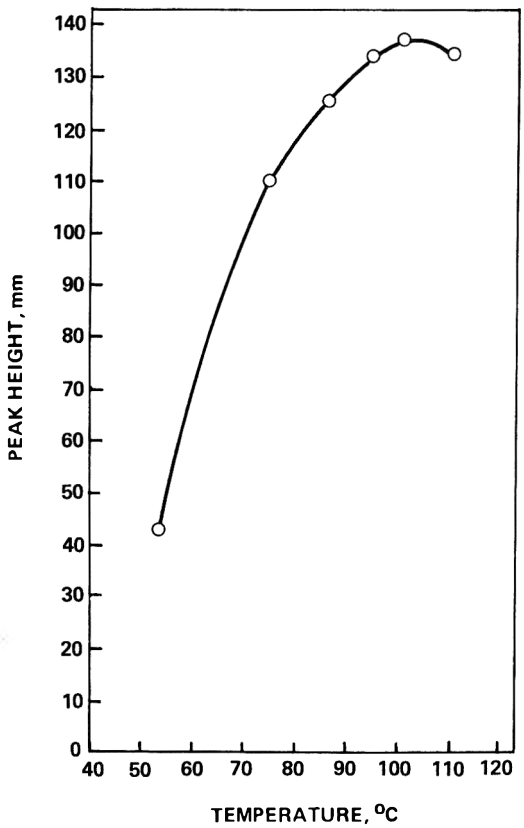

Figure 4. Response of LC-PCR system to sodium salinomycin as function of reactor coil temperature.

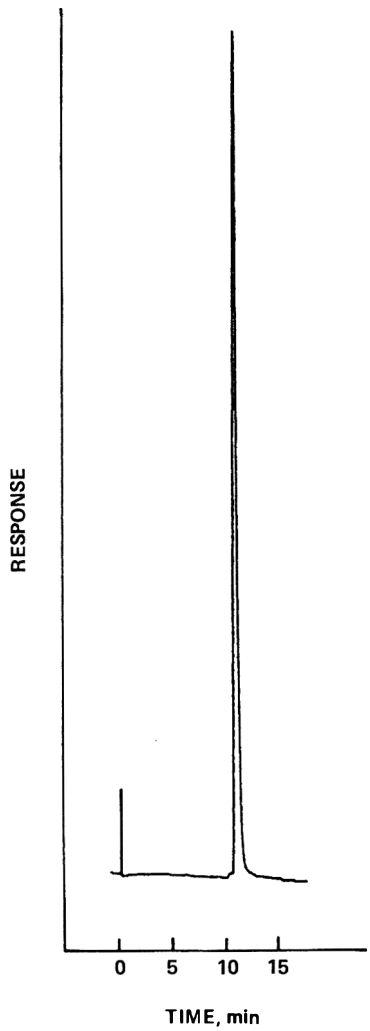

Figure 5. Response of LC-PCR system to sodium salinomycin reference solution. Concn = 10 μg/mL.

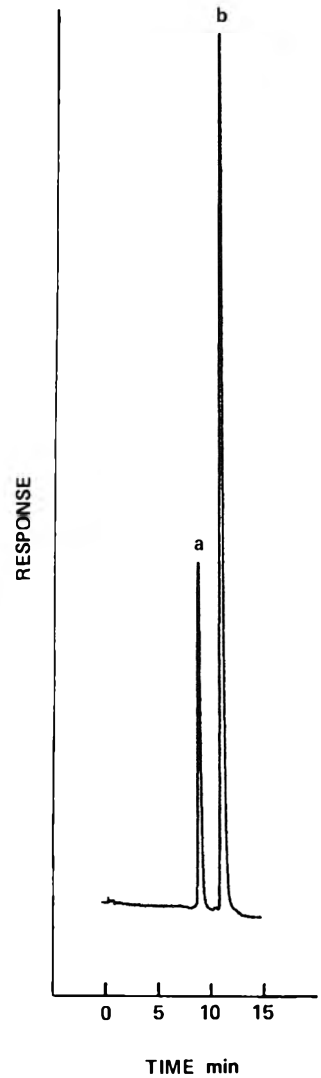

Figure 6. Separation of sodium salinomycin (b) from sodium 20-desoxy-salinomycin (a) in LC-PCR system. Concn = 10 μg/mL each component.

Note: mL S = 50 for 25 ppm feeds and 100 for 250 ppm supplements.

Results and Discussion

The response of sodium salinomycin in the system is affected by detection wavelength, vanillin reagent flow rate, and reactor coil temperature. Each of these parameters was optimized to provide maximum response to drug. The visible region absorption spectrum of the salinomycin-vanillin reaction product (Figure 2) shows the detector response is greatest at the absorption maximum at 527 nm. Measurement of drug response as a function of vanillin reagent flow rate shows that response is greatest at 0.27 mL/min (Figure 3). Color development is sub-optimal at lower flow because of unfavorable reagent-to-drug ratios, and at higher flow because of shorter residence time in the reactor coil. Measurement of drug response as a function of reactor coil temperature shows that response is close to maximum at 95°C (Figure 4). At lower temperatures color development is incomplete, and at higher temperatures the colored complex degrades and the effluent stream vaporizes.

Under the optimized conditions, sodium salinomycin responds as a single sharp peak eluting approximately 11.5 min after injection (Figure 5). Four columns from the same manufacturer exhibited retention times ranging from 10.8 to 14.1 min. Fifty percent full scale deflection (0.1 AUFS, 10

Table 1. Feed additives used to demonstrate absence of assay interference

Additive	Use level, ppm
Avoparcin	40
Bacitracin	500
Carbadox	55
Chlortetracycline	550
Copper sulfate	550
Flavomycin	4
Furazolidone	220
Gentian violet	8
Lasalocid acid	125
Lincomycin	110
Monensin	110
Morantel citrate	30
Neomycin	150
Olaquindox	100
Oleandomycin	250
Oxytetracycline	550
Pyrantel tartrate	106
Roxarsone	75
Sodium propionate	625
Tiamulin	200
Tylosin	1100
Valinomycin	125
Virginiamycin	110

mV recorder input) was obtained for 0.34 μg sodium salinomycin. Baseline stability was satisfactory with the equipment described. The added pulse dampeners were necessary, particularly in the reagent line, to suppress pump pulsations. Dampening requirements depend on the type of pumps used.

The system separates sodium salinomycin from 2 closely related compounds, sodium 20-desoxysalinomycin and sodium 20-oxosalinomycin. The separation of sodium salinomycin from sodium 20-desoxysalinomycin by LC-post-column reaction is shown in Figure 6. Since sodium 20-oxosalinomycin does not react with vanillin, its separation cannot be demonstrated by this system. However the separation of all 3 compounds was demonstrated by installing a 280 nm detector immediately after the analytical column. Although the compounds absorb weakly at this wavelength, response to a mixture containing 200 μg/mL of each was sufficient to demonstrate the separation (Figure 7). Separation factors, relative to sodium salinomycin, were 1.3 and 1.8 for sodium 20-oxosalinomycin and sodium 20-desoxysalinomycin, respectively. The capacity factor (k') for sodium salinomycin was 0.7 in this experiment.

The residence time of sodium salinomycin in the reactor coil is approximately 0.9 min. This value was determined using the LC-post-column reaction system with a 280 nm detector in line between the analytical column and the mixing tee. The residence time was taken as the difference in retention times of sodium salinomycin on the 2 detectors.

Numerous drug and nondrug feed additives (Table 1) were examined for potential interference at their normal use levels. None of these substances, with the exception of monensin, responded in the system. The monensin peak was resolved from that of sodium salinomycin (Figure 8). No attempt was made to investigate the applicability of this methodology to monensin because it was beyond the scope of this work.

A wide variety of nonmedicated feed and supplement formulations, (Table 2) representing formulas indigenous to major international marketing areas for this drug were examined for potential interference. None of these materials exhibited a peak eluting at or near the retention time of sodium salinomycin. Typical chromatograms for nonmedicated feed and supplement formulations are shown in Figure 9. A typical chromatogram for a medicated feed is shown in Figure 10.

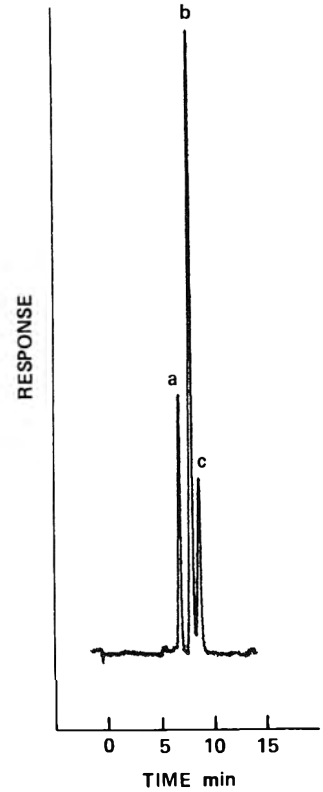

Figure 7. Separation of sodium salinomycin (c) from sodium 20-desoxysalinomycin (a) and sodium 20-oxosalinomycin (b) shown by direct monitoring of LC column effluent at 280 nm. Concn = 200 μg/mL each component.

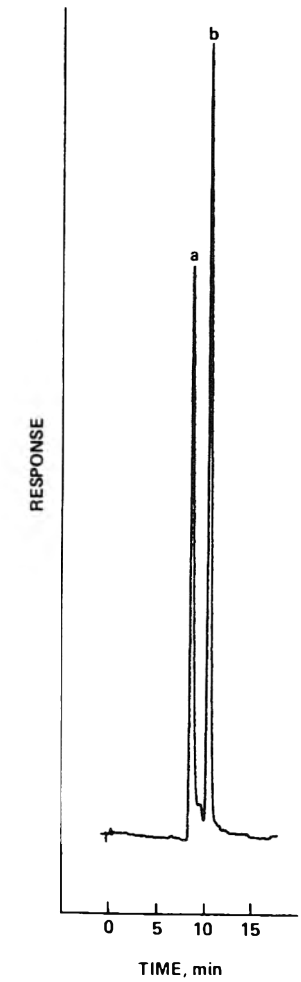

Figure 8. Separation of sodium salinomycin (b) from sodium monensin (a) in LC-PCR system. Concn = 10 μg/mL each component.

Table 2. Nonmedicated formulations tested for assay interference

Ration	Protein, %	Type of ration	Major components, >5%[a]
a	16	swine grower	corn, soybean meal
b	13	swine finisher	sorghum, rice bran, soybean meal, molasses
c	17	swine grower	corn, fishmeal, wheat bran
d	16	swine grower	barley, wheat, rapeseed meal, soybean meal
e	17	swine grower	wheat, barley, meat and bone meal
f	22	broiler starter	wheat screenings, corn, wheat, soybean meal, meat and bone scraps
g	23	broiler starter	corn, soybean meal
h	40	swine supplement	soybean meal, meat and bone scraps, alfalfa meal
i	42	swine supplement	soybean meal, meat meal

[a]Listed from higher to lower percentage. All rations contained iodized salt and vitamin and mineral premixes. Lower level ingredients in some formulations included limestone, dicalcium phosphate, animal fat, soybean oil, and choline chlorice.

Table 3. Linearity test in presence and absence of sample matrix[a]

	Feed assay		Supplement assay	
Statistic	With ration[b]	No ration	With ration[c]	No ration
Slope	0.415	0.407	0.040	0.039
× intercept, ppm	0.0	0.4	1.3	2.8
Correlation coefficient	0.9986	0.9997	0.9983	0.9998

[a]Salinomycin added by liquid spiking to represent 17.0, 25.0, and 31.5 ppm levels in feeds and 175, 250, and 325 ppm levels in supplements.
[b]Ration c, Table 2.
[c]Ration i, Table 2.

Table 4. Assay of feed and supplement spiked with sodium salinomycin solution[a]

	Sodium salinomycin found, ppm	
	Feed[b]	Supplement[c]
	24.2	245
	21.8	245
	22.4	245
	22.1	247
	23.0	245
	22.7	245
Av. ± SD	22.7 ± 0.8	245 ± 0.8
% of added	101	100

[a]Added: feed = 22.5 ppm, supplement = 245 ppm.
[b]Ration f, Table 2.
[c]Ration i, Table 2.

Table 5. Description of laboratory and commercial samples

Lot	Size, kg	Ration[a]	Form
1	0.25	f	meal
2	0.25	b	meal
3	0.25	e	meal
4	0.25	c	meal
5	35	h	meal
6	1361	b	meal
7	900	a	pellets

[a]Composition shown in Table 2.

Table 6. Assay results for laboratory and commercial samples

	Salinomycin, ppm[a]			CV,	%
Lot	Added	Found	Av. ± SD	%	of added
1	25.0	25.8,25.8,24.8,25.6,25.0,25.2	25.4 ± 0.4	1.6	102
2	25.0	25.0,25.2,25.0,25.0,25.0,25.3	25.0 ± 0.1	0.4	100
3	25.0	24.0,26.1,24.8	25.0	—	100
4	25.0	24.1,23.8,24.0	24.0	—	96
5	242	234,238,232	235	—	97
6	25.0	22.8,24.9,25.9,25.2,26.1,25.8 25.3,28.1,24.8,25.5,24.7	25.4 ± 1.3	5.1	102
7	23.5	22.4,21.9,22.3	22.2	—	94

[a]Results for large lots (5, 6, and 7) represent single determinations on individual, ground 500 g samples.

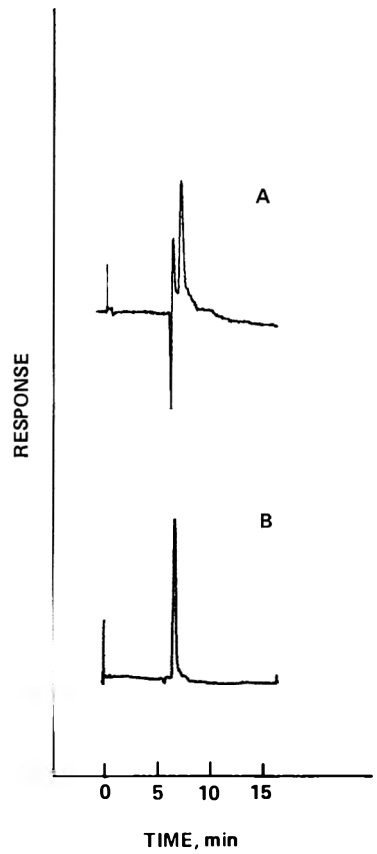

Figure 9. Response of LC-PCR system to nonmedicated feed (A) and supplement (B) samples.

Although the feed formulations did not contain interfering components, some of the rations caused a slight increase in the height-to-width ratio of the sodium salinomycin peak. This alteration of peak shape would cause up to 10% positive bias in some cases if calculations were based on peak heights. The bias is avoided by using peak area measurements.

The linearity of the system was evaluated over a range of 70–130% of label potency of feeds and supplements. Known quantities of sodium salinomycin in hexane solution were added to samples of nonmedicated feed (Ration c) to simulate analytical samples containing 17.0, 24.3, and 31.5 ppm of drug, respectively. Additional hexane was added to each sample to bring the total volume of solvent to 50 mL. These samples were shaken and assayed as described under *Method*. Corresponding samples, without feed, were prepared in the same manner to evaluate the influence of the feed matrix on linearity. A parallel experiment was performed using unmedicated supplement (Ration i) to simulate analytical samples containing 175, 250, and 325 ppm of drug. Least squares linear regression analysis of the data for the feeds and supplements (Table 3) demonstrates that linearity is excellent over the

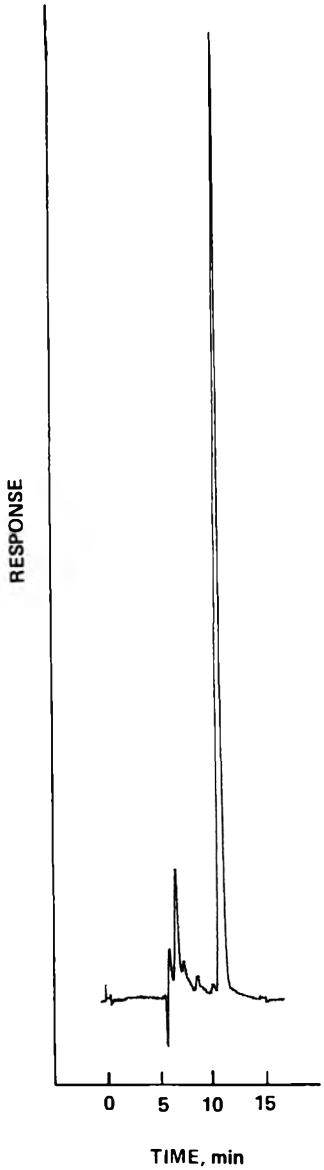

Figure 10. Response of LC-PCR system to feed containing 25 ppm sodium salinomycin.

range of 70–130% of label claim, intercepts are near zero, and the sample matrices do not affect slope, linearity, or intercept.

The solution spiking procedure described above was also used to evaluate accuracy and precision. Six samples each of nonmedicated feed (Ration f) and supplement (Ration i) were spiked with sodium salinomycin to simulate analytical samples containing 22.5 and 245 ppm of drug, respectively. The results, which are shown in Table 4, demonstrate good accuracy and precision.

Additional spiking experiments demonstrated the following: Drug recovery was not affected by a high level (7%) of added animal fat. The additional fat did not produce a chromatographic response. However, the recovery of sodium salinomycin was significantly decreased by the addition of 5% bentonite to feed.

Laboratory and commercial scale lots of medicated rations were assayed by the proposed procedure. Information relative to batch size, composition, and form of these medicated rations is shown in Table 5. The results (Table 6), show that

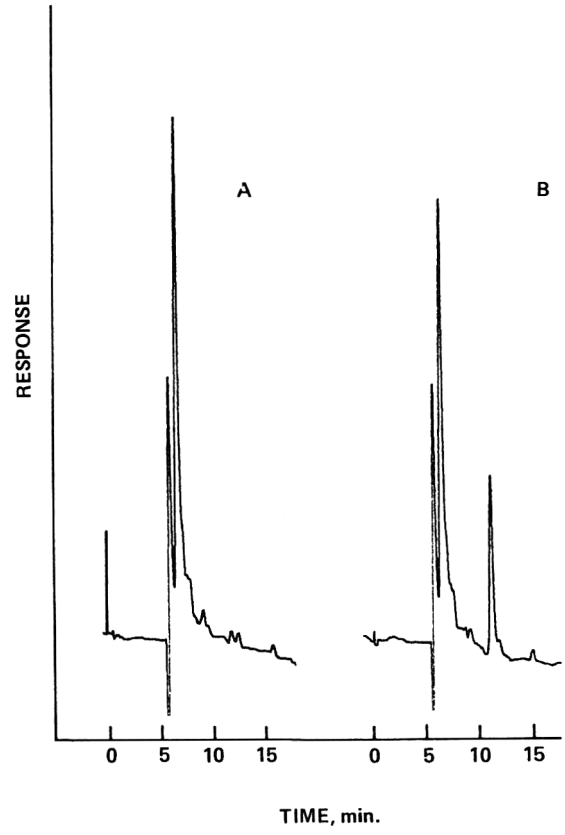

Figure 11. Response of LC-PCR system to nonmedicated feed (A) and feed containing 1 ppm sodium salinomycin (B). AUFS = 0.04, S = 25 mL.

in each case the amount of sodium salinomycin found was in good agreement with the amount added. The pelleted lot exhibited slightly lower recoveries relative to meals. Approximately 1% of that loss was due to uptake of moisture on pelleting. Coefficients of variation, calculated for samples with 6 or more replicates, demonstrated good precision.

A single preliminary experiment involving one feed suggested that the method has potential for low-level analysis. Using a final dilution volume of 25.0 ml and a detector setting of 0.04 AUFS, 1 ppm of sodium salinomycin in a spiked feed gave a significant response (Figure 11).

Acknowledgments

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Liquid Chromatographic Determination of Oxfendazole in Swine Feeds

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A relatively simple analytical method is presented for determination of oxfendazole (2-(methoxycarbonylamino)-5-phenylsulfinyl-benzimidazole) at levels as low as 0.012% in swine feeds, using cation exchange liquid chromatography (LC). The sample was extracted with a solvent mixture of methanol–glacial acetic acid (90 + 10) at 45°C, using a gyrorotory shaker. Plant pigments and other feed excipients were removed using zinc acetate treatment and pH-controlled extraction. Oxfendazole was further separated from the remaining interferences and quantitatively determined by LC on a Partisil SCX column with acetonitrile–0.01M phosphate buffer as mobile phase. The method is stability-specific, linear, precise, and accurate at 80–120% labeled strength (relative standard deviation 0.9–1.7 with mean recovery of 98–99%). Supporting data at a level of 0.0135% oxfendazole in swine feed indicated that this method is capable of complete recovery of oxfendazole from medicated swine feeds.

Oxfendazole (2-(methoxycarbonylamino)-5-phenylsulfinyl-benzimidazole) is a veterinary anthelmentic (1). It is available in suspension, paste, and top dressing pellets formulations. For swine treatment, oxfendazole has been developed in premix formulations of 1.35% and 6.75%. These are mixed with swine feed to result in final concentrations of 0.012–0.03% oxfendazole.

An analytical procedure was required to determine the dosage and to ensure homogeneity as well as stability of oxfendazole in feed. Liquid chromatography (LC) has been successfully used to quantitate benzimidazole-type compounds (2). Numerous other methods using LC for determination of mebendazole and its metabolites, as well as thiabendazole, in human plasma and other body fluids, banana, and citric fruits have been also reported (3–8). These methods generally use a variety of extraction and cleanup procedures before LC quantitation. The objective of this work was to develop a rapid, reliable, and stability-indicating analytical method for oxfendazole in various swine feeds. The method used a unique combination of procedures specifying zinc acetate treatment and pH-controlled extraction for cleanup. The final quantitation was done by strong cation exchange LC using 2-guanadinobenzimidazole as internal standard.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Model 3500 equipped with Model 8200 fixed wavelength detector set at 254 nm, System I integrator (Spectra Physics, Santa Clara, CA) and 100 μL loop injector (Altex, Berkeley, CA). General operating conditions: detector 254 nm, recorder 10 mV, chart speed 0.5 cm/min, and flow rate 1.5 mL/min.

(b) *Chromatographic column.*—25 cm × 4.6 mm id stainless steel column containing 10 μm bonded strong cation exchange packing (Partisil PSX-SCX®, Whatman Inc., Clifton, NJ).

(c) *Mobile phase.*—Acetonitrile (suitable for liquid chromatography)–0.01M phosphoric acid solution–0.01M monobasic potassium phosphate solution in water purified by ultrafiltration (50 + 25 + 25). Mix, filter, degas, and bring to ambient temperature.

(d) *Gyrorotary shaker with temperature control.*—Gyrorotary shaker model G24 (New Brunswick Scientific Co., Inc., NJ).

(e) *Heavy duty blender with 1 cup jars.*—(Osterizer Corp., Milwaukee, WI).

(f) *Oxfendazole reference standard.*—As received, not less than 98% pure (Institute of Organic Chemistry, Syntex Research, Palo Alto, CA). Prepare reference standard solution by dissolving oxfendazole in methanol, using sonication. Final concentration is about 16 μg/mL.

(g) *Internal standard.*—2-Guanadinobenzimidazole (Aldrich Chemical Co., Inc., Milwaukee, WI). Prepare internal standard solution by dissolving 2-guanadinobenzimidazole in methanol, using sonication. Final concentration is about 20 μg/mL.

(h) *Calibration standard solution.*—Deliver 15.0 mL oxfendazole reference standard (f) and 15.0 mL internal standard (g) solutions into 250 mL boiling flask. Evaporate to dryness, using nitrogen stream and steam bath. Dissolve residue in 10.0 mL mobile phase, using sonication.

(i) *Extracting solvent.*—Mix 100 mL glacial acetic acid, analytical reagent, and 900 mL methanol, analytical reagent.

(j) *Zinc acetate solution (20%).*—Dissolve 100 g zinc acetate dihydrate in ca 300 mL purified water, using magnetic stirrer. After dissolution of zinc acetate, add 1.5 mL glacial acetic acid, analytical reagent, and adjust volume to 500 mL, using purified water.

(k) *Salts solution.*—Dissolve 50 g sodium chloride and 35 g dibasic potassium phosphate in sufficient purified water to make 1 L solution.

Preparation of Sample Extract

Pulverize feed sample by precracking pellets with wooden mallet and then grinding in blender ca 2 min or until uniform powder is obtained. Accurately weigh 20 g sample into 250 mL glass-stopper Erlenmeyer flask. Add exactly 100 mL extracting solvent and incubate sample in gyrorotary shaker at 45°C for 30 min. Agitate sample at 250 rpm an additional 30 min and cool sample to ambient temperature. Decant ca 15 mL extract into test tube and centrifuge at 1000 rpm for 5 min. Accurately pipet 10.0 mL clear extract into 25 mL volumetric flask. Add 4 mL zinc acetate solution, mix thoroughly, and let stand 5 min to ensure complete precipitation of plant pigments. Fill flask to mark with deionized water and mix well. Transfer sample to test tube and centrifuge at 1000 rpm. Accurately pipet 15.0 mL extract into 250 mL separatory funnel containing 100 mL salts solution. Add 3.2 mL 1N sodium hydroxide solution to adjust to pH 8 and mix well. Add 50 mL dichloromethane and shake gently to extract oxfendazole. Let phases separate. Drain lower organic layer into 250 mL boiling flask through filtering funnel containing anhydrous sodium sulfate on cotton pledget. Repeat extraction procedure twice (for a total of 3 times), using 40 mL dichloromethane each time and always collecting organic layer in 250 mL boiling flask. Accurately add 15.0 mL internal standard solution and mix well. Evaporate to dryness with nitrogen stream and steam bath. Dissolve residue in 10.0 mL mobile phase, using sonic bath for 5 min. Filter sample solution into appropriate container through 2 μm polyvic filter (Millipore Corp., Bedford, MA) and inject 100 μL of each

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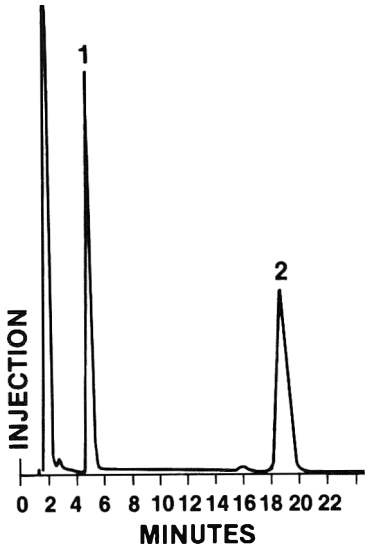

Figure 1. Chromatogram on strong cation exchange column of swine feed containing oxfendazole: (1) oxfendazole; (2) internal standard.

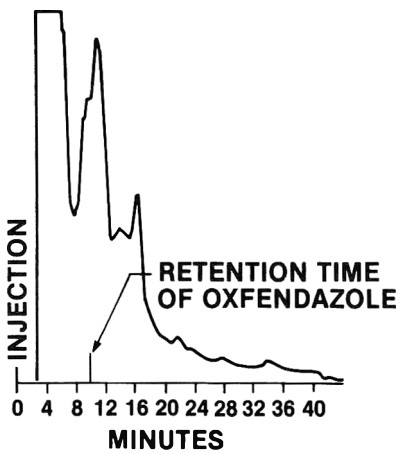

Figure 2. Chromatogram on strong cation exchange column of placebo swine feed methanolic extract.

sample and calibration standard solution. Figure 1 shows typical sample chromatogram. Quantitate drug by comparing oxfendazole/internal standard peak area ratios of extracted 127mples to calibration standard solutions.

Calculate percent oxfendazole as follows:

$$\text{Oxfendazole, \%} = (R_1/R_2) \times [(C/V) \times (15/10) \times 100]/[(SW/100) \times (10/25) \times (15/10)]$$

$$\text{Oxfendazole, \%} = (R_1/R_2) \times (C/SW) \times (25\,000/V)$$

where R_1 and R_2 are area ratios of oxfendazole/internal standard in sample and calibration standard, respectively; C = weight in g of oxfendazole used to prepare reference standard solution; SW = weight in g of feed sample; and V = volume of oxfendazole reference standard solution.

Results and Discussion

Animal feed is a very complex matrix including minerals, vitamins, and plant materials. Thus, a wide spectrum of organic compounds, such as flavonoids, carotenoids, xanthophylls, and many other pigments, could interfere with the quantitation of drugs in such matrixes. Therefore, the first step in performing a quantitative determination of a drug in a feed is the separation and removal of such interference.

Initial results of the assay procedure revealed that extraction with methanol at room temperature, using either an ultrasonic bath or a wrist-action shaker, failed to achieve complete

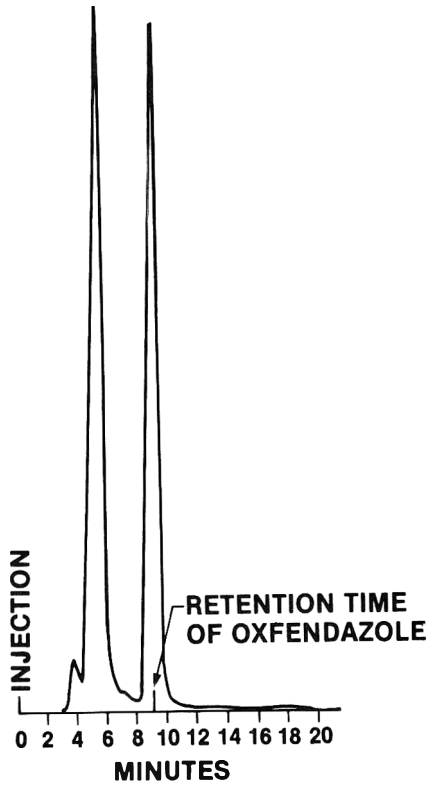

Figure 3. Chromatogram on strong cation exchange column of placebo swine feed methanolic extract after lead acetate treatment.

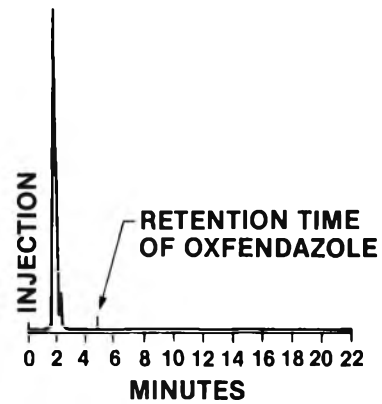

Figure 4. Chromatogram on strong cation exchange column of placebo swine feed methanolic extract after zinc acetate treatment and organic solvent extraction at pH 8.

recovery of oxfendazole. Further testing with a warm mixture of methanol and glacial acetic acid showed consistant but incomplete recoveries of oxfendazole. Possible reasons for the incomplete recovery were irreversible adsorption of oxfendazole to the feed matrix, losses during the extraction procedures, and incomplete dissolution of residues in mobile phase before injection onto the LC column. Several options, such as use of a radioactive tracer, use of an internal standard, and use of spiked placebo standards, are available to compensate for such losses. The internal standard option was chosen for convenience.

Originally, an analog synthesized in-house was used for the internal standard because it had extraction characteristics similar to oxfendazole. It was added at the beginning of the sample cleanup procedures, thus compensating for any losses during sample preparation. However, to make the method more versatile a commercially available internal standard was needed to replace the in-house compound. After an extensive search, a compound having both similar extraction characteristics and a suitable retention time was not found. It was

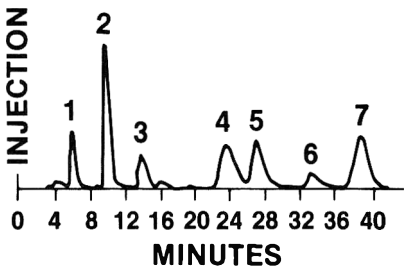

Figure 5. Chromatogram on cation exchange column showing resolution of oxfendazole and internal standard from possible degradation products of oxfendazole:
(1) 2-(methoxycarbonylamino)-5-phenylsulfonyl-benzimidazole; (2) 2-(methoxycarbonylamino)-5-phenylsufinyl-benzimidazole (oxfendazole); (3) 2-(methoxycarbonylamino)-5-phenylthio-benzimidazole; (4) 2-(amino)-5-phenylthio-benzimidazole; (5) 2-(amino)-5-phenylsulfonyl-benzimidazole; (6) 2-(amino)-5-phenylsulfinyl-benzimidazole; (7) 2-guanadinobenzimidazole (internal standard).

observed that the major losses of oxfendazole occurred during the evaporation process; therefore, the best of the chromatographically suitable compounds, 2-guanadinobenzimidazole, was chosen and added to the dichloromethane extracts before evaporation.

The use of lead acetate in the removal of interfering substances from agricultural products and plant extracts has been reported (9). For that reason, lead acetate treatment was tried first in removing some interfering components in the oxfendazole assay of feeds. Comparison of Figures 2 and 3 demonstrates the ability of lead acetate to remove some interfering compounds.

Zinc hydroxide was also successfully used in removing certain interfering components from feed extracts in determination of sulfanitran in animal feeds (10). Experiments in our laboratory showed that zinc acetate can replace lead acetate and zinc hydroxide in precipitation of interfering compounds.

After the zinc acetate treatment, further purification was accomplished by adjusting the methanolic extract to pH 8 and extracting with an organic solvent. Oxfendazole as an amine was extracted into the organic layer, while acidic interfering components were retained in the aqueous phase.

LC was chosen for final quantitation of oxfendazole because it offers sensitivity and a wide variety of chromatographic modes. The residue obtained after evaporation of the organic solvent extract was dissolved in mobile phase. The sample was chromatographed first on a C-18 reverse phase column (μBondapak C-18 by Waters Associates, Milford, MA). Such chromatograms showed numerous peaks including a large peak at the retention time of oxfendazole. When the same extract was chromatographed on a strong cation exchange column (Partisil-SCX), the chromatogram appeared clear of interfering peaks (Figure 4). Therefore, strong cation exchange chromatography was chosen for determination of oxfendazole in swine feeds.

Oxfendazole has been shown to have 5 possible degradation products (G. Thompson, Syntex Research, 1976). A mixture of these degradation products in the presence of oxfendazole and the internal standard was chromatographed using the LC parameters of the method. The chromatogram demonstrates that this method is capable of separating oxfendazole from all known degradation products, proving that the method is stability-indicating (Figure 5).

To test the efficiency and sensitivity of the method, recoveries of oxfendazole from swine feeds were studied. Twenty g aliquots of placebo swine feed were spiked with oxfendazole at the 0.012% level. The mean recoveries and standard deviations demonstrate that this method is accurate and precise (Table 1). A linearity plot of area ratio of oxfendazole to internal standard versus oxfendazole added showed that the response was linear between 0.0 and 7.2 μg oxfendazole injected.

Table 1. Recovery of 0.012% oxfendazole from swine feeds

	Analyst 1		Analyst 2	
Feed	Rec.,[a] %	No. of analyses	Rec.,[a] %	No. of analyses
Pig grower	99.0 ± 0.9	6	97.8 ± 1.7	6
Pre-farrowing	97.7 ± 1.7	6	—	—

[a] ± Standard deviation.

Table 2. Homogeneity of 0.0135% oxfendazole in swine feeds

Feed	Rec., %	SD, %	No. of analyses
Finisher	93.5	4.1	6
Growina	98.3	1.9	6
Startina	91.6	1.6	6

Table 3. Stability of 0.0135% oxfendazole in swine feeds (% recovery[a])

	Feed		
Time, mo.	Finisher	Growina	Startina
initial	93.0 (6)	98.6 (6)	91.6 (6)
0.5	96.3 (6)	—	97.3 (6)
4	96.0 (3)	104.6 (3)	96.6 (3)
7	95.0 (3)	99.6 (3)	99.3 (3)
9	95.0 (3)	104.0 (3)	98.6 (3)

[a] Number of analyses is in parenthesis.

Samples of swine feeds containing oxfendazole at 0.0135% level were analyzed using this method to evaluate the homogeneity of oxfendazole in the feeds. The results reveal that oxfendazole was evenly mixed in swine feeds (Table 2).

Tests were performed to determine the stability of oxfendazole in swine feed at 0.0135% level at room temperature. Samples of medicated swine feed were stored at controlled room temperature for several months and assayed periodically. The data demonstrated that oxfendazole was stable in swine feeds for a period of at least 9 months. No degradation products were observed in any of the samples (Table 3).

In conclusion, a relatively simple extraction, cleanup, and quantitation of oxfendazole in various swine feeds has been described. It should be noted that feeds used in the field differ from location to location such that minor modifications of the methodology may be necessary to remove extraneous interfering components.

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FOODS

Liquid Chromatographic Analysis of Sugars, Acids, and Ethanol in Lactic Acid Vegetable Fermentations

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Routine analysis of all the major substrates and products of homolactic or heterolactic acid vegetable fermentations was made possible by the use of 2 liquid chromatographic procedures. Sucrose, glucose, fructose, and mannitol were determined on a cation exchange column in the lead form. The coefficient of variation of the added compounds (2 to 30mM) in fermented cucumber juice with salt concentrations of 0–1.5% ranged from 2.0 to 3.1%. Sucrose (1–12.5mM), malic acid, lactic acid, acetic acid, and ethanol (2–25mM) added to cucumber juice containing 0–6.0% NaCl were separated on a reverse phase column; coefficients of variation ranged between 4.3 and 5.9%. Sample preparation requires only blending and filtration prior to injection.

Liquid chromatography (LC) has been applied to many analytical problems related to foods, including measurement of sugars (1–3) and organic acids (4, 5). Marsili (6) used gas chromatography to measure glucose, fructose, and lactic acid in cucumber juice fermentations. However, analysis of the major chemical changes that may occur during vegetable fermentation requires that at least 8 compounds, including sugars, organic acids, and ethanol, be measured. Salt, which is usually added to vegetables to direct the course of fermentation (7), is a serious interference problem for the analysis of sugars by LC. It will elute from both amine-derivatized silica columns and cation exchange carbohydrate columns as a large, broad peak with retention volumes similar to sugars. Modification of chromatographic characteristics of the salt (8) or removal of salt from the samples is required for analysis of sugars in the presence of salt. This paper describes a detailed procedure for the analysis of sucrose, glucose, fructose, mannitol, malic acid, lactic acid, acetic acid, and ethanol in vegetable fermentations.

Experimental

Apparatus

(a) *Pump.*—Model 6000A (Waters Associates, Inc., Milford, MA) or equivalent.

(b) *Injector.*—Rheodyne 7125 loop injector with a 10 μL loop (Rheodyne, Inc., Cotati, CA).

(c) *Detector.*—Refractive index, Model R401 (Waters Associates) or equivalent.

(d) *Integrator.*—Spectra-Physics Model 4100 (Spectra-Physics, San Jose, CA).

(e) *Column compression module.*—RCM-100 radial compression cell (Waters Associates).

(f) *Columns.*—Aminex HPX-87P 8% cross-linked strong cation exchange resin in the lead form, 9 μm particle size, 300 × 7.8 mm, column 68°C (Bio-Rad Labs, Richmond, CA) or equivalent for sucrose, glucose, fructose, and mannitol. Radial-Pak C_{18} reverse phase, 5 μm particle size, 100 × 8 mm (Waters Associates) or equivalent for sucrose, malic acid, lactic acid, acetic acid, and ethanol.

(g) *Circulating water bath.*—Model 2067 (Forma Scientific, Marietta, OH).

(h) *Guard columns.*—For the HPX-87P, dual-guard column system was required to remove NaCl from samples and protect analytical column. Micro-guard cation exchange cartridge packed with Aminex HPX-85H and anion exchange cartridge packed with Aminex A-25 were used (Bio-Rad Labs, Richmond, CA). Guard-Pak C_{18} pre-column insert (Waters Associates) was used for C_{18} Radial-Pak column.

(i) *Filters.*—Samples were clarified with either 0.2 μm (Metricel GA-8) or 0.45 μm (Metricel GA-6) pore size, 25 mm diameter membrane placed in reusable syringe filter holder (Gelman Sciences, Inc., Ann Arbor, MI).

Reagents

(a) *Mobile phases.*—Eluant for Aminex HPX-87P column was water, distilled and passed through Millipore MilliQ water purification system (Millipore Corp., Bedford, MA). Reverse phase column was eluted with 0.05M phosphoric acid with pH adjusted to 2.5 with concentrated NH_4OH.

(b) *Standard solutions.*—Combined standard for Aminex HPX-87P column contained 10mM sucrose and 20mM each of glucose, fructose, and mannitol. Combined standard solution for reverse phase column contained 10mM sucrose and 20mM each of malic acid, lactic acid, acetic acid, and ethanol. Impurities were not detected when 50mM concentrations of standard compounds were chromatographed. In all cases, sucrose was added at half the concentration of other compounds because its response factor for refractive index detection was higher than that for other sugars and its concentration in the commodities of interest was lower than that for glucose or fructose. Sucrose, glucose, fructose, mannitol, and L-malic acid were desiccated before use. Glacial acetic acid was used without treatment. Anhydrous, nondenatured ethanol was stored over Linde 4A molecular sieve before use. Crystalline L-lactic acid was stored dessicated and frozen. (It is very hygroscopic and must be weighed quickly to prevent significant moisture uptake.) All standards were obtained from Sigma Chemical Co. (St. Louis, MO), except acetic acid, Fisher Scientific Co. (Pittsburgh, PA), and mannitol, Aldrich Chemical Co. (Milwaukee, WI).

Spiked Juice Samples

To evaluate the effect of a typical sample matrix on accuracy and precision of the analysis, the compounds, which were analyzed on the reverse phase column, were added to juice extracted from fresh cucumbers. The cucumber juice contained only a small amount of malic acid and nondetectable levels of sucrose, lactic acid, acetic acid, and ethanol. Cucumbers ('Chipper' cultivar, 4–7 cm diameter) were cut

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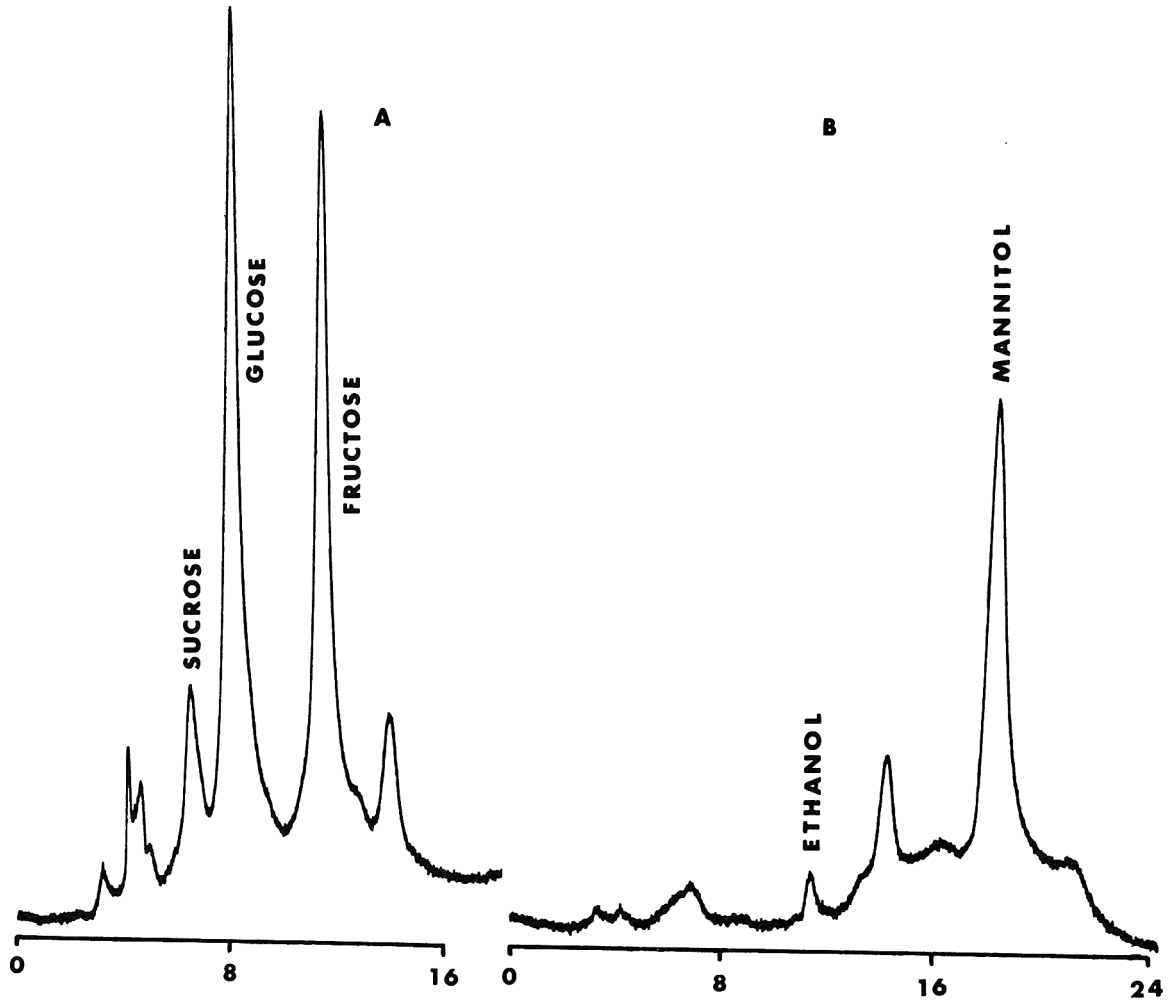

Figure 1. Sugar analysis of green bean juice before (A) and after (B) fermentation with *Lactobacillus cellobiosus*; 10 μL sample was chromatographed on Bio-Rad HPX-87P column with water as eluant at 0.9 mL/min. Concentrations of analyzed compounds were: sucrose, 2.6mM; glucose, 22.0mM; fructose, 27.8mM; mannitol, 26.5mM. Other peaks on chromatograms were unidentified compounds.

into 2–3 cm pieces and frozen. The pieces were thawed and put in a wine press for juice extraction. The juice was heated to boiling to inactivate enzymes, cooled, and frozen until use. Samples were prepared by mixing 25 mL juice, which had been adjusted to pH 3.4 with HCl, with the appropriate amount of an aqueous solution of 62.5mM sucrose, 125mM each of malic acid, lactic acid, acetic acid, and ethanol. Dry NaCl and water were added to bring the final volume to 50 mL. Samples were filtered through 0.2 μm filters and frozen until use. A set of samples was prepared which contained 4.0, 20.0, and 50.0mM of each compound (sucrose concentrations were half of this) and 0, 3.0, and 6.0% NaCl.

The samples without added NaCl were chromatographed on 4 occasions over a 2-day period. One set of triplicate analyses was performed on the samples with 3.0 and 6.0% added NaCl. Samples as prepared were mixed with an equal volume of 2 × concentrated elution buffer just before analysis. pH of these samples as injected into the LC column ranged from 2.27 to 2.67.

To evaluate matrix effects on sugar analysis with the HPX-87P column, sugars were added back to cucumber juice that has been fermented to remove all detectable sugars. Cucumber juice (700 mL) was filter-sterilized through a 0.2 μm sterile filter and inoculated with 1 mL of a 24-h culture of *Lactobacillus plantarum*, strain WSO, grown in MRS broth (9). Inoculated juice was incubated at 30°C. Once each day, a sample was titrated with NaOH. Sufficient 50% NaOH was added to the juice to readjust the pH to 4.5. This was continued until all detectable sugars were fermented as determined by LC. A total of 8.2 g 50% NaOH was added during fermentation. The completely fermented juice was filtered to remove bacterial cells. An aqueous stock solution of 50mM sucrose and 100mM glucose, fructose, and mannitol was prepared. Sugar stock solution was added to 25 mL fermented juice and water to prepare 50 mL solutions which contained 2, 15, and 30mM sugars (sucrose concentrations were half of this). Solutions with the same sugar concentrations were also prepared to contain 0.75 and 1.5% NaCl. These solutions were filtered through a 0.45 μm filter and frozen until use.

Samples were injected without dilution. Sugar concentrations were calculated relative to a 20mM aqueous solution of sugars (10mM sucrose). Four sets of triplicate injections of the samples without added NaCl were run over a 2-day period. Triplicate injections of the NaCl-containing samples were run once.

Preparation of Fermented Vegetable Samples

Fermented vegetables for analysis were prepared by blending samples without added liquid with a Tekmar tissue homogenizer. The homogenate was filtered through cheesecloth or centrifuged for 5 min at 2000 × g in a clinical centrifuge and then filtered by suction into a Vacutainer tube through a 0.45 μm, 25 mm filter fitted on a disposable syringe. Samples were frozen until analysis. Occasionally, fresh samples or samples collected early in a fermentation formed a precipitate after thawing. These samples were refiltered before chromatography.

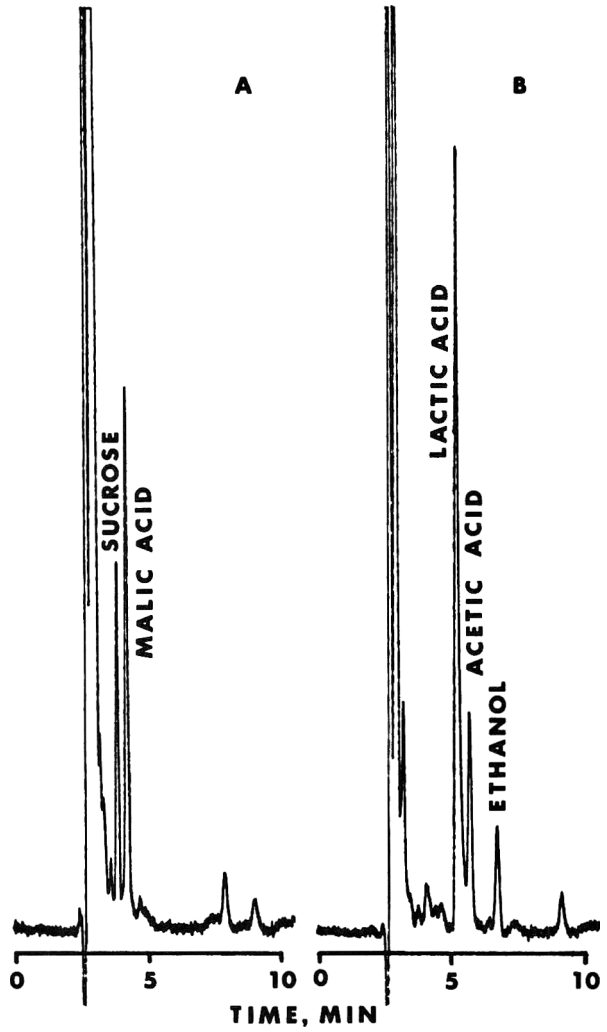

Figure 2. Sucrose, organic acid, and ethanol analysis of green bean juice before (A) and after (B) fermentation with *Lactobacillus cellobiosus*; 10 μL sample was chromatographed on Waters C_{18}, 5 μm Radial-Pak column with pH 2.5, 0.05M phosphoric acid as eluant at 1.0 mL/min. Concentrations of compounds were: sucrose, 2.6mM; malic acid, 9.3mM; lactic acid, 45.1mM; acetic acid, 20.3mM; ethanol, 21.7mM. Other peaks on chromatograms were not identified.

Brine samples were filtered through a 0.45 μm pore size filter directly into vacuum tubes and frozen. When brines were cloudy with suspended bacteria, the rate of filtration was improved by centrifugation of the sample for 5 min at 2000 × g to remove the bacterial cells.

Chromatographic Procedure

Sugar analysis on the HPX-87P column was done with the column in a water bath at 68°C with the guard columns at room temperature to prevent sucrose hydrolysis. A 10 μL sample was injected onto the column with a loop injector. Water was pumped through the column at 0.9 mL/min. Sugar concentrations were calculated by comparison to the peak height of 20mM standards (10mM for sucrose). If the samples contained NaCl, they were diluted with water so the salt concentration in the injected sample was 1.5% or less.

Samples for analysis of acids, ethanol, and sucrose on the C_{18} Radial-Pak column were mixed with an equal volume of 0.1M, pH 2.5 phosphate buffer before injection onto the column. This is done to reduce baseline upset at the solvent front and to protonate the organic acids. The flow rate was 1.0 mL/min. Sample component concentrations were calculated by comparison of the peak height with 20mM external standards (10mM sucrose).

For all analyses, the refractive index detector was set at an attenuation of 8× and 100 mV output. The SP4100 integrator was set for a 100 mV input. Peak height measurements were made with the integrator.

Results and Discussion

Chromatography of Sugars with Cation Exchange Column

Figures 1 and 2 show typical separations obtained from samples of fresh and fermented green beans, prepared by blending, filtration, and, in certain instances, dilution before injection into the chromatograph. Figure 1A shows the results of chromatography on the HPX-87P column. Sucrose, glucose, and fructose in the freshly brined beans were well separated. After fermentation (Figure 1B), mannitol was the major compound on the chromatogram. On this column, ethanol also eluted with a retention time almost identical to fructose. When both fructose and ethanol are present in a sample, the ethanol can be measured on the reverse phase column. The ethanol contribution can then be subtracted from the fructose peak on the HPX-87P column to estimate fructose concentration. Use of a column similar to the HPX-87P in the calcium instead of the lead form changes the relative retention of these compounds and may eliminate this problem.

The amount of NaCl in samples injected into the HPX-87P must be monitored because the guard columns have a limited exchange capacity. Approximately fifty 10 μL fermented vegetable sample injections, each with 1.25% NaCl, could be chromatographed before a visible salt peak would occur on the column. The guard columns can be used until the eluted NaCl interferes with a component of interest. When the component concentrates with high enough, samples were diluted to contain less than 1.5% NaCl before injection. Even at 1.5% NaCl, some baseline upset occurred which interfered with analysis of fructose.

New anion exchange guard columns typically will partially bind sugars. In one case, the peak heights of a standard solution of sugars in a new column were reduced by 13% for glucose, 14% for fructose and mannitol, and 22% for sucrose, relative to a conditioned guard column. A new guard column may be conditioned by pumping 68°C water through it for at least 5 h. For convenience, columns were often left in the water bath overnight before use. We do not know the mechanism by which heating the guard column eliminates sugar binding. As described above, guard columns must be kept at room temperature when analyses are performed.

Chromatography of aqueous solutions of glucose, fructose, and mannitol on the HPX-87P column resulted in a linear relationship between peak height and sugar concentrations over a 1–100mM concentration range. Table 1 shows results of the analysis of these compounds over a 2–30mM concentration range and sucrose over a 1–15mM range in fermented cucumber juice at each of 3 salt concentrations. The slope, intercept, coefficient of variation of the linear regression, and coefficient of determination for the relationship between added and analyzed sugar were calculated at each salt concentration. Overall values were those pooled over the 3 salt concentrations and calculated. The coefficients of variation at the 2mM concentration (1mM sucrose), pooled over the 3 salt concentrations, gave an estimate of variability at low sugar concentrations. Coefficients of determination (R^2) were 0.999 or higher for each compound at each salt concentration, indicating an excellent linear relationship between added and measured sugar concentrations in the fermented cucumber juice matrix. An ideal analysis should result in a linear rela-

Table 1. Regression analysis of analyzed vs added sucrose, glucose, fructose, and mannitol in completely fermented cucumber juice with 0, 0.75, and 1.5% NaCl added

Compound	NaCl concn, %	Slope[a]	Intercept[b]	CV over 2–30mM range, %	CV for 2mM concn, %
Sucrose	0	1.00	0.06	2.2[d]	
	0.75	1.03**	0.17*	1.4	
	1.5	1.03**	0.13*	1.1	
	Overall[e]	1.01	0.09	3.1	6.8[f]
Glucose	0	0.99**	0.25**	1.5	
	0.75	1.02	0.44	2.4	
	1.5	1.01	0.44*	1.9	
	Overall	1.00	0.31*	2.6	9.2
Fructose[c]	0	0.99	0.21**	1.5	
	0.75	1.01	0.43*	1.5	
	1.5	—	—	—	
	Overall	1.00	0.25*	2.0	8.4
Mannitol	0	0.99	0.11	1.8	
	0.75	1.02**	0.23	1.2	
	1.5	1.01	0.40	2.0	
	Overall	1.00	0.18	2.6	8.8

[a]Asterisks indicate that slope is statistically significantly different from expected 1.00.
[b]Asterisks indicate that intercept is statistically significantly different from 0.
[c]Fructose could not be accurately measured because of baseline upset at 1.5% NaCl.
[d]Sucrose concentration ranged from 1 to 15mM. Coefficient of variation is based on standard deviation of regression model.
[e]Calculated by pooling all data over 3 NaCl concentrations.
[f]Lowest sucrose concentration was 1.0mM.

tionship between added and analyzed compounds with a slope of 1.0 and an intercept of 0. Table 1 shows that the slopes were near 1.0 and the intercepts were near 0 for each compound analyzed. The high precision of the analysis resulted in statistically significant deviations of the slopes from 1.0 ($P \leq 0.01$) at some NaCl concentrations tested. However, when the regression was pooled over the three NaCl concentrations used, the overall slopes were not significantly different from 1.0. The intercept was significantly different from the expected zero in several cases, but the deviations were too small to be of practical concern in the analysis. The coefficients of variation for the 4 compounds analyzed over a 2-day period ranged from 2.0 to 3.1% when the regression was pooled over the 3 salt concentrations. The coefficients of variation for the lowest component concentrations analyzed ranged from 6.8 to 9.2%. Baseline upsets, which occurred when 1.5% NaCl was added to the samples, prevented the accurate analysis of fructose.

Chromatography of Sucrose, Acids, and Ethanol on Reverse Phase Column

Separation of compounds on the reverse phase column before and after fermentation is shown in Figure 2. Monosaccharides, salts, and other polar compounds elute at the solvent front and cause no interference with the separation of components of interest. Since sucrose separates on the C_{18} column, it can be measured on both of the columns used for analysis of fermentation components. Sucrose, malic acid, lactic acid, acetic acid, and ethanol can all be separated throughout the course of fermentations. The 2 LC procedures described here allow analysis of all major fermentation substrates normally formed in vegetable fermentations, except CO_2. The procedure for ethanol analysis does not work well with alcoholic fermentations, such as wine fermentations, because the large ethanol peak will interfere with analysis of minor acid components. This procedure can also be used to measure other organic acids, such as citric acid, succinic and fumaric acid, that elute after ethanol (5), although these compounds were not evaluated in these experiments.

One problem with the use of C_{18} reverse phase chromatography is that the retention times and resolution of the components gradually decline. This remains a general problem with this type of column packing material. In many cases, the useful life of a column can be extended by increasing the polarity of the elution solvent. This is not possible with an aqueous buffer as the eluant. By decreasing the temperature of the column to 12°C, when it began to lose resolution, useful column life can be extended significantly. This was done with Radial-Pak columns by placing the radial compression module in a double-walled plastic bag to protect it from water and submerging the whole module in a water bath. Adjustments must be made on the module to account for contraction and expansion of the hydraulic fluid in the compression cell during cooling and warming.

Table 2 shows the results of chromatography of samples with 0, 3, and 6% NaCl. The results were calculated at each salt concentration and then over all 3 salt concentrations, as was done for the sugar analysis. The coefficients of determination ranged from 0.995 to 0.999. Since the sucrose curve was linear with an overall slope of 1.0 for both columns, this indicated that it was not hydrolyzed under the acid conditions normally encountered in samples of fermented vegetables. Malic acid had a slope less than 1.0, and the lactic slope was greater than 1.0. We do not know the reason for this deviation from the expected slope for these compounds. Malic acid had a nonzero intercept because 3.8 mM malic acid was present in the starting cucumber juice. The other deviations of the intercepts were not large enough to be of practical consequence in the analysis. The coefficients of variation ranged from 4.3 to 5.9% when results were calculated over 3 component concentrations and 3 salt concentrations during a 2-day period. At the lowest concentration of the compounds analyzed, the coefficients of variation ranged from 2.7 to 7.7%.

The application of these 2 LC procedures has made it practical to routinely analyze all major substrates and products, with the exception of CO_2, that commonly occur in vegetable fermentations. This allows carbon balances to be calculated for complex heterolactic acid fermentations (10) and makes it possible to follow the course of lactic acid fermentations in detail (11).

We have recently purchased a column with an anion exchange packing material similar to the Bio-Rad HPX-87P column, except that the counter ion is calcium rather than lead. The column provided reproducibility similar to the lead

Table 2. Regression analysis of analyzed vs added sucrose, malic acid, lactic acid, acetic acid, and ethanol in cucumber juice with 0, 3, and 6% NaCl added

Compound	NaCl concn, %	Slope[a]	Intercept[b]	CV over 2–25mM range, %	CV for 2mM concn, %
Sucrose	0	0.99	0.23	4.0[c]	
	3.0	0.99	0.18	6.2	
	6.0	0.99	0.22	4.8	
	Overall[d]	1.00	−0.04	5.9	4.9[e]
Malic acid	0	0.99	3.59**	2.2	
	3.0	0.93*	3.83**	6.9	
	6.0	0.93*	3.99**	5.7	
	Overall	0.95*	3.80**	5.6	2.7
Lactic acid	0	1.09**	−0.37**	3.8	
	3.0	1.06	−0.32	8.6	
	6.0	1.07	−0.29	4.6	
	Overall	1.08**	−0.33**	5.9	4.3
Acetic acid	0	0.99	0.23	4.3	
	3.0	0.99	0.18	4.8	
	6.0	0.99	0.27	4.4	
	Overall	0.99	0.21*	4.3	6.3
Ethanol	0	0.99	0.19	3.0	
	3.0	0.98	0.22	7.6	
	6.0	0.99	0.28*	3.0	
	Overall	0.99	0.23*	4.8	7.7

[a]Asterisks indicate that slope is significantly different from expected 1.00 at 0.05 (*) or 0.01 (**) level.
[b]Asterisks indicate that intercept is significantly different from 0 at 0.05 (*) or 0.01 (**) level.
[c]Sucrose concentration ranged from 1 to 12.5mM. Coefficient of variation is based on standard deviation of regression model.
[d]Calculated by pooling all data over 3 NaCl concentrations.
[e]Lowest sucrose concentration was 1.0mM.

column, but with two advantages: Small amounts of NaCl did not interfere with sugar analysis because salt eluted from the column before the sugars, and interference with fructose did not occur because ethanol was separated from the other compounds.

Acknowledgment

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METALS AND OTHER ELEMENTS

Evaluation of Electron Capture Gas Chromatographic Method for Determination of Methyl Mercury in Freezer-Case Seafoods

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A method was recently adopted by AOAC for determination of methyl-bound mercury in canned and fresh-frozen seafood by electron capture gas chromatography. That method was applied to the analysis of commercially prepared freezer-case seafoods. None of the commercially added ingredients produced electron capture responses that interfered in the analysis for methyl mercury. Recoveries of 95.7–114% were obtained in fortification studies of methyl mercury at 0.2 and 1.0 ppm levels. The applicability of aqueous methyl mercuric chloride solution for fortification studies was demonstrated.

An electron capture (EC) gas chromatographic (GC) method developed in this laboratory for the determination of methyl-bound mercury in fish and shellfish was recently collaboratively studied and adopted official first action by AOAC (1). During method development and collaborative analyses, tissues of fresh-frozen and canned fish and shellfish were analyzed. However, commercially prepared, freezer-case seafoods were not included in the study.

In addition to tissue, commercial freezer-case seafoods contain all or most of the following ingredients (listed in 3 categories): *(1)* bleached wheat flour, soy protein, textured soy flour, and/or white corn flour; *(2)* shortening (vegetable oils), i.e., cottonseed, palm, and/or soybean oils (crab cakes—margarine instead of vegetable oil); *(3)* other ingredients, i.e., water, sugar, salt, whey, leavening agents, milk, eggs, preservatives, spices, and artificial flavors and colors.

It was not known to what extent the cooking process and the added ingredients, such as breading or batter coating, might interfere with the EC-GC method. For example, raw shrimp is reported to contain 0.8% naturally incurred fat, whereas french fried, batter-dipped shrimp contains approximately 10.8% fat (2). If absorbed by the seafood tissue during cooking, some of the added ingredients could give positive responses by EC detection.

Because a wide variety of commercially prepared freezer-case seafoods is available to the American consumer, ability to determine methyl mercury in these foods is desirable. Nearly 369 million pounds of fish sticks, fish portions, and breaded shrimp were processed in 1982 (3). We report here an evaluation of the collaboratively studied method for use in the determination of methyl mercury in commercially prepared freezer-case seafoods.

Experimental

Reagents

Reagents are given in ref. (1) except the following.

(a) *Methyl mercuric chloride stock fortification solution.*—400 μg Hg/mL. Weigh 0.1252 g methyl mercuric chloride into 250 mL volumetric flask. Dilute to volume with deionized water.

(b) *Methyl mercuric chloride working fortification solution.*—20 μg Hg/mL. Pipet 10.0 mL methyl mercuric chloride stock solution into 200 mL volumetric flask. Dilute to volume with deionized water.

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Sample Preparation

Commercially prepared, freezer-case seafood products were purchased from local supermarkets. Seven species of breaded or batter-dipped, cooked seafood were selected for analysis: shrimp, clams, scallops, crab cakes, cod sticks and/or fillets of cod, and fillets of pollock and whiting. The products were labeled as fried or oven-baked for heat- or cook-and-serve purposes.

One pound of each product was thawed and homogenized in a Waring blender. Duplicate 2 g portions of each homogenate were weighed into centrifuge tubes for analysis by the referenced method. Additional duplicate 2 g portions of each product were fortified at the 0.2 and 1.0 μg Hg/g levels by adding 20 and 100 μL, respectively, of the methyl mercuric chloride working fortification solution directly to the samples. The fortified portions were tightly stoppered in centrifuge tubes and stored overnight at 4°C before analysis.

To further evaluate the effect of the seafood coatings, additional samples of batter-fried cod and whiting fillets were carefully stripped of their coatings by the analyst using a knife. The coatings, fish tissues, and original portions of each product were separately homogenized, and duplicate 2 g portions of each homogenate were taken for analysis. Duplicate 2 g portions were also fortified and taken for analysis as previously described.

Analysis

All samples were analyzed as described in ref. 1: Methyl mercury is isolated from acetone-washed, homogenized tissue by adding hydrochloric acid and extracting into benzene the methyl mercuric chloride that is formed. The benzene extract is concentrated and analyzed for methyl mercuric chloride by EC-GC on 5% DEGS-PS treated with inorganic mercuric chloride solution. Details are given in the referenced method.

Results and Discussion

A new GC column was used to examine the chromatographic performance of the system. Chromatograms of benzene solvent, 0.5 ng methyl mercury standard in benzene, and a reagent blank revealed that the background was free of interferences even at attenuation × 16, the highest sensitivity used in this study. Fresh-frozen (i.e., no commercial ingredients added) halibut and oysters were also analyzed by the referenced method and no chromatographic interferences were noted.

The results of the analyses are presented in Table 1. Methyl mercury was not detected in clams, crab cakes, or scallops. Methyl mercury was detected in the remaining products, but at levels near the estimated detection limit of 0.01 μg Hg/g. At this level the methyl mercuric chloride peak is approximately twice the height of extraneous background peaks for a 5 μL injection of a 2 g sample in 20 mL benzene extract.

In the coating removal study, methyl mercury was found only in those portions containing fish tissue. This indicates, as suspected, that the seafood coating is not the source of the

Table 1. Methyl mercury in commercially prepared freezer-case seafoods

Sample	Found,[a] μg Hg/g	Rec., %[b] 0.2 μg Hg/g added	Rec., %[b] 1.0 μg Hg/g added
Shrimp, breaded, fried	0.01, 0.01	102	97.1
Clams, batter-dipped, fried	ND,[c] ND	98.4	103
Crab cakes, breaded	ND, ND	100	96.2
Scallops, french fried, crispy	ND, ND	99.6	95.7
Cod sticks, batter-dipped	0.03, 0.02	99.2	105
Whiting fillets, batter-fried	0.01, 0.01	97.2	97.1
Pollock fillets, toasted bread crumbs	0.01, 0.01	98.5	105
Cod fillets, batter-fried	0.04	101	106
Tissue only	0.07	102	101
Coating only	ND	96.4	107
Whiting fillets, batter-fried	0.01	99.3	114
Tissue only	0.02	104	108
Coating only	ND	102	107

[a]In unfortified duplicate samples.
[b]Values represent results for a single determination.
[c]ND indicates not detected (0.01 μg Hg/g detection limit).

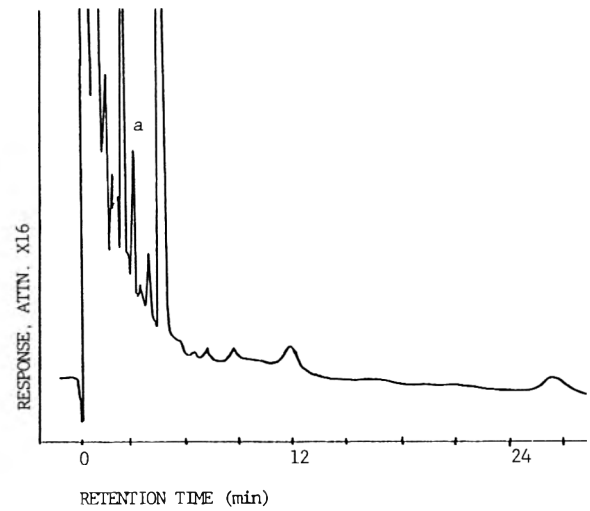

Figure 2. EC chromatogram for 0.5 mg equivalent of batter-fried cod fillets, a = 0.02 ng Hg.

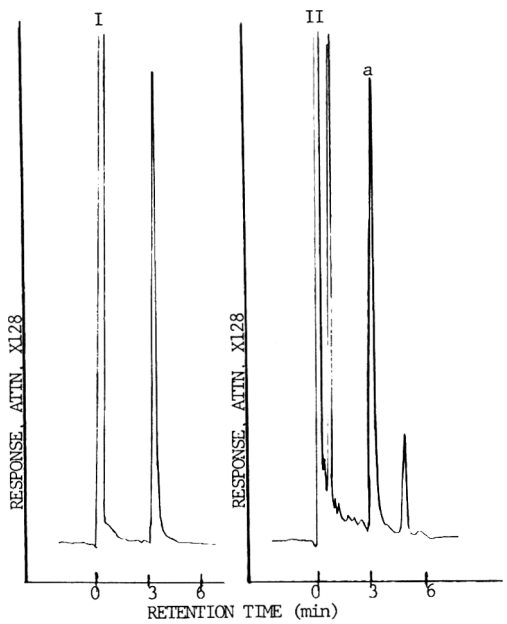

Figure 1. EC chromatograms for (I) 0.5 ng methyl mercuric chloride standard; (II) 0.5 mg equivalent of fortified, breaded, fried shrimp, a = 0.48 ng Hg.

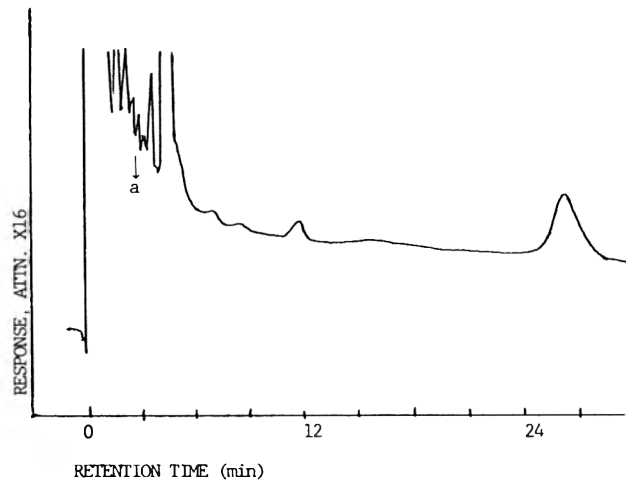

Figure 3. EC chromatogram for 0.5 mg equivalent of cod batter coating, a = methyl mercury peak absent.

methyl mercury. Mean recoveries for the fortified samples, including samples of batter coating only, were 100 and 103% at the 0.2 and 1.0 μg Hg/g fortification levels, respectively; actual recoveries ranged from 95.7 to 114%. Chromatograms obtained for methyl mercuric chloride standard and breaded, fried shrimp fortified with methyl mercuric chloride are shown in Figure 1.

Chromatograms of the commercially prepared freezer-case products revealed some extraneous peaks such as those produced by batter-fried cod fillets in Figure 2. Analysis of this sample at attenuation × 16 revealed several substances which elute before methyl mercury and those with relative retention times of 1.5, 4, and 9 with respect to methyl mercury. These may be attributable to some of the ingredients previously listed. A typical chromatogram of the batter coating for cod is shown in Figure 3. Analysis of the batter coating revealed large amounts of substances which elute before methyl mercury and those with relative retention times of 1.5, 4, and 9. Chromatograms obtained for cod and whiting tissue after removal of their coating revealed the absence of some substances which elute before methyl mercury and the presence of only small amounts of substances which elute at relative retention times of 1.5 and 4 with respect to methyl mercury. The small peaks at the relative retention times of 1.5 and 4 in the chromatograms of separated tissue are probably due to ingredients that are absorbed by the tissue from the batter during commercial cooking and are not removed by the stripping procedure. A typical chromatogram for separated cod tissue is shown in Figure 4. These results together with the findings obtained from fresh-frozen halibut and oysters (no commercial ingredients added) suggest that the batter is the source of the substances that produce the extraneous peaks.

It was observed in the analyses of all commercially prepared products that peaks produced by early eluters and substances emerging with a relative retention time of 1.5 did not interfere with the methyl mercury peak, even after repeated sample injections. However, interference from late eluters with relative retention times of 4 and 9, which have the chance of eluting near or with methyl mercury in subsequent sample injections, can be avoided by waiting to inject the next sample until the late eluters have emerged.

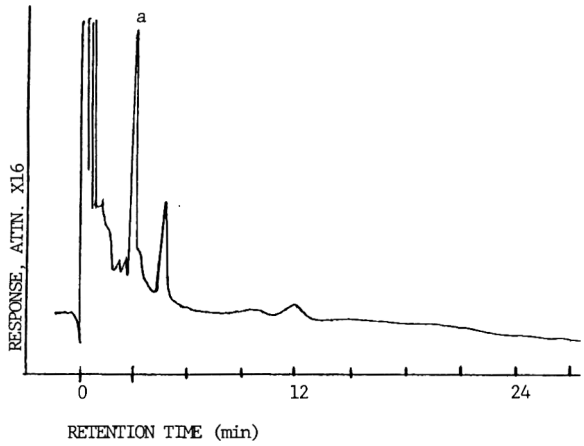

Figure 4. EC chromatogram for 0.5 mg equivalent of cod tissue stripped of batter coating, a = 0.03 ng Hg.

Conclusions

This study demonstrates that the official first action method is suitable for the direct determination of methyl-bound mercury in commercially prepared freezer-case seafoods. The results obtained in this study demonstrate that the acetone–benzene prewash treatment is sufficiently effective in removing seafood-incurred lipids and other organic compounds along with large amounts of commercially added ingredients that produce EC responses. However, because the method does not entirely remove all the fat and other ingredients, special attention should be paid to extraneous peaks and late eluting substances when successive sample injections are made, especially when samples are being examined for low levels of methyl mercury. The use of methyl mercuric chloride fortification solutions in water rather than in isopropanol is recommended for recovery studies, because change in concentration due to volatility of solutions is minimized.

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OILS AND FATS

Liquid Chromatographic Method for Quantitative Determination of Free Fatty Acids in Butter

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A liquid chromatographic method has been developed for the determination of free fatty acids in butter. The fatty acids are converted to the *p*-bromophenacyl esters, via a crown ether-catalyzed reaction, without separation from the other butter components. The esters are separated on a C_{18}-bonded silica column by using an acetonitrile–water solvent gradient and quantitated using the ester of heptadecanoic acid as internal standard. C_{16} and $C_{18:1}$ co-elute in the acetonitrile–water system but are separated using an isocratic methanol–acetonitrile–water system. Limits of detection range from 7 ng for butyric acid to 45 ng for linoleic acid. The average coefficient of variation (n = 10) for 10 free fatty acids from a butter was 5.83%.

Free fatty acids (FFA) contribute significantly to the desirable flavor of butter but when present in excessive concentration can impart a rancid flavor. Elevated FFA levels can arise from premanufacture lipolysis in cream or postmanufacture lipolysis in butter. The former is usually caused by the natural milk lipase while the latter is generally attributable to heat-stable bacterial lipases (1).

The short-chain acids, butyric (C_4) and caproic (C_6), are largely responsible for the rancid flavor because of their low flavor thresholds in butter (2). Hence, a measure of these acids is a good indication of the presence of a rancid flavor. Total FFA levels in butter are usually determined by titration of an aliquot of fat from butter (3). However, such a method measures only the fat-soluble acids (>C_{10}); consequently, the acid degree values obtained often show poor correlation with flavor scores (1, 4).

Several gas chromatographic (GC) methods have been used for estimating individual free fatty acids in milk-fat (5–7) and butter (4, 8). These methods require an initial step to separate FFA from fat. This can be done by using a column of KOH–silicic acid (9), anion exchange resin (10), or alumina (8). Erroneous results can be obtained if the fat hydrolyzes on the alkaline medium (4, 11).

Liquid chromatographic (LC) methods have also been used to determine individual fatty acids, usually by analysis of their UV-absorbing esters (12–16). However, these methods have not been used for analyzing FFA in butter. The LC method described in this paper is based on the crown ether-catalyzed derivatization of fatty acids to *p*-bromophenacyl (PBP) esters used by Durst et al. (12).

METHOD

Apparatus

(a) *Liquid chromatograph.*—Waters Model 218 consisting of 2 Model M45 solvent delivery systems, Model U6K injector, Model 660 solvent programmer, and Model 450 variable wavelength detector (Waters Associates, Milford, MA). Chromatographic column: Waters μBondapak C_{18} stainless steel column, 30 cm × 3.9 mm id (Waters Associates). Operating conditions: 20 μL injections; solvent gradient (unless stated otherwise) from 60% acetonitrile in water to 100% acetonitrile over 10 min at flow rate 1.6 mL/min; detection at 254 nm, 0.1 AUFS.

(b) *Reacti-vials.*—5 mL (Regis Chemical Co., Morton Grove, IL).

(c) *Water bath.*—Constant temperature controlled.

(d) *Sample filtration apparatus.*—Stainless steel Swinny filter holder with 0.45 μm Fluoropore filters (Millipore Corp., Bedford, MA).

Reagents

(a) *Solvents.*—Acetonitrile and methanol, LC grade (Waters Associates). Water, glass distilled. All solvents were filtered through 0.45 μm Fluoropore filter before use.

(b) *p-Bromophenacyl bromide (PBPB).*—(Hopkin & Williams, Chadwell Heath, Essex, UK). Reagent grade, recrystallized from pentane before use and prepared as 1 mg/mL acetonitrile solution.

(c) *1, 4, 7, 10, 13, 16-Hexaoxacyclooctadecane (18-crown-6).*—(Sigma Chemical Co., St. Louis, MO). Prepared as 1 mg/mL acetonitrile solution.

(d) *Standard fatty acids.*—>95% purity (by GC), obtained from various sources.

(e) *Standard fatty acid solutions.*—*1.* (C_n, mg/100 mL ethanol) C_4, 12.3; C_5, 13.2; C_6, 15.7; C_8, 17.2; C_{10}, 32.0. *2.* (C_n, mg/100 mL chloroform) C_{12}, 37.6; C_{14}, 47.8; C_{16}, 44.0; C_{17}, 42.1; C_{18}, 50.1; $C_{18:1}$, 38.6; $C_{18:2}$, 24.8.

(f) *Fatty acid internal standard solution.*—(100 mg C_{17} + 30 mg C_5)/100 mL chloroform–ethanol (1 + 1).

(g) *Sodium sulfate.*—Powder, anhydrous, analytical reagent grade.

(h) *Potassium carbonate.*—Anhydrous, analytical reagent grade.

(i) *Florisil PR.*—60–100 mesh (Floridin Co., Pittsburgh, PA).

Procedure

Derivatization of standard fatty acids.—Add 50 μL of each standard fatty acid solution, 400 μL PBPB solution, 40 μL 18-crown-6 solution, 20 mg potassium carbonate, and acetonitrile to total volume of 1 mL in 5 mL Reacti-vial. Cap and heat 30 min at 75–80°C, cool to room temperature, and filter before injection into LC apparatus. When derivatizing in presence of fat, adjust volume of acetonitrile accordingly.

Derivatization of FFA in butter.—Dissolve 500 mg butter in 4.8 mL chloroform–ethanol (1 + 1). Add 200 μL internal standard and 0.5 g anhydrous sodium sulfate to butter solution and shake mixture vigorously. Add 500 μL butter solution, 1 mL PBPB solution, 100 μL 18-crown-6 solution, and 70 mg potassium carbonate to 5 mL Reacti-vial. Cap and heat 30 min at 75–80°C, cool to 10°C, and filter before injection into liquid chromatograph.

Preparation of p-bromophenacyl esters (17).—Add 1 g fatty acid to 5 mL water in small flask and neutralize carefully with 10% sodium hydroxide solution. Make solution just acid by further addition of component acid. Add 10 mL alcohol and 1 g PBPB, then heat under reflux 1 h. Let solution cool and filter off precipitated ester. Recrystallize ester from alcohol

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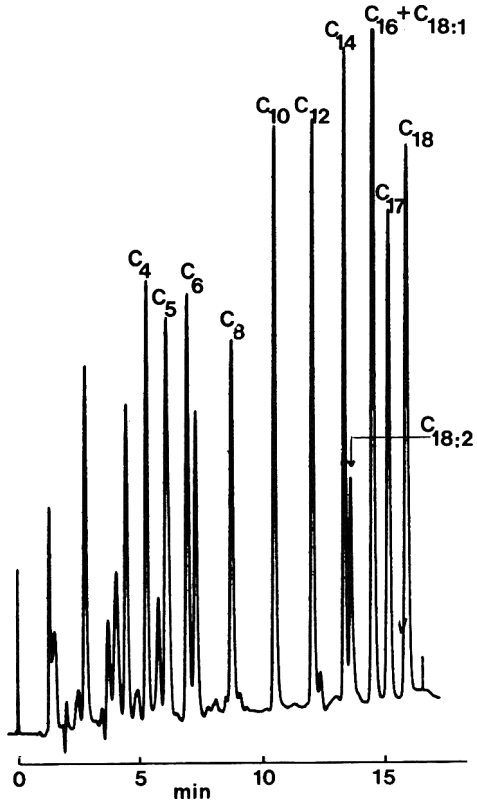

Figure 1. LC chromatogram of PBP esters of fatty acid standards. Conditions as described in text.

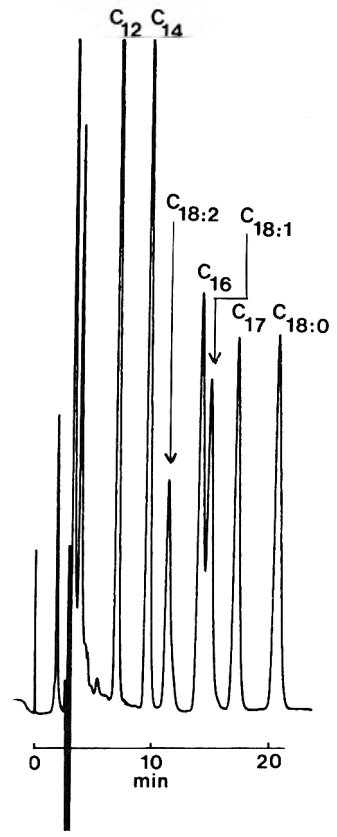

Figure 2. LC chromatogram of C_{12} and C_{18} esters, using mobile phase of methanol–acetonitrile–water (82 + 9 + 9) and flow rate of 1.0 mL/min. Other conditions as described in text.

Table 1. Recovery of fatty acids derivatized in the absence of fat

Fatty acid	Amount derivatized, nmol	Amount injected, nmol	LC response[a,b] /nmol	LC response[a,b] /nmol pure ester	Rec., %
C_5	265	3.98	39.4	38.1	103.4
C_{17}	233	3.50	39.4	39.3	100.2

[a]Peak height in mm.
[b]Response at 0.4 AUFS.

to constant melting point (PBP-C_5 mp 75.5–76°C; PBP-C_{17} mp 82.5–83°C).

Recovery studies.—(a) Derivatize known amounts of fatty acids (C_5, C_{17}) in absence of fat and compare LC responses with those of corresponding pure esters prepared by above procedure. (b) Derivatize 100 μL aliquots of internal standard in presence of 0, 5, 20, and 50 mg quantities of milk-fat which has been stripped of FFA by passing through a Florisil column. (c) Derivatize known amounts of C_5 and C_{17} in presence of stripped milk-fat (50 mg/mL reaction volume), using levels of PBPB to give molar PBPB:FFA ratios of 1:1, 2:1, 4:1, and 8:1. (d) Add aliquots of standard fatty acid solutions to quantities of stripped milk-fat to obtain total FFA concentrations of 1, 2, 3, and 4 mmol/100 g fat. Derivatize these samples by method outlined above for butter.

Limits of detection.—Determine by 3σ method of Kaiser (18).

Precision of method.—Determine coefficients of variation for individual FFAs in butter over 10 replicate assays.

Acid degree value (ADV) of butter.—Determine ADV, using method described by Deeth and Fitz-Gerald (3).

Calculations.—Determine concentration of each acid by comparison with C_5 and/or C_{17} peak heights.

Results and Discussion

Figure 1 illustrates a typical separation of the *p*-bromophenacyl esters of C_4–C_{18} fatty acid standards. Methanol and acetonitrile and their aqueous mixtures are commonly used eluants in separations on nonpolar stationary phases. However, resolution of esters above C_8 has been shown to diminish markedly for a methanol–water gradient compared with an acetonitrile–water gradient (19). Because of the superior resolution of longer-chain esters by using an acetonitrile–water gradient, it was selected for the mobile phase. However, with this gradient, resolution of C_{16} and $C_{18:1}$ was not effected, and a methanol–acetonitrile–water (82 + 9 + 9) mobile phase (20) was used to obtain this separation (Figure 2). This solvent system also improved resolution of C_{14} and $C_{18:2}$. Attempts to achieve a gradient elution using methanol, acetonitrile, and water which would separate all esters from C_4–C_{18}, including C_{16} and $C_{18:1}$, were unsuccessful.

The molar ratio of PBPB to FFA necessary for maximum derivative formation for standard fatty acid mixtures in the absence of fat was 1.6:1. Stoichiometric concentrations of 18-crown-6 are not necessary (21), and the molar ratio of PBPB:18-crown-6 was held at 10:1 as used by Durst et al. (12). A very large excess (500–1000 fold) of anhydrous potas-

Table 2. Effect of triglyceride on formation of PBP derivatives of fatty acids

Triglyceride, mg/mL	LC response[a,b] C_5	LC response[a,b] C_{17}
0	92	107
5	93	111
20	98	116
50	96	101

[a]Peak height in mm.
[b]Response at 0.4 AUFS.

Table 3. Effect of PBPB:FFA ratio on derivatization in the presence of fat

PBPB:FFA	LC response[a,b]/nmol C_{17}	Rec.[c] of C_{17}, %
1:1	9.5	24.2
2:1	16.9	43.0
4:1	29.7	75.6
8:1	39.9	101.5

[a]Peak height in mm.
[b]Response at 0.4 AUFS.
[c]Based on response of pure ester.

sium carbonate was necessary to obtain quantitative derivative formation.

Recoveries of the fatty acids C_5 and C_{17} derivatized in the absence of fat were quantitative (Table 1). Levels of at least 50 mg triglyceride /mL were tolerated in the reaction mixture (Table 2). The possibility of triglyceride hydrolysis occurring during the reaction was discounted after attempts to derivatize a sample of a pure triglyceride (trilaurin) and a sample of stripped milk-fat resulted in no detectable ester formation. To obtain quantitative recovery of fatty acids in the presence of milk-fat, it was necessary to increase the PBPB:FFA molar ratio to 8:1 (Table 3). The recoveries of individual fatty acids over a concentration range typical for butter were linear when derivatization was carried out in the presence of fat. The regression equations for y (peak height mm) on x (μg of added fatty acid) were as follows:

		r
C_4	$y = 22.4x - 3.0$	0.9978
C_6	$y = 23.7x + 2.5$	0.9926
C_8	$y = 19.5x + 8.0$	0.9918
C_{10}	$y = 31.6x + 0.5$	0.9964
C_{12}	$y = 51.1x - 13.9$	0.9898
C_{14}	$y = 50.8x - 12.0$	0.9992
C_{16}*		
C_{18}	$y = 43.3x - 6.5$	0.9996
$C_{18:1}$*		
$C_{18:2}$	$y = 18.6x - 6.0$	0.9974

*Peaks not separated

The method is therefore applicable for the quantitative determination of FFA in butter. Figure 3 shows a typical chromatogram of PBP esters of the FFAs in a butter (ADV 1.4).

The use of a large excess of PBPB to obtain quantitative recovery of FFA in butter results in the formation of a large number of reagent artifacts which cause quantitation problems in the C_4–C_6 region of the chromatogram. This problem was also encountered when using "*p*-Bromophenacyl-8," a solution of PBPB and crown ether marketed by Pierce Chemical Co., Rockford, IL, to derivatize fatty acids. The interference can be alleviated to a great extent by using much lower concentrations of PBPB (PBPB:FFA, 1:1). This results

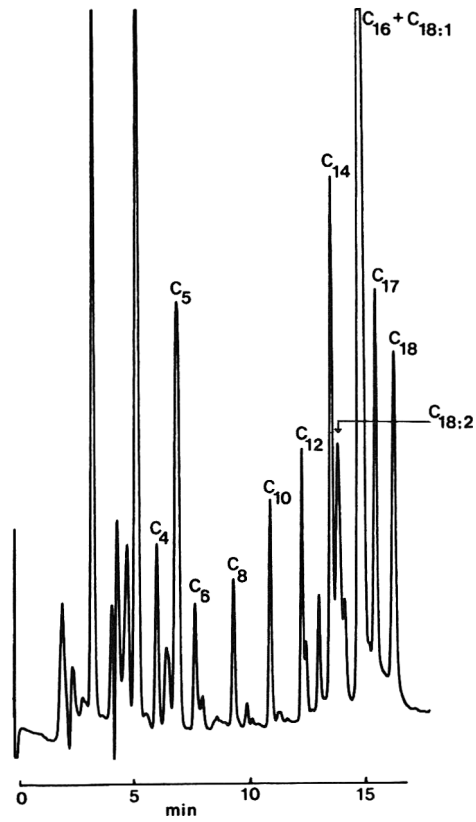

Figure 3. LC chromatogram of PBP esters of FFA in butter (ADV 1.4). Conditions as described in text.

in a lower sensitivity but a much clearer chromatogram, which can still be used for quantitation purposes, as no specificity for any particular acid was observed with esterification by PBPB. If a large excess of PBPB is used, an excess of formic acid can be added after the 30 min reaction time to convert excess PBPB to formate. This eliminates the PBPB peak, which tends to swamp the butyrate peak if PBPB is used in a large excess.

Although the butter was dissolved initially in chloroform–ethanol (1 + 1), the reaction solvent was predominantly acetonitrile. This was done to avoid solubility problems in LC, which may occur if a solvent unlike the mobile phase is used. Furthermore, triglycerides have low solubility in acetonitrile. Chilling of the reaction mixture to 10°C after completion causes maximal crystallization of the triglyceride, which is removed by filtration before injection, and ensures that negligible amounts of triglyceride, which may adhere strongly to the C_{18} column, are injected.

Interfering peaks occur in the low molecular weight (C_4–C_6) region of the chromatogram and, although such peaks have not been found to coincide with those of C_4 and C_6, it is recommended that blanks be run concurrently to eliminate this possible source of error. Extraneous peaks limit the usefulness of C_5 as an internal standard, particularly when a large excess of PBPB is used in derivatization. Consequently, C_{17} has been used as internal standard in this work. Some C_{17} occurs in milk-fat, but it has been found to be insignificant when calculating FFA concentrations.

The precision of the method is illustrated by the coefficients of variation (CV) for the individual FFAs given in Table 4. The average CV of the 10 acids was 5.83%. C_6 showed a relatively high CV (18.0%) which can be attributed to the presence of interfering peaks as discussed above.

Table 4. Coefficients of variation for PBP esters of butter free fatty acids

Fatty acid	Coefficient of variation, %
C_4	4.6
C_6	18.0
C_8	11.2
C_{10}	4.4
C_{12}	3.9
C_{14}	2.6
C_{16}	2.1[a]
C_{18}	3.2
$C_{18:1}$	1.8[a]
$C_{18:2}$	6.5[a]

[a]Calculated from chromatograms obtained using methanol–acetonitrile–water (82 + 9 + 9) solvent system.

Table 5. Limits of detection for each fatty acid

Fatty acid	Detection limit pmol	Detection limit ng
C_4	74	6.5
C_6	190	22.0
C_8	49	7.1
C_{10}	49	8.4
C_{12}	55	11.0
C_{14}	65	14.8
C_{16}[a]	—	—
C_{18}	111	31.5
$C_{18:1}$[a]	—	—
$C_{18:2}$	159	44.5

[a]Because of the lack of resolution of the esters of these fatty acids using the gradient elution program, limits of detection could not be obtained.

The advantages of the LC method over other methods, such as GC, are the minimal sample preparation required and the increased sensitivity toward the lower molecular weight esters. In the LC methods, the molar response of the UV detector increases sensitivity compared with the weight response of the flame ionization detector used in GC. This is particularly significant for the FFAs in butter, because the short-chain acids C_4 and C_6, which are largely responsible for the rancid flavors (2), are normally present at very low concentration (<10 ppm) in good quality butter. The levels of detection (Table 5) by the LC method are such that these acids can be readily determined at these low concentrations.

Acknowledgment

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SIMCA (Soft Independent Modeling of Class Analogy) Demonstrated with Characterization and Classification of Italian Olive Oil

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The multivariate technique SIMCA (soft independent modeling of class analogy) has been applied to the classification of foodstuffs on the basis of gas chromatographic profiles of some of their constituents. The data set used in this investigation consists of the percentage distribution of 7 fatty acids (7 variables) in 100 samples of virgin olive oil from 2 different regions (2 classes) of Italy, East and West Liguria. The SIMCA method can be used to compute whether an olive oil sample from Liguria originated in the western or eastern part of this region, while 98.8% of samples that do not originate in Liguria are correctly classified as outliers. The developed classification rules are adequate for identifying oils according to their origin. Standard decision diagrams (SDD) are very attractive tools for classification of new samples; the similarity between a new sample and each of the classes is easily computed. Consequently, the SDD visualizes any similarity toward each of the classes, and enables a decision on whether the new sample originates in one of the regions under study.

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Gas chromatographic profiles of foodstuffs can be used for classification according to origin or qualitative properties, and for the detection of adulteration. To obtain optimal information from those patterns, it is not sufficient to consider each parameter individually because important information may exist in the relationships existing between different constituents. Therefore, the use of multivariate techniques, such as pattern recognition, is necessary.

Table 1. Mean percentage concentration and standard deviation of 7 fatty acids in virgin olive oils from East and West Liguria

Fatty acid	Variable No.	East Liguria $\bar{X}_i$	East Liguria S_i	West Liguria $\bar{X}_i$	West Liguria S_i
Palmitic	1	11.45	1.04	10.53	0.72
Palmitoleic	2	0.84	0.14	1.08	0.24
Stearic	3	2.41	0.19	2.57	0.43
Oleic	4	77.46	1.57	76.74	1.16
Linoleic	5	6.89	0.69	8.97	0.94
Arachic	6	0.27	0.16	0.06	0.04
Linolenic	7	0.64	0.26	0.08	0.08
Eicosenoic	8	trace	—	trace	—

Table 2. Matrix for olive oil data set: a total of 572 samples, with 50 samples in class East Liguria and 50 samples in class West Liguria, and 7 variables per object

	Samples (objects)		
	Training set		Test set
Concn (%) of 7 fatty acids in each sample	East Liguria class 1 1 50	West Liguria class 2 51 100	101 ..... k
Var. 1			
2			
3	X_{ik}		
4			
5			
6			
7			

Several problems in food analysis can be approached with multivariate techniques. One of the purposes of such an approach is to develop decision rules that can be used to classify new samples. These rules will be developed on the basis of samples with known origin which form a "learning or training group." In food analysis, it is often not possible to examine a learning group for all possible classes because the number of classes can be large. There is a real possibility that an object belongs to a class for which no learning group was analyzed. These objects should be considered outliers with respect to the classes for which the decision rule was developed. Techniques such as linear discriminant analysis (LDA) and especially the K nearest neighbors method (KNN) classify a new sample necessarily in one of the given classes. The pattern recognition technique SIMCA (soft independent modeling of class analogy) provides the possibility of detecting outliers. It should, therefore, be better adapted to the realities of food analysis than are other pattern recognition methods. In this study, the usefulness of this method in the classification of virgin olive oil samples according to their origin is investigated. The emphasis of the article is on the study of the pattern recognition technique and not on the study of olive oil.

Procedure

Data

The data set used in this investigation was provided by Forina et al. (1, 2). It consists of the percentage distribution of 7 fatty acids in 100 samples of virgin olive oil from 2 different regions of Italy (East and West Liguria). Concentrations less than 0.05% were considered trace and were given a value of 0.02%. Table 1 shows the mean percentage concentration and the standard deviation of the fatty acids in the samples of both groups. In the first instance, samples with the same origin were supposed to form a homogeneous group (or class).

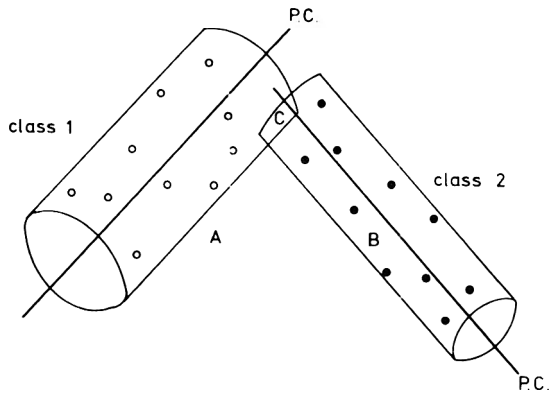

Figure 1. Illustration of SIMCA classification: object A is an outlier; object B is uniquely assigned to class 1; object C belongs to both classes, it is not uniquely classified. P.C. = principal component.

Programs

SIMCA analysis was performed with the package SIMCA-2T, which can be obtained from S. Wold (Institute of Organic Chemistry, Umea University, S-90187 Umea, Sweden). Calculations were performed on a CDC CYBER 170/750.

Methodology

In multivariate analysis, the data should be arranged in a data matrix as shown in Table 2. X_{ik} represents the percentage concentration of variable i in sample k. The training set consists of samples of known origin, namely, East and West Liguria. It is used to develop a decision model (or classification rule) that can be used to classify samples of unknown origin. The samples of the test set are used to evaluate the developed decision rule.

If multivariate data are available for a group of objects, each object can be seen as a point in an M-dimensional space (pattern space) with each dimension indicating one variable. In this space, objects which belong to the same group will be situated near each other; objects belonging to several groups will be situated far from each other. The more the objects differ, the more they will move away from each other

Up to now, most applications of pattern recognition methods to multivariate analysis of foodstuffs use the KNN and LDA methods. Both of these techniques are discrimination methods which are aimed at detecting differences between classes, i.e., they attempt explicitly or implicitly to construct boundaries between the classes. In this way, the multidimensional space is divided into as many regions as there are classes in the data set. An object with unknown classification is classified according to its position with respect to the boundaries.

The SIMCA method is a class-modeling technique which does not try to find differences between classes but tries to describe the similarities between the objects of a class. A separate mathematical description for each class, independent from the other classes in the training set, is obtained: a "class box" is constructed around the objects of each class. The classification of an object of the test set is made by comparing its place in the multidimensional space with respect to the situation of the class box.

According to the decision rules developed by the SIMCA method, 3 kinds of classification are possible: (1) an object is "uniquely" assigned to a class if it is situated inside the boundaries of only one class box; (2) an object can be situated inside the boundaries of more than one class box, i.e., it falls in the overlapping region of these boxes; in this case it is "not

uniquely'' classified; (3) an object is considered to be an outlier for that class if it falls outside the class box.

Figure 1 illustrates the SIMCA classification for 2 classes. Information on the mathematics of SIMCA methodology is given by Wold (3), Wold and Sjostrom (4), and Albano et al. (5).

An introduction to the philosophy of the method is necessary to elucidate the information obtained by applying SIMCA to a data set.

In multivariate analysis, the first step is usually scaling the variables to make the range between the different variables comparable. In discriminating methods as KNN and LDA, scaling should be performed over the variables of all the objects of all the classes. The SIMCA method, which develops a separate decision rule for each class, enables one to perform scaling on the whole data set or on each class separately. In the latter case, the decision rules developed for each class are completely independent from each other (6). In this investigation, autoscaling (also called Z-transformation) was used, meaning that from each variable X_{ik}, the average $\overline{X}_i$ is subtracted and the result is divided by the standard deviation S_i of that variable in a class. This is given by the equation

$$X'_{ik} = (X_{ik} - \overline{X}_i)/S_i$$

The second step in a SIMCA analysis is the construction of a principal components model for each class of the training set. Starting from the M original variables, M new uncorrelated variables or principal components are defined. The first principal component explains the largest part of the variance within a class. The second explains the greatest part of the residual variance, and so on. The axes of the original multidimensional pattern space are rotated in such a way that each dimension coincides with one principal component. Moreover, the origin is translated so that it corresponds with the centroid of the objects.

As the first few principal components explain the greatest part of the variance within a class, the relationship between the objects of a class can be estimated in a reduced multidimensional space, consisting of these first components only. The number of significant components A can be estimated according to different rules. The SIMCA method uses the cross validation criterion (7). If A is much smaller than M, the similarity between the objects of a class is high, i.e., this class is largely homogeneous.

The objects of class Q can be approximated by a principal component model (PC model) which is given by the equation

$$X'_{ik}{}^{(q)} = \alpha_i^{(q)} + \sum_{a=1}^{A_q} \beta_{ia}{}^{(q)}\, \Theta_{ak}{}^{(q)} + \epsilon_{ik}{}^{(q)}$$

where $\alpha_i^{(q)}$ is the mean of variable i in class Q. If separate scaling is performed, this parameter is equal to zero. $\beta_{ia}{}^{(q)}$ values are the loadings. These parameters, which depend only on the variables, give the correlation of variable i with principal component a. $\Theta_{ak}{}^{(q)}$ values are the factors. These parameters, which depend only on the objects, describe the place of object k in the A dimensional space. Important information is given by the range of the factors because this range is used to define the upper and lower boundaries of a class. $\epsilon_{ik}{}^{(q)}$ values are the residuals. These describe the part of the data that cannot be explained by the model.

The residuals consist of errors of measurement and also of model errors, i.e., imperfection of the approximation of class Q by a principal component model. The total variance between the objects of class Q can be divided into a part that is common to all the objects, i.e., it is specific for class Q, and a part that is random. The product terms $\beta_{ia}{}^{(q)}$ and $\Theta_{ak}{}^{(q)}$ describe the systematic part of the variance; the residuals describe the nonsystematic variance.

The total residual standard deviation of class Q is given by the equation

$$S_o^{(q)} = \left[\sum_{i=1}^{M} \sum_{k=1}^{N_q} \epsilon_{ik}{}^{(q)2} / (M - A_q)\,(N_q - A_q - 1) \right]^{1/2}$$

To evaluate the class model, each object p of class Q is fitted to the class model. The object p fits the class Q model if its residual standard deviation $S_p^{(q)}$ is not significantly larger than the total residual standard deviation $S_o^{(q)}$ for class Q.

$$S_p^{(q)} = \left[\sum_{i=1}^{M} \epsilon_{ip}{}^{(q)2} / (M - A_q) \right]^{1/2}$$

The comparison between $S_p^{(q)}$ and $S_o^{(q)}$ can be performed on the basis of an approximated F-test with $(M - A_q)$ and $(M - A_q)\,(N_q - A_q - 1)$ degrees of freedom.

In the geometrical interpretation, $S_o^{(q)}$ represents the typical distance of objects of the training set to the principal component for class Q, and is used to define a confidence interval around that model. $S_p^{(q)}$ measures the distance between object p and the model. An object is assigned to class Q if it falls inside the confidence interval or if it has an $S_p^{(q)}$ value less than a critical value calculated for a given level of significance. SIMCA-2T considers the object p an outlier if the F-value is greater than 3 for all classes. In our data set, this corresponds approximately with the 1% level of significance.

If outliers are detected, they should be removed from the training set, and a new class model must be developed with the remaining objects. This procedure is continued until all objects used in the development of a class model are assigned to that class. If outlying patterns are kept in the training set, the direction of the significant components will be influenced by these aberrant patterns. This leads to unstable class boxes, i.e., models that are sensitive to the deletion of one or more objects from the training set.

After all outliers have been removed from the training set, the developed classification rule is evaluated by deleting, at random, some objects from the training set and placing them into the test set. A decision rule is constructed with the remaining part of the training set, and the objects of the test set are fitted to the new class box. This procedure is followed until all the objects of the training set have been deleted once and only once. The validity of the class model is given by the prediction rate (rate of correctly classified test objects).

After the development of representative class boxes for each class of the training set, objects with unknown origin can be classified with respect to these class boxes. To facilitate classification, standard decision diagrams can be constructed for each pair of classes. In such a diagram, each axis corresponds with the distance or residual standard deviation of an object to the class boxes of each class (Figure 2). In this diagram, 2 lines can be drawn according to the critical value. These 2 lines divide the diagram into 4 parts: 2 regions for ''unique'' classification, one part indicating the outlier region, and one part indicating ''not unique'' classification. These last 2 regions can be divided into subparts which indicate the more suitable class of an object. To make a comparison between classification results obtained with SIMCA and those obtained with discriminating techniques that are not able to detect outliers, objects can be more closely classified

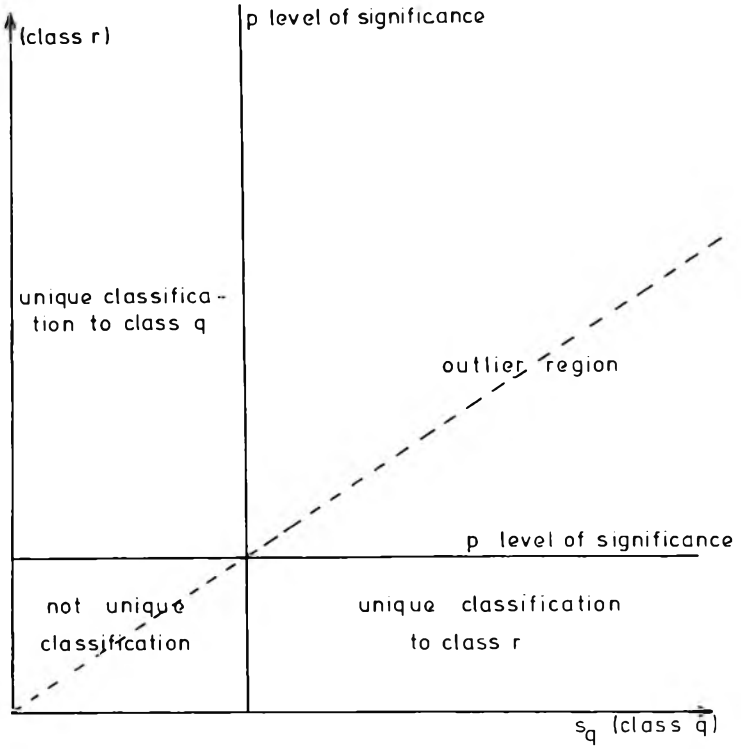

Figure 2. Standard decision diagram for differentiation between class *q* and class *r*.

Table 3. Stepwise development of class models

Class	Step	NPAT[a]	% variance[b] PC1	% variance[b] PC2	NOUT[c]
1	1	47	47	22	3
	2	44	49	22	1
	3	43	49	24	0
2	1	49	34	24	1
	2	48	34	26	0

[a]NPAT = number of objects used to develop the class model.
[b]% variance = percentage of total variance explained by each significant principal component (PC).
[c]NOUT = number of outliers that were detected in the corresponding step.

Table 4. Loadings β_{ia} of the variables on the 2 significant principal components in class 2

Factor-loadings	Perfected training set NPAT = 48[a]	Validation Step A NPAT = 32	Validation Step B NPAT = 32	Validation Step C NPAT = 32
β_{11}	−0.4784	0.4301	0.4583	0.4070
β_{21}	−0.5270	0.4478	0.5205	0.4852
β_{31}	0.0477	0.0257	0.0305	−0.2200
β_{41}	0.3257	−0.1499	−0.4307	−0.5678
β_{51}	0.1889	−0.4171	0.0704	0.4109
β_{61}	−0.3452	0.4784	0.2849	0.2069
β_{71}	−0.4798	0.4343	0.4965	0.1281
β_{12}	0.0643	−0.1978	−0.0682	−0.3174
β_{22}	0.2177	−0.3313	0.1960	−0.2943
β_{32}	−0.1989	0.0221	−0.4250	0.2174
β_{42}	−0.5427	0.7471	−0.3324	−0.1779
β_{52}	0.6483	0.4898	0.6593	0.4719
β_{62}	−0.4196	0.2100	−0.4794	−0.4593
β_{72}	−0.1343	0.0923	−0.0362	−0.5478

[a]NPAT = number of objects used to develop the class model.

in a class, according to the decision boundary indicated by the dotted line in Figure 2.

Results

To obtain completely independent decision rules (6), both classes were scaled separately. The *Z*-transformation was performed over the objects of the training set of each class separately.

The first information on the similarity between the objects of a class is obtained from the position of the objects of the training class in the plane formed by the first and second principal components. Samples 31, 32, and 50 for class East Liguria (further referred to as class 1), and sample 50 for class West Liguria (further referred to as class 2) were far removed from the rest of the samples of their class. For sample 50 of East Liguria, this was because of an extremely low concentration of palmitic acid (6.1%) and an extremely high concentration of oleic acid (84.1%). Samples 31 and 32 both had a high level of palmitic acid (14.0 and 13.5%, respectively). West Liguria sample 50 had a rather high level of oleic acid (79.5%). Obviously, these samples may be considered outliers and removed from the training set.

With the remaining 47 and 49 samples in class 1 and 2, respectively, a stable class box for each class was developed in stepwise fashion. Table 3 shows the number of patterns in the training set, the percentage of the total variance explained by the significant components, and the number of deleted objects for each step. For both classes, the cross-validation procedure indicated that 2 components were sufficient to explain the systematic part of the variance in each class. In class 1, 4 patterns were removed from the training set to obtain the stable class box. Only one nonfitting pattern was found in class 2. Both of the developed decision rules were evaluated according to the validation procedure previously described. Each time, a third of the training set was placed

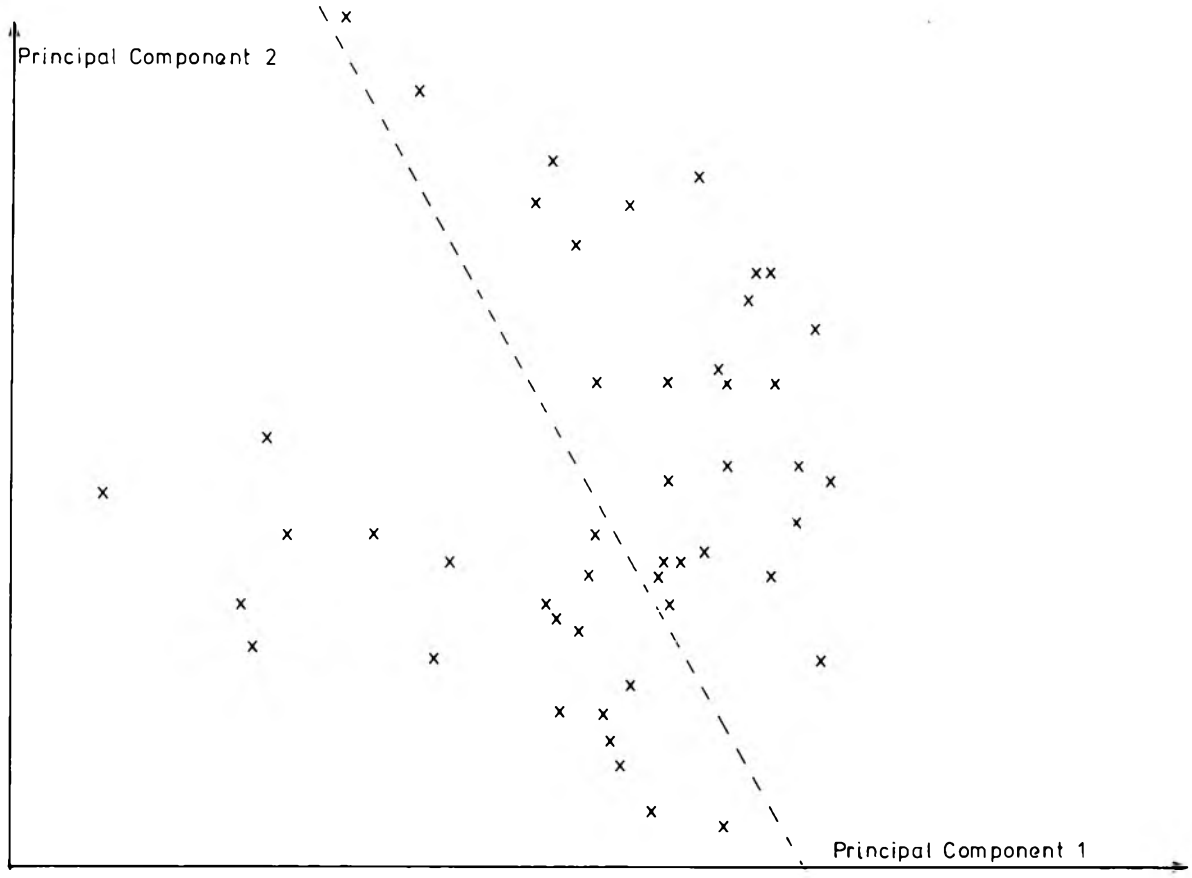

Figure 3. Principal component projection of the scaled objects of class 2 (West Liguria); dotted line indicates division of this class into 2 subgroups.

into the test set. The obtained class boxes overlap: 2 objects from class 1 are not uniquely classified, i.e., they are assigned to belong to both classes. For class 1, the prediction rate is 100%, but for class 2 only 95% was found. The loadings ($\beta_{ia}^{(q)}$), which indicate the direction of the principal components of the class box in the multidimensional pattern space, differed rather largely for each of the 3 validation steps for class 2 (Table 4). We concluded that subgroups must be present in this class. This was confirmed by the graphical display of the remaining objects (48) of class 2 on the plane of the first and second principal components. This plot indicates a high degree of dissimilarity between the objects of this class: the distribution of the objects was not homogeneous (Figure 3).

For this reason the class was divided into 2 parts, containing 30 and 18 objects. The objects of each part were scaled separately and a class model based on one principal component was developed for each part. In one part, 2 objects were outliers. Again both of the decision rules were validated. The recognition rate is 100% for both subgroups. The 2 class boxes overlap: 5 objects of part 1 are "not uniquely" classified; according to their distance to each of the submodels, they fit each of these models. This is visualized in the standard decision diagram for both subgroups (Figure 4).

From our investigation, it was obvious that class 2 could better be described by 2 class boxes than by a single box. None of the two PC models for class 2 overlap the PC model for class 1. This means that according to the 3 class boxes for the 2 classes, it can be computed whether a virgin olive oil with origin from Liguria, comes from the western or eastern part of this region.

One advantage of the use of a class modeling technique such as SIMCA is the development of separate mathematical decision rules that enable detection of outliers. This has a special interest in food analysis because the probability is great that a sample of unknown origin belongs to a group for which no training set was included. With regard to the developed decision rules, this sample should be indicated an outlier. In our investigation, this situation was simulated by placing into the test set the data of 472 samples of non-Ligurian Italian olive oils; oils originating in a region for which no class box was developed. All 472 samples were considered outliers for class 2, and only 8 (1.7%) fitted into the class box of class 1.

Forina et al. (1, 2) used parametric and nonparametric methods of multivariate analysis to classify the oil samples. The average percentage misclassified samples was 1% for West Liguria and 8% for East Liguria. Better prediction rates are obtained by applying the SIMCA technique to this data set. Samples that do not originate in Liguria are all distinct from the samples from West Liguria, and only 1.7% were considered to originate in East Liguria.

Conclusions

Until now, most applications of pattern recognition techniques in the field of food analysis involved LDA (linear discriminant analysis) and KNN (K nearest neighbours classification). Schatzki and Vandercook (8) tried to identify adulterations in concentrated orange juice. Saxberg et al. (9) examined the possibility of distinguishing expensive whiskies from less expensive brands by using KNN, LDA, and SIMCA. Leegwater and Leegwater (10) applied LLM (linear learning machine) for the classification of grape brandies. Kwan et al. (11) classified wines according to their quality with KNN and LDA. Scarponi et al. (12) analyzed samples of 3 groups of Venetian wines for several inorganic constituents; with LDA the 3 groups of wines could be reasonably well identified.

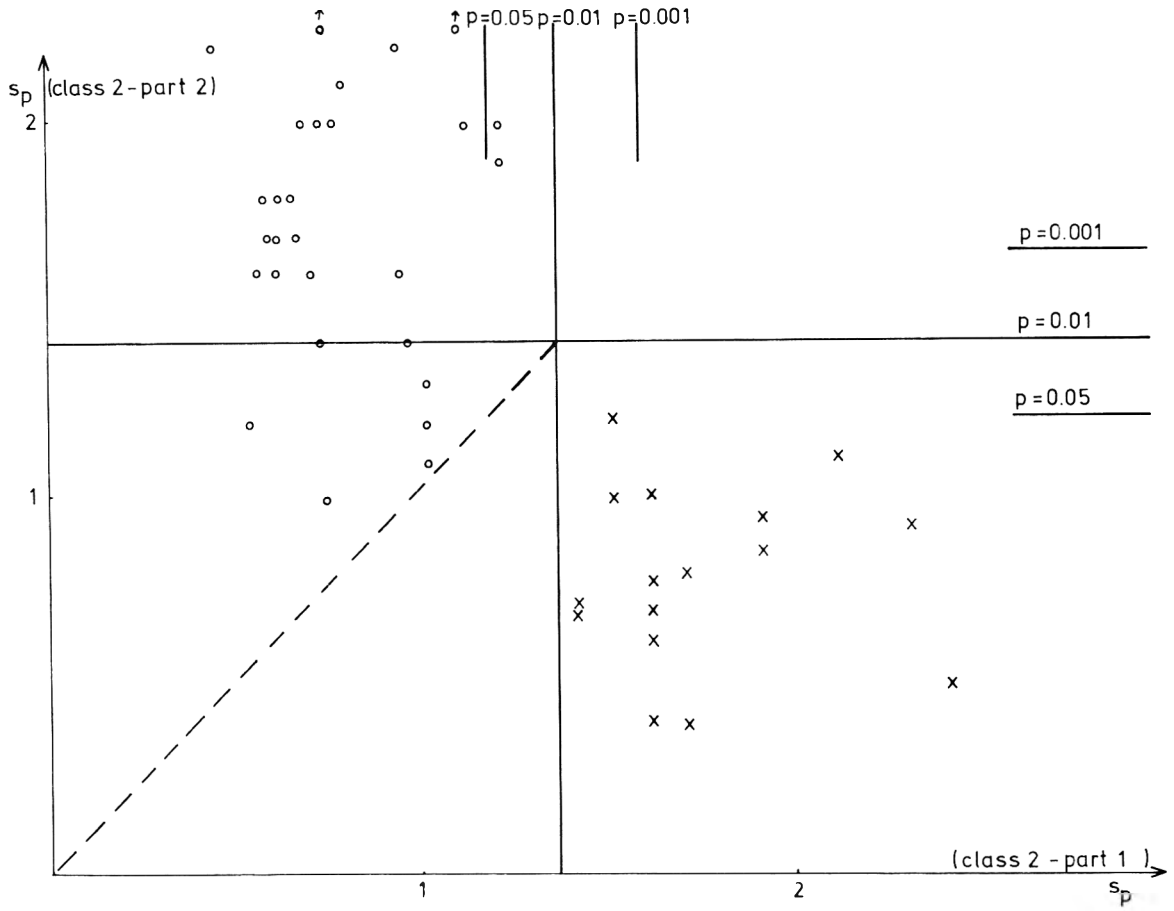

Figure 4. Standard decision diagram for differentiation between the 2 subgroups of class 2.

Powers and Keith (13) used LDA to classify coffee and chips according to their aromatic qualities on the basis of gas chromatographic profiles. Smeyers-Verbeke et al. (14) used LDA in the identification of milk samples from goats, sheep, and cows on the basis of the percentage concentration of 15 fatty acids. Coomans et al. investigated the same data set with other pattern recognition methods (15). Hartmann and Hawkes (16) were able to classify 45 samples of oils from 10 different regions according to their origin, on the basis of 10 parameters obtained by gas chromatographic analysis.

Our investigation proves that SIMCA is an adequate method for developing rules to classify olive oils according to their origin. This was recently confirmed by M. Forina (personal communication at NATA ASI on Chemometrics, Cosenza, Italy, Sept. 1983), who applied SIMCA to investigate season effects on the free fatty acid patterns of Portuguese olive oils. The ability to detect outliers makes this method of special interest in food analysis because it offers the possibility to differentiate samples from different specific origins. Our opinion that the SIMCA method can be successfully applied to other problems in food research is confirmed by a recent application of principal component analysis to a data base consisting of the amino acid patterns of French wines (17). Because the SIMCA method is based on principal components analysis, the discrimination between wines of different origins visualized in the principal component plots suggests that the SIMCA technique may be a useful tool for identifying wines according to origin.

Also, the applications of SIMCA are not confined to food analysis. A short review of applications in different fields is reported by Albano et al. (5).

In general, the following recommendations can be made for the optimal application of SIMCA: *(1)* Perform scaling over each class separately because this is the only way to obtain completely independent decision rules. Moreover, the development of decision rules for new classes does not interfere with the decision rules previously developed for other groups of samples.

(2) Take care in describing a class. If objects that were supposed to form a homogeneous group are obviously found to be dissimilar, the data set should be divided into subgroups, and each subgroup of more similar objects should be analyzed separately.

(3) Validate the class models developed for each class (or subset) by the use of the validation criterion, and test the performance of the model toward objects of other classes.

(4) If the developed class models seem to be adequate for the problem under study, use them for the classification of new samples. The classification is easily performed when standard decision diagrams are used because such diagrams enable visualization of the assignments.

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MYCOTOXINS

Liquid Chromatographic Analysis System for Cyclopiazonic Acid in Peanuts

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A liquid chromatographic procedure has been developed for analysis of cyclopiazonic acid in peanuts. The chromatographic system specifies a C_{18} or C_8 column loaded with 4-dodecyldiethylenetriamine and an aqueous mobile phase containing 4-dodecyldiethylenetriamine, zinc acetate, ammonium acetate, 2-propanol, and acetonitrile. Cyclopiazonic acid was extracted from peanuts with methanol-chloroform (20 + 80), partitioned into aqueous sodium bicarbonate, acidified, and back-extracted into dichloromethane. The limit of detection (280 nm UV) was approximately 4 ng and detector response was linear to at least 1 μg pure cyclopiazonic acid. The recovery of cyclopiazonic acid from peanuts spiked at 68.9, 210, and 955 μg/kg was 85.9% (12.86% CV), 72.9% (6.43% CV), and 81.4% (0.40% CV), respectively. Calculation of the chromatographic peak parameters based on the exponentially modified Gaussian model indicated that the C_{18} column produced less peak skewing than did the C_8 column.

Cyclopiazonic acid, a toxic indole tetramic acid (Figure 1), is a known metabolite of several *Penicillium* species (1–3) and *Aspergillus* species (4–6). The toxin has been shown to occur in contaminated corn (7), cheese (2), and peanuts (8). Although the mycotoxin is not considered to be carcinogenic, histopathologic alterations produced by cyclopiazonic acid in rats include lesions of the liver, kidney, pancreas, spleen, and heart (9). Chickens fed 100 ppm cyclopiazonic acid-contaminated rations had significant mortality rates and the surviving animals had proventriculitis, mucosal necrosis of the crop, chronic hepatitis, and myocardial inflammation (10).

Presently, cyclopiazonic acid in agricultural commodities has been separated and detected by thin layer chromatography (2, 7, 8). Previous laboratory experience has shown that the method suffers from variation in plate-to-plate reproducibility due to the requirement of oxalic acid impregnation for each thin layer plate and from inaccuracy due to inconsistent color development by reaction with Ehrlich reagent (dimethylaminobenzaldehyde and HCl).

A liquid chromatographic (LC) method for the determination of tenuazonic acid in tomato products has been reported by Scott and Kanhere (11) which uses a metal ion selective separation based on the work by Cooke et al. (12). A modification of the chromatographic system is presented here which allows the determination and quantitation of cyclopiazonic acid in peanuts. The chromatographic system parameters are also presented, using recently developed semiempirical equations (13).

Experimental

Instrumentation

The liquid chromatograph consisted of a Waters Associates (Milford, MA) Model 6000A pump, a Valco Instruments (Houston, TX) Model CV-6-μHPa valve loop injector fitted with a 100 μL loop, a Waters Associates Model 450 UV variable wavelength detector operated at 284 nm, and a Shimadzu Corp. (Kyoto, Japan) Model C-R1B Chromatopac data processor.

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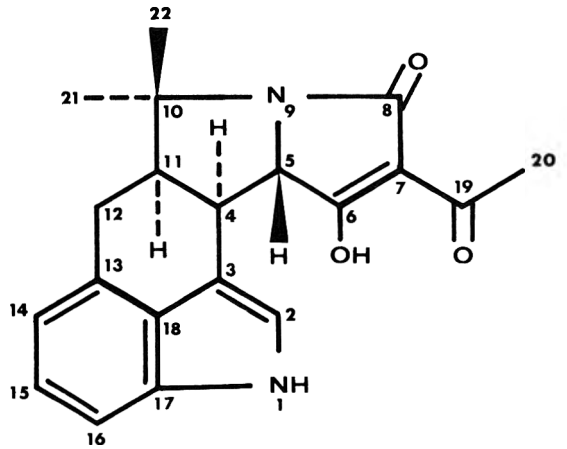

Figure 1. Structure of cyclopiazonic acid.

Materials

Pure cyclopiazonic acid (a gift from J. Dorner, National Peanut Research Laboratory, Dawson, Georgia) was isolated from *Aspergillus flavus* (CP27) liquid cultures. Tenuazonic acid, as the copper salt (a gift from P. S. Steyn, National Chemical Research Laboratory, Pretoria, South Africa), was dissolved in mild aqueous acid and extracted into chloroform for determination of the chromatographic parameters. 4-Dodecyldiethylenetriamine was obtained from Eastman Kodak (Rochester, N Y). Ammonium acetate and organic solvents were LC grade obtained from various distributors. Water was glass-distilled and all other chemicals were ACS grade.

Chromatographic System

The analysis of cyclopiazonic acid was conducted using 4.6 mm id × 25 cm Partisil-10 ODS or Partisil-10 C_8 columns (Whatman, Inc., Clifton, NJ) and a corresponding 4.6 mm id × 3 cm guard cartridge (Brownlee Labs, Inc., Santa Clara, CA). The mobile phase consisted of 40% acetonitrile, 30% isopropyl alcohol, 1% ammonium acetate, 0.025% 4-dodecyldiethylenetriamine, and 0.001M zinc acetate in water. The mobile phase was prepared by measuring 100 mL pH 7.3 stock solution containing 10% ammonium acetate, 0.25% 4-dodecyldiethylenetriamine, and 0.01M zinc acetate into a 1 L volumetric flask and diluting with 200 mL water, followed by adding 300 mL isopropyl alcohol and 400 mL acetonitrile. The contents were mixed and allowed to equilibrate to room temperature before being diluted to the mark with water. The resulting solution was degassed by a combination of intermittent vacuum and ultrasonication for ca 5 min. The mobile phase flow rate was maintained at 1.5 mL/min during this study.

Sample Extraction

Samples were processed according to the method previously described (8) with few changes. Essentially, 100 g samples of previously ground and blended peanuts were defatted with petroleum ether and allowed to air-dry. The defatted samples were extracted with 250 mL chloroform–methanol

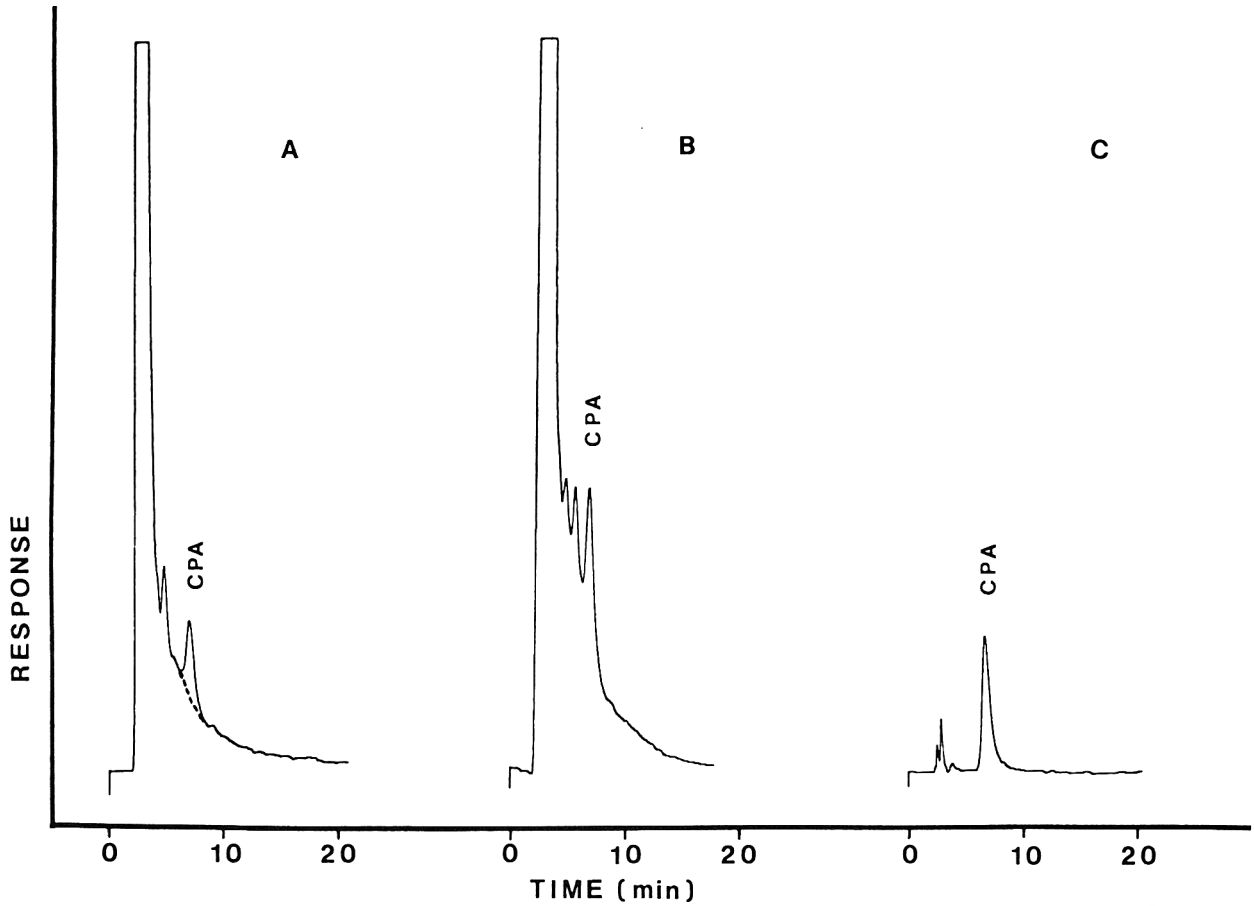

Figure 2. Liquid chromatogram of sample extracts of cyclopiazonic acid from (A) spiked peanuts (955 μg/kg), dotted line represents sample background of unspiked peanuts; (B) naturally contaminated peanuts (1847 μg/kg); and (C) standard cyclopiazonic acid (0.391 μg).

(80 + 20) plus 0.5 mL 85% phosphoric acid. Fifty mL extract was collected by gravity filtration and partitioned with 50 mL 0.5N sodium bicarbonate. The aqueous phase was washed with 50 mL chloroform and acidified with 6N HCl. Cyclopiazonic acid was recovered by partitioning with dichloromethane (2 × 25 mL) and flash-evaporation at 40°C. The extract was dissolved in acetonitrile, filtered using 0.45 μm pore nylon-66 filters (Rannin Instrument Co., Woburn, MA) into small vials, and evaporated to dryness under nitrogen. Before analysis, samples were redissolved in 500 μL mobile phase devoid of ammonium acetate and chelated metal ions.

Samples used for determining the recovery efficiency were obtained by pooling 3 segregated lots of peanuts from a previous study (8). The lots were known to be aflatoxin-contaminated but were considered to be cyclopiazonic acid-free. Four 100 g subsamples were processed to determine the background response and to ensure that there was, in fact, no residual cyclopiazonic acid. Samples for recovery experiments were 100 g subsamples which were defatted before spiking with a methanol solution of cyclopiazonic acid. The concentration of the cyclopiazonic acid was determined from UV measurements at 284 nm, using log ϵ = 4.31 (1). After evaporation of the solvent, the spiked samples were processed as normal samples. Four replications were performed at 2 levels.

Chromatographic Analysis

After equilibration of the column, samples were analyzed by variable-volume valve loop injection, and cyclopiazonic acid was detected at 284 nm. For most analyses, the detector was operated at 0.04 AUFS with a 2-s time constant. Detector output was digitally processed by the integrator system in a 2-point external calibration mode (5 replicate standard injections for each point) for peak area. The content injected, μg/μL, was found from the expression:

$$\text{content injected} = [(\text{response factor})(\text{peak area}) + \text{content}_0]/\text{injection volume}$$

where content_0 is the threshold level of detection. The concentration of cyclopiazonic acid in the sample was then calculated from

$$\text{content (μg/kg)} = \text{content injected (μg/μL)} \times 25\,000$$

Results and Discussion

Before the development of a liquid chromatographic system for tenuazonic acid by Scott and Kanhere (11), an acceptable liquid chromatographic system was not available for compounds containing the tetramic acid functionality. For reasons similar to those cited in that report, the more common reverse phase systems were unusable for cyclopiazonic acid. Gradient elution techniques did not offer significant improvement. Two inherent problems that could not be resolved with these systems were severe peak tailing and dependence of the peak retention time on the amount of cyclopiazonic acid injected. Initial investigations of the Scott-Kanhere mobile phase system using either acetonitrile or methanol produced unacceptably long retention times and peak band widths; however, the inclusion of 2-propanol in the acetonitrile mobile phase system led to the development of an acceptable chromatographic system. The concentrations of 2-propanol and acetonitrile were chosen by visual comparison of experimen-

Table 1. Chromatographic figures of merit for cyclopiazonic acid (CPA) and tenuazonic acid (TA)

		Parameters[a]											
Column	Compound	^tR (min)	B/A	$W_{0.1}$ (min)	Nsys (plates)	$\overline{M}_2$	^tG	$^\sigma G$	Nmax (plates)	τ	γs	RSE (%)	$\tau/^\sigma G$
C_{18}	CPA	6.57	1.82	1.58	235	0.1843	6.37	0.2208	831	0.3682	1.262	26	1.67
	TA	4.95	1.93	1.23	219	0.1159	4.79	0.1639	854	0.2984	1.347	23	1.82
C_8	CPA	7.65	3.04	2.18	120	0.4567	7.41	0.1958	1433	0.6468	1.753	8	3.30
	TA	4.85	3.04	1.94	61	0.3617	4.64	0.1741	710	0.5757	1.754	8	3.31

[a]Abbreviations: ^tR, peak retention time; B/A, empirical asymmetry factor; $W_{0.1}$, peak width at 0.1 peak height; Nsys, number of theoretical plates; $\overline{M}_2$, second central moment of the peak; ^tG, retention time of parent Gaussian peak; $^\sigma G$, standard deviation of parent Gaussian peak; Nmax, number of theoretical plates for parent Gaussian peak; τ, exponential modifier; γs, peak skew; RSE, relative system efficiency; $\tau/^\sigma G$, tau-sigma ratio.

tal chromatograms and calculation of the peak parameters which are discussed later.

Typical chromatograms for both spiked and naturally contaminated peanuts are presented in Figures 2A and B. The peak retention time for cyclopiazonic acid, using 2-propanol–acetonitrile (30 + 40) mobile phase on a C_{18} column at a flow rate of 1.5 mL/min is 6.60 ± 0.10 min, Figure 2C. The retention time is very sensitive to the amount of organic solvent in the mobile phase. A change from 30% to 25% 2-propanol concentration increased the retention time to 10.31 min. The separation is less sensitive to the pH of the mobile phase. A change of pH from 7.3 to 6.9 increased the retention time to 6.79 min without significantly altering any other parameters. The detector response is linear to at least 1 μg cyclopiazonic acid with a limit of detection at approximately 4 ng (2 × baseline noise, 0.01 AUFS). Similar results were obtained with a C_8 column; the retention time was slightly lengthened to 7.15 ± 0.17 min.

The extraction and sample cleanup procedure used in this study is essentially the same as that used previously for detecting cyclopiazonic acid in peanuts (8). The extraction efficiency was reported at that time to be approximately 93% at levels of 100–200 μg/kg. Re-examination of the efficiency, using 4 replications at spiked levels of 68.9, 210, and 955 μg/kg, gave 85.9% (12.86% CV), 72.9% (6.43% CV), and 81.4% (0.40% CV), respectively. The difference in efficiency is attributed in part to the lack of precision in the quantitation of cyclopiazonic acid on thin layer plates. A more realistic approach was taken for the determination of efficiency in the present study than in the previous one by using a composite sample lot which was known to be naturally contaminated by fungi. The sample lot gave a background chromatogram similar to that for cyclopiazonic acid-contaminated samples. The background chromatogram is shown by the dotted line in Figure 2A. The aflatoxin concentration of the sample lot was estimated to be 15 μg/kg (3 replications) by the Holaday-Lansden minicolumn procedure (14).

Cyclopiazonic acid in naturally contaminated samples can be confirmed by spotting appropriate amounts of the sample and a cyclopiazonic acid standard on thin layer plates as outlined by LeBars (2).

Since the mobile phase and mode of separation are somewhat novel, chromatographic parameters have been calculated using the semiempirical equations developed by Foley and Dorsey (13) for exponentially modified Gaussian (EMG) peaks. Kirkland et al. (15) have determined that calculations of column plate counts suffer serious error if the peak shape is not extremely close to Gaussian shape (i.e., skew $<<0.7$). The validity of the EMG model for the chromatographic system was checked by calculating equivalent peak parameters at 0.1 and 0.3 of peak height, using the appropriate equations (13). All parameters tested were in agreement within experimental error, indicating that the EMG peak model is a valid model for the chromatographic system.

The chromatographic figures of merit (CFOMs) for cyclopiazonic acid and tenuazonic acid on C_{18} and C_8 columns are presented in Table 1. A theoretical discussion of these various CFOMs is beyond the scope of this paper, but can be found in Foley and Dorsey (13), Kirland et al. (15), Yau (16), and Barber and Carr (17). To avoid confusion, symbols and abbreviations are those used by Foley and Dorsey (13). The peak retention time (^tR), the empirical asymmetry factor (B/A) of the peak and the peak width at 0.1 of peak height ($W_{0.1}$) were manually measured in accordance with the suggestions of Foley and Dorsey (13). The remaining CFOMs were calculated with the appropriate equations of that reference.

The purpose for including Table 1 in this work is to provide a basis for comparison of the present system and possible future improvements in the chromatographic system rather than as a basis for discussion of the principles under which the separation occurs. A comparison of the parameters shows that the chromatographic system using a C_8 column gives a less satisfactory peak shape than does the C_{18} column. The wide peak width and large amount of skew are responsible for the low number of theoretical plates. Replacement of the column and guard column with low dead volume connectors gave a $W_{0.1}$ value of 0.23 min for a 0.5 μL injection of cyclopiazonic acid which indicates that the CFOMs are largely controlled by the kinetics of the mobile and stationary phases. The use of CFOMs is also profitable in periodically checking for column degradation and irregularities between solvent batches. In these cases the measurement of ^tR, B/A, $W_{0.1}$ and the calculation of Nsys, and $\overline{M}_2$ is considered sufficient. Over a period of several weeks of continuous use, neither the C_8 or C_{18} columns have shown any significant deterioration as measured by these parameters. It should be noted that the C_{18} column used in this study is reported by the manufacturer to have 50% residual silanol groups compared with 5% or less for the C_8 column. The use of a fully capped C_{18} column may require a substantially different solvent composition than is reported here.

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Gas Chromatographic Determination of Deoxynivalenol in Wheat with Electron Capture Detection

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A method is described for the determination of deoxynivalenol (DON) in wheat. The method involves sample extraction with chloroform–ethanol (8 + 2), column chromatographic cleanup on silica gel of small particle size, and derivatization with heptafluorobutyric acid anhydride using 4-dimethylaminopyridine as a catalyst. The derivative DON tris-heptafluorobutyrate is determined by gas chromatography using an electron capture detector. Recoveries of DON added to wheat at levels of 118–1184 ng/g averaged 88% with a coefficient of variation of 8.6%.

Deoxynivalenol (DON), also known as vomitoxin, belongs to a group of chemically related toxins that are characterized by the 12,13-epoxytrichothecene ring system and are produced by several species of *Fusarium*. DON is most often associated with grains that have become infected with *Gibberella zeae*. It was first isolated in the United States in 1973 by Vesonder et al. (1) and in Japan by Yoshizawa and Morooka (2) from grain infected by *Fusarium graminearum*. The isolated toxin, when fed to animals, induced emesis (vomiting) and caused symptoms of feed refusal. Vesonder et al. (3) described these symptoms in swine that had been fed moldy grains infected with *G. zeae* during large-scale outbreaks of *G. zeae* infection in the U.S. grain belt in 1966, 1972, and 1975. Recently, Canada's crop of white winter wheat was extensively contaminated with DON (4, 5). In the late summer of 1982, wheat ("scabby wheat") and corn in the grain-producing belt of the United States were infected with *G. zeae*. On analysis, samples from infected fields showed high levels of DON contamination.

The acute and chronic toxicological properties of DON have been reviewed (6, 7) and linked with various diseases in animals and humans (6). Vomiting and feed refusal are observed in laboratory animals fed DON. Symptoms such as feed and water refusal are used as the basis of a mouse bioassay procedure for DON determination (8–10).

Existing chemical methods for determining DON in grain products are based on extraction with aqueous methanol (4, 11); the extract is cleaned up by protein precipitation and subjected to liquid-liquid partitioning and column chromatography. Other methods use packed (12, 13) and capillary column (5) gas chromatography with electron capture detection (GC-EC) to quantitate the fluoracyl derivative of DON in grain samples. A gas chromatograph coupled to a mass spectrometer operated in a single ion monitoring mode has been used (4, 13) as a selective and sensitive detection system for DON quantitation. A GC method (12) based on the detection of the trimethylsilyl ether derivative with a flame ionization detector has been developed for the determination of DON at high levels. Recently, a method (14) was developed that is based on acetonitrile–water extraction, cleanup on an activated charcoal/alumina column, and quantitation by thin layer chromatography using aluminum chloride as a spray reagent.

In general, these methods require extensive sample cleanup to remove interfering constituents. Methods (4, 11) based on aqueous methanol extraction that have detection limits of <300 ng/g require at least 3 or 4 separate cleanup steps, e.g., protein precipitation, liquid-liquid partition, and column chromatography, to obtain a sufficiently clean extract to allow determination of DON at low levels. Methods that incorporate a multistep cleanup procedure make analysis for DON very time consuming and costly.

We have developed a method for the determination of DON in wheat which is based on a rapid and efficient sample cleanup and GC-EC quantitation of the fluoracyl derivative of DON.

METHOD

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5880 A, or equivalent, equipped with ^{63}Ni electron capture detector and 183 cm × 2 mm id glass tube packed with 3% OV-101 on 80–100 mesh Chromosorb W HP.

(b) *Centrifuge.*—IEC EXO centrifuge for Class 1, Group D, Hazardous Locations (International Equipment Co., Damon Corp., Needham Heights, MA 02194).

(c) *Column for sample cleanup.*—Disposable polypropylene column fitted with plastic filter disk and 12 mL extension funnel, Quik-Sep Code QS-Q and QS-R (Bio Lab Products, San Jose, CA 95128).

(d) *Disposable test tube.*—125 × 16 mm id (Kimble Div., Owens-Illinois, Inc., Toledo, OH 43666).

(e) *Vortex tube mixer.*—Vortex-Genie Model K-550G (Scientific Industries, Inc., Bohemia, NY 11716), or equivalent.

(f) *Dry heating block.*—Reacti-Therm, equipped with Reacti-Block T-1 (Pierce Chemical Co., Rockford, IL 61105), or equivalent.

Reagents

(a) *Deoxynivalenol (DON) stock solution.*—100 ng/μL. Dissolve 1.0 mg DON (Myco-Lab Co., Box 321, Chesterfield, MO 63107) in 10 mL methanol. Store in freezer. Caution: DON may decompose in methanol after ca 30 days.

(b) *Heptafluorobutyric acid anhydride (HFBAA).*—Pierce Chemical Co.

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(c)) *Silica gel.*—E. Merck Silica Gel H, medium particle size (10–40 μm) (Brinkmann Instruments, Inc., Westbury, NY 11590). Use as received.

(d) *Solvents.*—Methanol, absolute ethanol. acetone, acetonitrile, toluene, *n*-hexane, methylene chloride, and chloroform, all distilled in glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442, or equivalent). (Caution: Chloroform is a possible carcinogen.)

(e) *Catalyst.*—4-Dimethylaminopyridine (4-DMAP), 99% minimum purity (Aldrich Chemical Co., Inc., Milwaukee, WI 53233). Prepare catalyst solution containing 2 mg/mL by dissolving 100 mg 4-DMAP in 50 mL toluene–acetonitrile (95 + 5).

Sample Extraction

Grind wheat sample to pass 2 mm screen. Weigh 25 g sample into 250 mL glass-stopper flask, add 10 mL water and 125 mL chloroform–ethanol (8 + 2), and shake 60 min, using wrist-action shaker set at fast rate. Filter sample through fluted paper. Transfer 10 mL of sample filtrate to 4 dram vial, and concentrate to dryness on dry heating block under stream of nitrogen. Save sample residue for column cleanup.

Column Cleanup

Prepare silica gel slurry by shaking 25 g silica gel and 100 mL methylene chloride. Insert 16 × 125 mm test tube into each metal centrifuge shield. Insert Quik-Sep column into each test tube. Pipet 5 mL silica gel slurry into each Quik-Sep column and centrifuge 2 min at 1000 rpm. Discard liquid collected in each test tube. (All elutions referred to in sample cleanup procedure are accomplished by centrifuging column assembly at 1000 rpm for 2 min.) Dissolve sample residue in 3 mL methylene chloride, using vortex tube mixer, and transfer to column. Rinse vial with 2 mL methylene chloride and add rinse to column. Centrifuge column assembly at 1000 rpm for 2 min. Discard liquid in test tube. In similar manner, wash column with 10 mL toluene–acetone (8 + 2) and discard liquid in test tube. Insert column into clean test tube. Elute DON from column with 8 mL methylene chloride–methanol (95 + 5). Quantitatively transfer sample eluate to 4 dram vial and concentrate to dryness on dry heating block at 60°C under stream of nitrogen. The final extract represents 2 g sample.

Derivatization

Transfer 10 μL DON stock solution to 3 dram vial and evaporate to dryness. Treat sample residue from column cleanup step and standard identically. Transfer 1.0 mL 4-DMAP catalyst solution to vial and add 50 μL HFBAA. Firmly cap vial and warm 20 min on dry heating block at 60°C. Let derivatized reaction mixture cool to room temperature. Add 1.0 mL 3% aqueous sodium bicarbonate solution to vial, mix 2 min on tube mixer, and let stand until layers are fully separated. Transfer 100 μL organic (upper) layer by syringe to 2 dram vial containing 900 μL *n*-hexane. Final concentration of standard solution, expressed as DON equivalent weight/μL, is 0.1 ng/μL. Final concentration of sample solution, expressed as underivatized sample equivalent weight/μL, is 0.0002 g/μL.

Gas Chromatography

Complete GC analysis on same day as derivatization.

Operating conditions.—Carrier gas 5% methane in argon; flow rate 60 mL/min; chart speed 0.5 cm/min; attenuation adjusted to give 10% full scale deflection for 100 pg standard; injection port temperature 200°C; oven temperature program—initial temperature 175°C, initial time 10 min, program rate 10°C/min, final temperature 250°C, final time 5 min.

Standard curve.—Inject 1–5 μL derivatized DON standard directly onto column to obtain peak response. Construct standard curve by plotting amount of derivatized DON vs detector response for the 100–500 pg range. The detector response (peak area) for the 100–500 pg range varies linearly. The retention time for the DON derivative under the operating conditions listed is ca 6.5 min.

Determination.—Inject 2 μL sample extract into gas chromatograph under same conditions used for preparing standard curve. Calculate amount of DON in sample by comparing peak area of sample with peak area of derivatized DON standard as follows:

$$\text{ng DON/g sample} = (A \times B \times D)/(C \times E \times F)$$

where A = concentration of DON standard (ng/μL), B = volume of DON standard (μL), C = peak area of standard, D = peak area of sample, E = concentration of sample (0.0002 g/μL, if 25 g sample is used), and F = volume of sample extract injected (μL).

Table 1. Recovery of deoxynivalenol added to wheat

Added, ng/g	Rec., ng/g	Rec., %
1184	1178	99.5
	1164	98.3
	968	81.8
592	476	80.4
	491	82.9
	572	96.6
296	229	77.4
	260	87.8
	246	83.1
118	104	88.1
	101	85.6
	113	95.8
Mean rec., %		88.1
SD		7.6
CV, %		8.6

Results and Discussion

This method is considerably faster and requires far less solvent than any other method presently available for the determination of DON. With the GC conditions described in the method, as little as 10 pg DON standard can be detected. The limit of detection in wheat is 118 ng/g. From the linear response established by the standard curve, the DON content of a sample can be rapidly determined with only a periodic check of the standard curve. Recoveries of DON added to wheat samples at levels ranging from 118 to 1184 ng/g are shown in Table 1. All samples were analyzed in triplicate along with a separate blank. Recoveries ranged from 77.4 to 99.5%. Chromatograms of a blank wheat sample in which no DON was detected and a wheat sample spiked with DON at the 118 ng/g level are shown in Figures 1 and 2, respectively.

The use of the catalyst 4-DMAP has been reported by Hofle et al. (15) to be a more effective reagent than imidazole in the acylation of alcohols. As shown in Figure 3, the formation of the trisheptafluorobutyrate derivative of a DON standard by using HFBAA/4-DMAP proceeds much faster than it does with the heptafluorobutyrylimidazole (HFBI) reagent presently used for DON derivatization in alternative methods (4, 5). Complete derivatization was accomplished in less than 10 min at 60°C for the 4-DMAP-catalyzed reaction, whereas the HFBI reagent did not effect complete derivatization after 2 h under the same conditions.

The sample cleanup procedure described in this method is rapid, efficient, and reproducible. The column cleanup requires

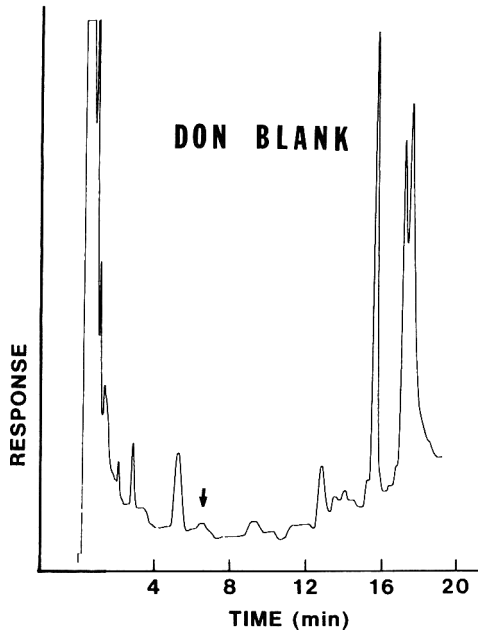

Figure 1. Chromatogram of wheat sample in which no deoxynivalenol (DON) was detected: initial column temperature 175°C, attenuation 2^{10}. Arrow indicates retention time of DON trisheptafluorobutyrate. Chromatogram represents 0.0004 g equivalent wheat injected.

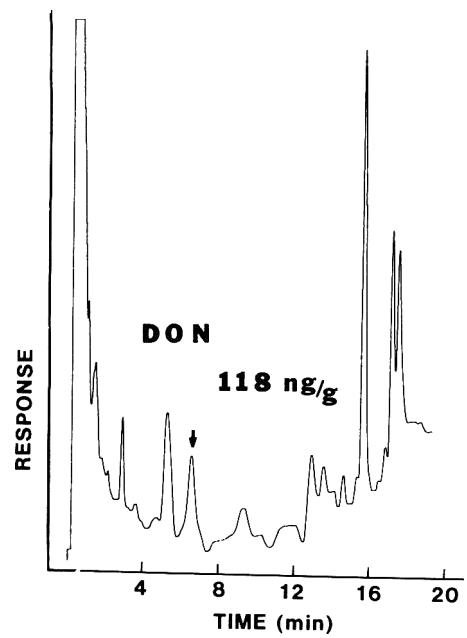

Figure 2. Chromatogram of wheat sample with deoxynivalenol (DON) added at the 118 ng/g level. See Figure 1 for GC conditions.

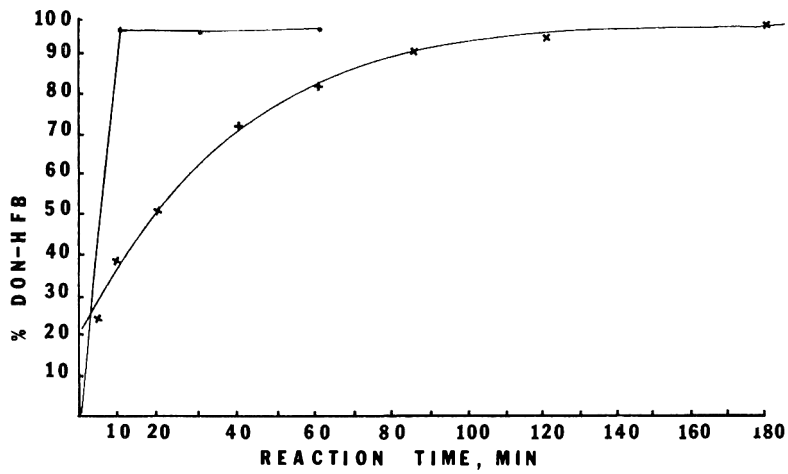

Figure 3. Reaction time vs deoxynivalenol trisheptafluorobutyrate formation. Reaction conditions: 1.18 μg DON reacted at 60°C; × reagent solution, 50 μL heptafluorobutyrylimidazole/mL toluene–acetonitrile (95 + 5); reagent solution, 50 μL heptafluorobutyric acid anhydride/mL toluene–acetonitrile (95 + 5) containing 2 mg 4-dimethylaminopyridine.

a total of only 23 mL solvent. Because of the small elution volume of the column, significant time is saved in the concentration of the sample extract. The column, although characterized as disposable, may be emptied and used again.

Silica gel of small particle size, when tightly packed, can provide a sample cleanup column with efficiency similar to that of high pressure liquid chromatographic columns of comparable length. The use of centrifugal force as a source of pressure to maintain sufficient column flow is a novel approach that has been described by Karasek and Rasmussen (16) and has been successfully used to fractionate complex matrices before mass spectrometric analysis. This approach permits the complete cleanup of 8 samples in less than 30 min with the use of an 8-place centrifuge rotor.

The cleanup procedure described here, coupled with chloroform–ethanol extraction and a more effective derivatization step, is a considerable improvement over presently available methods with respect to analysis time, cost, and consistency.

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Identification of Aflatoxins by Quadrupole Mass Spectrometry/Mass Spectrometry

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MS/MS daughter experiments were recorded for aflatoxins B_1, B_2, G_1, G_2, M_1, M_2, and aflatoxicol, using 3 ionization modes. Daughters were recorded from the molecular ion (M^+) using electron impact ionization (EI). Daughters from the protonated molecules (MH^+) were recorded in the positive ion mode and the daughters from the molecular anion (M^-) were recorded in the negative ion mode using chemical ionization (CI). These daughter spectra are all relatively simple. The EI daughters are quite similar to conventional EI spectra. The yield of (M^-) is about 100 times greater than the yield of M^+ in EI or MH^+ in isobutane CI spectrum. Negative ion daughter spectra were used to demonstrate the feasibility of determining the presence of aflatoxin B_1 in crude extracts of contaminated corn. Aflatoxin B_1 could be detected at 10 ppb.

Aflatoxins are compounds produced by the fungi *Aspergillus flavus* and *A. parasiticus*. They have been found in grains, peanuts, and tree nuts grown under favorable climatic conditions (1) and have been shown to be potent carcinogens in laboratory animals (2). Numerous methods have been devised to identify aflatoxins in complex mixtures, based on either biological activity or chemical/physical properties (UV, fluorescence, and chromatography). Because aflatoxins are found in low concentrations and are not amenable to gas chromatography, several steps are necessary to prepare samples pure enough for identification by mass spectrometry. Field desorption (FD) MS has been used to screen extracts for aflatoxins (3); however, FD has not found widespread application because it is a difficult technique to use. Haddon and coworkers identified aflatoxins (4) from purified fractions by using electron impact (EI) MS with low-resolution full scan and high-resolution selected ion monitoring (HRSIM) modes. They report confirmation of aflatoxin identities by EI-MS in samples at the 10–1000 ppb level after extensive sample cleanup involving isolation of at least 10–50 ng aflatoxin by TLC. One μg was reported to be near the detection limit for low-resolution MS even after extraction and TLC cleanup. HRSIM allowed less rigorously purified samples to be analyzed, and HRSIM detection limits for aflatoxins of 0.1 ng are reported (4). Nesheim and Brumley (5) and Brumley and coworkers (6) identified aflatoxins from negative ion chemical ionization (NICI) obtained from resonance capture conditions in the CI source. Aflatoxins M_1 and B_1 in food matrices at levels as low as 10 ppb were detected after extraction, silica gel column chromatography, 2-dimensional TLC, extraction, and NICI-MS. Because many of the components present in the partially purified 2-dimensional TLC fraction did not give intense negative ions under NICI conditions, whereas the aflatoxins did, the chemical noise was reduced to the point where about 20 ng aflatoxins recovered from TLC plates was sufficient to unambiguously identify aflatoxins B_1 and M_1 (6).

The major limitation to the confirmation of aflatoxin in the low ppb range has been attributed to chemical noise (5). The technique of tandem mass spectrometry or mass spectrometry/mass spectrometry has generated considerable interest as a method to help overcome the chemical noise problem in the analysis of target compounds in a complex matrix of organic compounds. MS/MS uses one mass filter to select the compound of interest from the matrix (usually as the molecular ion in EI, protonated molecule in CI, or molecular anion in NICI). These selected ions then undergo collisionally activated dissociation (CAD) to yield characteristic products, which are separated by the second mass filter and measured. In this paper, we discuss the CAD spectra of parent ions generated from aflatoxin in the EI and both positive and negative CI modes. The spectra were obtained with a commercial tandem quadrupole mass spectrometer, one of numerous instruments available for MS/MS studies that have been reviewed (7, 8). (Some MS/MS experiments with aflatoxin (unpublished) have been performed by SCIEX, Inc. (55 Glen Cameron Road, No. 202, Thornhill, Ontario, Canada L3T1P2; 416 881-4646). They have an application note for the APCI/MS/MS, using their TAGA 6000 instrument, analysis of mycotoxins.)

Experimental

A Finnigan 4535/TSQ triple quadrupole mass spectrometer capable of pulsed positive-negative ion chemical ionization was used for MS/MS analyses. Isobutane was used as the reagent gas or buffer gas (negative ion) at a pressure of 0.25 torr, as indicated by the thermocouple gauge connected to the source. Source temperature was maintained at 140°C and electron energy was 70 eV. Argon was the collision gas in the collision cell (Q_2) for CAD experiments. The pressure in Q_2 was measured by the thermocouple gauge on the collision cell. Collision energy, measured as the axial DC offset voltage of Q_2, was variable from +30 V to −30 V. For positive ions, it was typically set at −12 to −20 V; for negative ions, +20 V was used. Because daughter spectra produced in low-energy CAD experiments in quadrupole MS/MS are highly sensitive to both target gas pressure and collision energy, careful attention to instrument parameter settings is needed to achieve reproducible daughter spectra.

Samples were introduced via either the standard insertion probe or the direct exposure probe (DEP). With the standard

The mention of firm names or trade products does not imply that they are endorsed by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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Table 1. Electron impact mass spectra of aflatoxins

Compound	MW	M^+	Other ions[a]
Aflatoxin B_1	312	312(100)	284(20), 283(14), 281(6), 269(11), 256(16), 255(13), 241 (20), 228(30), 227(37), 213(22)
Aflatoxin B_2	314	314(100)	299(4), 285(20), 271(61), 256(7), 243(12), 229(10), 215(10)
Aflatoxin G_1	328	328(100)	300(13), 297(13), 256(13), 255(12), 241(20), 237(39), 213(19)
Aflatoxin G_2	330	330(100)	301(8), 287(19), 286(8), 269(7), 258(5), 243(16), 241(7), 229(8), 215(13), 213(6), 201(10)
Aflatoxin M_1	328	328(100)	299(65), 285(5), 283(8), 271(47), 237(8), 255(5), 243(51), 229(20), 227(13), 215(10), 201(10)
Aflatoxin M_2	330	330(100)	312(20), 301(22), 284(16), 273(32), 246(14), 245(11), 228(14), 217(17), 203(10)
Aflatoxicol	314	314(27)	296(12), 268(9), 257(9), 229(6), 224(5)[b]

[a]Above m/z 200 >4% relative intensity (number in parenthesis is percent relative intensity for that ion).
[b]Base peak m/z 45; many fragments below m/z 200.

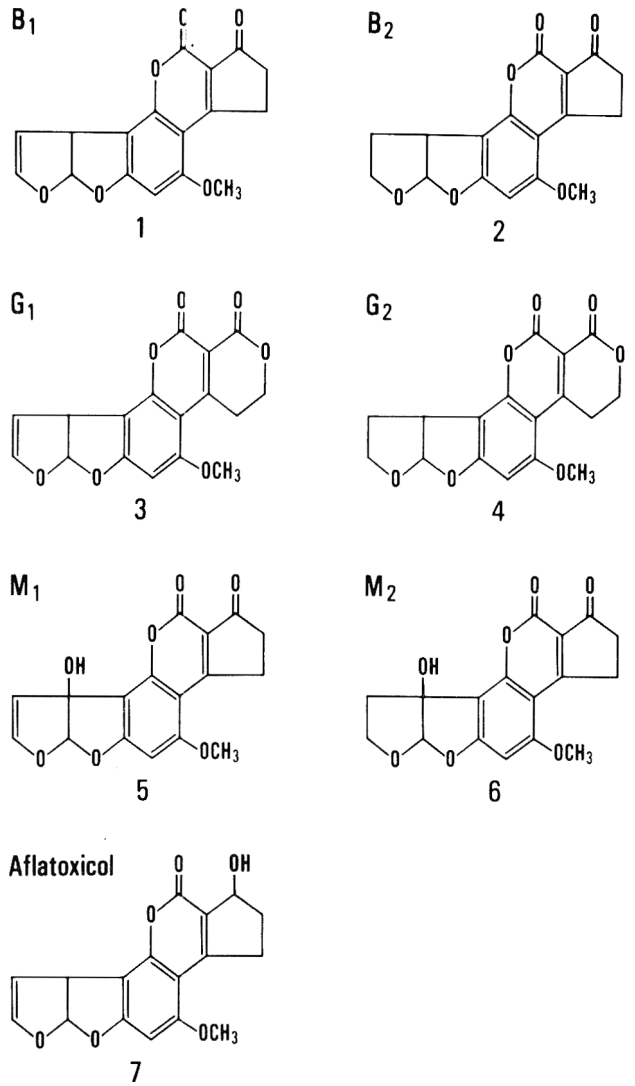

Figure 1. Structures of aflatoxins listed in tables.

probe, the sample was placed in a glass sample holder that was heated ballistically to 400°C in 4 min. With the DEP probe, the sample was deposited onto the tip of a sample filament that, under CI conditions, is located approximately 1 mm from the exit aperture of the ion source and is completely surrounded by reagent gas plasma. The sample is then rapidly heated by programming the filament current to 400 ma at 20 ma/s. Aflatoxins vaporize from the probe under these conditions as an approximately 2-s wide peak about 10 s after heating is begun. Because of the rapid vaporization from the probe, detection limits are an order of magnitude better than with the standard probe; however, the total amount of sample that can be used with this probe is proportionately less.

Data acquisition and mass spectrometer control were accomplished by an Incos 2300 data system using the vendor's specialized software to control scanning and mass setting of both quadrupoles in MS/MS experiments. Alternating the parent ions focused into Q_2 under computer control allowed simultaneous CAD profiles for multiple positive or negative parent experiments to be obtained in a single analysis. Conventional mass spectra were obtained by operating the first mass quadrupole filter (Q_1) as well as Q_2 in the all pass mode and scanning the second mass filter (Q_3).

Crude extracts were obtained by extraction of 50 g ground, blended corn sample with chloroform-water (250 mL = 25 mL) in the presence of Celite. Extracts were cleaned up using the CB method approved for corn by AOAC (9).

Results and Discussion

Because aflatoxins are fused-ring heterocyclic compounds with a relatively high degree of unsaturation and a high oxygen content, their EI spectra tend to show intense molecular ions and little fragmentation. Molecular ions from pure aflatoxins can be routinely detected by EI from 1 ng samples. However, the EI-MS of the complex matrix in which aflatoxins are found contains a signal at virtually every mass to charge (m/z) value from 50 to around 500, interfering with confirmation of the aflatoxins. Chemical noise interferences necessitate several orders of magnitude more sample to identify reliably the presence of aflatoxin M_1 in an extract over the amount of pure standard aflatoxin M_1 needed to produce a detectable molecular ion. Tandem MS provides a technique to filter chemical noise from the sample and thereby improve detection limits. To determine the viability of this technique for aflatoxin, the MS/MS daughter spectra for several standard aflatoxins were recorded for the EI molecular ions, the CI protonated molecules, and the NICI molecular anions.

The EI mass spectra of aflatoxins B_1, B_2, G_1, G_2, M_1, M_2, and aflatoxicol are reported in Table 1 (see Figure 1 for structures). To illustrate the variability in the daughter spectra obtained from quadrupole MS/MS with relatively small changes in pressure or ion energy, Figure 2 shows the CAD daughters for the M^+ (m/z 312) of aflatoxin B_1 at 2 collision cell pressures (1 and 2 mtorr) and 2 energies (−12 V and −20 V). Because of the high degree of variability in daughter spectra with changes in pressure or energy, careful attention to these parameters is required to obtain reproducible daughter fragment intensities. Table 2 presents the CAD daughters from the M^+ ions from electron impact of the aflatoxins at a collision cell pressure of 1 mtorr and an offset potential of −20 V. Recognizable daughter spectra could be obtained for samples as small as 5 ng. The MS/MS instrument, when used as a conventional mass spectrometer, is about 5 times more sensitive to the molecular ions of aflatoxins than it is when collecting CAD daughter spectra. This apparent loss in sensitivity is offset by the improvement in signal-to-noise ratio in the MS/MS mode. Setting Q_1 to pass only the molecular ion of the aflatoxin of interest and subsequently scanning for

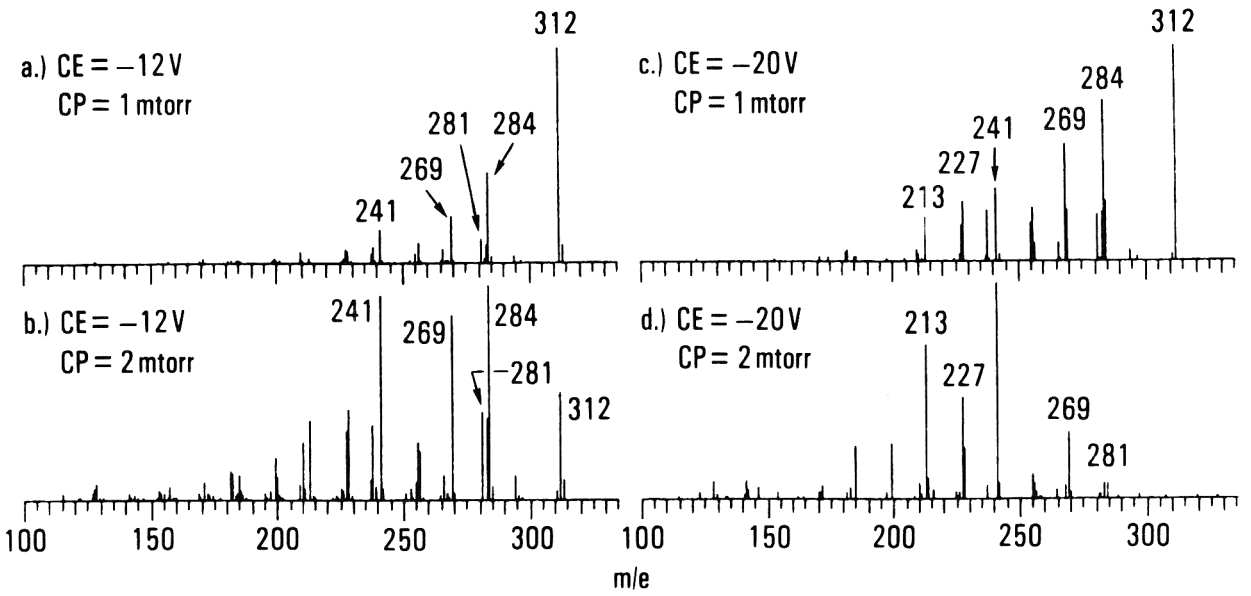

Figure 2. MS/MS CAD daughters of 312 from EI-MS of aflatoxin B_1 at different collision gas pressures and energies.

Table 2. Collisionally activated dissociation daughters of M^+ from electron impact mass spectra of aflatoxins (collision energy −20 V; pressure 1 mtorr)

Compound	MW	M^+	Daughters[a]
Aflatoxin B_1	312	312(100)	284(72), 283(26), 281(22), 269(69), 256(28), 255(14), 241(67), 238(14), 228(39), 213(20)
Aflatoxin B_2	314	314(50)	299(5), 285(29), 271(100), 256(12), 243(9), 241(3), 229(5), 227(7), 215(5)
Aflatoxin G_1	328	328(100)	310(8), 300(47), 297(43), 284(8), 282(7), 267(12), 257(16), 256(12), 241(46), 239(9), 227(16), 213(36)
Aflatoxin G_2	330	330(100)	315(4), 301(15), 287(54), 286(8), 269(15), 258(10), 257(4), 243(23), 229(7), 215(15), 201(11)
Aflatoxin M_1	328	328(49)	313(5), 299(100), 285(13), 283(19), 273(23), 271(15), 257(18), 243(31), 229(19)
Aflatoxin M_2	330	330(62)	312(33), 301(69), 299(14), 297(12), 284(37), 273(100), 271(19), 269(20), 256(15), 246(32), 228(12), 205(13)
Aflatoxicol	314	314(93)	313(59), 296(100), 286(23), 268(62), 257(51), 253(16), 229(12), 225(17), 215(5)

[a]Number in parenthesis is the percent relative intensity for that ion.

daughters in Q_3 yields less total signal, but much more of the instrument background and matrix signal is eliminated. There is an even more significant improvement in signal-to-noise ratio when samples that contain large amounts of impurities are analyzed.

The CAD daughters of M^+ for the aflatoxins occur at the same m/z values as the major fragments observed in the EI spectra. Under the conditions reported in Table 2, even the relative daughter intensities are similar to EI fragment intensities for all the aflatoxins, but, as demonstrated by Figure 2, the striking similarity of the daughter intensities to full EI spectra is a function of collision energy and collision cell pressure. The coincidence of m/z values in the daughter spectra and EI spectra indicates that similar fragmentations are probably occurring in the EI source and the CAD cell.

Metastable species, ions with sufficient energy to decompose during the $\sim 10^{-5}$ s they spend in the collision cell, can be examined if M^+ ions are selected by Q_1 and daughters are scanned by Q_3 with no collision gas added in the collision cell. As expected, most of the M^+ ions from the source that are focused in Q_2 pass unchanged through to the collector when collision gas is absent. A small percentage of the M^+ ions, however, has sufficient energy to form products in Q_2. Metastable daughter spectra are reported in Table 3. The intensity values have been normalized to the most intense metastable daughter. Taken together, these metastable species account for about 1–5% of the total signal collected from the M^+ ion. The metastable daughter spectra for aflatoxins show fewer ions than the CAD daughter spectra. For example, the m/z 312 M^+ ion from aflatoxin B_1 shows losses of 18 (m/z 294), 28 (m/z 284), 29 (m/z 283), 31 (m/z 281), and 46 (m/z 266) in this mode of operation. The CAD daughter spectrum indicates several additional losses, including intense daughter fragments at m/z 269 (loss of 43), m/z 255 (loss of 56), and m/z 241 (loss of 71). It is not readily apparent whether these additional losses arise from multiple collisions or rearrangements of the molecule under CAD conditions. Although the CAD fragmentation processes lead to daughters with the same m/z values as the EI fragments, these ions are not observed among the metastable daughters because molecular ions with sufficient energy to create these fragments do so while still in the source and do not survive for the time necessary to undergo metastable decompositions in Q_2. It is apparent from the data presented in Tables 2 and 3 that metastable daughter and CAD daughter spectra provide much useful information about the fragmentations observed in the EI-MS of various structurally similar aflatoxins. The presence of a double bond in the furan ring greatly affects the fragmentation of the molecule. Aflatoxins B_1 and G_1, both containing a double bond, show strong losses of CO (28) with a smaller loss of CHO (29), whereas B_2 and G_2, lacking this double bond, show strong losses of CHO and C_2H_3O (43) with a less significant loss of CO. The loss of CO probably occurs from the coumarin portion of the molecule since this loss is a primary fragmentation pathway in coumarin derivatives (10). Losses of CHO and C_2H_3O more likely are from the difuran rings.

Chemical ionization mass spectra typically show much less fragmentation than EI mass spectra. The decrease in fragmentation minimizes the noise contributed to the mass spec-

Table 3. Metastable daughters of M^+ of aflatoxins from EI-MS

		Daughters[a]						
	M^+	M-15	M-18	M-28	M-29	M-31	M-43	Other
B_1	312	—[b]	294(33)	284(100)	283(11)	281(22)	—	266(8)[M-46]
B_2	314	299(7)	296(1)	286(17)	285(46)	283(5)	271(100)	268(5)[M-46]
G_1	328	—	310(33)	300(100)	—	297(85)	—	284(10)[M-44]; 239(7)[M-59]
G_2	330	315(4)	312(1)	—	301(40)	299(4)	287(100)	258(6)[M-72]; 243(14)[M-87]
M_1	328	313(1)	310(2)	300(28)	299(100)	—	285(10)	284(55)[M-44]; 271(35)[M-57]
M_2	330	—	312(100)	302(17)	301(76)	299(11)	—	284(52)[M-46]; 274(93)[M-56]

[a]Number in parenthesis is percent relative intensity for that ion.
[b]Not detected.

Table 4. CAD daughters of MH^+ from isobutane chemical ionization mass spectra of aflatoxins (collision energy −20 V at 2 mtorr)

Compound	MW	MH^+	Daughters[a]
Aflatoxin B_1	312	313(100)	285(29), 270(6), 269(7), 257(5), 241(12), 214(4), 183(3)
Aflatoxin B_2	314	315(100)	299(1), 297(6), 287(23), 272(6), 271(8), 259(30), 243(7), 231(6), 227(7), 203(5)
Aflatoxin G_1	328	329(100)	311(18), 301(1), 300(1), 283(16), 269(4), 257(3), 243(62), 225(3), 215(16), 200(13), 185(4)
Aflatoxin G_2	330	331(100)	313(18), 303(3), 285(6), 275(5), 257(6), 245(11), 229(3), 217(4), 201(3)
Aflatoxin M_1	328	329(47)	301(11), 285(4), 283(5), 273(100), 259(49), 229(18), 216(5), 203(2)
Aflatoxin M_2	330	331(40)	313(18), 303(3), 285(27), 273(100), 259(33), 247(7), 229(7), 214(5), 203(3)
Aflatoxicol	314	315(—)	297(100), 269(11), 254(12), 226(15), 198(4), 173(6), 153(5), 135(4), 107(3)

[a]Number in parenthesis is percent relative intensity for that ion.

Table 5. CAD daughters of $M^{-\cdot}$ from resonance electron capture, negative ion chemical ionization mass spectra of aflatoxins (collision energy +20 V at 2 mtorr)

Compound	MW	$M^{-\cdot}$	Daughters[a]
Aflatoxin B_1	312	312(1)	297(100), 269(4), 253(5), 209(1)
Aflatoxin B_2	314	314(0.5)	299(100), 271(6), 243(8)
Aflatoxin G_1	328	328(—)	313(33), 269(100), 241(16)
Aflatoxin G_2	330	330(—)	315(28), 271(100), 243(11), 215(3)
Aflatoxin M_1	328	328(0.5)	313(100), 270(1), 269(1)
Aflatoxin M_2	330	330(0.5)	315(100), 287(1), 270(1), 259(1)
Aflatoxicol	314	314(0.5)	299(100), 281(11), 271(2), 253(3), 243(3), 237(6)

[a]Number in parenthes s is percent relative intensity for that ion.

trum by the sample matrix, gives a strong MH^+ ion for a target component, and thus improves the detectability of that component. Unfortunately, the protonated molecule (MH^+) is often the only significant signal observed in the CI spectrum. Unless characteristic fragmentation is observed or a chromatographic separation or other purification is made, there is no way to unambiguously determine whether very low signal levels at m/z = MH^+ are coming from the protonated target molecule or from some matrix component. Recording MS/MS daughter spectra for the MH^+ ions provides a greater degree of assurance that the signal measured is indeed from the target component. In the positive ion CI mode, all 6 aflatoxins give intense MH^+ signals with little fragmentation. CAD daughter spectra for these aflatoxins, at a collision pressure of 2 mtorr and an energy of −20 V, are reported in Table 4. The higher pressure for CAD was required to get appreciable fragmentation from the relatively stable MH^+ ions. All the aflatoxins can be identified by unique signals in their CAD daughter spectra of MH^+ signals in spite of the fact that the several pairs of aflatoxins (M_1/G_1 at m/z 329 and M_2/G_2 at m/z 331) cannot be differentiated by positive CI alone. Samples as small as 2–5 ng aflatoxins produce satisfactory spectra.

Under CI conditions, a population of electrons at near thermal energies is produced along with the positive ions in the gas plasma. These electrons are available to react by resonance electron capture with certain compounds and produce negative ions (11). Aflatoxins undergo resonance capture to produce molecular anions. Brumley et al. (6) reported abundant ions at $M^{-\cdot}$, $(M-H)^-$, and $(M-CH_3)^-$ from all aflatoxins studied when methane was the buffer gas in the CI plasma. In our experiments with isobutane as the buffer gas, we observe much less $(M-H)^-$ in the spectra. This difference may apparently be attributed to proton abstraction by a Bronsted base present in the methane plasma rather than to reaction with the bases OH^- and O_2^- from instrument background as Brumley et al. suggested. With a methane plasma in our instrument, our CI spectra are similar to those reported (6). In any event, with isobutane we see primarily 2 ions for each of the aflatoxins, $M^{-\cdot}$ and the phenoxide anion $(M-CH_3)^-$. The CAD daughter spectra of the $M^{-\cdot}$ for the aflatoxins at a collision energy of +20 V and 2 mtorr are presented in Table 5. Like their positive CI counterparts, these molecular anions are more stable than the EI molecular ions and required higher pressures to produce daughters in addition to the stable phenoxide anions. The CAD daughters of the phenoxide anions under the same conditions were virtually identical to the CAD daughters of the molecular anion for M_1 and B_1. As with positive CI, M_1 and G_1 could not be distinguished by NICI alone, but whereas the CAD daughters of $M^{-\cdot}$ from M_1 showed only an intense phenoxide anion at m/z 313 (loss of CH_3), the CAD daughters of $M^{-\cdot}$ from G_1 showed additional intense anions at m/z 269 $(M-59)^-$ and m/z 241 $(M-87)^-$. M_2 and G_2 can be similarly distinguished. The sensitivity for aflatoxins using resonance electron capture conditions and negative ion detection is greatly improved for 2 reasons: First, many matrix interferences do not undergo resonance electron capture, making them transparent in the analysis. Second, the ratio of negative to positive ions formed in compounds that undergo resonance capture is commonly 10^3 or greater (11). In the case of aflatoxins, the yield of negative ions is about 100 times as great as the yield of positive ions under the same conditions. Thus, CAD daughter spectra could be obtained from picogram quantities of aflatoxins. Because the loss of CH_3 was so predominant in the

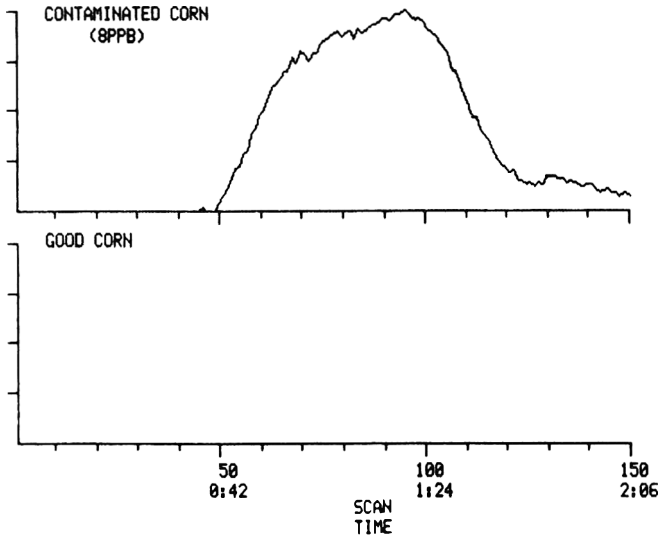

Figure 3. M/z 297 daughters of m/z 312. Upper curve contaminated corn (8 ppb) 0.025 g. Conditions: negative CI (isobutane) argon at 1 mtorr as collision gas and collision energy at +20 V.

CAD daughter spectra, sample sizes of about 100 pg were necessary to measure the additional daughter signals with reliable intensity ratios. This ensured that the daughter spectra recorded did indeed come from the M^- of the aflatoxin and did not come from a matrix contaminant.

To demonstrate the feasibility of NICI MS/MS to detect aflatoxins, chloroform extracts of 2 corn samples, one free of mycotoxin and the other containing 8 ng aflatoxin B_1/g were examined. The CAD daughter at m/z 297 from the m/z 312 parent was monitored from an extract aliquot equivalent to 25 mg corn. The sample was introduced by the direct insertion probe. Figure 3 shows the signal at m/z 297 (for daughters of m/z 312) for a 25 mg aliquot of the contaminated corn compared with the signal observed for the aflatoxin-free sample. When this much crude extract was put into the mass spectrometer, matrix effects tended to cause a highly variable loss in response for the 312 → 297 daughter signal. In this experiment, the signal was only about 25% as great as it should have been based on the response from a matrix free aflatoxin standard. If the amount of crude extract put into the mass spectrometer is below aliquots from 1 mg of crude corn extract, matrix effects are minimized and consistent recoveries are obtained. Careful attention to experimental detail and analysis of spiked clean corn extracts are needed to produce reliable quantitative results. Observing MS/MS signals of aflatoxin in corn extracts at these levels without any sample cleanup is a dramatic display of the improved separation power of the MS/MS technique, but much more definitive results are obtained by analyzing samples by MS/MS after cleanup on silica columns (9), where larger aliquots can be analyzed with better recoveries and lower detection limits can be achieved.

These experiments demonstrate that MS/MS, with NICI, can be used to confirm the presence of aflatoxins in real matrices. The advantage of the MS/MS procedure is that the preparative TLC separation step is not necessary; the whole matrix that is usually applied to the TLC plate can be put directly into the mass spectrometer. Good quantitative data from MS/MS require careful control of the amount of matrix that is put into the mass spectrometer. Studies aimed toward producing quantitative measurement of aflatoxins by MS/MS are now under way.

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Liquid Chromatographic Determination of Aflatoxin M_1 in Milk

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The official AOAC method for aflatoxin M_1 in milk was modified by replacing cellulose column chromatography with cartridge chromatographic cleanup and replacing thin layer chromatographic (TLC) determination with liquid chromatographic (LC) quantitation to yield a new method for bovine and porcine milk. An acetone extract of milk is treated with lead acetate and defatted with hexane, and M_1 is partitioned into chloroform as in the AOAC method. Chloroform is removed by evaporation under a stream of nitrogen at 50°C. The residue is dissolved in chloroform, the vessel is rinsed with hexane, and the 2 solutions are applied in sequence to a hexane-activated silica Sep-Pak cartridge. Less polar impurities are removed with hexane–ethyl ether, and M_1 is eluted with chloroform–methanol, and determined by C18 reverse phase LC using fluorescence detection. Recoveries of M_1 added to bovine milk at 0.25, 0.50, and 1.0 ng/mL were 90.8, 93.4, and 94.1%, respectively. The limit of detection was less than 0.1 ng M_1/mL for both bovine and porcine milk.

Aflatoxin M_1, a toxic, carcinogenic metabolite produced by animals from aflatoxin B_1, has been detected in the milk of lactating animals exposed to dietary B_1 (1–4). For this reason, there has been extensive interest in M_1 by regulatory agencies and research institutions.

When this laboratory was requested to analyze porcine milk for aflatoxin M_1, the official AOAC method (5) of extraction, purification, and thin layer chromatographic (TLC) determination was used on commercial, pasteurized, homogenized cow milk spiked with aflatoxin M_1. We found that the cellulose column chromatographic cleanup step yielded near zero recovery in our tests. We replaced that cleanup with the silica gel column procedure described by Trucksess and Stoloff (6), except 5% methanol in chloroform was used as the elution solvent for M_1. However, when TLC was used with spotmeter densitometry (7) for quantitation of M_1, we obtained recoveries exceeding 100%, indicating that significant background interferences remained in the extracts.

We report the development of a silica cartridge chromatographic cleanup step to replace time- and solvent-consuming silica gel column chromatographic cleanup, and automated sample analysis by reverse phase LC determination of M_1. The method provides improved separation and quantitation over TLC with spotmeter densitometry.

METHOD

Apparatus

(a) *Chromatographic cartridges.*—Silica Sep-Pak cartridges (Millipore-Waters, Milford, MA 01757).

(b) *Liquid chromatograph.*—Two M6000A pumps, Model 710 B WISP automatic injector, Model 720 system controller, Model 730 data module, with Intelink (Millipore-Waters).

(c) *Liquid chromatographic column.*—10 cm × 8 mm Radial-Pak 10 μm or 5 μm C18 cartridge fitted with C18 Guard-Pak guard column used with RCM-100 radial compression module (Millipore-Waters).

(d) *Fluorescence detector.*—Model FS-950 Fluoromat fluorescence detector equipped with FSA403 bandpass filter for excitation at 360 nm, using FSA111 lamp, and emission above 418 nm, using FSA426 longpass filter with FSA986A automatic overload reset control (Kratos/Schoeffel Instruments, Westwood, NJ 07675).

(e) *Spotmeter densitometer.*—Instrument as described by Dickens et al. (7).

(f) *Sample vials.*—Fisherbrand 7 mL scintillation vial with foil-lined screw cap (Fisher Scientific Co.). For LC, 4 mL vials fitted with 0.25 mL limited volume inserts and Teflon septa (Millipore-Waters).

(g) *Syringes.*—10 mL glass SP INTERFIT with glass plunger (American Scientific Products, Charlotte, NC 28210).

(h) *TLC plates.*—E. Merck No. 5763 20 cm × 20 cm glass plates coated with 0.25 mm silica gel 60 (American Scientific Products). Plates were spotted with samples by using Drummond "Microcaps" capillary pipets (Fisher Scientific Co.). Developing solvent was isopropanol–acetone–chloroform (5 + 10 + 85, v/v).

Reagents

(a) *Solvents.*—ACS-grade chloroform, acetone, trifluoroacetic acid, hexane, methanol, and ethyl ether (Fisher Scientific). LC grade acetonitrile, methanol (Fisher Scientific), and water from Model 1000 Hydro ultrapure water system (Hydro Service and Supplies, Inc., Research Triangle Park, NC 27709).

(b) *Aflatoxin M_1 standard.*—Stock prepared from crystalline M_1 (Calbiochem-Behring Corp., La Jolla, CA 92037), 1 μg/mL chloroform, and stored in freezer. Working standard of 0.1 μg/mL in methanol prepared daily; concentrations confirmed spectrophotometrically as described in secs. **26.004–26.011** (5).

(c) *LC mobile phase.*—Acetonitrile–acetic acid–water (15 + 3 + 82, v/v/v).

Preliminary Purification

Perform extraction of 100 mL milk, filtration, lead acetate precipitation, hexane partition, partition of M_1 into chloroform, and evaporation of solvent as in AOAC method **26.092** (5).

Cartridge Chromatography

Attach silica Sep-Pak cartridge to 10 mL syringe and rinse cartridge with ca 5–10 mL hexane. Transfer extract with chloroform (ca 6 mL) to syringe and apply to cartridge by depressing plunger. Rinse beaker with ca 6 mL hexane, transfer hexane rinse to syringe, and apply solution to cartridge by depressing plunger. Rinse cartridge with two 5 mL portions of hexane–ethyl ether (7 + 3, v/v) and discard rinses.

Elute aflatoxin M_1 with a single 7 mL portion of chloroform–methanol (9 + 1, v/v) into 7 mL scintillation vial. Evaporate purified extract, representing 47.6 mL fluid milk, to dryness under stream of nitrogen at 50°C.

LC Quantitation

Equilibrate instruments ca 30 min at flow rate of 2 mL/min. Adjust sensitivity of fluorescence detector to give 70–90% full scale deflection for 2–4 ng M_1 injected as desired. Set data module for integration with peak width of 80 s, noise

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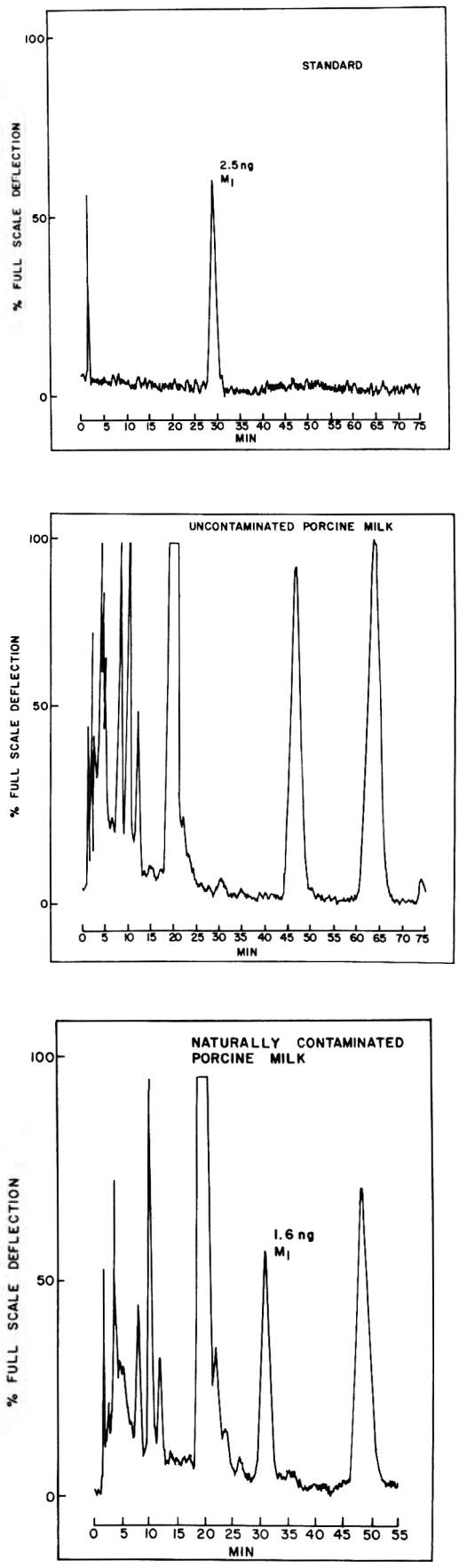

Figure 1. LC chromatograms on 5 μm C18 column of (top) aflatoxin M_1 standard: 25 μL injection containing 2.5 ng M_1 at detector sensitivity of about 62% full scale for 2.5 ng M_1 (March 22, 1983); (middle) extract of uncontaminated sow milk: 25 μL injection equivalent to 11.9 mL milk at detector sensitivity of about 70% for 2.5 ng M_1 (March 16, 1983); (bottom) naturally contaminated sow milk: 25 μL injection equivalent to 11.9 mL milk at detector sensitivity of about 85% for 2.5 ng M_1 (March 3, 1983).

Table 1. Statistical summary of LC analysis of spiked cow milk extracts purified by cartridge chromatography[a]

Statistic	Aflatoxin M_1 added, ng/100 mL		
	25	50	100
No. replicates[b]	5	10	9
Range	20.0–23.4	42.3–51.6	87.8–103.3
Mean	22.7	46.7	94.1
Standard error (SD/$\sqrt{N}$)	0.9	1.0	2.0
Coeff. of var., %	9.3	6.8	6.5
Recovery, %	90.8	93.4	94.1

[a]LC on 5 μm C18 radial compression column, mobile phase water–acetonitrile–acetic acid (82 + 15 + 3, v/v/v) at flow rate of 2.0 mL/min. Fluorescence detection, 360 nm excitation, and emission at 418 nm or above.
[b]Experiments carried out over 3 month period.

rejection 4, area rejection 50, run time 55–75 min, and chart speed 0.2 cm/min. Inject series of increasing volumes (up to 25 μL) of 0.1 μg/mL M_1 standard solution. Prepare standard curve by graphing average peak area or height vs ng M_1.

Dissolve purified milk extracts in 100 μL methanol and transfer solution to 0.25 mL (limited volume) LC vials. Inject 20–25 μL of extract representing 9.52–11.9 mL milk and identify M_1 by retention time.

Confirmation of Aflatoxin M_1 Identity

Proceed as described in sec. **26.095** (5).

Results and Discussion

Development of the cartridge cleanup step was similar to the approach used by Hutchins and Hagler (8). Solvent combinations were selected which ensured quantitative retention of aflatoxin M_1 on the cartridge during application and cleanup, and quantitative elution of M_1 from the cartridge in the final step while some more polar impurities were retained on the cartridge.

Extracts of milk which had been spiked at 1 ng M_1/mL and purified by the silica column method (6) yielded recoveries of more than 100% on TLC analysis with spotmeter densitometry. In 9 replicate TLC determinations, recoveries ranged from 103.0 to 146.6% with a mean of 127.0 ± 5.1 (SEM) and a coefficient of variation of 12.1%; background contributed materially to apparent recoveries of M_1. Silica cartridge chromatography of extracts of milk which had been spiked at 0.1–0.5 ng M_1/mL yielded purified extracts containing less background interference than extracts purified by silica column chromatography, and recoveries of about 90–95% (data not shown).

Figure 1 shows LC analyses of an M_1 standard and uncontaminated and naturally contaminated porcine milk. Standard M_1 (2.5 ng at about 62% full scale) is indicative of sensitivity; 0.16 ng would give a 2× baseline response equivalent to 0.013 ng M_1/mL (Figure 1A). The chromatogram of an extract of uncontaminated porcine milk (Figure 1B) shows an adequate analytical window for M_1 and chromatogram complexity. Analysis of naturally contaminated porcine milk (Figure 1C) gave an excellent response and chromatography for milk contaminated at 0.13 ng M_1/mL. No significant extract components eluted from the column after the large peak at about 65 min so analysis time was shortened to 55 min and the 65 min component was allowed to elute during the equilibration period before injection of the next sample.

A summary of 3 experiments conducted at monthly intervals on commercial bovine milk spiked at 0.25, 0.5, and 1.0 ng M_1/mL (Table 1) is a good indicator of method consistency over time. The mean recovery for the 3 M_1 concentrations was 92.8 ± 1% with an average coefficient of variation of 7.5

± 0.9%. An additional evaluation of the method by using 9 replicate bovine milk samples spiked at 0.5 ng M_1/mL yielded a mean percent recovery of 96.1 ± 1.1 with a coefficient of variation of 7.0%.

The limit of detection was less than 0.1 ng M_1/mL for both bovine and porcine milk.

This method was used in a study to determine the relationship of dietary aflatoxin B_1 concentration in sow diets to the concentration of aflatoxin M_1 in their milk (data to be presented elsewhere). Fifty-four samples were analyzed; 33 contained aflatoxin at concentrations which ranged from 0.1 to 3.9 ng/mL. In blind analyses, the milk of control sows receiving no dietary aflatoxin B_1 contained no detectable M_1.

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Short Liquid Chromatographic Method for Determination of Zearalenone and alpha-Zearalenol

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A rapid method has been developed for the determination of zearalenone and alpha-zearalenol, using liquid-liquid partition for sample extract cleanup followed by liquid chromatography (LC) with fluorescence detection. The same extract can also be used for determination of aflatoxin by LC. Relative standard deviations for the method based on duplicate analyses are 4.18% for artificially contaminated samples and 13.92% for naturally contaminated samples. The method was compared with the AOAC method of extraction and column chromatography extract cleanup, using the same LC conditions for quantitation for both methods. Results showed a correlation coefficient of 0.990 with a mean zearalenone content by this method of 987.8 ppb, and 732.6 ppb by the official AOAC method. The detection limit of this method is 2.2 ppb for zearalenone and 1.5 ppb for alpha-zearalenol.

Various methods have been developed to determine zearalenone in corn and other cereal grains, including thin layer chromatography (1–3), minicolumn (4), gas chromatography (5), liquid chromatography (LC) (6–12), and gas chromatography-mass spectrometry (GC-MS) (2, 11, 13). A short method was introduced by this laboratory for the determination of aflatoxin by LC using only liquid-liquid partition for sample extract cleanup (14). A similar short extraction and cleanup procedure has been developed for the determination of zearalenone and alpha-zearalenol. The following describes this method and its comparison with the current AOAC method for extraction and cleanup (3). Compounds were quantitated for both methods by LC using fluorescence detection.

METHOD

Reagents

(a) *Mobile phase.*—Combine 800 mL methanol and 200 mL water. Stir slowly and continuously during use.

(b) *Standards.*—Zearalenone and alpha-zearalenol were obtained from M. Machman (Commercial Solvents Corp., Terre Haute, IN 47808). alpha-Zearalenol was also obtained from C. J. Mirocha, University of Minnesota. Mix stock standards of zearalenone and alpha-zearalenol in mobile phase and dilute to volume in mobile phase so that final concentrations are 1 ng zearalenone/μL and 0.5 ng alpha-zearalenol/μL.

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Apparatus

(a) *LC pump.*—Model 110, constant flow (Beckman, 1450 Sixth St, Berkeley, CA 94710), or equivalent.

(b) *LC injector.*—Model LC 420 autosampler equipped with 20 μL loop (Perkin Elmer, Norwalk, CT 08856), or equivalent.

(c) *LC detector.*—Model 650-10LC fluorescence spectrophotometer (Perkin Elmer), or equivalent. Set excitation and emission for 280 and 465 nm, respectively, with 20 nm slit width, attenuation 1.

(d) *LC column.*—Reverse phase C18, 4.6 mm × 25 cm. Zorbax (DuPont, Wilmington, DE), or equivalent.

Sample Extraction

Grind samples to pass 30 mesh sieve and mix well. Weigh 50 g sample into 500 mL g-s Erlenmeyer flask. Add 25 mL water and disperse sample evenly in added water with spatula. Add 250 mL CH_2Cl_2 and again disperse sample evenly. Add 10 g cupric carbonate basic. Stopper flask and shake 30 min on mechanical wrist-action shaker. Filter through 24 cm 2V paper and collect ca 100 mL filtrate. Pipet 50 mL CH_2Cl_2-extract into 250 mL flask. Remaining filtered extract (14) can be analyzed for aflatoxin, if desired.

Sample Cleanup

Evaporate CH_2Cl_2 to dryness under vacuum and immediately transfer residue to 250 mL separatory funnel, using four 20 mL portions of acetonitrile. Extract acetonitrile solution with two 50 mL portions of petroleum ether (Note 1). Discard petroleum ether and transfer acetonitrile extract to 250 mL round-bottom flask. Evaporate acetonitrile solution to dryness. Immediately transfer residue to 1 dram vial with minimal amount of CH_2Cl_2. Evaporate to dryness under stream of nitrogen. Add via pipet 4.0 or 2.0 mL (for samples of low zearalenone or alpha-zearalenol concentrations) of mobile phase. Mix on vortex mixer ≥20 s; if oily residue is present, let solution settle after vortexing and draw off bottom layer for injection onto LC column.

Note 1: Do not use more than two 50 mL portions of petroleum ether when extracting acetonitrile solution, to min-

Table 1A. Determination of zearalenone (ppb) in naturally contaminated samples

	AOAC		This method	
Sample	α-Zearalenol	Zearalenone	α-Zearalenol	Zearalenone
Mixed feed	ND	12.7	ND	19.1
	ND	10.2	ND	18.7
Av.		11.45		18.9
Mixed feed	ND	936	ND	582
	ND	837	ND	875
Av.		886.5		728.5
Mixed feed	ND	77	ND	88
Mixed feed	5.0	174.9	23.0	188.2
	5.2	166.8	16.7	182.0
Av.	5.1	170.9	19.85	185.1
Corn	ND	44.3	ND	60.9
	ND	48.9	ND	54.1
Av.		46.6		57.5
Corn	ND	831	7.1	1186
	ND	827.7	7.7	1070
Av.		829.35		1128
Corn	ND	153	ND	222
	ND	132	ND	219
Av.		142.5		220.5
Mixed feed	12.7	1435	27.4	2083
	6.2	1554	31.4	2608
Av.	9.45	1494.5	29.4	2345.5
Mixed feed	6.1	2875	108	4124
	7.6	2994	105	4112
Av.	6.85	2934.5	106.5	4118
Mean		732.6		987.8

Between methods correlation coefficient = 0.990.

imize loss of zearalenone and alpha-zearalenol which are very slightly soluble in petroleum ether.

LC Quantitation

Set flow rate at 1.0 mL/min and let column equilibrate 45 min. Inject standard solution. Alpha-zearalenol and zearalenone should have baseline separation and have approximate retention times of 5.25 and 6 min, respectively. Adjust makeup of mobile phase, if necessary, to obtain adequate resolution. Repeat injection of standard solution until peak heights are constant. Inject sample extracts interspersed with standard as frequently as necessary to ensure accurate quantitation. Quantitate by using peak height method.

Table 2. Comparison of % recovery of zearalenone by various LC methods

Method	Sample	Recovery, %	Range added, ppb
Ware & Thorpe (6)	corn	89–96.5	10–200
Scott et al. (2)	corn	84–104	50–100
Moller & Josefsson (8)	cereal	73.9–102.3	65–52
Cohen & LaPointe (9)	feed	90–96.3	10–1000
Holder et al. (7)	feed	76–86.5	10–10 000
This method	mixed feed	65.6–91.1	10–2000

Table 3. Relative standard deviations (%) for zearalenone analyses

Sample	Method	Zearalenone	α-Zearalenol
Naturally contaminated	This method	13.92	6.99
	AOAC	6.04	38.2
Artificially contaminated	This method	4.18	4.21
	AOAC	4.29	—

Calculations

$$\alpha\text{-Zearalenol, } \mu g/kg = (SAMPH \times C \times V_1)/(W \times STPH)$$

$$\text{Zearalenone, } \mu g/kg = (SAMPH \times C \times V_1)/(W \times STPH)$$

where $SAMPH$ = sample peak height; $STPH$ = standard peak height; C = standard concentration in ng/μL, 0.5 ng alpha-zearalenol/μL, and 1.0 ng zearalenone/μL; W = weight of sample in g × fraction of methylene chloride filtrate used (i.e., W = 10 g for 50 mL filtrate); V_1 = μL mobile phase solvent used to dissolve sample, usually 4000 μL (2000 μL for low-level samples).

Results and Discussion

Nine samples of naturally contaminated corn and mixed feed (collected from farm fields by C. J. Mirocha, University of Minnesota) and 7 samples of artifically contaminated corn were selected for study. Each sample was analyzed in duplicate whenever sample size was sufficient, both by the official AOAC method for extraction and column chromatography

Table 1B. Determination of zearalenone (ppb) in artificially contaminated corn

Added		AOAC		This method	
Zearalenone	α-Zearalenol	Zearalenone	α-Zearalenol	Zearalenone	α-Zearalenol
10	0	10.4	ND	8.7	ND
		6.7	ND	9.0	ND
Av.		8.55		8.85	
50	0	49.5	ND	47.9	ND
		29.7	ND	43.2	ND
Av.		39.6		45.55	
100	0	63.1	ND	71.1	ND
		55.0	ND	78.0	ND
Av.		59.05		74.55	
300	0	167	ND	213	ND
		180	ND	206	ND
Av.		173.5		209.5	
500	10	320	ND	346	7.8
		291	ND	346	8.1
Av.		305.5		346	7.95
1000	20	434	ND	664	16.2
		429	ND	647	14.1
Av.		431.5		655.5	15.2
2000	50	698	ND	1439	42.4
		678	ND	1381	43.2
Av.		688		1410	42.8

Between methods correlation coefficient 0.995.

cleanup (3) and by this rapid method. The extracts from both methods were quantitated by LC.

The artificially contaminated samples were obtained by adding a culture solution in methanol (*Fusarium roseum* "Graminearum") containing a known concentration of zearalenone and alpha-zearalenol to corn and mixed feed samples. The ratio of alpha-zearalenol to zearalenone was adjusted to simulate the approximate ratio found in naturally contaminated corn samples, i.e., approximately 1:40. alpha-Zearalenol is 3 times more estrogenic than zearalenone, whereas the beta-isomer is equal in activity (13) to zearalenone but much less abundant in nature; thus, only zearalenone and alpha-zearalenol are analyzed in normal routine assays. Results are shown in Tables 1A and 1B for naturally contaminated mixed feed and corn and for artificially contaminated corn samples, respectively.

Table 1B shows that no alpha-zearalenol (10–50 ppb added) could be recovered from artificially contaminated samples by using the AOAC method of extraction and cleanup. For naturally contaminated samples, 3 contained alpha-zearalenol as determined by the AOAC method, but the quantities found were much lower than the respective values obtained by the rapid extraction method (Table 1A).

The average recovery of zearalenone by the AOAC extraction method in this study was 61.5%, compared with 75.6% by the rapid method with a correlation coefficient of 0.995. A previous collaborative study (15) showed an average recovery of 109% by the AOAC TLC method. The difference could be due to differences in quantitation by LC and TLC (using visual quantitation), or because the results of previous study included individual results substantially greater than the spiking levels used (i.e., recoveries ranged from 33 to 267%, with relative standard deviations of 27–53%, (Table 1, 15)). Examination of the recovery data in Table 1B indicates that as the added zearalenone range becomes larger, the percent recovery becomes smaller for both techniques; however, it decreases more rapidly for the AOAC extraction method. A comparison of recoveries obtained by various LC methods found in the literature (Table 2) indicates that this method gives comparable recoveries to those published.

Table 1A indicates that results obtained by the 2 methods showed a correlation coefficient of 0.99, although the mean zearalenone content by the rapid method was 987.8 ppb vs 732.6 ppb by the AOAC method for the naturally contaminated samples.

Precision data, based on the results of duplicate analyses performed, are shown in Table 3.

Detection limits of the described method were 2.2 μg/kg for zearalenone and 1.5 μg/kg for alpha-zearalenol. These were calculated based on 2 × noise/signal as shown in Figure 1. Figure 2 is a chromatogram of an artificially contaminated corn sample, and Figure 3 shows a chromatogram for a naturally contaminated mixed feed sample.

Based on the results obtained on identical samples and using the identical quantitation technique but different extraction techniques, we believe this rapid method provides more efficient zearalenone extraction and quantitation than does the AOAC method. In our laboratories, this method requires substantially less time per assay than any of the other current methods we have evaluated (6–10).

Acknowledgment

We express our thanks to C. J. Mirocha of the University of Minnesota for his guidance and assistance in this study.

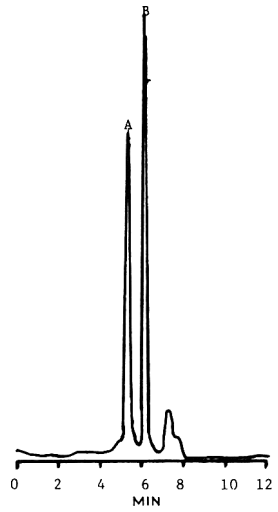

Figure 1. Chromatogram of standard α-zearalenol (10 ng) and zearalenone (20 ng). A = α-zearalenol; B = zearalenone.

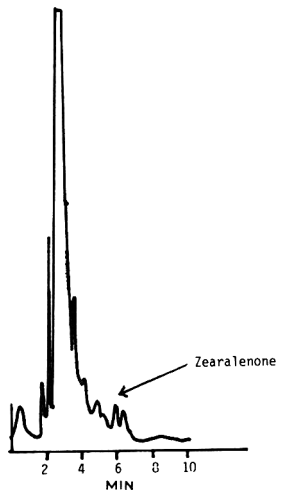

Figure 2. Chromatogram of artificially contaminated corn sample. Peak represents 0.87 ng zearalenone injected, equivalent to 8.7 ppb in sample. Final dilution of extract was 2.0 mL.

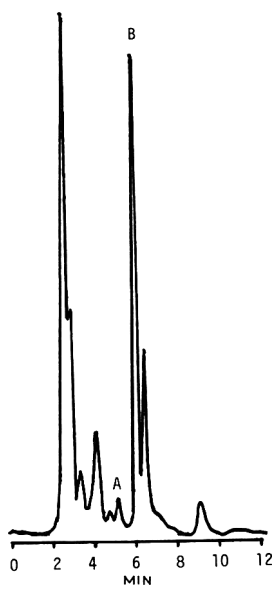

Figure 3. Chromatogram of naturally contaminated mixed feed sample. Peak represents 0.542 ng α-zearalenol and 20.6 ng zearalenone injected, equivalent to 108.3 ppb α-zearalenol and 4123.6 ppb zearalenone in sample. Final dilution of extract was 40.0 mL. A = α-zearalenol; B = zearalenone.

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FOOD ADDITIVES

Liquid Chromatographic Determination of Sodium Saccharin, Caffeine, Aspartame, and Sodium Benzoate in Cola Beverages

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A rapid method has been developed for the determination of sodium saccharin, caffeine, aspartame, and sodium benzoate in cola beverages. The sample is degassed, diluted in water, and injected onto a C18 column. The mobile phase consists of 15% acetonitrile in triethylammonium phosphate buffer adjusted to pH 4.3 with NaOH. The total run time is less than 10 min and the active compounds are determined using absorbance detection at 214 nm.

Aspartame, L-aspartyl-L-phenylalanine methyl ester, is a dipeptide having approximately 180 times the sweetness of sucrose (1). It is now being used as a table-sugar substitute, as an additive in dry powder mixes, and as a sweetener in carbonated beverages.

A search of the literature for aspartame methodology indicated methods based on gas chromatography (2), and the analysis of fluorescent derivatives (3). Since these methods are somewhat involved, analysis based on liquid chromatography (LC) would be the method of choice for many products.

A liquid chromatographic method for the determination of saccharin, caffeine, and benzoate in soda beverages has already been developed (4). Woodward et al. used a mobile phase containing acetic acid and sodium acetate. The wavelength of their analysis was 254 nm. In the procedure presented here, an acetonitrile–triethylammonium phosphate buffer is used as mobile phase to provide optical transparency at short wavelengths. Detection at 214 nm provides increased response to both saccharin and aspartame.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Altex Model 110A pump, or equivalent, operating at 2.0 mL/min; Altex Model 500 Autosampler, or equivalent, with 20 μL loop; Waters Associates 30 cm × 4.6 mm μBondapak C18 analytical column; Beckman Model 160 detector, or equivalent, operating at 214 nm and 0.05 AUFS; and Beckman Model CR1-A integrating recorder, or equivalent, operating at attenuation 3.

(b) *Chemicals and reagents.*—Organic-free water was prepared by passing reverse osmosis water through a Milli-Q™ water purification system (Millipore Co., Bedford, MA 01730). LC quality triethylamine, LC quality acetonitrile, reagent grade phosphoric acid, and 1N NaOH (Fisher Scientific Co.). Sodium saccharin, caffeine, and sodium benzoate (Sigma Chemical Co.). Aspartame (Searle Food Resources, Inc., Box 1045, Skokie, IL 60076).

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

(d) *Mobile phase.*—Dilute 10 mL buffer solution to ca 800 mL with water, add 150 mL acetonitrile, adjust pH to 4.3 with 1N NaOH, bring final volume to 1 L with water, and pass solution through 0.45 μm membrane filter. If desired, adjust concentration of acetonitrile and pH to optimize separation. Be sure, however, to keep pH within 2.2–6.0 to avoid damaging silica-based C18 column.

(e) *Stock standard solutions.*—Accurately weigh ca 200 mg each standard into separate 100 mL volumetric flasks. Dissolve sodium saccharin, caffeine, and sodium benzoate in water and dilute to volume with water. Dissolve aspartame in 80 mL ethanol–water (1 + 1) and dilute to volume with water. Prepare all standards fresh on day of use.

Table 1. Retention time (min) vs pH of mobile phase

Compound	pH = 2.70	pH = 3.05	pH = 4.30
Saccharin	2.85	2.70	2.54
Caffeine	3.73	3.75	3.76
Aspartame	5.30	5.40	5.34
Benzoate	11.56	11.36	7.34

Sample Preparation

Cautiously degas sample, using either sonication or mechanical agitation. Dilute degassed sample 5:100 with water to form sample solution.

Standard Preparation

Prepare mixed standard that closely matches actual sample. For example, for samples containing 20 mg sodium saccharin, 12 mg caffeine, 12 mg aspartame, and 20 mg sodium benzoate per 100 mL, pipet 10, 6, 6, and 10 mL respective stock solutions into same 100 mL volumetric flask and dilute to volume with water to form mixed standard. Dilute mixed standard 5:100 with water to form standard solution.

Procedure

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

$$\text{mg/100 mL} = 100 \times (\text{Cstd/Csam}) \times (\text{Asam/Astd})$$

where Cstd = conc. of standard solution in mg/100 mL; Csam = conc. of sample solution in mL/100 mL; and A = peak area. Report final results to 0.1 mg/100 mL.

Results and Discussion

Initial chromatographic work was done using a mobile phase of 150 mL acetonitrile and 10.0 mL buffer solution diluted to 1 L with water. This mobile phase provided excellent separation among saccharin, caffeine, and aspartame. Increasing the pH from 2.7 to 4.3 decreased the retention time of the benzoate without changing the retention times of the other components significantly. These results are shown in Table 1.

The chromatographic scan of a cola sample is shown in Figure 1. The large peak eluting in approximately 1.3 min contains citric acid and caramel color.

Assay values for several types of cola beverages are given in Table 2. Each sample was assayed in duplicate and the

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Table 2. Assay values for selected cola beverages, mg/100 mL

Sample	Na Saccharin	Caffeine	Aspartame	Na Benzoate
A. Diet cola, brand A, caffeine-free	41.3	0.0	0.0	0.0
B. Diet cola, brand A, with aspartame	18.7	9.5	9.7	10.1
C. Reg. cola, composite of F, G, and H	0.0	3.2	0.0	0.0
D. Diet cola, brand B	33.7	13.0	0.0	18.7
E. Diet cola, brand B, with aspartame	20.9	13.0	9.2	19.9
F. Reg. cola, brand A, 99% caffeine-free	0.0	0.1	0.0	0.0
G. Reg. cola, brand B, caffeine-free	0.0	0.0	0.0	0.0
H. Reg. cola, brand B	0.0	9.9	0.0	0.0

Table 3. Recovery data for 3 samples of cola beverages

		Na Saccharin		Na Benzoate	
Sample	Added, mg/100 mL	Found, mg/100 mL	Rec., %	Found, mg/100 mL	Rec., %
A	4	3.80	95.0	3.96	99.0
	10	9.64	96.4	10.16	101.6
	20	19.67	98.4	20.01	100.0
	40	39.60	99.0	40.37	100.9
B	4	3.90	97.5	4.02	100.5
	10	10.03	100.3	9.91	99.1
	20	19.88	99.4	20.18	100.9
	40	39.57	98.9	39.96	99.9
C	4	3.80	95.0	4.06	101.5
	10	9.64	96.4	10.02	100.2
	20	19.51	97.6	20.01	100.0
	40	39.63	99.1	40.28	100.7
Av.			97.8		100.4
		Caffeine		Aspartame	
A	2	2.03	101.5	2.00	100.0
	5	5.08	101.6	5.00	100.0
	10	10.04	100.4	10.00	100.0
	20	20.08	100.4	20.11	100.6
B	2	2.03	101.5	2.02	101.0
	5	5.06	101.2	5.02	100.4
	10	10.00	100.0	9.90	99.0
	20	19.87	99.4	19.97	99.9
C	2	2.05	102.5	1.98	99.0
	5	5.02	100.4	4.98	99.6
	10	9.91	99.1	9.94	99.4
	20	20.04	100.2	20.11	100.6
Av.			100.7		100.0

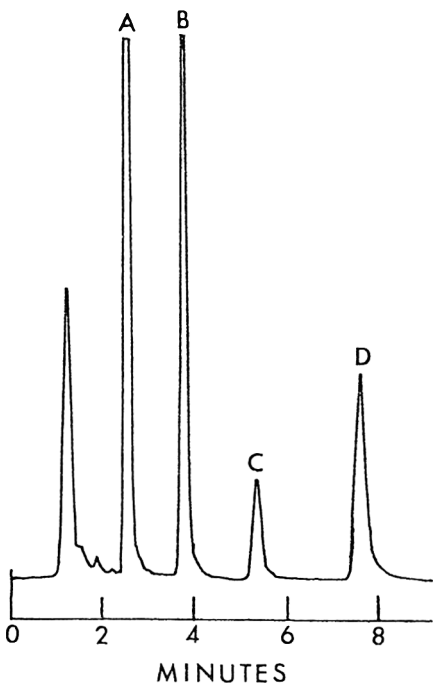

Figure 1. Chromatogram of cola sample containing 20 mg/100 mL of sodium saccharin (A), 12 mg/100 mL of caffeine (B), 12 mg/100 mL of aspartame (C), and 20 mg/100 mL of sodium benzoate (D).

average difference between duplicates in mg/100 mL was 0.11 for saccharin, 0.03 for caffeine, 0.10 for aspartame, and 0.08 for benzoate. Percent error was calculated using the formula: $\%\ \text{error} = 100 \times |X_2 - X_1|/\bar{X}$. Average values for % error were 0.32% for saccharin, 0.50% for caffeine, 1.10% for aspartame, and 0.50% for benzoate.

With respect to precision, this method does not use an internal standard and therefore quantitation depends on injection volume reproducibility. Reproducibility is assured by injecting a standard solution 10 times and determining the standard deviation of the resulting peak areas. This standard deviation is always less than 0.3% when the equipment is working properly. It is recommended that the analyst perform this type of test if the area differences of duplicate injections is more than 2%.

To test response linearity, standard curves were prepared using 0, 2, 5, 10, and 20 mL mixed intermediate standard solution diluted to 100 mL with water. Tabulations were made of peak area vs standard concentration. Correlation coefficients of better than 0.9998 were found for all standard curves.

To test spiking recovery, the first 3 samples from Table 2 (Samples A, B, and C) were spiked at 4 levels. The saccharin results indicated that approximately 0.2 mg/100 mL was lost at the 4.0 mg/100 mL level and that approximately 0.4 mg/100 mL was lost at the 40 mg/100 mL level. Because there was minimal opportunity for loss of saccharin—the sample

was simply diluted with water and injected—the low recovery result was probably due to integration error caused by interference from caramel color. This interference can be reduced by decreasing the concentration of acetonitrile in the mobile phase at the expense of increased retention time. The average recovery for saccharin was 97.8%.

Caffeine, aspartame, and sodium benzoate all yielded baseline resolved peaks. Their average recoveries were 100.7, 100.0, and 100.4%, respectively. These results are shown in Table 3.

This method is designed for typical working levels and not for trace or residue levels. The actual detection limits depend on the amount of sample matrix interference and on the precision of the instrumentation. However, as a guide, the detection limits are estimated to be 0.2 mg/100 mL for saccharin, aspartame, and benzoate and 0.05 mg/100 mL for caffeine. If assay values below 1.0 mg/100 mL are required, increasing electronic amplification, increasing injection volume, increasing sample concentration, and calculating by peak height rather than by peak area will result in some improvement in the quantitation.

Although this method applies well to cola beverages containing caramel color, citric acid, saccharin, caffeine, aspartame, and benzoate, the method does not necessarily apply to other carbonated beverages. These beverages may contain artificial colors, and the retention times of these colors would have to be determined individually to assure that they do not co-elute with other components of the sample.

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Liquid Chromatographic Determination of Flavor Enhancers and Chloride in Food

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A liquid chromatographic method is described for the simultaneous determination of flavor enhancers (glutamate, inosine-5′-monophosphate, and guanosine-5′-monophosphate) and chloride (salt) added to food. This rapid method exhibits excellent recoveries, and does not require derivatization or gradient elution by refractive index and UV detection in series.

Production of this important food additive began shortly after the discovery of the taste-enhancing properties of glutamate (glutamic acid) by Ikeda (1). In 1979, the world monthly production capacity of glutamate (2) was estimated at 30 kilotons. Spurned by interest in this flavor compound, food processing analytical needs, and concern over "Chinese restaurant syndrome" and other nervous system effects (3, 4), a variety of analytical methods have been developed to determine glutamate in foods. Enzymatic methods (5), microbiological assays (6), paper chromatography (7), volumetric methods (8), fluorometric methods (9), and gas chromatography (10) have all been used to detect glutamate in food. With the advent of liquid chromatography (LC), methods based on glutamate derivatization (11, 12) and direct measurement by refractive index (13) have also been developed.

Glutamate, mainly as monosodium glutamate (MSG), has usually been added to food at levels of 0.2–0.8% (3) to achieve the best flavor enhancement. Recently, the nucleotides inosine-5′-monophosphate (IMP) and guanosine-5′-monophosphate (GMP), which also have flavor-enhancing effects (first recognized by Kodama (14)), have become commercially available as food additives. The nucleotides are added to foods at low levels (typically 0.01%) with MSG and, because of a synergistic flavor enhancement effect (15), foods require much smaller amounts of MSG (16). Food processors are changing product formulations to include nucleotide–MSG mixtures that provide the same flavor enhancement as MSG alone, at lower cost.

Food analysis methods for nucleotide flavor enhancers include colorimetric (17), ion exchange (18), and LC (12, 19) determinations. Although these methods are available, a total analysis in food of all the flavor-enhancers described would require more than one procedure, and would often require derivatization or complex workup of the food. We have developed a rapid analysis that can be used to identify and quantitate the flavor enhancers (glutamate, IMP, and GMP), chloride (salt), aspartate (aspartic acid), and pyroglutamic acid. Chloride content of food measures salt (NaCl) content often added to foods containing flavor enhancers. Also, by measuring chloride (rather than sodium), sodium input from salt and flavor enhancers can be estimated. Aspartate is important because its metabolism is interrelated with glutamate metabolism (20). Pyroglutamic acid, an important derivative of glutamic acid, has been reported as tasteless (21, 22), bitter (23), and a flavor enhancer (24) that could be formed from glutamate during processing or reconstitution of dried food products (25), and is not detected by most glutamate analyses (13).

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METHOD

Apparatus and Reagents

(a) *LC system.*—Beckman Model 110A pump and 50 μL loop injector, Laboratory Data Control Spectromonitor III UV detector (set at 254 nm) attached to Waters R401 differential refractometer maintained at 25°C with constant temperature ethylene glycol bath, and detectors attached to separate strip chart recorders. Whatman 25 cm × 4.6 mm id Partisil SAX column protected by 7 cm × 2.1 mm id guard column containing pellicular anion exchanger and pre-injector; 25 cm × 4.6 mm id precolumn (Solvecon) containing silica gel.

(b) *Evaporator.*—Buchi Rotavapor-R with water bath maintained at ≤ 50°C.

(c) *Solvents.*—Purify LC water by reverse osmosis (Milli-RO), and further purify by using Milli-Q system. All other solvents and reagents reagent grade or better.

(d) *Buffer.*—0.017M potassium dihydrogen phosphate (LC grade). Prepare fresh daily in LC water, adjust to pH 4.0 with phosphoric acid, degas, and filter through Millipore paper (0.45 μm).

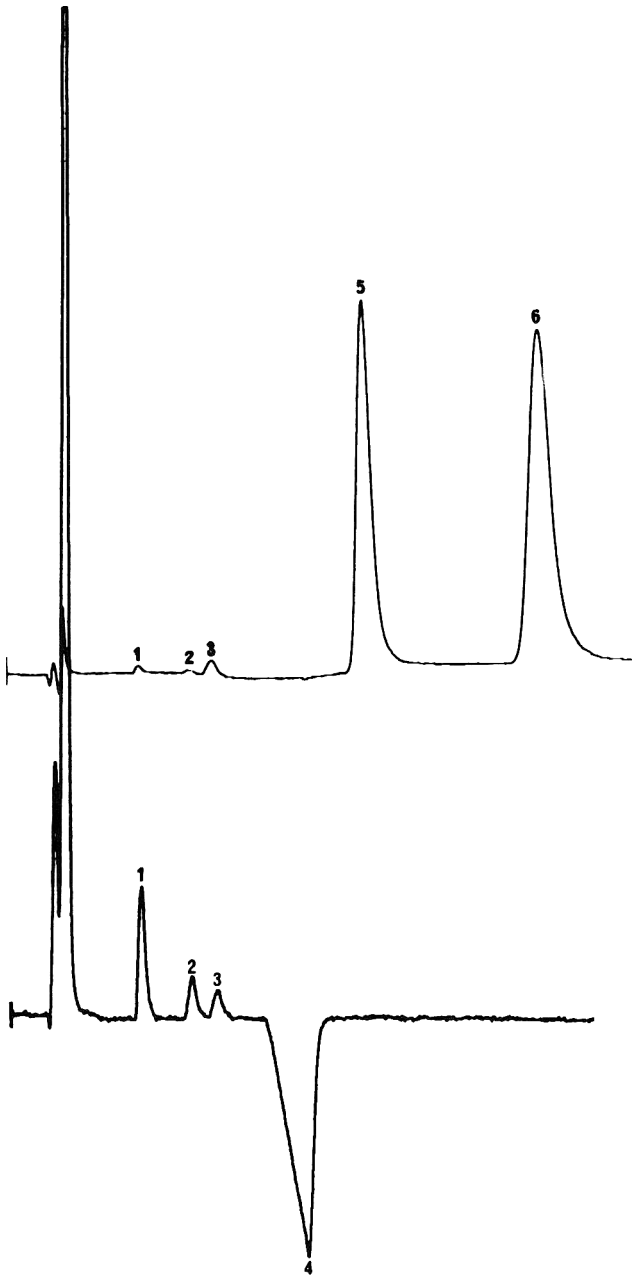

Figure 1. LC chromatogram from RI (lower) and UV (upper) detectors of 1, glutamic acid (0.4 mg/mL, retention time 6.66 min); 2, aspartic acid (0.2 mg/mL, retention time 8.93 min); 3, pyroglutamic acid (0.2 mg/mL, retention time 10.25 min); 4, chloride (3.0 mg NaCl/mL, retention time 13.05 min); 5, IMP (8.0 μg/mL, retention time 17.18 min); and 6, GMP (8.0 μg/mL, retention time 25.14 min) at flow rate of 1 mL/min with 0.017M pH 4.0 phosphate buffer.

(e) *Celite.*—Celite 545 (Fisher Scientific).

(f) *Standards.*—L-Glutamic acid (A grade, Calbiochem-Behring Corp., San Diego, CA), pyroglutamic acid (Puriss, Koch-Light Laboratories, Ltd, Burkshire, UK), salt (Fisher Scientific Co.), L-aspartic acid, disodium IMP, and disodium GMP (all from Sigma Chemical Co., St. Louis, MO). Dissolve in LC water and dilute to desired concentrations.

Sample Preparation

Weigh canned products before and after emptying to determine total can contents. Homogenize both liquid (soups) and other canned products in Waring blender before analysis. Grind dried products to powder with mortar and pestle. Use 10 g liquid product or 1 g dry product for analysis.

Table 1. Linearity of peak area measurements

Compound	Concn. range tested	Corr. coeff.	Detection limit, μg/mL
Glutamate	0.2–0.025 mg/mL	0.99998	10
Aspartate	0.2–0.05 mg/mL	0.99999	20
Pyroglutamic acid	0.2–0.05 mg/mL	0.99999	20
Chloride	4.0–0.25 mg/mL	0.99995	20
IMP	10–0.625 mg/mL	0.99999	0.1
GMP	10–0.625 mg/mL	0.99993	0.1

Table 2. Recovery of compounds in rice soup

Compound	Amt Present	Amt added	Amt found	Rec., %
IMP	ND	40 μg	40.9 μg	102
		80 μg	81.0 μg	101
		100 μg	96.4 μg	96
		120 μg	115.4 μ	96
GMP	ND	40 μg	42.5 μg	106
		80 μg	78.3 μg	98
		100 μg	101.0 μg	101
		120 μg	124.8 μg	104
Glutamate	ND	20 mg	19.6 mg	98
		40 mg	39.1 mg	98
		50 mg	50.5 mg	101
		60 mg	59.1 mg	101
Aspartate	ND	10 mg	10.1 mg	101
		20 mg	20.7 mg	103
		25 mg	23.7 mg	95
		30 mg	29.0 mg	99
Pyroglutamic acid	ND	20 mg	20.4 mg	102
		40 mg	40.4 mg	101
		50 mg	48.0 mg	96
		60 mg	57.8 mg	99
Chloride	ND	40 mg	42.4 mg	106
		80 mg	77.6 mg	97
		100 mg	100.0 mg	100
		120 mg	120.0 mg	100

Table 3. Recovery of compounds in consomme

Compound	Amt present	Amt added	Amt found	Rec., %
IMP	592.3 μg	10 μg	602.3 μg	100
		20 μg	613.8 μg	105
		40 μg	633.2 μg	100
		80 μg	673.9 μg	101
GMP	48.8 μg	10 μg	58.6 μg	98
		20 μg	67.3 μg	93
		40 μg	89.6 μg	102
		80 μg	128.9 μg	100
Glutamate	36.0 mg	5 mg	41.0 mg	100
		10 mg	45.8 mg	98
		20 mg	57.3 mg	106
		40 mg	77.0 mg	102
Aspartate	3.0 mg	5 mg	7.9 mg	98
		10 mg	13.1 mg	101
		20 mg	22.8 mg	99
		40 mg	42.7 mg	99
Pyroglutamic acid	4.6 mg	5 mg	9.0 mg	89
		10 mg	14.1 mg	95
		20 mg	23.7 mg	95
		40 mg	43.3 mg	97
Chloride	130.0 mg	10 mg	139.9 mg	99
		20 mg	150.7 mg	103
		40 mg	168.5 mg	96
		80 mg	211.0 mg	101

Table 4. Analysis for nucleotides at various wavelengths

	IMP found, mg/100 g		GMP found, mg/100 g	
Wavelength, nm	Instant noodle soup	Chicken vegetable soup	Instant noodle soup	Chicken vegetable soup
239	25.10	4.78	21.85	3.26
254	25.15	4.90	22.05	3.28
269	25.07	5.63	21.99	3.26

Table 5. Precision of analysis procedure

Compound	Amt found, mg/100 g	Av. found, mg/100g	SD
	Chicken Vegetable Soup		
Glutamate	76,80,76,79,76	77.4	1.9
Aspartate	18,18,17,18,18	17.8	0.4
Chloride	1142,1127,1125,1117,1122	1126.6	9.4
IMP	4.98,4.61,4.93,5.11,4.88	4.90	0.18
GMP	3.12,3.36,3.53,3.61,3.57	3.45	0.17
	Chicken Rice Soup		
Glutamate	243,239,244,245,245,243	243.2	2.2
Chloride	1714,1738,1700,1726, 1738,1756	1728.7	19.8

Sample Analysis

The extraction procedure is a modification of the AOAC method for analysis of glutamate (26), developed by Fernandez-Flores et al. (8).

Add prepared sample to 150 mL beaker containing magnetic stir bar and 30 mL buffer. Stir contents of beaker 15 min and then add 40 mL acetone. Stir an additional 5 min, then filter beaker contents through Celite pad that has been washed with 20 mL 50% acetone in water. Wash beaker and residue on Celite pad with six 20 mL portions of 50% acetone in water. Transfer filtrate to round-bottom flask, evaporate to ca 10 mL, and then dilute with water to 50 mL in volumetric flask. Filter sample through 0.45 μm Millipore paper before injection of sample into LC system with buffer flow rate of 1 mL/min.

Identify peaks from chromatogram by retention time and quantitate by using area comparison (peak height × width at half height), with average area of standard injected before and after sample.

Results and Discussion

Two detectors were required for the analysis of flavor enhancers, because although glutamate and salt (chloride) are added to food in amounts that can be detected by refractive index, the small quantities of the nucleotides IMP and GMP usually added could not be detected by refractive index methods. Conversely, although IMP and GMP could easily be detected by UV absorbance, glutamate, aspartate, and pyroglutamic acid do not have significant UV response in a region free of interference from other compounds. Thus, glutamate, aspartate, pyroglutamic acid, and chloride were detected by refractive index, and the nucleotides were monitored by UV detection at 254 nm. The wavelength of 254 nm, chosen as a compromise between the UV maxima of IMP and GMP, is also a convenient wavelength for less expensive filter UV detectors.

The solvent conditions were adjusted so that gradient elution was not required for separation of the 6 compounds studied (Figure 1). Moreover, the possible interfering compounds adenosine-5′-monophosphate, xanthosine-5′-monophosphate, sorbic acid, benzoic acid, cinnamic acid, citric acid, tartaric acid, malic acid, succinic acid, lactic acid, and acetic acid were chromatographed and found to have different retention times than the compounds studied. The compounds guanine, hypoxanthine, lysine, asparagine, alanine, phenylalanine, proline, histidine, tyrosine, tryptophan, 4-amino butyric acid, and glutamine also did not interefere and, as expected, appeared at or near the solvent front.

Measurement of peak areas gave a linear response for all compounds studied (Table 1). Detection limits in Table 1 are given for peaks with peak heights twice the noise level. Tables

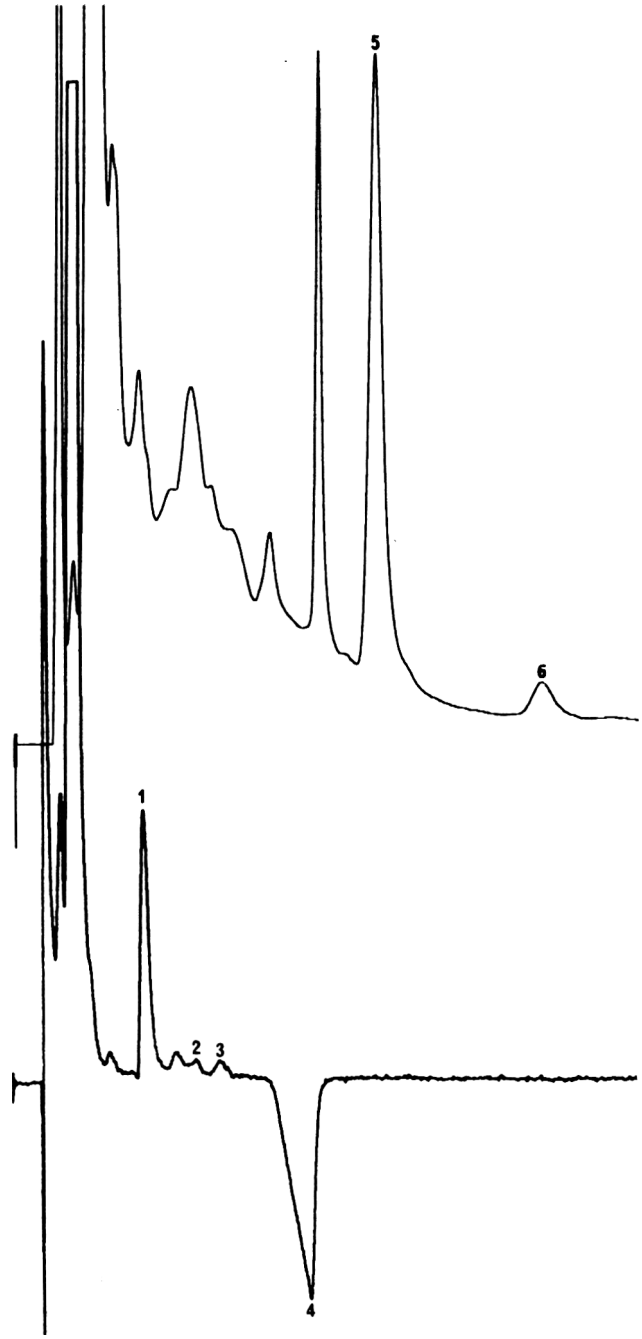

Figure 2. LC chromatogram of beef consomme (Table 3) at flow rate of 1 mL/min with 0.017M pH 4.0 phosphate buffer. See Figure 1 for peak identification.

2 and 3 show the results of recovery studies. To further show that interfering compounds were not co-eluting with IMP and GMP, quantitation of 2 samples containing nucleotides was carried out at various wavelengths (Table 4). Two groups were analyzed 5 times to further test the precision of the extraction and analysis procedure (Table 5). All other samples were analyzed at least in duplicate. Typical analyses of food samples are given in Table 6 and Figures 2, 3, and 4.

We found that liquid food samples such as consomme could be diluted, filtered, and injected directly for analysis, however, this procedure could shorten LC column life. Charcoal decolorization, described in an earlier paper for MSG and pyroglutamic acid analysis (13), was eliminated because of adsorption of nucleotides by the charcoal. This change did not seem to detrimentally affect the analysis of the other nonnucleotide compounds. We found that initial dilution of the sample in water rather than buffer resulted in cloudy suspen-

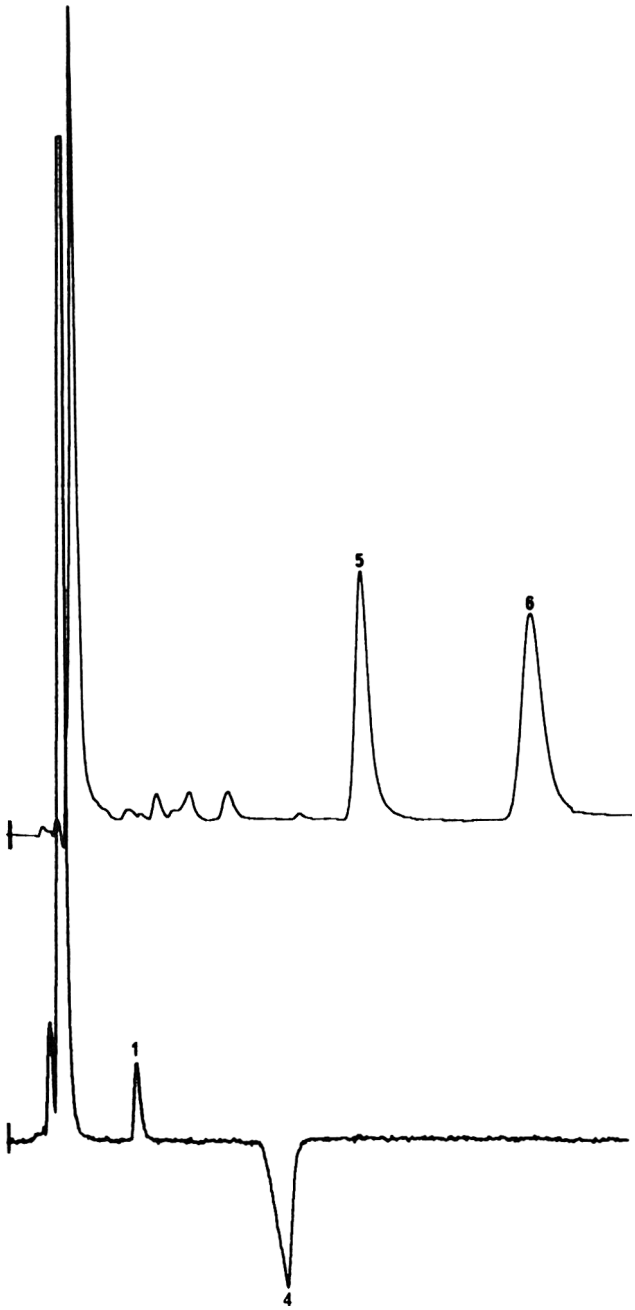

Figure 3. LC chromatogram of Japanese instant noodles (Table 6) at flow rate of 1 mL/min with 0.017M pH 4.0 phosphate buffer. See Figure 1 for peak identification.

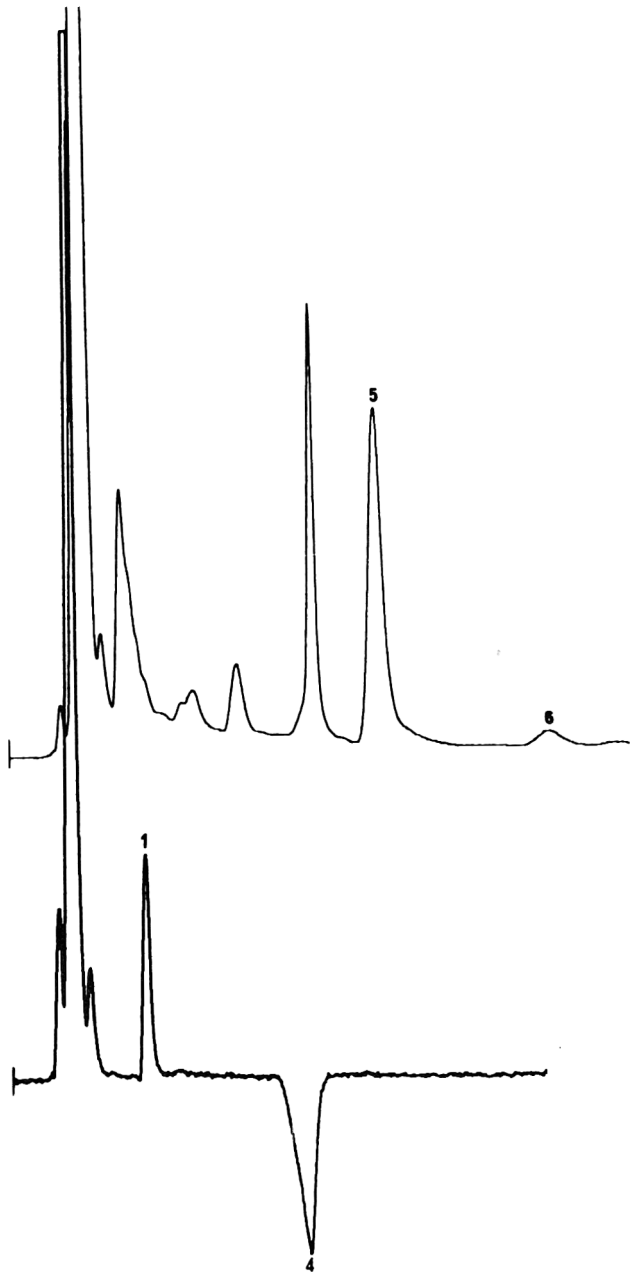

Figure 4. LC chromatogram of Chinese restaurant soup (Table 6) at flow rate of 1 mL/min with 0.017M pH 4.0 phosphate buffer. See Figure 1 for peak identification.

Table 6. Determination of flavor enhancers in food

Food sample	Glutamate, mg/100 g	Aspartate, mg/100 g	Chloride, mg/100 g	Pyroglutamic acid, mg/100 g	IMP, mg/100 g	GMP, mg/100 g
Tomato soup (canned)	77	41	1015	53	0.94	1.26
Beef consomme (canned)	414	30	1300	46	5.95	0.49
Potato chips	191	41	3620	218	ND	0.94
Japanese instant noodles (dried form)	1035	ND	5637	ND	25.15	22.05
Chinese restaurant soup (meat and vegetable soup)	373	ND	772	ND	3.47	0.20
Japanese restaurant soup (miso-seatangle soup)	241	114	1588	59	0.34	ND
Beef-in-A-Mug	2494	755	33067	ND	28.12	22.83
Tempura sauce	1561	460	13675	195	48.80	31.27

sions that caused filtration problems with non-salted foods. For this reason, the buffer was used as the initial diluting solution. If the Celite was not pre-washed with water–acetone, material (non-interfering) was eluted from the Celite. In contrast with earlier work (13), with the new buffer conditions, aspartic acid and pyroglutamic acid were completely separated.

The procedure represents a rapid analysis for flavor enhancers, salt, and aspartic and pyroglutamic acids which should greatly simplify food analysis of these compounds.

Acknowledgment

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Confirmation of *N*-Nitrosodimethylamine and *N*-Nitrosopyrrolidine in Foods By Conversion to their Nitramines with Pentafluoroperoxybenzoic Acid

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A method is presented, wherein concentrated dichloromethane extracts from malt, beer, and nonfat dried milk powder containing *N*-nitrosodimethylamine (NDMA), and fried dry-cured and pump-cured bacon containing NDMA and *N*-nitrosopyrrolidine (NPYR) undergo 3N $HClO_4$–Celite microcolumn cleanup followed by peroxidation with pentafluoroperoxybenzoic acid, a stable solid peroxyacid, to *N*-nitrodimethylamine and *N*-nitropyrrolidine, as an aid to nitrosamine confirmation. The nitramine-containing solution underwent further cleanup with 6N $HClO_4$–Celite and acid alumina-6% H_2O microcolumns for subsequent analysis by gas chromatography interfaced with thermal energy analyzer and ^{63}Ni electron capture detectors. With a 20 g sample, 1 ppb NDMA and 2.5 ppb NPYR could be confirmed by this method. The extract can be concentrated further before analysis, so the detection level can be reduced to <1 ppb NDMA and 1 ppb NPYR.

The confirmation of volatile nitrosamines in food and nonfood products by mass spectrometry is currently considered the most reliable technique (1). Because of its cost, however, the mass spectrometer is not available to many investigators who perform nitrosamine analysis. Furthermore, nitrosamines are often detected in various substrates in quantities too low for mass spectral confirmation. For these reasons, alternative methods are needed which, though not as definitive as mass spectrometry, will confirm the presence of nitrosamines. Peroxidation of nitrosamines to their corresponding nitramines with trifluoroperoxyacetic acid, prepared by the reaction of trifluoroacetic acid or anhydride with 30–90% hydrogen peroxide, was used in the past for detecting nitrosamines; this was because the electron capture (EC) detector showed greater sensitivity and selectivity for nitramines than detectors then available for nitrosamines (2–4). This peroxidation reaction has been applied, on a limited basis, as a confirmatory procedure by a few investigators, primarily for the detection of *N*-nitrosodimethylamine (NDMA) in nitrite-treated fish (2, 5), cured meat products (6), and ambient air and cigarette smoke (7).

The thermal energy analyzer (TEA), which shows a high degree of selectivity and sensitivity for detecting nitrosamines when interfaced to a gas chromatograph (GC), has simplified nitrosamine analysis and is used widely for this purpose. The TEA detector is also responsive to nitramines. Several investigators have reported nitramine to nitrosamine molar responses of 0.35 to 0.87, depending on the particular volatile nitramine, and a significantly different response for the same nitramine (8–10). Despite the variation in TEA response, in general, the retention times of the nitramines are longer than the corresponding nitrosamines. The recognition of the presence of the nitrosamine peak before peroxidation, and, of its reduction or disappearance afterward coupled with the simultaneous appearance of the nitramine peak, was recommended as an aid to confirmation of volatile nitrosamines in a recent IARC publication (11). While our research was in progress, Sen et al. (12) reported the trifluoroperoxyacetic acid peroxidation of nitrosamines to nitramines in various food products. In this study, the nitramines were detected by

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GC-TEA at an elevated furnace temperature of 625°C rather than the range 450°–475°C, normally used for nitrosamine analysis.

The objectives of this investigation were to develop a reliable aid to nitrosamine confirmation by a peroxidation reaction that would minimize repeated use of concentrated hydrogen peroxide, and to use TEA for detecting nitramines under normal conditions so that routine nitrosamine analysis would not be disrupted by changes in furnace temperature.

METHOD

Reagents

(a) *Solvents.*—Glass-distilled dichloromethane (DCM; redistilled), hexane and pentane both passed through silica gel 60 columns (Burdick & Jackson Laboratories, Muskegon, MI 49442), and anhydrous ethyl ether (redistilled, or reflux with 3–5 g $LiAlH_4$/L for 2 h, distill, and store under nitrogen at −20°C; J. T. Baker Chemical Company, Phillipsburg, NJ 08860).

(b) *Sodium sulfate.*—Anhydrous, granular, reagent grade (Mallinckrodt, Inc., St. Louis, MO 63147).

(c) *Silica gel 60.*—70–230 mesh. Place in chromatographic column and wash with anhydrous ether (5 mL/g from unopened can) previously saturated with water. Reactivate by drying 14 h in 150°C oven (Brinkmann Instruments, Inc., Westbury, NY 11590).

(d) *Alumina.*—Acid, 100–200 mesh. Deactivate to activity 3 with 6% water, place in chromatographic column, and wash column with redistilled anhydrous ethyl ether (4 mL/g) for cleanup. Activate 14 h in 180°C oven, then deactivate to activity 3 with 6 g water/94 g alumina (Bio-Rad Laboratories, Rockville Center, NY 11571). *Caution:* Remove residual ethyl ether before activating alumina or silica gel 60 in the oven, to avoid potential explosive hazard.

(e) *Perchloric acid.*—70%. Dilute to 3N and 6N (Eastman Kodak Co., Rochester, NY 14650).

(f) *Celite 545.*—Heat 4h in 180°C oven (Fisher Scientific Co., Fair Lawn, NJ 07410).

(g) *Boiling chips.*—Carborundum, small size (or equivalent).

(h) *Lithium aluminum hydride.*—Used as obtained commercially (Alfa Products, Danvers, MA 01923).

(i) *Pentafluoroperoxybenzoic acid.*—React pentafluorobenzoic acid and 90% hydrogen peroxide in the presence of methanesulfonic acid and trifluoromethanesulfonic acid at 55°–65°C. Cool reaction mixture, extract with DCM, and concentrate extract. Recrystallize crude peroxyacid from DCM; peroxyacid will precipitate as long needles, with 99–100% peroxide content as determined by starch-I_2-thiosulfate method (14). Store peroxyacid at −20°C until use. Caution: 90% H_2O_2 is an extremely strong oxidizer and must be handled appropriately. A more detailed procedure for the preparation will be reported elsewhere (13).

(j) *N-Nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPYR).*—Working standard, 0.1 ng/μL DCM. Caution: NDMA and NPYR are potent animal carcinogens and must be handled appropriately.

(k) *N-Nitrodimethylamine (DMN) and N-nitropyrrolidine (PYRN).*—Prepare with trifluoroacetic acid and 30% hydrogen peroxide by the method of Emmons (15). Working standard, 0.1 ng/μL hexane.

(l) *Hydrogen peroxide.*—90% (FMC Corp., Philadelphia, PA 19103).

Apparatus

(a) *Disposable Pasteur pipets.*—Heavy wall, 5¾ in. length, 5.5 mm id (Fischer Scientific Co., Cat. No. 13-678-6A).

(b) *Evaporative concentrator.*—Kuderna-Danish, 250 mL with 4 mL concentrator tube and Snyder and micro-Snyder distilling columns (Kontes Glass, Vineland, NJ 08360).

(c) *Glass wool.*—Pyrex or equivalent.

(d) *Microflex vial.*—1 mL (Kontes Glass).

(e) *Gas chromatograph-thermal energy analyzer (GC-TEA).*—Previously described (16), except reduce GC He flow rate to 17 mL/min and program oven temperature from 130° to 220°C at 4°C/min. Inject 8 μL sample at attenuation 8.

(f) *Gas chromatograph-^{63}Ni electron capture detector.*—Hewlett-Packard Model 5880A. GC instrument conditions: 1.83 m × 3.2 mm stainless steel column packed with 15% Carbowax 20M-TPA on 60–80 Gas-Chrom P; 95% argon–5% methane carrier gas, 30 mL/min; injector 200°C; detector 300°C; column, 140°C for DMN, 180°C for PYRN. Inject 1 μL sample at attenuation 2^4 or 16.

Samples

(a) *Cure-pumped bacon.*—Analyze 20 g fried samples for NDMA and NPYR by dry column method (17).

(b) *Dry-cured bacon.*—Analyze 10 g or 25 g fried samples for NDMA and NPYR by dry column (17), multidetectional (18), and mineral oil distillation (19) methods.

(c) *Beer.*—Analyze 25 g or 50 g samples for NDMA by another dry column method (20).

(d) *Nonfat dried milk powder.*—Analyze 25 g samples for NDMA by a third dry column method (21).

(e) *Malt.*—Analyze samples for NDMA by DCM extraction (50 g) method, different dry column method (25 g), and mineral oil distillation (25 g) methods from those described above (22).

For the current study, one beer and all of the fried cured-pumped bacon samples had previously been analyzed by GC-low resolution mass spectrometry method (23) to ensure that nitrosamines were present.

Procedures

(a) *Preperoxidation cleanup.*—Place DCM extracts of food product (0.2–0.5 mL) in concentrator tube and, if necessary, concentrate to 0.2 mL and dilute with 4–5 mL pentane. Pack disposable Pasteur pipet containing glass wool plug with 3 cm layer of 3N $HClO_4$–Celite mixture. To prepare acid–Celite, place 1 g Celite 545 and 0.8 mL 3N $HClO_4$ in 30 mL beaker and mix with 3 mm od stirring rod 2 min. Wash column with 2 mL pentane, and then add food extract–pentane mixture. Wash column with ca 10 mL pentane, and place column on top of second pipet column containing glass wool plug and 0.5 cm layer of acid alumina (activity 3), washing column with 3 mL DCM before use. Elute nitrosamines from acid–Celite column with 4 mL DCM onto acid alumina column which will retain pigmented compounds. Collect eluate from alumina column in concentrator tube, add boiling chip, fit tube with micro-Snyder column, place in 70°C water bath, and concentrate eluate to ca 0.5 mL. Remove boiling chip and concentrate eluate to 0.2–0.3 mL, or to its starting volume with gentle stream of nitrogen if GC-TEA analysis is desired.

(b) *Peroxidation.*—Place eluate in 1 mL microflex vial containing 10–15 mg pentafluoroperoxybenzoic acid and reduce volume to ca 0.1 mL with stream of nitrogen. Cap vial, shake by hand to obtain homogeneous solution, and store in dark at ambient temperature for 4 h. Add ca 0.1 mL each of DCM and 1N NaOH, cap vial, shake vigorously by hand in rocking

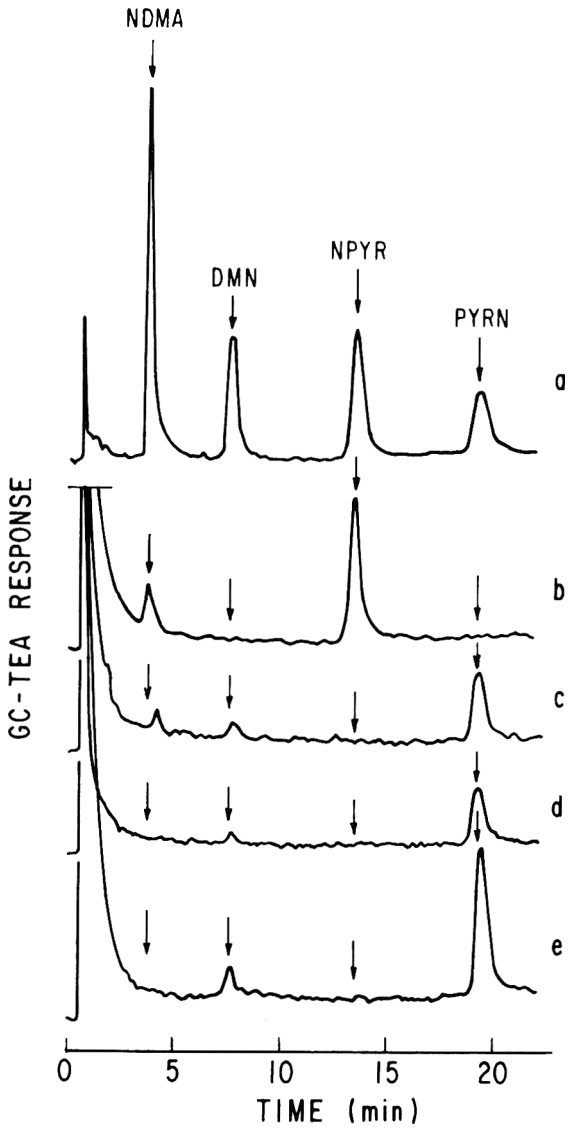

Figure 1. GC-TEA chromatogram of (a) 0.4 ng each of NDMA, DMN, NPYR, and PYRN standards; (b) fried cure-pumped bacon sample extract after acid–Celite column cleanup; (c) sample after peroxidation; (d) sample after acid–Cellte and acid alumina cleanup, (e) sample concentrated to ca ⅓ original volume.

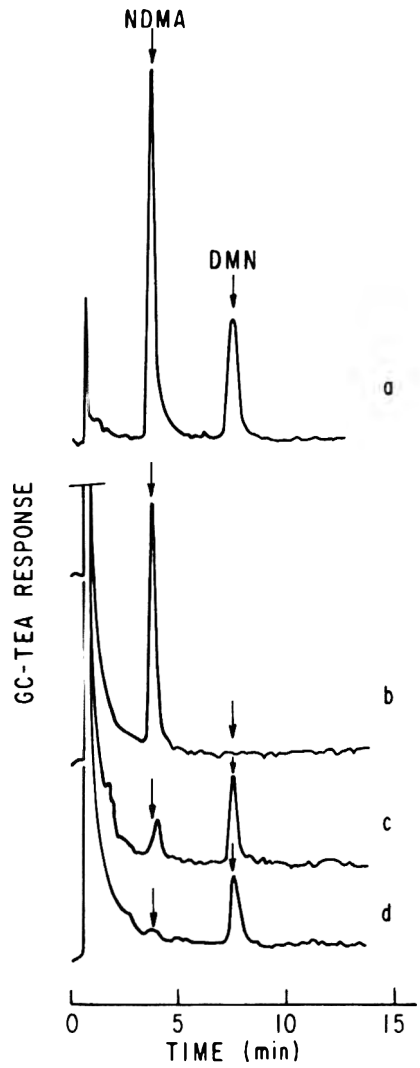

Figure 2. GC-TEA chromatogram of (a) 0.4 ng each of NDMA and DMN standards; (b) beer sample extract after acid–Celite cleanup; (c) sample after peroxidation; (d) sample after acid–Celite and acid alumina cleanup.

motion for 10–15 s, and let mixture stand 10–15 min to permit phases to separate. Remove usually cloudy aqueous upper layer with pipet, add Na_2SO_4, dilute extract with DCM, pass this through pipet column containing 1–2 cm layer of anhydrous Na_2SO_4, and collect filtrate in concentrator tube. Wash vial twice with DCM and also pass washings through Na_2SO_4 column. Concentrate filtrate first on water bath and then with stream of nitrogen to 0.2 mL, or to same volume before peroxidation if GC-TEA analysis is desired.

(c) *Postperoxidation cleanup.*—Add 4–5 mL pentane to 0.2 mL concentrated extract, and pass mixture through pipet column containing glass wool plus 1 cm layer of Na_2SO_4, glass wool, and 2 cm layer of mixture of 6N $HClO_4$–Celite (0.8 mL/g). Wash column with 5 mL pentane, elute nitramines with 4 mL DCM, concentrate eluate on water bath to ca 0.5 mL, then add 4 mL pentane and pass eluate through 1.5 cm layer of acid alumina (activity 3) pipet column. Wash column with ca 6 mL pentane and elute nitramines with 3 mL $LiAlH_4$-treated ethyl ether. Collect eluate in concentrator tube containing hexane equal to predetermined volume for concentrated eluate, then concentrate eluate in water bath to 0.4–0.5 mL, remove boiling chip, and reduce volume further with stream of nitrogen. Subject reagent and solvent blanks minus the food extracts to the same procedure to determine the presence of possible interfering peaks after GC detection.

Results and Discussion

Preliminary peroxidation reactions with the stable peroxyacid, *p*-nitroperoxybenzoic acid, indicated that it was unsuitable: Nitrosamine standards required several hours at 70°C for their conversion to nitramines. A stronger peroxyacid was therefore required. Pentafluoroperoxybenzoic acid was suitable because the peroxidation reaction could be run at room temperature. Many of the peroxidation reactions, including some of the products of these reactions, which are represented in Figures 1–4, were carried out after storage of pentafluoroperoxybenzoic acid at −20°C for more than a year. The stability of the peroxyacid appeared unaffected by longterm storage at −20°C. Certain food extracts yielded non-nitramine TEA-responsive peaks after peroxidation with pentafluoroperoxybenzoic acid, thereby requiring sample cleanup before peroxidation. The use of $HClO_4$–Celite columns for cleanup was based on the results of investigations on the retention of various nitrosamines on Celite 545 con-

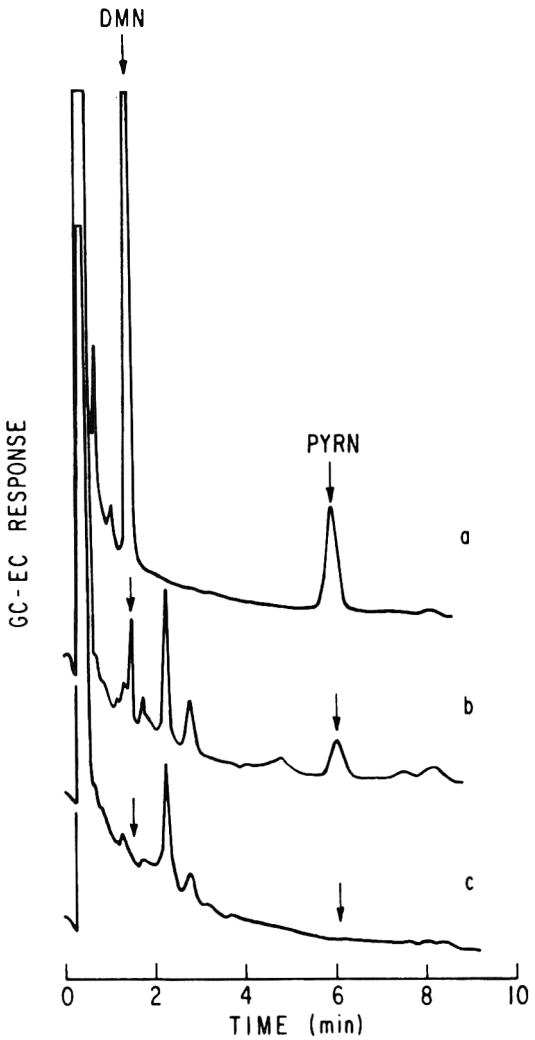

Figure 3. GC-EC chromatogram at column temperature of 180°C: (a) 0.1 ng each of DMN and PYRN standards; (b) fried cure-pumped bacon sample extract after peroxidation and cleanup steps; (c) blank.

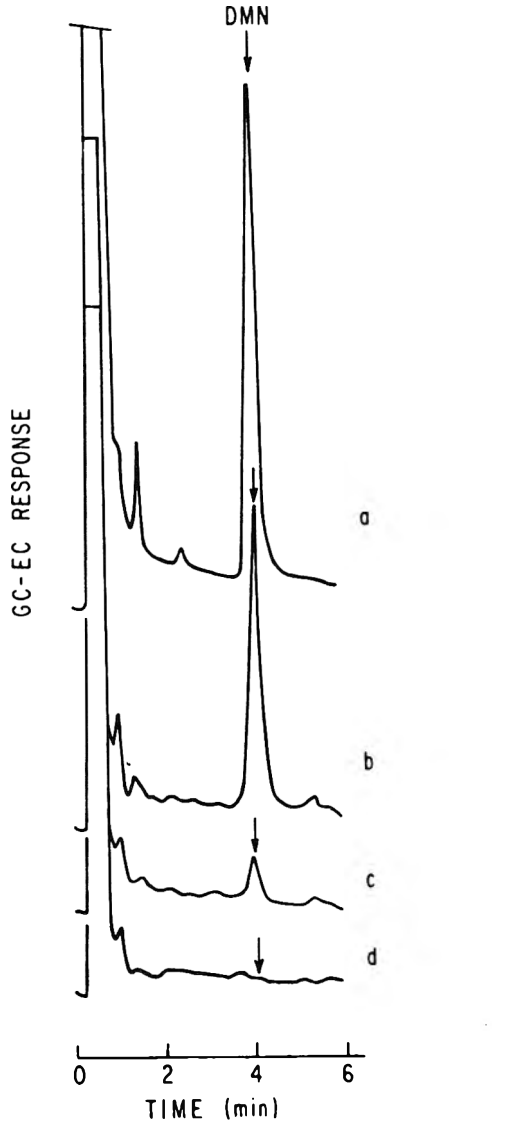

Figure 4. GC-EC chromatogram at column temperature of 140°C: (a) 0.1 ng DMN standard; (b) beer sample extract after peroxidation and cleanup steps; (c) fried cure-pumped bacon sample extract after peroxidation and cleanup steps; (d) blank.

taining different concentrations of $HClO_4$ (G. W. Harrington and H. M. Pylypiw, Jr, unpublished data, Temple University, Philadelphia, PA 1982). The $HClO_4$–Celite gave better results in preliminary studies than the HCl–Celite column we used previously (24), and was therefore used for this study.

The GC-TEA chromatograms for the before and after peroxidation reaction of cure-pumped bacon and beer are shown in Figures 1 and 2, respectively. Figure 1b shows the relatively clean chromatogram of a bacon extract after acid–Celite cleanup. In contrast, the chromatogram of the original extract (not shown) contained a large initial peak, with a small apparent NDMA tailing peak, thereby making quantitation of NDMA difficult. The original extract contained 3.5 ppb NPYR and approximately 0.3 ppb NDMA as determined by GC-TEA analysis. The chromatogram resulting after the peroxidation reaction is shown in Figure 1c. In this chromatogram, the PYRN peak was prominent, whereas the DMN peak was very small, and a contaminant peak eluting slightly after NDMA, which was also present after the peroxidation reaction in other food extracts and the blanks, was removed by the postperoxidation cleanup procedure (Figure 1d). Acid–Celite cleanup of the peroxidized extract resulted in some loss of DMN and PYRN (Figure 1d). Concentrating the postperoxidation acid-Celite cleanup extract to approximately ⅓ its volume increased the peak size of PYRN, and of DMN from very weak to prominant, but not the background level. The overall sensitivity was thereby increased (Figure 1e). The results for beer, in which the original NDMA content was 1.6 ppb, are shown in Figure 2. These results were similar to those for bacon in Figure 1.

For GC-TEA analysis, the acid–Celite cleanup step followed by peroxidation and washing the reaction mixture with 1N NaOH may be sufficient; reasonably clean chromatograms were obtained for bacon and beer extracts as indicated by Figure 1c and Figure 2c. When GC-EC analysis was performed, the reaction mixture needed to be cleaned up further with an acid-Celite column containing a stronger acid solution than that used for cleaning the preperoxidation extracts. This is necessary because the amine nitrogen in the nitramine is less basic than that of the corresponding nitrosamine. The acid alumina (activity 3) column was also needed to replace the DCM nitramine solvent with hexane–ethyl ether because the former produces about a 100 times greater EC detector response than the latter.

GC temperature programming was not possible with GC-EC analysis because of a rapid increase in the baseline with

an increase in the oven temperature. Two isothermal GC oven temperatures were used, 140°C for DMN and 180°C for PYRN. At 180°C, the retention times for DMN and PYRN were about 1.5 and 6 min, respectively. DMN and PYRN were detected in the cure-pumped bacon sample (Figure 3b), which was the same as the bacon sample represented in Figure 1, with no interfering peaks indicated in the blank (Figure 3c). At an oven temperature of 140°C, for the beer sample (Figure 4b), which was the same as the beer sample represented in Figure 2d, the DMN EC resonse was strong, but weak for the bacon sample (Figure 4c), whereas the blank contained no peaks in this same region (Figure 4d). This was similar to the relative TEA response for these same 2 samples (compare Figures 1d and 2d); therefore, the results for the GC-TEA and GC-EC were in agreement. Since the TEA and EC detectors operate on different principles, detection of nitramines on both detectors increased the confidence that nitrosamines were actually present.

For discussion and illustrative purposes, the beer and bacon samples were selected as representative examples because the NDMA in beer and NPYR in cure-pumped bacon had been previously confirmed by GC-MS (19). In addition, the 0.3 ppb apparent NDMA in bacon could be confirmed by this method as DMN only after concentration of the extract to increase sensitivity. Up to now, confirmation of 1 ppb NDMA was difficult. This method permitted confirmation of apparent 0.3 ppb NDMA in bacon since the sample extract could be reduced to increase the nitramine response without a corresponding increase in background response.

The method, as reported, may be limited to certain volatile nitrosamines because others will not be retained by the acid–Celite columns used. Among some of the other volatile nitrosamines reported in food and nonfood products, *N*-nitrosomorpholine can be analyzed by the method described in this paper, whereas *N*-nitrosodiethylamine (NDEA) and *N*-nitrosopiperidine (NPIP) require higher concentrations of $HClO_4$. For analyzing NDMA and/or NPYR in the presence of NDEA and/or NPIP, the effluent from the 3N $HClO_4$–Celite column has to be passed through a 6N $HClO_4$–Celite column, which retains NDEA and NPIP. After peroxidation the nitramines of NDEA and NPIP are retained by a 70% $HClO_4$–Celite column that is effective for cleanup before GC-EC analysis.

The lowest confirmatory levels for this method by GC-TEA were approximately 20 and 50 ng/mL in food extracts containing NDMA and NPYR, respectively, with a TEA signal-to-noise ratio of 4:1. For a 20 g sample, this is equivalent to 1 ppb for NDMA and 2.5 ppb for NPYR. The extracts can be concentrated as indicated by Figure 1e, so the actual NDMA and NPYR levels can be reduced to <1 ppb and 1 ppb, respectively. The overall recovery of nitrosamine in the concentrated DCM extract to nitramine including the cleanup steps was 50–60% by the GC-TEA analysis, based on the assumption of a stoichiometric conversion of nitrosamine to nitramine. Despite using only ⅛ of the total sample size for analysis, the GC-EC analysis has greater sensitivity for DMN and about the same for PYRN compared to the GC-TEA analysis. However, the GC-EC detector is less selective and therefore more responsive to contaminants than GC-TEA. Some food extracts gave TEA responsive peaks other than nitramines after peroxidation with pentafluoroperoxybenzoic acid when the cleanup procedure was omitted. In general, the use of cleanup procedures will decrease the possibility that the nitrosamine and nitramine peaks observed are due to contaminants. In our opinion, if the nitramine peak height is greater than 30% AUFS under the conditions reported (about 5 ppb NDMA or 10 ppb NPYR), the probability of this and the larger parent nitrosamine peak being due to contaminants is greatly reduced. Therefore, the GC-TEA analysis alone is considered sufficient as a confirmatory aid. However, as the nitramine peak height becomes smaller than this arbitrary 30% level, the probability that the nitramine and the parent nitrosamine peaks are due to potential contaminants also increases. To compensate for this situation we recommend the use of both GC-TEA and GC-EC detection, in addition to the utilization of the previously reported ultraviolet photolytic technique applied directly to nitrosamine-containing solutions (25).

In conclusion, a large number of bacon, malt, nonfat dried milk powder, and beer samples containing varying levels of nitrosamines, analyzed by various isolation procedures commonly in use, were employed to demonstrate the general applicability of the nitramine confirmatory method. On a qualitative basis, analyses by both the GC-TEA and GC-EC techniques were in agreement for the presence of nitramine when these compounds could be detected by GC-TEA. Once prepared, the ease of handling pentafluoroperoxybenzoic acid makes this peroxyacid superior to the peroxidation reagents, hydrogen peroxide and trifluoroacetic acid, that have been commonly used.

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COFFEE AND TEA

Headspace Gas Chromatographic Determination of Methylene Chloride in Decaffeinated Tea and Coffee, with Electrolytic Conductivity Detection

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A headspace gas chromatographic procedure is described for the determination of methylene chloride (MC) in decaffeinated tea and coffee. The tea or coffee sample, with added methylene bromide (MB) internal standard, is equilibrated for 1.5 h at 100°C in aqueous sodium sulfate before manual headspace sampling. MC and MB are separated on a Porasil A column at 160°C and detected by using a Coulson electrolytic conductivity detector. For coffee and tea samples spiked at 1.3 ppm MC, as well as commercially decaffeinated teas and coffees containing up to 8 ppm MC, coefficients of variation were 10% or less. For decaffeinated teas, problems involving sample homogeneity and loss of MC before sealing the headspace vial had to be overcome. Similar problems with decaffeinated instant and ground coffees were minimal. The headspace procedure was superior to a previously reported distillation technique. MC was readily detected at 0.05 ppm. Fourteen decaffeinated teas and 15 decaffeinated coffees were analyzed; MC was detected at levels that ranged up to 15.9 and 4.0 ppm, respectively.

In 1977, trichloroethylene (TCE) was delisted as a permitted decaffeination solvent for coffee in Canada (1). Two years earlier, in the United States, its use had been voluntarily discontinued (2), and thus methylene chloride (MC) became the only chlorinated solvent used for decaffeination in Canada and the United States. In the past few years, decaffeinated teas have joined the widely accepted decaffeinated coffees in the marketplace. However, solvent residues in decaffeinated teas are not presently regulated in Canada or the United States. Furthermore, little information is available on decaffeination solvent usage or on residual solvent levels in these teas. Gas chromatographic (GC) procedures have been developed over the last 15 years to measure chlorinated solvent residues in coffee, yet published GC procedures are lacking for decaffeinated teas. Existing analytical procedures for residual solvents in decaffeinated coffees could be evaluated for their application to teas and, if they are suitable, a unified procedure for both decaffeinated products would then be available. However, if they are not suitable, new procedures would have to be developed for tea, and then applied to coffee in an attempt to develop one procedure for both products.

Methods for solvent residues in decaffeinated coffees have received little attention over the past few years. The most recent reports (3, 4) were published in 1977 and 1982 and reviewed previous work. Methods developed over the past 12 years (3, 5, 6) normally used distillation procedures to isolate the solvent residue(s) and GC with electron capture (EC) or Coulson electrolytic conductivity detectors (CECD) for detection and quantitation. Although these distillation procedures reported good recoveries and/or sensitivities, they also required specially fabricated distillation equipment that was not commercially available.

Headspace gas chromatography (HSGC) has become a well recognized technique for the determination of volatile compounds in liquid and solid matrices (7–9). Headspace procedures have been applied to solvent residues in spice oleoresins (10), oilseed meals and flours (11, 12), and hop extracts (13), but they have not been evaluated for decaffeinated coffees and teas. The capillary HSGC procedure reported by Van Rillaer et al. (4) for the determination of a number of coffee decaffeination solvents uses flame ionization as well as mass spectrometric detection. It is apparent from their published chromatograms of decaffeinated coffee headspace that interferences would prevent the determination of MC by using a flame ionization detector. A similar comment has been made by Saxby and Pratten (14). A general procedure for volatile halocarbons in foods has recently been reported by Entz and Hollifield (15). In their procedure, water-immiscible solid food samples are sealed in a headspace vial with 20N sulfuric acid and are equilibrated/digested for 1 h at 90°C. A headspace aliquot is then analyzed by GC with EC detection. Unfortunately, their procedure has not been evaluated for halocarbons as volatile as MC. Their studies do, however, again demonstrate the versatility of HSGC.

Preliminary studies to determine MC in decaffeinated teas used vacuum (3) or steam distillation (5) procedures to isolate MC, several different column packing materials to separate MC from interferences, and both EC and CECD. These studies were largely unsuccessful. We evaluated HSGC, using a number of column packing materials and both EC and CECD, and developed a successful procedure.

METHOD

Apparatus

(a) *Gas chromatograph.*—Varian Aerograph Model 2100, or equivalent equipped with 6 ft × 4 mm id U-shape column packed with 80–100 mesh Porasil A (Waters Associates Inc.) or Spherosil XOA (Foxboro/Analabs) and fitted with a Coulson electrolytic conductivity detector. Operating conditions: temperatures (°C)—injector 200, column 160, and detector 250; helium carrier gas for CECD at 80 mL/min, CECD furnace at 800–820°C with hydrogen flow at 80 mL/min; 6 × 1/16 in. od glass-lined stainless steel tube connecting GC column to vent valve heated to 245°C; vent valve block temperature 250°C. To optimize CECD response, 0.003 in. diameter stainless steel wire was partially inserted into glass capillary leading to mixing chamber (16). Retention times for methylene chloride (MC) and methylene bromide (MB) should be 2–3 and 5–6 min, respectively.

(b) *Syringe.*—1 mL gas-tight syringe Model 050033, Series A-2 (Precision Sampling Corp.), or equivalent equipped with syringe heating mantle as described previously (17), or equivalent. Heat syringe with steam. Use boiling water bath to supply steam and water aspirator to draw steam through syringe, or use steam generator. Connect syringe with ball joints and clips or tubing connectors. Check heating efficiency of mantle by inserting thermometer and corks for syringe. Temperature should be ≥ 98°C. Use 2 in. 22 gauge side port needle.

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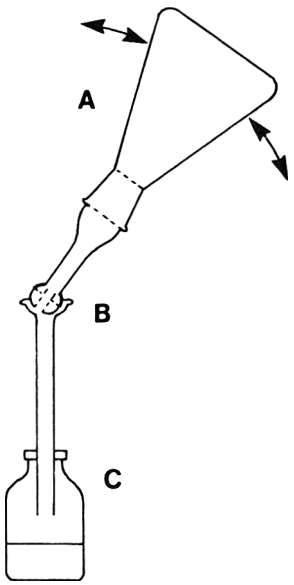

Figure 1. Sample dispensing apparatus: A, 125 mL Erlenmeyer flask with ℨ 24/40 joint; B, ℨ12/5 ball and socket joint with male end joined to ℨ 24/40 male joint; and C, 30 mL headspace sample vial.

(c) *Water bath.*—As described previously (18).

(d) *Headspace sample vials.*—As described previously (18).

(e) *Blender.*—Sunbeam Model BL4000, or equivalent, equipped with 8 oz glass sealable blender jars fitted with Teflon Gasket or seal cut from Teflon sheet.

(f) *Glassware.*—Rinse with acetone and blow dry with nitrogen. Clean headspace vials with soap and brush, rinse with water, and dry in oven at 160°C.

(g) *Sample dispensing apparatus.*—See Figure 1.

Reagents and Materials

(a) *Regular and decaffeinated tea and coffee (ground and instant).*—As purchased in local retail stores.

(b) *Chlorinated hydrocarbons.*—Distilled-in-glass grade methylene chloride (MC) and methylene bromide (MB, dibromomethane, 98%, Aldrich Chemical Co.). For calculation of concentrations use densities (20°C) of 1.327 and 2.477 g/mL, respectively.

(c) *Solvents.*—Distilled-in-glass grade methanol and reagent grade acetone.

(d) *MB internal standard solutions.*—Store all solutions under refrigeration and prepare working internal standard solution fresh every week or when flask is less than two-thirds full. (1) *Stock solution.*—0.743 mg/mL. Pipet 1 mL MB into 100 mL volumetric flask, dilute to volume with methanol, and mix. Pipet 3 mL aliquot into 100 mL volumetric flask, dilute to volume with water and mix. (2) *Working solution.*—74.3 μg/mL. Pipet 10 mL stock solution into 100 mL volumetric flask, dilute to volume with water, and mix.

(e) *MC stock solution.*—0.133 mg/mL. Store all solutions under refrigeration. Pipet 1 mL MC into 100 mL volumetric flask, dilute to volume with methanol, and mix. Pipet 1 mL aliquot into 100 mL volumetric flask, dilute to volume with water, and mix. Prepare fresh for use in (f).

(f) *Mixed standard solution.*—Store all solutions under refrigeration. Prepare fresh for each calibration curve 6 standard solutions each containing 74.3 μg/mL MB and 0.265, 1.33, 2.65, 6.64, 11.9, and 19.9 μg MC/mL by pipetting 10 mL MB stock solution and 0.2, 1, 2, 5, 9, or 15 mL MC stock solution into each of six 100 mL volumetric flasks. Dilute to volume with water, and mix.

(g) *Sodium sulfate.*—Anhydrous reagent grade.

(h) *Saturated sodium sulfate solution (19).*—Dissolve 264 g Na_2SO_4 in 945 mL water, with magnetic stirring and heating as necessary. Let cool. Add a few crystals of Na_2SO_4 to prevent supersaturation.

Preparation of Calibration Curves

Weigh 2.0 g nondecaffeinated tea, instant coffee, or ground coffee into each of 14 headspace sample vials. Using graduated cylinder, add 15 mL saturated Na_2SO_4 solution to each vial. Into 12 of these vials, pipet 1 mL of the appropriate mixed standard solution (f) in duplicate and into 2 vials pipet 1 mL MB working solution (d)(2) to give MB concentration of 37.2 ppm and MC concentration of 0, 0.133, 0.664, 1.33, 3.32, 5.97, or 9.95 ppm in each flask with respect to the tea or coffee. Immediately cap vial with closure after adding each 1 mL aliquot. Mix vial by rotation.

Equilibrate sample vials in boiling water bath for 1.5 h and sample headspace as described below under *Headspace Technique* after 1.5 h in bath.

Using chromatogram of blank sample as guide, draw baseline and estimate peak heights of MC (H_C) and MB (H_B) to nearest 0.1 mm on chromatogram, correcting for any interfering peak in blank sample. Determine ratio H_C/H_B for the 12 headspace injections. Plot H_C/H_B vs ppm MC and draw best line through points. Alternatively, determine peak areas and plot area ratio A_C/A_B vs ppm MC and draw best line through points.

Preparation of Samples

(a) *Tea.*—Chill unopened tea package and blender jar and blade assembly in freezer for 2 h at < -10°C. Open tea package, quickly transfer 40–50 g tea to blender and replace and tighten blade assembly. For tea bags, cut open each bag and quickly transfer tea to blender as above. Blend frozen tea 5 min or until pulverized at highest speed, stopping every 1–2 min to shake blender to dislodge tea adhering to blender walls. Return blender and contents to freezer for 2 h. Open blender jar and quickly transfer contents to Erlenmeyer flask by using glass powder funnel. Replace ball-joint fitting of sample dispensing apparatus and keep inverted in ring clamp (Figure 1). Let tea warm to room temperature.

With graduated cylinder, transfer 15 mL saturated Na_2SO_4 solution into headspace vial. Place septum and aluminum seal over opening but do not crimp. Immerse headspace vial in shallow pan containing water and ice with level of water just above liquid in headspace vial. Let stand 0.5 h. For each sample, remove septum and aluminum seal and pipet 1 mL MB solution (74.3 μg/mL) into headspace vial. Holding lower part of sample dispensing apparatus (Figure 1) in vertical position, move Erlenmeyer flask toward vertical while tapping Erlenmeyer flask with finger to gradually dispense tea. Discard first 4 or 5 g of tea. Place vial on top-loading balance, insert sampler tube just into headspace vial, and dispense 2 g of tea, weighing to nearest 0.01 g. Immediately replace septum and seal and crimp tightly so that seal cannot be turned. Immerse vial in ca 50°C water bath, swirling occasionally until precipitated Na_2SO_4 crystals are redissolved and tea is mixed.

Equilibrate in boiling water bath for exactly 1.5 h and sample headspace as described in *Headspace Technique.*

(b) *Coffee (ground or instant).*—With graduated cylinder, transfer 15 mL saturated Na_2SO_4 solution into headspace vial. Pipet 1 mL MB solution (74.3 μg/mL) into headspace vial. Thoroughly mix contents of jar or can by shaking. Open can, jar, or package of coffee and weigh 2 g of coffee to nearest

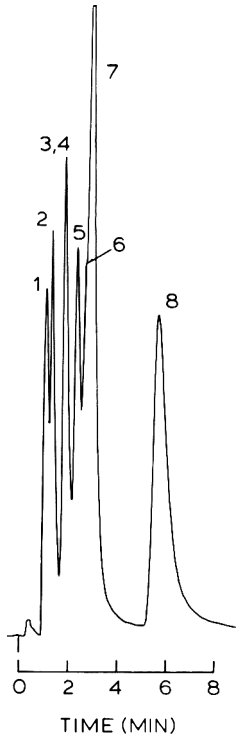

Figure 2. Gas chromatogram using CECD of headspace from 15 mL aqueous solution at 16 ×: 1, 1,1-dichloroethane, 0.314 ppm; 2, 1,1,1-trichloroethane, 0.089 ppm; 3, 4, carbon tetrachloride, 0.531 ppm, 1,2-dichloroethylene, 0.084 ppm; 5, methylene chloride, 0.088 ppm; 6, chloroform, 0.099 ppm; 7, 1,1,2-trichloroethylene, 0.097 ppm; 8, methylene bromide (internal standard), 0.691 ppm.

0.01 g onto aluminum foil, using metal spoon or spatula to transfer coffee to foil. Transfer coffee to headspace vial and immediately place septum and seal over opening and crimp tightly so that seal cannot be turned. Swirl to wet coffee.

Equilibrate in boiling water bath for exactly 1.5 h and sample headspace as described in *Headspace Technique*.

Headspace Technique

Heat syringe mantle and syringe with steam for at least 15 min before sampling. Keep plunger in steam-heated test tube. Briefly flush syringe with nitrogen to ensure needle is clean of bits of septum.

Use following technique to sample headspace: Replace heated plunger in syringe and depress fully. Disconnect mantle and syringe and pierce septum of vial with needle about 1 cm. Withdraw plunger slowly (5 s) to 1 mL. (A mark on plunger may be helpful.) Wait 30 s to let barrel fill. Lock syringe. Withdraw needle and immediately insert needle into gas chromatograph. Unlock syringe and inject sample. Reconnect mantle to steam source and prepare for next sample. Identify MC and MB from retention times.

Calculation

Measure peak height or determine areas of MC and MB as for standards and determine average ratio H_C/H_B or A_C/A_B from duplicate samples. Determine ppm MC from calibration curve and correct to 2.00 g sample.

Results and Discussion

Initial attempts to develop a procedure to determine MC in tea were carried out using steam distillation (5) with EC and Coulson electrolytic conductivity detection. Steam distillation was chosen over the closed system vacuum distillation procedure (3) because of the general availability of the distillation apparatus compared with the customized fabrication necessary for the closed system procedure. Several decaffeinated and nondecaffeinated teas were distilled and their toluene extracts were injected onto the GC system. However, interferences arising from the teas, especially with EC detection, made identification and quantitation of MC difficult. Several GC columns, including Porasil A, Chromosorb 102, Carbowax on Carbopak C, and Porapak Q, failed to satisfactorily resolve the interferences from MC.

HSGC was then tried following the procedure of Entz and Hollifield (15). EC detection was quickly ruled out because of many interfering peaks at or near the retention time of MC. With CECD, however, chromatograms with fewer interferences were obtained. With the porous polymer GC packing materials such as Chromosorb 102 and Porapak QS, as well as the conventional liquid stationary phases such as OV-101 and Carbowax 20M-TPA, a large tailing early-eluting peak occurred that was attributed to water vapor. MC eluted on the tail of this peak. Chromosorb 102 gave the best resolution between MC and the large water peak, yet peak height measurements were somewhat unreliable. With Porasil A as the column packing material, however, much better chromatography was attained. The water vapor apparently is absorbed to the silica and does not give any peaks on the chromatogram. As a result of these preliminary studies, a procedure using HSGC, a Porasil A column, and CECD was chosen for further study and refinement.

Using this column and detector, we studied the chromatographic behavior of MC and several other volatile halogenated solvents. With a toluene standard solution of halocarbons, the chromatography reported previously was obtained (20). However, in the described HSGC procedure, the water-saturated injections partially deactivate the silica column and MC elutes earlier and tails. The separation of some volatile hydrocarbons injected as a headspace from an aqueous solution is shown in Figure 2. The column had to be stabilized by injecting water to obtain reproducible retention times. Normally, after leaving the column overnight at 225°C (to purge late-eluting tea components) or after 5 or 6 h at 160°C with no water-containing injections, a 6 μL injection of water was sufficient. For a new column, however, repeated 6 μL injections of water were necessary before the retention times of MC and MB decreased to the desired 2–3 and 5–6 min, respectively.

The headspace sample treatment described by Entz and Hollifield (15) involves the digestion of 1–2 g of water-immiscible samples with 20N H_2SO_4 for 1 h at 90°C. Although we used this procedure with heating at 100°C to evaluate the chromatographic column and detector as described above, the charred tea residue made the headspace vials difficult to clean. However, saturated aqueous sodium sulfate produced headspace partition for standards similar to the corrosive 20N H_2SO_4, as well as similar headspace patterns for decaffeinated teas. A 1 mL gas-tight locking syringe fitted with a heating mantle (17) was used to sample the pressurized headspace vial. This syringe allowed injection of a pressurized sample that gave about twice the response as that of a non-locking unpressurized syringe. For a non-locking syringe, glass heating mantles are commercially available. Because of possible variations in the volume of the headspace vial as well as volume injected, MB was chosen as an internal standard. Separation of MB from the common chlorinated solvents is shown in Figure 2.

Using the described technique, MB as an internal standard, and individual tea samples containing MC, HSGC responses for MB and MC were determined at various times. A plot of

Table 1. Effect of deviations from stated procedure on repeatability (N = 5 or 6)

Tea	Deviation	CV, %		MC, ppm	
Breakfast	unblended	28.4	(4.0)[a]	0.254	(0.281)
Spice	unblended	42.1	(6.5)	10.9	(7.62)
Breakfast	weighed in open	24.0	(4.0)	0.243	(0.281)
Spice	weighed in open	16.2	(6.5)	6.62	(7.62)
Spice	Na_2SO_4 soln, rm temp.	12.0	(6.5)	5.89	(7.62)
Spice	tea added first, then Na_2SO_4 soln, rm temp.	11.4	(9.2)[b]	5.26	(5.83)[b]
Earl Grey	tea added first, then Na_2SO_4 soln, rm temp.	20.7	(6.9)[b]	1.04	(0.845)[b]

[a]Values in parentheses obtained by following proposed procedure except as noted by *b*.
[b]Values obtained by following proposed procedure except Na_2SO_4 solution was at room temperature.

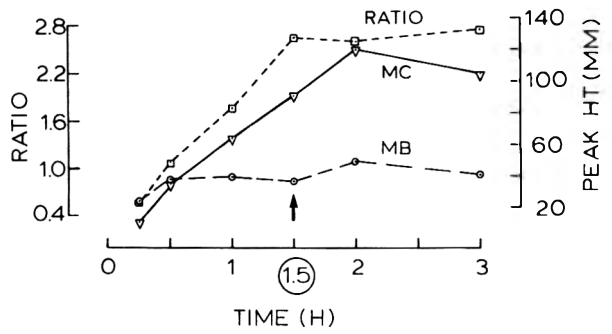

Figure 3. Equilibration curves for tea containing about 1.3 ppm MC spiked with 9.92 μg MB.

peak heights and peak ratios against time is shown in Figure 3. It is apparent that added MB equilibrates in 0.5–1 h, yet MC, which must first desorb from the tea takes 1.5 h before the peak heights become relatively constant. Thus, 1.5 h was taken as the equilibration time. Sampling when equilibrium is attained should provide the best repeatability because the peak height before equilibrium is time dependent and will be less. The effect of shaking during equilibration was not investigated.

During the study of equilibration times using decaffeinated teas containing residual MC, considerably more variation in duplicates was apparent with MC than with the MB internal standard. Thus, investigations of sample homogeneity and/or MC loss on handling were carried out, and a sampling procedure which gave acceptable repeatability was developed. To minimize the loss of MC by volatility, the tea was kept cold during transfer from one container to another except for the final addition to the headspace vial. Tea leaves were blended to a coarse dust in a blender to minimize sample inhomogeneity. The necessity of these procedures is demonstrated in Table 1.

The relative importance of the various steps in preparing the sample for HSGC can be evaluated from Table 1. Blending the tea to give a finely divided sample is mandatory to obtain good repeatability. For both breakfast and spice teas the coefficients of variation for replicate samples are about 7 times less when the tea is blended before sampling. In the spice tea, which contains whole cloves not containing MC, the need for blending is obvious; yet for the breakfast tea, which contains only tea leaves, blending also appears necessary. The loss of MC during blending cannot be evaluated from these results because of the large coefficient of variation (CV) in the unblended sample. Variation between replicates can also increase if the blended sample is weighed in the open on a piece of paper rather than using the sample dispensing apparatus. Variable loss of MC may occur if weighed in the open; however, with the large CV the actual amount lost is difficult to assess. We found that if the sample in the ball-joint sampler was chilled, the sample was not free flowing and was difficult to dispense. Loss of MC, and a resulting increase in CV, may also occur if the tea is added to a room temperature sodium sulfate solution. The tea may release MC, and it may volatilize and be lost. Similarly, increased variation and loss of MC occurs when the tea is added to the headspace flask first, followed by the sodium sulfate solution. Of the components of the procedure described, blending the tea to ensure homogeneity is the most important step to reduce variability; use of the sample dispensing apparatus, order of addition, and cooling the headspace flask are also important considerations to reduce variable loss of MC.

Table 2. MC residues found in decaffeinated teas and coffees by headspace gas chromatographic method

Product	Labeled origin	Container	MC, ppm
Tea			
English breakfast	N. Am.	tea bag	0.70
English breakfast (2)[a]	N. Am.	cardboard can	10.3, 15.9
English breakfast	Switz.	tea bag	0.49
English breakfast	unknown	bulk	0.34
Spice (3)[a]	N. Am.	cardboard can	5.97, 8.93, 7.62
Earl Grey (2)[a]	N. Am.	tea bag	0.61, ND[b]
Earl Grey	N. Am.	tea bag	ND
Earl Grey	unknown	bulk	1.04
Almond	unknown	bulk	0.98
Strawberry	unknown	bulk	0.38
Coffee			
Instant	N. Am.	glass jar	0.91
Instant	N. Am.	glass jar	0.80
Instant (6)[c]	N. Am.	glass jar	ND
Roasted and ground (2)[a]	N. Am.	metal can	1.54, 2.13
Roasted and ground	N Am.	metal can	1.00
Roasted and ground	N Am.	foil bag	ND
Roasted and ground (2)[c]	Germany	metal can	ND
Roasted and ground	Austria	foil bag	4.04
Roasted and ground	unknown	bulk	0.45

[a]Number of different samples analyzed, same manufacturer.
[b]Not detected, < 0.05 ppm.
[c]Number of different samples analyzed, different manufacturers.

The precautions necessary for teas were not needed for decaffeinated roasted and ground and instant coffees. For example, when these coffees were weighed on paper, repeatabilities were 6.5 and 6.4%, respectively. When room temperature sodium sulfate solution was added to the coffee in the headspace vial, repeatabilities were 9.7 and 10.9%, respectively.

In all these investigations, both peak areas and peak heights were used to calculate their respective ratios. In nearly all cases, the repeatability of area ratios was slightly better than for peak heights, and was therefore the method of choice.

We then evaluated the sensitivity of the procedure using the CECD. Figure 4 shows the headspace analysis of 2 g of

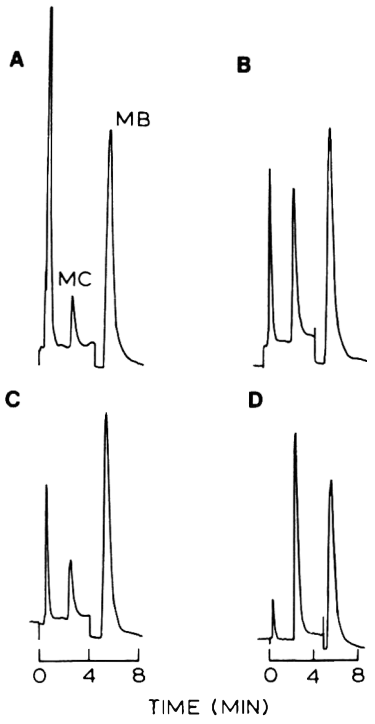

Figure 4. Gas chromatograms using CECD of headspace: A, blank tea spiked with 0.13 ppm methylene chloride (MC) at 16 × and 37.2 ppm methylene bromide (MB) internal standard at 256 ×; B, almond tea containing 0.98 ppm MC at 64 ×; C, strawberry tea containing 0.38 ppm MC at 64 ×; D, roasted and ground coffee containing 2.13 ppm MC at 128 ×.

Earl Grey tea spiked at 0.133 ppm. A 5% FSD at attenuation 16 would correspond to about 0.050 ppm. Sensitivity is similar for roasted and ground coffee, and for instant coffee the 5% FSD corresponds to about 0.040 ppm. This sensitivity for MC is similar to that obtained using a distillation technique (3).

Figure 4 also shows chromatograms of several tea and coffee samples purchased at local retail stores. The peak for MC must be attenuated for the different levels of MC found in the products, while the peak for MB is attenuated at 256× for all samples. These results and additional data are presented in Table 2. Fourteen tea samples, 7 roasted and ground coffees, and 8 instant coffees were analyzed and had MC levels ranging up to 15.9, 4.0 and 0.91 ppm, respectively. MC was not detected in 2 tea samples, 3 roasted and ground coffee samples, and 6 instant coffee samples. There was no evidence for the presence of any other volatile halogenated decaffeination solvent.

The HSGC procedure presented in this paper has been shown to be applicable to both decaffeinated teas and coffees, although more care and special equipment is needed to obtain repeatable results for tea. The procedure is simple, rapid, and inexpensive compared with distillation procedures. The major drawback of the method is the need for the selective Coulson electrolytic conductivity detector. The Hall detector, although not evaluated, would probably also prove satisfactory.

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COLOR ADDITIVES

Thin Layer Chromatographic Detection and Spectrophotometric Determination of the Trisodium Salt of 1,3,6-Pyrenetrisulfonic Acid in D&C Green No. 8

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A thin layer chromatographic method is presented for separating the reaction by-product 1,3,6-pyrenetrisulfonic acid (trisodium salt) (PTS) from D&C Green No. 8 (8-hydroxy-1,3,6-pyrenetrisulfonic acid). PTS is detected visually, extracted from the adsorbent, and determined spectrophotometrically. Recoveries of PTS added at 0.75–6.73% levels to 8-hydroxy-1,3,6-pyrenetrisulfonic acid ranged from 80.0 to 94.8%.

D&C Green No. 8, the trisodium salt of 8-hydroxy-1,3,6-pyrenetrisulfonic acid (Figure 1), is listed in the Code of Federal Regulations (CFR) (*Code of Federal Regulations* (1982) Title 21, Part 74, U.S. Government Printing Office, Washington, DC, sec. 74.1208) for use in externally applied drugs and cosmetics. The color is prepared by sulfonating pyrene to produce the tetrasulfonic acid. This product is fused with aqueous sodium hydroxide to yield the monohydroxy-trisulfonate, which is isolated as the trisodium salt. During this process the 1,3,6-pyrenetrisulfonate (PTS) (Figure 2) is also formed. The CFR limits the amount of PTS to 6.0% by weight of D&C Green No. 8.

In the past, PTS was determined in the laboratories of the Division of Color Technology (DCLT), Food and Drug Administration (FDA), by using a partition column chromatographic procedure (A. B. Leatherman, FDA, 1965). This method was time consuming and it was difficult to achieve a good separation. The thin layer chromatographic (TLC) method presented here is simple, fast, and adequate for the quantitative examination of samples of D&C Green No. 8 submitted for certification.

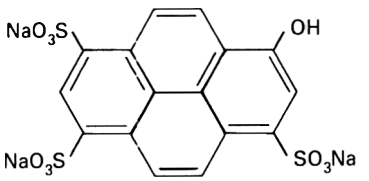

Figure 1. D&C Green No. 8.

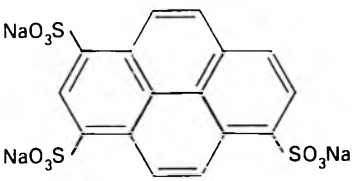

Figure 2. 1,3,6-Pyrenetrisulfonic acid (trisodium salt).

Table 1. Recovery of PTS added to 8-hydroxy-1,3,6-pyrenetrisulfonic acid

Added, %	Found,[a] %	Rec., %
0	0.03	—
0.75	0.60	80.0
	0.69	92.0
	0.69	92.0
	0.65	86.7
1.50	1.34	89.3
	1.39	92.7
	1.40	93.3
	1.41	94.0
2.99	2.77	92.6
	2.81	94.0
	2.77	92.6
	2.82	94.0
6.73	6.14	91.2
	6.29	93.5
	6.32	93.9
	6.33	94.1
5.24	4.97	94.8

[a]After subtraction of 0.28% PTS content of sample.

Experimental

Apparatus

(a) *Thin layer chromatographic accessories.—(1)* Chromatographic tank suitable for ascending development of 20 × 20 cm TLC plates and lined with sheet of Whatman Grade No. 1 chromatography paper. *(2)* TLC plates, 20 × 20 cm, pre-coated with silica gel GF and MN 300 cellulose with fluorescent indicator in proportion of 3 to 1, 250 μm thick (Analtech, Inc., PO Box 7558, 75 Blue Hen Dr, Newark, DE 19711). *(3)* TLC mechanical streaker, delivering 0.100 mL/pass.

(b) *Disposable filter assembly.*—Gelman Acrodisc, 0.45 μm pore size (Gelman Sciences, Inc., 600 South Wagner Rd, Ann Arbor, MI 48106).

(c) *Spectrophotometer.*—Suitable for use in ultraviolet (UV) region.

(d) *Optical cells.*—Matched pair, 2.5 cm.

(e) *UV lamp.*—Long wave, suitable for use in illuminating TLC plates.

Reagents

(a) *Solution A.*—Dissolve 2.0 g NaCl, 0.25 g Na_2CO_3, and 0.25 g $NaHCO_3$ in 110 mL water.

(b) *Developing solvent.*—Solution A–methanol (110 + 15).

(c) *Sample solvent.*—Acetone–water (3 + 2).

(d) *Extracting solvent.*—0.76N NH_4OH. Add 5 mL concentrated NH_4OH to water and dilute to 100 mL.

Determination

Weigh 1.000 g D&C Green No. 8 into beaker and dissolve in ca 60 mL acetone–water (3 + 2). Transfer quantitatively to 100 mL volumetric flask and dilute to volume. Using mechanical streaker, apply 0.100 mL of solution onto TLC plate. Let plate air-dry. Place plate in lined chromatographic tank that has been equilibrated with developing solvent ca 30 min. Develop plate until solvent front is 1 in. from upper edge. Remove plate from tank and let dry.

Examine plate under UV lamp. PTS appears as a violet band just above the band of the main component. Remove adsorbent containing PTS from plate and extract PTS with 0.76N NH_4OH. Filter through Luer-Lok tip syringe to which

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Table 2. Averages and coefficients of variation for amounts of PTS found in the recovery study

Spike level, %	Av. found, %	CV, %
0.75	0.66	6.5
1.50	1.39	2.2
2.99	2.79	0.94
6.73	6.27	1.4

Table 3. Determination of PTS in D&C Green No. 8 by TLC and column chromatographic (CC) methods[a]

	PTS found, %	
Sample	TLC	CC
1	7.96	6.9
	7.83	
2	7.74	7.1
	7.90	
3	7.93	7.8
	7.89	

[a]Spectrophotometric measurement used in the determinative step of each method.

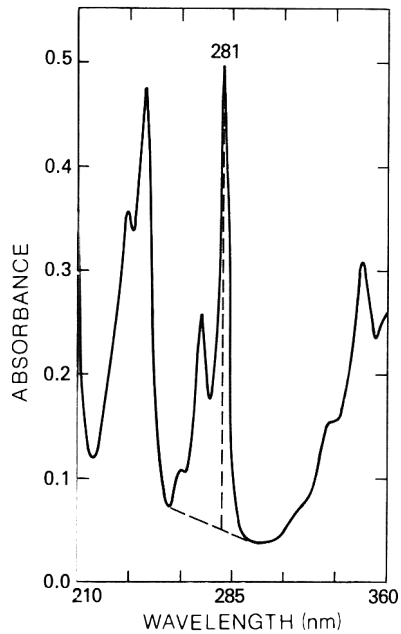

Figure 3. UV spectrum of 1,3,6-pyrenetrisulfonic acid in 0.76N NH_4OH, with baseline and perpendicular drawn.

disposable filter assembly has been attached. Note volume. Obtain UV spectrum from 360 to 210 nm, using 2.5 cm cell.

Calculation

The UV spectrum of PTS shows multiple peaks. Measure absorbance at 281 nm by using a baseline technique (Figure 3). Draw a line that connects the lowest points on either side of the peak at 281 nm. Then construct a perpendicular line from the top of the peak to the point at which it intersects the baseline. Subtract the absorbance reading at this intersection from the absorbance reading at the peak maximum. This value is the absorbance of PTS. Calculate % by weight of PTS in sample of D&C Green No. 8 as follows:

$$\% \text{ PTS} = (A \times V \times 100)/(A' \times L \times W)$$

where A = absorbance at 281 nm, V = volume of solution containing PTS extracted from TLC band (mL), A' = 78.5 mL/(mg × cm) = approximate absorptivity of PTS standard in 0.76N NH_4OH at 281 nm, L = cell length (cm), and W = 1.00 mg = sample weight on plate.

Recovery Study

A solution containing ca 200 mg PTS (Hilton-Davis Chemical Co., Division of Sterling Drug Inc., Cincinnati, OH 45237) in 100 mL 0.76N NH_4OH was used in a recovery study. The exact concentration of the PTS solution was determined spectrophotometrically from aliquots diluted with 0.76N NH_4OH.

The PTS solution was added to 8-hydroxy-1,3,6-pyrenetrisulfonic acid (trisodium salt), which is available from Kodak Laboratory Chemicals (Eastman Kodak Co., Rochester, NY 14650) as a fluorescent protein stain and laser dye. The concentration of PTS in this product, which contains less contaminants than certified batches of the color additive, was 0.28 ± 0.035% as determined by the TLC method.

Four spiking levels were chosen for the recovery study: slightly above the specification limit, and one-half, one-quarter, and one-eighth the specification limit. Four recovery solutions at each spiking level were prepared and analyzed. (One additional recovery solution that was slightly below the specification limit was also analyzed.) Also included in the recovery study was 1 unspiked sample.

Results and Discussion

Table 1 presents the recovery data and individual percentages found in the recovery study. The amount of PTS found in the unspiked sample, 0.03%, is well within the standard deviation of the blank values.

The averages and coefficients of variation (CVs) (standard deviation times 100 divided by the average) for the amounts of PTS found at each spiking level are presented in Table 2. These CV values are low, indicating good repeatability. The highest CV (6.5%), which is for the lowest level, indicates that the variation is greatest at that level.

Samples of commercial batches of D&C Green No. 8 submitted for certification to DCLT were analyzed by this TLC method over a 10-month period. These batches contained between 1.89 and 7.96% PTS. Three of the samples were analyzed twice by the TLC method and once by the column chromatographic method (A. B. Leatherman, FDA, 1965) because the PTS content exceeded the specification limit of 6.0%. Table 3 shows the results.

In addition to the PTS band, the developed TLC plates for most of the commercial samples revealed violet bands, visible under UV light, which separated below the band of the main component. These bands may correspond to 1-pyrenesulfonic acid and tc isomers of pyrenedihydroxydisulfonic acid and pyrenemonohydroxydisulfonic acid. A band for 1,3,6,8-pyrenetetrasulfonic acid, which was identified from the R_f value by comparison with a standard, was also detected in a few samples just below the solvent front.

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SUGAR AND SUGAR PRODUCTS

Liquid Chromatographic Determination of Sugars in Licorice Extracts: Collaborative Study

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A collaborative study to determine fructose, glucose, sucrose, and maltose in a variety of licorice extracts has been performed using liquid chromatography. Six collaborators participated in the study, and each collaborator was sent 5 samples which were analyzed in blind replicates. A practice sample was used with a specific range of 8–10% fructose, 10–12% glucose, and 5–7% sucrose. The individual sugar concentration of the 5 samples ranged from 2.0 to 10%. All samples were quantitated by comparing results with a pure sugar standard containing fructose, glucose, sucrose, and maltose and analyzed under the same conditions. No significant problems were encountered running the practice or other samples despite the wide range of instruments and columns used. Results showed less deviation within laboratories compared with results obtained among laboratories. The method has been adopted official first action.

Licorice root, the basic raw material of licorice extract, grows wild in river valleys from the Middle East to Central Asia. Licorice extract is prepared by grinding the root, extracting with hot water, and evaporating the extract solution. The evaporated extract solution is then finished to a spray-dried or paste form.

Classical methods for determining sugars in licorice extracts are based on reduction of copper salts (1). These methods are nonspecific for individual sugars and are time-consuming, requiring about 2½ h to analyze one sample. An accurate, simple, and rapid procedure for determining sugars in licorice by liquid chromatography (LC) (2) was reported in 1982.

Sugars in Licorice Extracts
Liquid Chromatographic Method
First Action

(Applicable to paste or spray-dried products)

Principle

Sample is dissolved in H_2O, filtered, and subjected to column cleanup. Aliquot is injected into LC app. equipped with differential refractometer detector. Response is compared with that of std soln.

Apparatus

(a) *Liquid chromatograph.*—Model M6000A solv. delivery system, Model U6K universal injector, Model R401 differential refractometer, and 30 cm × 4 mm μBondapak carbohydrate column (Waters Associates, Inc.), or equiv.

(b) *Computing integrator.*—Model 3390A (Hewlett Packard), or equiv.

(c) *Guard column.*—Packed with 37–50 μm AX/Corasil (Waters Associates, Inc.), or equiv.

(d) *Sample and solvent clarification kit.*—Aq. and org. (Waters Associates, Inc.), or equiv.

(e) *Cartridge.*—Sep-Pak™ C_{18} (Waters Associates, Inc.), or equiv. Condition just before use by passing 10 mL MeOH followed by 10 mL H_2O thru cartridge.

Reagents

(a) *Mobile phase.*—Use LC grade reagents, CH_3CN–H_2O (83 + 17). Filter and degas mobile phase with solv. clarification kit. Flow rate 2.0 mL/min.

(b) *Sugar std soln.*—2.5 mg/mL each fructose, glucose, sucrose, and maltose (Sigma Chemical Co.). Accurately weigh 250 mg each fructose, glucose, sucrose, and maltose into 100 mL vol. flask and dil. to vol. with H_2O, heating gently until dissolved. Using aq. sample clarification kit, filter thru 0.45 μm filter, followed by preconditioned C_{18} cartridge.

Sample Preparation

Accurately weigh 1.0 g licorice product into 250 mL erlenmeyer and add 100.0 mL H_2O. Place flask in 85–90° H_2O bath until sample dissolves, then cool to room temp. Filter thru 0.45 μm filter. Pass 5 mL sample thru preconditioned C_{18} cartridge, and collect eluate. Sugars in sample should pass thru C_{18} cartridge and any nonpolar components in sample will be retained.

Liquid Chromatography

Let LC column equilibrate 30 min at flow rate of 2.0 mL/min. Inject 10 μL mixed sugar std soln. Approx. retention times are fructose 6.6 min, glucose 7.7 min, sucrose 12.4 min, and maltose 15.2 min (retention times vary from one column to another). Inject 60 μL sample soln. If samples are analyzed in series, re-inject std soln at regular intervals.

Calc. concn of each sugar by comparing either peak hts or peak areas of std with corresponding peak hts or peak areas of sample. Calc. amt of each sugar by the following equation:

$$\%\ \text{Sugar} = (C'/C) \times (Ar/Ar') \times (V'/V) \times 100$$

where C' and C = concn of std and sample in mg/mL, resp.; Ar' and Ar = av. peak area of std and sample, resp. (Ht'/Ht could be used for ht instead of area); V' and V = μL injected of std and sample, resp.

Results and Discussion

Five different licorice extract samples, known by their trademark as POLE, "S," SAIL, SHIP, and COMET, were prepared in blind duplicates and sent to 9 laboratories. Samples were sent in sealed polypropylene bottles. Bottles were then encased in plastic bags to prevent any changes in moisture content. Blind duplicate samples were numbered in a way that no participant analyzed the samples in the same sequence nor were any duplicates analyzed in a specific order. Explicit instructions were sent to each laboratory along with a 4-sugar standard, a practice sample, and forms for reporting results. The practice sample contained a specific amount of fructose, glucose, and sucrose. Each laboratory was required to obtain results within this range before starting the study. Results of duplicate determinations of 5 samples by 6 labo-

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The recommendation of the Associate Referee was approved by the General Referee and Committee D and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) 67, March.

Table 1. Collaborative data for LC determination of fructose, glucose, sucrose, and maltose in licorice products

	Sample 1				Sample 2				Sample 3				Sample 4				Sample 5			
Coll.	F	G	S	M	F	G	S	M	F	G	S	M	F	G	S	M	F	G	S	M
1	2.1	2.9	7.0	7.2	1.9	2.7	4.3	—	2.3	2.7	5.4	—	5.7	7.9	4.0	—	5.7	7.6	6.9	—
	2.1	2.9	6.8	7.0	2.0	2.7	4.4		2.0	2.4	5.3		5.7	7.8	5.0		5.8	7.8	6.8	
2	3.0	3.3	9.8	8.3	3.5	2.6	5.7	—	4.4	3.5	7.6	—	7.9	9.5	6.1	—	8.7	9.0	10.4	—
	3.1	3.5	9.2	8.5	3.3	2.7	5.7		4.0	2.7	7.5		7.5	8.2	5.7		8.4	8.6	10.3	
3	3.1	3.1	6.7	6.9	2.7	2.1	4.4	—	2.1	2.3	5.5	—	6.8	7.6	4.0	—	6.5	7.7	6.3	—
	2.3	2.7	6.3	6.8	3.1	2.0	4.5		3.1	2.1	5.5		5.6	6.4	3.2		7.3	7.1	6.7	
4	2.3	2.8	8.3	7.6	2.6	3.2	5.4	—	3.1	3.8	6.9	—	6.7	8.4	5.3	—	7.0	8.6	8.9	—
	2.5	2.8	8.4	7.8	2.8	3.0	5.3		3.1	4.2	6.8		6.8	8.6	5.3		6.8	8.4	8.5	
5	2.7	3.1	9.3	9.9	2.4	3.5	6.3	—	3.0	3.0	7.8	—	7.6	10.0	6.3	—	8.0	10.1	10.4	—
	2.6	3.4	9.7	10.3	2.7	3.2	6.0		2.7	3.3	8.5		7.4	9.6	5.7		7.9	10.0	9.8	
6	3.0	3.7	9.2	8.4	3.3	2.9	6.0	—	3.4	2.6	7.7	—	8.0	9.9	5.7	—	8.2	10.0	9.2	—
	3.3	3.4	8.6	8.3	3.0	2.4	6.0		3.3	2.2	7.7		7.8	9.4	5.5		8.0	9.6	9.5	

Table 2. Exclusion of outlying data from Table 1

	Sample 1				Sample 2				Sample 3				Sample 4				Sample 5			
Coll.	F	G	S	M	F	G	S	M	F	G	S	M	F	G	S	M	F	G	S	M
1	—[a]	2.9	7.0	7.2	—[a]	2.7	4.3	—	—[a]	2.7	5.4	—	—[a]	7.9	4.0	—	—[a]	7.6	6.9	—
		2.8	6.8	7.0		2.7	4.4			2.4	5.3			7.8	5.0			7.8	6.8	
2	3.0	3.3	9.8	8.3	3.5	2.6	5.7	—	4.4	3.5	7.6	—	7.9	9.5	6.1	—	8.7	9.0	10.4	—
	3.1	3.5	9.2	8.5	3.3	2.7	5.7		4.0	2.7	7.5		7.5	8.2	5.7		8.4	8.6	10.3	
3	—[a]	3.1	6.7	6.9	2.7	2.1	4.4	—	3.1	2.3	5.5	—	—	7.6	4.0	—	—[a]	7.7	6.3	—
		2.7	6.3	6.8	3.1	2.0	4.5		3.1	2.1	5.5			6.4	3.2			7.1	6.7	
4	2.3	2.8	8.3	7.6	2.6	3.2	5.4	—	3.1	3.8	6.9	—	6.7	8.4	5.3	—	7.0	8.6	8.9	—
	2.5	2.8	8.4	7.8	2.8	3.0	5.3		3.1	4.2	6.8		6.8	8.6	5.3		6.8	8.4	8.5	
5	2.7	3.1	9.3	9.9	2.4	3.5	6.3	—	3.0	3.0	—[a]	—	7.6	10.0	6.3	—	8.0	10.1	10.4	—
	2.6	3.4	9.7	10.3	2.7	3.2	6.0		2.7	3.3			7.4	9.6	5.7		7.9	10.0	9.8	
6	3.0	3.7	9.2	8.4	3.3	2.9	6.0	—	3.4	2.6	7.7	—	8.0	9.9	5.7	—	8.2	10.0	9.2	—
	3.3	3.4	8.6	8.3	3.0	2.4	6.0		3.3	2.2	7.7		7.8	9.4	5.5		8.0	9.6	9.5	

[a]Outlier (3).

Table 3. Statistical analysis of collaborative data

	Sample 1				Sample 2				Sample 3			
Statistic	F	G	S	M	F	G	S	M	F	G	S	M
Including Outlying Individual Results												
Mean	2.7	3.1	8.3	8.1	2.8	2.8	5.3	—	3.1	2.9	6.8	—
Repeatability CV, %	9.6	5.8	3.6	2.0	6.8	6.6	1.9	—	5.5	10.8	3.0	—
Reproducibility CV, %	16.2	10.9	15.9	16.5	18.8	16.8	14.5	—	21.7	23.9	17.3	—
Std dev.	0.42	0.33	1.3	1.12	0.50	0.45	0.74	—	0.65	0.67	1.14	—
Excluding Outlying Individual Results												
Mean	2.8	3.1	8.3	8.1	2.9	2.8	5.3	—	3.3	2.9	6.6	—
Repeatability CV, %	4.9	5.8	3.6	2.0	7.0	6.6	1.9	—	5.0	10.8	1.0	—
Reproducibility CV, %	12.9	10.9	15.9	16.5	12.6	16.8	14.5	—	16.0	23.9	17.0	—
Std dev.	0.34	0.34	1.3	1.1	0.36	0.45	0.74	—	1.51	0.67	1.05	—

	Sample 4				Sample 5			
	F	G	S	M	F	G	S	M
Including Outlying Individual Results								
Mean	7.0	8.6	5.2	—	7.4	8.7	8.6	—
Repeatability CV, %	5.4	6.4	8.3	—	3.6	2.9	3.0	—
Reproducibility CV, %	13.3	13.2	19.0	—	14.2	12.5	19.0	—
Std dev.	0.89	1.10	0.94	—	1.00	1.04	1.57	—
Excluding Outlying Individual Results								
Mean	7.5	8.6	5.2	—	7.9	8.7	8.6	—
Repeatability CV, %	2.4	6.4	8.3	—	1.90	2.9	3.0	—
Reproducibility CV, %	6.9	13.2	19.0	—	8.9	12.5	19.0	—
Std dev.	0.48	1.10	0.94	—	0.66	1.04	1.57	—

Table 4. Summary of statistics

Sugar	Sample	Lab. outliers eliminated	Est mean	Repeat.	Reprod.
Fructose	1	1[y], 3[c]	2.81	0.1369 (CV = 4.87%)	0.3629 (CV = 12.90%)
	2	1[y]	2.94	0.2049 (CV = 6.97%)	0.3711 (CV = 12.62%)
	3	1[y]	3.32	0.1612 (CV = 4.86%)	0.5350 (CV = 16.12%)
	4	1[y], 3[c]	7.46	0.1768 (CV = 2.37%)	0.5176 (CV = 6.94%)
	5	1[y], 3[d]	7.81	0.1500 (CV = 1.90%)	0.7062 (CV = 8.97%)
Dextrose	1	none	3.13	0.5102 (CV = 5.77%)	0.9680 (CV = 10.95%)
	2	none	2.75	0.1826 (CV = 6.64%)	0.4644 (CV = 16.89%)
	3	none	2.90	0.3136 (CV = 10.81%)	0.6958 (CV = 23.99%)
	4	none	8.61	0.5470 (CV = 6.35%)	1.1386 (CV = 13.23%)
	5	none	8.71	0.2533 (CV = 2.91%)	1.0893 (CV = 12.51%)
Sucrose	1	none	8.28	0.3014 (CV = 3.64%)	1.3165 (CV = 15.96%)
	2	none	5.33	0.1000 (CV = 1.88%)	0.7770 (CV = 14.57%)
	3	5[c]	6.59	0.0548 (CV = 0.83%)	1.1128 (CV = 16.89%)
	4	none	5.15	0.4282 (CV = 8.31%)	0.9791 (CV = 19.01%)
	5	none	8.64	0.2566 (CV = 2.97%)	1.6439 (CV = 19.02%)
Maltose	1	none	8.08	0.1581 (CV = 1.96%)	1.1778 (CV = 14.57%)

y: Youden outlier.
c: Cochran outlier
d: Dixon outlier.

ratories using the LC method are presented in Table 1. Three laboratories, after receiving the samples, decided not to participate in the study due to lack of time and personnel changes in their organization.

Statistical Analysis

Results were analyzed statistically to determine if any laboratory should be eliminated.

(a) *Elimination of extreme results.*—*(1)* Outlying laboratories: The Youden test (3) to determine if any laboratory shows consistently high or low values indicated that Laboratory 1/fructose did not satisfy the criteria and, therefore, should be eliminated. *(2)* Outlying individual results: Dixon's test (3) to determine outlying individual results indicated that Laboratory 3/fructose sample 5 did not satisfy the criteria. *(3)* The Cochran test (3) indicated that Laboratory 3/fructose sample 1, Laboratory 3/fructose sample 4, and Laboratory 5/sucrose sample 3 did not satisfy the criteria. *(4)* Variation between laboratories: All laboratories were within the range, indicating no outlying laboratories. *(5)* Variation between replicates: All laboratories were within the range, indicating no outlying laboratories.

(b) *Analysis of variance of the data.*—A one-way analysis of variance was carried out both with and without the outlying results to compare repeatability between replicates and reproducibility between laboratories. The coefficient of variation between replicates (repeatability), including all outlying results, were in the same range, with fructose having a coefficient of variation from 3.6 to 9.6%, glucose from 2.9 to 10.8%, and sucrose from 1.9 to 8.3%. Only sample 1 contained maltose, so not enough data were available for comparing the coefficients of variation with other sugars. Reproducibility between laboratories, including all outlying results, indicated slightly higher coefficients of variation. Fructose had a coefficient of variation between laboratories from 13.3 to 21.7%, glucose from 10.9 to 23.9%, and sucrose from 14.5 to 19.0%. Table 2 represents the exclusion of outlying results. The exclusion of outlying results had little effect on the repeatability or reproducibility values obtained for glucose and sucrose. Elimination of outlying results for fructose, however, resulted in an increase in precision of both repeatability and reproducibility (Table 3). Table 3 also presents the means, coefficients of variation within laboratories (repeatability), coefficients of variation between laboratories (reproducibility), and standard deviations for each of the samples, including outlying results and excluding outlying results. A summary of all statistics is given in Table 4.

Slight modifications were made after discussions with the Associate Referee by all collaborators to increase the sample concentration and amount of sample injected. This was done because collaborators thought the peaks were too small, making quantitation difficult. To increase the peak height, the concentration of sample was increased. One collaborator deviated from the method by dissolving the sample in acetonitrile–water (90 + 10) rather than in 100% water. This was

done so samples would dissolve easier. None of these modifications had any significant effect on either the test repeatability or reproducibility. One collaborator complained of an unknown peak appearing just before sucrose. This peak was thought to be some type of contaminant since no other collaborator noted this peak. Another collaborator also found very slight amounts of maltose in 3 samples which did not contain maltose. The Associate Referee thought this was due to baseline noise coming from the refractive index detector. No effect on reproducibility or repeatability resulted by using different columns from different manufacturers. The use of an internal standard was recommended and used by one collaborator; however, based on overall results, it may not be necessary.

Results indicate that the precision of the technique is excellent within laboratories as indicated by coefficients of variation (repeatability). Precision between laboratories is slightly lower than within laboratories. The study also indicates excellent clean, sharp peaks with no interfering substances, and most collaborators were able to obtain baseline or nearly baseline resolution between fructose and glucose.

One of the factors leading to large coefficients of variation between laboratories can be traced to collaborators using different techniques for measuring peak heights between the sugar standard and samples. This is the first time collaborators have used this method and, due to lack of practice with the method, larger coefficients of variation may have resulted. With additional practice in using the method, coefficients of variation between laboratories should improve.

Recommendation

It is recommended that this method be adopted official first action.

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FERTILIZERS

Liquid Chromatographic Determination of Nitrogen Derived from Urea and Water-Soluble Methylene Ureas in Urea Formaldehyde Fertilizers: Collaborative Study II

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A second collaborative study with 6 collaborators was conducted on the AOAC official first action method for measuring nitrogen derived from urea, methylenediurea (MDU), and dimethylenetriurea (DMTU). No variations were made. The collaborators made single determinations on 2 separate days on 5 sets of paired samples. The mean coefficients of variation for the completed study were 4.22% for urea, 5.08% for MDU, and 5.48% for DMTU, which are only slightly higher than the values reported in the first study: urea, 3.24%; MDU, 3.48%; and DMTU, 6.56%. The method has been adopted official final action.

In 1982, a collaborative study was conducted on a method which measured the nitrogen derived from urea and water-soluble methylene ureas in urea formaldehyde fertilizers. Results of the study were reported at the 96th AOAC Annual International Meeting held October 25–28, 1982, in Washington, DC. The method was adopted official first action (1).

To give a wider degree of exposure to the method and to confirm the results of the initial collaborative study (2), a second collaborative study was conducted. Collaborators other than those that participated in the first study were obtained. A different set of urea formaldehyde samples was also used; one was a fertilizer/pesticide combination product, subjecting the method to a further test of its ruggedness.

Method Committee A had recommended that an internal standard be sought for the method. In response to this recommendation, some 50 commercially available water-soluble organic compounds were evaluated as potential internal standards. None fit the criteria of an internal standard of stability, solubility, purity, retention time, and peak shape. Efforts to obtain an internal standard are continuing.

Collaborative Study

Six collaborative laboratories were furnished with 10 samples (5 matched pairs) and asked to perform a single analysis on each sample on 2 different days, using solutions of samples prepared on that day. The method, external standards of urea, methylenediurea (MDU), and dimethylenetriurea (DMTU), a practice sample, and report forms were furnished to each collaborator. Collaborators were instructed to use peak height measurements for calculations and to return all data and chromatograms.

MDU and DMTU used as external standards were obtained by preparative liquid chromatography. Identity was confirmed by elemental analysis, molecular weight, and infrared spectroscopy. The urea was Baker Analyzed reagent grade, used as purchased.

The sample pairs analyzed were selected to cover the range of urea formaldehyde ratios (1.3–2.5) which might be encountered in typical products. The fertilizer grade for each of the 5 matched pair of samples was as follows: Samples 1 and 10 (27-3-3), Samples 2 and 9 (36-4-4), Samples 3 and 7 (27-3-3), Samples 4 and 6 (31-5-6), and Samples 5 and 8 (26-3-3). Samples 5 and 8 also contained the herbicides 2,4-D and MCPP. The study was designed to follow Youden's procedure for closely matched pairs (3).

The collaborative study samples were analyzed in our laboratory, following the method as written. Analyses were run on 7 different days over a 3-week period, with fresh samples prepared each day. Standards were prepared as needed. Mean coefficients of variation for the 5 matched pairs were 3.5, 4.2, and 5.5% for urea, MDU, and DMTU, respectively.

Collaborators used a variety of columns for the study: 1, Whatman Partisil ODS 3; 2, Excalibar ODS (10 μm); 3, Sperisorb 5 μm ODS; 4, Anspec 5 μm C18 RP; 5, Brownlee RP-18 Speri-5; 6, C18 μBondapak 10 μm.

The detection limits and recoveries obtained for urea, MDU, and DMTU were: urea av. rec. at 1–19%, 99.8%; MDU av. rec. at 1–12%, 97.2%; DMTU av. rec. at 0.5–8%, 86.2%. The percent recovery for urea and MDU are good at the levels expected in most urea formaldehyde fertilizers. Both have solubilities in water greater than that expected to be present. DMTU has limited solubility in water (0.1 g/100 mL) and it is, therefore, difficult to dissolve pure DMTU above the solubility limit without extended agitation or heat. This is in contrast to a fertilizer sample: Under the conditions of the method, levels of DMTU in solution in excess of 0.2 g/100 mL are possible.

Results and Discussion

Collaborative results for nitrogen derived from urea, MDU, and DMTU are given in Tables 1, 2, and 3, respectively. Urea, MDU, and DMTU values for sample pair 3 from Collaborator 1 were omitted. Apparently, Collaborator 1 injected Sample 2 twice and did not inject Sample 3 on day 1. Nitrogen values for MDU from Collaborator 5 on sample pair 2 for both days were also rejected. Collaborator 5 could not get baseline separation of urea and MDU with the column used and had difficulty quantitating the MDU peaks because of high concentrations of urea present.

The average coefficients of variation obtained in this study, with the exception of DMTU, are slightly higher than in the previous study (see Table 4), but are still well within acceptable limits for a method using external standards.

Although several pairs of nitrogen results for each component could have been rejected, thereby improving the coefficients of variation, we decided to include the values rather than give a possible false impression of the method.

Collaborators, in general, did not have any problem with the method. Collaborator 1 substituted a variable speed, horizontal circular shaker for 50 min (140–160 rpms) and increased the injection size from 10 to 20 μL on day 2 because of small peak heights obtained for DMTU on day 1. A defect in the pumping system prevented increasing sensitivity without an

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The recommendation of the Associate Referee was approved by the General Referee and Committee A and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) 67, March.

Table 1. Collaborative results for determination of urea by LC

	Sample									
	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5	
Coll.	1	10	2	9	3	7	4	6	5	8
					Day 1					
1	11.10	10.67	18.28	17.46	18.32[a]	9.22	4.43	4.37	9.33	9.41
2	11.12	10.94	18.19	18.23	8.96	9.32	4.30	4.24	9.05	9.52
3	11.56	10.74	19.47	18.57	9.86	9.51	5.30	5.01	9.98	9.89
4	11.36	11.07	18.00	16.30	9.52	9.30	4.30	4.34	9.52	9.62
5	11.08	10.78	18.45	17.43	9.05	9.33	4.30	4.20	8.62	9.46
6	10.79	10.57	17.94	17.40	8.94	9.10	4.22	4.32	9.29	9.14
					Day 2					
1	10.96	10.65	17.75	17.64	9.04	9.15	4.33	4.36	9.15	9.31
2	11.46	10.26	17.30	18.43	9.26	8.88	4.10	4.51	9.26	9.92
3	10.90	10.85	18.21	18.39	9.27	9.24	4.54	4.83	9.81	9.80
4	10.47	10.87	16.67	17.91	8.30	9.19	3.97	4.34	8.96	9.61
5	10.87	11.04	18.04	17.57	8.38	8.97	4.06	4.15	8.25	9.50
6	10.77	10.56	17.99	17.90	8.37	9.06	4.45	4.22	9.22	9.42
N	12		12		11		12		12	
Mean	10.89		17.90		9.09		4.38		9.38	
S_d	0.252		0.682		0.432		0.399		0.429	
S_r	0.294		0.603		0.298		0.156		0.303	
S_b	—		0.224		0.221		0.259		0.215	
$S = \sqrt{S_r^2 + S_b^2}$	0.273		0.643		0.371		0.303		0.371	
CV, %	2.5		3.6		4.1		6.9		4.0	

[a]Rejected due to injection of wrong sample.

Table 2. Collaborative results for determination of MDU by LC

	Sample									
	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5	
Coll.	1	10	2	9	3	7	4	6	5	8
					Day 1					
1	7.91	7.85	4.13	3.95	4.24[a]	6.31	3.70	3.64	6.11	6.48
2	7.78	7.60	4.00	3.75	5.56	6.12	3.05	3.10	5.92	6.30
3	8.00	7.63	4.88	4.52	5.99	6.27	3.92	3.76	6.16	6.44
4	7.78	7.79	3.73	3.50	5.56	5.96	3.44	3.35	5.98	6.41
5	7.99	7.89	4.30[b]	4.15[b]	5.77	6.36	3.57	3.48	6.02	6.53
6	7.53	7.52	3.65	3.81	5.41	5.86	3.43	3.38	5.75	5.85
					Day 2					
1	7.72	7.44	4.04	3.98	5.61	6.04	3.51	3.54	6.00	6.18
2	8.06	7.46	3.89	4.17	5.60	5.89	3.44	3.78	6.03	6.70
3	7.57	7.79	4.28	4.49	5.68	6.11	3.49	3.55	5.98	6.30
4	7.55	7.89	4.11	4.58	5.47	6.41	3.69	4.07	6.15	6.70
5	8.32	8.35	3.60[b]	1.69[b]	5.55	6.43	3.39	3.47	5.76	6.82
6	7.68	7.59	3.88	3.83	5.17	6.02	3.75	3.52	5.98	6.36
N	12		10		11		12		12	
Mean	7.78		4.06		5.86		3.54		6.20	
S_d	0.299		0.467		0.237		0.303		0.232	
S_r	0.178		0.190		0.166		0.129		0.177	
S_b	0.170		0.302		0.120		0.194		0.106	
$S = \sqrt{S_r^2 + S_b^2}$	0.246		0.357		0.205		0.233		0.206	
CV, %	3.2		8.8		3.5		6.6		3.3	

[a]Rejected due to injection of wrong sample.
[b]Rejected due to poor resolution of peaks.

increase in noise level. The simplicity and rapid assay of the method was thought to be quite advantageous.

Collaborator 2 had to replace rotor seals in the injector used and, had time permitted, would have liked to repeat analyses and replace day 1 results. The change did not appear to contribute to any disparity in the reported results.

Methanol–water is generally used to flush columns after use with water or for storage. Collaborator 3 apparently could not initially flush all of the methanol from the system. This resulted in higher than anticipated values for MDU on the practice sample because methanol has a retention time essentially the same as MDU. It is suggested that this be noted in the method.

The question of peak area vs peak height measurement, although not raised, was investigated on a limited basis. Values from one run were obtained using both peak height and peak area measurements and are shown in Table 5. Results are essentially the same and indicate that either peak height or peak area could be used.

Recommendation

The results of this second collaborative study essentially duplicate the results of the previous study. As indicated previously, the coefficients of variation from the collaborative

Table 3. Collaborative results for determination of DMTU by LC

	Sample									
	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5	
Coll.	1	10	2	9	3	7	4	6	5	8
					Day 1					
1	3.23	3.24	1.68	1.57	1.72[a]	2.34	1.72	1.76	2.06	2.34
2	3.23	3.33	1.61	1.52	2.00	2.37	1.31	1.59	2.47	2.41
3	3.36	3.30	1.61	1.61	2.16	2.42	1.77	1.70	2.23	2.38
4	3.27	3.52	1.52	1.43	2.27	2.38	1.61	1.61	2.43	2.35
5	3.43	3.46	1.69	1.73	2.27	2.51	1.61	1.69	2.34	2.51
6	3.18	3.22	1.49	1.42	2.15	2.48	1.57	1.59	2.27	2.23
					Day 2					
1	3.29	3.41	1.62	1.64	2.21	2.54	1.52	1.76	2.21	2.34
2	3.35	3.29	1.58	1.66	1.97	2.56	1.50	1.71	2.15	2.37
3	3.26	3.31	1.54	1.58	2.06	2.40	1.58	1.60	2.12	2.27
4	3.01	3.13	1.44	1.44	1.81	2.35	1.44	1.57	2.06	2.22
5	3.46	3.46	1.69	1.60	2.01	2.38	1.48	1.48	2.02	2.59
6	3.31	3.43	1.55	1.51	1.99	2.50	1.84	1.72	2.34	2.52
N	12		12		11		12		12	
Mean	3.55		1.57		2.26		1.61		2.30	
S_d	0.154		0.118		0.128		0.147		0.142	
S_r	0.062		0.045		0.099		0.087		0.123	
S_b	0.100		0.077		0.057		0.083		0.051	
$S = \sqrt{S_r^2 + S_b^2}$	0.117		0.089		0.114		0.121		0.133	
CV, %	3.3		5.7		5.1		7.5		5.8	

[a]Rejected due to injection of wrong sample.

studies are low and approach values obtained for many chromatographic methods using internal standards. Therefore, the Associate Referee recommends that the LC method collaboratively studied for the determination of nitrogen derived from urea and water-soluble methylene ureas in urea formaldehyde fertilizers be adopted official final action.

Table 4. Mean coefficients of variation for urea, MDU, and DMTU: comparison of previous and current collaborative studies

	Av. CV, %	
Component	Study I	Study II
Urea	3.2	4.2
MDU	3.5	5.1
DMTU	6.6	5.5

Table 5. Peak height vs peak area measurements for quantitation of urea and water-soluble methylene ureas in fertilizer samples

	Urea		MDU		DMTU	
Sample	Ht	Area	Ht	Area	Ht	Area
1	10.81	10.95	8.02	8.23	3.50	3.41
2	17.77	17.83	4.30	4.69	1.76	1.65
3	9.09	9.23	5.51	5.90	2.30	2.18
4	4.61	4.89	3.73	3.82	1.60	1.60
5	9.13	9.23	6.45	6.62	2.43	2.39
6	4.52	4.57	3.73	3.82	1.65	1.60
7	9.23	9.23	6.02	6.19	2.55	2.47
8	9.23	9.32	6.62	6.83	2.47	2.43
9	17.77	17.77	4.02	4.30	1.59	1.65
10	10.62	10.62	7.72	7.85	3.41	3.46

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Simple and Rapid Portable Chromatographic Method for Separation and Detection of Phenylmercuric Acetate in Seeds and Water

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Phenylmercuric acetate can be detected by horse liver acetone powder succinate dehydrogenase inhibition, using a mixture of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT), sodium succinate, and *N*-methylphenazonium methosulfate as the chromogenic reagent. The simple cleanup involves extraction of phenylmercuric acetate in chloroform and concentration by evaporation. In the extract, the compounds in seeds or water could be separated and identified by paper chromatography in the field or laboratory at microgram levels with an acetone–water (70 + 30) solvent system.

Thin layer chromatographic and paper chromatographic enzymatic methods are simple, sensitive, and specific for separation and detection of heavy metal compounds like mercuric chloride, copper sulfate, cadmium sulfate, silver nitrate, and potassium chromate from fresh water (1–4). Inorganic mercury was separated by paper and thin layer chromatography by using a 0.1% sodium chloride solution as the solvent system, with detection by a succinate dehydrogenase inhibition technique (3). However, this solvent system or concentration technique (by evaporation) is not suitable for chromatographic separation and detection of the organic mercury compound phenylmercuric acetate, a commonly employed fungicide in seed dressing and herbicide.[2] Phenylmercuric acetate is a hazardous neurotoxic agent that may contaminate agricultural commodities during spraying operations when used as a seed dressing, or from industrial effluents. The chromatographic technique reported here for detection of phenylmercuric acetate is a new, rapid, and sensitive method. It is simple compared with the cumbersome conversion of organic mercury to elemental inorganic mercury and its subsequent analysis by nonenzymatic and other chemical methods (5–8). Detection of phenylmercuric acetate in field conditions is also described.

METHOD

Apparatus and Reagents

All chemicals were analytical grade.

(a) *Phenylmercuric acetate.*—(BDH, India). Use 98% pure for preparation of various concentrations in chloroform.

(b) *Enzyme.*—Prepare 2% homogenate of horse liver acetone powder (Sigma Chemical Co., St. Louis, MO 63178) in ice cold water, filter as described earlier (1, 3), and use immediately.

(c) *Chromogenic reagent.*—Mixture of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT), sodium succinate, and *N*-methylphenazonium methosulfate (PMS). Prepare INT (BDH, India) 0.4% in water, sodium succinate (BDH, Poole, UK) 2.5% in water, and PMS (BDH, Poole, UK) 0.1% in water. Mix 10 + 10 + 2 mL.

(d) *Filter paper strips.*—Whatman No. 3, 2.5 × 7.0 cm (3).

Seed Dressing

Seeds dressed (20 ppm) with technical (98% pure) and commercial phenylmercuric acetate powder ("Parasan," 1% Hg obtained from Hyderabad Chemical Supplies Private Ltd, India), as well as field samples, namely seeds from local seed sellers (Rayalaseema Seeds Corp., India) were analyzed for phenylmercuric acetate.

Extraction Procedure

Accurately weigh 50 g dressed seed samples and transfer to conical flask. Add 50 mL chloroform and stir well. Decant chloroform into clean petri dish. Repeat process with 25 mL chloroform. Evaporate contents of petri dish at room temperature to 5 mL or appropriate volume (1–5 mL). Sample is now ready for chromatography.

Phenylmercuric Acetate in Water

Fortify 1 L fresh water with phenylmercuric acetate by adding 10 mg technical or commercial product to give 10 ppm concentration, and use for analysis. Mix fortified fresh water with 100 mL chloroform and shake well 5 min in separatory funnel. Withdraw chloroform layer into petri dish. Repeat process with 25 mL chloroform. Evaporate chloroform to 5 mL or appropriate volume (1–5 mL). Sample is ready for chromatographic analysis. (Evaporate chloroform further to increase sensitivity if necessary.)

Screening of Phenylmercuric Acetate by Detector Strips

Use portable detector strips (2), which respond to phenylmercuric acetate in 900 ng amounts, to confirm presence of phenylmercuric acetate in prepared or unknown samples, and then proceed with chromatographic separation and identification.

Paper Chromatography

Spot phenylmercuric acetate samples in chloroform 2 cm above base of Whatman No. 3 filter paper strip with graduated microcapillary pipet. Hang paper strips in acetone–water (70 + 30) solvent system contained in 12 × 4 cm glass jar described earlier (1, 3). Remove paper strips and let dry. Spray with 2% horse liver acetone powder enzyme suspension. Enzyme should be sprayed sufficiently to wet paper strip without causing leaching. Place paper strips on glass plate and let stand at room temperature (28°C) 4 min to allow inhibition of enzyme by organic mercury compound. Paper strips should not dry. To avoid leaching, do not spray excess enzyme solution. Spray INT–sodium succinate–PMS (10 + 10 + 2) mixture as fine mist (1). To avoid leaching, do not spray excess mixture. Place paper strips in hot air oven (80°C) along with glass plate. Phenylmercuric acetate inhibition of succinate dehydrogenase appears as white spot in pink farmazon (1–3) background within 10–15 min. The lower limit of detection is 4 μg by the spot test and 8 μg when separated by paper chromatography with recommended solvent system.

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[2]Phenylmercuric acetate is no longer cleared for use in the United States.

Table 1. Screening of surface deposits of phenylmercuric acetate in seeds and water samples by chromatographic-enzymatic method

Sample	Source[a]	Detection[b]
Triticum vulgare (100 g)	local market	–
	seeds corp.	+
	fortified	+
Phaseolus vulgaris (100 g)	local market	+
	seeds corp.	+
	fortified	+
Cicer arientium (100 g)	local market	–
	seeds corp.	–
	fortified	+
Phaseolus mungo (100 g)	local market	+
	seeds corp.	+
	fortified	+
Phaseolus areus (100 g)	local market	–
	seeds corp.	+
	fortified	+
Freshwater sample (10 ppm)	fortified	+

[a]Dressed seeds were obtained from Rayalaseema Seeds Corp., Tirupati, A.P., India. Fortified samples were treated with technical grade and 1% phenylmercuric acetate commercial grade. Market samples were obtained from local seed sellers; the seed surface deposits were eluted with chloroform.

[b]Present or absent. Detection limit: with and without chromatographic separation, 8 and 4 μg, respectively; portable Biodetector strips: 900 ng.

Field Analysis

Instead of placing in hot air oven, warm paper strips on glass slide with cigarette lighter (2) for enzymatic inhibition. Other tools are portable as described earlier (2, 3, 9).

Results and Discussion

In the present method, phenylmercuric acetate can be chromatographically separated with acetone–water (70 + 30) as solvent system. The R_f value for phenylmercuric acetate was 0.22 ± 0.011. The interference of inorganic mercury with phenylmercuric acetate is not possible in the present procedure because of the following: inorganic mercury is insoluble in chloroform; phenylmercuric acetate is insoluble in 0.1% NaCl aqueous solvent solution used for inorganic mercury separation; the R_f values for the 2 compounds are different; and other heavy metal inhibitors like $CuSO_4$, $CdSO_4$, $HgCl_2$, and $AgNO_3$ (3) are insoluble in chloroform, and thus, interference is eliminated by the extraction procedure.

The principle of the chemical reaction is that the succinate dehydrogenase of horse liver acetone powder acts on sodium succinate substrate in a dehydrogenation reaction (oxidation in Krebs cycle where the succinate substrate is converted to fumarate) (10). During this chemical reaction the electrons liberated are accepted by tetrazolium salt (INT), reducing it to a pink complex, formazan. Wherever phenylmercuric acetate is present on the paper strip, the white inhibition zones appear amid the pink formazan background (1–4). The phenylmercuric acetate inhibits succinate dehydrogenase, so that electrons are not liberated to form the pink formazan complex.

The present method is simpler than methods reported earlier in which cumbersome procedures are involved for analysis by nonenzymatic and other chemical methods (5–8, 11–14). Noninterference of inorganic mercury with phenylmercuric acetate in the present method has the advantage that both organic and inorganic mercury compounds can be detected separately. The inorganic mercury can be chromatographically separated using the 0.1% NaCl solvent system with the enzyme inhibition technique described earlier (3).

Phenylmercuric acetate was detected in fortified laboratory and field samples by the present method (Table 1).

The merits of the present method are as follows: The detection can be done in 30 min, either in the laboratory or in the field. The method can be used with minimal cleanup for direct detection either in fresh water or in seeds. Without using chromatographic separation, the limit of detection of phenylmercuric acetate by spot test on a paper strip is 4 μg, 8 μg after chromatographic separation and nanogram amounts with biodetector strips (2). The disadvantages are that the method is useful for detection and identification only and not for quantitation. The method is simpler, though less sensitive than instrumental methods (6–8).

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Improved Storherr Tube for Assisted and Sweep Co-Distillation Cleanup of Pesticides, Polychlorinated Biphenyls, and Pentachlorophenol from Animal Fats

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A procedure is presented for the simultaneous extraction from animal fat and quantitation of 15 pesticide residues, 4 polychlorinated biphenyl (PCB) formulations, and pentachlorophenol (PCP). Silanization of the Storherr tube packing reduces thermal decomposition and allows for residue recoveries in excess of 88% from pork, beef, chicken, sheep, and rabbit fat. This Storherr tube performs well in either the assisted distillation or sweep co-distillation mode of operation. Detection limits of 2–5 ppb for the pesticides and 10 ppb for the PCBs are readily attained.

Storherr et al. (1) described a technique for the sweep co-distillation cleanup of fat samples for pesticide residue analyses. Since the publication of their work, various modifications to the technique have been reported. The effect of distillation tube diameter (2, 3), nitrogen flow rate (3, 4), method and rate of solvent introduction (3), and condenser efficiency (1, 3) on the recovery of pesticide residues have been studied. The development of the assisted distillation technique by Heath and Black (3) simplified the procedure and reduced analysis time. By combining condensation and cleanup into a single step, McDougall (5) further reduced analysis time.

Our experience with both the assisted and sweep co-distillation versions of the technique indicated that the excellent recoveries achieved at the published fortification levels were unattainable at significantly lower fortification levels. Terminal pesticide residues in Canadian livestock rarely match these higher levels (6), and serious problems have been encountered in the application of these techniques to routine residue monitoring. In addition, the problem of on-column decomposition of some pesticides (1, 3) during the cleanup has limited the acceptance of these techniques by workers in the multiple-residue field. This report describes a treatment of the Storherr tube packing which allows for improved pesticide recovery at these lower levels. The problems of on-column thermal decomposition are virtually eliminated with the modified Storherr tube, and a wider range of pesticides and PCBs (polychlorinated biphenyls) can be recovered than indicated in previous reports.

Experimental

Apparatus

(a) *Sweep co-distillation apparatus.*—Kontes Glass Co., Catalog No. K-500750.

(b) *Gas chromatograph.*—Hewlett-Packard Model 5830A or equivalent, equipped with electron capture detector.

(c) *Distillation tubes.*—Kontes Glass Co., Catalog No. K-898650-0020.

(d) *Glass beads.*—3 mm diameter, Pyrex.

(e) *Sand.*—20–30 mesh, Ottawa sand standard, Fisher Scientific Co.

(f) *Silanized glass wool.*—Chromatographic Specialties Co., Brockville, Ontario, Canada K6V 5W1.

(g) *Collection tube.*—Kontes Glass Co., Catalog No. K-500750-0354.

(h) *Chromatographic column.*—Pasteur pipet with 6 cm 60–100 mesh Florisil®, pesticide residue grade, contained between 2 silanized glass wool plugs.

(i) *Pesticide gas chromatography column.*—2 m × 2 mm id glass column packed with equal parts of 15% QF-1 on 80–100 mesh Gas-Chrom Q and 10% DC-200 on 80–100 mesh Gas-Chrom Q.

(j) *Pentachloroanisole gas chromatography column.*—2 m × 2 mm id glass column packed with 5% OV-7 on 80–100 mesh Gas-Chrom Q.

(k) *Solvent pump.*—Pye Unicam Model LC3 chromatograph, or equivalent.

(l) *Solvent injector.*—19 gauge stainless steel tube connected with Teflon tubing to solvent pump.

(m) *Condenser pipet.*—Pasteur pipet with 4 cm of 80–100 mesh Anakrom ABS contained between 2 silanized glass wool plugs.

Reagents

(a) *Solvents.*—Hexane, heptane, acetone (distilled-in-glass grade, Caledon Laboratories, Georgetown, Ontario, Canada L7G 4R9).

(b) *Ethyl ether.*—Preserved with 2% ethanol, distilled-in-glass grade.

(c) *Siliconizing fluid.*—1% solution of AquaSil® in water (AquaSil is available as 20% solution from Chromatographic Specialties).

(d) *Pesticide primary standards.*—Min. 98% purity.

(e) *Polychlorinated biphenyl standards.*—Analabs Inc., 80 Republic Dr, North Haven, CT.

(f) *Pentachlorophenol standard.*—97.8% purity, Chem Service Inc., West Chester, PA.

(g) *Diazomethane/ether solution.*—Prepared by distillation of diazomethane (Caution: carcinogen.) from decomposition of 0.8 g Diazald® into 25 mL cold ethyl ether.

Preparation of Storherr Tubes

Soak distillation tubes, glass beads, and Ottawa sand in separate containers of hot (80°C) nitric acid (Caution: toxic fumes.). Stir beads and sand periodically during 8 h soaking time. Decant and replenish hot acid from sand at 2 h intervals. Filter sand and glass beads from nitric acid, using large vacuum-assisted fritted funnel. Successively rinse with water, acetone, and hexane. Rinse distillation tubes in the same manner.

Silanize inner wall of distillation tube, glass beads, and sand with 1% solution of AquaSil. Expose the sand and glass beads to 2 volumes of siliconizing fluid for 0.5 h. Filter through large fritted funnel to remove siliconizing fluid. Apply slight vacuum and rinse thoroughly with methanol, acetone, and hexane. Air-dry tubes and packing materials for several hours at room temperature.

Push silanized glass wool plug through injection port and into outlet end of distillation tube. Add silanized glass beads

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to depth of 24 cm. Push second silanized glass wool plug onto glass beads. Add silanized Ottawa sand to depth of 2.5 cm. Fill remainder of distillation tube (about 1.5 cm) with silanized glass wool. Place septum in injection port of tube. Use this Storherr tube in the following procedure.

Sample Preparation

Sample preparation is identical to techniques of Heath and Black (3).

Sweep Co-Distillation

Technique employed is similar to Storherr method (1) for chlorinated pesticides. Prepare Storherr tube, Florisil tube, and Anakrom condenser pipet as described above. Inject clear melted fat (1.00 g maximum) into Ottawa sand. Use depth guide on syringe to assure that point of injection is 1 cm below top of sand layer. Position solvent injector tip 1 cm below point of injection of fat. Immediately start injection of hexane at a rate of 1.0 mL/min. Continue sweep co-distillation at 247 ± 3°C and nitrogen flow of 600 mL/min for 30 min. Rinse condenser tubing and pipet as described by Storherr (1) for organophosphate pesticides. Evaporate eluate just to dryness. Add 0.5 mL hexane and transfer solution to Florisil tubes. Elute Florisil tubes with 15 mL of 2.5% ether/hexane (fraction 1), followed by 15 mL of 60% ether/hexane (fraction 2), followed by 10 mL acetone (fraction 3). Collect each fraction in separate 20 mL tubes. Evaporate fraction 1 to dryness, add 1.00 mL hexane, and inject appropriate aliquot into pesticide and PCB channel of gas chromatograph. Discard fraction 2. Evaporate fraction 3 just to dryness and methylate with diazomethane/ether solution. Add 10 μL glacial acetic acid to destroy excess diazomethane and adjust to final volume of 4.00 mL with hexane. Inject appropriate aliquot into PCP channel of gas chromatograph.

For pesticide and PCB analysis, gas chromatograph operating conditions are as follows: oven temperature, 220°C; injection port, 235°C; detector, 300°C; 5% methane/argon carrier gas, 30 mL/min.

For pentachloroanisole analysis, gas chromatograph operating conditions are as follows: oven temperature, 180°C; injection port, 200°C; detector, 300°C; 5% methane/argon carrier gas, 30 mL/min.

Assisted Distillation

This technique is similar to McDougall's modification (5) of Heath and Black's method (3). Prepare Storherr tube and Florisil column as described above. Attach a transfer line consisting of 60 mm length of collection tubing (Kontes K-500750-0354) between end of Storherr tube and Florisil column. Maintain distillation temperature at 235°C and nitrogen gas flow rate at 600 mL/min. After 30 min distillation, disconnect transfer line from Storherr tube and rinse line with two 50 μL portions of hexane onto Florisil column. Elute Florisil column as described above.

Calculations

All recoveries were determined on samples fortified with individual standards. Peak area was used to measure detector response. Individual standard curves appropriate to the fortification levels were used to determine recovery of the pesticides. Polychlorinated biphenyls were determined on the basis of total peak areas of all isomers.

Results and Discussion

Preliminary investigations of these distillations revealed that use of the injection method of Storherr et al. (1) results in the most consistent recoveries. It was not possible to achieve consistent recoveries with either technique if the injection of fat was made into a glass wool plug rather than sand. Replicate injections of aldrin resulted in uncorrected recoveries of 102, 101, and 100% for injections into silanized sand with assisted distillation cleanup. However, the same sample when injected into a Storherr tube in which the sand was replaced with silanized glass wool resulted in recoveries of 94, 100, and 80%. With the glass wool, fat formed pools which remained essentially undisturbed by the flow of carrier gas through the column. Such dead spaces did not exist when sand was used in place of glass wool. It appears that the essential advantage of the sand is the better dispersion of the carrier gas over the entire cross sectional area of the distillation tube. This advantage is lost if untreated sand is used because many of the pesticides then undergo some thermal decomposition.

Heath and Black (7) introduced significant improvements to the original technique by an acid washing of the distillation tube, and obtained reasonable results for a limited number of pesticides. However, the recovery of *p,p'*-DDT remained low. Some authors have suggested that an active surface catalyzes the conversion of *p,p'*-DDT to *p,p'*-TDE (8). Others (1, 3) have attributed the interconversion of *p,p'*-DDT and its metabolites to a property of the fat or oil in which they occur. A variation of *p,p'*-DDT recovery due to species differences was not observed when data obtained for pork, beef, chicken, sheep, and rabbits were compared. However, decomposition from 7 to 15% *p,p'*-DDT occurred in all cases when untreated Storherr tube packings were used. *p,p'*-DDT was partially converted to *p,p'*-DDE in all cases. Table 1 shows that the silanized Storherr tube allows the quantitative recovery of *p,p'*-DDT from animal fats with either the assisted or sweep co-distillation technique.

The improved recovery of *p,p'*-DDT from the silanized Storherr tube was not related to the faster elution of the pesticide from these tubes. Experimental evidence clearly indicated that decomposition of *p,p'*-DDT was not a function of time in the heated zone. Instead it appeared to be the result of a rapid degradation of *p,p'*-DDT to *p,p'*-DDE in the hot distillation tube. The ratio of *p,p'*-DDE/*p,p'*-DDT was the same in the fraction collected from 0 to 10 min as in the fraction collected from 10 to 20 min after injection into the Storherr tube. If *p,p'*-DDT decomposition were a function of time, a relatively larger amount of *p,p'*-DDE would be in the fraction collected over the second 10 min interval. Silanization prevented the breakdown of *p,p'*-DDT to *p,p'*-DDE. The conversions of *p,p'*-DDT to *p,p'*-TDE (1, 8) and *p,p'*-TDE to *p,p'*-DDE (3) were not observed in any of the animal fats studied.

Another example of the very significant improvement in recovery from the silanized column can be seen in the case of PCP. PCP was eluted from the Florisil column in fraction 3. It was totally separated from the pesticides and polychlorinated biphenyls which were in fraction 1 and the co-distilling lipids which were discarded in fraction 2. Table 1 indicates that the silanized Storherr tube allows for a very high recovery of PCP in either assisted or sweep co-distillation mode. However, the untreated Storherr tube resulted in a quantitative decomposition. A simple silanization of the Storherr tube materials without initial acid wash resulted in a recovery of 26% PCP. In addition, a nitric acid wash without subsequent silanization resulted in a recovery of 82% PCP. The nitric acid wash alone has a major effect on PCP recovery. However, for quantitative recovery, subsequent silanization is also necessary. A mere nitric acid wash had very little effect on the recovery of the other residues in this report.

Table 1. Residue recoveries from fortified chicken fat

Pesticide	Added, mg/kg	% Recovery[a]			
		Assisted distn		Sweep co-distn	
		A[b]	B[c]	A[b]	B[c]
Lindane	0.020	95 ± 1	90 ± 2	100 ± 1	85 ± 3
Heptachlor	0.030	90 ± 2	78 ± 3	92 ± 2	86 ± 2
Aldrin	0.030	101 ± 1	100 ± 1	101 ± 1	100 ± 1
Heptachlor epoxide	0.050	94 ± 2	90 ± 2	102 ± 1	93 ± 1
p,p'-DDE	0.060	100 ± 1	96 ± 2	101 ± 1	100 ± 1
p,p'-TDE	0.100	101 ± 0	94 ± 1	99 ± 1	94 ± 2
p,p'-DDT	0.100	101 ± 1	89 ± 2[d]	97 ± 1	85 ± 2[e]
Dieldrin	0.065	101 ± 1	90 ± 2	100 ± 1	100 ± 1
Fenchlorophos	0.050	93 ± 3	78 ± 4	98 ± 2	81 ± 2
Chlorpyriphos	0.075	100 ± 2	85 ± 4	100 ± 1	56 ± 4
Ethion	0.200	93 ± 2	80 ± 3	101 ± 1	57 ± 4
Methoxychlor	0.300	88 ± 2	87 ± 3	100 ± 1	74 ± 4
Chlordane	0.100	90 ± 2	—	95 ± 1	—
Endosulfan I	0.060	95 ± 2	—	92 ± 2	—
Endosulfan II	0.060	75 ± 3	—	90 ± 3	—
Pentachlorophenol	0.025	100 ± 1	0	96 ± 2	0
PCB Aroclor 1248	0.100	100 ± 2	—	99 ± 2	—
Aroclor 1254	0.100	99 ± 2	90 ± 2	99 ± 2	83 ± 3
Aroclor 1260	0.100	99 ± 2	—	98 ± 3	—
Aroclor 1268	0.100	97 ± 2	—	98 ± 3	—

[a]Mean ± standard deviation for 3 replicates.
[b]With silanized Storherr tube packing.
[c]With identical Storherr tube containing untreated packings.
[d]Plus 15% *p,p'*-DDE.
[e]Plus 11% *p,p'*-DDE.

Other types of silanizing reagents proved to be less effective. Trimethylsilane adducts afforded no net improvement over untreated tubes when measured in terms of pesticide recovery.

The Storherr columns can be re-used up to 10 times without significant deterioration if a simple hexane backwash to remove the fat is employed after each analysis. This is effective for all residues except PCP. If PCP is to be analyzed, the sand must be replaced after every sample injection and the glass beads must be changed after every 5 injections or as soon as any discoloration is noted. Liver and muscle extracts can be analyzed using the sweep co-distillation mode; however, the Storherr tube must be totally repacked after each injection.

While there was a moderate increase in background observed on the gas chromatographic tracings, the major problem with deteriorating Storherr columns was reduced recovery of the pesticides. With liver and muscle extracts, this reduction in recovery was sufficient after one injection to preclude re-use of the same columns. Aside from PCP which is discussed above, the pesticide which underwent most rapid loss of recovery was *p,p'*-DDT. Storherr tubes were considered suitable for further use as long as the recovery of *p,p'*-DDT remained greater than 80%. In the worst case, this level of deterioration was not observed until after the tenth injection of fat samples.

The preliminary work on this procedure made use of Florisil which was activated 3 h at 130°C. Recently, we have noticed variations in the quality of the results obtained with Florisil from different lots. The limit of detection was adversely affected because background noise attributable to the Florisil was observed in the gas chromatograms. Bulk pre-washing of the Florisil with pesticide grade ethyl ether before activation is usually sufficient to remove these interfering compounds.

A detection limit of <0.005 mg/kg is readily achieved for the pesticides and PCP in any of the species tested. For PCB residues, the detection limit is <0.010 mg/kg in these species.

Conclusions

The merits of these techniques to the residue chemist have been described by several authors (1, 3, 5). The method described in this paper allows the cleanup of a large number of residues by using commercially available equipment. This investigation shows that decomposition during cleanup need not be a problem for many pesticides and pollutants. Table 1 shows that sweep co-distillation offers better analytical results; however, the inherent simplicity of assisted distillation represents a very useful alternative.

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Interim Method for Determination of Volatile Organic Compounds in Hazardous Wastes

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An analytical protocol is presented for the determination of volatile organic compounds in hazardous wastes that are amenable to the purge-and-trap method. The protocol features a tetraethylene glycol dimethyl ether (tetraglyme) extraction of the liquid or solid waste, addition of an aliquot of the tetraglyme waste to water, and purging of the tetraglyme–water mixture with subsequent gas chromatographic/mass spectrometric (GC/MS) analysis. The size of the tetraglyme aliquot is determined by gas chromatographic screening of a hexadecane waste extract. Quality assurance/quality control procedures are included within the analytical protocol. Spike extraction recoveries of industrial wastes obtained by following this protocol are given.

The Resource Conservation and Recovery Act of 1976 (RCRA) and its amendments require the U.S. Environmental Protection Agency (EPA) to regulate hazardous waste activities (1–3). Implementation and enforcement of the RCRA requires reliable monitoring tools which must provide the capability for identification and delineation of waste sites, characterization of waste composition and waste sites, and detection of environmental contamination resulting from hazardous waste operations. Of special concern to this regulatory effort is the development and testing of analytical procedures for the identification and quantification of hazardous organic compounds in industrial wastes. These analytical procedures must be applicable to a variety of organic compounds in diverse waste types. The waste may occur as a solid, semisolid, liquid, or multi-phase form. Organic compounds may be incorporated within the waste at a wide range of concentrations. These analytical problems pose a severe challenge to current analytical methodology.

With these analytical constraints in mind, we developed a protocol which is applicable to a wide range of sample matrices, applicable to a wide range of purgeable compounds, capable of detecting compounds in the range of 1 ppm to 100%, and easily and economically performed. The protocol has been designed to work on aqueous sludges, dry solids, soils, sediments, tars, oil, and oily sludges. Organic compounds which may be determined by this method include purgeable halocarbons, hydrocarbons, ketones, ethers, esters, sulfides, and nitriles having boiling points in the range of 30–150°C.

The main features of the method are solvent extraction with tetraglyme, a nonvolatile, water-miscible and oil-miscible solvent; dilution of the extract with solvent if necessary; and purge-and-trap analysis of an extract aliquot by the method of Bellar and Lichtenberg (4, 5). The tetraglyme extract dilution factor is determined by gas chromatographic (GC) screening a hexadecane sample extract.

The protocol covers the determination of volatile organic compounds in a variety of solid waste matrices and may be applied to nearly all types of samples, regardless of water content, including aqueous sludges, caustic liquors, acid liquors, waste solvents, oily wastes, mousses, tars, fibrous waste, polymeric emulsions, filter cakes, spent carbons, spent catalysts, soils, and sediments. The detection limit of the method for determining an individual compound is estimated to be approximately 0.5–5 μg/g (wet weight), but for samples which contain more than 1 mg/g of total volatile material, the detection limit should be proportionately higher. The method is based on a purge-and-trap, gas chromatographic/mass spectrometric (GC/MS) procedure and should be restricted to use by, or under the supervision of analysts experienced in the use of purge-and-trap systems and gas chromatography/mass spectrometry and skilled in the interpretation of mass spectra.

A brief summary of the method follows: A portion of solid waste is dispersed in tetraglyme to dissolve the volatile organic constituents. A portion of the tetraglyme solution is combined with water in a specially designed purging chamber. An inert gas is bubbled through the solution at ambient temperature and the volatile components are efficiently transferred from the aqueous phase to the vapor phase. The vapor is swept through a sorbent column where volatile components are trapped. After purging is completed, the sorbent column is heated and backflushed with inert gas to desorb the components onto a gas chromatographic column. The gas chromatographic column is heated to elute the components which are then detected with a mass spectrometer (4–9). Low molecular weight impurities in tetraglyme can be volatilized during the purging procedure. Thus, the tetraglyme employed in this method must be purified before use and stabilized to prevent peroxide formation as described in the *Experimental* section. Impurities in the purge gas and organic compounds out-gassing from the plumbing ahead of the trap account for the majority of contamination problems. The analytical system must be demonstrated to be free from contamination under the described analytical conditions by running laboratory reagent blanks. The use of non-TFE plastic tubing, non-TFE thread sealants, or flow controllers with rubber components in the purging device should be avoided. Samples can be contaminated by diffusion of volatile organic compounds (particularly fluorocarbons and methylene chloride) through the septum seal into the sample during shipment and storage. A field reagent blank prepared from reagent water and carried through the sampling and handling protocol can serve as a check on such contamination. Contamination by carry-over can occur whenever high-level and low-level samples are analyzed sequentially. Whenever an unusually concentrated sample is encountered, it should be followed by an analysis of reagent water to check for cross-contamination. After each use, the purging chamber is cleaned as described in the purge-and-trap procedure. The trap and other parts of the system are also subject to contamination; therefore, frequent additional heating and purging of the entire system may be required.

The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be

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Table 1. Recovery of spiked compounds from wastes

Waste matrix	Recovery, %[a,b]		
	Fluorobenzene	1,2-Dibromotetra-fluoroethane	Bis(perfluoro-isopropyl) ketone
Soil	84 (1)	78 (9)	73 (12)
Treated POTW sludge	86 (2)	82 (1)	77 (3)
Oxychlorination catalyst	84 (4)	53 (4)	64 (2)
Acetone/water	90 (7)	83 (17)	94 (8)
Coal gasification tar	88 (3)	80 (11)	90 (7)
Latex paint	104 (9)	86 (12)	99 (4)
Chlorinated solvent	118 (9)	100 (1)	115 (4)

[a]Spiked at level to give 250 ng in purge apparatus.
[b]Average of triplicate extractions; percent relative standard deviation is given in parentheses.

minimized by whatever means available; this warning is particularly important when reference compounds are handled. The laboratory is responsible for maintaining a current awareness file of OSHA (U.S. Occupational Safety and Health Administration) regulations regarding the safe handling of the chemicals used in applying this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified for the information of the analyst (10–12).

The following volatile organic compounds that may be determined by this method have been tentatively classified as known or suspected human or mammalian carcinogens: benzene, carbon tetrachloride, chloroform, and vinyl chloride. Whenever primary standards containing any of these or other toxic compounds are prepared, the operation should be performed in a hood. A toxic gas respirator approved by the U.S. National Institute of Occupational Safety and Health Administration should be worn when the analyst handles high concentrations of these toxic compounds.

Experimental

Apparatus

(a) *Sampling equipment (for discrete sampling).*—Vials of 25 mL capacity or larger, equipped with screw cap (Pierce 13075 or equivalent) should be used. Detergent wash, rinse with tap and distilled water, and dry 1 h at 105°C before use. Seal vials with Teflon-faced silicone septums (Pierce 12722 or equivalent). Detergent wash, rinse with tap and distilled water, and dry 1 h at 105°C before use.

(b) *Purge-and-trap device.*—Consists of 3 separate pieces of equipment: purging chamber, trap, and desorber. Several complete devices are now commercially available. Purging chamber must be designed to accept 5 or 25 mL samples with a water column at least 3 cm deep. Gaseous head space between water column and trap must have a total volume < 15 mL. Purge gas must pass through water column as finely divided bubbles and must be introduced no more than 5 mm from base of water column. Purging chamber, illustrated in Figure 1 of reference 5, meets these design criteria. The trap must be ≥ 25 cm long with inside diameter ≥ 2.5 mm. Trap must be packed to contain following minimum amounts of adsorbents: 1.0 cm methyl silicone-coated packing and 15 cm silica gel. Minimum specifications for trap are illustrated in Figure 2 of reference 5. Desorber must be capable of rapidly heating trap to 180°C within 30 s. Polymer section of trap should not be heated higher than 180°C and remaining sections should not exceed 220°C. Desorber design, illustrated in Figure 2 of reference 5, meets these criteria. Purge-and-trap device may be assembled as a separate unit or be coupled to a gas chromatograph as illustrated in Figures 3 and 4 in reference 5.

Gas Chromatograph/Mass Spectrometer System

(a) *Gas chromatograph.*—Analytical system complete with temperature-programmable gas chromatograph and all required accessories including syringes, analytical columns, and gases. GC column should be 2 m × 2 mm id stainless steel or glass and packed with 1% SP-1000 on 60–80 mesh Carbopack B or equivalent.

(b) *Mass spectrometer.*—Capable of scanning from 40 to 250 amu every 7 s or less, utilizing 70 V (nominal) electron energy in electron impact ionization mode, and producing mass spectrum which meets all criteria in Table 1 when 50 ng 4-bromofluorobenzene (BFB) is injected through GC inlet.

(c) *GC/MS interface.*—Any GC-to-MS interface that gives acceptable calibration points at 50 ng per injection for each compound of interest and achieves acceptable tuning performance criteria (see *Daily GC/MS Performance Tests*) may be used. GC-to-MS interfaces constructed of all-glass or glass-lined materials are recommended. Glass can be deactivated by silanizing with dichlorodimethylsilane.

(d) *Data system.*—Computer system interfaced to mass spectrometer must allow continuous acquisition and storage on machine-readable media of all mass spectra obtained throughout duration of chromatographic program. Computer must have software that allows searching any GC/MS data file for ions of specific mass and plotting such ion abundances vs time or scan number. This type of plot is defined as extracted ion current profile (EICP). Software must also be available that allows integrating abundance in any EICP between specified time or scan number limits.

(e) *Sample transfer implements.*—Implements are required to transfer portions of solid, semisolid, and liquid wastes from sample containers to laboratory glassware. Transfer must be accomplished rapidly to avoid loss of volatile components during transfer step. Liquids may be transferred using hypodermic syringe with wide-bore needle or no needle attached. Solids may be transferred using conventional laboratory spatula, spoon, or coring device. Coring device that is suitable for handling some samples can be made by using glass tubing saw to cut away closed end of barrel of glass hypodermic syringe.

(f) *Syringes.*—5 mL and 25 mL gas-tight with shut-off valve, equipped with narrow-bore needle, ≥ 15 cm long.

(g) *Microsyringes.*—10 μL, 25 μL, 100 μL, 250 μL, 500 μL, and 1000 μL. These syringes should be equipped with narrow-bore needles long enough to extend from sample inlet to within 1 cm of glass frit in purging device (see Figure 1 of reference 5). Needle length required will depend on dimensions of purging device.

(h) *Centrifuge tubes.*—50 mL round bottom glass centrifuge tubes with Teflon-lined screw caps. Tubes must be marked before use to show approximate 20 mL graduation (Kimble

No. 45212 or equivalent). Centrifuge should be capable of accommodating 50 mL glass tubes.

(i) *Bottle.*—15 mL screw-cap, Teflon cap liner.

(j) *Balance.*—Analytical, capable of accurately weighing 0.0001 g.

(k) *Rotary evaporator.*—Equipped with Teflon-coated seals (Buchi Rotavapor R-100, or equivalent).

(l) *Vacuum pump.*—Mechanical, 2-stage.

(m) *Sonifier.*—375-watt, fitted with ½ in. probe and half-wave extension, capable of pulsed operation at variable power settings (Heat Systems–Ultrasonics Sonicator Model W-375 with No. 200 ½ in. disruptor horn and 406-HW-050-T half-wave extender, or equivalent).

Reagents

(a) *Reagent water.*—Water in which no interference is observed at method detection limit for compounds of interest. Reagent water may be generated by passing tap water through carbon filter bed containing ca 500 g activated carbon (Calgon Corp., Filtrasorb-300, or equivalent). Water purification system (Millipore Super-Q or equivalent) may be used to generate reagent water. Reagent water may also be prepared by boiling water 15 min. Subsequently, while maintaining temperature at 90°C, bubble contaminant-free inert gas through water 1 h. While still hot, transfer water to narrow-mouth screw-cap bottle and seal with Teflon-lined septum and cap.

(b) *Reagent tetraglyme.*—Tetraglyme in which no interference is observed at method detection limit for compounds of interest. Purify tetraglyme (tetraethylene glycol dimethyl ether, Aldrich 17, 240-5 or equivalent) by treatment at reduced pressure in rotary evaporator. Tetraglyme should have peroxide content < 5 ppm as indicated by EM Quant Test Strips (available from Scientific Products Co., Catalog No. P1126-8, and other suppliers). Remove peroxides by passing tetraglyme through column of activated alumina. Place tetraglyme in round-bottom flask equipped with standard taper joint, and affix flask to rotary evaporator. Immerse flask in water bath at 90–100°C and maintain vacuum at <10 mm Hg ≥ 2 h, using 2-stage mechanical pump. Equip vacuum system with all-glass trap, which is maintained in dry ice/methanol bath. Cool tetraglyme to ambient temperature and add 0.1 mg/mL of 2,6-di-tert-butyl-4-methylphenol to prevent peroxide formation. Store tetraglyme in tightly sealed screw-cap bottle in area free of solvent vapors. Demonstrate that all interfering volatiles have been removed from tetraglyme by analyzing reagent water/tetraglyme blank.

Trap Materials

(a) *2,6-Diphenylene oxide polymer.*—60–80 mesh Tenax, chromatographic grade or equivalent.

(b) *Methyl silicone packing.*—3% OV-1 on 60–80 mesh Chromosorb W or equivalent.

(c) *Silica gel.*—Davison Chemical (35–60 mesh), grade-15 or equivalent.

Standards

(a) *Calibration standards, stock solutions.*—Prepare stock solutions of calibration standards from pure standard materials or purchase as certified solutions. Prepare stock standard solutions of individual compounds in tetraglyme, using assayed liquids or gases as appropriate.

(b) *Liquids.*—Using 100 μL syringe, immediately add 2 drops of assayed reference material to flask, then reweigh. Liquid must fall directly into tetraglyme without contacting neck of flask.

(c) *Gases.*—To prepare standards for any compounds that boil below 30°C (e.g., bromomethane, chloroethane, chloromethane, or vinyl chloride), fill 5 mL valved gas-tight syringe with a reference standard to 5.0 mL mark. Lower needle to 5 mm above tetraglyme meniscus. Slowly introduce reference standard above surface of liquid. The heavy gas rapidly dissolves in tetraglyme. Reweigh, dilute to volume, stopper, then mix by inverting flask several times. Calculate concentration in μg/μL from net gain in weight. When compound purity is assayed to be ≥ 96%, weight may be used without correction to calculate concentration of stock standard. Commercially prepared stock standards in methanol may be used at any concentration if they are certified by manufacturer or independent source. Transfer stock standard solution to Teflon-sealed screw-cap bottle. Store, with minimal headspace, at −10 to −20°C and protect from light. Prepare fresh standards weekly for gases or for reactive compounds such as 2-chloroethylvinyl ether. All other standards must be replaced after 1 month, or sooner if comparison with check standards indicates a problem.

(d) *Calibration standards, secondary dilution solutions.*—From stock solutions, prepare secondary dilution standards in tetraglyme that contain compounds of interest, either singly or mixed. Secondary dilution standards should be prepared at concentrations such that aqueous calibration solutions prepared as described in *Calibration* section will bracket working range of analytical system (9). Secondary dilution standards should be stored with minimal headspace.

(e) *Surrogate standards.*—Add surrogate standards to all samples and calibration solutions; use 1,2-dibromotetrafluoroethane, bis(perfluoroisopropyl) ketone, fluorobenzene, and *m*-bromobenzotrifluoride added to each sample to assess effect of sample matrix on recovery efficiency. Prepare tetraglyme solutions of surrogate standards, using procedures described herein. Prepare concentrations and add amount of solution to each sample as required to give amount of each surrogate in purging device equal to amount of each internal standard added, assuming 100% recovery of surrogate standards.

(f) *Internal standards.*—In this method, internal standards are used during analysis of all samples and during all calibration procedures. Analyst must select one or more internal standards similar in analytical behavior to compounds of interest. Analyst must further demonstrate that measurement of internal standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples. However, for general use, D_4-1,2-dichloroethane, D_6-benzene, and D_5-ethylbenzene are recommended as internal standards covering a wide boiling point range. Add 4-bromofluorobenzene (BFB) to internal standard solution to permit mass spectrometer tuning for each GC/MS run to be checked. Using procedures described in *Calibration standards and Surrogate standards* sections, prepare tetraglyme solution containing BFB and each internal standard at 20.0 μg/mL.

(g) *Sodium monohydrogen phosphate.*—2.0M in water.

(h) *n-Nonane and n-dodecane.*—98 + % purity.

(i) *n-Hexadecane.*—Distilled-in-glass (Burdick and Jackson, or equivalent).

Calibration

Assemble purge-and-trap device that meets specifications in *Purge-and-trap* section and connect device to GC/MS system. Condition trap overnight at 180°C by backflushing with inert gas flow ≥ 20 mL/min. Before use, condition trap daily for 10 min while backflushing at 180°C. Operate gas chromatograph using conditions described in *Gas chromatograph*

section and operate mass spectrometer using conditions described in *Daily GC/MS Performance Test section.*

Conduct calibration procedures using minimum of 3 concentration levels for each calibration standard. One concentration level should be 2–5 times method detection limit (5–50 ng); one should be at a level near but below concentration that causes saturation of mass spectrometer; and the third should be at a level in middle of this working range of GC/MS system. Prepare solution containing required concentrations of calibration standards, including surrogate standards, to 5 mL reagent water contained in 5 mL gas-tight syringe having shut-off valve and fitted with 15 cm narrow-bore needle. Add volume of secondary dilution solution containing appropriate concentrations of calibration standards to reagent water, using microsyringe. When discharging contents of microsyringe, be sure that end of syringe needle is well beneath surface of water. Similarly, add 12.5 μL internal standard solution. Add contents of 5 mL syringe to purging device by inserting needle as far as possible through septum of purging device and discharging contents slowly.

Conduct purge-and-trap analysis procedure and tabulate area response of primary characteristic ion against concentration for each compound, including internal standards. Calculate a response factor (RF) for each compound, using Equation 1:

$$RF = (A_s C_{is})/(A_{is} C_s) \quad (1)$$

where A_s = area of primary characteristic ion for compound to be measured; A_{is} = area of primary characteristic ion of internal standard; C_{is} = concentration of internal standard; and C_s = concentration of compound to be measured.

Internal standard selected for calculation of RF of compound and subsequent quantitation of compound is generally internal standard that has retention time closest to that of compound. It is assumed that linear calibration plot will be obtained over range of concentrations used. If RF value over working range is constant (<10% RSD), RF can be assumed to be invariant, and average RF can be used for calculations. Alternatively, results can be used to plot calibration curve of response ratios, A_s/A_{is}, vs RF.

RF must be verified on each working day by measurement of 2 or more calibration standards, including one at beginning of day and one at end of day. Concentrations selected should be near midpoint of working range. Response factors obtained for calibration standards, analyzed immediately before and after a set of samples, must be within ±20% of response factor used for quantitation of sample concentrations.

Quality Control

Each laboratory that uses this method should operate a formal quality control program. Minimum requirements of this program consist of initial demonstration of laboratory capability and analysis of spiked samples as continuing check on performance. Laboratory is required to maintain performance records to define quality of data generated. Ongoing performance checks must be compared with established performance criteria to determine if results of analyses are within accuracy and precision limits expected of method. Before performing any analyses, analyst must demonstrate ability to generate acceptable accuracy and precision with this method. This ability is established as described in this section. Laboratory must spike all samples including check samples with surrogate standards to continuously monitor laboratory performance. To establish ability to generate acceptable accuracy and precision, analyst must perform the following operations, using representative sample as check sample: Analyze 4 aliquots of unspiked check sample according to method in *Sample Extraction and Analysis* section. For each compound to be measured, select spike concentration representative of twice the level found in unspiked check sample or level equal to 10 times expected detection limit, whichever is greater. Prepare spiking solution by dissolving compounds in tetraglyme at appropriate levels. Spike minimum of 4 aliquots of check sample with spiking solution to achieve selected spike concentrations. Spike samples by adding spiking solution to tetraglyme used for extraction, and analyze.

Calculate average percent recovery, *(R)*, and standard deviation of percent recovery, *(s)*, for all compounds and surrogate standards. Background corrections must be made before R and s are calculated. Average percent recovery must be > 20 for all compounds to be measured and > 60 for all surrogate compounds. Percent relative standard deviation of percent recovery ($s/R \times 100$), must be < 20 for all compounds to be measured and all surrogate compounds.

The analyst must calculate method performance criteria for each surrogate standard. Calculate upper and lower control limits for method performance for each surrogate standard, using values for R and s calculated by the following equations:

$$\text{Upper control limit (UCL)} = R + 3s$$
$$\text{Lower control limit (LCL)} = R - 3s$$

UCL and LCL can be used to construct control charts that are useful in observing trends in performance. For each surrogate standard, laboratory must maintain record of R and s values obtained for each surrogate standard in each waste sample analyzed. An accuracy statement should be prepared from these data and updated regularly.

Laboratory is required to spike all samples with surrogate standards to monitor spike recoveries. Spiking levels used should be that which will give an amount in purge apparatus equal to amount of internal standard, assuming 100% recovery of surrogate standards. For unknown samples, spiking level is determined by performing extraction and estimating major volatile compounds content. Then, amount of extract to be analyzed is determined by calculating MVCC. If recovery for any surrogate standard does not fall within control limits for method performance, results reported for that sample must be qualified as being outside of control limits. Laboratory must monitor frequency of data so qualified to ensure that frequency remains at or below 5%. Four surrogate standards, namely, 1,2-dibromodifluorethane, bis(perfluoroisopropyl) ketone, fluorobenzene, and *m*-bromobenzotrifluoride, are recommended for general use to monitor recovery of volatile compounds varying in volatility and polarity.

Each day, analyst must demonstrate through analysis of process blank that all glassware and reagent interferences are under control. It is recommended that laboratory adopt additional quality assurance practices for use with this method. Specific practices that are most productive depend on needs of laboratory and nature of samples. Field replicates may be analyzed to monitor precision of sampling technique. Whenever possible, laboratory should analyze standard reference materials and participate in relevant performance evaluation studies.

Sample Storage

All samples must be stored in Teflon-lined screw-cap vials. Sample containers should be filled as completely as possible

to obtain minimum headspace or void space. Vials containing liquid sample should be stored in inverted position. All samples must be refrigerated from time of collection to time of analysis, and should be protected from light.

Daily GC/MS Performance Tests

At beginning of each day that analyses are to be performed, GC/MS system must be checked to see that acceptable performance criteria are achieved for BFB (13). BFB performance test requires the following instrumental parameters: electron energy of 70 V (nominal), mass range of 40–250 amu, and scan time of ≥ 6 scans per peak but not to exceed 7 s per scan.

Bleed BFB vapor into mass spectrometer and tune instrument to achieve all key ion criteria for mass spectrum of BFB given in reference 13. BFB is included in internal standard solution added to all samples and calibration solutions. If any key ion abundance observed for BFB during analysis of sample differs > 20% from that observed during analysis of calibration solution, then analysis in question is considered invalid. Instrument must then be returned or sample and/or calibration solution must be reanalyzed until above condition is met.

Peak intensity of D_6-benzene is used to monitor mass spectrometer sensitivity. Peak intensity for D_6-benzene observed during each sample analysis must be between 0.7 and 1.4 times D_6-benzene peak intensity observed during applicable calibration runs. For example, if peak intensity of D_6-benzene observed during calibration was 170 000 area counts, then each subsequent sample or blank must give D_6-benzene peak intensity 120 000 and 240 000 area counts. If D_6-benzene peak intensity is outside of specified range, sample must be reanalyzed. If peak intensity is again outside specified range, analyst must investigate cause of variability in sensitivity and correct problem.

Sample Extraction and Analysis

Analytical procedure involves extracting sample with tetraethylene glycol dimethyl ether (tetraglyme) and analyzing portion of extract by purge-and-trap GC/MS procedure. Amount of extract to be taken for GC/MS analysis is based on estimated major volatile compounds content (MVCC) of sample. MVCC is estimated by extracting sample with *n*-hexadecane and analyzing *n*-hexadecane extract by gas chromatography. Estimate is based on area response, relative to that of *n*-nonane, for the 5 major components that elute before retention time of *n*-dodecane. Response factor and retention time of *n*-nonane are determined by analyzing 2 μL aliquot of *n*-hexadecane solution containing 100 μg/mL of *n*-nonane.

Conduct GC analyses using flame ionization detector and 3 m × 2 mm id glass column packed with 10% OV-101 on 100–120 mesh Chromosorb W-HP. Program column temperature from 80 to 280°C at 8°/min and hold at 280°C 10 min. Determine area response for *n*-nonane and divide by 100 to obtain area response factor. Record retention time of *n*-nonane.

Add 1.0 g sample to 20 mL *n*-hexadecane and 2 mL 0.5M Na_2HPO_4 contained in 50 mL glass centrifuge tube and cap securely with Teflon-lined screw cap. Shake mixture vigorously 1 min. If sample does not disperse during shaking process, sonify mixture 2 min by inserting sonifier horn 1–2 cm below surface of hexadecane and using power setting of 5 and a 25% pulsed duty cycle. Cap tube tightly and let mixture stand until clear supernate is obtained. Centrifuge if necessary to facilitate phase separation.

Analyze 2 μL aliquot of *n*-hexadecane supernate, using conditions described herein. Determine total area response for 5 major components that elute before retention time of *n*-nonane, and subtract any areas for the same 5 components that appear in *n*-hexadecane blank. Calculate MVCC by using Equation 2:

$$\text{MVCC} = [(\text{MVCAR}_{\text{sample}} - \text{MVCAR}_{\text{blank}})/ \text{ } n\text{-nonane area response factor}] \times 20 \quad (2)$$

where MVCC is major volatile compounds content of sample in μg/g; $\text{MVCAR}_{\text{sample}}$ is major volatile compounds area response obtained for sample; and $\text{MVCAR}_{\text{blank}}$ is major volatile compounds area response. Transfer aliquot of sample for extraction with tetraglyme as quickly as possible to minimize loss of volatiles from sample.

To 50 mL glass centrifuge tube with Teflon-lined cap, add 40 mL reagent tetraglyme. Add appropriate volume of surrogate standard solution; insert needle of syringe ca 4 cm below surface of tetraglyme during addition. Weigh capped centrifuge tube and tetraglyme on analytical balance.

Using appropriate implement, transfer ca 2 g sample to tetraglyme in centrifuge tube in such a fashion that sample is dissolved in or submerged in tetraglyme as quickly as possible. Take care not to touch sample-transfer implement to tetraglyme. Recap centrifuge tube immediately and weigh on analytical balance to determine accurate sample weight.

Disperse sample by vigorous agitation for 1 min. Mixture may be agitated manually or with the aid of vortex-mixer. If sample does not disperse during this process, sonify mixture for 2 min by inserting sonifier horn 1–2 cm below surface of tetraglyme and using power setting of 5 and 25% pulsed duty cycle. Cap tube tightly and let mixture stand until clear supernate is obtained as sample extract. Centrifuge if necessary to facilitate phase separation. Sample extract may be stored for future analytical needs. If storage is desired, transfer solution to 10 mL screw cap vial with Teflon cap liner. Store at −10 to −20°C, and protect from light.

Reagent water, internal standard solution, and sample extract are added to purging chamber connected to purge-and-trap device that has been flushed with helium during a 7-min trap reconditioning step. The additions are made using appropriate size syringe equipped with 15 cm narrow-bore needle. Insert needle through septum of purging device as far as possible.

Add 5.0 mL reagent water to which 12.5 μL internal standard solution has been added to purging device. Insert needle of microsyringe ca 4 cm below surface of water during addition of internal standard solution. Inject aliquot of sample extract below surface of water in purging device. Total quantity of the 5 major volatile compounds injected should not exceed ca 2 μg. If major volatile compound content (MVCC) of sample is 200 μg/g or less, use 200 μL aliquot of sample aliquot. If MVCC is greater than 200 μg/g, use aliquot of sample extract that contains 2–4 μg of the 5 major volatile compounds. Dilute sample extract with tetraglyme, as necessary, to prepare diluted extract that contains 10–400 μg/mL of the 5 major volatile compounds. Concentration (in μg/mL) of the 5 major volatile compounds in sample extract can be calculated by dividing MVCC by 20 times dilution factor (DF). Volume (in μL) of aliquot of diluted extract to be taken can be calculated by dividing 20 000 × DF by MVCC and multiplying by 2–4. For example, if MVCC is 14 000 μg/g and DF of 10 is used, 20 000 DF/MVCC = 14.3; multiplying by 2–4 gives 28.6–57.2; therefore, a 40 μL aliquot (representing 0.0002 g original sample) would be appropriate volume of diluted extract to take for analysis. If major volatile compounds are expected to be halogenated compounds, use only

Table 2. Recovery of spiked compounds from clean matrix

Compound	Relative retention time[a]	Characteristic ion, m/z[b]	Response factor[c]	Recovery at given level,[d] %	
				5 μg/g	25 μg/g
Acrylonitrile	0.54	53	0.07	99(22)	85(8)
Propionitrile	0.60	54	0.03	ND[e]	65(21)
Diethyl ether	0.74	59	0.09	103(7)	126(5)
2-Butanone	0.78	72	0.03	93(5)	74(6)
Dibromomethane	0.79	93	0.38	104(5)	100(1)
2-Chloroacrylonitrile	0.81	87	0.20	93(2)	98(2)
Methyl acrylate	0.91	55	0.18	85(16)	102(14)
1-Chlorobutane	0.93	56	0.16	99(8)	141(7)
Dimethyl disulfide	0.96	94	0.02	ND	124(2)
Dibromoethane	1.06	107	0.50	99(2)	86(2)
Methyl methacrylate	1.13	100	0.07	92(9)	86(3)
4-Methyl-2-pentanone	1.16	58	0.08	85(10)	79(13)
2-Hexanone	1.23	58	0.07	84(10)	76(18)
Styrene	1.58	104	0.66	99(9)	92(3)
o-Xylene	1.61	106	0.42	104(7)	89(3)
m-Bromobenzotrifluoride	1.70	145	0.63	120(15)	77(10)

[a]Relative to that of D_6-benzene.
[b]Area response of characteristic ion was used for quantitation.
[c]Response factor = (Area of characteristic ion of compound purged from distilled water/area of characteristic ion of D_6-benzene purged from distilled water) × (concentrated of D_6-benzene/concentration of compound).
[d]Average of triplicate extractions; percent relative standard deviation is given in parentheses. Percent recovery = (Area of characteristic ion of compound from the sample run × 100)/area of characteristic ion of compound from the calibration run.
[e]Not detected.

one-fifth amount of diluted extract determined above. If MVCC is less than 200 μg/g and greater sensitivity is desired, use large purging chamber containing 25 mL reagent water and use 1.0 mL aliquot of sample extract. Volume of diluted extract to be taken for analysis can be used to determine appropriate volume of surrogate standard solution to be used. Appropriate volume of surrogate standard solution to add to 2 g sample can be calculated using Equation 4. Equation 4 is derived from Equation 3:

$$V_{ss} = V_{0.25} \times V_e \times DF/V_a \quad (3)$$

where V_{ss} is appropriate volume, μL, of surrogate standard solution to add to 2 g sample; $V_{0.25}$ is volume of surrogate standard solution that contains 0.25 μg; V_e is volume of tetraglyme extract, μL, from original extraction of 2 g sample; DF is dilution factor; and V_a is volume of diluted extract to be taken for analysis.

Since $V_{0.25}$ equals 0.25 μg divided by concentration, μg/μL, of surrogate standard solution and V_e = 40 000 μL, Equation 4 can be obtained:

$$V_{ss} = (0.25/C_{ss}) \times (40\,000DF/V_a) = 10\,000DF/(C_{ss} \times V_a) \quad (4)$$

where C_{ss} is concentration, μg/μL, of surrogate standard solution.

Purge sample in purging device with helium to transfer volatile components to trap. Heat trap to desorb volatile components which are swept by helium carrier gas onto GC column for analysis. Adjust gas (helium) flow rate to 40 ± 2 mL/min. Set purging device to purge, and purge sample for 11.0 ± 0.1 min at ambient temperature. At conclusion of purge time, adjust device to desorb mode, and begin GC/MS analysis and data acquisition using 6 ft × 2 mm id glass GC column packed with 1% SP-1000 on Carbopack B (60–80 mesh). Operate GC isothermally at 45°C for 3 min, then program at 8°/min to 240°C, and maintain at 240°C for 15 min.

Concurrently, introduce trapped materials to GC column by rapidly heating trap to 180°C while backflushing trap with helium at flow rate of 30 mL/min for 4 min. If this rapid heating requirement cannot be met, GC column must be used as secondary trap by cooling it to 30°C or lower during 4 min desorb step and starting GC program after desorb step. Return purge trap device to purge mode and continue acquiring GC/MS data. Let trap cool 8 min. Replace purging chamber with clean purging chamber fitted with new septum. Clean purging chamber after each use by sequential washing with acetone, methanol, detergent solution, and water and drying at 105°C.

Purge trap at ambient temperature 4 min. Recondition trap by heating to 180°C. Do not let trap temperature exceed 180°C, since the sorption/desorption is adversely affected by heating trap to higher temperatures. After heating trap ca 7 min, turn off trap heater. When cool, trap is ready for use with next sample. If response for any ion exceeds working range of MS system, repeat analysis using correspondingly smaller aliquot of sample.

Qualitative Identification

Obtain EICP for primary characteristic ion and at least 2 other characteristic ions for each compound when practical. The following criteria must be met to make qualitative identification: Characteristic ions of each compound of interest must maximize in same scan or within one scan of each other; retention time must fall within ±30 s of retention time of authentic compound; and relative peak heights of characteristic ions in EICPs must fall within ±20% of relative intensities of these ions in a reference mass spectrum.

Quantitative Determination

When a compound has been identified, quantitation of that compound will be based on integrated abundance from EICP of primary characteristic ion. In general, primary characteristic ion selected should be relatively intense ion, as free of interference as possible, and as close as possible in mass to characteristic ion of internal standard used. Use internal standard technique for performing quantitation and calculate concentration of each individual compound of interest in sample, using Equation 5:

$$\text{Concentration, } \mu g/g = (A_s C_{is})/(A_{is})(RF) \quad (5)$$

where A_s is area of primary characteristic ion of compound to be measured; A_{is} is area of primary characteristic ion of internal standard; C_{is} is concentration of internal standard in μg/g; and RF is response factor of compound being quanti-

Table 3. Average recovery and percent RSD of all spiked volatile compounds[a] (high and low) in each waste sample

Sample type	Physical description	Recovery, av. %		RSD, %		Spike level range,
		High spike	Low spike	High spike	Low spike	μg/L
Creosote-contaminated soil	wet solid	35	49	8	3	5–30
Latex paint waste	semisolid	103	134	9	5	200–1200
Ethanes spent catalyst	oily powder	21	34	38	21	500–3000
Coal-gasification tar	tar	106	110	13	13	100–600
Oxychlorination spent catalyst	pelletized solid	30	20	22	31	5–30
POTW sludge	wet filter cake	29	44	18	40	5–30
Herbicide acetone-water	liquid	68	38	2	5	100–600
Chlorinated ethanes waste	liquid	90	82	3	3	4000–24 000
Contaminated river sediment	powder					

[a]Each sample was spiked at 2 concentration levels (high and low) with the following pairs of volatile compounds: 1,1,1-trichloroethane and 1,2-dichloropropane, 1,1,2,2-tetrachloroethane and bromoform, 2-chloroacrylonitrile and propionitrile, cyclopentanone and 2-hexanone, ethylbenzene and chlorobenzene.

tated. Report results in μg/g without correction for recovering data. When duplicate and spiked samples are analyzed, report all data obtained with sample results. If surrogate standard recovery falls outside control limits (see *Quality Control* section), data for all compounds in that sample must be labeled as suspect.

Results and Discussion

The analytical protocol was first optimized for precision, accuracy, and practicality in a single laboratory evaluation (6). Spike recoveries that were obtained by following this protocol are listed in Table 1. The best recoveries for this spectrum of hazardous waste types were obtained for neutral, aprotic compounds. Analysis before spiking had revealed that each of these wastes contained a significant number of naturally incorporated compounds; thus, a real background was present for the spike compounds. The recovery values for 16 volatile compounds from a spiked 1 + 1 mixture of wet clay and diatomaceous earth is presented in Table 2. This matrix was chosen because it could be prepared as a homogeneous mixture and contained no purgeable compounds. A 200 μL aliquot of tetraglyme was chosen to test the purging efficiency under conditions of the theoretical maximum ratio of tetraglyme to water.

To reduce analytical costs for the interlaboratory comparison test, a spiking scheme was used that minimized the number of GC/MS analyses required for each waste. The scheme involved spiking each waste with pairs of chemically compatible compounds. Each pair represents a different compound class. The 2 compounds in each pair were selected on the basis of similar physicochemical properties (volatility, solubility, polarity, or acidity) that should lead to similar recovery efficiencies. One compound from each pair was spiked at a relatively low level and the other at an approximately 5-fold higher level. With this approach, data for the determination of unspiked components and data for the recoveries of different classes of compounds at 2 spike levels were obtained simultaneously by a single GC/MS run. The high and low spike levels used corresponded to those levels that would give approximately 50 and 250 ng volatile compounds or 10 and 50 ng semivolatile compounds on the GC column during analysis if extraction recovery were 100%. Since the degree of dilution or concentration required for each waste varied widely from waste to waste, the actual spike level used also varied widely. Before the interlaboratory comparison test, the spiked test samples were analyzed in a single laboratory to test the recovery efficiencies for each spike pair. The results of the single laboratory analysis are listed in Table 3 and these data confirm the similar recovery efficiencies of each spike pair.

After protocol revisions, based on single laboratory testings, a final protocol was prepared and evaluated in a formal interlaboratory comparison test. Results of this interlaboratory test have been published in an EPA report and are being prepared for submission to a peer-reviewed journal (7). The method quantitation limits have been determined in a separate EPA effort (8). Preliminary indications are that the method quantitation limits will be approximately 0.5 to 5 μg/g. It should be noted that the protocol is labeled "interim" because further work is underway which may result in modifications of protocol defects revealed by the interlaboratory comparison test (14).

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Automated Sample Cleanup for Pesticide Multiresidue Analysis. Part II—Design and Evaluation of Column Chromatography Module

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An automated, continuous flow system is described for Florisil column chromatography of pesticide residues from food extracts. Evaluation of the system using 5 common organochlorine and organophosphorus pesticides in 2 crop matrices demonstrates essentially no difference in recovery or precision between automated and currently used manual analyses. The automated procedure uses only 20% of the solvents and adsorbents used in the manual procedure and is 3 times faster.

Pesticide multiresidue analysis is conducted in countless laboratories throughout the world, using methods identical or similar to those published in the U.S. Food and Drug Administration's *Pesticide Analytical Manual* (1). For high moisture, low fat foods, a cleanup procedure based on the one first proposed by Mills et al. (2) is used. This procedure removes substances that interfere with the electron capture detection of the pesticides by a combination of solvent partitioning and adsorption column chromatography on activated Florisil. Unfortunately, this procedure is time-consuming, labor-intensive, and increasingly costly in terms of distilled-in-glass solvents and chromatographic adsorbents.

In an attempt to overcome these problems, we developed an automated cleanup system consisting of both a solvent partitioning module and a column chromatography module. The first module has recently been described (3) and provides results that are as accurate and precise as those obtained manually. In this report, we describe the column chromatography module (CCM) and evaluate its performance in the determination of pesticide residues in nonfatty foods.

Automated column chromatography techniques have been applied successfully to several analytical methods. Spackman et al. (4) first reported the development of an amino acid analyzer that specified automated ion exchange chromatography. Ion exchange resins are easily reactivated, so it has been possible to use these resins in numerous commercially available instruments. Tindle and Stalling (5) applied automated gel permeation chromatography to the separation of pesticides from lipids and were able to use the same column for long periods before replacement became necessary. Ramstad et al. (6) automated the silica gel chromatography cleanup of herbicide formulations for the analysis of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The silica gel was regenerated by back-flushing with a series of solvents and at least 400 samples could be analyzed on a single column. Using a modular approach, Stalling et al. (7) reported an automated chromatography cleanup system suitable for gel permeation, cesium silicate, and dispersed-carbon chromatography.

Florisil (activated magnesium silicate) is the adsorbent of choice for chromatographic separation of pesticides from food pigments and lipids. Methods involving Florisil column chromatography have undergone extensive evaluation and have been studied collaboratively (8–11). McMahon and Burke (12) compiled data on the analytical behavior of more than 300 pesticides, pesticide metabolites, and many common industrial chemicals on Florisil. Furthermore, the multiresidue procedure calls for column elution first with 6% ethyl ether/petroleum ether, followed by 15% ethyl ether/petroleum ether. Tentative identification of a pesticide residue depends, in part, on knowing the eluate in which it is normally found. For example, under normal gas chromatographic (GC) operating conditions, *p,p'*-DDE and dieldrin have identical retention times on OV-101 columns. However, they may be distinguished by the fact that *p,p'*-DDE is eluted from Florisil with 6% ethyl ether/petroleum ether, while dieldrin is eluted with 15% ethyl ether/petroleum ether.

Activity of the adsorbent depends on its activation temperature (13, 14) and exclusion of water. Moddes (13) studied regeneration of "spent" Florisil and concluded that regeneration was too time-consuming to be of practical value in routine work.

Because the use of Florisil has many advantages and regeneration of this adsorbent would probably lead to inconsistencies in pesticide recovery, column miniaturization was investigated as a viable alternative for achieving economies in time, solvents, and adsorbents. Law and Goerlitz (15) found that miniaturized alumina, silica gel, and Florisil columns were satisfactory for use in several applications. Erney (16) demonstrated that miniaturized (4 g) Florisil columns gave the same elution pattern as full-size (20 g) columns for pesticide mixtures, provided that elution volumes were properly chosen.

Our approach uses one miniaturized Florisil column for each sample analysis. Automated, continuous flow sample processing is made possible by the use of a 10-port sampling valve with 2 large-volume holding coils and a 60-port switching valve of our own design. This system allows for the elution of the contents of one column with 2 different eluting solvents at the same time that a subsequent column is being loaded.

Experimental

Apparatus

The column chromatography module is constructed primarily of commercially available components, several of which are available from Technicon Industrial Systems (Tarrytown, NY). A schematic diagram of the entire module is shown in Figure 1.

The 10-port pneumatically actuated sampling valve is part of a Technicon FAST-LC chromatographic cartridge. The valve body is made of Nitronic-60 alloy steel and the seals are fabricated from an inert fluorocarbon polymer. Operation of the valve is controlled by a Technicon FAST-LC programmer. The sampling valve incorporates 2 holding coils. Each holding coil is assembled from two 40-turn glass coils (Technicon part No. 190-0051-10D) connected together in series

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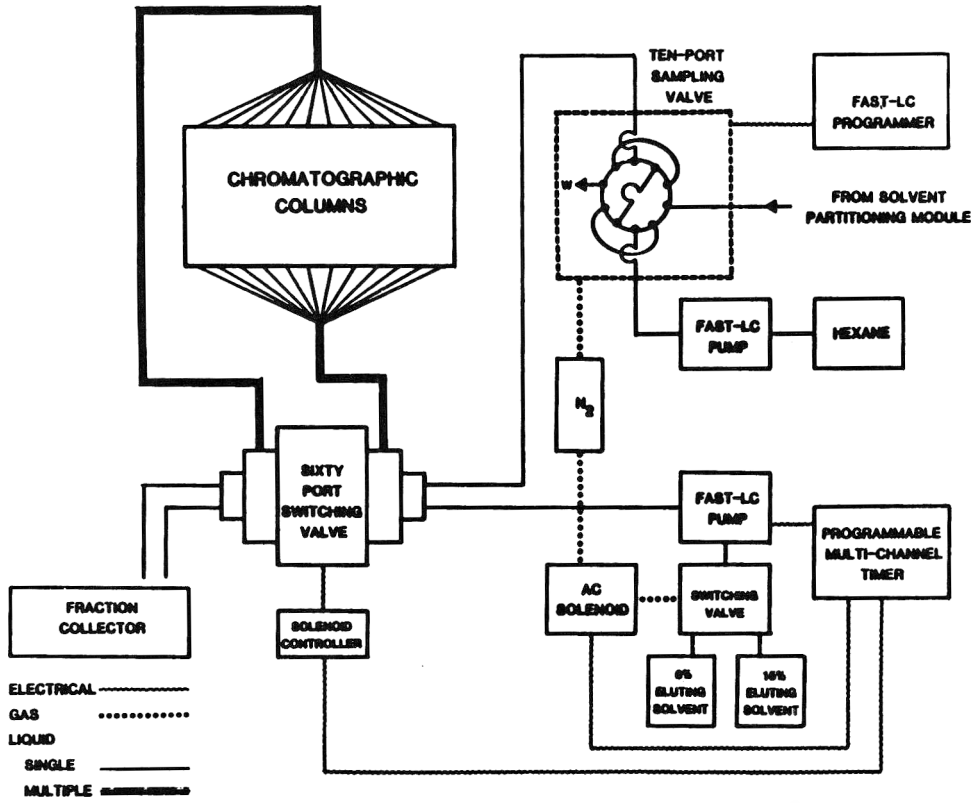

Figure 1. Schematic diagram of column chromatography module.

and to the sampling valve with stainless steel Swagelok unions fitted with Teflon ferrules.

The 10-port sampling valve can exist in 2 configurations; the internal flow paths are shown in Figure 2. The two holding coils are represented pictorially by loops 1 and 2.

The 60-port switching valve (Figure 3) is a dual rotary valve of unique design manufactured to our specifications by Scanivalve Corp. (San Diego, CA). It is now commercially available as Model No. KEM SAM/2X2P-30T. Two 30-port rotary "samplivalves" are fitted with Teflon rotors which press against the flat surfaces of Tefzel stators, and are advanced by a common solenoid drive. Appropriate flow passages are drilled in the rotors and stators to give the desired switching pattern. The complete valve has 2 in ports and 2 out ports and 60 peripheral ports.

Each pair of peripheral ports establishes a loop which includes a chromatographic column. The valve design allows for independent access to 2 adjacent columns at any given time. Thus, while the contents of one column are being eluted, the next column is pre-washed and loaded with a sample extract.

The chromatographic columns are assembled as shown in Figure 4. The glass columns (K420165) and polypropylene Luer tips (K420160-9004) are available from Kontes (Vineland, NJ); the polypropylene stepped connectors (F19563) are available from Bel-Art Products (Pequannock, NJ).

The polypropylene Luer tips fit into stainless steel hypodermic syringe needles (19-gauge Luer type) which are connected to 3 ft lengths of 1/16 in. od Teflon tubing. The opposite ends of the Teflon tubes are connected to the peripheral ports of the 60-port switching valve with Cheminert fittings.

The position of the polypropylene stepped connector on the glass column can be adjusted, within certain limits, to permit use of different amounts of adsorbent as necessitated by variations in adsorbent activity. The adsorbent used in this application is Florisil (60–100 mesh, PR grade), com-

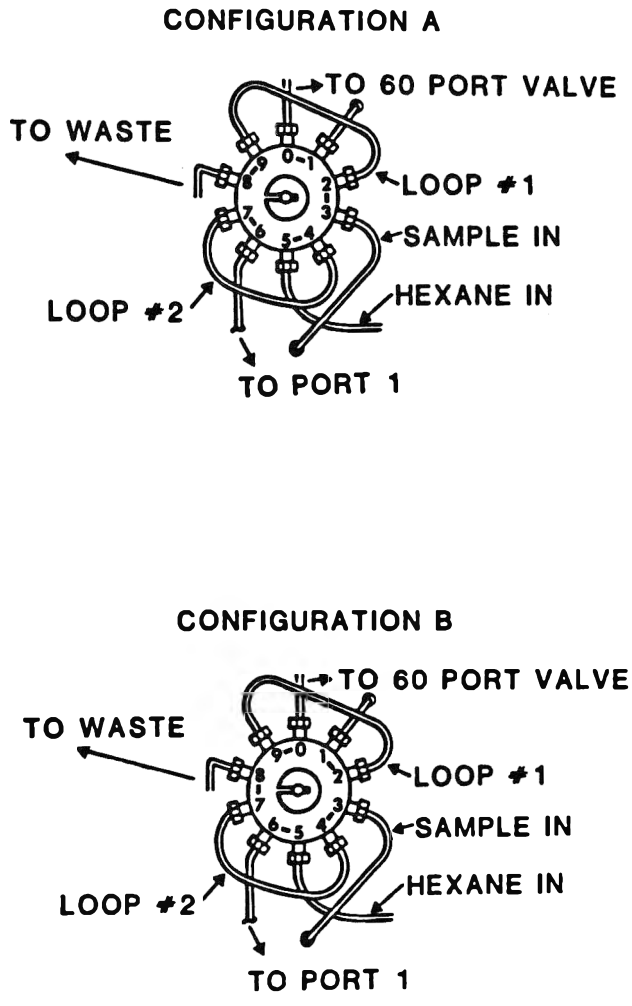

Figure 2. Ten-port sampling valve.

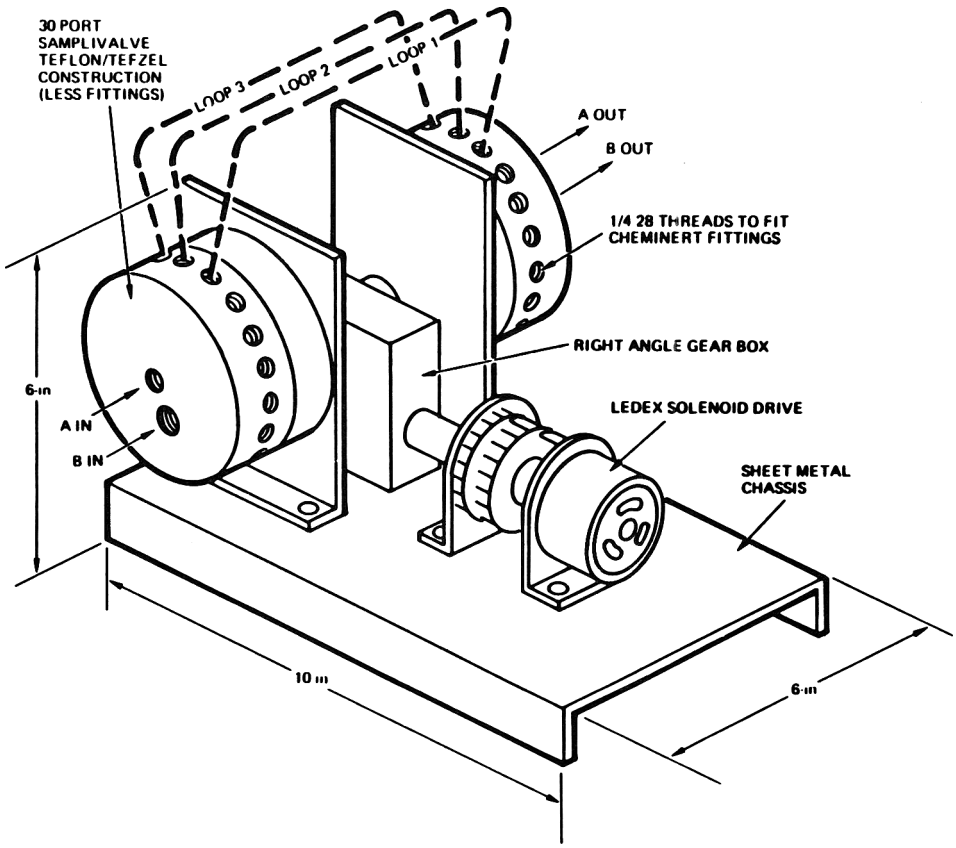

Figure 3. Operational diagram of 60-port switching valve.

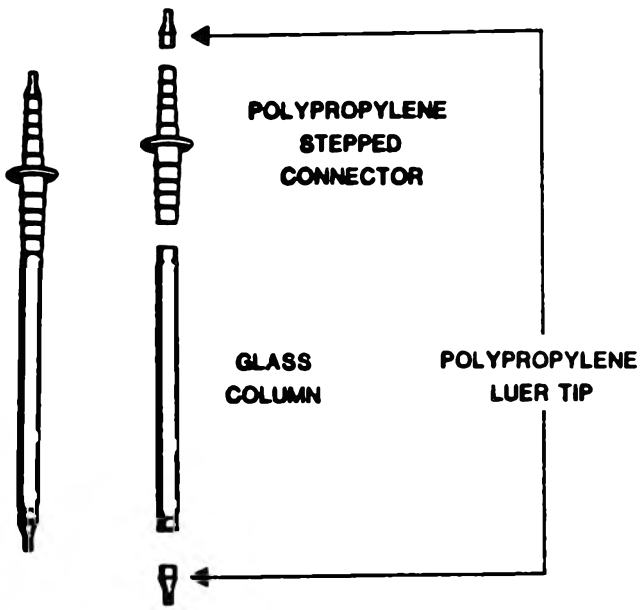

Figure 4. Chromatographic column: assembled, and unassembled.

mercially activated at 650°C and stored at 130°C for a minimum of 96 h before use. A lauric acid value (an indicator of the activity of the adsorbent) is calculated for each lot of Florisil as specified by Mills (17) and the quantity of adsorbent equivalent to 4 g of a Florisil lot with a lauric acid value of 110 mg/g is used for each column.

A small plug of glass wool is used at each end of the chromatographic column and a ½ in. layer of anhydrous Na_2SO_4 is placed in the bottom of the column before addition of Florisil.

Two Technicon FAST-LC pumps are used for precise metering of solvents. These pumps feature operation at a choice of either of 2 preset flow rates which are manually selectable in 0.1 mL/min increments from 0.1 to 9.9 mL/min via digital flow rate controls. The desired preset flow rate is automatically selected by appropriate contact closures provided by a programmable multi-channel timer (Minarik Model WP-6001, Los Angeles, CA).

The programmable timer also controls both the switching valve, which selects the eluting solvent, and the solenoid controller (Scanivalve Model CTLR 10 P/S2-S6), which advances the 60-port valve.

Details of the solvent switching circuit are shown in Figure 5. In the configuration shown, the appropriate contact in the programmable timer (B) is open and the 4-way valve (E) provides nitrogen to the upper chamber of the pneumatic actuator (F). The piston is maintained in the lower position and the metering pump accepts the 6% eluting solvent from reservoir (I). When timer contact (B) closes, relay (C) allows an AC current to energize solenoid (D) which, in turn, is connected to the 4-way valve (E). Nitrogen pressurizes the lower chamber of the pneumatic actuator and the upper chamber is vented. The piston rises and the 15% eluting solvent in reservoir (H) flows to the metering pump. This solvent switching system permits the isolation of volatile solvents from potential electrical hazards.

A Technicon large industrial sampler (equipped with its own dedicated timer) is modified for use as a dual channel fraction collector. The mechanical sampling arm is removed and 2 stationary delivery probes are mounted at appropriate points above the rotating tube rack. The tube rack can accommodate up to one hundred 50 mL test tubes in 2 concentric rows of 50 tubes each. All liquid flow connections between components in the CCM are 1/16 in. od Teflon tubing, except for the Teflon tubing connecting the solvent reservoirs to the metering pumps, which is ⅛ in. od.

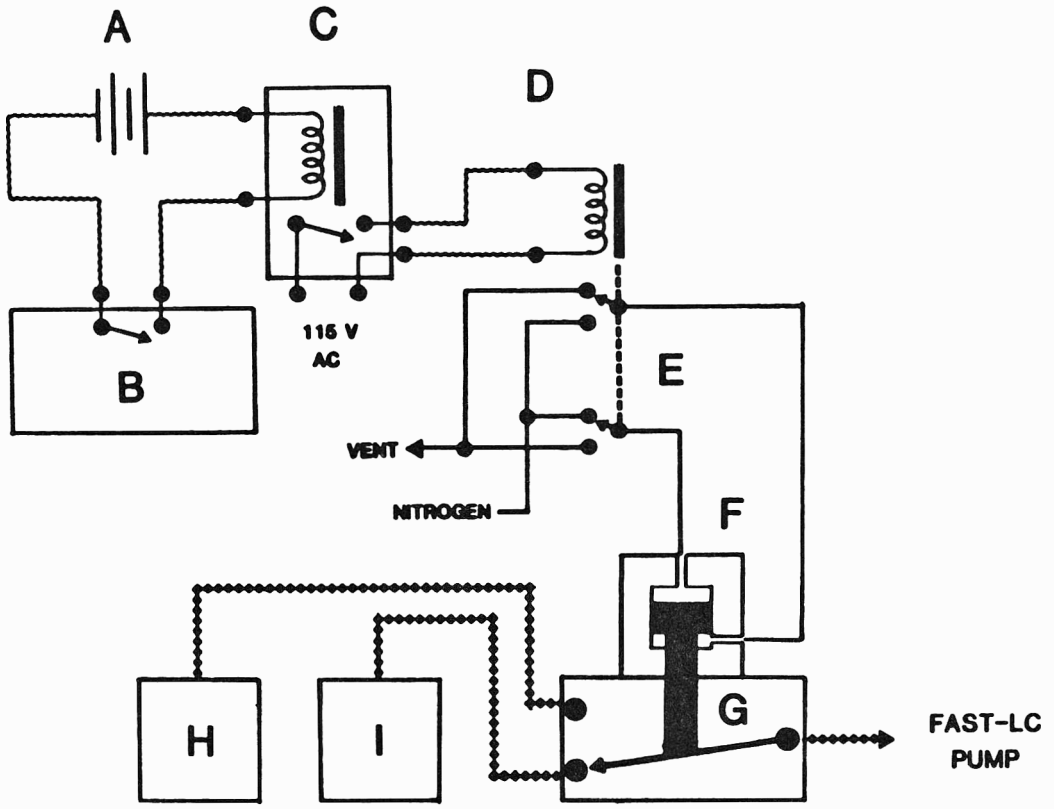

Figure 5. Solvent switching circuit: (A) 6 V DC power supply; (B) programmable multi-channel timer; (C) relay (Teledyne P/N 601-1403, Hawthorne, CA); (D) AC solenoid (Clippard Model AVSC, Cincinnati, OH); (E) 4-way spool valve (Clippard Model MAV-4); (F) pneumatic actuator (Rheodyne Model 5300); (G) 3-way slider valve (Rheodyne Model 5302, Berkeley, CA); (H) 15% eluting solvent reservoir; (I) 6% eluting solvent reservoir.

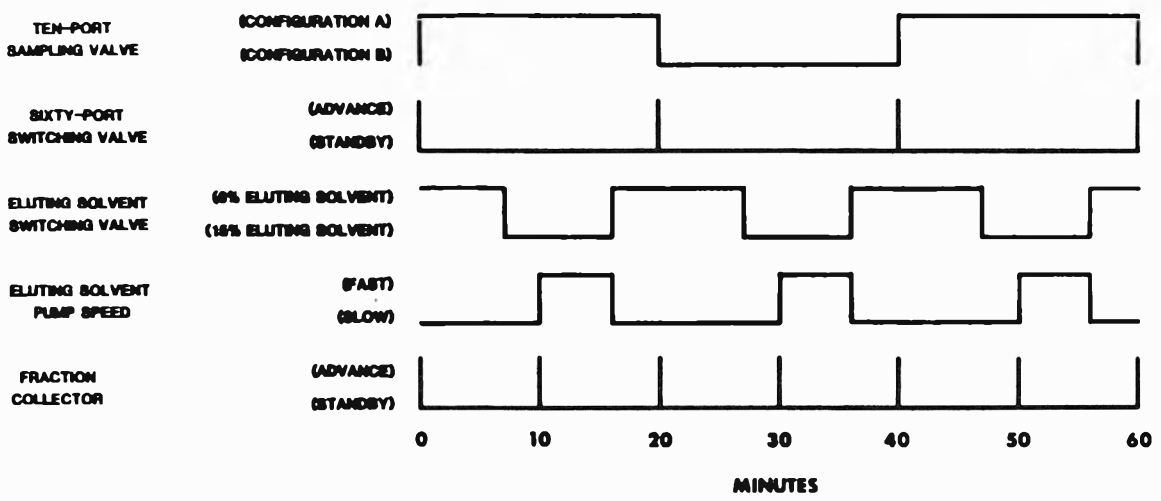

Figure 6. Timing sequence.

Operation of the System

Three timing devices automatically control 5 component functions that are necessary for unattended operation of the column chromatography module. The timing sequence is shown in Figure 6. The 10-port sampling valve and the fraction collector are each independently controlled by their own dedicated timers. The remaining 3 functions are under the control of the programmable multi-channel timer.

A sample cycle is initiated by activating the 3 timing devices. The first sample extract in hexane is delivered to the 10-port sampling valve at a flow rate of 1.08 mL/min by the peristaltic action of a Technicon pump III, a component of the previously described solvent partitioning module (3). With the sampling valve in configuration A (Figure 2), the extract enters at port 3, proceeds internally to port 2, and is collected in loop 1 (the first holding coil). The sample extract forces most of the pure hexane (which had been occupying loop 1 from the previous cycle) to go to waste via ports 9 and 8. Simultaneously, pure hexane is delivered to port 5 at a rate of 1.4 mL/min by the first metering pump. The hexane proceeds to port 4 and forces the contents of loop 2 (the second holding coil) sequentially through ports 7, 6, 1, and 0 and on to a chromatographic column via the 60-port switching valve. After 20 min, the valve changes to configuration B and loop 2 is filled with the next sample extract while the sample extract in loop 1 is displaced with pure hexane and loaded onto a chromatographic column. In normal operation, the holding coils, which have capacities of approximately 24 mL,

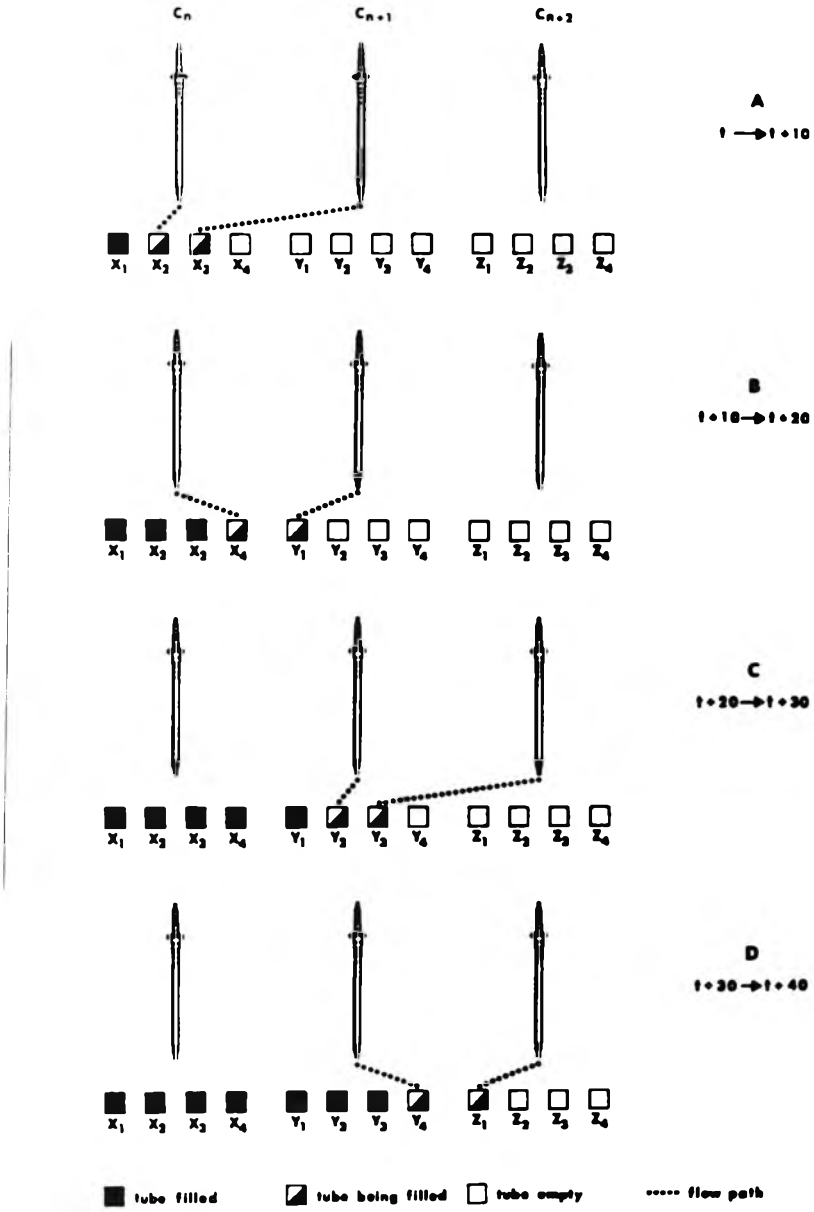

Figure 7. Elution flow diagram: t = time in minutes; C = Florisil column. (Note: For simplicity, eluates are shown coming from bottom of columns. In reality, solvent is pumped from bottom and exits from top.)

are loaded with 21.5 mL sample extract and subsequently flushed with 28 mL pure hexane.

At the 60-port valve, the sample extract enters through "A IN" (Figure 3) and is diverted to the bottom (sodium sulfate end) of one of 30 chromatographic columns mounted on a rack. The pesticides and most plant coextractives remain on the column and the hexane leaves through the top. The solvent completes its passage through the loop by returning to the 60-port valve and passes through "A OUT" to one of the delivery probes of the fraction collector. After 20 min, the 60-port valve advances one step, and while the next sample extract is loaded onto a new column, the previous column is eluted, first with 6% ethyl ether/hexane, and then with 15% ethyl ether/hexane. All solvents are Burdick and Jackson distilled-in-glass quality.

The eluting solvent delivery system has a hold-up volume of 10 mL between the metering pump inlet and the chromatographic columns. Typical fast and slow eluting solvent flow rates are 5.0 and 3.0 mL/min, respectively (Figure 6). The multi-channel timer is programmed to allow for switching from the 6% to 15% eluting solvent 3 min before advance of the fraction collector. In a similar manner, the change from 15% to 6% eluting solvent occurs 4 min before the fraction collector advances. This timing sequence ensures that no residual 15% eluting solvent remains in the supply lines when the 60-port switching valve advances to the next chromatographic column.

The eluting solvents enter the 60-port switching valve at "B IN" and exit to the second delivery probe of the fraction collector through "B OUT." Figure 7 is a schematic representation of the events involving 3 adjacent Florisil columns during four 10-min intervals. The fraction collector is programmed to advance at 10-min intervals, allowing for the collection of 4 fractions every 20 min. During the first time interval shown (Figure 7A), Column C_n is being eluted with 6% ether and this eluate is collected in tube X_2 through the "B OUT" delivery probe. At the same time, column C_{n+1} is being loaded with the next sample extract in hexane through "A IN." Column C_{n+1} retains the solutes and the hexane passes through the column and the "A OUT" delivery probe into X_3. This arrangement is necessitated by the fact that some nonpolar pesticides from the previous column loading (C_n) may still be in the "A OUT" transfer line after the advancement of the 60-port valve. In the next interval (Figure

Table 1. Comparison of automated and manual Florisil chromatography procedures for analysis of pesticides in fortified pea extracts

	Automated			Manual		
Pesticide	Mean, μg[a]	CV, %[b]	Rec., %	Mean, μg[a]	CV, %[b]	Rec., %
Aldrin	1.93	5.6	93	1.90	4.4	92
Heptachlor epoxide	3.87	8.8	94	3.82	4.9	93
p,p'-DDT	10.20	1.0	100	9.46	7.6	93
Methyl parathion	7.83	3.9	94	6.08	5.5	73
Dieldrin	5.87	1.0	98	5.27	6.1	88

[a]Total quantity recovered; calculated from 5 replicates.
[b]Coefficient of variation.

Table 2. Comparison of automated and manual Florisil chromatography of pesticides in fortified tomato extracts

	Automated[a]			Manual		
Pesticide	Mean, μg[b]	CV, %[c]	Rec., %	Mean, μg[b]	CV, %[c]	Rec., %
Aldrin	1.51	6.4	73	1.89	4.1	91
Heptachlor epoxide	3.85	4.2	94	3.80	3.9	93
p,p'-DDT	10.15	4.0	99	9.75	7.1	95
Methyl parathion	6.56	2.1	79	6.01	9.0	72
Dieldrin	5.18	4.8	87	5.72	1.8	96

[a]Results were obtained with 15% ether elution at 3.5 mL/min, rather than 5.0 mL/min as specified in *Experimental* section.
[b]Total quantity recovered; calculated from 5 replicates.
[c]Coefficient of variation.

7B), column C_n is eluted with 15% ether and this eluate is collected in X_4 while column C_{n+1} continues to be loaded with the next sample. This hexane fraction is collected in Y_1. At the end of this interval, the 60-port valve advances and new flow paths to columns C_{n+1} and C_{n+2} are established while the flow path to column Cn is severed. The third and fourth time intervals (Figures 7C and 7D) are repeats of the first and second time intervals, except that column C_{n+1} and column C_{n+2} replace columns C_n and C_{n+1}, respectively. Also, tubes Y_2, Y_3, Y_4 and Z_1 are filled instead of respective tubes X_2, X_3, X_4, and Y_1. For each sample, the contents of tubes 1, 2, and 3 are combined in a 125 mL Kuderna-Danish evaporative concentrator (Kontes, K570025). After evaporation to an appropriate volume on a steam bath, the concentrate is analyzed by GC. The contents of tube 4 (the 15% ether fraction) is treated similarly.

Evaluation of the System

The performance of the automated chromatography procedure was evaluated by comparison with the manual column chromatography procedure commonly used by FDA for pesticide multiresidues analysis (18). Samples of food crops were extracted with acetonitrile and partitioned into hexane, using standard methodology (19).

The extracts in hexane were diluted to 100 mL. Aliquots (20 mL) were then transferred to 25 mL volumetric flasks. To each portion (representing either 20% or 80% of each of the original extracts) was added 2.0 mL of a solution containing 5 pesticides commonly found as residues in foods. This solution was prepared in hexane and contained 1.038 μg aldrin, 2.984 μg dieldrin, 2.054 μg heptachlor epoxide, 4.150 μg methyl parathion, and 5.118 μg *p,p'*-DDT/mL. The pesticides were obtained from the Pesticides and Industrial Chemical Repository, Environmental Protection Agency, Research Triangle, NC.

The pesticide-fortified extracts in the 25 mL volumetric flasks were adjusted to volume with hexane; these extracts were subsequently aspirated into the CCM for 16 min at a nominal flow rate of 1.08 mL/min (followed by a 4-min wash with pure hexane). Aspiration, for purposes of evaluating the performance of the CCM, was accomplished using a Technicon pump III with a 2 ft length of 1/16 in. od Teflon tubing connected to the front end of an Acidflex pump tube. The free end of the Teflon tubing was manually moved between the sample extracts and the wash solution. The actual volume of each extract aspirated into the CCM was determined by measuring the volume of solution remaining in the volumetric flask.

The remaining pesticide-fortified extracts were analyzed by the manual column chromatography procedure (18).

Pesticide residues were quantitated on a Tracor Model 550 gas chromatograph equipped with a constant current ^{63}Ni electron capture detector and a 6 ft × 2 mm id glass column packed with 5% OV-101 on Chromosorb WHP, 80–100 mesh. Carrier gas (nitrogen) flow rate was 30 mL/min. Column temperature (ca 200°C) was adjusted to permit elution of *p,p'*-DDT at 3.13 relative to chlorpyrifos; detector sensitivity was adjusted to provide ½ full scale deflection for 1.5 μg chlorpyrifos.

Results and Discussion

The evaluation of the column chromatography module for the analysis of fortified green pea extracts is summarized in Table 1. In the automated part of this study, the fast and slow elution solvent flow rates were 5.0 and 3.0 mL/min, respectively. Aldrin, heptachlor expoxide, and *p,p'*-DDT eluted in the 6% ether fractions and dieldrin and methyl parathion in the 15% ether fractions, as expected.

Agreement between the 2 procedures in terms of both recovery and precision was excellent, except for methyl parathion, where the automated system clearly gave superior results. This occurred because the lot of Florisil used in these experiments was known (from previous analyses) to give incomplete recoveries for methyl parathion when standard elution volumes were used. We were able to overcome this deficiency in the Florisil by eluting with 15% ether at a relatively high flow rate (5 mL/min), a rate that effectively increases eluate volume without increasing elution time. Elution at 3.5

mL/min (lesser volume of solvent) gave methyl parathion recoveries equivalent to those of the manual procedure, as evidenced by our results with fortified tomato extracts (Table 2). Analysis of recovery and precision data for quantitation of fortified tomato extracts showed excellent agreement between the 2 methods except for aldrin. These analyses were conducted before the pea extract analyses. At that time our elution protocol did not include collecting the hexane pre-wash, an error which resulted in loss of aldrin (an exceptionally nonpolar material) to waste. In the manual procedure, the hexane pre-wash is collected in the same receiver as the 6% ether eluate. Adjustment of the elution protocol to collection of the hexane pre-washes, as described above, allows for essentially complete aldrin recovery (see Table 1).

If desired, the system can be modified to include a third eluting solvent (50% ethyl ether/petroleum ether) for the elution of more polar pesticides such as malathion (11).

The automated column chromatography module can process 30 sample extracts in 11 h with minimal user intervention. The first sample is completed 1 h after introduction and each additional sample extract requires 20 min. In contrast, it would require the full attention of an experienced analyst to manually process only 10 similar sample extracts in this period. The system requires 80% less solvents and adsorbents. In routine use, the savings in labor and material costs would more than offset the initial purchase price of the equipment. Most important, government regulatory agencies would improve their performance in protecting the consumer from potential pesticide hazards.

Acknowledgments

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Chemical Derivatization Analysis of Pesticide Residues. VIII. Analysis of 15 Chlorophenols in Natural Water by In Situ Acetylation[1]

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A rapid analytical method is presented for quantitative analysis of 15 chlorophenols in natural waters by in situ acetylation. In the presence of $KHCO_3$, phenols in water are acetylated by acetic anhydride directly without pre-extraction. The resultant acetates are extracted by petroleum ether and analyzed by electron capture gas chromatography. The investigation to optimize the conditions for in situ acetylation of these phenols is also described. This method has been validated and shown to be applicable over a range from 100 to 0.01 ppb with a 1 L water sample.

Gas chromatographic (GC) analyses of chlorophenols in water are done by 2 general approaches. One involves the direct analysis of free phenols (1), while the other involves analysis of derivatized phenols (2). The former route is generally faster because no extra derivatization steps are included in the procedure. Methods involving derivatization are longer, but the derivatives formed are usually easier to chromatograph, more sensitive to the electron-capture detector (ECD), and more amenable to column cleanup techniques than their parent phenols. However, both approaches normally include solvent extraction of the free phenols from acidified samples, and this step has been shown by other authors to give low recoveries for some phenols (2–4).

In 1974, Chau and Coburn reported a method for analysis of pentachlorophenol (PCP) in natural and waste water (5). In their method, PCP is extracted from water by benzene. The phenol is back-extracted into 0.1M K_2CO_3 and then acetylated in aqueous medium by acetic anhydride. The resulting PCP acetate is extracted by hexane and analyzed by packed column chromatography with electron capture detection. Later, Krijgsman and van de Kamp applied this method to all 19 chlorophenols, using a 30 m SE-30 glass capillary column (6). Although these authors claimed recoveries of chlorophenols between 80 and 100%, no validation data were given. Coutts et al. reported a method of direct acetylation of phenols in aqueous solution (4). This method eliminates the deficiencies of incomplete recovery of free phenols from water samples by the solvent extraction technique. Recovery of the phenol acetates from water has been shown to be quantitative.

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Although Coutts et al. applied the method to a wide range of phenols including nitro- and nonchlorinated phenols at ppm levels, their validation data were limited to just one chlorophenol, namely, 2,4-dichlorophenol (4, 7).

In this paper, we describe a simple and reproducible method for the analysis of 15 chlorophenols in natural waters by an in situ acetylation technique. This method has a range of application from 100 to 0.01 ppb chlorophenols.

Experimental

(a) *Gas chromatographs.—(1)* Hewlett-Packard Model 5710A equipped with ^{63}Ni electron capture detector, Model 7671A autosampler, and Model 3390A reporting integrator. Operating temperatures: injector, 200°C; detector, 300°C; column, see below. Carrier gas, methane–argon (5 + 95). *(2)* 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: injector, 200°C; detector, 300°C; column, see below. Carrier gas, helium. (All instruments available from Hewlett-Packard, Avondale, PA 19311.)

(b) *Columns.—(1)* 1.8 m × 2 mm id glass, packed with 3% OV-17 on Gas-Chrom Q, 120–140 mesh (Applied Science Laboratories, Inc., State College, PA 16801). Column temperature, 150°C. Flow rate, 25 mL/min.

(2) 1.8 m × 2 mm id glass, packed with 3% OV-1 on Gas-Chrom Q, 100–120 mesh (Chromatographic Specialties Ltd, Brockville, Ontario, Canada K6V 5W1). Column temperature, 140°C. Flow rate, 28 mL/min.

(3) 12 m × 0.2 mm id fused silica capillary column (FSCC) coated with crosslinked dimethyl silicone and surface-deactivated by siloxane (Part No. 19091-60312, Hewlett-Packard). Temperatures (°C): initial, 70°, hold for 0.5 min, programming rate 1, 10°/min (70° to 120°) hold at 120° for 5 min, programming rate 2, 2°/min (120° to 160°). Splitless valve on for 0.5 min. Linear velocity, 25 cm/s.

(c) *Gas chromatography–mass spectrometry (GC-MS).*—Finnigan Model 4000 GC-MS-DS operating in EI mode. Column, 30 m × 0.25 mm id DB-1 FSCC, 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°C/min (70° to 100°), hold at 100° for 5 min, programming rate 2, 2°/min (100° to 140°). Carrier gas, helium at 15 psi.

(d) *Reagents.*—Use all pesticide grade solvents. *(1) 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 at 5 mg/mL. Keep in the dark at 4°C. Prepare mixture of phenols in acetone at 50 μg/mL for spiking purposes. *(2) Acetic anhydride.*—Distill AnalaR grade (BDH Chemicals, Toronto, Ontario, Canada M8Z 1K5) reagent 3 times and collect 138–140°C fraction for acetylation reactions.

In Situ Aqueous Acetylation of Phenols and Cleanup

To 1 L water sample containing phenols in 1.14 L whisky bottle or other similar size bottle, add 5.0 g anhydrous $KHCO_3$ and stir until solid is completely dissolved. Add 5.0 mL acetic anhydride and 60 mL petroleum ether (30–60°C) to sample and stir *slowly* until evolution of CO_2 subsides. Then stir sample vigorously 60 min. Separate layers in separatory funnel and drain water sample back into whisky bottle. Collect organic layer in 250 mL round-bottom flask. Repeat extractive acetylation process twice with 40 mL aliquots of petroleum ether. Dry combined organic layers through anhydrous sodium sulfate. To dry extract, add 2 mL isooctane as keeper and evaporate extract to 3–5 mL.

Prepare mini cleanup column by plugging 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.

Prewet column with 5 mL hexane and discard washings. With Pasteur pipet, quantitatively transfer concentrated sample extract in isooctane to silica gel column. Elute column with 5 mL toluene–hexane (2 + 98) and discard washing. Continue elution with 10 mL toluene–hexane (50 + 50). Collect this fraction and dilute to 10.0 mL. Analyze sample by injecting 2 μL extract, splitless mode, into the OV-1 FSCC connected to electron capture detector.

Results and Discussion

GC-MS Analysis of Phenol Acetates

Many of the phenol acetates were not available commercially for peak identification, so confirmation of structures was done by GC-MS analysis. A mixture of 22 phenols containing all 19 chlorophenols, 2-chloro-5-methyl-, 4-chloro-3-methyl-, and phenol (10 μg each) was acetylated and examined by GC-MS operating in the EI mode. Close inspection of the individual mass spectrum confirms the formation of acetates under the conditions used. Identification of peaks was based on the presence of *(1)* the molecular ion (M^+), *(2)* the CH_3CO^+ fragment (m/z = 43), *(3)* the parent phenol ion $(M-42)^+$, and *(4)* the isotopic pattern of chlorine atoms in the regions of M^+ and $(M-42)^+$. As expected, the isomeric acetates gave nearly identical mass spectra and their identities were assigned mainly on the basis of their retention times.

Gas Chromatography with Electron Capture Detector (ECD)

Because of poor sensitivity of the ECD toward monochlorophenol acetates, these phenols were not included in our subsequent work. The isothermal GC separation of the 16 di-, tri-, tetra-, and pentachlorophenol acetates on a 3% OV-17 packed column at 150°C (Figure 1a) was not satisfactory. Because of overlapping peaks, only 9 peaks could be resolved from the 16 compounds on this column. Decreasing the column temperature to 130°C only broadened the peaks with no improvement on peak resolution. Switching to a 3% OV-1 packed column improved the resolution. Thirteen peaks were observed from the 16 acetates chromatographed at 140°C (Figure 1b). The unresolved pairs were (i) 2,5- and 2,4-dichlorophenol acetates, (ii) 2,3,5- and 2,4,5-trichlorophenol acetates, and (iii) 2,3,5,6- and 2,3,4,6-tetrachlorophenol acetates. Resolution of the same mixture on a 12 m OV-1 FSCC was even better (Figure 2). Under the conditions used, acetates of all the 16 chlorophenols with the exception of 2,5- and 2,4-dichlorophenols could be resolved on this column. It was also reported that all 19 chlorophenol acetates could be resolved on a longer (30 m) OV-101 capillary column (6; M. Fox, personal communication). 2,5-Dichlorophenol does not appear in environmental samples too often, and also it is not a U.S. Environmental Protection Agency priority pollutant; this phenol was therefore omitted in the following recovery studies. The identities of peaks in Figures 1a, 1b, and 2 were determined by injecting the individual phenol acetates.

The order of elution of the phenol acetates on the nonpolar OV-1 FSCC was the same as that for the PFB ethers of the same phenols (8) on the same column with only one excep-

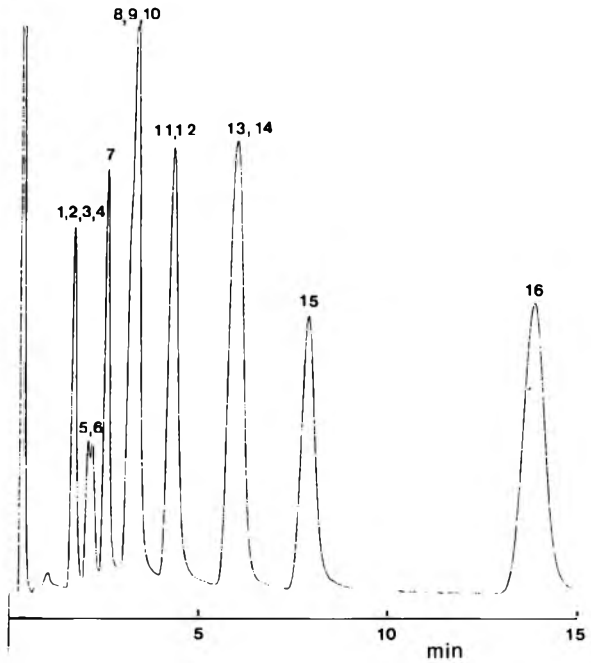

Figure 1a. Gas chromatogram of standard mixture of 16 chlorophenol acetates on 3% OV-17 column. See Table 1 for identification of peaks. See *Experimental* for GC conditions.

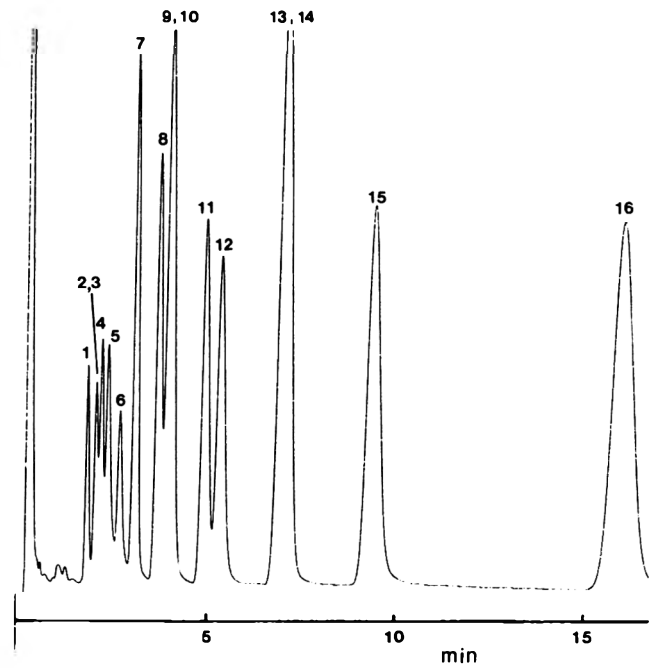

Figure 1b. Gas chromatogram of standard mixture of 16 chlorophenol acetates on 12m OV-1 FSCC. See Table 1 for identification of peaks. See *Experimental* for GC conditions.

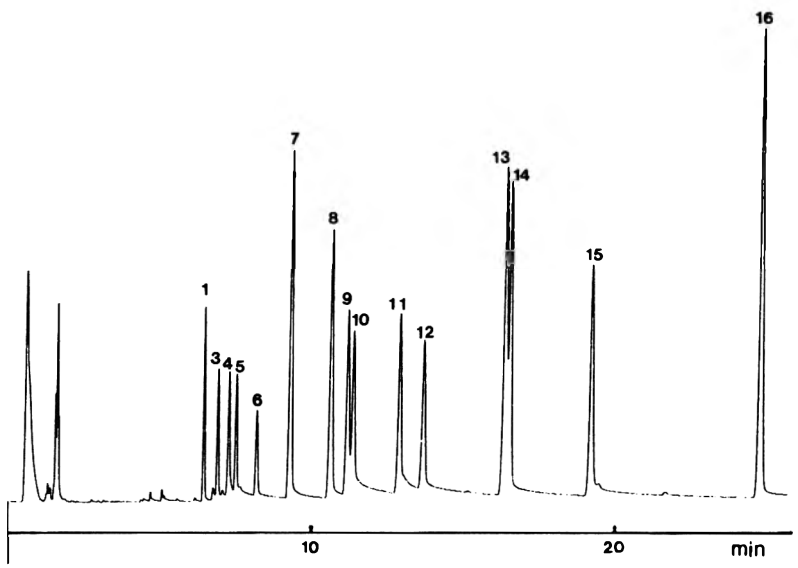

Figure 2. Gas chromatogram of standard mixture of 16 chlorophenol acetates on 12m OV-1 FSCC. See Table 1 for identification of peaks. See *Experimental* for GC conditions.

tion: 2,4,5-Trichlorophenol acetate eluted after 2,3,5-trichlorophenol acetate while the PFB ethers of 2,4,5- and 2,3,5-trichlorophenols had the reverse elution order. Also note that on this OV-1 FSCC, the 2,5- and 2,4-dichlorophenols were better resolved in the form of PFB ethers than their acetates, while the opposite was true for 2,3,5- and 2,4,5-trichlorophenols.

After about 6 injections, both the packed and capillary columns had to be baked at a higher temperature (e.g., 220°C) to clean the GC columns; otherwise, the high boiling co-extractives (long retention times) of the previous samples interfered with subsequent analyses. To reduce tailing of the acetates on the OV-1 FSCC, it was necessary to cut off the first 10–20 cm of the column at the injection port end after about 100 injections. Table 1 gives the relative sensitivities of the individual chlorophenol acetates determined on the OV-1 FSCC. The difference in ECD sensitivities from di- to penta- chlorophenols was roughly a factor of 7.

Evaporative Losses

Solutions of chlorophenol acetate (1 μg each) in hexane and petroleum ether (30–60°C) were evaporated from 150 mL to about 5 mL, using a rotary evaporator at 30°C, and diluted to 10.0 mL before GC analysis. The results of these simulated extraction/evaporation experiments indicated that evaporative losses (recoveries of 80–90%) were detectable for acetate solutions in hexane, even in the presence of a keeper such as isooctane. However, for petroleum ether solutions with or without a keeper, evaporative losses were not significant (recoveries $\leq$ 90%) if the volume was reduced to about 5 mL. Because petroleum ether quantitatively recovered the chlorophenol acetates from water, this solvent was used instead of hexane in the extraction of the acetates.

In Situ Aqueous Acetylation of Chlorophenols

Not all the individual chlorophenol acetates are available commercially and in-laboratory synthesis was not feasible;

Table 1. Retention times and relative retention times of chlorophenol acetates on 3 GC columns[a]

Parent phenol	3% OV-17 RT (RRT)	3% OV-1 RT (RRT)	OV-1 FSCC RT (RRT)	OV-1 FSCC Rel. sens.
1. 2,6-Dichloro-	1.64 (1.19)	1.91 (1.17)	6.52 (2.60)	1.99
2. 2,5-Dichloro-	1.67 (1.21)	2.10 (1.28)	6.97 (2.78)	—
3. 2,4-Dichloro-	1.64 (1.19)	2.14 (1.31)	6.99 (2.79)	1.44
4. 3,5-Dichloro-	1.62 (1.18)	2.29 (1.40)	7.35 (2.93)	1.77
5. 2,3-Dichloro-	2.15 (1.56)	2.46 (1.50)	7.60 (3.03)	1.65
6. 3,4-Dichloro-	2.03 (1.47)	2.79 (1.70)	8.29 (3.31)	1.40
7. 2,4,6-Trichloro-	2.52 (1.83)	3.21 (1.96)	9.40 (3.75)	5.83
8. 2,3,6-Trichloro-	3.31 (2.40)	3.83 (2.34)	10.75 (4.29)	5.29
9. 2,3,5-Trichloro-	3.13 (2.27)	4.06 (2.48)	11.31 (4.51)	3.59
10. 2,4,5-Trichloro-	3.27 (2.37)	4.18 (2.55)	11.50 (4.59)	4.78
11. 2,3,4-Trichloro-	4.29 (3.11)	5.10 (3.12)	13.04 (5.20)	4.54
12. 3,4,5-Trichloro-	4.20 (3.05)	5.52 (3.37)	13.84 (5.52)	3.95
13. 2,3,5,6-Tetrachloro-	5.82 (4.22)	7.16 (4.37)	16.54 (6.60)	6.72
14. 2,3,4,6-Tetrachloro-	6.00 (4.35)	7.31 (4.47)	16.71 (6.67)	6.66
15. 2,3,4,5-Tetrachloro-	7.78 (5.65)	9.64 (5.89)	19.43 (7.75)	4.92
16. PCP	13.78 (10.00)	16.37 (10.00)	25.07 (10.00)	10.00

[a]ECD response factors for the acetates relative to PCP acetate are calculated on OV-1 FSCC.

Table 2. Relative recoveries of chlorophenols under various acetylation conditions[a]

[Phenol], ppb	1	1	1	1	1	10
[$KHCO_3$], M	0.5	0.5	0.5	0.5	0.1	0.05
Ac_2O, mL	5	5	5	10	10	5
Volume, L	1	1	1	1	1	0.1
Time, h	3 × 1	3 × 0.5	3 × 2	3 × 1	3 × 1	3 × 1
Parent phenol	(Ref.)	—	—	—	—	—
2,6-Dichloro-	100	95.5	98.6	90.1	98.6	94.4
2,4-Dichloro-	100	93.7	101.4	93.0	101.1	100.3
3,5-Dichloro-	100	98.6	100.8	95.2	98.5	100.7
2,3-Dichloro-	100	101.2	99.1	93.8	95.8	97.6
3,4-Dichloro-	100	94.5	100.3	94.3	96.1	100.4
2,4,6-Trichloro-	100	97.2	102.9	91.1	102.1	99.0
2,3,6-Trichloro-	100	104.7	102.8	89.3	101.0	99.7
2,3,5-Trichloro-	100	90.0	103.9	92.1	103.8	102.9
2,4,5-Trichloro-	100	99.7	102.0	92.0	102.2	102.7
2,3,4-Trichloro-	100	89.0	105.8	91.0	103.1	103.9
3,4,5-Trichloro-	100	85.8	104.0	92.4	97.3	102.1
2,3,5,6-Tetrachloro-	100	92.6	102.5	89.7	100.7	98.3
2,3,4,6-Tetrachloro-	100	94.2	103.8	88.6	99.2	99.3
2,3,4,5-Tetrachloro-	100	98.4	103.8	90.2	98.1	104.9
PCP	100	91.7	101.0	86.4	99.2	97.3

[a]Average of 4 trials for each condition.

Table 3. Mean recoveries of chlorophenols (as acetates) in distilled water samples fortified at various levels[a]

Parent phenol	Fortification level, ppb (No. of replicates) 200 (4)	100 (4)	10 (4)	1 (6)	0.1 (6)	0.01 (4)
2,6-Dichloro-	97.0	98.5	99.3	100 (2.8)	91.4 (3.7)	126.0
2,4-Dichloro-	88.6	94.0	97.3	100 (2.4)	98.5 (3.3)	98.3
3,5-Dichloro-	99.3	102.8	101.7	100 (1.8)	98.3 (4.4)	87.6
2,3-Dichloro-	92.5	101.9	102.5	100 (5.1)	103.4 (2.9)	80.0
3,4-Dichloro-	99.5	102.2	101.5	100 (7.4)	97.5 (3.5)	84.6
2,4,6-Trichloro-	96.5	100.0	100.5	100 (2.1)	90.2 (2.8)	77.3
2,3,6-Trichloro-	98.1	103.1	104.3	100 (1.2)	97.3 (2.7)	80.8
2,3,5-Trichloro-	75.6	97.0	100.0	100 (1.6)	101.5 (3.9)	87.1
2,4,5-Trichloro-	72.2	97.0	98.4	100 (3.4)	101.9 (6.2)	84.2
2,3,4-Trichloro-	59.7	91.9	99.2	100 (1.2)	96.4 (6.4)	83.1
3,4,5-Trichloro-	63.4	100.7	105.4	100 (0.94)	90.4 (13.0)	81.1
2,3,5,6-Tetrachloro-	97.8	100.7	101.8	100 (1.2)	96.6 (2.5)	83.7
2,3,4,6-Tetrachloro-	98.4	103.6	102.7	100 (2.8)	95.2 (4.6)	73.3
2,3,4,5-Tetrachloro-	66.3	95.4	101.2	100 (0.80)	89.0 (2.1)	77.8
PCP	95.1	99.8	101.3	100 (4.5)	101.5 (2.3)	97.8

[a]All recoveries are relative to the 1 ppb level. Relative standard deviations, %, are given in parentheses.

Table 4. Mean recoveries (%) of chlorophenols in fortified natural water samples (n = 3) after correction for blanks

	Toronto Harbour (ppb)		Hamilton Bay (ppb)			Lake Erie (ppb)		
Parent phenol	1	0.1	10	1	0.1	10	1	0.1
2,6-Dichloro-	98.2	95.4	98.9	99.1	92.4	99.3	97.2	89.0
2,4-Dichloro-	98.3	96.2	101.1	98.5	104.8	106.0	99.2	107.0
3,5-Dichloro-	94.7	99.3	98.3	99.2	103.6	101.6	96.5	102.9
2,3-Dichloro-	88.6	98.8	97.0	102.7	101.8	100.4	91.4	97.7
3,4-Dichloro-	96.6	102.2	94.5	97.4	86.0	98.3	89.7	92.0
2,4,6-Trichloro-	97.8	94.0	97.7	99.4	105.3	100.0	97.6	92.8
2,3,6-Trichloro-	97.2	87.9	98.0	97.2	94.9	97.2	94.8	106.7
2,3,5-Trichloro-	99.6	101.1	97.8	99.1	96.5	102.8	99.1	98.1
2,4,5-Trichloro-	87.9	100.8	93.9	94.6	98.8	96.3	99.1	98.1
2,3,4-Trichloro-	100.0	100.0	93.4	95.9	94.9	106.6	101.9	103.9
3,4,5-Trichloro-	100.6	108.6	91.3	99.3	97.8	102.6	105.0	104.7
2,3,5,6-Tetrachloro-	96.7	97.6	95.1	98.5	96.4	99.8	97.4	98.1
2,3,4,6-Tetrachloro-	92.4	87.3	93.0	97.6	107.4	96.8	97.8	91.4
2,3,4,5-Tetrachloro-	101.8	99.7	96.5	95.6	93.8	104.4	99.5	93.8
PCP	97.1	96.3	93.7	100.7	100.8	99.7	97.8	92.2

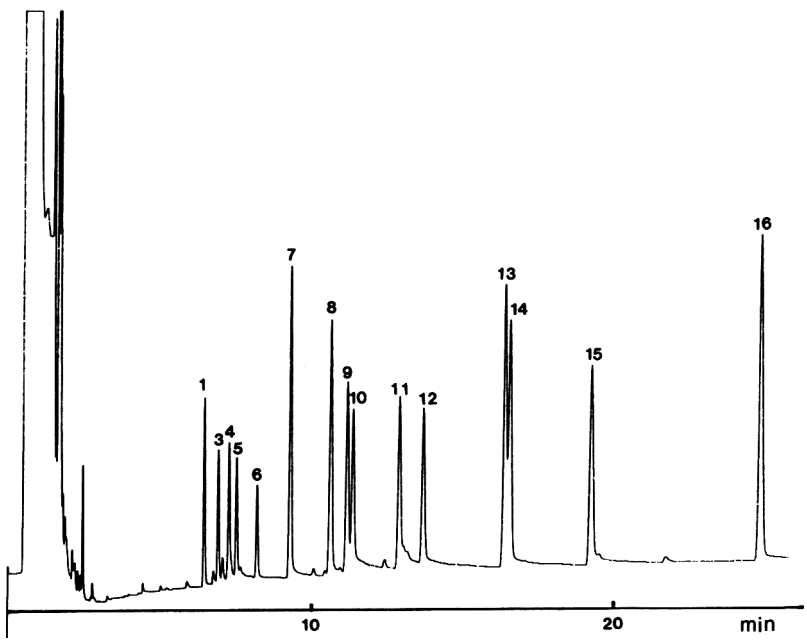

Figure 3. Gas chromatogram of extract from Lake Erie water sample with chlorophenols fortified to 1 ppb. GC conditions and peak identification same as Figure 2.

therefore, the absolute recoveries of chlorophenols as their acetates via this derivatization/extraction method were not determined. Consequently, relative recoveries of the phenols were reported by comparison with those obtained under a reference condition as detailed in the *Experimental* section. This approach will not affect the quality of real-life data because the sample and standard will be treated and compared chromatographically under identical conditions.

Several authors (2–4) have found that many phenols, especially the more water-soluble ones, could not be quantitatively recovered from acidified water samples by the solvent extraction technique. However, recoveries of all chlorophenol acetates from water at the 1 ppb level have been shown in this work to be quantitative. Therefore the present in situ aqueous acetylation method eliminates the drawbacks of low recoveries in previously published methods involving solvent extraction of free phenols.

To demonstrate that the conditions we chose for acetylation of water samples were optimal, relative recoveries of chlorophenols under various acetylation conditions were determined (Table 2). Slightly lower yields were obtained for several tri- and tetrachlorophenols as well as PCP if the reaction time was decreased from 3 to 1.5 h (Column 2, Table 2). However, a doubling of the reaction time to 6 h did not improve the recoveries significantly (Column 3). For all subsequent acetylation of water samples, a 3 h reaction time was used. It was surprising to find that recoveries of all 15 chlorophenols were slightly lower (about 90%) if the amount of acetic anhydride was doubled from 5 to 10 mL while the $KHCO_3$ concentration was kept constant (Column 4). However, recoveries of chlorophenols were basically unchanged if both the $KHCO_3$ and acetic anhydride concentrations were doubled (Column 5). Acetylation of chlorophenols was also carried out in 100 mL 0.05M $KHCO_3$ so that the concentrations of each phenol and acetic anhydride were 10 times of the reference condition. Again, recoveries of chlorophenols remained the same (Column 6).

In an attempt to find the applicability of the method, recoveries of chlorophenols were determined over a wide range of phenol concentrations. It is obvious from Table 3 that essentially quantitative recoveries were obtained from 0.1 to 100 ppb chlorophenols. Under the conditions used, low recoveries of 2,3,5-, 2,4,5-, 2,3,4-, and 3,4,5-trichlorophenols as well as 2,3,4,5-tetrachlorophenol were observed when the

individual chlorophenol concentrations were 200 ppb. Similarly, at 0.01 ppb, generally lower (75–85%) recoveries were obtained for all chlorophenols other than a few exceptions. Recoveries of chlorophenols at 200, 100, and 10 ppb were obtained by diluting the extracts 200, 100, and 10 times, respectively, before they were compared with the 1 ppb standard. On the other hand, at 0.1 ppb level, the extract was compared with the 0.1 ppb standard obtained by a 10-fold dilution of the 1 ppb standard. For 0.01 ppb level, the sample extracts were concentrated from 10 to 1 mL before they were compared with the 0.1 ppb standard.

Recovery of Chlorophenols in Fortified Natural Water Samples

Water samples from Toronto Harbour, Hamilton Bay, and Lake Erie fortified at 10, 1, and 0.1 ppb were acetylated and extracted. Extracts were then analyzed by capillary column chromatography with electron capture detection using the 12 m OV-1 FSCC as described. Recoveries were calculated by comparison with a distilled water sample fortified at 1 ppb. Recoveries of all chlorophenols in each case were generally between 90 and 105% (Table 4). The above 3 natural waters had chlorophenol blanks less than 0.01 ppb for most isomers. However, a few exceptions showed the blank between 0.01 and 0.05 ppb. Silica gel column cleanup was applied to natural water samples fortified at 1 ppb or lower. For the natural water samples fortified to higher chlorophenol levels, this cleanup was usually not necessary. Figure 3 shows a typical chromatogram from a natural water sample fortified at 1 ppb.

Summary

A method is presented for the quantitative analysis of 15 chlorophenols in natural waters by in situ acetylation. This method is simple and involves no more steps than a method which requires no derivatization. The major advantages of the present method are that phenol acetates are much easier to recover from water samples and they are also easier to chromatograph on GC columns than free phenols. Both factors improve the reliability of the analytical data generated. This method applies to the di-, tri-, tetra-, and pentachlorophenols in natural waters from 0.01 to 100 ppb. Because of sensitivity limitations, this method does not apply to monochloro- and alkyl phenols at levels commonly found in environmental samples.

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Gas Chromatographic Method for Analysis of 2,4-D in Wheat: Interlaboratory Study

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A procedure is described for the determination of 2,4-D (2,4-dichlorophenoxyacetic acid) in dried green plant material. Samples are first extracted with dilute sodium hydroxide, and then after acidification and solvent extraction, the residues are methylated using boron trifluoride–methanol reagent. The methyl ester of 2,4-D is cleaned up on a Florisil column and quantitated using a gas chromatograph equipped with an electron capture detector. Six laboratories made quadruplicate determinations on control, dried green wheat check samples, on 4 similar samples fortified at the 0.50 ppm level, and on 4 samples fortified at the 1.00 ppm level with 2,4-D. Based on the data from 5 laboratories, the plant fortifications of 0.50 and 1.00 ppm yielded average interlaboratory recoveries of 2,4-D of 83.3 and 88.2%, respectively. The procedure also has potential for the determination of 2,4-D in wheat straw and wheat grain.

Ester and amine formulations of 2,4-D have been used as herbicides for almost 40 years; over this period, many methods have been described for their extraction from plant tissue and analysis (1–12). Although acidic, alkaline, and neutral organic solvents have been reported as suitable extractants for the recovery of 2,4-D residues from plant material, studies have shown (2, 7) that, because 2,4-D forms conjugated products with plant substituents, a hydrolytic stage is necessary to completely release the herbicide from plant material. For this reason, alkaline solutions are preferred for the extraction of 2,4-D from treated plant material (1, 2, 5). An added advantage to using alkaline extractants is that all ester and amine residues are converted to the phenoxyalkanoate anion (10).

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The present investigation was conducted to develop a procedure for determination of 2,4-D in dried green wheat tissue, wheat straw, and wheat grain; and then to subject the procedure to an interlaboratory analytical study by supplying several laboratories with samples of dried green wheat material fortified with 2,4-D.

Development of Method

The extraction procedure selected for further study was that of Jensen and Glas (5), whose method described the determination of 2,4-D in fresh green plant tissue. These authors reported recoveries of approximately 95% from samples fortified with 2,4-D at rates between 1.0 and 2000 ppm. These rates are high, and on a dry weight basis would be equivalent to about 4–5 times greater because the moisture content of grass is nearly 80%. Thus, it was felt that a more sensitive method was required that would allow detection of 0.2 ppm 2,4-D on dry plant material.

The procedure to be described is essentially that of Jensen and Glas (5) with 2 changes. One was the use of borontrifluoride–methanol reagent instead of the toxic diazomethane methylating reagent; however, experiments conducted by the author indicated that, within experimental error, the efficiency of methylation of 2,4-D acid was the same for borontrifluoride-methanol reagent (described below) as for diazomethane. The second modification was the use of a Florisil column for cleanup of 2,4-D methyl ester, because cleanup with the recommended alumina column (5) was unsuccessful.

Table 1. Results from control samples (blank) and samples fortified with 1.0 ppm 2,4-D

Substrate	Amount found, ppm[a]	
	Blank	Fortified samples[b]
Dried green wheat	0.04 ± 0.01	0.888 ± 0.038
Dried wheat straw	0.03 ± 0.02	0.913 ± 0.035
Wheat grain	0.03 ± 0.01	0.907 ± 0.038

[a]Mean and standard deviations from 4 replicate analyses.
[b]Mean blank subtracted.

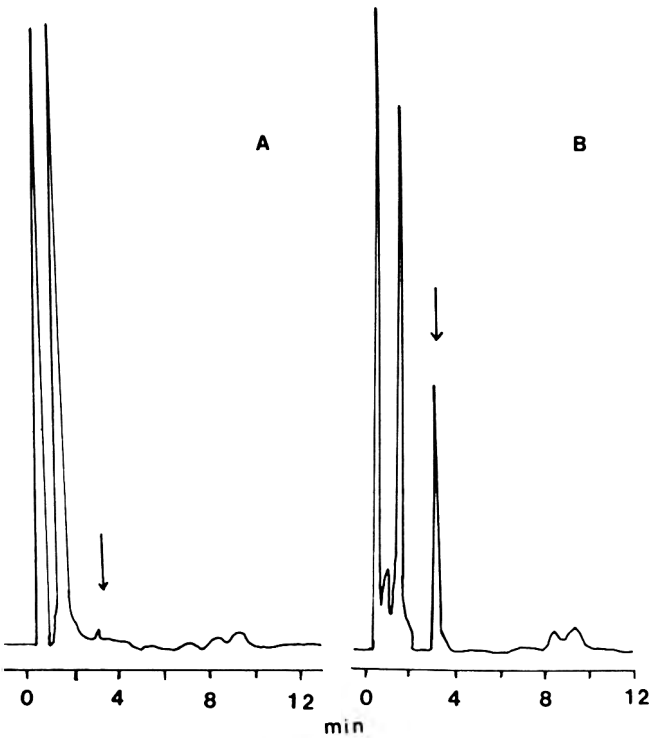

Figure 1. Gas chromatograms derived from A, control sample; and B, dried green wheat fortified with 1.0 ppm 2,4-D. A ^{63}Ni detector was used.

An Ultrabond 20M column was advised because of its excellence for analysis of phenoxy esters.

The present procedure was satisfactory for the recovery of 1.0 ppm 2,4-D from fortified samples of dried green wheat, from wheat straw collected at harvest, and from wheat grain. All samples were analyzed in quadruplicate and fortified 48 h before extraction and analysis. Extraction and subsequent analysis of untreated control samples indicated background amounts less than 0.05 ppm of 2,4-D. These results are summarized in Table 1. Typical chromatograms derived from control and fortified dried green wheat samples are shown in Figure 1. As a result of these preliminary studies, it was decided to further test the procedure by submitting samples of dried green whea
study.

Each participat
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4.0 g dried green
during July of 19
stage, by cutting at ground level. After being dried at 50 °C for 48 h in a vented oven, the plant material had achieved constant weight and was ground to pass a 1 mm screen. Of the 12 vials sent to each laboratory, 4 contained check (blank) samples, 4 contained samples fortified at the 0.50 ppm level, and 4 contained samples fortified at the 1.00 ppm level with 2,4-D. The samples were fortified by adding 2,4-D in solution (0.1 mg/mL methanol) directly to the dried plant material in the vials. No indication was given as to which vials contained untreated material and which contained fortified samples. Laboratories were requested to freeze the samples when received. A separate bag of untreated plant material was sent so that the analysts could use this material to familiarize themselves with the procedure.

Because of the difficulty in obtaining homogeneity with the fortified samples, analysts were requested to use the entire sample for each analysis, and to rinse the container with the 0.1N NaOH extraction solvent. Of the 12 laboratories that had initially agreed to participate in the study, results were received from six.

METHOD

Apparatus

(a) *Miscellaneous.*—Waring blender (or equivalent), wrist-action shaker, bench top centrifuge, hot water bath, and tank of purified nitrogen.

(b) *Glassware.*—250 mL glass-stopper flasks Ŧ 24/40, 50 mL glass-stoppered tubes Ŧ 24/40, 50 mL pyrex centrifuge tubes, 15 mL glass tubes graduated in 0.1 mL, glass chromatography columns (20 cm × 8 mm id) fitted with tap and reservoir, disposable Pasteur pipets, 25 mL separatory funnels.

Reagents

All chemicals should be ACS grade or equivalent.

(a) *Sodium bicarbonate.*—0.1N aqueous solution.

(b) *Sodium hydroxide.*—0.1N aqueous solution.

(c) *Hydrochloric acid.*—Concentrated.

(d) *Sodium chloride.*—Heated at 600°C for 24 h. Prepare saturated solution in water.

(e) *Organic solvents.*—Acetone, benzene, diethyl ether, and *n*-hexane should all be glass-distilled quality.

(f) *Diethyl ether–n-hexane.*—30 + 70.

(g) *n-Hexane–acetone.*—*n*-Hexane containing 0.5% acetone (v/v), *n*-hexane containing 1.5% acetone (v/v).

(h) *Borontrifluoride–methanol reagent.*—14% by weight.

(i) *Florisil.*—(Fisher Scientific Co., Fair Lawn, NJ). Heat at 600°C for 24 h. Deactivate by taking 95.0 g Florisil and adding 5.0 g water. Shake 1 h on wrist-action shaker, and then periodically for 24 h. Keep in tightly stoppered flask for no more than 2 weeks before preparing new batch from freshly heated Florisil.

(j) *2,4-D acid.*—(Dow Chemical Co., Midland, MI), ana-
stallized from mixture of ace-
ite needles. Needles dried at

ve recrystallized 2,4-D acid in
ntaining 1.0 and 0.1 mg acid/

I or ^{63}Ni electron detector and
lass column packed with 100–
rrier gas flow 40 mL/min, col-
tion time of 2,4-D methyl ester
is 5.50 min. Under typical conditions, with attenuation × 32, 1 ng 2,4-D methyl ester should give one-half scale deflection.

Extraction

Weigh 4.0 g sample and add to Waring blender; add 100 mL 0.1N NaOH. Blend at full speed for 2 min. Add contents of blender to 250 mL glass-stopper flask and shake on wrist-action shaker 30 min at full speed. Centrifuge ca 40 mL extract at 3500 rpm for 5 min. Take 25.0 mL extract (equivalent to 1.0 g plant material) and add to 50 mL glass tube; add 5 g sodium chloride, 1.5 mL HCl, and 20 mL ether–hexane mixture. Stopper tube and shake vigorously by hand for 1 min; add contents of tube to clean centrifuge tube and spin at 3500 rpm 2 min. Remove organic layer by using disposable pipet and place in fresh glass-stopper tube containing 15 mL 0.1N $NaHCO_3$. (After acidfication, proceed with ether–hexane extraction as fast as possible because plant components are precipitated on acidification and 2,4-D acid can adsorb to these components.)

Shake ether–hexane–bicarbonate mixture 1 min by hand and, after settling, discard organic layer by using pipet. Repeat extraction of centrifuged plant extract with additional 20 mL portion of ether–hexane mixture as before; then partition with the same bicarbonate solution used above. Discard organic layer. Add 10 mL diethyl ether to bicarbonate solution, shake by hand 1 min, and then discard ether layer by using pipet. Add 5 g NaCl to bicarbonate solution, acidify with 1.5 mL HCl, and shake gently until no more CO_2 evolves. Add 15 mL diethyl ether and shake by hand 1 min. After settling, remove ether by using pipet and place in 50 mL glass tube. Add additional 15 mL portion of diethyl ether to acidified bicarbonate solution and shake as before, adding ether extract to previous extract. Evaporate combined ether extracts to dryness at 45°C, using water bath and stream of nitrogen. Add 2 mL benzene to tube and evaporate as above to completely remove any remaining moisture.

Methylation

Add 5.0 mL borontrifluoride–methanol reagent down sides of glass tube to rinse all residues to bottom. In fume hood, immerse tube in hot water bath at 65°C for 40 min. Cool tube in iced water. Add 10 mL saturated NaCl solution to tube and add contents to 25 mL separatory funnel. Rinse tube with 10 mL water and add rinse to funnel. Extract with 5.0 mL *n*-hexane, shaking 1 min. Pour aqueous phase into fresh 25 mL separatory funnel. Add hexane layer, using disposable pipet, to top of Florisil column. Add 3.0 mL *n*-hexane to first separatory funnel and rinse funnel, adding rinsings to second separatory funnel. Shake for 1 min, and then add hexane layer to Florisil column by using pipet.

Florisil Cleanup

Put glass wool plug in chromatography column and fill column with *n*-hexane. Add 2.0 g 5% deactivated Florisil. Add 1 cm heated NaCl to top of column. Drain away hexane. Prepare columns immediately before use. As soon as hexane has drained, add 5.0 mL hexane solution containing possible 2,4-D methyl ester. When this has eluted, add 3.0 mL rinsings. Elute with 10 mL hexane containing 0.5% acetone at 2 mL/min. Discard this fraction. Elute 2,4-D methyl ester with sufficient hexane containing 1.5% acetone at 2 mL/min so that all ester is eluted.

(Florisil (5% deactivated) columns seem to be satisfactory, but 2,4-D methyl ester must remain on the column less than 10 min, or hydrolysis of the ester occurs. If the ester remains on such columns for 10 min or less, recovery of ester is 98%. If residence time is 30 min, then recovery is less than 80%. Each batch of deactivated Florisil must be calibrated for elution pattern of 2,4-D methyl ester to ensure that no elution occurs with the 10 mL hexane containing 0.5% acetone, and to determine the amount of hexane containing 1.5% of acetone necessary to elute the 2,4-D methyl ester.)

Collect this fraction in graduated tube. Evaporate solvent in gentle stream of nitrogen, using water bath set at 45°C, to 5.0 mL. Inject 5 μL portions onto gas chromatograph.

Table 2. Results from control samples (blank) in interlaboratory test of method for 2,4-D in dried green wheat

Lab.	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean
1	0.139	0.056	0.044	0.044	0.071
3[a]	0.215	<0.05[b]	<0.05	0.275	0.135
4	0.00	0.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00	0.00
7	0.060	0.063	0.055	0.039	0.054
10	<0.05	<0.05	<0.05	<0.05	0.025

[a]Cochran outlier.
[b]All data reported as <0.05 taker as 0.025 for calculations.

Preparation of 2,4-D Methyl Ester Standards

Take 25 μL 2,4-D acid standard (25 μg) and methylate with borontrifluoride–methanol reagent as described above for 40 min. After cooling, add 10 mL saturated NaCl solution and 25.0 mL *n*-hexane and shake for 1 min. Decant hexane layer and use this as standard. This solution contains the methyl ester of 2,4-D equivalent to 1.0 ng acid/μL hexane. Prepare standard containing 0.1 ng/μL by dilution.

Results and Discussion

For the interlaboratory study, the fortification levels of Samples 5–8 (0.50 ppm 2,4-D acid) and samples 9–12 (1.00 ppm 2,4-D acid) were selected to reflect the much smaller amounts of herbicide that may be present under field conditions. At the time of collection, the green wheat plants were at the heading stage and had a moisture content of 79%. Since the 4.0 g dried samples represented 19 g freshly collected field sample, the fortification rates based on undried field matter were thus equivalent to 0.105 and 0.210 ppm, respectively.

To recover 2,4-D from plant material, it is important to use an alkaline extractant which can also hydrolyze any conjugated materials that may be formed between the herbicide and plant constituents, and convert any 2,4-D esters on the crop to the phenoxyalkanoate anion. For this reason the combination of an alkaline extraction involving a 2 min blending procedure followed by a 30 min shaking, as recommended by Jensen and Glas (5), was considered necessary.

Laboratories 3 and 6 noted that the method was time consuming and laboratory 10 considered that a high degree of technical ability was required. However, the use of stoppered tubes and centrifuge tubes kept the transfer of liquids between glassware to a minimum. The ether–hexane extractant was both effective in recovering 2,4-D from acidified plant extract and prevented the formation of intractable emulsions. Partitioning of the ether–hexane extracts with bicarbonate solution removed the 2,4-D into the aqueous medium while colored plant pigments were retained in the organic phase.

Differences were experienced between the various batches of Florisil used by the different laboratories. No laboratories reported any elution of 2,4-D methyl ester with 10 mL hexane containing 0.5% acetone. Laboratories 3, 6, and 10 used 10 mL hexane containing 1.5% acetone to completely elute the methyl ester of 2,4-D from their Florisil columns, while Laboratories 1, 4, and 7 had to use 22, 20, and 18 mL, respectively, of eluting solvent. In all cases, elution of the methyl

Table 3. Interlaboratory results from samples fortified with 0.5 ppm 2,4-D

	Amount found, ppm				Amount (ppm) with lab. mean blank subtracted			
Lab.	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 1	Rep. 2	Rep. 3	Rep. 4
1	0.407	0.407	0.432	0.432	0.336	0.336	0.361	0.361
3[a]	0.510	1.30	1.02	0.525	—	—	—	—
4	0.325	0.345	0.330	0.315	0.325	0.345	0.330	0.315
6	0.470	0.521	0.488	0.502	0.470	0.521	0.488	0.502
7	0.479	0.466	0.464	0.446	0.425	0.412	0.410	0.392
10	0.50	0.50	0.50	0.60	0.475	0.475	0.475	0.575

Statistical Analysis

Added, ppm	0.5
Av. recovery, ppm	0.416
Av. recovery, %	83.3
Data sets used	5
S_o	0.027
S_L	0.079
S_x	0.083
% CV_o	6.37
% CV_L	18.91
% CV_x	19.95

[a]Cochran outlier; results not included in statistics.

Table 4. Interlaboratory results from samples fortified with 1.0 ppm 2,4-D

	Amount found, ppm				Amount (ppm) with lab. mean blank subtracted			
Lab.	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 1	Rep. 2	Rep. 3	Rep. 4
1	1.040	1.041	1.144	0.907	0.969	0.970	1.073	0.836
3[a]	—[b]	1.43	0.608	1.38	—	—	—	—
4	0.645	0.690	0.675	0.710	0.645	0.690	0.675	0.710
6	0.856	0.884	0.884	0.928	0.856	0.884	0.884	0.928
7	0.826	0.900	0.900	0.847	0.772	0.846	0.846	0.793
10	0.93	1.10	1.14	1.20	0.905	1.075	1.115	1.175

Statistical Analysis

Added, ppm	1.0
Av. recovery, ppm	0.882
Av. recovery, %	88.2
Data sets used	5
S_o	0.072
S_L	0.142
S_x	0.160
% CV_o	8.16
% CV_L	16.14
% CV_x	18.09

[a]Cochran outlier; results not included in statistics.
[b]Samples lost.

ester of 2,4-D appeared to be quantitative indicating that, provided the residence time of ester on columns prepared from different batches of Florisil was less than 10 min, minimal hydrolysis to 2,4-D acid occurred.

The results from the interlaboratory study are summarized in Tables 2–4. According to the Cochran test, Laboratory 3 was judged to be an outlier and the data from this source were omitted from all statistical analyses.

For the control samples, where the results were reported to be <0.05 ppm, a figure of 0.025 ppm was used in the calculations. The mean results from each laboratory for the analysis of the blank control samples (Table 2) indicated a range from 0.000 to 0.135 ppm, probably reflecting interferences from laboratory reagents. The mean control values for each laboratory (Table 2) were subtracted from each of its own analytical results obtained for the 8 fortified samples. In this way, results from laboratories experiencing interferences were corrected to a greater degree than those from laboratories that did not.

Table 3 statistically summarizes the reported analytical data from analysis of dried wheat samples fortified at the 0.50 ppm level with 2,4-D. An average recovery of 83.3% was obtained as the mean of 20 analyses with an interlaboratory standard deviation (S_L) of 0.079, and an intralaboratory standard deviation (S_o) of 0.027. For the samples fortified with 1.00 ppm 2,4-D, the average recovery was 88.2% (Table 4), and the inter- and intralaboratory standard deviations were 0.142 and 0.072, respectively.

Although all laboratories reported use of a ^{63}Ni electron capture detector for the gas chromatographic analysis, not all laboratories were able to use Ultra-Bond 20M gas chromatographic columns. Laboratory 3 used a 5% Carbowax 20M TPA column, while Laboratories 7 and 10 both used 5% Dexsil 300 coated on Chromosorb W HP. Laboratory 3 proved to be an outlier, so it is possible that some of the experimental errors may be attributed to the choice of column packing. The results obtained by Laboratories 7 and 10 using Dexsil gas chromatographic columns (Tables 3 and 4) indicate that, at both fortification levels, 2,4-D recoveries recorded by Laboratory 7 were slightly lower than the average recovery figures, while those for Laboratory 10 were slightly higher. This suggests that the choice of column between between Ultra-Bond 20M and Dexsil 300 was not likely to affect the recovery results.

Using the described procedure, an estimated quantitation limit of 0.20 ppm of 2,4-D in dried green wheat tissue was

considered feasible. Thus, the method appears to have potential as an analytical procedure for the analysis of 2,4-D in wheat material.

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Simple Analytical Method for Organophosphorus Pesticide Determination in Unpolished Rice, Using Removal of Fats by Zinc Acetate

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A rapid and simple method is developed for the determination of organophosphorus pesticides in unpolished rice. The new method incorporated acetonitrile–water (1 + 1) extraction, removal of fats by zinc acetate, and further cleanup on an activated charcoal chromatographic column. The higher fatty acids in the extract react rapidly with zinc acetate to form insoluble zinc carboxylates, which precipitate. Additional interferences were cleaned up on an activated charcoal chromatographic column, and organophosphorus pesticides adsorbed on the activated charcoal were eluted with acetone–hexane. Dimethoate is not retained on the activated charcoal and must be extracted with dichloromethane from the first acetonitrile–water eluate. Pesticides are measured by flame photometric gas chromatography. Recoveries from 50 g unpolished rice samples fortified with 5–50 μg diazinon, 6–30 μg parathion, 8–40 μg fenitrothion and IBP, 10–50 μg dimethoate and fenthoate, 20–100 μg malathion, or 40–200 μg EPN ranged from 75.7 to 95.8%.

The analytical methodology for extraction, cleanup, and determination of chlorinated and organophosphorus pesticide residues in a variety of food samples had been developed using liquid-liquid partition and column chromatography prior to gas chromatography. Bertuzzi et al. (1) and Burke et al. (2) confirmed that the use of 35% water in acetonitrile resulted in improved extraction of residues from low-moisture plant material. It was further demonstrated by Wheeler et al. (3) that the effectiveness of acetonitrile as an extracting solvent for nonfatty foods increased with increasing water content and that the optimum water content of acetonitrile–water extraction mixtures was 40–50%. It was determined in the present study that these extracting solvents were also effective for extraction of organophosphorus pesticides from unpolished rice.

Cleanup techniques have been focused on the separation of pesticides from fats and oils. The ordinary cleanup methods that have been adopted, such as the AOAC method (4) and the Japanese official method (5), were based on acetonitrile-hexane partition and adsorption chromatography with activated charcoal. Although a major proportion of fats is removed by the partition technique, the removal of fats remains a problem.

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Higher fatty acids react with metal ions, with the exception of Na and K, to form insoluble metal carboxylates that precipitate as "metal soap." To remove fats from extracts, this reaction was applied to the cleanup technique and a simple and rapid analytical method for organophosphorus pesticide residue determination in unpolished rice was developed.

METHOD

Reagents

(a) *Pesticide grade solvents.*—Acetonitrile, acetone, hexane, and dichloromethane (Wako Pure Chemical Industry).

(b) *Activated charcoal.*—As described in Ref. (6).

(c) *Zinc acetate.*—$Zn(OAc)_2$ analytical reagent grade.

(d) *Extracting solution.*—Acetonitrile–water (1 + 1).

(e) *Eluting solution.*—Acetone–hexane (1 + 1).

(f) *Filter paper.*—Whatman No. 42.

(g) *Pesticide standard solution.*—Standards (>95%) were obtained from Wako Pure Chemical Industry. Prepare acetone solution at the following levels (μg/mL): diazinon, 5; fenitrothion, 8; parathion, 6; malathion, 20; dimethoate, 10; IBP (*S*-benzyl-diisopropyl phosphorothioate), 8; fenthoate, 10; and EPN, 40.

Apparatus

(a) *Extraction cylindrical separatory funnel.*—250 mL, 22 × 4 cm id glass tube with glass stopper, stopcock, coarse fritted glass disk in lower part, and Ŧ 24/40 lower joint.

(b) *Pulverizer.*—Type SK-M3 (Kyoritsu Riko Ltd, Tokyo, Japan).

(c) *Mechanical shaker.*—Models V-S and V-P (Iwaki Co., Ltd, Tokyo, Japan).

(d) *Activated charcoal chromatographic column.*—As described in ref. (6).

(e) *Gas chromatograph.*—Model 7A-PFP (Shimadzu Corp., Kyoto, Japan) with FPD (flame photometric detector) (P-mode) and 2 m × 3 mm id glass column packed with 10% DC-200 on 60–80 mesh Chromosorb WAW DMCS (Gas Kuro Kogyo Ltd, Tokyo, Japan). Operating conditions: temperatures (°C)—column, 210; injection port and detector, 230;

gas flow (mL/min)—nitrogen carrier, 50; hydrogen, 60; and air, 50.

Procedure

Weigh 50 g finely ground unpolished rice into 250 mL extraction cylindrical separatory funnel, add 100 mL acetonitrile–water (1 + 1), and shake vigorously 10 min on mechanical shaker. Connect side-arm vacuum adapter and 500 mL glass-stopper Erlenmyer flask to separatory funnel. Let stand 10 min and drain extract into receiving flask by gravity. When rate of dripping is slow, apply gentle suction. Re-extract residue with 100 mL acetonitrile–water (1 + 1) and collect ca 180 mL extract. Add 120 mL water and 1 g zinc acetate. Shake vigorously 10 min on wrist-action mechanical shaker. To remove zinc carboxylate precipitate, filter extract mixture through paper into 500 mL Erlenmyer flask. After washing precipitate on filter paper with small portion of acetonitrile–water (1 + 1), pour extract into 0.5 g activated charcoal column, wash column with additional 10 mL water. Transfer eluate to 500 mL separatory funnel and extract dimethoate with 100 mL dichloromethane by shaking 5 min on mechanical shaker. Add 100 mL acetone–hexane (1 + 1) to column and collect this fraction. Transfer this fraction to 500 mL separatory funnel containing 100 mL 5% NaCl solution. Shake 5 min on mechanical shaker and separate layers. Re-extract aqueous layer with 50 mL hexane and wash combined hexane extracts with 50 mL water. Dry dichloromethane and hexane extracts over anhydrous sodium sulfate and concentrate to 2–5 mL in Kuderna-Danish concentrator. Inject suitable aliquot of the 2 concentrates into gas chromatograph and compare retention times and peak heights of samples with those of standards for quantitation. The fatty acid content was determined as oleic acid by titration with 0.1N KOH.

Results and Discussion

Because dried and bulky unpolished rice absorbs the extracting solution, filtration must be aided by vacuum to separate as much extract as possible. Therefore, we devised a separatory funnel fitted with a fritted glass thimble to extract twice successively and rapidly. The extracting solution used could extract the tested organophosphorus pesticides efficiently without co-extracting large amounts of fats from unpolished rice.

Fifty g unpolished rice contains approximately 1 g fat. Two extractions with 100 mL acetonitrile–water (1 + 1) contain 150–200 mg fat as oleic acid. An almost equal amount of fat was found in the acetonitrile extract after the acetonitrile–hexane partition.

Less than 50% acetonitrile in water caused difficulty in filtering the extract because of water-soluble co-extracting substances.

Rice oil usually contains 58% oleic acid, 22% palmitic acid, and 17% linoleic acid. To remove these higher fatty acids, zinc acetate was used as a suitable reagent in the present study. Because zinc acetate reacts easily and quantitatively with these higher fatty acids, the fats can be removed from the extract by adding excess zinc acetate and filtering the precipitate through filter paper. Acetic acid is generated by this reaction, but the dissociation of acetic acid is suppressed by excess zinc acetate. Accordingly, the pH remains in the range 5–6 and does not cause degradation of organophosphorus pesticides. Zinc chloride generates hydrogen chloride and the pH decreases to about 3, so the precipitation reaction could not proceed without the addition of alkali.

Table 1. Adsorption (%) on 0.5 g activated charcoal of organophosphorus pesticides from 300 mL acetonitrile–water at different ratios

Pesticide	Added, μg	Acetonitrile concn, v/v% 50	40	30
Dimethoate	20	0	0	0
Diazinon	10	60	98	104
IBP	16	44	92	98
Fenitrothion	16	84	94	97
Malathion	40	42	94	98
Parathion	12	88	98	102
Fenthoate	20	89	100	100
EPN	80	74	81	84

Table 2. Recovery (%) of organophosphorus pesticides from fortified unpolished rice samples

Pesticide	Fortification level, ppm	Recovery, mean ± SD[a]
Dimethoate	0.2	95.8 ± 4.0
	1	94.6 ± 3.2
Diazinon	0.1	92.8 ± 2.3
	0.5	93.7 ± 2.2
IBP	0.16	91.3 ± 2.0
	0.8	93.6 ± 2.7
Fenitrothion	0.16	89.2 ± 0.8
	0.8	91.6 ± 3.0
Malathion	0.4	93.2 ± 1.4
	2	94.0 ± 1.5
Parathion	0.12	87.5 ± 1.8
	0.6	88.8 ± 0.8
Fenthoate	0.2	89.9 ± 1.7
	1	91.5 ± 1.3
EPN	0.8	77.7 ± 3.3
	4	78.0 ± 2.4

[a]Based on triplicate analyses.

To assure the pesticide extraction efficiency during cleanup of samples containing high quantities of free fatty acids, 5 g zinc acetate was added to 200 mL acetonitrile–water (30 + 70) containing 5 g oleic acid and 2 mL pesticide standard solution. The recoveries of pesticides from the procedure as described above were greater than 80%. Moreover, losses of organophosphorus pesticides caused by addition of zinc acetate and adsorption on the precipitate were not observed. Also, the oleic acid peak did not appear in FID gas chromatogram.

As a further cleanup procedure, the activated charcoal column chromatography described in the estimation of chlorinated pesticide residues in milk was adopted (6). It was quite effective for elimination of colored substances and foaming water-soluble substances. They adsorbed strongly on activated charcoal and were not removed with the 100 mL acetone–hexane (1 + 1) eluting solution, which yielded good recovery of the organophosphorus pesticides tested.

To confirm the adsorption efficiency of activated charcoal, 300 mL acetonitrile–water at different ratios fortified with 2 mL organophosphorus pesticide standard solution was added to a 0.5 g activated charcoal column. The percentages of organophosphorus pesticides retained on the activated charcoal are shown in Table 1. To retain the organophosphorus pesticides tested, the acetonitrile concentration must be adjusted to 30–40%. Dimethoate was entirely eluted with 300 mL acetonitrile–water (30 + 70); therefore, it must be extracted with dichloromethane from this eluate. Further washings of dichloromethane extract might give poor recovery of dimethoate.

To show the advantages of the proposed method, we compared it with the existing Japanese official method (5).

The recoveries of organophosphorus pesticides from actual unpolished rice were studied at 2 fortification levels. These

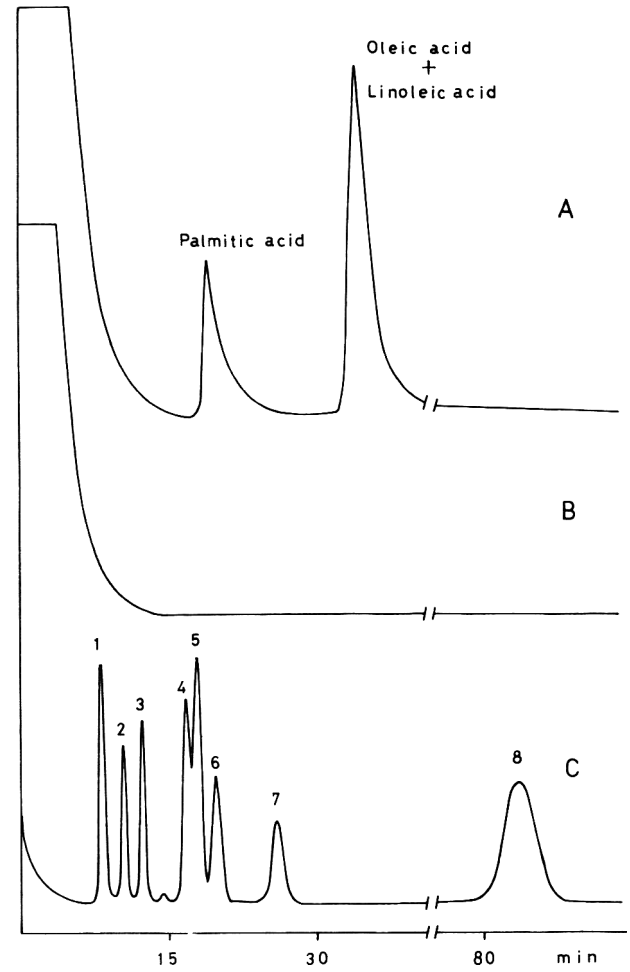

Figure 1. Gas chromatograms: A, FID, existing method; B, FID, proposed method; C, FPD, 1 μL pesticide standard solution. Peaks: 1, dimethoate; 2, diazinon; 3, IBP; 4, fenitrothion; 5, malathion; 6, parathion; 7, fenthoate; 8, EPN.

average recoveries were satisfactory, as shown in Table 2, and almost identical to those of the existing method. But when IBP was present as an incurred residue in unpolished rice, the proposed method determined 166 ppb, whereas the existing method determined 96 ppb. Both were the average of 3 analyses. This difference might be ascribed to the efficiency of the extracting solution used and the simplicity of the procedure.

As shown in Figure 1, the proposed method has no peaks of free fatty acids in the FID (flame ionization detector) gas chromatogram, but the existing method gives peaks for palmitic acid, oleic acid, and linoleic acid. Although such a small portion of fatty acids would probably not affect the FPD gas chromatographic determination of organophosphorus pesticide residues, it would have some adverse effect on the gas chromatographic column and detector, as pointed out by Sonobe et al. (7). The final solution prepared by the existing method was oily, but such was not observed in the proposed method. Accordingly, the proposed method would allow lower detection limits of organophosphorus pesticides because of the ability to concentrate samples further and/or use larger injection volumes.

For 50 g sample, 2 mL final solution, and 5 μL injection, detection limits for organophosphorus pesticides determined by extrapolation are diazinon, 4; fenitrothion, 6.4; parathion, 4.8; malathion, 16; dimethoate, 8; IBP, 6.4; fenthoate, 8; and EPN, 32 ppb.

Finally, the simple and rapid proposed method involved only a single concentration step, and the complete removal of fats may be applicable to other sample types and to the analysis of food additives and contaminant chemicals.

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MICROBIOLOGICAL METHODS

Genetic Methods for the Detection of Microbial Pathogens. Identification of Enterotoxigenic *Escherichia coli* by DNA Colony Hybridization: Collaborative Study

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Enteropathogenic *Escherichia coli* strains may produce a cholera-like, heat-labile enterotoxin (LT) as a virulence factor. The gene that codes for LT can be purified by recombinant DNA techniques and used as a genetic probe for DNA hybridization. These probes detect enterotoxigenic strains as well as strains that may not manifest toxin production but carry the genetic information to do so. In this study, 13 laboratories tested 3 known and 25 unknown (10 positive and 15 negative) cultures of *E. coli* for the presence of the LT gene. The isolates had been tested and classified by the mouse Y-1 adrenal cell test and an enzyme-linked immunosorbent assay. Cultures were spotted on nitrocellulose filters on MacConkey agar and incubated. Colonies were lysed in situ and their DNA was hybridized to ^{32}P-labeled, purified LT gene DNA (provided to the collaborators). Positive colonies were identified by autoradiography. Of 325 samples, 315 (96.9%) were identified correctly and 10 were misclassified; there were 6 false negative and 4 false positive identifications. Chi-square values indicated that the method agreed with the previous classification and was equally efficient in distinguishing positive and negative samples (95.7 and 98.1%, respectively). The method has been adopted official first action.

Not all isolates of *Escherichia coli* should be considered "normal flora" or mere indicators of fecal pollution. Some strains cause disease in humans (1) and in certain domestic animals (2). Several mechanisms may play a role in the pathogenicity of *E. coli* (3): enterotoxin production (4–6), species-specific colonization factors (7–9), and the ability to invade tissues and cause parenteral infections (10–12). However, the actual sequence of events and interactions of these characteristics has not been determined in human disease (13–16). Because these properties are plasmid-mediated (9, 17–22), the phenotypes may be unstable (23, 24).

Enterotoxigenic *E. coli* cells have been isolated from sources implicated in foodborne outbreaks (25–27) and are not uncommon in certain foods (28, 29). There are several methods to test for production of enterotoxins. The cholera-like, heat-labile enterotoxin (LT) (30–32) can be detected by morphological changes in cultured mouse Y-1 adrenal cells (33–35), by an enzyme-linked immunosorbent assay (36–38), or by radioimmunoassay (39, 40). Heat-stable toxins (ST) may be detected by measuring intestinal fluid accumulation in the suckling mouse (41, 42) or suckling pig (43, 44). Comparison of these methods (45) is confounded by strain differences in both the LT and ST produced (46–49), i.e., the toxins themselves may show heterogeneity, and laboratory conditions that provide consistent production are often difficult to establish (50–54).

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The recommendation of the Associate Referee was approved by the General Referee and Committee F and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) **67**, March.

W. E. Hill received the award for the best Associate Referee Report of the Year at the 1983 Annual International Meeting of AOAC.

Advances in recombinant DNA methodology have made it possible to clone some virulence factors of *E. coli* (55, 56). The responsible gene fragments are isolated, purified, radioactively labeled (57), and used as probes in DNA colony hybridization to detect isolates that carry the genetic information for pathogenic determinants. The method has been applied to clinical isolates (58, 59) and recently used to detect and enumerate enterotoxigenic strains in seeded food samples (60).

The relative advantages and disadvantages of DNA colony hybridization have been compared with traditional assays for virulence (61). Among the most important advantages are the detection of virulent strains in mixed cultures without selective enrichment, convenience in screening large numbers of isolates, and freedom from variable gene expression in the laboratory. Eliminating selective enrichment could lead to more frequent recovery of pathogenic *E. coli*, because present methods may select against these strains (24, 62, 63). To determine if pathogenic bacteria can be identified reliably by DNA colony hybridization, a collaborative study was conducted to classify cultures of *E. coli* according to their potential for producing LT.

In theory, these procedures may be used for any segment of DNA that has been cloned. However, the details presented here refer to the detection of *E. coli* strains that possess the genetic potential to produce LT. The screening of other bacterial species for virulence factors may involve critical changes in parameters, such as conditions for cell growth, the restriction endonucleases used to produce the specific DNA fragment(s), and the electrophoretic procedures used to purify the fragment(s).

It is critical that the final reagent (i.e., the radioactive DNA fragment used for DNA hybridization) be tested with known positive and negative cultures to ensure that it is specific for the particular trait being examined. In the present study, collaborators were supplied with the radioactively labeled LT gene DNA and sonicated calf thymus DNA.

Bacterial Culture Handling

Collaborators were instructed to prepare all microbiological media according to the manufacturer's instructions. To maintain virulence, bacterial cultures were shipped on agar slants, such as brain heart infusion (BHI) agar, by overnight carrier. Immediately upon receipt, they were subcultured into rich broth medium, e.g., BHI broth, and incubated overnight at 37°C. A 2 mL portion of the overnight culture was added to 1 dram vials containing 0.5 mL 50% (w/v) sterile glycerol. Several replicates of each culture were made. Vials were labeled and stored frozen at ≤ -70°C. If an ultralow temperature freezer was not available, cultures were stored several weeks at -20°C, provided that the freezer was *not* frost-free, to avoid repeated freezing and thawing. When cultures were to be tested, a small amount of material was scraped from

the surface of the frozen culture and inoculated into broth for overnight incubation at 37°C. Vials could be used about 10 times in this manner.

Detection of *Escherichia coli* Producing Heat-Labile Enterotoxin

DNA Colony Hybridization Method

First Action

(*Caution:* This procedure uses radioactive and mutagenic compounds. Personnel must receive adequate training and monitoring and have proper facilities available for handling these substances.)

Method Performance

Results	*Percent*	*95% Confidence Range (approx.)*
Correct	96.9	95–99
False positive	2.1	1–5
False negative	4.6	1–11

Of 13 laboratories, 8 (62%) correctly identified all unknown samples (25/25); 11 laboratories (85%) identified ≥96% of the samples.

Principle

Isolated and purified genes (DNA) that code for determinants of bacterial virulence can be used to detect pathogenic strains. Specific fragments of DNA are isolated by cleaving plasmid DNA with appropriate restriction endonucleases and sepg resulting pieces by gel electrophoresis. Purified fragments are radioactively labeled in vitro. Bacterial cultures to be tested are spotted on nitrocellulose filters in agar medium and incubated until colonies are visible. Cells are lysed in situ, DNA is fixed to filter, and radioactive virulence gene DNA fragments are added. Colonies which contain same gene as radioactive DNA will bind this DNA and become radioactive. These colonies are detected by autoradiography.

Reagents

(Prep. all media according to manufacturer's instructions.)

(a) *10X M9 Salts.*—Dissolve 10 g NH_4Cl, 60 g Na_2HPO_4, 30 g KH_2PO_4, and 5 g NaCl in final vol. of 1 L H_2O. Dispense into 100 mL aliquots and autoclave 15 min at 121°.

(b) *Amplification medium.*—Sterilize all components sep. Aseptically combine 100 mL 10X M9 salts (a), 835 mL H_2O, 10 mL 0.1M $MgSO_4$, 10 mL 0.01M $CaCl_2$, 25 mL 20% (w/v) casamino acids, 20 mL 20% (w/v) glucose, and 0.2 mL thiamine (10 mg/mL).

(c) *TE buffer.*—Combine 10 mL 1.0M Tris-HCl (tris-hydroxy-methyl aminomethane HCl) and 2 mL 0.5M Na_2EDTA. Adjust to pH 8.0 with 10N NaOH. Add H_2O to final vol. of 1 L.

(d) *TES buffer.*—Combine 30 mL 1.0M Tris, 10 mL 0.5M Na_2EDTA, and 10 mL 5.0M NaCl. Add H_2O to final vol. of 1 L.

(e) *CsCl saturated isopropanol.*—Add ca 50 mL TE buffer (c) to ca 350 mL isopropanol. Add solid CsCl (reagent or optical grade) until bottom layer is satd.

(f) *Triton lytic mix.*—Add 0.1 mL Triton X-100, 5 mL 1.0M Tris, pH 8.0, and 12.5 mL 0.5M Na_2EDTA, pH 8.0, to H_2O (final vol. 100 mL).

(g) *10X TBE electrophoresis buffer.*—Dissolve 108 g Tris, 9.3 g Na_2EDTA, and 55 g boric acid in ca 800 mL H_2O. Adjust pH to 8.2 with concd HCl and bring final vol. to 1 L with H_2O.

(h) *10X HindIII reaction buffer.*—Combine 50 mL 1.0M Tris, pH 8.0, 10 mL 1.0M $MgCl_2$, 10 mL 5.0M NaCl, and 10 mL 100 mM dithiothreitol in final vol. of 1 L H_2O.

(i) *Stop soln.*—Combine 1.0 mL 10% (w/v) sodium dodecyl sulfate, 10 mg bromophenol blue, 2 mL 0.5M Na_2EDTA, pH 8.0, 5 g glycerol in a final vol. of 10 mL of H_2O.

(j) *10X nick translation buffer.*—Combine 500 μL 1.0M Tris, pH 7.8, 50 μL 1.0M $MgCl_2$, 7 μL 2-mercaptoethanol, and 500 μL nuclease-free bovine serum albumin (1 mg/mL). (Commercially available nick translation kits contain similar reagents. Follow supplier's instructions.)

(k) *Hybridization mixture.*—Combine 22 mL distd formamide, 12.5 mL 20X SSC (l), 0.5 mL 10% (w/v) sodium dodecyl sulfate, 5.0 mL 10X Denhardt's soln (m), 0.1 mL 0.5M Na_2EDTA, pH 8.0, and 9.9 mL H_2O.

(l) *20X Standard saline citrate soln (SSC).*—Add 175.4 g NaCl and 88.2 g Na citrate to final vol. of 1 L H_2O. 5X and 2X SSC may be prepd by dilg 20X SSC with H_2O.

(m) *10X Denhardt's soln.*—Combine 2.0 g Ficoll (400,000 mol. wt), 2.0 g polyvinyl pyrrolidone (360,000 mol. wt), and 2.0 g nuclease-free bovine serum albumin in 1 L H_2O. Store 5 mL aliquots at −20°.

(n) *Calf thymus DNA.*—Dissolve 1 g purified calf thymus DNA in 100 mL H_2O by stirring for several hours. Sonicate until av. mol. wt is 300,000–500,000 which may be detd by electrophoresis with appropriate stds. Store in 1 mL portions at −20°.

(o) *Brain heart infusion broth.*—Prep. and sterilize according to supplier's instructions.

(p) *DE 52 column chromatography medium.*—Prep. according to manufacturer's instructions in loading buffer (q).

(q) *DE 52 loading buffer.*—Combine 30 mL 5.0M NaCl, 0.2 mL 0.5M Na_2EDTA, and 10 mL 1.0M Tris (pH 8.0) in final vol. of 1 L H_2O.

(r) *DE 52 eluting buffer.*—Combine 200 mL 5.0M NaCl, 0.2 mL 0.5M Na_2EDTA, and 10 mL 1.0M Tris (pH 8.0) in final vol. of 1 L H_2O.

(s) *Sephadex G-50 column chromatography medium.*—Prep. according to manufacturer's instructions in TE buffer (c).

Apparatus and Materials

(a) *Preparative ultracentrifuge and fixed angle rotor.*—100,000 × g and 13 mL tubes.

(b) *Shaker.*—In 37 ± 1° H_2O bath with clips for holding 1 L erlenmeyers.

(c) *Longwave ultraviolet lamp.*—302 nm transilluminator preferred. Camera for photographing gels is useful.

(d) *Refrigerated superspeed centrifuge and fixed angle rotor.*—37,000 × g and −20°, capable of holding 50 mL tubes and adapters for 15 or 30 mL tubes.

(e) *Siliconized glass tubes.*—15 or 30 mL capable of withstanding 10,000 × g.

(f) *Spectrophotometer or colorimeter and sample holder.*—Measure bacterial cell growth at 550 or 600 nm.

(g) *Escherichia coli strain C600(pEWD299)(ATCC 37218).*—Contains cloned heat-labile enterotoxin gene. Pos. and neg. strains such as *E. coli* H10407(ATCC 35401) and plasmid pBR313 (ATTC 37018) are needed as controls during hybridization.

(h) *Alpha-^{32}P deoxycytosine triphosphate.*—dCTP, 2000–3000 Ci/mmole, aq. stabilized (ICN Pharmaceuticals, Irvine, CA; New England Nuclear, Boston, MA; Amersham, Arlington Heights, IL).

(i) *Ultralow temperature freezer.*—Capable of −70° is preferred; however, −20° (not frost-free) may be substituted.

(j) *Vacuum desiccator.*—Large enough to contain 15 or 30 mL tubes.

(k) *Polycarbonate tubes.*—50 mL.

(l) *Variable volume micropipettors and tips.*—To cover range of 1–1000 μL.

(m) *Electrophoresis apparatus.*—Horizontal and vertical units with bed dimensions ca 12 × 12 cm and appropriate power supplies (to 125 mA; 200 V).

(n) *Incubator.*—H_2O bath or dry heating block capable of maintaining 37 ± 1°.

(o) *Plastic conical centrifuge tubes.*—500 and 1500 μL sizes able to withstand 15,000 × g with appropriate racks.

(p) *Centrifuge.*—For spinning tubes (o) at greater than 10,000 × g.

(q) *Dialysis tubing.*—¼ in. diam., 10–12,000 molecular weight cut-off. Boil 3 min before use.

(r) *Glass wool.*—Boiled or siliconized.

(s) *Disposable plastic syringes.*—1 mL.

(t) *Vacuum side arm flask.*—250 mL for degassing.

(u) *Cooling block or refrigerated H_2O bath.*—15 ± 1°.

(v) *Plastic column.*—Disposable, ca 4 × 0.9 cm.

(w) *Scintillation counter.*—Or Geiger-Mueller counter if calibrated in cpm.

(x) *Nitrocellulose filters.*—0.45 μm pore size, 82 mm diam.

(y) *Absorbent paper filters.*—82 mm diam.; similar in characteristics to Whatman No. 1.

(z) *Petri dishes.*—100 × 15 or 20 mm, plastic.

(aa) *Vacuum oven.*—Maintain 80 ± 3°.

(bb) *X-ray film.*—8 × 10 in. is convenient size.

(cc) *X-ray film holder cassette.*—With intensifying screens (Kodak regular or Dupont Cronex lightening plus).

Isolation of Plasmid DNA

Inoculate 25 mL brain heart infusion broth (o) contg 10 μg ampicillin (filter-sterilized)/mL with frozen stock of strain C600 (pEWD299). Incubate overnight at 37° with shaking. Read *A* at 550 nm, using 25-fold diln. Inoculate 1.5 L amplification medium (b) to A_{550} = 0.02. [Note: This procedure can be scaled up to 10 L.] Shake or aerate well at 37°. When A_{550} = 0.4, add solid chloramphenicol to 100 μg/mL. Reduce shaking to 75 rpm or aeration to 2 Lpm. Incubate overnight. Harvest cells by centrifugation at 4°, resuspend pellets in TES buffer (d), and centrf. again. Resuspend cells in 8 mL 25% sucrose (w/v, nuclease-free) in TE buffer (c) in 50 mL polycarbonate centrf. tube. Add 1 mL 1% lysozyme (egg white, grade 1), mix gently, and let sit on ice 5 min. Add 13 mL Triton lytic mix (f), stir briefly to mix, and incubate on ice 30 min. Centrf. 30 min at 27,000 × g. Decant supernate thru gauze. If pellet is very soft, centrf. again at 37,000 × g for 30 min and combine this supernate with first one. Measure vol. of supernate (to 0.1 mL) and add 0.97 g solid CsCl for each mL. Add soln to ultracentrf. tubes and layer on surface 0.1 mL ethidium bromide, 10 mg/mL, for each mL supernate (before addn of CsCl) on liq. surface [*Caution:* Ethidium bromide is mutagenic. Handle with care.] Fill tubes with light mineral oil, balance to 50 mg, and cap or seal. Centrf. 40 h in fixed angle rotor at 100,000 × g or 18 h in vertical rotor at 180,000 × g at room temp. (23°).

Observe ultracentrifuge tube in subdued room light, without fluorescent lights. Locate lower, orange band with longwave UV light and remove band with needle and syringe. Place band into polystyrene or siliconized glass tube. Ext and discard ethidium bromide with isopropanol satd with TE buffer and CsCl (e). Repeat until pink color is gone and then ext twice more. Measure remaining sample vol. and add 3 vols H_2O and 25 μg yeast transfer RNA (2.5 mg/mL, stored at −20°). After addition of water, add one-ninth total vol. of 3.0M Na acetate–10mM $MgCl_2$. Add 2.5 vols −20° alcohol and hold at −20° 1 h. Centrf. 10 min. at 9,000 × g at 0°. Discard supernate and let pellets drain until alcohol odor is gone. Tubes may be dried 15 min in vac. desiccator but do not over-dry. Gently resuspend pellet in 1 mL TE buffer (c). Est. DNA concn by electrophoresis against known stds. If A_{258} is measured, DNA concn will be over-estd because of presence of RNA. [For pure DNA, A_{258} = 1.0 corresponds to 50 μg/mL and ratio $A_{258}/A_{280} \simeq 1.8$]. Store DNA in plastic or siliconized glass tubes at 4°.

Enterotoxin Gene DNA Isolation

Enzyme Titration

Titr. restriction endonuclease against plasmid (pEWD299), using estd DNA concn to det. correct amt of enzyme. Usually, one unit of enzyme will digest about 1 μg DNA. However, this can vary by several fold, depending on plasmid, enzyme, or impurities. Generally, it is best to follow methods suggested by supplier.

If *Hind*III is used to cleave pEWD299, an 850 base-pair fragment will be generated which contains nucleotide sequence for entire B subunit and about one-third of the A subunit of the heat-labile enterotoxin. Dispense ca 1 μg DNA into four 500 μL conical plastic tubes. Add 2.5 μL 10X—*Hind*III reaction buffer (h). Add 0, 2, 5, or 25 units of enzyme to each tube. Add 2.5 μL bovine serum albumin (1 mg/mL, nuclease-free). Add H_2O to bring vol. to 25 μL. Incubate 1 h at 37°. Add 5 μL stop soln (i) and electrophorese 25 μL of each mixture for 3 h at 100 V in 0.7% agarose in 1X TBE dild from (g). As control, run 30–50 ng linear bacteriophage lambda DNA. Stain gel with ethidium bromide (2 μg/mL) until lambda DNA band is visible under longwave UV light. If record is desired, rinse gel briefly with H_2O, and photograph with 302 nm transilluminator and camera with Wratten No. 23A or 9 filter.

Preparative Digest

Scale up titrn digest using lowest amt of enzyme that achieves complete digestion. After 1 h of incubation at 37°, add one-tenth vol. of stop soln (i). Prep. 10% polyacrylamide gel. For 50 mL gel, combine 32.5 mL H_2O, 5.0 mL 10X TBE (g), 12.5 mL 40% acrylamide (w/v; caution: acrylamide monomer is a neurotoxin). Degas 15–30 min in sidearm flask under vac. Add 0.5 mL freshly prepd 10% (w/v) ammonium persulfate soln. Add 50 μL TEMED (*N,N,N′,N′*-tetramethylethylenediamine) but mix gently so as not to aerate degassed soln. Pour vertical gel which should harden in 10–20 min. Layer digest on gel and electrophorese for 3 h at 100 V in 1X TBE (diluted from (g)). Stain with ethidium bromide (2 μg/mL) until bands are visible with longwave UV. Slice 850 base-pair band (nearest bottom) from gel and place into dialysis tubing with 1–2 mL 1X TBE. Place bag in horizontal electrophoresis unit and cover with 1X TBE. Electroelute band from gel at 50 V for 16 h. Reverse polarity of electrodes and turn on power for 15 s at 150 V. Remove buffer contg DNA from dialysis bag with plastic pipet. Add one-tenth vol. of stop soln (i). Repeat electrophoresis and electroelution as described above.

DE52 Chromatography

Prep. DE52 according to manufacturer's instructions, using loading buffer (0.15M NaCl, 1mM Na_2EDTA, pH 8.0, 0.01M Tris, pH 8.0). Construct 0.3 to 0.4 mL DE52 column in 1 mL plastic syringe plugged with boiled or siliconized glass wool.

Wash column with 2 mL loading buffer. Apply 1–2 mL DNA to top of column bed. Wash column with 3–4 mL loading buffer. Elute DNA with 10 column vols of eluting buffer (1.0M NaCl, 1mM Na_2EDTA, pH 8.0, 10mM Tris, pH 8.0) in 0.3 mL aliquots. Collect 0.3 mL fractions in 500 μL plastic tubes. Most of DNA should be in first 2 or 3 fractions. Spot 2 μL of each fraction onto 1% agarose with 2 μg/mL ethidium bromide and illuminate with UV light. Fractions contg DNA will fluoresce; pool these fractions and alcohol-ppt by measuring total vol. and adding 10 μL transfer-RNA (2.5 mg/mL) and one-ninth soln vol. of 3.0M NaOAC-10mM $MgCl_2$. Add 2.5 vols of −20° alcohol and hold at −20° ≥1 h. Centrf. at 10,000 × g for 10 min. Discard supernate and gently rinse pellet (which may not be visible) with 0.5 mL −20° alcohol. Drain well until alcohol odor is gone but do not dry completely. Gently resuspend DNA pellet in 200–300 μL TE buffer (c).

In Vitro DNA Labeling

Kits are available commercially for nick translation reaction. Following procedure uses 50 ng DNA rather than 1 μg often suggested by suppliers. To 500 μL conical plastic centrf. tube, add 50 ng DNA (as prepd above in max. vol. of 5 μL). Add 3 μL 10X reaction buffer (j). Add 1.5 μL of soln 333 μM in each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and thymidine triphosphate. Add 16 μL alpha-^{32}P deoxycytosine triphosphate (dCTP) (h). Add H_2O to final vol. of 26 μL. Add 2 μL DNase 1 (100 ng/mL, dild immediately before use). Incubate 10 min at 15°. Add 2 μL DNA polymerase I 1 U/μL). Incubate 1 h at 15°. Add 2 μL 0.5M Na_2EDTA, pH 8.0. Prep. 2 mL column of Sephadex G-50 (prepd according to manufacturer's instructions, using TE buffer (c)). Load reaction mix onto column and elute by adding 100 μL portions of TE buffer (c). Collect twenty 2-drop fractions into 500 μL tubes. Spot 2 μL of each fraction onto 2 × 2 cm paper squares (e.g., Whatman 3MM), dry, add scintillation fluid (e.g., 5 g 2,5-diphenyl-oxazole/L toluene), and count. Geiger-counter may suffice to assay fractions. Labeled DNA is eluted from column usually between fractions 6 and 12. Unincorporated dCTP elutes as larger peak, starting between fractions 12 and 15. Pool fractions from earliest peak and count 2 μL as previously described. Using 3000 Ci/mmol of dCT^{32}P, specific activities of 2–8 × 10^8 cpm/μg usually result.

Colony Hybridization Filter Preparation

When received, transfer sample cultures to 5 mL rich broth and incubate at 37° for 18–24 h. Aseptically add 2 mL culture to 0.5 mL sterile 50% (v/v) glycerol. Store at −70° if possible. [Note: Frost-free freezers will decrease culture viability. If cultures must be stored at −20°, use non-frost-free unit. This caveat holds for all frozen material in this procedure.]

Boil nitrocellulose filters (0.45 μm, 82 mm diam.) for 2–3 min in ca 2 L H_2O. While still wet, flatten filters (to minimize wrinkles) between paper filters (such as Whatman No. 1 or Schleicher and Schuell No. 597), using forceps to avoid touching filters. Loosely wrap filters in A1 foil and sterilize at 121°, 15 lb, for 10–20 min on liq. (slow exhaust) cycle. Store at room temp.

Aseptically inoculate ca 5 mL rich broth with portion of frozen bacterial culture. [It is not necessary to completely thaw culture and it may be re-used several times.] Incubate cultures 18–24 h at 37°. Aseptically, place sterile nitrocellulose filter on dry MacConkey agar plate. Ensure that no bubbles are trapped under filter and that it wets completely. Discard filters that do not lie flat. Label filters, using soft lead pencil or by perforating filter in distinctive pattern with needle. This may be more easily done after baking at 80° (see below). Filters marked with 5 mm square grid are useful for arranging cultures in orderly array. Inoculate filters with sterile microbiological needle, using 1:100-fold diln, in sterile normal saline, of overnight culture. Always inoculate each filter with known pos. and neg. control cultures. Record location of each culture; 30–50 cultures should fit on each filter. It is vital that filters and the resulting autoradiogram can be oriented unambiguously. Make duplicate filters, since procedure may have to be repeated. Incubate filters 18–24 h at 37°. Mark cultures which have failed to grow, or a false-neg. result may be reported.

Table 1. Strains of *E. coli* used in the study

Strains	Reference	LT production[a]
	Control	
H10407	64	+
C600 (pEWD299)	65	+
HB101 (pBR313)	66	−
	Unknown	
K334C2	23	+
TD427C2	45	+
K135C5	45	−
TD225C4	45	+
K324C1	45	−
TD514C1	45	−
35897	45	−
53892A2	45	+
TD462C1	45	−
M415C5	23	−
B44	67	−
E2534	68	+
711 (pSR19)	69	+
M407	23	−
H10407	64	+
H446	23	−
214-4	12	−
PB200P	70	+
PB200	70	+
GV50B	71	+
TRA6	72	−
TRA7	72	−
78-IEC-1	73	−
PM152	74	−
ATCC 25922	75	−

[a]Heat-labile enterotoxin (LT) production was determined by both biological (modified mouse Y-1 adrenal cell) and immunological (ELISA) tests (60).

Lyse colonies by transferring filters for 10 min onto paper filters (in 100 × 15 mm plastic petri plates) wetted with 1.5 mL 0.5M NaOH. Ensure that no bubbles are trapped under filters. Transfer nitrocellulose filters for ≥1 min each, to series of 3 paper filters each wetted with 1.5 mL 1.0M ammonium acetate–0.02N NaOH. Shift nitrocellulose filter to fourth ammonium acetate–NaOH filter for 10 min. Keep filters horizontal during transfers so that lysed colonies will not run together. Air dry nitrocellulose filter on absorbent paper ≥30 min. Bake in vac. oven 2 h at 80°. Cool filters to room temp. and label with H_2O-proof ink or pencil. Store between paper filters under vac.

Colony Hybridization

Freshly prep. 50 mL hybridization mixt. (k). Boil 0.5 mL sonicated calf thymus DNA (n) 10 min and add to hybridization mixt. (k). Pre-incubate each nitrocellulose filter 3 h at 37° in 100 × 15 mm plastic petri dish contg 5 mL hybridization mixt. with boiled calf thymus DNA. After 3 h, alkali-denature radioactive toxin gene DNA. Det. vol. of DNA required to contain 1 × 10^6 cpm. Correct for 14.2-day half-life of ^{32}P. Add 1 × 10^6 cpm DNA to 500 μL plastic conical tube and bring total vol. to 300 μL with H_2O. Add 6 μL 10N NaOH and mix briefly with pipet tip. After 10 min, neutze with 6 μL

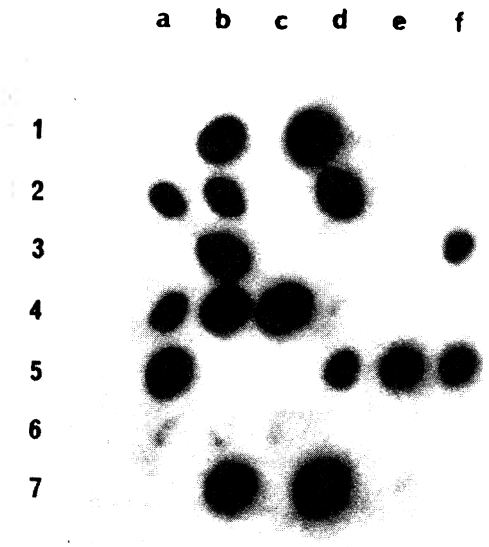

Figure 1. Autoradiogram of colony hybridization filter after incubation with DNA probe for LT gene of *E. coli*.

Dark spots represent positive results. Culture locations: 1-b, H10407; 1-c, C600 (pEWD299); 1-e, HB101 (pBR313); 2-a, K334C2; 2-b, TD427C2; 2-c, K135C5; 2-d, TD225C4; 2-e, K324C1; 2-f, TD514C1; 3-a, 35897; 3-b, 53892A2; 3-c, TD462C1; 3-d, M415C5; 3-e, B44; 3-f, E2534; 4-a, 711 (pSR19); 4-b, H10407; 4-c, C600 (pEWD299); 4-d HB101 (pBR313); 4-f, M407; 5-a, H10407; 5-b, H446; 5-c, 214-4; 5-d, PB200P; 5-e, PB200; 5-f, GV50B; 6-a, TRA6; 6-b, TRA7; 6-c,78-IEC-1; 6-d, PM52; 6-e, ATCC 25922; 7-b, H10407; 7-d, C600 (pEWD299); 7-e, HB101 (pBR313). Other positions were not inoculated.

10N HOAc. Boil 0.5 mL sonicated calf thymus DNA for 10 min and add 50 mL hybridization mixt. (k). Place nitrocellulose filter into 5.0 mL fresh hybridization mixt. and 1×10^6 cpm alkali denatured and neutzd probe DNA. Incubate 18–24 h at 37°.

Rinse filters 5–10 s in 10–15 mL 5X SSC (dild from 20X SSC (l))–0.1% (w/v) sodium dodecyl sulfate (SDS). Place filter into clean petri dish and cover with 10–15 mL 5X SSC–0.1% SDS and incubate 1 h at 70°. Place filter in fresh 5X SSC–0.1% SDS and incubate addnl 1 h. Rinse filter 5–10 s in 2X SSC (dild from 20X SSC (l)). Air-dry 15–30 min. Mount filter with small pieces of tape onto paper and cover with plastic sheet (such as document holder).

Autoradiography

In dark room, place film on plastic-covered filters in cassette film holder with intensifying screens. Enclose film holder in plastic bag and expose film preferably at −70° but at least −20°. Exposure length is dictated by amt of radioactive DNA bound to filter. If increase of 2–3 cps is observed when Geiger-Mueller counter is held over filter, it is likely that pos. reaction will be visible after 1 d exposure. After exposure, let cassette reach room temp. Develop following manufacturer's instructions. If spots are too faint for analysis, expose new film for longer period.

Interpretation of Results

DNA of cells having gene for heat-labile enterotoxin of *Escherichia coli* bind radioactive toxin gene DNA. Film will be exposed and dark spots will appear after development. Since colony size and hybridization efficiency can vary, this test is best used qual. and not quant. If there are dark areas on film where no colonies should be, unhybridized radioactive DNA has probably not been completely washed away. Rewash filter twice in 5X SSC–0.1% SDS at 70° for 1 h. Let dry and expose film. After film development, make pos. or neg. detn of each unknown culture by comparing intensity of spot with pos. and neg. control cultures. Neg. control should show no darkening of film or, at most, very faint darkening. Pos. control should show distinct darkening of film clearly discernable above background.

Troubleshooting

If autoradiograms are unsatisfactory, a number of factors might be responsible. False-neg. results could be due to spontaneous loss of virulence determinants, insufficient growth of colonies on filters, failure to bake filters to fix DNA, or insufficient radioactivity during hybridization. False-pos. results may result from insufficient filter washing after hybridization, failure to add Denhardt's soln or sonicated calf thymus DNA, or use of probe DNA fragment which was not purified adequately. Possible remedies include use of new bacterial cultures, prepg new filters with lysed colonies, reviewing procedures and reagent composition, rewashing filters, or checking darkroom methods.

Results and Discussion

Each of the 13 laboratories participating in this study received 3 labeled control cultures and 25 coded unknown cultures (10 positive, 15 negative). These cultures (64–75) are described in Table 1. Each collaborator also received from 2 to 5×10^6 cpm ^{32}P-labeled LT gene probe, prepared as described previously, and 1 mL sonicated calf thymus DNA. Redistilled formamide and nitrocellulose filters were supplied to some participants. Collaborators were instructed to identify the culture as positive for the LT gene if the intensity of spots on autoradiograms equalled or was greater than the control culture (H10407) and was more intense than C600 (pBR313). An autoradiogram is shown in Figure 1.

Of the 325 samples tested in the complete study, 315 (96.9%) were identified correctly. The 10 misclassified cultures were as follows: Coll. B, 1 false positive in K324C1; Coll. D, 1 false negative in 711(pSR19); Coll. E, 1 false positive in 78-IEC-1; Coll. H, 4 false negatives in K334C2, E2534, 711(pSR19), PB200; Coll. I, 1 false negative in TD225C4 and 2 false positives in 35897 and B44. The percentage of correctly identified positive and negative cultures was 95.4% (124/130) and 97.9% (191/195), respectively. Computed chi-square statistics indicated that the method agreed (>95% confidence) with the previous classification of the samples and that the method was equally efficient at classifying negative and positive samples.

Conclusion

This collaborative study marks the first attempt of the Food and Drug Administration to establish a test for pathogenicity based on genetic principles. Radioactive DNA coding for a pathogenic determinant (in this case, *E. coli* LT) was used to detect cultures with the same gene. This detection does not require the expression of virulence genes in the laboratory as do the previous methods (76–78). The colony hybridization technique does not appear to be subject to the sources of variability discussed by Mehlman et al. (79). Recent results also indicate that selective enrichment may not be required (60) and that other organisms, such as *Yersinia enterocolitica*, may be detected and enumerated by DNA colony hybridization (80).

Based on the results of this collaborative study (correct classification of 96.9% of the samples), we recommend that this procedure be adopted as a screening method to detect strains of *E. coli* with the genetic potential to elaborate LT.

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Recovery of *Salmonella* Species from Nonfat Dry Milk Rehydrated Under Rapid and Reduced Pre-enrichment Conditions: Collaborative Study

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A collaborative study was conducted to compare the relative efficiency of the AOAC rapid rehydration method with the reduced rehydration soak method for the recovery of *Salmonella* species from nonfat dry milk (NFDM). In the AOAC method, a 25 g sample of NFDM is rapidly rehydrated at a 1:9 sample/water ratio and mixed by swirling. After 60 min, the flask contents are adjusted to a pH of 6.8, and 0.45 mL of 1% aqueous brilliant green dye solution is added. The flasks are then incubated at 35°C. In the soak method, a 25 g sample of NFDM is gently added to the sterile brilliant green (BG) water at a 1:9 sample/BG water ratio and allowed to soak undisturbed for 60 min at room temperature before incubation. Twelve collaborators analyzed 3 shipments of samples with the following results for the AOAC and soak methods: shipment 1—31 and 46 positive samples, respectively, with a 48% increase in detection by the soak method; shipment 3–45 and 66 positive samples, respectively, with a 47% increase in detection by the soak method; shipment 2—no significant difference in recovery of *Salmonella* species by the 2 methods. It is recommended that the official final action method for the detection of *Salmonella* species, 46.054–46.067, **be revised to use the soak method for the analysis of nonfat dry milk.**

Several investigators (1, 2) have reported that repair of damage to cells injured by heating and drying can be improved by slowly rehydrating the dried product containing damaged cells. Ray et al. (3) obtained higher most probable number (MPN) recoveries of *Salmonella* species when dry milk MPN portions were rehydrated initially at a 1:2.5 sample/broth ratio and diluted after 1 h to a 1:10 sample/broth ratio than when MPN portions were rehydrated initially at a 1:10 sample/broth ratio. van Schothorst et al. (4) also reported higher recovery of *Salmonella* species from dry milk when samples were reconstituted in buffered peptone water (BPW), using a soaking procedure in which the milk powder was poured carefully onto the medium but not mixed, or when samples were reconstituted in BPW at a sample/volume ratio of 1:2, followed 30 min later by diluting to a final 1:9 sample/volume ratio.

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The microbiological method of analysis for dry milk contaminated with *Salmonella* species as currently specified in *Official Methods of Analysis* (5) requires mixing the milk powder well by swirling with the appropriate pre-enrichment medium at a 1:9 sample/broth ratio. Andrews et al. (6) reported enhanced recovery of *Salmonella* from nonfat dry milk (NFDM) powder pre-enriched under conditions of reduced rehydration. In that study the soak method consisted of adding 25 g samples of NFDM powder gently and slowly to 225 mL volumes of sterile distilled water and letting the samples soak undisturbed for 60 min at room temperature. Accordingly, this collaborative study compared the efficiency of pre-enriching NFDM samples analyzed by the rapid (AOAC) and slow (soak) methods of rehydration.

Collaborative Study

For this study, each of the 13 participating collaborators was furnished with 24 (20 collaborative and 4 control) sample units of NFDM powder, in each of 3 shipments, for a total of 72 sample units. Each sample unit weighed approximately 30 g. Samples for shipment 1 were inoculated with *S. anatum* and *S. kentucky*, for shipment 2, with *S. tennessee* and *S. new brunswick*, and for shipment 3, with *S. infantis* and *S. newington* (Table 1). For each shipment, 4 replicate sample units were taken from each of the inoculated levels 1–4, and 4 replicate sample units were taken from the uninoculated level 5, for a total of 20 collaborative sample units. In addition, 4 control sample units of duplicate known positive (level 6) and duplicate known negative (level 7) sample units were included in each shipment of 24 sample units.

To prepare inoculated samples, a 200 mL volume of brain-heart infusion (BHI) broth was inoculated with 1 of 6 *Salmonella* species cultures (Table 1) grown on BHI agar slants for 18–24 h at 35 ± 2°C, the incubation temperature used throughout this study. After the broth was incubated for 18–24 h, 5 mL of each of 2 *Salmonella* serotypes was mixed in a centrifuge tube and centrifuged at about 3090 × g for 10 min. The cell sediment was washed twice with Butterfield's phosphate buffer (pH 6.8–7.2) and resuspended in 10 mL of buffer. Serial 10-fold dilutions were made from the washed cell suspension to the 10^{-5} dilution, and 2-fold dilutions were

Table 1. Levels of *Salmonella* species organisms[a] in artificially inoculated nonfat dry milk samples

Level	Shipment 1: *S. anatum* (E_1) and *S. kentucky* (C_2) (MPN/g)	Shipment 2: *S. tennessee* (C_1) and *S. new brunswick* (E_2) (MPN/g)	Shipment 3: *S. infantis* (C_1) and *S. newington* (E_2) (MPN/g)
1	0.04	0.23	0.04
2	0.02	0.09	0.02
3	0.02	0.08	0.02
4	<0.003	0.02	0.004
5	–[b]	–[b]	–[b]
6	+[c]	+[c]	+[c]
7	–[d]	–[d]	–[d]

[a]Determined by soak method only on day analyses were initiated.
[b]A 100 g sample from uninoculated level 5 was qualitatively found to be negative for *Salmonella* species by the originating laboratory.
[c]A 25 g sample from a known positive control level was qualitatively found to be positive for *Salmonella* species by the originating laboratory.
[d]A 100 g sample from a known negative control level was qualitatively found to be negative for *Salmonella* species by the originating laboratory.

prepared to obtain the desired level of organisms for inoculating the milk powder. Each of the levels 1–4 of NFDM was inoculated from a serial 2-fold dilution of pooled *Salmonella* species cells as specified for each sample shipment. Corresponding *Salmonella* serotypes from a 10^{-2} dilution were used to inoculate level 6 with a high level of contamination to provide known positive control samples. A volume of 2.5 mL of the diluted cell suspension and an equal volume of double strength NFDM (20 g dry milk/100 mL distilled water) were mixed in a screw-cap tube and the contents were placed in a serum vial. The contents were shell-frozen in a dry ice–ethanol bath and immediately placed in a Virtis freeze dryer (Virtis Co., Gardiner, NY) for 24 ± 2 h. The lyophilized contents were transferred to a mortar and pulverized to a fine powder.

Retail packages of NFDM were purchased locally and pooled to form a lot contained in a plastic tray (61 × 36 × 21 cm) with foil cover. A 100 g portion of each retail package was examined by the soak method described in this study to ensure the absence of any naturally occurring *Salmonella* species. All individual retail packages of NFDM that were combined to form a particular level of inoculation were of the same manufacturer's control code number. Pulverized powder containing the lyophilized cells was used to inoculate 50 g of dry milk powder in a sterile Stomacher 400 bag (Tekmar Co., Cincinnati, OH). The 50 g of inoculated NFDM was used as a seed inoculum for the 3000 g bulk sample and mixed manually to distribute the inoculated microorganisms. The inoculated bulk samples were stored at room temperature for 7–8 days before packaging. During the storage period each bulk sample of milk powder was mixed 2 additional times to increase the potential of uniform distribution of the inoculated microorganisms. Replicate sets of individual 24/30 g samples were prepared for each collaborator and stored at room temperature until shipped. Each sample was assigned a different number selected from a standard statistical table of random numbers (7). Collaborators were instructed to initiate their examination on a specified day and to report results for each sample as positive or negative for *Salmonella* species on the standardized sheets furnished with samples.

The originating laboratory determined most probable number (MPN) values of samples on the day that collaborators initiated their analyses. This study was performed in 3 segments. Samples of NFDM were prepared during 3 consecutive months, and each of the 3 sample shipments was sent 12 days later so that sample analyses could be initiated on the 19th day after inoculation. Collaborators were asked to pre-enrich one of the duplicate series of NFDM samples in sterile distilled water, using the AOAC method, and to pre-enrich the corresponding series of replicate samples in distilled water with 0.002% (final concentration) brilliant green dye, using the soak method. For the AOAC method, collaborators swirled rehydrated samples 25 times clockwise, 25 times counterclockwise, 25 times clockwise, and 25 times counterclockwise. They were directed to use AOAC methods **46.054–46.067** for isolation and confirmation of *Salmonella* species recovered by either pre-enrichment method.

METHOD

All media and reagents used in this study were prepared as described in *Official Methods of Analysis* (5), **46.054–46.067,** except for the following modifications:

(a) Add as new section in **46.054:**

(w) *Brilliant green (BG) water.*—Prep. sterile H_2O as in **46.055(m)** and add 2 mL 1% aq. brilliant green dye, **46.055(n),** per L sterile H_2O and mix well.

(b) In **46.056(b),** change heading to *"Dry whole milk.—"*

(c) Add as new section in **46.056:**

(f) *Nonfat dry milk (NFDM).*—Aseptically open sample container and aseptically weigh 25 g sample into sterile beaker (250 mL) or other appropriate container. Cover with sterile foil cover or sterile cap to prevent contamination. Using sterile glass or paper (made with tape to withstand autoclaving) funnel, pour 25 g analytical unit gently and slowly over surface of 225 mL brilliant green H_2O, **46.054(w),** contained in sterile 500 mL erlenmeyer or other appropriate container. Let container with sample pre-enrichment broth stand undisturbed 60 ± 5 min. Incubate loosely capped container, without mixing or pH adjustment, for 24 ± 2 h at 35°.

Results and Recommendations

The MPN levels of *Salmonella* species in the collaborative samples of NFDM were determined by the soak method on the 19th day after sample inoculation, the day when actual collaborative analyses were initiated. For each of the 3 shipments, levels 1–4 were the inoculated collaborative samples, level 5 was the uninoculated collaborative samples, level 6 was the known positive control samples, and level 7 was the known negative control samples.

Table 2 shows the recovery of *Salmonella* species from 3 shipments of NFDM powder on an individual sample basis by the collaborators. Data for the 4 control samples (levels 6 and 7) were included in Tables 1 and 2, but not in Tables 3 and 4, because this information was used by collaborators as an analytical guideline and not in the evaluation of the 2 pre-enrichment methods. For shipment 1, the results of Collaborator 13 were excluded from subsequent tables because this Collaborator reported *Salmonella* species in an uninoculated collaborative sample in level 5. For shipment 3, the results of Collaborator 11 were excluded from subsequent tables because *Salmonella* species were reported both in an uninoculated sample of level 5 and in a known negative control sample of level 7. This table shows a declining number of positive samples recovered from succeeding inoculated levels 1–4 in shipments 1 and 3. Each analyst, except Analyst 11, confirmed the presence of *Salmonella* species by both methods of sample preparation in the inoculated (positive) control samples (level 6) and the absence of *Salmonella* species by both methods of sample preparation in the uninoculated (neg-

Table 2. Collaborators' results for recovery of *Salmonella* species from nonfat milk samples

Salmonella species level[a]	Collaborators 1		2		3		4		5		6		7		8		9		10		11		12		13	
	A[b]	S[c]	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S
Shipment 1																										
1	+−[d]	++	++	++	++	++	−+	++	++	++	++	++	++	++	+−	−−	++	++	+−	++	+−	++	++	++	−−	++
2	+−	−+	−−	−+	−−	−−	++	++	−−	++	+−	+−	++	−+	−−	−−	−−	++	−+	−−	−−	−+	−−	−+	−−	−+
3	−+	−+	−−	+−	−−	−−	++	+−	−+	−+	−−	+−	−−	−+	−−	−−	−−	−−	−+	−−	−−	−−	−−	+−	−+	−+
4	−−	−−	−−	−−	−−	+−	−−	−−	−−	+−	−−	−−	−−	+−	−−	−−	−−	++	−−	−−	−−	−−	−−	−−	−−	−−
5[e]	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	+−	−−
6[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7[g]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Shipment 2																										
1	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+−	++	++	++	++	++	++	++	++	++	++
2	++	++	−+	++	++	+−	+−	+−	++	++	+−	++	++	−−	−+	+−	−−	++	−+	++	++	++	++	++	++	+−
3	++	++	++	−+	++	++	++	++	++	++	++	++	++	++	++	++	+−	+−	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+−	+−	++	++	++	++	++	++	+−	++
5[e]	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−
6[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7[g]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Shipment 3																										
1	++	++	++	+−	−−	−+	−+	++	++	−+	++	++	+−	++	−−	+−	+−	++	+−	+−	−+	+−	++	++	−+	++
2	++	++	+−	+−	−−	++	++	++	++	++	++	+−	++	++	−+	++	−−	++	+−	++	+−	−+	++	++	++	++
3	++	−+	+−	+−	−−	++	+−	+−	+−	++	+−	+−	−+	−+	−+	++	−−	++	+−	−+	−+	++	−−	++	++	−+
4	+−	−−	−−	−+	−−	−−	−−	+−	−−	−−	−−	−−	−−	−−	−−	++	−−	++	−+	−+	−−	++	−−	−+	−−	−−
5[e]	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	−−	+−	−−	−−	−−	−−
6[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7[g]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−

[a]Number of *Salmonella* species cells per level given in Table 1.
[b]A, AOAC method.
[c]S, soak method.
[d]+, *Salmonella* species recovered; −, *Salmonella* species not recovered.
[e]Uninoculated collaborative samples.
[f]Known positive control samples.
[g]Known negative control samples.

Table 3. Recovery of *Salmonella* species from nonfat dry milk collaborative samples, presented according to level of inoculation

Level of inoculation	No. of samples positive for *Salmonella* species[a] Shipment 1 AOAC	Shipment 1 Soak	Shipment 2 AOAC	Shipment 2 Soak	Shipment 3 AOAC	Shipment 3 Soak
1	19	22	26	26	15	19
2	7	12	19	21	17	22
3	5	7	25	25	11	17
4	0	5	24	26	2	8
5[b]	0	0	0	0	0	0

[a]Of 24 samples, 12 duplicate collaborative samples were compared at each level for shipments 1 and 3. Of 26 samples, 13 duplicate collaborative samples were compared at each level for shipment 2.
[b]Uninoculated level.

ative) control samples (level 7). Results reported for samples in shipment 2 indicated a relatively consistent positive-sample detection rate, as shown by the similarity of results obtained for samples in the lowest to the highest inoculated levels. The greatest differences among the pre-enrichment methods were apparent from analytical results of the lower contamination level (levels 3 and 4) in shipment 3.

Table 3 shows the recovery of *Salmonella* species from NFDM samples by level of inoculation as reported by 12 collaborators for shipments 1 and 3 and by 13 collaborators for shipment 2. Results of analyses by the AOAC method and the soak method, listed by level, show the number of samples positive for *Salmonella* species that were recovered for 24 NFDM samples from shipments 1 and 3 and for 26 NFDM samples from shipment 2. Greater differences between the methods were observed when results of relatively low level samples in shipments 1 and 3 were compared with results of higher level samples in shipment 2. The soak method showed a higher recovery of *Salmonella* than the AOAC method for each of the 4 inoculated levels, 1–4, from shipments 1 and 3. The AOAC method failed to recover any positive samples from level 4, the lowest inoculated level from shipment 1, whereas the soak method recovered 5 positive samples. Moreover, the soak method detected 4, 5, 6, and 6 additional positive samples for levels 1–4, respectively, in shipment 3.

Table 4 shows the recovery of *Salmonella* species from 8 inoculated NFDM collaborative samples examined per method by each participating laboratory. For shipments 1 and 3, 10 of 12 collaborators reported that the soak method gave an equal or higher number of positive samples than the AOAC method. For shipment 2, 11 of 13 collaborators reported that the soak method gave an equal or higher number of positive samples than the AOAC method.

Results from 13 collaborators for samples from 3 shipments were evaluated by using the sign test for differences, which indicated a significant difference between methods ($P \leq 0.05$). The data from 10 collaborators for the soak method showed significantly fewer erroneous results. Based on the same eval-

Table 4. Recovery of *Salmonella* species from nonfat dry milk collaborative samples, as determined by collaborators

	No. of *Salmonella*-positive samples/8 inoculated samples					
	Shipment 1		Shipment 2		Shipment 3	
Coll.	AOAC	Soak	AOAC	Soak	AOAC	Soak
1	3	4	8	8	7	5
2	2	4	7	7	4	4
3	2	3	8	7	0	5
4	5	5	7	7	4	6
5	3	6	8	8	5	5
6	3	4	7	8	5	4
7	4	5	8	6	4	5
8	1	0	5	8	2	7
9	2	6	6	8	1	8
10	3	2	7	8	4	5
11	1	3	8	8	E[a]	E
12	2	4	8	8	4	7
13	E	E	7	7	5	5

[a]E, excluded.

uation test, data from 1 of the other 2 collaborators indicated equal ability of both analytical methods, but data from the second collaborator indicated fewer erroneous results with the AOAC method. Inclusion of data from Collaborator 13 for shipment 1 and Collaborator 11 for shipment 3 did not affect the sign test result. Collaborative recovery data reported by level of inoculation from 12 participating laboratories for samples from shipments 1 and 3 and from 13 participating laboratories for samples from shipment 2 were evaluated using the Chi-square (χ^2) test to determine differences between methods. This analysis revealed a significant difference ($P \leq 0.05$) with level 4 samples from shipments 1 and 3, and indicated a significantly greater number of positive samples detected with the soak method. There was no significant difference, even at the 90% level of confidence, between results obtained with the 2 analytical methods for shipment 2 samples.

Results from 13 collaborators for samples from 3 shipments were analyzed using a Chi-square test for matched samples. When the combined results from 13 collaborators for each of the 5 levels per shipment were evaluated, the soak method was significantly ($P \geq 0.05$) more efficient than the AOAC method for the determination of positive samples in shipments 1 and 3. A similar evaluation of results reported for samples in shipment 2 indicated that both methods were almost equally efficient for the detection of positive samples.

Results from 12 collaborators for samples in shipments 1 and 3 indicated that the soak method demonstrated the greater efficiency for recovering *Salmonella* species from low contamination levels of NFDM. In a comparison of the AOAC method with the soak method, 31 and 46 positive samples, respectively, were recovered in shipment 1 (a 48% increased detection by the soak method) and that 45 and 66 positive samples, respectively, were recovered in shipment 3 (a 47% increased detection by the soak method). For shipment 2, 94, and 98 positive samples were recovered by the AOAC and soak methods, respectively. There was no statistical difference between results reported for either of the 2 analytical methods with samples from shipment 2.

Besides giving a higher recovery of *Salmonella* species than the AOAC method, the soak method requires less manual manipulation than the AOAC method, which requires pH adjustment and mixing NFDM powder with the pre-enrichment broth. The only substantive adverse comment received from the collaborators was an objection to the use of a sterile glass funnel or sterile paper funnel for adding the NFDM powder to the pre-enrichment medium for the soak method. This would seem to be a minor objection in view of the improved efficiency of analysis and the absence of mixing and pH adjustment requirements for this improved method.

It is recommended that the official final action method for the detection of *Salmonella* species, **46.054 – 46.067,** be revised to use the soak method for the analysis of nonfat dry milk. No revision in the AOAC method of analysis for dry whole milk is recommended at this time.

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Enumeration of Total Coliforms, Fecal Coliforms, and *Escherichia coli* in Foods by Hydrophobic Grid Membrane Filter: Supplementary Report

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Raw, comminuted poultry meat was used to determine the specificity of the media and incubation conditions used in the hydrophobic grid membrane filter method. Confirmation rates for target colonies were 100% for total coliforms, 98% for fecal coliforms, and 97–99% for *Escherichia coli*. The results of total coliform enumeration in 30 pasteurized milk samples by both the hydrophobic grid membrane filter method and AOAC method 46.013–46.016 are also reported.

In preparation for a collaborative study of hydrophobic grid membrane filter methods for enumerating total coliforms, fecal coliforms, and *Escherichia coli*, preliminary investigations were carried out to determine the specificity of the selective media and incubation conditions used in the proposed methods. Also, the performance of the proposed total coliform method was compared with that of the reference method, AOAC **46.013–46.016**, on 30 spiked samples of pasteurized milk. The results of these preliminary studies are contained in this report.

Experimental

Specificity of Selective Media and Incubation Conditions

Ten samples of naturally contaminated comminuted poultry meat were used to determine specificity for each individual analysis. Each sample was homogenized in peptone/Tween 80 diluent (1) and an amount of homogenate equivalent to 0.1 g sample was filtered. For total coliforms, the filters were placed on mFC agar (1) and incubated 24 h at 35°C. In the case of fecal coliforms, filters were first placed on tryptic soy-magnesium sulfate agar (TSAM) (1) and incubated 4–5 h at 35°C; then filters were transferred to mFC agar and incubated 24 ± 2 h at 44.5°C. For *E. coli*, one filter from each sample was placed directly on tryptone bile agar (TBA) (1) and incubated 24 ± 2 h at 44.5°C. A second filtration was made from 5 of the samples. These filters were first placed on TSAM and incubated 4–5 h at 35°C; then the filters were transferred to TBA and incubated 24 ± 2 h at 44.5°C. After incubation, growth on the filters was evaluated as described below.

Total coliforms.—Ten lactose-positive (blue) colonies were selected from each filter and streaked to MacConkey agar for purification. Each purified isolate was subcultured to tryptic soy agar (TSA) and to LST broth. The LST culture was incubated 48 ± 3 h at 35°C and examined for growth and gas. If growth had occurred, but no gas was present, one drop of phenol red solution was added to the LST culture to check for acid production. An oxidase test and gram stain were also carried out on the TSA culture.

Fecal coliforms.—These filters were evaluated identically to the total coliform filters, except that EC broth, incubated 48 ± 3 h at 45.5°C, was substituted for the LST broth.

E. coli.—Up to 10 colonies were selected at random from each filter and the row and column coordinates of these colonies on the filter were noted. The colonies were streaked to MacConkey agar for purification. Then the indole reaction (1) was developed on each filter and the reaction (positive or negative) that developed in each of the noted grid coordinates was recorded. Once purified, each isolate was identified by the IMVIC test according to AOAC method **46.013–46.016.**

Enumeration of Total Coliforms in Pasteurized Milk

Thirty samples of pasteurized milk were used. Separate suspensions were prepared from 24 h TSA cultures of *E. coli* (mixture of 5 strains), *Citrobacter freundii* (mixture of 2 strains), and *Enterobacter* spp. (mixture of *E. aerogenes* and *E. agglomerans*). Three replicate control counts were made from each suspension by spread-plating on TSA and by filtering and incubating the filters on TSA for 24 ± 2 h at 35°C. Each suspension was used to inoculate 10 samples. Each sample was analyzed by both the hydrophobic grid membrane filter method (1) and by the reference method, AOAC **46.013–46.016.**

Results and Discussion

Specificity of Selective Media and Incubation Conditions

All 100 isolates from the total coliforms filters were oxidase-negative, gram-negative rods. All 100 isolates produced gas from LST within 48 ± 3 h at 35°C.

All 100 isolates from the fecal coliform filters were also oxidase-negative, gram-negative rods. Sixty-five isolates produced gas from EC broth within 48 ± 3 h at 45.5°C. An additional 33 isolates produced acid but no gas. Two isolates produced neither acid nor gas within 48 h (98% confirmation rate).

Eighty-four indole-positive isolates were obtained from the 10 filters incubated directly on TBA. Eighty-three of these isolates were confirmed by the IMVIC test to be *E. coli* (99% confirmation). One isolate which appeared on the filter to be indole-negative was also determined to be *E. coli* biotype I. The 5 filters that were incubated on TSA before being transferred to TBA provided 38 indole-positive isolates. Thirty-seven of these were identified as *E. coli* (97% confirmation). Again, one "indole-negative" isolate was also identified as *E. coli* biotype I. These results are consistent with the 95%

Table 1. Enumeration of total coliforms in pasteurized milk by hydrophobic grid membrane filter and AOAC 46.013–46.016 methods[a]

Inoculum	Expected count (log 10 MPN/mL)	Geometric mean (log 10 MPN/mL)		SD	
		HGMF	**46.013–46.016**	HGMF	**46.013–46.016**
E. coli	1.8711	1.9610	2.2295	0.1525	0.4137
C. freundii	1.8494	2.2139	2.4705	0.2446	0.4806
Enterobacter spp.[b]	1.6329	1.6525	2.0512	0.1942	0.1443

[a]Means represent analysis of 10 samples.
[b]*E. aerogenes* and *E. agglomerans*.

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confirmation rate reported by Anderson and Baird-Parker (2) for this medium following a study of 555 indole-positive colonies.

Enumeration of Total Coliforms in Pasteurized Milk

Mean counts per milliliter and corresponding standard deviations for each series of 10 samples are contained in Table 1. Although the mean results obtained by the hydrophobic grid membrane filter method tended to be lower than those of the reference method, results obtained by filtration were much closer to the expected counts, based on replicate counts done on the inoculum suspensions. In 2 of the 3 series, the standard deviations of the filter counts were appreciably lower than those of the reference method.

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Enumeration of Total Coliforms, Fecal Coliforms, and *Escherichia coli* in Foods by Hydrophobic Grid Membrane Filter: Collaborative Study

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A collaborative study was conducted in 18 laboratories to assess the performance of the hydrophobic grid membrane filter method against that of the AOAC official first action method 46.013–46.016 for enumerating total and fecal coliforms and *Escherichia coli*. The study was carried out on frozen breaded fish, raw comminuted poultry, unroasted walnut pieces, ground black pepper, and cheddar cheese. The hydrophobic grid membrane filter method recovered significantly larger numbers of target bacteria in 7 of the food/analysis combinations: fecal coliforms in fish; *E. coli* in poultry; fecal coliforms and *E. coli* in walnuts; and total coliforms, fecal coliforms and *E. coli* in black pepper. Random error (S_r^2) associated with the hydrophobic grid membrane filter method was significantly lower than that of the reference method in over 30% of the paired sample series. The hydrophobic grid membrane filter method for total coliform, fecal coliform, and *E. coli* enumeration in foods has been adopted official first action.

In 1982, a collaborative study was conducted to evaluate the performance of the hydrophobic grid membrane filter method for enumerating total coliform bacteria in selected foods (nonfat dry milk and canned custard). As a result of the study, this method was adopted official first action. At the recommendation of the General Referee, a second collaborative study was organized to extend the evaluation of the method to other foods and to study the performance of hydrophobic grid membrane filter methods for fecal coliform and *Escherichia coli* enumeration. This report presents the results of that second collaborative study.

Collaborative Study

Eighteen laboratories served as collaborators. The portion(s) of the study carried out by each laboratory are shown in Table 1. Each collaborator received a complete set of instructions, data sheets, and a set of samples for each portion of the study in which he or she took part. Special materials and media required for the hydrophobic grid membrane filter method were provided by the Associate Referee; all other materials were furnished by the collaborating laboratories.

Table 1. Laboratories collaborating in each food category

Coll.	Fish	Poultry	Nuts	Pepper	Cheese
1	Y[a]	Y	Y	Y	Y
2	Y	Y	Y	Y	Y
3	N	Y	N	N	N
4	Y	N	N	N	Y
5	N	N	Y	Y	Y
6	N	N	Y	Y	N
7	N	Y	Y	Y	N
8	N	Y	Y	Y	Y
9	N	N	N	Y	Y
10	Y	Y	Y	N	N
11	N	N	Y	Y	N
12	N	Y	N	N	N
13	N	Y	N	N	N
14	Y	Y	Y	Y	Y
15	Y	Y	N	N	N
16	Y	Y	Y	Y	Y
17	N	N	N	Y	N
18	Y	Y	Y	Y	Y
Total	8	12	11	12	9

[a]Y: Collaborator participated
N: Collaborator did not participate.

Five food products were used in the study, namely, frozen breaded fish, raw comminuted poultry meat, unroasted walnut pieces, ground black pepper, and cheddar cheese. The poultry meat was naturally contaminated with a variety of coliform bacteria and was analyzed without inoculation. Ground pepper was inoculated with lyophilized nutrient broth cultures by the Associate Referee; the inoculum was dispersed through the pepper by grinding overnight on a ball mill. The remaining foods were inoculated by collaborators using pre-calibrated inoculum suspensions provided by the Associate Referee. These suspensions were prepared as described previously (1). The contents of each inoculum mixture and the correlation of inoculum number to sample number are provided in Table 2. The Associate Referee retained representative portions of all samples and inoculum suspensions.

The study was carried out over a 5-week period, with each food scheduled to be run at the start of a different week. On

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The recommendation of the Associate Referee was approved by the General Referee and Committee F and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) **67**, March issue.

Table 2. Replicate sample pairs and inocula used

Food	Inoculum used in samples[a]							
	1	2	3	4	5	6	7	8
Fish	IV	II	I	III	II	IV	I	III
Poultry[b]	III	I	II	II	I	III	—	—
Nuts	IV	I	III	II	III	II	I	IV
Pepper	III	II	II	IV	III	I	IV	I
Cheese	I	III	IV	III	I	II	II	IV

[a]Inoculum I: *Escherichia coli* (mix of 5 strains)
Inoculum II: *Escherichia coli* (mix of 5 strains)
Citrobacter freundii (mix of 2 strains)
Inoculum III: *Escherichia coli* (mix of 5 strains)
Citrobacter freundii (mix of 2 strains)
Enterobacter aerogenes (one strain)
Enterobacter agglomerans (one strain)
Inoculum IV: *Hafnia alvei* (one strain)
[b]Naturally contaminated material. I = low; II = moderate; III = high levels of natural coliform population at time of blending and subdivision of samples. This series consisted of 6 samples only.

Table 3. Day-of-use control counts on each sample series: total coliforms

Food	Inoculum/series number (count/g)[a]		
	I	II	III
Frozen fish	1.1761	1.4771	3.0414
Raw poultry	1.7076	2.1761	2.0414
Unroasted walnuts	2.7853	2.8129	4.7924
Black pepper	0.5315	0.5185	0.4914
Cheddar cheese	>3.0414	2.9138	2.4624

[a]All counts were rounded to 2 significant figures and converted to log 10; walnut counts are reported per 100 g.

Table 4. Day-of-use control counts on each sample series: fecal coliforms

Food	Inoculum/series number (count/g)[a]		
	I	II	III
Frozen fish	1.1761	0.6532	3.0414
Raw poultry	1.6532	1.7324	1.5563
Unroasted walnuts	2.5051	2.7160	4.5682
Black pepper	0.5185	0.5185	0.4914
Cheddar cheese	>3.0414	2.9138	2.3979

[a]See footnote to Table 3.

Table 5. Day-of-use control counts on each sample series: *E. coli*

Food	Inoculum/series number (count/g)[a]		
	I	II	III
Frozen fish	1.1761	0.6532	3.0414
Raw poultry	1.6532	1.7324	1.5563
Unroasted walnuts	2.5051	2.7160	4.5682
Black pepper	0.5185	0.5185	0.4914
Cheddar cheese	>3.0414	2.9138	2.3424

[a]See footnote to Table 3.

the designated set-up date for each food, the coordinating laboratory performed 6 replicate counts from each inoculum mixture or sample series, using the official first action method **46.013–46.016** to determine the approximate total coliform, fecal coliform, and *E. coli* levels in each case. Results of these "day-of-use" control counts are shown in Tables 3–5.

Each collaborator analyzed all samples by both the HGMF method described below and by the official first action method **46.013 – 46.016** (reference method). The procedures outlined in the proposed method and the reference method were followed throughout, except for the walnuts. For walnuts, 10^0, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were used to inoculate the 3-tube MPN series to cover the full range of coliform counts anticipated in the samples. The 3 consecutive dilutions used to calculate the MPN index for the walnuts were chosen as described in *Standard Methods for the Examination of Dairy Products*, 14th edition (2). Collaborators calculated the most probable number (MPN) of total coliforms, fecal coliforms, and *E. coli* for both methods and reported all raw data (unconverted counts, number of gas-positive tubes, biochemical confirmations, etc.) in addition to the MPN/g.

Total Coliforms, Fecal Coliforms, and *Escherichia coli* in Foods

Hydrophobic Grid Membrane Filter Method

First Action

Principle

Hydrophobic grid membrane filter (HGMF) uses membrane filter imprinted with hydrophobic material in grid pattern. Hydrophobic lines act as barriers to spread of colonies, thereby dividing membrane filter surface into sep. compartments of equal and known size. Number of squares occupied by colonies is enumerated and converted to most probable number value of organisms by using formula given below.

Apparatus, Culture Media, and Reagents

(a) *Hydrophobic grid membrane filter (HGMF).*—Membrane filter has pore size of 0.45 μm and is imprinted with nontoxic hydrophobic material in grid pattern. ISO-GRID (available from QA Laboratories Ltd, 135 The West Mall, Toronto, Ontario, Canada M9C 1C2) or equiv. meets these specifications.

(b) *Filtration units for HGMF.*—Equipped with 5 μm mesh prefilter to remove food particles during filtration. One unit is required for each sample. ISO-GRID (available from QA Laboratories Ltd) or equiv. meets these specifications.

(c) *Pipets.*—1.0 mL serological with 0.1 mL graduations; 1.1 mL or 2.2 mL milk pipets are satisfactory. 5.0 mL serological with 0.1 mL graduations.

(d) *Blender.*—Waring Blender, or equiv., multispeed model, with low-speed operation at 10 000–12 000 rpm, and 250 mL glass or metal blender jars with covers. One jar is required for each sample.

(e) *Vacuum pump.*—Water aspirator vac. source is satisfactory.

(f) *Manifold or vacuum flask.*

(g) *Filter paper.*—Whatman No. 1 or No. 4, or equiv.

(h) *Peptone/Tween 80 diluent.*—Dissolve 1.0 g peptone (Difco 0118) and 10.0 g Tween 80 in 1 L H_2O. Dispense enough

vol. into diln bottles to give 90 ± 1 mL after autoclaving 15 min at 121°.

(i) *M-FC agar.*—10.0 g tryptose, 5.0 g proteose peptone No. 3, 3.0 g yeast ext, 5.0 g NaCl, 12.5 g lactose, 1.5 g bile salts No. 3, 0.1 g aniline blue, and 15.0 g agar dild to 1 L with H_2O (M-FC Agar, Difco 0677, is satisfactory). Heat to boiling. Temper to 50–55°. Adjust pH to 7.4 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(j) *Tryptone bile agar (TBA).*—20.0 g tryptone, 1.5 g bile salts No. 3, and 15.0 g agar dild to 1 L with H_2O (Tryptone bile agar, Oxoid CM595, is satisfactory). Heat to boiling. Autoclave 15 min at 121°. Temper to 50–55°. Adjust pH to 7.2 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(k) *Tryptic soy-magnesium sulfate agar (TSAM).*—15.0 g tryptone, 5.0 g phytone (or soytone), 5.0 g NaCl, 1.5 g $MgSO_4.7H_2O$, and 15.0 g agar dild to 1 L with H_2O. Heat to boiling. Autoclave 15 min at 121°. Temper to 50–55°. Adjust pH to 7.3 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(l) *Indole reagent.—(1) Soln A:* Dissolve 2.5 g *p*-dimethylamino benzaldehyde and 10 mL HCl in 90 mL alcohol. *(2) Soln B:* Dissolve 2.0 g potassium persulfate in 200 mL H_2O. Mix equal vols of Soln A and Soln B just before use.

(m) *Tris buffer.*—1.0M, pH 7.6. Dissolve 121.1 g tris(hydroxymethylamino)methane and dil. to 1 L with H_2O. Adjust pH to 7.6 with 1N HCl.

(n) *Trypsin stock soln.*—Dil. 10 g trypsin to 100 mL with tris buffer. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material, then filter-sterilize using 0.45 μm membrane filter.

Sample Preparation

(a) *Nut meat pieces.*—Aseptically weigh 50 g sample into sterile jar. Add 50 mL peptone/Tween 80 diluent (h) and shake vigorously (50 times thru 30 cm arc). Let stand 3–5 min and shake just before doing filtrations.

(b) *Cheese.*—Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (h) and blend 2 min at low speed (10 000–12 000 rpm). Aseptically combine, in 16 × 150 mm tube, 3.5 mL of this 1:10 homogenate and 3.5 mL trypsin soln (n). Incubate 20–30 min at 35 ± 0.5° in H_2O bath. Vortex to remix suspension just before doing filtrations.

(c) *Other foods needing digestion.*—Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (h) and blend 2 min at low speed (10 000–12 000 rpm). Aseptically combine in 16 × 150 mm tube 5.0 mL of this 1:10 homogenate and 1.0 mL trypsin soln (n). Incubate 20–30 min at 35 ± 0.5° in H_2O bath. Vortex to remix suspension just before doing filtrations.

(d) *Other foods.*—Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (h) and blend 2 min at low speed (10 000–12 000 rpm).

Analysis

(See Fig. **46:01.**) Turn on vac. source. Place sterile filtration unit on manifold or vac. flask. Open clamp A. Pull forward on latch B, and rotate back funnel portion C. Aseptically place sterile HGMF on surface of base D. Rotate funnel forward and clamp unit shut by pressing with thumb on latch B.

Aseptically add ca 15–20 mL sterile H_2O to funnel. Pipet required volume (see Table 6) of sample suspension into funnel. Apply free end of vac. tubing E to suction hole F to draw liq. thru prefilter mesh G. Aseptically add addnl 10–15 mL H_2O to funnel and draw thru mesh as before. Close clamp A to direct vac. to base of filtration unit and draw liq. thru HGMF.

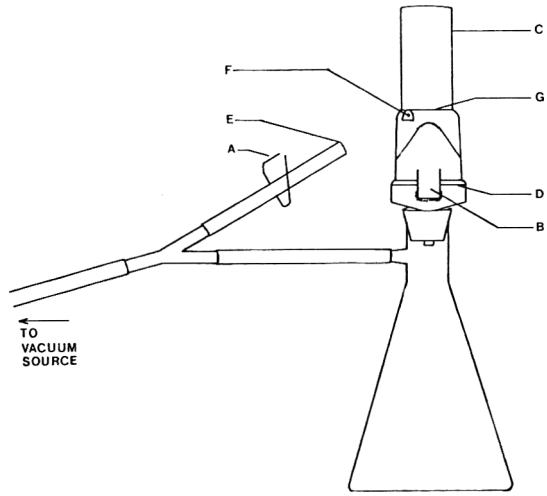

Fig. 46:01 Schematic of hydrophobic grid membrane filtration unit.

Table 6. Filtering volumes and multiplication factors

Food	Filtering diln	Filtering vol., mL	Multiplication factor
Nut meat pieces	10^0	0.5	2
Cheese	10^{-1}	2.0[a]	10
Other foods needing digestion	10^{-1}	1.2[a]	10
Other foods	10^{-1}	1.0	10

[a]Filtering volume of enzyme-digested suspension.

Open clamp A. Release latch B, and rotate back funnel C. Aseptically remove HGMF and place on surface of pre-dried agar plate (see below). Avoid trapping air bubbles between filter and agar.

(a) *Total coliform count.*—Place HGMF on surface of pre-dried M-FC agar (i). Incubate 24 ± 2 h at 35°. Count all squares contg one or more blue colonies. Include any shade of blue. Score each square as either pos. (blue) or neg. Convert pos. square count to MPN with the formula

$$\text{MPN} = [N \log_e (N/(N - x))]$$

where N = total number of squares and x = number of pos. squares. Multiply by reciprocal of diln factor and report as MPN of total coliform bacteria/g.

(b) *Fecal coliform count.*—Place HGMF on surface of pre-dried TSAM (k). Incubate 4–5 h at 25° for dry foods and 4–5 h at 35° for all other foods. Transfer HGMF to surface of pre-dried M-FC agar (i) and incubate 24 ± 2 h at 44.5 ± 0.5° in closed container. Proceed as in (a), and report as MPN of fecal coliform bacteria/g.

(c) *E. coli count.*—Place HGMF on surface of pre-dried TSAM (k). Incubate 4–5 h at 25° for dry foods and 4–5 h at 35° for all other foods. Transfer HGMF to surface of pre-dried TBA (j) and incubate 24 ± 2 h at 44.5 ± 0.5° in closed container. Prepare indole reagent (l) by combining equal vol. of Soln A and Soln B. Place 9 cm filter paper disk in petri dish lid and flood with 1–2 mL indole reagent (l). Transfer HGMF to filter paper, ensuring that no air bubbles are trapped between HGMF and paper. Let stand 10–15 min, then transfer HGMF back to surface of TBA. Count all squares contg one or more pink (indole pos.) colonies. Score each square as either pos. (pink) or neg. Convert pos. square count to

MPN with formula above. Multiply by reciprocal of diln factor and report as MPN of *E. coli* (biotype 1)/g.

Results and Discussion

All reported data were checked for correctness of the most probable number (MPN) indices and dilution factor calculations and rounded to 2 significant figures. Data from the walnut samples were expressed as MPN/100 g to avoid negative logarithms and all data were converted to $\log_{10}$ values for statistical analysis. The data are displayed in Tables 7–21.

Two collaborators reported significant method deviations. Collaborator 2 encountered difficulties in performing IMVIC confirmations on isolates from EMB agar. Therefore, it was necessary to disregard *E. coli* data from Collaborator 2 for all 5 foods. Total and fecal coliform data were unaffected. Collaborator 4 carried out the reference method analysis of the frozen breaded fish using a dilution series beginning at 10^{-3} instead of 10^{-1} as specified. All data from this collaborator for the fish series were discarded.

Two collaborators (No. 1 and No. 13) reported beginning some or all analyses on dates other than those specified. Since both the HGMF and the reference methods were initiated at the same time, data from these collaborators were not discarded unless determined to be outliers by a rank score test (3).

Data obtained from samples inoculated with Mixture IV, *Hafnia alvei* (see Table 2), were excluded from all statistical analyses.

Each food/analysis combination was then tested independently for the presence of outlying laboratories by rank score test (3). Where found, outliers have been indicated by footnotes to the appropriate data tables. Data from outlying laboratories were excluded from statistical evaluation of the food/analysis series in which they occurred.

Despite the efforts of the Associate Referee to obtain products with little or no indigenous coliform population for the inoculated sample series, the walnuts, and to a much lesser extent, the black pepper, were naturally contaminated with coliforms. This was evidenced by the occurrence of counts in some of the samples inoculated with *H. alvei* (Mixture IV), a lactose-negative bacterium. Both the HGMF and the reference methods recovered significant numbers of coliforms and even the occasional *E. coli* from these "negative control" samples. Therefore the counts were clearly not a "false positive" result associated with a single method but represented the detection and enumeration of an indigenous coliform flora.

The presence of these naturally occurring coliforms did not preclude carrying out a statistical evaluation of the data from samples inoculated with Mixtures I, II, and III. While the distribution of the natural coliform population was not homogeneous, a pocket of contamination would have been equally likely to occur in the sample portion analyzed by either method. Thus, the results would not have been biased in favor of one or the other method and a comparison of mean recoveries could be made. Similarly, while the existence of a heterogeneously distributed coliform population would almost certainly result in elevated repeatability and reproducibility coefficients of variation, here, again, the impact would have been similar on both methods.

Random error (S_r^2) and systematic error (S_b^2) were determined on each paired sample series for which at least 2 collaborators reported finite data for both samples of the pair using both methods. These variances are summarized in Tables 22–24. An *F*-test was used to compare the random errors of the methods. In 25 of the 31 replicate series analyzed, random error of the hydrophobic grid membrane filter method was less than that of the reference method. The difference between the 2 methods was statistically significant in 10 of the 25 series ($P \leq 0.05$). In no instance was the random error of the reference method significantly less than that of the hydrophobic grid membrane filter method. These results are consistent with the findings of a previous collaborative study of total coliform enumeration by hydrophobic grid membrane filter (1).

The data from each food/analysis combination were subjected to a 3-way analysis of variance (ANOVA) and precision estimates of repeatability and reproducibility were determined. The results of these evaluations are contained in Tables 25–27. In almost every instance, the coefficients of variation of repeatability and reproducibility were smaller for the hydrophobic grid membrane filter than for the reference method. This is not surprising, considering the relative magnitudes of the random errors (S_r^2) associated with both methods.

The hydrophobic grid membrane filter methods recovered significantly higher numbers of the target bacteria in 7 cases: fecal coliforms in fish ($P \leq 0.05$); *E. coli* in poultry ($P \leq 0.05$); fecal coliforms and *E. coli* in walnuts ($P \leq 0.05$); and total coliforms, fecal coliforms, and *E. coli* in black pepper ($P \leq 0.01$). Overall mean recovery was numerically higher than that of the reference method in all cases except total coliforms in cheddar cheese.

The impressive performance of the hydrophobic grid membrane filter method was probably due to 2 factors. A major advantage of any membrane filter method is the ability to resuscitate injured bacteria on a nonselective medium before those cells are exposed to selective agents or to elevated incubation temperatures. While LST broth is often considered by microbiologists to be a resuscitation medium, it contains sodium lauryl sulfate, a selective agent known to be harmful to injured gram-negative bacteria (4). The 4–5 h resuscitation step incorporated into the *E. coli* hydrophobic grid membrane filter method has been shown to double the recovery of injured cells over that obtained by direct incubation of the filter on tryptone bile agar (5).

A second important advantage of the hydrophobic grid membrane filter method is the ability to exclude from the culture medium interfering substances contained in the sample. The benefits of this were made evident by the results of the ground pepper sample series. Black pepper has been reported to be a weak inhibitor of some bacteria (6). In the reference method, the inhibitory constituents of pepper were inevitably added to the LST broth, most likely increasing the selectivity of the LST and impairing the ability of injured cells to repair and multiply. In contrast, with the hydrophobic grid membrane filter method, the ground pepper solids were removed from the sample suspension by the 5 μm prefilter, and any water-soluble inhibitors were rinsed through the filter and diluted out. This, combined with the resuscitation step for fecal coliforms and *E. coli*, permitted the detection and enumeration of injured target bacteria, and produced significantly higher counts than the reference method.

The results of this collaborative study are consistent with those of a recent Canadian study reported by Sharpe et al. (7). In that study, a hydrophobic grid membrane filter *E. coli* method was evaluated against a 5-tube MPN procedure by 5 regional government laboratories. The study was carried out on ground beef, parmesan cheese, and frozen green beans. In almost every instance, the hydrophobic grid membrane filter method recovered significantly higher numbers of *E. coli* than the reference method (ANOVA; $P \leq 0.05$).

Table 7. Total coliform counts[a] in frozen, breaded fish

Coll.	MPN/g by HGMF method								MPN/g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	<1.0000	2.4771	2.4624	3.1761	2.2304	<1.0000	<1.0000	2.3617	<0.4771	>3.0414	1.3617	>3.0414	1.6435	1.1761	2.6628	>3.0414
2	1.0000	2.2304	2.1461	1.7782	2.9685	1.4771	M[b]	M	<0.4771	2.1761	0.8633	2.6628	2.6628	0.9590	M	M
4[c]	1.3010	1.3010	<1.0000	2.2304	1.6021	<1.0000	1.3010	2.3010	<2.4771	<2.4771	<2.4771	<2.4771	3.3617	<2.4771	<2.4771	2.5563
10	<1.0000	1.7782	<1.0000	2.6021	1.6990	<1.0000	1.0000	2.7559	<0.4771	1.9685	0.5563	2.3802	1.6335	0.5663	1.6335	3.0414
14	<1.0000	1.0000	1.6990	2.3010	1.7782	<1.0000	<1.0000	>4.9638	0.5563	0.9590	1.9685	2.6628	1.9685	0.5563	0.9590	2.3802
15	<1.0000	2.0414	1.0000	2.3979	1.7782	<1.0000	<1.0000	2.3617	1.3617	2.0792	>3.0414	2.3802	1.9685	0.5563	<0.4771	2.6628
16	<1.0000	1.6021	2.3424	<1.0000	1.4771	1.0000	<1.0000	2.3617	0.9590	1.9685	1.9685	0.9590	1.8751	1.6335	0.5563	2.3802
18	<1.0000	2.0414	<1.0000	2.5315	2.1461	<1.0000	<1.0000	2.5315	<0.4771	2.3222	0.5563	3.0414	2.1761	0.5563	0.5563	2.3222

[a] All counts were rounded to two significant figures and converted to $\log_{10}$.

[b] Missing value - analysis not done or incomplete.

[c] Collaborator used wrong dilution series for reference method.

Table 8. Fecal coliform counts[a] in frozen, breaded fish

Coll.	MPN/g by HGMF method								MPN/g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	<1.0000	2.4771	2.4624	3.1761	2.2304	<1.0000	<1.0000	2.3617	<0.4771	2.6628	0.9590	>3.0414	1.6435	<0.4771	1.3617	3.0414
2	2.7709	2.1761	2.1139	3.8451	3.0792	M[b]	M	M	<0.4771	1.1761	0.8633	2.3802	2.1761	<0.4771	M	M
4[c]	<1.0000	1.4771	<1.0000	1.8451	1.7782	<1.0000	<1.0000	2.3010	<2.4771	<2.4771	<2.4771	<2.4771	2.5563	<2.4771	<2.4771	2.5563
10	<1.0000	1.4771	1.3010	2.4314	1.6021	<1.0000	<1.0000	2.6335	<0.4771	1.6335	<0.4771	2.3802	1.3617	<0.4771	0.9590	2.6628
14	<1.0000	<1.0000	1.6990	2.3010	1.9031	<1.0000	<1.0000	>4.9638	<0.4771	0.5563	1.9685	2.6628	1.9685	<0.4771	0.9590	2.3802
15	<1.0000	2.0000	<1.0000	2.3617	1.6021	<1.0000	<1.0000	2.3424	<0.4771	2.0792	0.9590	2.3802	0.5563	<0.4771	<0.4771	2.6628
16	<1.0000	1.0000	2.4624	<1.0000	1.4771	<1.0000	<1.0000	2.0414	<0.4771	1.3222	1.6335	0.5563	1.8751	<0.4771	0.5563	1.3617
18	<1.0000	1.8451	<1.0000	2.3222	1.9542	<1.0000	<1.0000	2.2553	<0.4771	1.1761	<0.4771	2.1761	1.6335	<0.4771	<0.4771	2.1761

[a,b,c] See footnotes to Table 7.

Table 9. *Escherichia coli*[a] counts in frozen, breaded fish

Coll.	MPN/g by HGMF method								MPN/g by A.O.A.C. 46.013-016							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	<1.0000	2.4771	<1.0000	3.1461	2.0792	<1.0000	<1.0000	2.3617	<0.4771	2.6628	0.9590	1.5441	1.6435	<0.4771	1.3617	1.0414
2	<1.0000	1.3010	<1.0000	2.0000	1.3010	<1.0000	M[b]	M	<0.4771	M	M	M	M	<0.4771	M	M
4[c]	<1.0000	1.3010	<1.0000	2.1139	1.4771	<1.0000	<1.0000	1.8451	<2.4771	<2.4771	<2.4771	<2.4771	2.5563	<2.4771	<2.4771	2.5563
10	<1.0000	1.6990	<1.0000	2.3222	1.7782	<1.0000	<1.0000	2.2788	<0.4771	1.6335	<0.4771	2.3802	1.3617	<0.4771	0.9590	2.6628
14	<1.0000	1.0000	1.4771	2.1139	1.3010	1.0000	1.0000	>4.9638	<0.4771	<0.4771	1.9685	2.3802	1.9685	<0.4771	0.9590	2.3802
15	<1.0000	1.9542	<1.0000	2.3424	1.6021	<1.0000	1.0000	2.3617	<0.4771	2.0792	0.5563	2.3802	0.5563	<0.4771	<0.4771	1.1761
16	<1.0000	2.0000	2.2304	<1.0000	1.4771	<1.0000	<1.0000	1.9031	<0.4771	1.3222	1.6335	0.5563	1.8751	<0.4771	0.5563	1.3617
18	1.0000	1.7782	1.0000	2.2553	1.6021	<1.0000	<1.0000	2.2788	<0.4771	1.1761	<0.4771	2.1761	1.6335	<0.4771	<0.4771	2.1761

[a,b,c] See footnotes to Table 7.

Table 10. Total coliform counts[a] in frozen, comminuted poultry

Coll.	MPN/g by HGMF method						MPN/g by AOAC 46.013-016 method					
	1	2	3	4	5	6	1	2	3	4	5	6
1	2.2304	2.1139	2.0414	1.4771	2.2041	2.3424	2.6628	2.3302	2.3802	2.3802	2.6628	2.6628
2	2.2304	2.0414	2.3617	2.6128	2.7924	3.2041	1.0414	1.3222	2.3802	1.9685	2.0792	2.6628
3	2.6335	2.3424	2.5911	2.4624	2.3424	2.4771	2.6628	2.3302	1.6335	1.8751	3.0414	1.8751
7	2.4771	2.2788	2.3010	2.3617	2.2553	2.3424	2.3802	1.9685	1.9685	2.3802	1.6335	1.9685
8	2.6021	2.5441	2.5051	2.5563	2.4472	2.2788	2.3802	1.9685	1.9685	2.6628	2.3802	1.4624
10	2.5051	2.4314	2.4624	2.4150	2.1139	2.6435	2.3802	2.1761	2.3802	2.6628	2.3802	2.3222
12	2.2553	2.4472	2.0414	2.4150	2.4314	2.4472	3.0414	1.9685	2.6628	>3.0414	2.6628	2.3802
13[b]	2.1461	1.8451	1.6990	1.9031	1.6990	2.1139	1.6335	1.3617	1.9685	1.9685	1.3617	1.6335
14	2.2304	2.0000	1.9031	2.2304	1.7782	2.2304	3.0414	1.3617	2.3802	2.3802	2.1761	>3.0414
15	2.3010	2.3424	3.0000	2.3222	2.4150	>4.9638	2.6628	1.8751	2.6628	3.0414	1.9685	>3.0414
16	2.3617	1.8451	2.3617	2.0000	2.0000	2.3979	2.3802	1.8751	1.3617	2.3802	2.3802	2.6628
18[b]	2.0792	1.6990	1.6990	1.6990	1.9031	1.9031	1.9685	1.9685	2.1761	1.8062	1.3617	2.6628

[a] All counts were rounded to two significant figures and converted to $\log_{10}$.

[b] Statistical outlier for HGMF method, based on rank scores (3).

Table 11. Fecal coliform counts[a] in frozen, comminuted poultry

Coll.	MPN/g by HGMF method						MPN/g by AOAC 46.013-016 method					
	1	2	3	4	5	6	1	2	3	4	5	6
1	2.1761	1.6990	2.2553	1.6021	2.0792	1.4771	1.3617	0.9590	1.9685	1.9685	0.9590	1.6335
2	2.0414	2.4314	2.3979	2.1461	2.1761	2.3979	1.0414	1.3222	1.6335	1.6335	1.6335	2.3802
3	2.2788	2.2553	2.1461	2.4314	2.2553	2.1461	1.9685	2.3802	1.3617	1.8751	1.6335	0.9590
7	2.4771	2.3010	2.3010	2.4150	2.2553	2.3617	2.3802	1.9685	1.9685	1.9685	1.6335	1.9685
8	2.4150	2.4150	2.2788	1.6021	2.4771	2.3979	1.9685	1.9685	1.9685	2.3802	1.4624	1.4624
10	2.4314	2.3802	2.5051	2.3802	2.0792	2.4624	1.9685	2.1761	2.3802	1.8751	1.6335	2.1761
12	2.5051	2.4771	2.1761	2.4624	2.4624	>4.9638	2.3802	1.6335	2.6628	2.3222	1.9685	1.6335
13[b]	1.3010	1.4771	1.4771	1.0000	1.4771	1.4771	1.6335	1.3617	1.3222	1.1761	0.9590	1.1761
14	2.2788	2.0000	1.6990	1.9542	1.7782	2.1761	2.0792	1.3617	2.3802	1.9685	1.3617	2.6628
15	2.2788	2.0000	2.2788	2.0792	2.7160	>4.9638	1.6335	1.3010	3.0414	1.4314	1.9685	2.3802
16	2.3802	1.7782	2.3010	1.9031	1.8451	2.2788	2.3802	1.5911	1.3617	1.6335	1.6335	1.3617
18[b]	1.4771	1.6021	1.4771	1.4771	1.0000	1.3010	0.9590	1.6335	1.5911	1.5911	0.5563	0.9590

[a], [b] See footnotes to Table 10.

Table 12. Escherichia coli counts[a] in frozen, comminuted poultry

Coll.	MPN/g by HGMF method						MPN/g in AOAC 46.013-016 method					
	1	2	3	4	5	6	1	2	3	4	5	6
1	2.0792	1.3010	1.0000	1.0000	1.7782	2.0792	1.3617	0.9590	1.9685	1.9685	0.9590	1.3617
2	1.0000	1.6021	1.6021	<1.0000	1.8451	1.8451	<0.4771	M[b]	M	M	M	M
3	2.3222	2.1139	2.2788	2.2553	2.2304	2.2304	1.9685	2.3802	1.3617	1.8751	1.1761	0.9590
7	2.3979	1.8451	2.0792	2.0000	1.6021	2.3010	1.9685	1.9685	1.1761	1.9685	1.1761	1.9685
8[c]	3.4150	3.4150	3.3222	3.4314	2.6021	3.3617	<0.4771	0.8633	0.5563	<0.4771	0.4771	0.4771
10	2.0000	2.1139	2.1761	2.1461	1.6990	2.2553	1.3222	2.1761	1.3222	1.3010	1.6335	1.4472
12[d]	2.4624	2.3617	2.5051	2.5185	2.6232	>4.9638	2.3802	1.6335	2.6628	1.3010	0.8633	1.3617
13[d]	1.7782	1.4771	1.3010	1.0000	1.0000	1.6990	1.6335	1.3617	1.3222	1.1761	0.9590	1.1761
14	2.0414	1.3010	2.0414	1.7782	2.0000	2.0000	0.5563	1.3617	1.6335	1.6335	0.9590	1.9685
15	1.9542	2.0414	2.1139	1.3010	1.9542	2.4771	0.9590	1.0414	2.1761	1.4314	1.9685	1.0414
16	2.1139	1.8451	1.9031	1.8451	1.4771	2.1761	1.9685	1.5911	1.3617	1.6335	1.6335	1.3617
18[d]	<1.0000	1.6021	<1.0000	1.0000	<1.0000	1.3010	0.9590	1.3617	1.5911	1.1461	0.5563	0.9590

[a] All counts were rounded to two significant figures and converted to $\log_{10}$.

[b] Missing value - analysis not done or incomplete.

[c] Statistical outlier for both methods, based on rank scores (3).

[d] Statistical outlier for HGMF method, based on rank scores (3).

Table 13. Total coliform counts[a] in walnut pieces

Coll.	MPN/100g by HGMF method								MPN/100g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	3.3802	3.6628	3.7160	4.8633	3.7160	<2.3010	4.3010	3.7924	4.3802	4.6628	2.9685	>5.0414	3.9685	3.2041	4.3222	4.6628
2	3.3802	3.0000	3.9638[b]	4.2304[b]	3.8451[b]	2.3010	2.6021	<2.3010	1.5563	2.6335	3.3802	2.3617	2.1761	1.9590	2.3617	1.5563
5	3.1461	3.8195	3.6628	2.9031	3.4472	2.3010	4.4150	4.3222	2.6335	2.6335	3.1761	2.1761	3.1761	2.9685	4.3802	2.9685
6	3.3802	3.9542	3.6021	3.7324	3.7924	5.6021	M[c]	M	5.0414	2.9685	2.9685	2.6335	3.3617	2.9685	3.6335	3.6335
7	4.0000	4.5185	4.1461	3.5798	3.8325	3.5051	4.7634	M	2.3617	2.3617	3.3617	2.6335	3.6335	3.3617	2.5911	M
8	M	M	M	M	M	M	M	M	4.3802	4.3802	4.3802	3.1761	3.6335	2.5563	3.3222	2.8751
10	3.4150[b]	4.4914[b]	4.0792	4.0414	3.7634	4.2304	3.1461[b]	2.6021	3.1761	2.9685	4.1761	4.3802	3.3617	2.3617	2.1761	3.3617
11	4.2253	2.3010	3.7324	4.8921	3.9731	3.7160	<2.3010	4.0414	4.3802	3.6335	3.9685	3.3617	4.3802	2.9685	3.3222	2.9685
14	<2.3010	4.9445	3.6990	3.7782	3.4150	<2.3010	<2.3010	<2.3010	4.3222	5.0414	2.9685	5.0414	3.1761	4.3802	4.3802	>5.0414
16	3.8325	3.7482	3.4771	3.7634	3.8062	3.8573	3.9823	4.3010	5.0414	4.6628	3.6335	3.6335	3.6335	3.6335	3.6335	5.0414
18	<2.3010	<2.3010	3.6628	4.2304	3.6990	5.0792	2.6021	4.1761	3.8751	4.6628	3.6335	3.5335	3.6335	4.3802	5.0414	4.6628

[a] All counts were rounded to two significant figures and converted to $\log_{10}$. Counts expressed per 100g to avoid use of negative logarithms.
[b] Mold reported on the filter.
[c] Missing value - analysis not done or incomplete.

Table 14. Fecal coliform counts[a] in walnut pieces

Coll.	MPN/100g by HGMF method								MPN/100g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	3.3802	3.6628	3.4150	4.5798	3.6628	<2.3010	4.3010	3.6628	2.8751	1.9590	2.9685	>5.0414	3.9685	2.6335	2.9685	1.9590
2	3.7482[b]	4.0000	3.5563	3.5051[b]	3.8195	3.6435	3.4472[b]	2.6021[b]	1.5563	1.9590	3.3802	2.1761	2.6335	2.3617	<1.4771	2.0414
5	2.6021	3.3802	3.6435	2.3010	3.3010	<2.3010	4.5441	2.7782	<1.4771	2.1761	2.9685	1.5563	2.9685	1.9590	1.9590	1.5563
6	3.2041	3.2553	3.8062	3.5315	4.2788	5.7160	M[c]	M	3.3617	2.1761	2.6335	1.9590	2.9685	<1.4771	<1.4771	<1.4771
7	3.9638	4.5185	4.0000	3.2041	3.7924	3.3802	<2.3010	M	<1.4771	1.5563	2.6335	<1.4771	3.6335	1.9590	1.9590	M
8	M	M	M	M	M	M	M	M	<2.4771	3.3617	4.3802	<2.4771	3.6335	<2.4771	<2.4771	<1.4771
10	4.2553[b]	4.6021[b]	4.0792	2.9031[b]	4.0414	4.3222[b]	3.0000[b]	<2.3010[b]	<1.4771	2.9685	4.1761	2.6335	2.9685	1.5563	1.9590	2.3617
11	3.9542	4.0414	3.6435	4.7160	4.0414	2.7782	<2.3010	2.9031	3.9685	3.1761	3.9685	2.6335	4.3802	2.6335	<1.4771	2.3617
14	<2.3010	4.3424	3.6990	3.7324	3.6021	<2.3010	2.3010	2.3010	4.1761	<1.4771	2.3617	5.0414	3.1761	2.1461	<1.4771	<1.4771
16	<2.3010	3.6628	3.4472	3.7324	3.5051	3.4150	3.6990	3.3010	<1.4771	1.5563	3.3617	<1.4771	3.6335	<1.4771	1.5563	<1.4771
18	2.3010	<2.3010	3.6628	3.2553	3.5315	4.7324	2.3010	3.9445	<1.4771	<1.4771	3.6335	<1.4771	3.6335	1.9590	1.8633	<1.4771

[a],[b],[c] See footnotes to Table 13.

Table 15. *Escherichia coli* counts[a] in walnut pieces

Coll.	MPN/100g by HGMF method								MPN/100g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1[b]	3.3802	3.6628	3.4472	4.5798	3.6628	<2.3010	4.3010	3.6628	2.8751	1.9590	2.9685	>5.0414	3.9685	2.6335	2.9685	1.9590
2	<2.3010	3.0000	3.4771	2.3010	3.0792	<2.3010	<2.3010	2.3010	M[c]	M	M	M	M	M	<1.4771	M
5	<2.3010	3.0792	3.4771	2.6021	3.1461	<2.3010	<2.3010	<2.3010	<1.4771	1.9590	1.9685	1.5563	1.9590	1.9590	1.5563	<1.4771
6	<2.3010	2.3010	3.3802	<2.3010	3.3424	<2.3010	M	M	<1.4771	2.1761	2.6335	1.5563	2.9685	<1.4771	<1.4771	<1.4771
7	2.3010	<2.3010	3.4472	<2.3010	3.3424	<2.3010	2.3010	M	<1.4771	1.5563	2.6335	<1.4771	3.6335	1.9590	1.9590	M
8[d]	2.3010	3.8325	3.9138	3.5051	4.1461	2.3010	4.6435	2.3010	<2.4771	<2.4771	3.0414	<2.4771	3.3617	<2.4771	<2.4771	<1.4771
10	<2.3010	<2.3010	3.7924	2.3010	3.6435	<2.3010	<2.3010	<2.3010	<1.4771	<1.4771	4.1761	2.6335	2.9685	1.5563	<1.4771	1.9590
11	<2.3010	<2.3010	3.4771	2.3010	3.4771	2.3010	<2.3010	3.5315	2.1761	1.9590	3.9685	2.3617	3.6335	1.8633	<1.4771	2.3617
14	<2.3010	<2.3010	2.9031	<2.3010	3.2041	<2.3010	<2.3010	2.3010	2.4314	<1.4771	<1.4771	2.3010	2.3010	<1.4771	<1.4771	<1.4771
16	<2.3010	2.6021	3.0792	2.6021	3.2553	<2.3010	<2.3010	<2.3010	<1.4771	1.5563	3.3617	<1.4771	3.6335	<1.4771	1.5563	<1.4771
18	<2.3010	<2.3010	3.3424	<2.3010	3.2553	<2.3010	<2.3010	2.9031	<1.4771	1.5563	3.6335	<1.4771	3.6335	<1.4771	<1.4771	<1.4771

[a] All counts were rounded to two significant figures and converted to $\log_{10}$. Counts expressed per 100g to avoid use of negative logarithms.
[b] Statistical outlier for reference method (A.O.A.C. 46.013-46.016), based on rank scores (3).
[c] Missing value - analysis not done or incomplete.
[d] Statistical outlier for H.G.M.F. method, based on rank scores (3).

Table 16. Total coliform counts[a] in ground black pepper

Coll.	MPN/g by HGMF method								MPN/g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	2.3979	1.8451	1.3010	1.9031	1.9031	1.9542	1.4771	1.9031	1.6335	<0.4771	<0.4771	0.5563	1.6335	0.9590	<0.4771	1.5911
2	1.0000	1.6990	1.4771	1.8451	2.6721	1.9542	1.7782	2.4472	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
5	1.9031	2.7993	2.1761	1.6990	1.3010	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
6	1.4771	1.6021	1.9542	1.6990	1.6990	2.0000	1.0000	1.6021	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
7	1.4771	1.7782	1.9031	1.0000	1.6990	1.3010	1.8451	1.3010	0.5563	<0.4771	0.5563	<0.4771	<0.4771	0.5563	<0.4771	0.5563
8	2.3010	<1.0000	2.8573	2.9395	<1.0000	1.4771	1.6990	<1.0000	0.5563	0.5563	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
9	2.0792	2.0792	1.9031	2.2041	1.9031	1.9031	2.3802	2.7634	1.3617	0.9590	0.9590	<0.4771	0.5563	<0.4771	<0.4771	0.5563
11	2.0000	2.0792	2.5185	2.5185	2.2041	2.0000	1.9542	2.2788	0.5563	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
14[b]	1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	0.5563	<0.4771	<0.4771	<0.4771
16	2.2553	2.2304	1.3010	1.6021	2.5563	1.8451	2.3010	2.3010	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
17	2.5441	1.8451	1.9542	1.7782	2.4150	1.9542	1.8451	2.4314	1.3617	<0.4771	0.5563	0.5563	1.1761	0.5563	<0.4771	1.3617
18	2.1461	1.7782	1.0000	2.0000	2.0414	2.0792	1.8451	1.6021	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771

[a] All counts were rounded to two significant figures and converted to $\log_{10}$.

[b] Statistical outlier for HGMF method, based on rank scores (3).

Table 17. Fecal coliform counts[a] in ground black pepper

Coll.	MPN/g by HGMF method								MPN/g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	1.7782	2.1461	1.4771	<1.0000	1.4771	2.1461	1.0000	1.4771	0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	1.3617
2	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	0.5563
5	<1.0000	2.7853	<1.0000	1.6990	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
6	1.4771	<1.0000	1.6021	1.4771	1.7782	1.6990	1.6990	1.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
7	<1.0000	1.6021	1.7782	1.6021	1.9031	1.9542	1.8451	1.0000	0.5563	<0.4771	0.5563	<0.4771	<0.4771	0.5563	<0.4771	0.5563
8	3.0414	3.3424	<1.0000	1.7782	<1.0000	<1.0000	1.6021	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
9	<1.0000	<1.0000	<1.0000	M[b]	1.0000	<1.0000	1.3010	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	0.5563	<0.4771	<0.4771	0.5563
11	1.6021	1.9031	2.0000	2.1761	<1.0000	<1.0000	1.7782	1.7782	0.5563	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
14	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	1.3010	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
16	1.6021	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	1.4771	1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
17	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
18	<1.0000	<1.0000	<1.0000	<1.0000	1.6990	1.4771	1.6990	1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771

[a] All counts were rounded to two significant figures and converted to $\log_{10}$.

[b] Missing value - analysis not done or incomplete.

Table 18. *Escherichia coli* counts[a] in ground black pepper

Coll.	MPN/g by HGMF method								MPN/g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	1.3617
2	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	M[b]
5	<1.0000	1.3010	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
6	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
7	<1.0000	<1.0000	1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	0.5563	<0.4771	0.5563	<0.4771	<0.4771	0.5563	<0.4771	0.5563
8	<1.0000	3.5051	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
9	<1.0000	<1.0000	<1.0000	M[b]	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	0.5563	<0.4771	<0.4771	0.5563
11	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	0.5563	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
14	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
16	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
17	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771
18	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771	<0.4771

[a,b] See footnotes to Table 17.

Table 19. Total coliform counts[a] in cheddar cheese

Coll.	MPN/g by HGMF method								MPN/g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1[b]	1.3010	1.0000	<1.0000	1.0000	1.6021	1.6990	<1.0000	<1.0000	>3.0414	1.6335	<0.4771	<0.4771	>3.0414	1.9685	1.9685	0.5563
2	2.0000	1.3010	<1.0000	1.4771	2.2041	1.8451	1.9031	<1.0000	2.3222	1.6335	1.3617	1.3010	2.1761	3.0414	2.1761	<0.4771
4	2.6021	1.7782	<1.0000	1.4771	2.6335	1.6021	2.3010	<1.0000	3.0414	2.0792	<0.4771	2.3802	2.6628	2.3802	2.3802	<0.4771
5[b]	2.5315	2.1461	<1.0000	2.0792	2.8976	2.4771	2.5911	<1.0000	2.3802	1.3010	<0.4771	1.9685	2.6628	2.3802	2.6628	<0.4771
8	2.5051	1.6021	1.0000	1.3010	1.9031	2.6128	1.3010	1.0000	1.3617	1.3617	<0.4771	2.3222	>3.0414	2.1761	1.5563	<0.4771
9	2.1139	1.6021	<1.0000	1.7782	2.0000	1.8451	1.6021	<1.0000	3.0414	2.1761	<0.4771	1.6335	2.3802	3.0414	3.0414	<0.4771
14	3.5798	1.0000	<1.0000	1.7782	2.3979	2.0792	2.0792	<1.0000	2.3802	1.6335	<0.4771	1.9685	2.3802	2.6628	2.3802	1.3617
16	2.1461	1.3010	<1.0000	1.4771	2.3010	2.0792	2.3617	<1.0000	3.0414	1.9685	0.8633	1.9685	2.1761	2.6628	2.6628	0.8633
18[b]	2.6128	1.9542	<1.0000	3.0000	2.7782	2.3617	2.5315	<1.0000	2.3802	1.5911	1.3617	1.8751	3.0414	2.6628	2.3802	1.6335

[a] All counts were rounded to two significant figures and converted to $\log_{10}$.

[b] Statistical outlier for HGMF method based on rank scores (3).

Table 20. Fecal coliform counts[a] in cheddar cheese

Coll.	MPN/g by HGMF method								MPN/g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1[b]	<1.0000	<1.0000	<1.0000	1.0000	2.0414	1.4771	<1.0000	<1.0000	1.8751	1.6335	<0.4771	<0.4771	2.3222	1.3617	1.6335	<0.4771
2[c]	1.9031	1.3010	<1.0000	1.3010	2.1461	1.7782	1.4771	<1.0000	1.1761	1.1761	0.9590	1.3010	1.8751	1.1461	1.3010	<0.4771
4	2.4771	1.6990	<1.0000	1.6990	2.5185	1.8451	2.2788	<1.0000	3.0414	1.5911	<0.4771	2.3802	2.6628	1.9685	1.9685	<0.4771
5[b]	2.4914	2.0000	<1.0000	1.7782	2.8325	2.0792	2.4314	<1.0000	1.9685	1.3010	<0.4771	1.6335	2.3802	2.3802	2.6628	<0.4771
8	2.5682	1.7782	<1.0000	1.4771	2.0000	2.3979	1.8451	<1.0000	1.3617	1.3617	<0.4771	2.0792	2.4624	1.8751	2.6628	<0.4771
9	2.2553	1.6021	<1.0000	1.4771	2.2304	1.6021	1.9031	<1.0000	2.3802	2.1761	<0.4771	1.6335	2.3802	2.1761	3.0414	<0.4771
14	2.4150	1.6021	<1.0000	1.6021	2.4624	2.2553	2.3617	<1.0000	2.3802	1.6335	<0.4771	1.9585	2.3802	2.3802	1.9685	<0.4771
16	2.0414	1.6021	<1.0000	1.6021	1.7782	1.9031	1.9542	<1.0000	3.0414	1.6335	<0.4771	1.6335	1.9685	1.3617	2.3802	<0.4771
18	2.6232	1.6990	<1.0000	2.7559	2.6128	2.2041	2.2788	<1.0000	2.3802	1.3617	<0.4771	1.3617	3.0414	1.9685	1.9685	<0.4771

[a], [b] See footnotes to Table 19.

[c] Statistical outlier for reference method (A.O.A.C. 46.013-016) based on rank scores (3).

Table 21. *Escherichia coli* counts[a] in cheddar cheese

Coll.	MPN/g by HGMF method								MPN/g by A.O.A.C. 46.013-016 method							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	<1.0000	<1.0000	<1.0000	1.0000	1.6021	1.0000	<1.0000	<1.0000	1.3617	1.1761	<0.4771	<0.4771	2.1761	1.3617	1.6335	<0.4771
2	1.6990	1.0000	<1.0000	1.3010	1.7782	1.3010	1.0000	<1.0000	M[b]	M	M	M	M	M	M	<0.4771
4	2.5682	1.7782	<1.0000	1.6990	2.6232	1.4771	2.0414	<1.0000	3.0414	1.5911	<0.4771	2.3802	2.6628	1.9685	1.9685	<0.4771
5[c]	2.4624	2.0000	<1.0000	1.7782	2.8388	2.0414	2.1761	<1.0000	1.9685	1.3010	<0.4771	1.6335	2.3802	2.3802	2.6628	<0.4771
8	2.2304	1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	<1.0000	0.9590	<0.4771	<0.4771	2.0792	2.4624	1.8751	2.6628	<0.4771
9	2.0000	<1.0000	<1.0000	1.0000	1.0000	1.4771	1.0000	<1.0000	2.3802	2.1761	<0.4771	1.6335	2.3802	2.1761	3.0414	<0.4771
14[d]	2.3010	1.6021	<1.0000	1.3010	2.5911	2.2304	2.0000	<1.0000	<0.4771	0.5563	<0.4771	<0.4771	<0.4771	0.7924	<0.4771	<0.4771
16	1.7782	<1.0000	<1.0000	1.7782	1.4771	1.3010	1.3010	<1.0000	3.0414	1.6335	<0.4771	1.6335	1.9685	1.3617	2.3802	<0.4771
18	2.6532	1.9031	<1.0000	1.6021	2.6902	2.0000	2.1461	<1.0000	2.3802	1.3617	<0.4771	0.9590	3.0414	1.9685	1.9685	<0.4771

[a] All counts were rounded to two significant figures and converted to $\log_{10}$.

[b] Missing value - analysis not done or incomplete.

[c] Statistical outlier for HGMF method based on rank score (3).

[d] Statistical outlier for AOAC 46.013-46.016 method based on rank score (3).

Table 22. Comparison of random (S_r^2) and systematic (S_b^2) errors associated with total coliform counts done by hydrophobic grid membrane filter and AOAC 46.013–46.016 methods

Food	Sample pair[a]	N^b	Random error by method			Systematic error by method	
			HGMF	46.013–46.016	F^c	HGMF	46.013–46.016
Fish	II	6	0.1598	0.1301	1 2283	0.1083	0.0489
	III	3	0.0111	0.2544	22 9189[d]	0.1412	−0.1168
Poultry	I	10	0.0415	0.0639	1 5398	0.0232	0.0809
	II	9	0.0608	0.0862	1 4178	0.0499	0.0659
	III	8	0.0770	0.3337	4 3338[d]	−0.0186	−0.0651
Walnuts	I	6	0.2845	0.4946	1.7385	0.2204	0.5595
	II	8	0.6899	0.4438	1.5545	0.1685	0.1274
	III	10	0.0290	0.1971	6.7966[d]	0.0096	0.0620
Cheese	I	5	0.1641	0.0636	2.5802	0.0759	0.0281
	II	6	0.2307	0.0692	3.3338	−0.0864	0.1148
	III	6	0.0797	0.1235	1.5496	−0.0217	−0.0223

[a]Only those sample pairs with finite values for both elements of the pair by both methods are included.
[b]Number of sample pairs.
[c]$F = [S_r^2(\text{method A})]/[S_r^2(\text{method B})]$, where method A is that method with the larger S_r^2 value.
[d]Significant value for F ($P \leq 0.05$).

Table 23. Comparison of random (S_r^2) and systematic (S_b^2) errors associated with fecal coliform counts done by hydrophobic grid membrane filter and AOAC 46.013–46.016 methods

Food	Sample pair[a]	N^b	Random error by method			Systematic error by method	
			HGMF	46.013–46.016	F^c	HGMF	46.013–46.016
Fish	II	5	0.0558	0.4120	7 3835[d]	0.1456	−0.0872
	III	3	0.0103	0.0133	1 2913	0.0109	0.0331
Poultry	I	10	0.0477	0.1009	2 1153	0.0343	0.0417
	II	10	0.0673	0.1869	2.7771	0.0101	0.0075
	III	8	0.0423	0.3354	7.9291[d]	0.0188	−0.0625
Walnuts	I	4	0.7193	0.3456	2.0813	−0.3394	0.0124
	II	3	1.4350	0.2325	6.1720	−0.7093	0.4992
	III	10	0.0370	0.2601	7.0297[d]	0.0304	0.0668
Cheese	I	6	0.0296	0.2927	9.8885[d]	0.0471	−0.0401
	II	6	0.0575	0.1725	3.0000	0.0132	−0.0125
	III	6	0.1157	0.1263	1.0916	0.0075	−0.0140

[a–d]See footnotes to Table 22.

Table 24. Comparison of random (S_r^2) and systematic (S_b^2) errors associated with *Escherichia coli* counts done by hydrophobic grid membrane filter and AOAC 46.013–46.016 methods

Food	Sample pair[a]	N^b	Random error by method			Systematic error by method	
			HGMF	46.013–46.016	F^c	HGMF	46.013–46.016
Fish	II	5	0.0272	0.4120	15.1471[d]	0.0461	−0.0975
	III	4	0.0773	0.2126	2.7503	0.0126	0.1734
Poultry	I	7	0.0916	0.2367	2.5841	0.0049	−0.0072
	II	7	0.0427	0.1189	2.7845	0.1543	−0.0149
	III	7	0.0257	0.2837	11.0389[d]	0.0003	−0.0503
Walnuts	III	9	0.0176	0.2281	12.9602[d]	0.0594	0.2025
Cheese	I	4	0.1216	0.2626	2.1595	0.3244	−0.0871
	II	4	0.0923	0.1498	1.6230	0.1102	0.0404
	III	2	0.0123	0.3551	28.8699	−0.0061	0.1630

[a–d]See footnotes to Table 22.

Table 25. Means and precision estimates for total coliform enumeration by hydrophobic grid membrane filter (HGMF) and AOAC 46.013–46.016 methods

Food	Method	Geometric mean, MPN/g	Precision estimates			
			S_A[a]	S_B[b]	CV_A[c]	CV_B[d]
Fish	HGMF	45	0.6459	0.7171	39.1	43.4
	46.013–46.016	39	0.7146	0.7266	44.8	45.5
Poultry	HGMF	200	0.3799	0.4716	16.6	20.6
	46.013–46.016	170	0.3912	0.4791	17.6	21.6
Walnuts	HGMF	41	0.6983	0.7822	19.3	21.6
	46.013–46.016	34	0.5842	0.9056	16.5	25.6
Pepper	HGMF	63[e]	0.4306	0.5268	24.0	29.3
	46.013–46.016	3.8	0.1366	0.2601	23.8	45.3
Cheese	HGMF	55	0.3107	0.4285	17.8	24.6
	46.013–46.016	76	0.3926	0.4316	20.9	23.0

[a]Repeatability standard deviation.
[b]Reproducibility standard deviation.
[c]Repeatability coefficient of variation.
[d]Reproducibility coefficient of variation.
[e]Significantly higher than reference method (ANOVA; $P \leq 0.01$).

Table 26. Means and precision estimates for fecal coliform enumeration by hydrophobic grid membrane filter (HGMF) and AOAC 46.013–46.016 methods

Food	Method	Geometric mean, MPN/g	Precision estimates			
			S_A[a]	S_B[b]	CV_A[c]	CV_B[d]
Fish	HGMF	56[e]	0.5628	0.6780	32.2	38.8
	46.013–46.016	20	0.4522	0.5073	34.6	38.9
Poultry	HGMF	140	0.4837	0.6350	22.5	30.0
	46.013–46.016	54	0.4236	0.4959	24.4	28.6
Walnuts	HGMF	29[e]	0.8021	0.8155	23.2	23.6
	46.013–46.016	2.8	0.7225	0.7915	29.6	32.4
Pepper	HGMF	21[f]	0.4581	0.5191	34.7	39.3
	46.013–46.016	3.1	0.0924	0.0925	18.8	18.8
Cheese	HGMF	50	0.2348	0.3500	13.8	20.6
	46.013–46.016	39	0.3688	0.4320	23.1	27.1

[a–d]See corresponding footnotes to Table 25.
[e]Significantly higher than reference method (ANOVA; $P \leq 0.05$).
[f]Significantly higher than reference method (ANOVA; $P \leq 0.01$).

Table 27. Means and precision estimates for *Escherichia coli* enumeration by hydrophobic grid membrane filter (HGMF) and AOAC 46.013–46.016 methods

Food	Method	Geometric mean, MPN/g	Precision estimates			
			S_A[a]	S_B[b]	CV_A[c]	CV_B[d]
Fish	HGMF	35	0.4800	0.5357	31.1	34.7
	46.013–46.016	15	0.4725	0.5471	40.6	47.1
Poultry	HGMF	96[e]	0.3929	0.7086	19.8	35.8
	46.013–46.016	24	0.4471	0.5416	32.5	39.3
Walnuts	HGMF	6.2[e]	0.3754	0.5334	13.4	19.1
	46.013–46.016	1.6	0.4920	0.6530	22.4	29.8
Pepper	HGMF	11[f]	0.2602	0.2602	20.9	25.3
	46.013–46.016	3.1	0.0930	0.0932	18.9	18.9
Cheese	HGMF	30	0.2599	0.4501	17.6	30.4
	46.013–46.016	27	0.4282	0.6360	29.8	44.2

[a–d]See corresponding footnotes to Table 25.
[e,f]See corresponding footnotes to Table 26.

Recommendation

It is recommended that the proposed hydrophobic grid membrane filter method for enumeration of total coliforms, fecal coliforms, and *Escherichia coli* in foods be adopted official first action.

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CHEMICAL CONTAMINANTS MONITORING

Gas Chromatographic Determination of Fenvalerate Insecticide Residues in Processed Tomato Products and By-Products

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The results of a 5-laboratory collaborative determination of residues of the synthetic pyrethroid insecticide fenvalerate in tomato products are presented. Tomatoes from plants treated in the field at 2–4 day intervals (13 foliar applications) were processed into chopped fresh tomatoes, canned quarters, juice, paste, and the by-product skins plus seeds. Gas chromatographic analysis of the commodities for fenvalerate showed the fresh produce to contain 0.26 ppm, and the skins plus seeds contained 1.9 ppm. Residues were barely detectable in canned peeled quarters and juice, but averaged 0.12 ppm for paste, the concentration product of juice. High residues were associated with the skin content of the product. Five laboratories using modifications of the same analytical technique obtained good collaborative agreement.

Fenvalerate (cyano(3-phenoxyphenyl)methyl 4-chloro-alpha-(1-methylethyl)benzeneacetate), also known as Pydrin, Sumicidin, or Ectrin, is an effective broad spectrum synthetic pyrethroid insecticide with adequate stability in the field. This compound is used extensively on a variety of crops, including fruits, field crops, and ornamentals. It is effective on pests resistant to other chemicals, has a low mammalian toxicity, and is used at low dosage rates compared with other agricultural chemicals (1).

This is our second study aimed at broadening the data base for fenvalerate residues on processed commodities, the first having been conducted on apples (2). It provided another opportunity for the 5 cooperating laboratories, all participants in Northeast Regional Project No. 115(NE-115), to compare data generated either by strict adherence to the manufacturer's method, or by modifications of the method involving changes in solvents, procedures, or equipment that a given laboratory might routinely use as a matter of preference, convenience, or necessity.

Representative tomato products (juice, paste, and canned quarters) produced under commercial conditions, the juicing by-product, skins plus seeds, and chopped fresh tomatoes were analyzed for residues of fenvalerate by the laboratories designated I–V. All sample production, processing, and distribution was done under the direction of Laboratory I.

Experimental

Samples

(a) *Tomatoes.*—Early Girl tomatoes, *Lycopersicon esculentum* Mill., were grown at the University of Massachusetts experimental farm, Sunderland, MA. Beginning at the blossom stage, plants received treatments of fenvalerate at the recommended 1 × rate of 0.1 lb active ingredient (in 70 gal. of water)/acre. Pesticide was applied at 50 psi with a CO_2 sprayer on July 10, 14, 16, 22, 24, 28, and 31, and August 4, 7, 10, 13, 17, and 20, 1981, for a full season cycle of 13 applications. The commercial formulation, Pydrin 2.4 EC (2.4 lb a.i./gal. of emulsifiable concentrate), was used.

Tomatoes from both the treated plot (1 ×) and a check plot (CK) were harvested 24 h after final application and immediately transported fresh to Geneva, NY, for processing.

(b) *Chopped whole tomatoes.*—Approximately 6 kg of each tomato sample was chopped in a Hobart food chopper. After thorough hand mixing to ensure homogeneity and to minimize juice formation, 1 kg subsamples were bagged and frozen (−20°C).

(c) *Tomato juice and skins plus seeds.*—Twenty-five to 50 kg fresh tomatoes were soaked and rinsed in cold water, stemmed, and chopped in a staggered blade chopper, from which they were continuously pumped through a pulper preheater at 79°C (see Table 1 for processing details). From the preheater, the pulp went directly into a Langseenamp finisher operated at 1000 rpm with a 0.033 in. screen that extracted juice from skins and seeds. The latter were divided into 6 subsamples, bagged, and frozen at −20°C. Juice was then put through a pasteurizer of NYSAES design which preheated at 82°C, sterilized at 114°C, and delivered liquid at 88°C to the filling port of an American Can Co. 34-A 006 vacuum closing machine, in which the juice was sealed into #303 (15½ oz) cans. NaCl, 3.9 g/400 g can of tomato product, was added to produce a tomato product having the traditional 1% salt content. Canned products were stored at room temperature until analyzed.

(d) *Tomato paste.*—Tomato juice was concentrated by boiling at atmospheric pressure with agitation. The product was then sterilized on-line at 100°C and canned, with the addition of 1% NaCl, as described above.

(e) *Canned tomato quarters.*—Fresh whole tomatoes (7–8 kg) were scalded 1 min at 100°C and then plunge-cooled in water. Stems and skins were removed by hand and tomatoes were quartered and hand-packed into #303 cans. After the addition of 1% NaCl, cans were sealed and processed 30 min to an internal temperature of 93°C followed by water cooling to 38°C.

Determination of Solids

One can each of check and 1 × treated products for both juice and paste were opened and the contents were freeze-dried in a Virtis Model 50-SRC to a final shelf temperature of −50°C at 52 atm. for the determination of total solids. Paste was calculated to be the 3.2-fold concentration product of juice; other results are given in Table 1.

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Table 1. Production of tomato juice, paste, and seeds plus skins

Detail	Check	1× treatment
Fruit to juicer (kg)	25.28	47.48
Juice produced (kg—%)	22.05–87.2	41.17–86.7
Seeds plus skins (kg—%)	1.14–4.5	3.34–7.0
Juice brix (% solids)	5.7	5.6
Paste brix (% solids)	18.4	18.4
Concentration factor	3.2	3.2

Extraction and Cleanup of Samples

Identical sets of 10 samples were distributed to each of the 5 participating laboratories. Each set consisted of Pydrin-treated samples at the 1× rate and check. Copies of the residue method and analytical standards (99%) were provided courtesy of the manufacturer, Shell Development Co., Biological Sciences Research Center, Modesto, CA 95352 (3).

Laboratory I.—Blend 25 g samples with 130 mL hexane–isopropanol (3 + 1) for 3 min, filter, and transfer filtrate to 250 mL separatory funnel. Shake with 70 mL water plus 70 mL saturated Na_2SO_4 solution, separate, and discard aqueous phase. Evaporate organic phase to dryness on rotary film evaporator. For juice and canned quarters, dissolve residue in 20 mL hexane and place on Florisil Sep-Pak cartridge (Waters Associates); wash with additional 20 mL hexane and 20 mL benzene–hexane (3 + 2); collect fenvalerate in final elution with 35 mL benzene.

For fresh tomatoes, seeds plus skins, and paste, drain separated organic phase through 50 g pad of anhydrous Na_2SO_4, evaporate to dryness, and redissolve in 20 mL hexane. Prepare 20 × 80 mm column of 70–230 mesh silica gel (EM Laboratories, No. 7734), activated overnight at 100°C. Add 20 mm layer of anhydrous Na_2SO_4 and prewet with 50 mL hexane, drained to top of column. Add residues in 20 mL hexane and wash with 120 mL hexane. Elute column with 150 mL benzene–hexane (3 + 2), collecting fenvalerate from fresh tomatoes and seeds plus skins in third 50 mL fraction. For paste, discard third fraction and elute fenvalerate with final 125 mL of benzene. Concentrate collected fractions to volumes suitable for GC analysis.

Laboratory II.—Transfer blended products (3 g seeds plus skins, 5 g all others) to Teflon-pestle tissue grinder with 10 mL hexane–isopropyl alcohol (3 + 1). Add additional water (10 mL) to skins plus seeds. After 2 min maceration, remove pestle and centrifuge grinder to bring clear hexane layer to top. Remove hexane layer with Pasteur pipet and save. Add fresh hexane (5 mL) and repeat maceration, centrifugation, separation steps. Repeat cycle and evaporate combined extracts to volume suitable for GC analysis.

Laboratory III.—Blend 20 g product with 100 mL methylene chloride and 50 mL methanol. Filter through sintered glass and partition filtrate with 50 mL 4% NaCl solution. Separate phases and wash filter and NaCl solution twice with additional 50 mL methylene chloride. Flash-evaporate combined CH_2Cl_2 fractions at 50°C and, to ensure complete removal of CH_2Cl_2, add 10 mL petroleum ether to the flask twice and flash-evaporate each time at 50°C. Dissolve residue in 10 mL isooctane and add 1 mL of this to column prepared from 3 g Florisil (activated at 145°C) topped with 2 cm Na_2SO_4, and washed with 10 mL hexane. Elute sample with 25 mL hexane–ethyl acetate (95 + 5) and flash-evaporate collected eluate at 50°C. Dissolve residue in 2 mL isooctane for GC analysis.

Laboratory IV.—Blend 50 g sample 3 min with 150 mL hexane–isopropanol (3 + 1). After filtration, wash 90 mL aliquot in separatory funnel 3 times with 100 mL water. Elute resulting hexane fraction through 6 g Florisil column (60–100 mesh, activated overnight at 140°C) and wash column with additional 50 mL hexane. Elute fenvalerate with 50 mL 5% ethyl acetate in hexane, and concentrate to 1.0 mL for GC analysis.

Laboratory V.—Chopped whole tomato: Blend 100 g sample (canned quarters, 200 g sample) with 200 mL methylene chloride and filter. Dry filtrate over anhydrous Na_2SO_4. Concentrate 50 mL aliquot to near dryness on Rinco evaporator and dissolve residue in 10 mL hexane for GC analysis. Tomato paste: Blend 50 g sample as above, concentrate 5 mL aliquot of filtrate to near dryness with nitrogen purge, and dissolve residue in 5 mL hexane for GC analysis. Seeds and skins: Blend 100 g sample as above and concentrate 25 mL aliquot of filtrate to near dryness on Rinco evaporator. Dissolve residue in 100 mL hexane for GC analysis. Juice: Shake 200 g sample with 250 mL methylene chloride in separatory funnel. Concentrate 50 mL aliquot of methylene chloride layer to near dryness on Rinco evaporator and dissolve residue in 10 mL hexane for GC analysis.

Chromatographic Analyses

Laboratory I.—Analyze samples on Tracor Model 222 gas chromatograph equipped with ^{63}Ni electron capture detector, using column of 5% OV-101 on Gas-Chrom Q, 80–100 mesh, 61 cm × 3.2 mm id. Thermal parameters: column 225°C; detector 290°C; and inlet 220°C; nitrogen carrier gas. Samples will elute as single peaks. Integrate and calculate by Hewlett-Packard 3352 Labdata System operating in external standard mode.

Laboratory II.—Analyze samples on Tracor Model 222 gas chromatograph equipped with ^{63}Ni detector, using column of 3% OV-17 on Gas-Chrom Q, 100–120 mesh, 180 cm × 4 mm id glass. Thermal parameters: column, 240°C; detector, 315°C; and inlet, 225°C; nitrogen carrier gas. Fenvalerate is resolved as 2 peaks. Combine areas for each sample and compare with those of standard curve.

Laboratory III.—Analyze samples on Tracor Model 220 gas chromatograph equipped with ^{63}Ni detector, using column of 3% SP-2100 on Supelcoport, 100–120 mesh, 50 cm × 4 mm id. Thermal parameters: column, 240°C; detector, 350°C; and inlet, 250°C; nitrogen carrier gas. Integrate isomers of fenvalerate as one peak with Varian Vista 401.

Laboratory IV.—Analyze samples on Tracor Model 220 gas chromatograph equipped with thermionic (N-P) detector, using column of 3% Dexsil 300 on Supelcoport, 100–120 mesh, 120 cm × 2 mm id glass. Thermal parameters: column, 250°C; detector, 275°C; helium carrier gas. Integrate and compare both peaks independently with corresponding peaks derived from standard; report residues as average of the 2 values.

Laboratory V.—Analyze samples on Tracor Model 550 gas chromatograph equipped with ^{63}Ni electron capture detector, using column of 3% SP-2110 on Supelcoport, 100–120 mesh, 180 cm × 4 mm id. Thermal parameters: column, 260°C; detector, 300°C; inlet, 260°C. Inject 5 μL aliquots and measure peak heights for both isomers, compare with those obtained for 100 and 500 pg standard injections in 5 μL hexane.

Results and Discussion

Fenvalerate residues appear to be associated primarily with those commodities containing tomato skins (Table 2). While the skins plus seeds fraction contains high residues, the seed-containing canned product, which had been peeled before packing, was residue-free, indicating that fenvalerate adheres to the skin, and remains adhered during processing. Identical results were obtained in our study of apple products (2). Small

Table 2. Fenvalerate residues in tomato products (ppm)

Product	Rate	Laboratory[a] I	II	III	IV	V	$\bar{X}$	S
Chopped whole	CK	0.002	<0.03	<0.01	<0.01	<0.001	—	—
tomatoes	1×	0.39	0.48	0.16	0.17	0.10	0.26	± 0.16
Canned tomato	CK	<0.001	<0.03	<0.01	<0.01	<0.001	—	—
quarters	1×	0.022	<0.03	<0.01	<0.01	0.003	—	—
Tomato	CK	0.003	<0.03	<0.01	<0.01	<0.001	—	—
juice	1×	0.002	0.04	0.03	<0.01	0.023	—	—
Tomato	CK	0.002	<0.03	<0.01	<0.01	<0.03	—	—
paste	1×	0.093	0.11	0.11	0.11	0.18	0.12	± 0.034
Tomato seeds	CK	0.002	<0.03	0.02	<0.01	<0.03	—	—
plus skins	1×	1.1	3.0	2.9	1.4	1.2	1.9	± 0.94

[a] I = Cornell University, Geneva, NY
II = Cornell University, Ithaca, NY
III = Pennsylvania State University, University Park, PA
IV = Rutgers University, New Brunswick, NJ
V = U.S. Dept of Agriculture, Beltsville, MD

Table 3. Fortification level (ppm) and recovery (%) of fenvalerate

Product	Laboratory I Fort.	I Rec.	II Fort.	II Rec.	III Fort.	III Rec.	IV Fort.	IV Rec.	V Fort.	V Rec.
Chopped whole tomatoes	0.04	104	0.21	100	0.20	96	—[b]	112	—[c]	100
Canned tomato quarters	0.04	111	0.52	102	0.02	94	—[b]	112	—[c]	100
Tomato juice	0.04	112	—	—	0.02	96	—[b]	112	—[c]	100
Tomato paste	0.04	96	0.21	105	0.02	101	—[b]	112	—[c]	100
Tomato seeds + skins	0.04	106	—	—	0.20	92	—[b]	112	—[c]	100

[a] See footnotes, Table 2.
[b] Overall average of 0.1, 0.5, and 1.0 ppm.
[c] No fortification level reported.

skin fragments carry over into the juice and its concentration product, paste, which accounts for the barely detectable residues in the former and the slightly higher levels in the latter. The high standard deviation noted for the skins plus seeds could have occurred if varying ratios of seeds to skins occurred in the subsamples. While care was taken to produce homogenous samples, the tough, thin, pesticide-bearing skins may have thwarted this.

The agreement between laboratories for residues and recoveries was generally good (Tables 2 and 3), again verifying that the basic analytical method, even though used with modifications by each laboratory, is sound. Other reported methods for fenvalerate determination follow this same analytical scheme for crop materials (4, 5). However, additional solvent partitionings or gel permeation chromatography cleanups are usually required for animal tissues or high-fat products (6, 7).

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Monocrotophos and Dicrotophos Residues in Birds as a Result of Misuse of Organophosphates in Matagorda County, Texas

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About 1100 birds of 12 species died from organophosphate poisoning in Matagorda County on the Texas Gulf Coast in March and May 1982. Birds died from feeding on rice seed that was illegally treated with dicrotophos or monocrotophos and placed near rice fields as bait to attract and kill birds. Brain acetylcholinesterase inhibition of affected birds averaged 87% (range 82–89%), and contents of gastrointestinal tracts contained residues of dicrotophos (5.6–14 ppm) or monocrotophos (2.1–13 ppm). Rice seed collected at mortality sites contained 210 ppm dicrotophos or 950 ppm monocrotophos. Mortality from dicrotophos poisoning continued for almost 3 weeks. The practice of illegally treating rice seed with either of these 2 organophosphates appears to be infrequent but widespread at present.

The persistent adverse affects on bird populations from the agricultural use of the organochlorine aldrin in Texas from 1967 to 1974 were reported by Flickinger and King (1) and Flickinger (2). Aldrin was registered for insect control in newly planted rice fields, but was strongly suspected to be heavily and widely used as an avicide. Following the suspension of aldrin in 1974 by the U.S. Environmental Protection Agency (EPA), uses of organophosphates toxic to birds, such as monocrotophos, have increased. Between 1980 and 1982, 4 incidents of mortality of birds and other wildlife occurred following agricultural uses of monocrotophos in the Panhandle (B. Warren, personal communication; E. Flickinger, unpublished report) and Rio Grande Valley (C. Chapman, personal communication) of Texas, and on rice seed in southwestern Louisiana (3). Two additional bird poisoning incidents occurred near newly planted rice fields in Matagorda County on the upper Texas Gulf Coast in 1982. Mortalities from these 2 incidents were determined to occur following unregistered treatments of rice seed with dicrotophos in March and monocrotophos in May. The rice seed treated with these 2 compounds was not planted in rice fields but was placed near newly planted rice fields as bait to kill birds. Because of extreme toxicities of monocrotophos and dicrotophos to birds (4, 5) and incomplete residue persistence data, they are not registered by EPA for legal treatment of rice. Rice fields are planted and flooded from March through May. The mechanics of rice farming are described by Flickinger and King (1).

In March and May 1982, reports were received of 2 die-offs several days after rice planting began in Matagorda County. The die-offs were initially reported to the Texas Parks and Wildlife Dept by anonymous callers and the information was subsequently relayed to the senior author. This study of these 2 incidents was undertaken to identify potentially hazardous pesticides and methods of application, and to appraise and document adverse impacts on wildlife.

METHODS

Field

Debilitated and dead birds were counted at the 2 die-off sites. Eight dead blackbirds were collected near a rice field in the die-off area in central Matagorda County in March to determine cause of mortality. Two dead blackbirds and 6 teal were collected near a rice field in the die-off area in southwestern Matagorda County in May. Healthy blackbirds of the same species were collected in Willacy County, Texas, to serve as controls for AChE (acetylcholinesterase) determinations. Rice seed was collected at 5 sites near a rice field in each die-off area where it was scattered or found in piles. Rice seed was also collected in a newly planted field in March.

Laboratory

Three blackbirds were sent to the National Wildlife Health Laboratory (NWHL) Madison, Wisconsin, for necropsy and pathological examinations. Brain AChE was determined in 5 blackbirds from the March die-off, 2 from the May die-off, and 3 controls by the method of Ellman et al. (6) as modified by Hill and Fleming (7). Brains of the teal were too decomposed to assay. The anterior portion of gastrointestinal tracts (ventriculus and proventriculus) of 7 blackbirds and 6 teal were opened and the contents were analyzed for organophosphates at the Patuxent Wildlife Research Center. Rice seed was also analyzed for organophosphates.

The contents of the GI tracts or contents plus GI tracts were extracted by swirling in a flask containing 10 mL methylene chloride. An aliquot of the methylene chloride extract was analyzed by a gas chromatograph equipped with a flame photometric detector and a 1% Reoplex 400 column. The lower limit of reportable residues was 1.0 ppm on a wet-weight basis. Dicrotophos and monocrotophos were confirmed by gas chromatography–mass spectrometry. Rice (10 g) was extracted by swirling in a flask containing 50 mL methylene chloride and analyzed in the same manner.

Results

Birds began dying in central Matagorda County about March 11, 1982. On March 16, we estimated approximately 800 birds being covered with earth during a soil disking operation in a field near rice fields. We subsequently traced dead and debilitated birds in the direction of the first 2 rice fields to be planted in the area. Table 1 shows species and numbers of birds found dead near one rice field between March 16 and 30. Commercial rice seed colored pink had been drilled into furrows or harrowed into soil of the 2 newly planted fields. Sick and dead birds were found largely on a section of road on 2 sides of the rice field where uncolored rice seed was found scattered or in many small piles. Few dead birds were found in the rice fields, but mortality occurred along the road through March 30, following what appeared to be one incident of poisoned rice seed placement.

No indication of infectious disease or significant histopathology was found in birds necropsied at NWHL. Symptoms exhibited by dying birds were loss of muscular coordination, prostration, tetany, outstretched wings, and convulsions. Table 2 shows brain AChE inhibition of affected birds. GI tract contents of 3 of 5 blackbirds from the March die-off consisted largely of uncolored rice seed. Table 2 also shows residues of dicrotophos detected in GI tracts of birds. Rice seed collected from the rice field road contained 210 ppm dicrotophos.

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Table 1. Number of birds found dead or debilitated near 2 newly planted rice fields

Species	No. of sick or dead
Central Matagorda County: March 16–30	
Red-winged blackbird *(Agelauis phoeniceus)*	173
Great-tailed grackle *(Quiscalus mexicanus)*	23
Brown-headed cowbird *(Molothrus ater)*	14
Mourning dove *(Zenaida macroura)*	23
Eastern meadowlark *(Sturnella magna)*	5
Vesper sparrow *(Pooecetes gramineus)*	2
Common snipe *(Gallinago gallinago)*	1
Southwestern Matagorda County: May 2–7	
Blue-winged teal *(Anas discors)*	24
Mottled duck *(Anas fulvigula)*	4
Red-winged blackbird	4
Great-tailed grackle	2
Common moorhen *(Gallinula chloropus)*	2
Mourning dove	1
Redhead *(Aythya americana)*	1
Ruddy turnstone *(Arenaria interpres)*	1

No organophosphate residues were found in rice seed from the newly planted field.

Again, around May 2, birds were reported dying along a ditch near a newly planted rice field in southwestern Matagorda County. Table 1 shows species and numbers of birds found dead on May 7 near uncolored rice seed scattered along the ditch and in piles in the ditch water. Rice seed colored pink had been drilled into furrows in the newly planted field. Few birds were found dead in the field. Mortality continued from May 2 through May 7. Brain AChE depression of 2 blackbirds and contents of GI tracts of 6 teal, consisting almost entirely of rice seed, are shown in Table 2. Rice seed collected along the ditch contained 950 ppm monocrotophos.

Discussion

Residues of organophosphates inhibit AChE in the nervous system, thereby disrupting synaptic transmission of nerve impulses. AChE depression greater than 50% may be diagnostic of death in birds (8). Death usually occurs from asphyxiation because of failure of the respiratory center of the brain (7). No organophosphate residues were found in 2 blackbirds from the March die-off or 2 from the May die-off. These birds probably died from organophosphate-induced anorexia resulting from reduced food consumption after exposure to organophosphates, as discussed by Grue (9) in a study of the effects of organophosphates on grackles.

The evidence that birds died from feeding on intentionally poisoned rice seed was the proximity of most dead or debilitated birds to uncolored rice seed found on the road or in the ditch near the 2 rice fields, uncolored rice seed containing monocrotophos or dicrotophos residues in GI tracts of birds, high residues of monocrotophos or dicrotophos in rice seed near the fields, greatly inhibited AChE activities in brains of birds found dead, and no evidence of infectious disease or pathology in dead birds.

Rice seed collected from the newly planted field in March contained no organophosphate residues. The owner of this

Table 2. Brain AChE inhibition, and organophosphate residues in GI tract contents of red-winged blackbirds and blue-winged teal found dead, and in rice seed collected near 2 newly planted rice fields

Sample	Sex	AChE inhibition, %	OP residues (ppm wet wt)
C. Matagorda Co.: March			Dicrotophos
Red-winged blackbird	M	82	14
	F	86	6
	M	87	6
	M	89	ND[a]
	M	89	ND
Rice seed			210
S.W. Matagorda Co.: May			Monocrotophos
Red-winged blackbird	M	85	ND
	M	85	ND
Blue-winged teal[b]	M		13
	M		10
	F		10
	M		9
	M		3
	F		2
Rice seed			950

[a]ND = none detected at 1.0 ppm level of sensitivity.
[b]No AChE analyses; birds were decomposed when found.

field and other rice growers in the area planted rice seed that was not treated with pesticides to prevent poisoning of game birds such as pheasants, quail, and doves. The rice fields around which the bird kills occurred were planted with seed commercially treated with Vitavax-R fungicide and Kenco-zinc (zinc oxide) fertilizer, but rice seed placed near the fields was treated with monocrotophos or dicrotophos as determined by laboratory analyses. Dicrotophos is applied to other crops along the Texas Coast near the habitat of the endangered Attwater's prairie-chickens *(Tympanuchus cupido attwateri)* (10) and could become a threat to low numbers of this species. The illegal application of these organophosphates to rice was done to kill birds in an area where other rice growers were attempting to protect them. The practice does not appear to be frequent at present, but may expand if monocrotophos is cleared for treatments on corn grown near rice fields in counties along the Texas Coast (J. Day, Texas Dept of Agriculture, 1982). Results of this study demonstrate that further restrictions should be imposed on the uses and distribution of toxic insecticides with histories of wildlife mortality to prevent continued wildlife losses from their illegal use.

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PLANT TOXINS

Liquid Chromatographic Determination of Glucosinolates in Rapeseed as Desulfoglucosinolates

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A method was developed for the quantitative determination of rapeseed glucosinolates as the desulfo derivatives, using liquid chromatography. Glucosinolates were desulfated at 37°C with the enzyme aryl sulfatase in Tris buffer, pH 8.0. All glucosinolates present in rapeseed were separated in 30 min on a Waters C_{18} Z-module using an acetonitrile gradient at 4 mL/min. Recoveries of benzyl, 4-hydroxybenzyl, and allyl glucosinolates added to plant extracts were quantitative.

Much effort is currently expended by plant breeders in attempts to decrease levels of glucosinolates present in rapeseed. Procedures for the estimation of glucosinolates by myrosinase digestion and determination of released isothiocyanates (1) may be equivocal because hydrolysis can lead to a range of different products unless digestion conditions are carefully controlled. In addition, the isothiocyanates arising from the indole glucosinolates are unstable and further degrade to produce thiocyanate ion (2).

The 2 methods available for separation of intact glucosinolates involve preliminary ion exchange purification. The liquid chromatographic (LC) method (3) using ion-pair chromatography enables partial separation of glucosinolates present in rapeseed. Whereas gas chromatography (GC) can also be used to separate these compounds (4), the breakdown of certain glucosinolates on derivatization is a major drawback to its use for the separation of silyl derivatives (5).

We have described an LC method for separating glucosinolates as the corresponding desulfoglucosinolates (DS glucosinolates), following hydrolysis with the enzyme aryl sulfatase (6). This procedure, which can be applied to seed, root, and leaf tissue, offers a number of advantages (7) over previously published methods. We have now extended this procedure by modifying the extraction, digestion, and separation conditions to enable quantitative recovery of glucosinolates.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Spectra-Physics 8000 single pump ternary gradient instrument (Spectra-Physics, Inc., Santa Clara, CA) equipped with Valco injector and 200 μL loop, coupled to Spectromonitor III (Laboratory Data Control Riviera Beach, FL) variable wavelength UV detector set at 226 nm. Spectra-Physics 4000 data system was used to plot chromatograms and measure peak areas.

(b) *LC column.*—Waters Z-module (Waters Associates, Inc., Milford, MA) fitted with 100 × 8 mm Radial-Pak ODS (5 μm particle size) cartridge. Flow rate 4.0 mL/min at ambient temperature. Waters semipreparative (300 × 7.8 mm) ODS column.

(c) *Ion exchange column.*—Bio-Rad polypropylene Econocolumn (Bio-Rad Laboratories, Richmond, CA) fitted with short length of Silastic tubing and plastic clip-on flow-controller. Add 100 mg DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) to 2 mL 0.5M ammonium acetate. Wash gel with five 1 mL portions of water.

(d) *Centrifuge.*—MSE GF-6 centrifuge (MSE Ltd, London, UK) fitted with 7-73 aluminum fixed-angle rotor.

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(e) *Microbalance.*—Perkin-Elmer AD-2Z Autobalance (Perkin-Elmer Co., Norwalk, CT).

(f) *GC column.*—25 m BP5 flexible fused silica (Scientific Glass Engineering Pty Ltd, Ringwood, Australia).

Reagents

(a) *Solvents.*—LC grade acetonitrile (Waters Associates).

(b) *LC water.*—Pass distilled water through 0.45 μm filter membrane. Use throughout.

(c) *Glucosinolates.*—Glucoerucin, glucotropaeolin, and glucosinalbin from Karl Roth (Karlsruhe, GFR). Sinigrin from Serva (Heidelberg, GFR).

(d) *Extraction solution.*—50mM mercaptoethanol, 1mM EDTA. Weigh 0.390 g 2-mercaptoethanol (BDH) into 100 mL volumetric flask. Add 10 mL 10mM EDTA and dilute to volume with water.

(e) *Tris buffer.*—50mM Tris buffer, pH 8.0 at 25°C or 37°C, prepared by mixing Trizma base and Trizma HCl (Sigma Chemical Co., St. Louis, MO) according to Sigma Technical Bulletin No. 106B. Dilute solution to 20mM with water. Prepare buffer used for enzyme digestions by combining 18 mL 20mM Tris buffer with 2 mL extraction buffer.

(f) *Internal standard solution.*—Accurately weigh 18.2 mg *o*-nitrophenyl-β-D-galactopyranoside (ONPGal) (Sigma Chemical Co.) and 18.3 mg sulfanilic acid (BDH Chemicals Ltd, Poole, UK) into 100 mL volumetric flask, dissolve, and dilute to volume with LC water.

Preparation of Desulfoglucosinolate Standards

Use seeds of rapeseed (*Brassica napus* L. cv. Midas) as the source of glucosinolates. Prepare extract of defatted, dried meal (50 g) as described previously (5). Elute non-indole glucosinolates with 0.375M pyridine acetate. Elute indole glucosinolates from column by washing with 0.75M pyridine acetate (8). Convert glucosinolates to corresponding desulfo derivatives as described elsewhere (6).

LC Collection

Separate and collect milligram quantities of DS glucosinolates, using Waters Bondapak semipreparative C_{18} reverse phase column operating at 3 mL/min with solvent program described below. Confirm identity of DS glucosinolates, after lyophilization, by GC/mass spectrometry (MS) (6).

Prepare standard solutions of each DS glucosinolate by accurately weighing ca 2 mg purified standard, then transferring to 5 mL volumetric flask and diluting to volume with water.

Standard Curve

Mix aliquots of 20–400 μL DS glucosinolate solutions with 800 μL mixed internal standard solution and inject a tenth of the total volume into the liquid chromatograph.

Prepare graphs of ratio of DS glucosinolate area to area of internal standard vs weight of DS glucosinolate for each DS glucosinolate over the range 0–15 μg. From these graphs, calculate response factors for each DS glucosinolate, and incorporate these figures into a program of the data system to enable automatic quantitation of each glucosinolate.

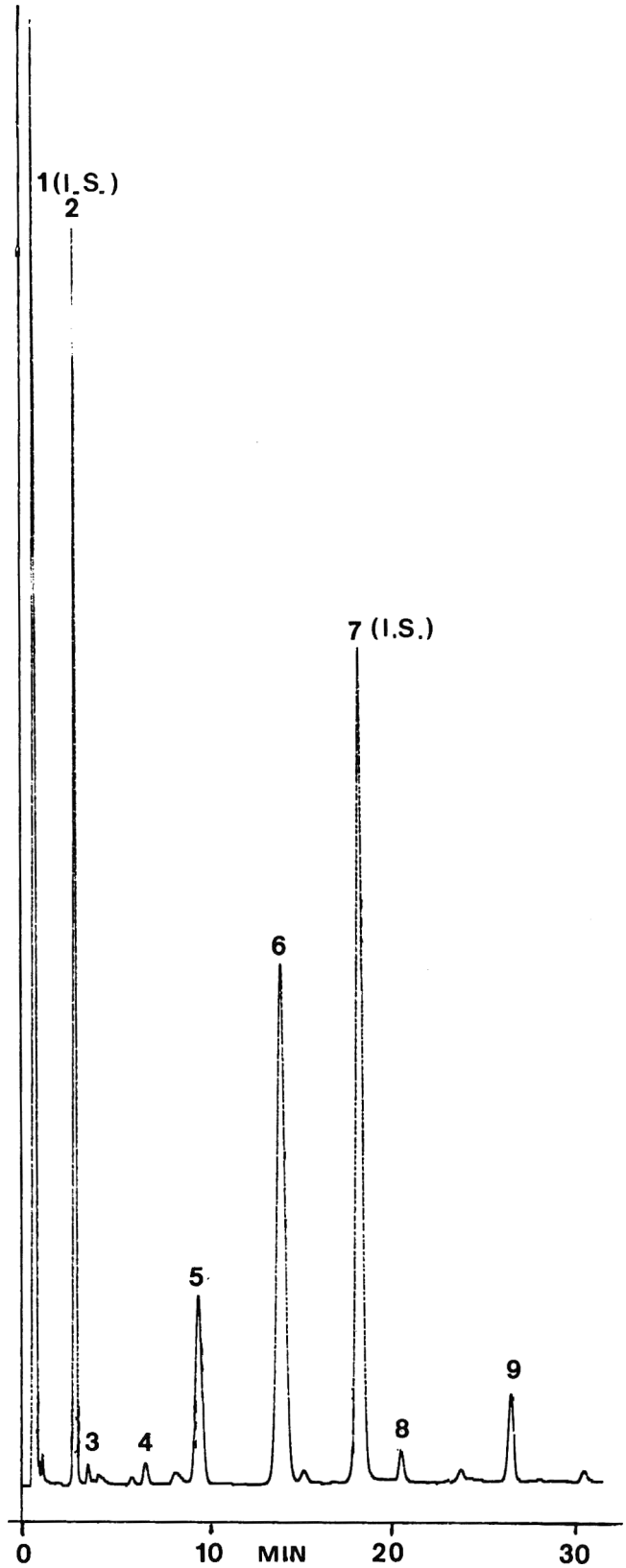

Figure 1. A chromatogram of DS glucosinolates from Tower rapeseed. Peaks were identified as follows: 1, sulfanilic acid (internal standard); 2, 2-hydroxybut-3-enylglucosinolate; 3, allylglucosinolate; 4, 2-hydroxypent-4-enylglucosinolate; 5, but-3-enylglucosinolate; 6, 4-hydroxy-3-indolylmethylglucosinolate; 7, ONPGal (internal standard); 8, pent-4-enylglucosinolate; 9, 3-indolylmethylglucosinolate. Detection wavelength: 226 nm, 0.2 AUFS.

Sample Preparation

(a) *Extraction of glucosinolates from rapeseed meal.*—Accurately weigh 200 mg oil-free meal into Kimax culture tube (Kimble Co., Toledo, IL). Heat tubes containing meal in boiling water bath 1 min. Add 4 mL boiling 50mM mercaptoethanol–1mM EDTA solution. Remove tubes from bath and mix thoroughly with vortex mixer. Loosely cap tubes and return them to bath 5 min. Remove from bath and let stand 10–15 min at room temperature. Centrifuge at 2000 rpm (600 x/g) 10 min. Transfer 2 mL aliquot to 10 mL centrifuge tube and add 0.5 mL solution containing 0.5M lead and 0.5M barium acetate. Mix and centrifuge. Use aliquot of supernate for desulfation.

(b) *Desulfation.*—Transfer 2 mL aliquot of supernate to 10 mL tube and dilute to 8 mL with water. Mix well and add to DEAE-Sephadex A-25 column. Wash with two 1 mL portions of 18mM Tris buffer (pH 8.0 at 37°C) containing 5mM 2-mercaptoethanol and 0.1mM EDTA and discard eluate. Add 500 μL 0.2% w/v aryl sulfatase (Type H-1, Sigma Chemical Co.) dissolved in the same Tris buffer and run this solution into resin, leaving meniscus above resin. Cap column and place in incubator at 37°C for 16 h. Elute DS glucosinolates with four 1 mL portions of water into 5 mL volumetric flask and dilute to volume with water. For LC analysis, mix 1 mL aliquot of sample with 800 μL mixed internal standard solution and inject 180 μL into liquid chromatograph.

(c) *LC analysis.*—Separate DS glucosinolates in rapeseed using C_{18} Radial-Pak cartridge at 4 mL/min. Use solvent meal extract program consisting of 100% water for 10 min followed by gradient of 0–12% acetonitrile over the next 30 min.

Results and Discussion

The elution profile of DS glucosinolates obtained from the meal of *B. napus* L.cv. 'Tower' (a low glucosinolate variety), together with 2 internal standards, sulfanilic acid and ONPGal, is shown in Figure 1. All DS glucosinolates were well separated in a total run time of 30 min.

Glucosinolate or DS glucosinolate standards corresponding to those present in rapeseed are not commercially available; therefore, glucosinolates were extracted from rapeseed and separated into indole and nonindole fractions by using ion exchange chromatography (8). These fractions were then desulfated on-column with aryl sulfatase, and the resulting DS glucosinolates were purified on a semipreparative C_{18} LC column. DS benzyl, DS allyl, DS hydroxybenzyl, and DS 4-methylthiobutyl glucosinolate standards were prepared from commercially available glucosinolates.

Milligram amounts of the DS glucosinolates were weighed on a microbalance and calibration curves were constructed for each DS glucosinolate. The response factors obtained relative to sulfanilic acid are shown in Table 1. The standard curves were linear in the range 0–16 μg. The purity of selected DS glucosinolates, for which sufficient material was available (hydroxybutenyl and allyl), was determined by microanalysis and found to be > 97%. The purity of all DS glucosinolates, with the exception of 4-hydroxy-3-indolylmethyl glucosinolate, was also examined by GC after silylation, as described by Minchinton et al. (6), using a BP-5 fused silica capillary column. In each case, the collected compound chromatographed as a single peak with a peak area greater than 98% of the total peak area of the chromatogram. The water content of several of the DS glucosinolates was examined by drying under vacuum overnight at 50°C. The loss in weight for DS allyl, DS 3-hydroxybut-3-enyl, and DS 3-indolylmethyl glucosinolates was less than 1.3%. On our system, the minimum detectable amounts were 10 ng on-column for DS-3-indolylmethyl glucosinolate and 30 ng for DS-3-hydroxybutenyl glucosinolate (30:1, S/N).

The conditions necessary for quantitative determinations of glucosinolates present in rapeseed were then investigated

Table 1. LC retention times and response factors used for quantitation of DS glucosinolates

Glucosinolate	Retention time, min	Response factor[a]
2-Hydroxybut-3-enyl (progoitrin)	2.7	9.23
Allyl (sinigrin)	3.3	9.17
2-Hydroxypent-4-enyl (napoleiferin)	6.3	9.34
But-3-enyl (gluconapin)	8.9	9.87
4-Hydroxy-3-indolylmethyl (4-hydroxyglucobrassicin)	13.3	2.44
Pent-4-enyl (glucobrassicanapin)	20.0	9.87
3-Indolylmethyl (glucobrassicin)	25.8	2.62
4-Hydroxybenzyl (sinalbin)	9.1	4.68
Benzyl (glucotropaeolin)	21.1	8.40
4-Methylthiobutyl (glucoerucin)	23.3	9.23

[a]Determined relative to sulfanilic acid at 226 nm. (Area DS glucosinolate/area sulfanilic acid) × response factor = μmol DS glucosinolate present in 200 mg original rapeseed sample.

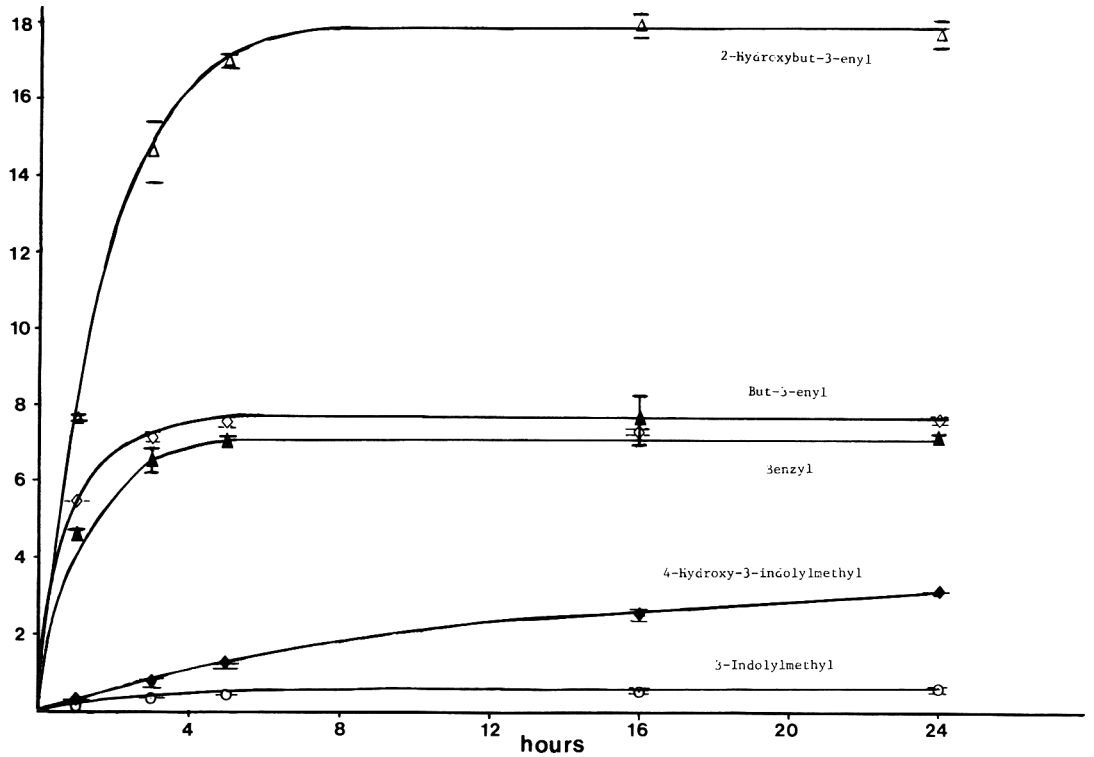

Figure 2. Time course of digestion of glucosinolates with aryl sulfatase at room temperature. Glucosinolates were extracted from rapeseed, benzyl glucosinolate was added, and mixture was hydrolyzed in 18mM Tris buffer, pH 8.0.

using the method of Thies (9) as a basis. We found that the acetate concentration in the sample loaded onto DEAE-Sephadex, following protein precipitation, must be 0.05M or less to ensure no appreciable loss of glucosinolates. Also, for quantitative recovery of indole DS glucosinolates, the 100 mg DEAE columns must be eluted with four 1 mL portions of water, whereas the aliphatic DS glucosinolates were eluted in a total volume of 1.5 mL.

When 4-methylthiobutyl and benzyl glucosinolates were hydrolyzed on-column with aryl sulfatase in water at room temperature, according to the method of Thies (9), and the time course of digestion was followed by LC, the DS glucosinolates peaked at approximately 30 min and then decreased at a rate of 0.12–0.16 μmole/h. Glucosinolates from rapeseed showed a similar time course. Attempted purification of the aryl sulfatase by ion exchange chromatography on DEAE-Sepharose (10) by gel filtration on Sephadex G-100 (Pharmacia) or Ultrogel AcA 22 (LKB, Bromma, Sweden), or by chromatofocusing {PBE 94 (Pharmacia), pH 6.0–4.0} did not markedly affect the rate of glucosinolate decrease.

It was suspected that the chief contaminants in the commercial aryl sulfatase preparation responsible for the observed decrease in glucosinolate content were glucosidase or galactosidase enzymes that were active against glucosinolates or desulfoglucosinolates. Accordingly, a search was made for conditions of digestion which would eliminate this activity. Published data (11) indicated that glucosidases and other related enzymes show no appreciable activity at pH values above 8.0. Rapeseed glucosinolate digestion was repeated at pH 8.0 (Figure 2). All glucosinolates reached a plateau stage after 10 h of digestion except for 4-hydroxy-3-indolylmethyl glucosinolate. The rate of digestion of this component was increased by performing the digestion at 37°C (Figure 3). In the course of this work, it was discovered that greater amounts (an approximate 2-fold increase) of 4-hydroxy-3-indolylmethyl glucosinolate could be detected if mercaptoethanol or ascorbic acid were included in the extraction and digestion buffers as described in the Method section. This finding is consistent with our previous observations on the marked susceptibility of this compound to oxidation. The recoveries of commercially available aromatic and aliphatic glucosinolates taken through this procedure are depicted in Table 2.

The LC method was applied to a sample of Tower rapeseed and the results were compared with those obtained by the

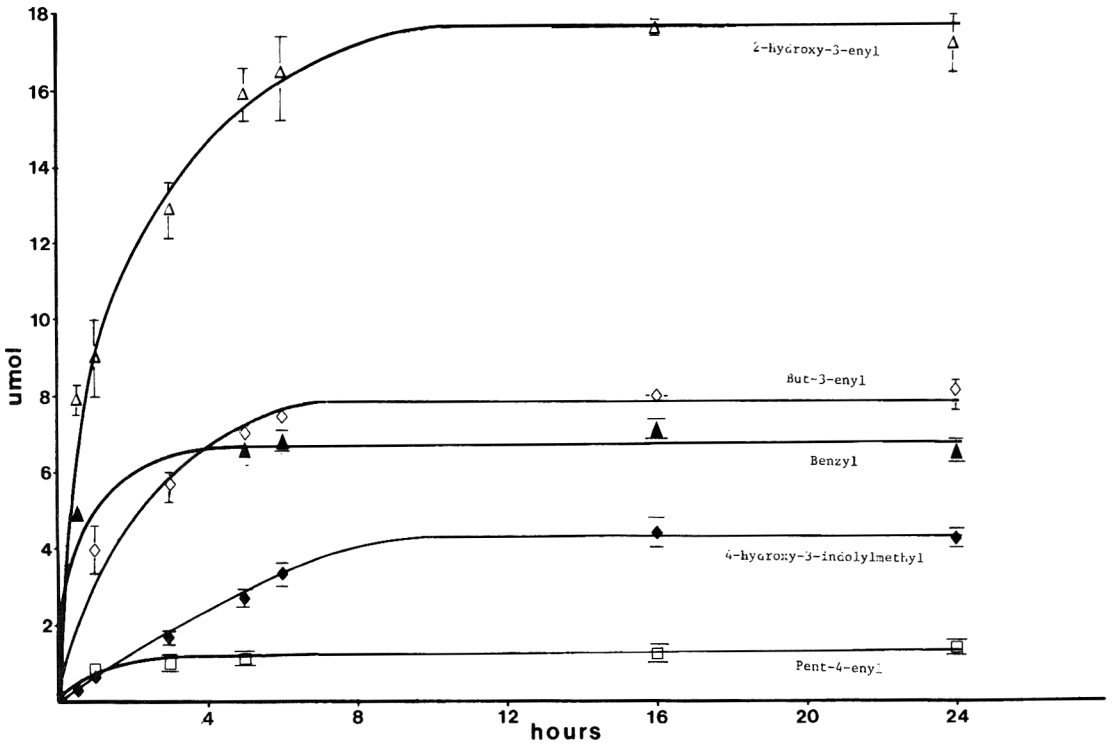

Figure 3. Time course of digestion of glucosinolates with aryl sulfatase at 37°C. Digestion was performed in 18mM Tris buffer, pH 8.0, containing 5mM mercaptoethanol and 0.1mM EDTA.

Table 2. Recovery of commercially available glucosinolates

Glucosinolate	Amount added, mg	Recovery, %[a]	CV, %
4-Hydroxybenzyl	0.354	102.9	5.5
Allyl	0.936	97.5	4.6
Benzyl	0.564	100.7	3.3
4-Methylthiobutyl	0.483	87.1	5.0

[a]Average of 5 determinations.

Table 3. Comparison of LC method with myrosinase digestion method for glucosinolate quantitation

Glucosinolate	LC[a]		GC/UV[b]	
	μmol/g	CV %	μmol/g	CV %
2-Hydroxybut-3-enyl	16.6	3.0	15.3	4.0
But-3-enyl	6.3	3.8	6.2	8.6
Pent-4-enyl	0.9	7.2	0.9	7.4
Allyl	0.6	9.5		
2-Hydroxypent-4-enyl	0.5	5.2		
4-Hydroxy-3-indolylmethyl	5.7	11.5		
3-Indolylmethyl	0.8	2.0		
Total	31.4			

[a]Average of 7 determinations.
[b]Average of 5 determinations.

myrosinase digestion procedure (1). Using the myrosinase procedure we obtained quantitative recovery of sinigrin as allyl isothiocyanate in the presence or absence of rapeseed meal. Good agreement was observed between the 2 methods (Table 3). The LC data were obtained using sulfanilic acid as internal standard. Very similar coefficients of variation were observed when ONPGal was used as internal standard (data not shown). For comparison, a sample of the Tower meal was also analyzed for glucose (12) and thiocyanate ion (13) following myrosinase digestion. The results were glucose, 30.1 ± 0.7 μmol/g, and thiocyanate, 10.2 ± 0.3 μmol/g.

In conclusion, on-column desulfation of glucosinolates with aryl sulfatase at pH 8.0 coupled with reverse phase LC separation of the resulting DS glucosinolates can be used for the quantitation of glucosinolates present in rapeseed. Purification of aryl sulfatase does not seem to be necessary under these conditions. This method has 3 advantages over other methods for glucosinolate determination: No extensive prepurification of glucosinolates before analysis is required; glucosinolate derivatization is not necessary; and all glucosinolates present in rapeseed, including the individual indole glucosinolates, can be measured in one analysis.

As reported by McGregor (13), indole glucosinolates, which break down on digestion with myrosinase to produce thiocyanate ion, represent a significant component of the total in low glucosinolate rapeseed varieties such as Tower. In relation to this, inclusion of mercaptoethanol in the extracting solutions increased the amount of 4-hydroxy-3-indolylmethyl glucosinolate detected by LC; however, we cannot be certain that this represents the total present in the meal. The results listed in Table 3 show that there is at least a 7-fold greater amount of 4-hydroxy-3-indolylmethyl glucosinolate than 3-indolylmethyl glucosinolate in Tower meal.

Because of the facile oxidation of the hydroxyindole glucosinolate, quantitation of this compound is difficult. Of the conditions so far tested, inclusion of 2-mercaptoethanol has given the best recovery. A small peak derived from the mercaptoethanol has been observed in the region of DS butenyl glucosinolate; however, because the peak area for this component is constant, it can be allowed for in a blank run. It should be noted that mercaptoethanol need only be added in those cases where measurement of 4-hydroxy-3-indolylmethyl glucosinolate is desired. For quantitation of sulfoxide containing glucosinolates, mercaptoethanol should not be included in the buffers because this reagent can reduce sulfoxides.

High recovery of added glucosinolate standards plus a good correlation with the myrosinase procedure indicates that this LC method can be used for the single step quantitation of all glucosinolates in rapeseed. The excellent separation of the

glucosinolates derived from the seed, root, and leaf of 5 cruciferous plants and the apparent freedom from interference by nonglucosinolate compounds (7) suggests that it is possible that this procedure could be applied to other glucosinolate-containing plant tissues.

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PESTICIDE FORMULATIONS

Gas Chromatographic Methods for Determination of γ-BHC in Technical Emulsifiable Concentrates and Water-Dispersible Powder Formulations and in Lindane Shampoo and Lotion: Collaborative Study

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Although the gas chromatographic separation of the isomers of BHC was demonstrated two decades ago, the present AOAC method of analysis of BHC for gamma-isomer (lindane) content is based on a separation carried out on a liquid chromatographic partition column. A method of analysis has been developed that uses an OV-210 column for separation of the gamma-isomer from the other isomers and impurities in technical BHC. Di-*n*-propyl phthalate was chosen as an internal standard. The same system allows quantitation of lindane in lotion and shampoo after these products are extracted with ethyl acetate–isooctane (1 + 4). The analytical methods were subjected to a collaborative trial with 10 laboratories. The coefficient of variation for technical BHC was 2.83%. For the water-dispersible powder and emulsifiable concentrate, the coefficients of variation were 2.89% and 4.62%, respectively. Coefficients of variation for 1% lindane lotion and shampoo were 4.36% and 11.92%, respectively. The method has been adopted official first action.

Although BHC is no longer manufactured or used in the United States, it is still used in rather large quantities in other countries. The gamma-isomer (lindane) is widely used in the United States to control a variety of insect pests on vegetables and ornamental plants. It also has applications in veterinary practice to control flies, lice, and ticks. In the public health sector, lindane is used in shampoos and lotions for control of head lice.

Methods adopted by the AOAC and the Collaborative International Pesticides Analytical Council (CIPAC) for analysis of BHC-containing products for gamma-isomer content are based on the separation of the gamma-isomer from other isomers on a partition column developed by Ramsey and Patterson (1) and modified by Harris (2). The isolated gamma-isomer is quantitated by weighing. The World Health Organization (WHO) has adopted the above technique for separation of the gamma-isomer; however, WHO permits quantitation by either polarography or infrared spectrophotometry (3).

The above methods are tedious and time-consuming. They also require large quantities of solvents and other reagents. In contrast, the isomers of BHC can be easily separated by gas chromatography (4). The development of a GC method of analysis of BHC formulations required a column that would isolate the gamma-isomer from other isomers and impurities and an internal standard that was resolved from all components in BHC and its formulations.

An OV-210 column separated the gamma-isomer from other isomers, although it did not resolve the delta- and epsilon-isomers. A homologous series of dialkyl phthalates was chromatographed in an effort to find an appropriate internal standard. Di-*n*-propyl phthalate, which eluted on the OV-210 column about 4 min after the delta- and epsilon-isomers, proved to be quite satisfactory (see Figure 1).

In the case of shampoo and lotion formulations, procedures for extraction of the gamma-isomer were developed. A mixture of ethyl acetate–isooctane (1 + 4) was satisfactory for extraction of the gamma-isomer; however, for the lotion a second extraction with acetonitrile was required to remove a large interfering peak of unknown origin. After one extraction of the ethyl acetate–hexane extract with acetonitrile, only a trace of the interfering impurity remained (see Figure 2).

Following the development of methods for separation and quantitation of the gamma-isomer, a variety of technical and formulated products were analyzed. Minor modifications were made in the procedures, and the methods were then subjected to collaborative study.

Collaborative Study

Each collaborator was furnished samples of technical BHC, water-dispersible powder, and emulsifiable concentrate, as well as samples of lindane shampoo and lotion. The technical material and the water-dispersible powder were furnished by Union Carbide Co., Manila, Philippines. The emulsifiable concentrate was prepared in our laboratory by dissolving technical BHC in xylene with the addition of Atlox 3404 and 3409 as surfactants. The shampoo and lotion were furnished by Reed and Carnrick Pharmaceuticals, Piscataway, NJ. Collaborators were also furnished γ-BHC analytical standard, di-*n*-propyl phthalate, isoamyl alcohol, and a supply of column packing. Participants were requested to make 2 weighings of each sample and to make duplicate injections of each sample extract.

gamma-BHC in Technical BHC, Pesticide Formulations, and Lindane Shampoos and Lotions

Gas Chromatographic Method

First Action

CIPAC–AOAC Method

(Applicable to tech., emulsifiable conc., and H_2O-dispersible powd. formulations and in lindane shampoo and lotion)

Principle

Samples of tech. BHC (benzene hexachloride) and formulations are dissolved in EtOAc with dipropyl phthalate added as internal std. Lindane (γ-BHC) shampoo and lotion samples are extd with EtOAc-isooctane contg dipropyl

These studies were conducted as part of a contractual agreement between the Centers for Disease Control and the World Health Organization.

Use of trade names or commercial sources does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

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This report of the Associate Referee, J. W. Miles, was presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee A and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) **67**, March.

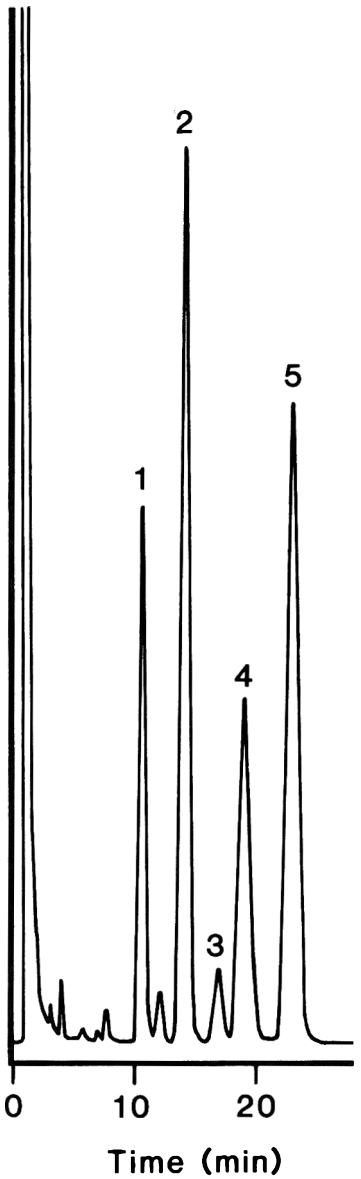

Figure 1. Gas chromatogram of technical BHC, δ-isomer (1), γ-isomer (2), β-isomer (3), δ- plus ε-isomers (4), di-*n*-propyl phthalate (5).

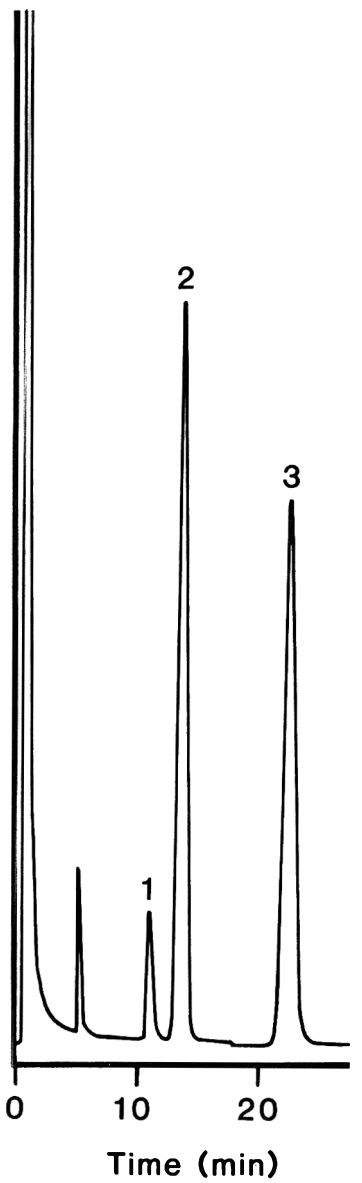

Figure 2. Gas chromatogram of lindane lotion. Impurity (1), γ-isomer (2), di-*n*-propyl phthalate (3).

phthalate internal std. EtOAc-isooctane layer is then extd with CH_3CN to remove interferences. Std and sample solns are carried thru same extn procedures. γ-BHC content is detd by GC using flame ionization detector.

Apparatus and Reagents

(a) *Gas chromatograph.*—Suitable for on-column injection; equipped with flame ionization detector.

(b) *GC column.*—Acid-washed and dry, 2 mm id × 1.83 m glass column packed with 7.5% OV-210 on 100–120 mesh Chromosorb W-HP. Operating conditions: injector 220°; detector 250°; column 160°; N carrier gas flow, ca 10 mL/min.

(c) *Internal std soln for technical BHC and formulations.*—Accurately weigh ca 2.5 g di-*n*-propyl phthalate into 100 mL vol. flask, dil. to vol. with EtOAc, and mix.

(d) *Internal std soln for lindane lotion and shampoo.*—Accurately weigh ca 0.5 g di-*n*-propyl phthalate into 100 mL vol. flask, add 20 mL EtOAc, and dil. to vol. with isooctane and mix.

Sample Preparation

(a) *Technical BHC and formulations.*—Accurately weigh samples of pure std γ-BHC and BHC tech., emulsifiable conc., and H_2O-dispersible powd. contg ca 250 mg γ-BHC into sep. 50 mL screw-cap bottles. To each bottle add by pipet 5.0 mL internal std soln and 20.0 mL EtOAc and shake 30 s. For H_2O-dispersible powd., filter or centrf. to remove particulates.

(b) *Lindane lotion and shampoo.*—Accurately weigh ca 100 mg pure γ-BHC into 50 mL screw-cap centrf. tube. Add by pipet 10.0 mL internal std soln. Agitate to dissolve γ-BHC and add 20 mL H_2O. Accurately weigh 10 g 1% lindane lotion or shampoo into 50 mL screw-cap centrf. tube. Add 20 mL H_2O. Gently agitate contents by tapping tube with fingers to mix H_2O and shampoo or lotion. Add by pipet 10.0 mL internal std soln. Vigorously shake std and samples by hand 1 min. Add 5 drops isoamyl alcohol to each tube and centrf. Transfer by pipet 3.0 mL each ext (top layer) to sep. 15 mL-screw-cap centrf. tubes. Add by pipet 5.0 mL isooctane followed by 3.0 mL CH_3CN. Vigorously shake each tube by hand 30 s. After phases sep., withdraw by disposable pipet a portion of CH_3CN phase (lower layer) for GC analysis.

Analysis of Solutions

(a) *Technical BHC and formulations.*—Inject 1 μL portions of std soln of γ-BHC until response ratios (area or peak ht) for γ-BHC to internal std agree ±2%. Make duplicate

Table 1. Data obtained in collaborative trial of GC method for analyzing technical BHC and formulations and lindane lotion and shampoo

	Technical		25% WDP		12% EC		1% Lotion		1% Shampoo	
Coll.	1	2	1	2	1	2	1	2	1	2
1	43.99	44.34	25.16	24.77	12.29	12.22	0.9982	1.062	0.953	1.018
	43.87	43.92	24.94	25.58	12.28	12.15	0.9795	1.009	1.025	0.9895
2	44.16	43.97	24.81	24.56	12.46	12.42	0.984	0.950	0.991	0.991
	43.51	43.47	24.77	25.05	12.44	12.40	0.984	0.956	1.005	0.991
3	43.93	43.58	25.42	25.44	12.55	12.67	0.989	0.977	0.964	0.971
	43.88	43.71	25.66	25.31	12.58	12.57	0.989	0.972	0.967	0.965
4	42.19	42.44	24.40	24.34	12.14	12.39	0.931		0.935	0.936
	42.25	42.60	24.37	24.35	12.34	12.17	0.931		0.935	0.940
5	43.28	43.73	24.94	25.12	12.55	12.63	1.13[a]	1.19[a]	1.06	1.03
	43.32	43.27	25.25	25.17	12.68	12.60	1.14[a]	1.20[a]	1.07	1.04
6	44.05	43.97	25.35	25.44	12.86	12.89	0.981	0.973	0.938	0.908
	43.87	44.05	25.45	25.44	12.79	12.93	0.981	0.973	0.930	0.908
7	43.94	43.47	25.22	25.36	12.70	12.78	0.978	0.976	0.981	0.957
	43.93	43.67	25.28	25.49	12.92	12.74	0.984	0.974	0.976	0.955
8	44.5	44.0	25.4	25.0	12.5	12.7	0.99	0.98	0.86	0.88
	44.3	43.5	25.3	25.1	12.5	12.9	1.0	0.96	0.84	0.86
9	44.80	44.09	25.65	25.60	13.15	13.23				
	45.29	44.31	25.62	25.73	13.33	13.23				
10	44.69	44.24	24.98	25.25	12.83	12.65				
	44.41	44.46	24.84	25.57	12.59	12.69				

[a]Omitted from calculations. Observations outside 1 SD (99% confidence interval).

Table 2. Statistical analysis of data presented in Table 1

Statistic	Tech.	WDP	EC	Lotion	Shampoo
n	40	40	40	26	32
Mean	43.82	25.16	12.64	0.98	0.96
Var. (total)	0.4198	0.1503	0.0851	0.0007	0.0032
SD	0.6479	0.3877	0.2917	0.0265	0.0562
Var. (within-labs)	0.0839	0.0358	0.0084	0.0003	0.0003
SD	0.2197	0.1893	0.0920	0.0179	0.0160
Var. (among-labs)	1.5397	0.5318	0.3404	0.0018	0.0131
SD	1.2408	0.7290	0.5835	0.0427	0.1146
CV	2.83	2.89	4.62	4.36	11.92

injections of std soln followed by duplicate injections of sample solns. Recalibrate after not more than 4 injections of sample solns.

(b) *Lindane lotion and shampoo.*—Inject 1 μL portions of ext of std soln of γ-BHC until response ratios (area or peak ht) for γ-BHC to internal standard agree ±2%. Make duplicate injections of std soln followed by duplicate injections of unknown exts. Recalibrate after not more than 4 injections of unknown exts.

Calculations

For each injection, calc. response ratio (R) = area (for peak ht) of γ-BHC peak to area (or peak ht) of internal std peak.

$$\gamma\text{-BHC, wt\%} = (R/R')(W'/W) \times P$$

where R' and R = av. response ratio for std and sample solns, resp.; W' and W = wt (mg) of γ-BHC std and sample, resp.; and P = purity of γ-BHC std (%).

Results and Discussion

Agreement among the 10 collaborating laboratories was excellent on the technical grade BHC and its formulations, and no values fell outside the 95% confidence interval (Tables 1 and 2). Inter-laboratory coefficients of variation ranged from 2.83% for technical BHC to 4.62% for the emulsifiable concentrate. Wider variance was observed in the results obtained on the 1% lindane lotion and shampoo. For lotion, the results from one laboratory fell outside the 99% confidence interval and were rejected. The coefficient of variation among laboratories was 4.36% for lotion and 11.92% for shampoo.

Two laboratories failed to run the samples of lotion and shampoo. One collaborator changed the column temperature from 160 to 170°C. Three participants changed the nitrogen flow rate. One reported 11 mL/min, another 30, and a third 60. Retention times ranged from 7.4 to 14 min for the gamma-isomer and from 12.4 to 23.8 min for the internal standard. Two collaborators noted that they were not able to completely resolve the impurity in the lindane lotion from the gamma-isomer; however, their values did not differ significantly from the others. One participant complained of foaming when the shampoo was shaken with the internal standard solution. The column efficiency of each participant was calculated from their chromatograms. Values ranged from 1070 to 3770 theoretical plates. Statistical analyses of the data are presented in Table 2.

The above methods were adopted by CIPAC at its 27th annual meeting in Brisbane, Australia. The Associate Referee recommends adoption of the methods by AOAC as official first action.

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Liquid Chromatography of Liquid Formulations Containing 2,4-D, Dicamba, and MCPP as Their Salts: Collaborative Study

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A liquid chromatographic method for any liquid mixture of the 3 herbicides 2,4-D, MCPP, and dicamba, found in commercial formulations, has been collaboratively studied. Each collaborator received 4 samples, 3 of which were ternary formulations and one a binary formulation of amine salts. Concentrations in the formulations range from 0.02 to 2.32% for dicamba, 0.32 to 21.7% for 2,4-D, and 0.22 to 11.6% for MCPP. A binary solvent system and the use of a specified 25 cm column of RP 10 μm in the reverse phase ion suppression mode will selectively quantitate each herbicide, separating many impurities found in the technical products. Standard deviations in each of 11 columns of results obtained indicate good precision. The method has been adopted official first action.

The development of a method for binary and ternary formulations of herbicides was reported previously (*J. Assoc. Off. Anal. Chem.* **66,** 1220–1225 (1983)). The method is specific for 2,4-D, dicamba, and MCPP in any combination found in currently marked amine salt liquid formulations of these 3 herbicides. Dicamba is always in the lowest percentage concentration, usually 10 times less than 2,4-D and in a similar ratio to MCPP. The rationale in development of the method is detailed in the 1983 report.

For the method to be applicable to a variety of mixed formulations, the detector response must be linear with respect to amount present. A series of different concentrations corresponding to the appropriate levels present were tested. The peak height ratios of component to internal standard were plotted against milligrams present. A linear relationship was demonstrated in the expected ranges for each of the components as is shown in Figure 1.

This report of the Associate Referees was presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

The recommendation of the Associate Referees was approved by the General Referee and Committee A and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) **67,** March.

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Collaborative Study

A variety of samples were sent to each collaborator, including a ready-to-use formulation already mixed with water, a brush controller sample with the highest concentration of all 3 herbicides, and 2 concentrates for lawn weed control. Analytical standards and the internal standards salicylic acid and butyrophenone were included in the shipment. Also included was a test solution of salicylic acid at 0.950 g/L to check detector peak height response before preparation of the internal standard solution. Instructions were explicit, which was deemed necessary for this specific and complex analysis which gives 3 herbicides on the same chromatogram (Figure 2).

Collaborators were asked to calculate dicamba in the 33% eluate in addition to that found in the 22% eluate specified in the method. The object of this request was to determine the extent of interference by *p*-chlorophenoxyacetic acid which originates from technical 2,4-D. This impurity coelutes with dicamba in the 33% eluant in an isocratic solvent system and is separated from dicamba in the 22% eluant.

Dicamba, 2,4-D, and MCPP in Pesticide Formulations
Liquid Chromatographic Method
First Action

Principle

Combinations of dicamba, 2,4-D, and MCPP in liq. formulations as their salts are detd in any combination, using binary mobile phase 22% and 33% CH_3CN–H_2O, phosphate buffered, pH 2.68–2.70, on reverse phase bonded microparticulate column. Specified column seps known impurities, analytes, and internal stds salicylic acid and butyrophenone.

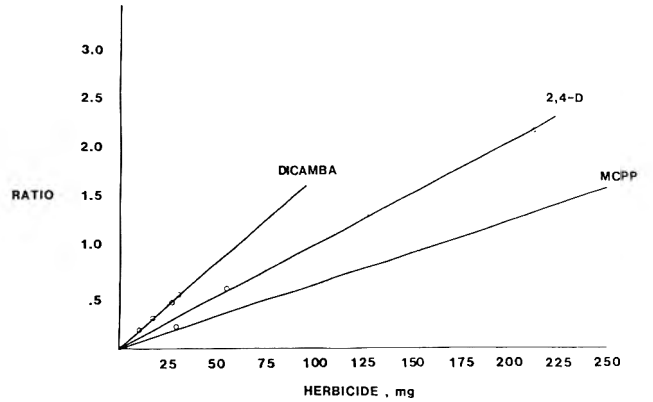

Figure 1. Linearity of response for each herbicide is shown as ratio of peak heights of herbicide to internal standard vs weight of herbicide.

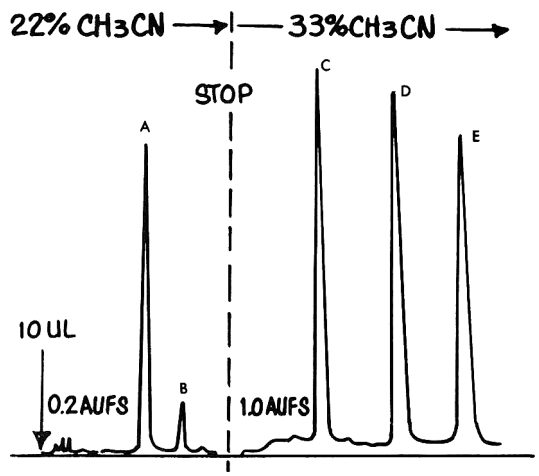

Figure 2. Sample chromatogram showing separations of A, salicylic acid; B, dicamba; C, 2,4-D; D, MCPP; and E, butyrophenone.

Apparatus

(a) *Liquid chromatograph.*—Fitted with 5000 psi pressure gage, 280 nm detector, 10 μL injector, eluant reservoir line filter, and 10 mV FS strip chart recorder; flow rate 2.0 mL/min.

(b) *Liquid chromatographic column.*—250 × 4.6 (id) mm Partisil 10/25 ODS-3 with Co:Pell ODS pellicular guard column (Whatman Inc.).

(c) *Operating conditions.*—Flow rate 2.0 mL/min; detector 280 nm; injection vol. 10 μL; temp. ambient (≤76°F); setting 0.2 AUFS for dicamba and 1.0 AUFS for 2,4-D and MCPP; chart speed 0.5 cm/min.

(d) *Performance characteristics.*—10 μL injection of 0.95 g salicylic acid/L causes ca 50% recorder deflection at 1.0 AUFS. Optimum conditions for column sepn are obtained when *o*-chlorophenoxyacetic acid impurity, from technical 2,4-D, is sepd between salicylic acid and dicamba in 33% eluant and immediately after dicamba in 22% eluant.

Reagents

Prep. each eluant in 1 L Erlenmeyer and re-circulate to conserve mobile phase.

(a) *Eluant I.*—22% mobile phase: 725 mL H_2O (0.4 μm-filtered), 220 mL CH_3CN (distd in glass), 16.9 mL NaOH (ca 17.7N, 1 + 1, **50.033(b)**); add H_3PO_4 to pH 2.69 ± 0.01 (note vol.) and dil. to 1 L with H_2O.

(b) *Eluant II.*—33% mobile phase: 610 mL H_2O, 330 mL CH_3CN, 16.9 mL NaOH (ca 17.7N, 1 + 1, **50.033(b)**); add H_3PO_4 to pH 2.69 ± 0.01 (note vol.) and dil. to 1 L with H_2O.

(c) *Diluting solvent.*—Isopropanol–H_2O (2 + 1).

(d) *Internal std soln.*—0.95 g salicylic acid (Aldrich Chemical Co.) + 9.00 mL butyrophenone (Aldrich Chemical Co.); dil. to 1 L with dilg solv.

(e) *Std soln.*—Governing factor is highest peak. For expected 2,4-D in highest concn, weigh 0.150 g; for expected MCPPA in highest concn, weigh 0.225 g. Weigh stds according to expected sample composition and ratio of herbicides to each other. Weigh into graduated, conical centrf. tube (Corning, Cat. No. 25330-50) or other stoppered, leak-proof container marked at 25 mL. Pipet 20.0 mL internal std soln; dil. to 25.0 mL with dilg solv.

Preparation of Sample Solution

Use same container as std soln and accurately weigh sample equiv. to 0.150 g 2,4-D or 0.225 g MCPP according to which is expected to be in highest concn in formulation. For sample having vol. >5 mL, dil. to 50 mL after addn of 20 mL internal std soln. If sample is dild to 50 mL, also dil. internal std soln to approx. same concn. Increase sensitivity to obtain approx. same peak ht. Filter samples and stds thru Whatman 9.0 cm glass fiber GF/C, or equiv. Use same pipet for adding internal std soln to stds and samples.

Determination

Flush LC column of previous solv. with H_2O for several minutes to stable baseline.

Dicamba: With eluant I flowing at 2.0 mL/min, inject 10 μL salicylic acid (0.95 g/L in dilg solv.) to condition column. Set detector to maximize peak (ca 0.2 AUFS), inject 10 μL std soln, and, when pen returns to baseline after elution of dicamba, inject 10 μL sample soln.

2,4-D and/or MCPP must be flushed from column after sample soln injection by the sequence: H_2O 2 min, 50% CH_3CN until pen returns to stable baseline. Elution order is salicylic acid first and dicamba second.

2,4-D and MCPP: With eluant II flowing at 2.0 mL/min, and attenuation at 1.0 AUFS, inject 10.0 μL std soln. When pen returns to baseline after elution of butyrophenone, inject 10.0 μL sample soln. Elution order is salicylic acid, dicamba, 2,4-D, MCPP, and butyrophenone.

Calculation

Use salicylic acid as internal std for calc. of dicamba; use butyrophenone for calcg 2,4-D, MCPP. Either peak ht or computer-integrated areas may be used.

$$\% \text{ Compd} = (W'/W) \times (R/R') \times P$$

where W' = mg std; W = mg sample; P = % purity of std; R and R' = peak ht or area ratios of compd to internal std for sample and std, resp.

Results and Discussion

Of interest is that *ortho*-chlorophenoxyacetic acid elutes just before dicamba in the 33% eluant and just after dicamba in the 22% eluant. An examination of all 4 chromatograms in this study shows *o*-CPAA to be the major impurity. On the other hand, *p*-CPAA, judging from results by difference between the 33% and 22% eluates, produces only a slightly higher bias for dicamba in the 33% eluate. Collaborators used the 33% eluant in an isocratic solvent system the first day to determine 2,4-D and MCPP, then repeated with replicate sample injections in the 22% eluant on the second day to determine dicamba. Two samples can be injected succes-

Table 1. Collaborative results for LC assay of liquid amine salt formulations for percent acid equivalent in ternary formulation Samples A, B, C and binary formulation Sample D

	Dicamba in 22% eluate			2,4-D in 33% eluate				MCPP in 33% eluate			
Coll.	A	B	C	A	B	C	D	A	B	C	D
1	0.020	0.77	2.49	0.31	6.69	21.71	9.28	0.21	3.22	11.54	9.46
	0.022	0.77	2.42	0.31	6.78	21.71	9.34	0.21	3.29	11.52	9.56
2	0.018	0.75	2.36	0.31	6.71	21.80	9.34	0.21	3.23	11.70	9.84
	0.018	0.75	2.40	0.31	6.67	21.70	9.38	0.21	3.18	11.60	9.88
3	0.021	0.73	2.29	0.31	6.74	22.16	9.23	0.21	3.21	11.72	9.56
	0.021	0.76	2.28	0.31	6.79	21.92	9.35	0.21	3.23	11.61	9.65
4	0.021	0.71	2.37	0.31	6.74	21.37	9.48	0.21	3.23	11.46	9.62
	0.022	0.72	2.38	0.32	6.73	21.57	9.38	0.21	3.20	11.45	9.55
5	0.021	0.73	2.29	0.30	6.72	21.46	8.96	0.21	3.27	11.78	8.98
	0.020	0.71	2.24	0.30	6.63	21.63	9.01	0.21	3.26	11.43	8.92
6	0.018	0.71	2.08	0.34	7.26[a]	21.54	8.95[a]	0.21	3.33	11.00	8.84
	0.020	0.69	2.26	0.35	7.08	22.50[a]	9.60	0.21	3.29	11.42	9.78
7	0.019	0.80	2.36	0.30	6.80	21.26	8.97	0.20	3.22	11.61	9.42
	0.018	0.74	2.17	0.31	6.72	21.21	8.97	0.20	3.14	11.21	8.88
8	0.016	0.78	1.87[a]	0.32	6.64	21.74	9.44	0.21	3.20	11.58	9.91
	0.038[a]	0.71	2.27	0.31	6.67	21.94	9.36	0.21	3.23	11.61	9.72
9	0.015	0.77	2.31	0.31	6.74	21.70	9.25	0.21	3.26	11.67	9.61
	0.016	0.76	2.35	0.32	6.77	21.83	9.40	0.22	3.26	11.74	9.83
10	0.019	0.79	2.32	0.38	6.71	21.70	9.26	0.26	3.24	11.71	9.73
	0.019	0.78	2.43	0.38	6.64	21.52	9.30	0.26	3.14	11.53	9.79
11[b]	0.039	0.95	2.91	0.27	8.41	24.05	11.65	0.21	3.41	11.02	10.12
	0.040	0.95	2.90	0.42	8.41	27.15	11.66	0.57	3.37	12.24	10.11
$\bar{X}$	0.019	0.75	2.32	0.32	6.72	21.66	9.26	0.22	3.23	11.58	9.53
S_d	0.002	0.032	0.097	0.024	0.052	0.240	0.191	0.016	0.047	0.138	0.350
CV_d	10.0	4.2	4.2	7.4	0.8	1.1	2.1	7.5	1.2	1.2	3.6
S_r	0.001	0.023	0.071	0.005	0.043	0.113	0.156	0.002	0.037	0.138	0.254

[a]Outlier at 99% confidence limits. Both results in pair containing outlier have been omitted.
[b]All results from Collaborator 11 excluded from statistical calculations.

sively in the latter eluant, without interference, to determine dicamba, followed by flushing with water and then 50% acetonitrile.

As shown in Table 1, each collaborator reported 11 results in pairs, for a total of 121 pairs. The coefficient of variation (CV) is consistent in all samples and indicates good precision. There is a generally very good agreement between laboratories; however, in several instances, agreement within a duplicate does not show the same precision as most other laboratories for the same sample. The percent CV for dicamba is considerably higher in Sample A because of the very low percent present. Collaborator 6, in answer to inquiry on the disparity of that laboratory's results, noted a problem in integrator-calculation of area; therefore, results were recalculated on the basis of manually measured peak height. There is still a degree of inconsistency or imprecision in the figures supplied by Collaborator 6 that can not be explained at this writing.

Comments by collaborators on the method were favorable, although one collaborator noted that 1 L of 33% eluant was not enough for all samples without recycling. One collaborator remarked on having to attend the instrument to change attenuation after elution of dicamba. Manual peak height measurement requires increased sensitivity setting for dicamba; however, computerized integration probably would not require monitoring.

Recommendation

Because of the specificity of the method and the good results obtained, it is recommended that the LC method be adopted official first action.

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Rodger W. Stringham, State Chemical Laboratory, West Lafayette, IN

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Gas Chromatographic Determination of Metribuzin in Formulations: Collaborative Study

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A collaborative study was conducted on a gas chromatographic method for determining metribuzin. Two 75% dry flowable and two 42% liquid flowable formulations were analyzed by 18 laboratories. Formulations were extracted by shaking or ultrasonic mixing for 1–5 min with methylene chloride which contained di-*n*-butyl phthalate as an internal standard. The extracts were injected into a gas chromatograph equipped with an OV-225 column. Peak area ratios and peak height ratios showed no significant difference. The reproducibility coefficient of variation was 0.75% for the 75% formulation and 1.41% for the 42% formulation. This GC method has been adopted official first action.

Metribuzin, 4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one, is a herbicide that has been used for the control of a large number of grass and broadleaf weeds. A gas chromatographic (GC) procedure using a thermal conductivity detector has been published (1). This method was modified slightly to permit the use of a flame ionization detector to widen its acceptability. A collaborative study was undertaken to evaluate its precision and reproducibility among several laboratories. This paper describes the results of the collaborative study.

Metribuzin in Pesticide Formulations
Gas Chromatographic Method
First Action

(Method is suitable for tech. metribuzin and formulations.)

Principle

Sample is extd with CH_2Cl_2 contg di-*n*-butyl phthalate as internal std and metribuzin is detd by gas chromatgy.

Apparatus and Reagents

(a) *Gas chromatograph.*—Equipped with flame ionization detector (FID).

(b) *Chromatographic column.*—1–2 m × 2 mm id glass column packed with 3% OV-225 on 60–80 or 80–100 mesh Gas-Chrom Q, or equiv.

(c) *Di-n-butyl phthalate.*—Eastman No. 1403 or equiv. that contains no impurities eluting at retention time of metribuzin.

(d) *Reference std metribuzin.*—Mobay Chemical Corp., Agricultural Chemicals Division, PO Box 4913, Hawthorn Rd, Kansas City, MO 64120.

Preparation of Standards

(a) *Internal std soln.*—Weigh 4.5 g di-*n*-butyl phthalate, dil. to 1 L with CH_2Cl_2, and mix well. If necessary, adjust concn so that peak hts of metribuzin and internal std are within 20%.

(b) *Metribuzin std soln.*—Accurately weigh ca 200 mg ref. std metribuzin into ca 250 mL glass bottle. Add by pipet 100.0 mL internal std soln. Mix well.

Preparation of Sample

Shake liq. flowable formulations vigorously ≥ 1 min before sampling. Accurately weigh sample contg ca 200 mg metribuzin into glass bottle (ca 250 mL). Pipet in 100.0 mL internal std soln. Stopper and mech. shake or ultrasonify 1–5 min. Let insol. materials settle and use supernate for injection.

Determination

Adjust operating parameters to cause metribuzin to elute in 3–5 min. Maintain all parameters const thruout analysis. Typical values are as follows: temps (°)—inlet 250, column 210, detector 250; carrier gas 20–40 mL/min (either He or N); air and H flows as recommended for FID. Measure peak areas by electronic integration, or alternatively, peak hts. Retention times (min)—*N*-methyl isomer (impurity in tech. metribuzin) ca 1.5–2, di-*n*-butyl phthalate ca 2–3, metribuzin ca 3–5. If internal std and *N*-methyl isomer are not resolved on 1 m column, substitute longer column, but do not exceed 2 m.

Make repetitive 2 μL injections of metribuzin std soln until response is stable and ratios of metribuzin peak area (or ht) to internal std peak area (or ht) for successive injections agree within ± 1% of their mean.

Make duplicate 2 μL injections of each sample. Metribuzin/internal std ratios for 2 sample injections must agree within ± 1% of their mean. If not, repeat detn, starting with std injections. After every 4–6 sample injections and after last sample injection, make 2 injections of calibration soln. Av. metribuzin std soln ratios preceding and following samples must lie within ± 1.0% of the mean; otherwise, repeat series of injections.

Calculations

Calc. ratios for each injection. Average 2 sample ratios and 4 std ratios (std injections immediately before and after sample injections).

$$\% \text{ Metribuzin} = (R/R') \times (W'/W) \times P$$

where R = av. sample ratio (metribuzin peak/internal std peak); R' = av. std ratio (metribuzin peak/internal std peak); W' = mg std; W = mg sample; P = % purity of metribuzin std.

Method Development

The main impurity in technical metribuzin is the *N*-methyl isomer, 4-amino-6-(1,1-dimethylethyl)-3,4-dihydro-2-methyl-3-thioxo-1,2,4-triazin-5(2*H*)-one. This compound elutes just before the internal standard. A typical chromatogram of technical metribuzin on an OV-225 column is shown in Figure 1.

Another impurity occasionally found in metribuzin is butylthion, 4-amino-6-(1,1-dimethylethyl)-3,4-dihydro-3-thioxo-1,2,4-triazin-5(2*H*)-one. This compound lacks a methyl group in the 3 position. On an OV-225 column, butylthion has a retention time of 5.5 min compared with 4.0 min for metribuzin. On methyl silicone columns such as SE-30, DC-200,

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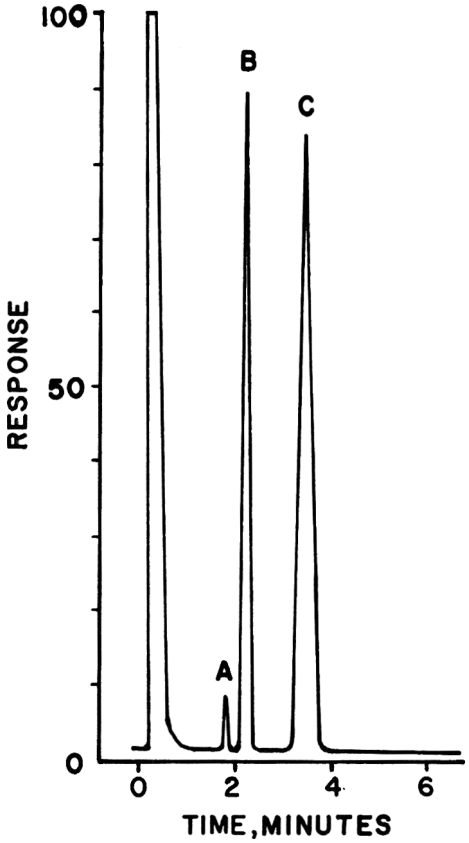

Figure 1. GC separation of *N*-methyl isomer (A), di-*n*-butyl phthalate (B), and metribuzin (C).

and OV-101, butylthion separates poorly from metribuzin and therefore such columns could not be used.

A linearity test on the response of flame ionization detection to metribuzin was conducted by weighing 200, 250, and 300 mg standard metribuzin into separate bottles, adding 25 mL internal standard and 100 mL of CH_2Cl_2, and comparing peak area ratios. The ratios of the 200 and 300 mg standards compared with the 250 mg standard (normal standard weight) were 100.0 and 100.8% of theoretical, which is within the repeatability of the method. The metribuzin peak response of the thermionic detector was previously found to be linear over a 2-fold range (1).

A test for recovery of metribuzin was carried out by accurately weighing 250 mg standard metribuzin into 10 separate bottles. To 5 of these bottles was added the proper amount of a blank consisting of all of the inert ingredients in the 4 lb/gal. liquid flowable (LF) formulation, and to the other 5 the proper amount of the 75% dry flowable (DF) blank. These preparations were mixed thoroughly by shaking and rotating, then analyzed according to the method. The recoveries from the 4 lb/gal. LF formulation ranged from 99.3 to 100.0% with an average of 99.8%. The recoveries from the 75% DF preparations ranged from 99.4 to 101.1% with an average of 99.9%.

Collaborative Study

Each collaborator was sent standard metribuzin (93.2%), di-*n*-butyl phthalate internal standard, 2 samples of 75% DF formulations, and 2 samples of 4 lb/gal. LF formulations. Both pairs of formulations had metribuzin values about 1% apart as recommended by Youden and Steiner (2). Collaborators were asked to report one determination per sample, that value being the average of 2 injections of each sample extract.

Results and Discussion

The GC method permits measurements of peak areas by electronic integration or measurement of peak heights if electronic integration is not available. Ten collaborators reported peak area data only, 5 reported both peak area and peak height data, and 3 reported peak height data only. For evaluation of the GC method, peak area results were used for the 15 collaborators who submitted only peak height data. These results are shown in Table 1 (Collaborators 1–15, peak area data; Collaborators 16–18, peak height data). Results were interpreted following the outline of Youden and Steiner (2).

Collaborator 11 had the highest results on all 4 samples and those results were rejected on the basis of the ranking test (2). Three of the 4 results of Collaborator 7 were rejected as outliers after the Dixon test (2) was applied; the other value was not used because one of the pair was rejected. The remaining results showed a repeatability standard deviation of 0.36% for the 75% DF and 0.46% for the 4 lb/gal. LF based on 32 observations (16 pairs) for each. The reproducibility coefficients of variation were 0.75% for the 75% DF and 1.41% for the 4 lb/gal. LF, based on 32 observations for each.

Enough data were available to compare results obtained by using peak areas with those obtained by using peak heights. Table 2 lists the results of the 8 collaborators who submitted peak height data. These results were compared with the results of the first 15 collaborators in Table 1, which were obtained using peak area measurements. The statistical evaluation of the 2 sets of data is shown in Table 3. No significant difference can be seen between the means or the standard deviations of peak area vs peak height measurements. Therefore, either method of measurement can be used.

The method is rugged as indicated by the wide variety of gas chromatographic instruments, integrators, columns, and extraction methods used by the collaborators. The method was written for use with either a flame ionization or thermionic conductivity detector. The author routinely uses a thermionic conductivity detector and found good agreement between the 2 detectors. However, flame ionization was used by all 18 collaborators in this study. All collaborators used glass columns although stainless steel columns can be used (1). Lengths of the columns used ranged from 3 to 6 ft. All loadings were 3% OV-225, except Collaborator 10 used 1½% OV-225 and Collaborator 6 used 5% OV-225. Supports included Gas-Chrom Q, Supelco, Chromosorb G, and Chromosorb WHP. Two collaborators used helium as carrier gas; the remainder used nitrogen. Flows ranged from 20 to 60 mL/min. Extraction methods included reciprocating shakers, wrist-action shakers, magnetic stirring, and hand shaking, and 3 collaborators used ultrasonic mixing.

The most frequent comment made by the collaborators was that the internal standard peak height was almost twice the metribuzin peak height. This has been corrected and an adjustment is permitted for any instrument that may still give dissimilar peak heights.

Recommendation

It is recommended that the GC method be adopted official first action.

Acknowledgments

I express my gratitude to the following collaborators and their associates for taking part in this study:

D. Anderson and J. E. Forrette, Velsicol Chemical Corp., Chicago, IL

Table 1. Results of metribuzin (%) collaborative study

Coll.	Sample 1	Sample 2	Diff. (1 – 2) plus 0.74	Sample 3	Sample 4	Diff. (3 – 4) minus 1.46
1	75.01	75.73	+0.02	42.52	40.84	+0.22
2	74.99	75.84	−0.11	42.49	40.83	+0.20
3	75.24	75.91	+0.07	42.56	40.94	+0.16
4	75.72	75.73	+0.73	43.16	42.59	−0.89
5	74.29	75.32	−0.29	41.65	40.70	−0.51
6	75.27	75.83	+0.18	42.03	40.35	+0.22
7	76.10[a]	77.16[b]	—	30.90[b]	32.19[b]	—
8	75.29	75.49	+0.54	42.95	41.02	+0.47
9	73.64	75.04	−0.66	42.15	42.04	−1.35
10	75.50	75.92	+0.32	41.72	40.29	−0.03
11	77.65[c]	78.39[c]	—	44.75[c]	43.58[c]	—
12	75.22	75.82	+0.14	42.55	40.77	+0.32
13	74.83	75.61	−0.04	43.02	40.36	+1.20
14	74.46	76.14	−0.94	42.55	41.08	+0.01
15	76.74	76.56	+0.92	43.22	41.30	+0.46
16	75.02	76.26	−0.50	41.96	41.04	−0.54
17	75.17	76.52	−0.61	43.52	41.23	+0.83
18	74.71	75.22	+0.23	42.66	41.90	−0.70
Mean	75.07	75.81		42.54	41.08	
Sample 1 − Sample 2 = −0.74						
Sample 3 − Sample 4 = 1.46						
N		32			32	
S_r		0.36			0.46	
S_b		0.43			0.37	
S_d		0.56			0.59	
CV, %		0.75			1.41	

[a]Not used because other half of pair was rejected.
[b]Rejected by Dixon test (2).
[c]Rejected by ranking test (2).

Table 2. Results of metribuzin (%) collaborative study by peak height measurements

Coll.	Sample 1	Sample 2	Diff. (1 – 2) plus 0.95	Sample 3	Sample 4	Diff. (3 – 4) minus 1.34
1	75.15[a]	75.61[a]	+0.49	42.58[a]	40.90[a]	+0.34
5	74.81	75.88	−0.12	41.79	40.70	−0.25
6	73.67[b]	74.84[b]	—	41.37[b]	39.91[b]	—
7	74.70	75.17	+0.48	31.63[c]	32.02[c]	—
9	74.55	76.09	−0.59	42.18	40.89	−0.05
16	75.02[d]	76.26[d]	−0.29	41.96[d]	41.04[d]	−0.42
17	75.17	76.52	−0.40	43.52	41.23	+0.95
18	74.71	75.22	+0.44	42.66	41.90	−0.58
Mean	74.87	75.82		42.45	41.11	
Sample 1 − Sample 2 = −0.95						
Sample 3 − Sample 4 = 1.34						
N		14			12	
S_r		0.33			0.40	
S_b		0.24			0.36	
S_d		0.40			0.53	
CV, %		0.53			1.28	

[a]Average of manual and electronic peak height measurement.
[b]Rejected by ranking test (2).
[c]Rejected by Dixon test (2).
[d]By electronic peak height measurement.

Table 3. Comparison of peak area vs peak height measurements for metribuzin (%)

Comparison	By peak area	By peak height	Diff.
Sample 1	75.09 (*N* = 13)	74.87 (*N* = 7)	+0.22
Sample 2	75.76 (*N* = 13)	75.82 (*N* = 7)	−0.06
Sample 3	42.51 (*N* = 13)	42.45 (*N* = 6)	+0.06
Sample 4	41.01 (*N* = 13)	41.11 (*N* = 6)	−0.10
75% DF:			
N	26	14	
S_r	0.36	0.33	
S_b	0.46	0.24	
S_d	0.59	0.40	
CV, %	0.78	0.53	
4 lb/gal. LF:			
N	26	12	
S_r	0.46	0.40	
S_b	0.37	0.36	
S_d	0.59	0.53	
CV, %	1.41	1.28	

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TECHNICAL COMMUNICATIONS

Decomposition of 4,4′-(Diazoamino)-bis(5-methoxy-2-methylbenezenesulfonic Acid) in Solutions of FD&C Red No. 40

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4,4′-(Diazoamino)-bis(5-methoxy-2-methylbenzenesulfonic acid), when present as a reaction by-product in FD&C Red No. 40, is shown to decompose rapidly in aqueous solutions of the color additive. The decomposition is halted by the addition of sodium borate buffer. Quantitationly liquid chromatography shows that decomposition is nonlinear with time and follows approximate first order kinetics.

4,4′-(Diazoamino)-bis(5-methoxy-2-methylbenzenesulfonic acid) (DMMA) has been identified as a reaction by-product of FD&C Red No. 40 (1). This compound, as well as another reaction by-product and 2 intermediates, is determined in batches of FD&C Red No. 40 submitted for certification to the Division of Color Technology (DCLT), Food and Drug Administration (FDA), by using a liquid chromatographic (LC) method. On the basis of the results of a collaborative study of this method reported by Cox and Reed (2), the method was adopted official first action by AOAC.

However, Bailey and Cox (1) reported that in an acid medium DMMA is unstable and decomposes to 4-amino-5-methoxy-2-methylbenzenesulfonic acid, which is commonly known as cresidine sulfonic acid (CSA), and to CSA diazonium ion. Furthermore, the aqueous solutions used for DCLT analyses of FD&C Red No. 40 samples fall within the pH range at which DMMA is known to decompose (3). Although Marmion (4) studied the decomposition of DMMA in solution at various pH levels, he did not investigate the decomposition within the first few minutes after the dye was dissolved. Thus an experiment was conducted to determine how rapidly DMMA decomposes in a solution of the color additive prepared in water.

Experimental

The apparatus and reagents were the same as those described in ref. 2. The FD&C Red No. 40 sample used in the decomposition study was a commercially manufactured batch containing 0.26% DMMA.

Twenty-one solutions were prepared from the FD&C Red No. 40 sample, and the method in ref. 2 was followed as described. However, the length of time between the preparation of each solution and the addition of sodium borate buffer (pH 9) was varied.

A 10 mL portion of 0.10M $Na_2B_4O_7$ was added to the first sample; 50 mL water was added to dissolve the sample, and the solution was diluted to 100 mL with water. A 50 mL portion of water was added to the next sample; after 1 min, 10 mL 0.10M $Na_2B_4O_7$ was added and the solution was diluted to 100 mL with water. For the remaining samples, the length of time between the preparation of the solution and the addition of 0.10M $Na_2B_4O_7$ ranged from 2 min to 6 h.

After each solution was chromatographed as described in ref. 2, the amounts of DMMA were quantitated and a plot was constructed for DMMA concentration vs time that solution was unbuffered.

Table 1. DMMA concentrations and times FD&C Red No. 40 solutions remained unbuffered during decomposition study

Sample	Time, min	Concn,[a] %
1	0	0.260
2	1	0.250
3	2	0.232
4	3	0.229
5	4	0.220
6	5	0.203
7	10	0.175
8	20	0.146
9	30	0.114
10	45	0.108
11	60	0.089
12	90	0.075
13	120	0.056
14	150	0.045
15	180	0.035
16	210	0.032
17	240	0.021

[a]By weight of FD&C Red No. 40.

Results and Discussion

The FD&C Red No. 40 sample used in this study is typical of certified batches of the color additive in that the pH of the aqueous solution is approximately 6.0. However, it differs from most samples of FD&C Red No. 40 in the amount of DMMA present. Certified batches of the color additive generally contain 0–0.02% by weight of the reaction by-product, whereas the sample used in this study contained 0.26% DMMA.

Table 1 presents the concentrations of DMMA in the first 17 sample solutions tested. The DMMA peaks in the last 4 sample chromatograms (4.5, 5.0, 5.5, and 6.0 h) were detected but were unresolved from other peaks. Therefore, the DMMA concentrations of the last 4 samples were not measured. In the 6.0 h sample chromatogram, the DMMA peak appeared only as a small shoulder.

The plot in Figure 1, which shows how the decomposition of DMMA under these experimental conditions proceeded rapidly in the first 30 min and then slowed down, demonstrates the nonlinearity of the decomposition. Regression calculations indicated that the reaction follows approximate first order kinetics.

As the DMMA in each sample solution was destroyed, the amount of CSA in the sample solution increased. In Solution No. 1 (buffer added first), 0.08% CSA was quantitated. In the sample solution prepared 4 h before the addition of buffer (Solution No. 17), the level of CSA increased to 0.16%. This increase corresponds to the amount of CSA that would be expected from the observed DMMA concentration change. The addition of the buffer halted the DMMA decomposition.

Conclusions

On the basis of the work described in this decomposition study, the wording of AOAC official method **34.B01–34.B06** (5) was changed to specify addition of sodium borate first in

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On the recommendation of the Associate Referee, E. A. Cox, and the approval of the General Referee and Committee G, this change was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.

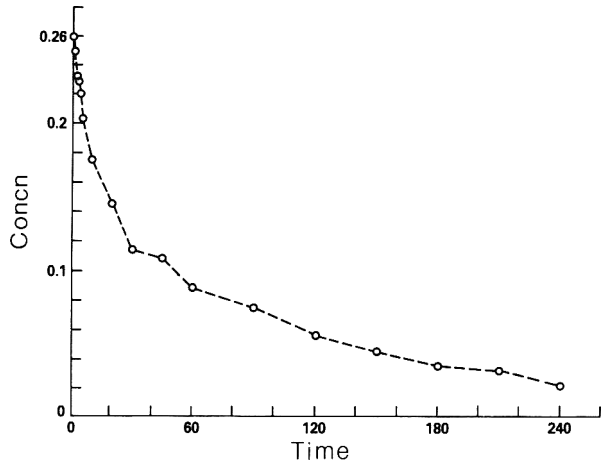

Figure 1. Concentration of DMMA (% by weight in solution of FD&C Red No. 40) vs time that solution remained unbuffered (min).

the determinative step. The first sentence of **34.B06** now reads:

"Weigh 0.250 g sample, add 10 mL 0.1M $Na_2B_4O_7$ first, then add ca 50 mL H_2O, and, when dissolved, dil. to 100 mL with H_2O."

Failure to add the sodium borate first could result in decomposition of DMMA.

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Identification and Semiquantitation of Monensin Sodium in Liver Tissue

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A bioautographic technique for the determination of monensin sodium in animal feed has been applied to chicken and beef liver tissues. The reliable limit of sensitivity is 100 ppb, but 10 ppb can be detected. This technique can be used to semiquantitate monensin in tissues by comparing the zones of inhibition of unknown test samples against monensin standards.

Monensin is active against coccidiosis in fowl (1) and increases feed efficiency and weight gain in beef cattle (2). Questions have been raised about the effective detection of low-level monensin residues (50 ppb) in edible tissues, and, therefore, a method is needed for detection of residues in animal tissue in the lower ppb range.

Donoho and Kline (3) effectively used a thin layer bioautographic technique to determine monensin in chicken tissues. The basic technique, as reported by Kline and Golab (4), was recently modified (5) for use with animal feeds. This present communication extends the application of the modified method to liver tissues of chickens and cattle. The modified method can detect 10 ppb monensin in animal liver tissue, but results were not always consistent at this level.

METHOD

Reagents and Apparatus

See reagents (**a–h**) and apparatus (**a–f**) (5), except use 100 mL pear-shape flask (**c**).

Column Preparation

Alumina and Sephadex LH-20 columns are prepared as in ref. 5.

Sample Preparation

Weigh 10 g frozen liver tissue into 50 mL Pyrex glass tube with ground-glass stopper. Add 20 mL methanol–water (8 + 2). Homogenize tissue ca 5 min and then shake 30 min on mechanical shaker. Centrifuge homogenate 15 min at 1500–1800 rpm. Decant extract directly into alumina column if concentration is less than 1.0 ppm. Wash sides of column with additional 25 mL methanol–water (8 + 2) and collect eluate in 250 mL separatory funnel. If concentration of monensin is greater than 1.0 ppm, decant extract into alumina column and elute. Transfer aliquot equivalent to 100 ppb monensin to separatory funnel and add 100 mL 5% NaCl. Shake vigorously and let mixture stand 5 min. As described in ref. 5, partition monensin into methylene chloride, evaporate to dryness, dissolve residue, and continue with further purification on Sephadex LH-20 column.

Thin Layer Chromatography (TLC) and Bioautography

Proceed with TLC and bioautography as described in ref. 5. Air-dry plates 10–15 min for TLC or 1.5–2 h when continuing with bioautography.

Results and Discussion

Recovery determinations were conducted on beef and chicken liver tissues with the bioautographic technique. In each study, 10 g portions of frozen blank tissue were spiked with 0.1, 0.05, or 0.025 μg monensin sodium. Based on the results of many bioassays, the best recovery data were obtained at the 0.1 μg (100 ppb) spike level; the recoveries were reproducible and were estimated to be 50–60% for chicken liver tissue and 60–70% for beef liver tissue when 20 μL of extract was spotted (Tables 1 and 2).

On the TLC plates with monensin at levels of 50 ppb (0.05 μg/g) or less, the spots were slightly diffused and lighter in color. The plates with more than 50 ppb monensin had brighter red spots, which when viewed under ultraviolet (UV) light appeared as bright orange. After the TLC plate was sprayed a second time and viewed again under UV light after 5–10 min, the orange spots appeared to be brighter. These plates

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Table 1. Recovery data for beef liver tissue samples, each spiked with 100 ppb monensin sodium, for semiquantitative determination by bioautography

Sample[a]	R_f × 100	Zone diam., mm[b]	Est. rec., %[c]
Blank	—	—	—
1	73	7	70
2	71	6	60
3	70	6	60
4	70	7	70
5	71	7	70
6	77	7	70
7	74	6	60
8	73	5	50
9	73	5	50
10	73	6	60
Ref. std rec.,	75	10	100
Ref. std	78	10	—

[a]Blank, liver tissue without added monensin. Reference standard recovery sample, 5.0 μg monensin sodium/mL carried through procedure starting with 5% NaCl–methylene chloride partition. Reference standard, 5.0 μg monensin sodium/mL spotted directly from dilution flask. Volumes spotted, 20 μL for both tissues and standards.
[b]Zone diameters obtained by using sample spotting and quantitating template.
[c]Percent recovery estimated based on TLC determinations, assuming 100% resolution of reference standard.

Table 2. Recovery data for chicken liver tissue samples, each spiked with 100 ppb monensin sodium, for semiquantitative determination by bioautography

Sample[a]	R_f × 100	Zone diam., mm[b]	Est. rec., %[c]
Blank	—	—	—
1	70	5	63
2	69	4	50
3	68	5	63
4	67	4	50
5	68	6	75
6	70	5	63
7	68	5	63
8	66	3	38
9	67	5	63
10	69	5	63
Ref. std rec.,	71	8	100
Ref. std	74	8	—

[a]Blank, tissue sample without added monensin. Reference standard recovery sample, 5.0 μg monensin sodium/mL carried through procedure starting with 5% NaCl–methylene chloride partition. Reference standard, 5.0 μg monensin sodium/mL spotted directly from dilution flask. Volumes spotted, 20 μL for both tissues and standards.
[b]Zone diameters obtained by using sample spotting and quantitating template.
[c]Percent recovery estimated based on TLC determinations, assuming 100% resolution of reference standard.

were used to determine sensitivity by comparing both methods of detection.

The bioautographic technique showed sensitivity at levels of 10 ppb monensin in beef liver and 50 ppb in chicken liver tissues. The technique was used to semiquantitate monensin at levels of 100 ppb in the tissues by comparing the zones of inhibition of test samples to those of the reference standards; the sensitivity of the method was determined to be 50 ppb monensin for both tissue types tested.

Liver tissues spiked at levels of 100 ppb monensin showed no interferences on the bioautography plate and gave the best results. The R_f values for monensin on TLC plates (K-6 and Uniplates) ranged between 0.66 and 0.73 for both chicken and beef liver tissues. The Uniplate gave consistent R_f values in the bioautographic technique, with R_f values for monensin ranging between 0.70 and 0.74 for both types of tissue.

Other antibiotics used as preventive medications for broilers and beef cattle were tested in combination with monensin, using the TLC procedure with K-6 Whatman plates. These antibiotics included lincomycin, zinc bacitracin, flavomycin (bambermycin), chlortetracycline (HCl), oxytetracycline, lasalocid sodium, and tylosin. None interfered with monensin, confirming the previous results (5). Lasalocid sodium was also tested on bioautography plates, and the R_f values were higher by at least 0.1 than those of monensin sodium.

Although bioautography results cannot be obtained as quickly as TLC results, 10 ppb levels of monensin residues in liver tissues are detectable. This modified technique can be used as a screening test for monensin and also as a semiquantitative method.

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Elimination of Aliquoting in Automated Flame Photometric Determination of K_2O in Fertilizers

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Current automated instrument systems used in conjunction with the official AOAC flame photometric method for K_2O in fertilizer, 2.D06, all require a dilution step to bring the fertilizer extract to the appropriate concentration range. Two CFA (Continuous Flow Analysis) automated instrument systems are described which together eliminate aliquoting over the full range of fertilizer potassium, a significant saving of analyst effort if large numbers of samples are analyzed. The systems have performance characteristics well within the limits in the official method, and produce results comparable to current methods on routine samples.

The official AOAC automated flame photometric method for K_2O in fertilizers has undergone several modifications, reflecting changes in instrumentation and in the philosophy of how automated methods should be written (1–4). All of these variations have included a labor-intensive aliquoting step. After extraction, it has been necessary to dilute the potassium solution to bring its concentration into range of the standards used on the automated equipment. This requires a significant amount of analyst time when large numbers of samples are routinely determined.

During a recent collaborative study (4), an instrumental system that eliminated aliquoting came to the attention of the Associate Referee. This system, as it is presented here, was developed by the Commonwealth of Virginia, Department of General Services, Division of Consolidated Laboratory Services, and slightly modified in the Associate Referee's laboratory.

To eliminate aliquoting, it is necessary to dilute more on the automated manifold and to use a wider range of standard concentrations. Two concentration ranges were selected, 0–600 mg K_2O/L for samples with K_2O guarantees <30%, and 580–1280 mg K_2O/L for sample guarantees of 30 to 63%.

In this laboratory, most samples with a K_2O guarantee <30% are also analyzed for P_2O_5, so an ammonium citrate extraction is performed. Almost all of the samples that fall into the higher range are 60% potash samples, for which no P_2O_5 analysis is needed; an ammonium oxalate extraction is performed on these. This leaves only a few higher range K_2O samples that also require a P_2O_5 analysis, and in these few instances an ammonium citrate extraction is performed, followed by 1:1 dilution and quantitation using the lower range standards.

METHOD

The instrument parameters are essentially the same for the 2 standard ranges. Where there are differences, specifications for the lower 0–600 mg K_2O/L range are given first and for the higher 580–1280 mg K_2O/L range, second.

Apparatus

AutoAnalyzer system with following modules (available from Technicon Instruments Corp.): Sampler II or IV, proportioning pump III, flame photometer IV, and recorder. Computer or calculator capable of least square fits is desirable.

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Reagents

(Note: Brij 35™ has been substituted for Flaminox™ 1% solution as the wetting agent because it reduces baseline noise slightly.)

(a) *Lithium nitrate solution.*—Dissolve 1.0947 g La_2O_3 in 30 mL HNO_3, add 0.6623 g dried (2 h at 105°C) $LiNO_3$ and 1 mL Brij 35. Dilute to 1 L with water.

(b) *Sample wash and dilution water solution.*—Dilute 1 mL Brij 35 to 1 L with water.

(c) *Potassium stock standard solutions.*—Low range: 1 g K_2O/L. Dissolve 5.7736 g dried (2 h at 105°C) KH_2PO_4 (NBS SRM 200) in water (or use other primary standard material and correct for purity), and dilute to 2 L. High range: 10 g K_2O/L. Dissolve 14.4342 g dried KH_2PO_4 in water and dilute to 500 mL.

(d) *Potassium working standard solutions.*—Low range: Accurately measure 50, 100, 150, 200, 250, and 300 mL of 1 g K_2O/L stock solution into 500 mL volumetric flasks. Add 100 mL ammonium citrate, **2.044** (3), and dilute to volume with water. Mix well. Add 3 mL $CHCl_3$ to preserve standard solutions for long periods. High range: Accurately measure 29, 36, 43, 50, 57, and 64 mL of 10 g K_2O/L stock solution into 500 mL volumetric flasks. Add 2.0 g $(NH_4)_2C_2O_4$ and dilute to volume.

Preparation of Samples

Use available P_2O_5 citrate extraction, **2.050** (3), for low range standards. Use K_2O ammonium oxalate extraction, **2.099(a)** (3), for high range standards.

Analytical System

Assemble manifold as in Figure 1 for low range standards. Use clear standard pump tubes for air and solution stream flows. Use 1.6–2.0 mm id glass transmission tubing downstream from photometer aspiration fitting. (Technicon part numbers are given in parentheses.) Air, water, and sample are combined through injection fitting A (116-0489-01). Sample and water are mixed in a 20-turn coil, B (157-0248), and a portion of the solution is resampled at AO fitting C. Insert 0.035 mm tubing into tee arm of AO fitting and insert a metal nipple into this tubing for connection to resample pump tube. Cut resample tube connection as short as possible. Excess solution from resample goes to waste via 0.035 mm tubing. Water and $LiNO_3$ reagent are mixed at injection fitting D (116-0489-01). Resample is injected into this dilute $LiNO_3$ stream at A10 fitting E. Solution is mixed in 15-turn coil F (190-0751-425-01 plus 157-0248-01), to which 15 mm length of glass manifold tube is attached. A 35 mm length of hard thin-wall polyethylene tubing (ca 0.034 mm id) connects manifold tube to another AO fitting G. A portion of solution is aspirated to flame photometer through 15 mm of hard thin-wall polyethylene tubing (ca 0.034 mm id) attached to tee arm of AO fitting by same means as resample pump tube was attached to its AO fitting. AO fitting is oriented so that no bubbles are aspirated. Unaspirated solution goes from top of AO fitting to pump via 75 mm of 0.065 mm tubing. Note that capillary backpressure tubing used in previous manifold designs is omitted. This device was useful with Flame III models, but Flame IV models with independently adjustable aspiration rates function with less noise without this tubing.

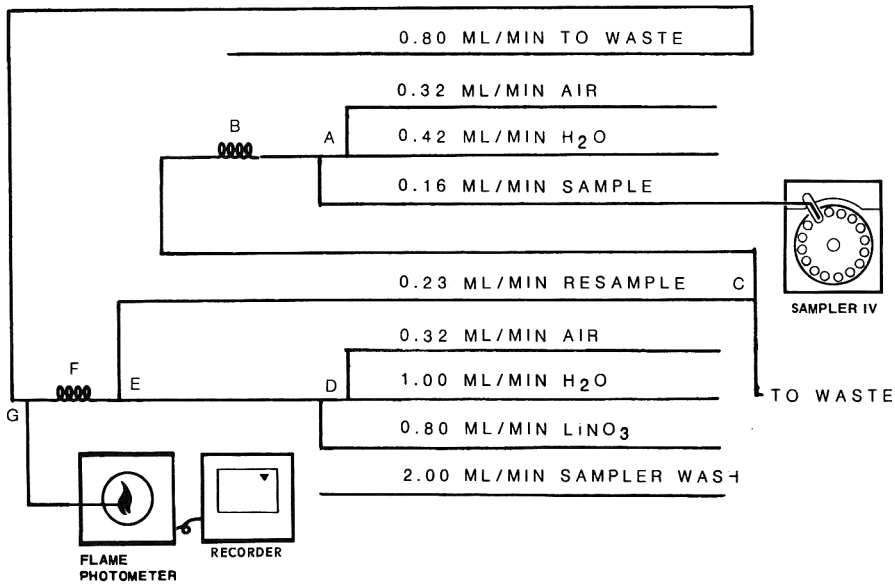

Figure 1. K_2O low range standard flow diagram (Technicon part numbers in parentheses): A, injection fitting (116-0489-01); B, 20-turn coil (157-0248); C, AO fitting; D, injection fitting (116-0489-01); E, A10 fitting; F, 15-turn coil (190-0751-426-01 plus 157-0248-01); G, AO fitting.

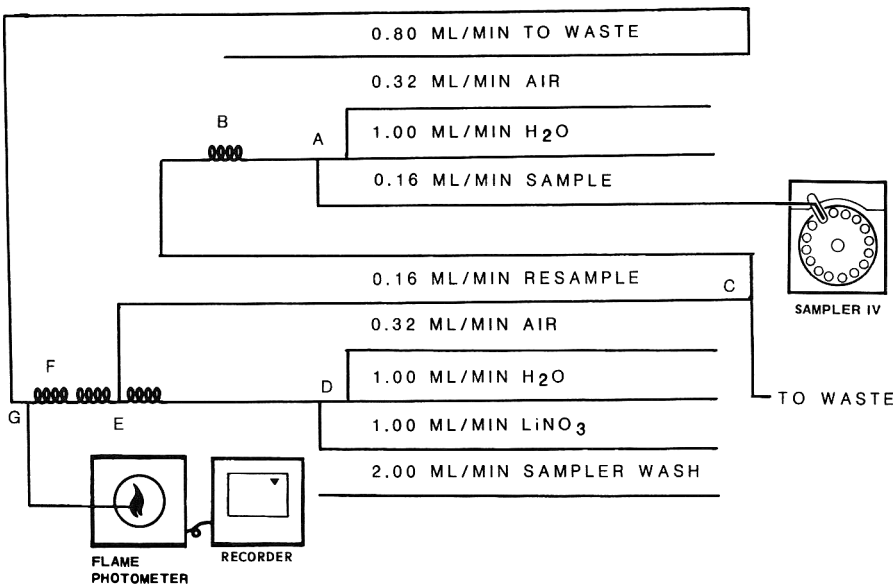

Figure 2. K_2O high range standard flow diagram (Technicon part numbers in parentheses): A, injection fitting (116-0489-01); B, 20-turn coil (157-0248); C, AO fitting; D, injection fitting (116-0489-01); E, 20-turn coil with insert (157-8089-01); F, 15-turn coil (190-0751-426-01 plus 157-0248-01); G, AO fitting.

For high range standards, a 20-turn coil with insert (157-8089-01) was connected between $LiNO_3$–water injection fitting and the 15-turn coils. The A10 fitting was removed and the resample was connected to the 20-turn coil insert. Other differences in the high range configuration include changes in pump tubes: Dilution water pump tube was changed from 0.42 to 1.00 mL/min, resample from 0.23 to 0.16 mL/min, and $LiNO_3$ from 0.80 to 1.00 mL/min (Figure 2).

Startup and Shutdown Procedures

Start system and place reagent lines in proper solutions. Let equilibrate 30 min before beginning calibration. Adjust flame photometer as follows:

1. Damping control to damp 2 position for low range standard or damp 3 for high range standard.
2. Methane flame height of main cone ca 4 cm.
3. Atomizer adjust control set to give atomization rate of ca 1.8 mL/min for low range standard or ca 1.7 mL/min for high range standard.
4. For low range standards, use 0.3 and 0.6 neutral density filters for Li and K detectors, respectively. For high range standards use 0.15 and 0.9, respectively.

At end of each run pump water through reagent lines for at least 15 min.

Checkout and Calibration

For low range standards, equilibrate while pumping water and set baseline to zero using baseline control. Pump 600 mg K_2O/L standard through system and adjust standard calibration control to read about 90% full scale. Check for noise and drift.

For high range standards, pump 580 mg K_2O/L through system and adjust baseline to 30% full scale (as low as possible), using baseline control. Pump 1280 mg K_2O/L standard and adjust standard calibration control to read about 90%.

Determination

If low range standards are used (ammonium citrate extraction), analyze samples with guarantees < 30% undiluted.

Table 1. Instrumental system performance specifications

Performance criteria	Found		Allowed, AOAC
	Low range	High range	
K and Li response	equal	equal	equal
Noise	0.5%	1%	2%
Carryover	0.8%	0.34%	1 %
Drift	negligible	0.2%/10 samples	1%/10 samples
Precision	0.5%	0.5%	2%
Standard curve			
Diff., known and calcd	0.4%	0.3%	2%
Av. abs. values	0.3%	0.2%	1%
Phosphate effect	0.3%	0.15%	1%
20 Magruder samples			
Av. bias	0.06%	0.04%	0.1%
Av. of absolute value of differences	0.2%	0.15%	0.4%
5 KH_2PO_4 samples			
Diff. of calcd mean and theoretical value	0.15%	0.02%	0.2%
Std dev.	0.2%	0.1%	0.25%

Samples with guarantees ≥ 30% are diluted 50 mL to 100 mL with water. Add an additional 10 mL ammonium citrate solution before adjusting to volume so that concentration of ammonium citrate is same as in standards.

For high range standard (ammonium oxalate extraction), analyze all samples undiluted.

Sample tray configuration: standards, Nos. 1, 6, 6, 1, 1, 2, 3, 4, 5, 6, (30 samples, Nos. 1, 1, 2, 3, 4, 5, 6)*N*, 1, 1, 6, 6, where *N* can be any integer.

Sample at rate of 40/h, 2:1 sample-to-wash ratio for low range standards or 4:1 sample-to-wash ratio for high range standards.

Calculations

In the Associate Referee's laboratory, the sample tray sequence of the Nos. 1 and 6 standards at the beginning and end of the run allows a computer correction for carryover. Also, the 2 complete standard sequences allow a computer correction for drift. Using the corrected peak heights, determine a least squares fitted curve of emission vs K_2O concentration. Calculate mg K_2O/mL from corrected sample peak heights using least squares fitted equation.

$$\% K_2O = (\text{mg } K_2O/\text{mL}) \times (\text{dilution factor}) \times (50)/(\text{g sample})$$

where dilution factor is 1 for nondiluted samples or 2 for diluted samples.

Instrument Performance Specifications

The official method (2) includes instrument performance specifications in various categories. Table 1 shows a comparison of the performance of the 2 instrument systems with official allowed performance levels. In each category, both systems have performance levels well within limits set in the official method.

Results and Discussion

A number of routine samples were analyzed both by the low range standard system and by the example instrument system from the recent collaborative study (4). Results of 183 samples ranging from 1 to 51% K_2O were determined, representing 3 independent runs by both of the systems.

A Student *t*-test resulted in a mean difference of 0.005%, a standard deviation of the differences of 0.198, and a *t*-value of −0.34 with 182 degrees of freedom. There is no significant difference at the 95% confidence level.

A similar comparison of the high range standard method was made with 72 samples ranging from 30 to 63% K_2O, representing 2 independent runs by both systems. A Student *t*-test resulted in a mean difference of 0.007%, a standard deviation of the differences of 0.268, and a *t*-value of −0.21 with 71 degrees of freedom. Again, there was no significant difference at the 95% confidence level.

Note that the additional mixing coil used with the high range standard system had the effect of eliminating a precipitate which formed when the system was used with ammonium oxalate extraction chemistry. In practice, this extra coil is now left in place when the system is switched back to the low range standards.

Also note that all instrument performance levels reported in Table 1 were achieved without the use of computer correction for carryover and drift. Correction would only improve the performance levels. The routine sample results were from computer corrected peak heights. Laboratories without computer facilities, wishing to implement these instrumental systems, may prefer to analyze at about 30 samples/h rather than 40/h to reduce carryover. This is the primary difference between the system as used in the Virginia laboratory and in the Associate Referee's laboratory.

However, this is not a serious disadvantage because the automated system runs by itself and the limiting step is the sample preparation.

Conclusion

Eliminating aliquoting represents a significant saving of analyst time. A possible source of analyst mistakes as well as one source of method error are also eliminated. This is accomplished within the framework of the official AOAC methodology for K_2O.

Acknowledgments

We again acknowledge the Virginia Division of Consolidated Laboratory Services, where the basic development of the system was done. We especially thank Loretta Kirk for her generous help. The authors, however, bear responsibility for any deficiencies.

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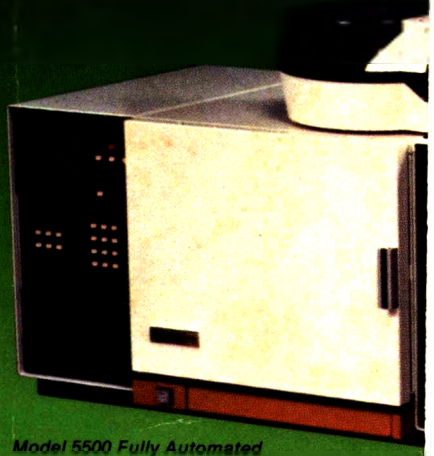

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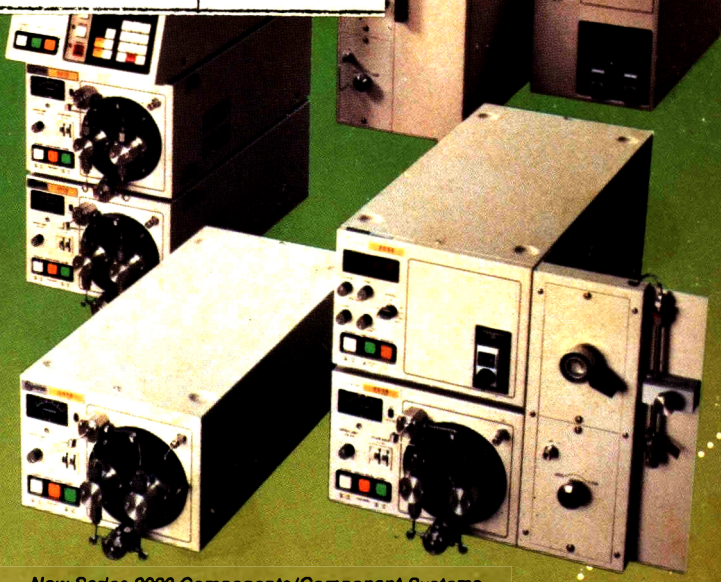

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