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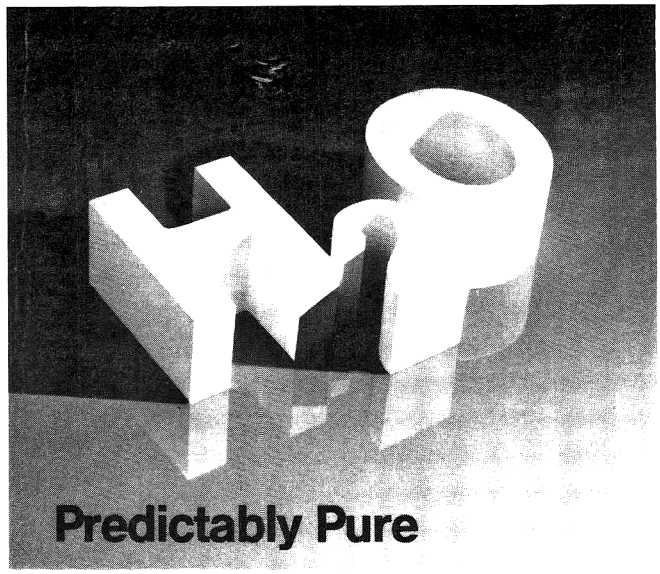

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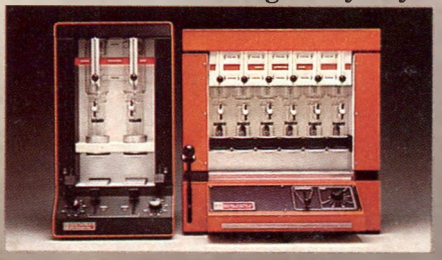

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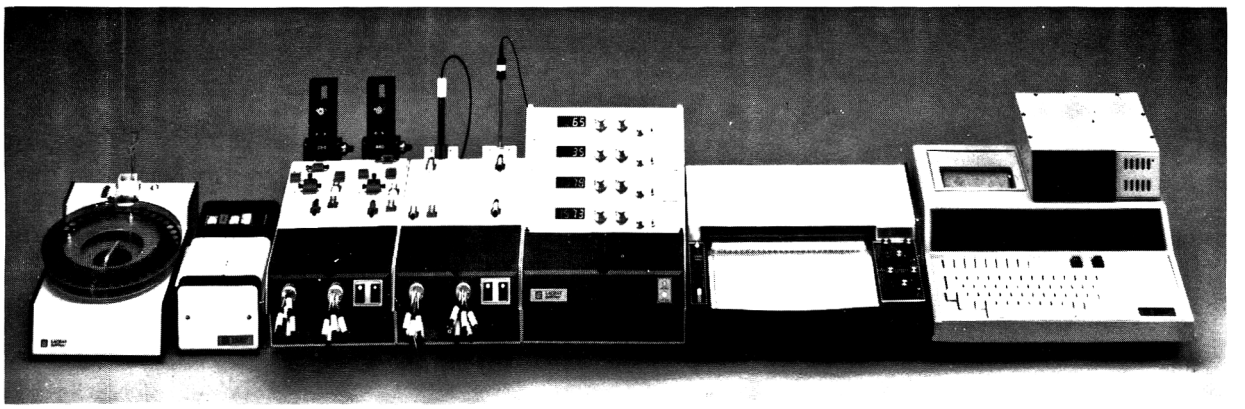

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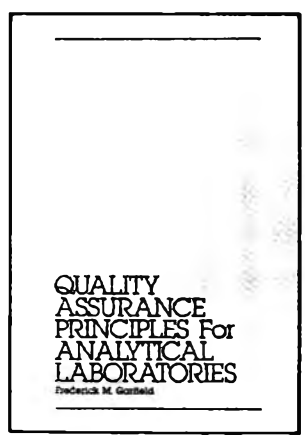

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BOOKS IN BRIEF

Analysis of Food Contaminants. Edited by J. Gilbert. Published by Elsevier Science Publishing Co., Inc., PO Box 1663, Grand Central Station, New York, NY 10163, 1984. 400 pp. Price: $74.00. ISBN 0-85334-255-5.

This book discusses selection of specialized techniques, including size exclusion and gel chromatography, immunoassay, gas chromatography, high performance liquid chromatography, mass spectrometry, and chemiluminescence, that can be used in the analysis of food contaminants. Each technique is described in simple terms and is critically appraised in context of one or more specific food contaminant problems. Key references are supplied for those researchers who wish to explore these methods in more detail.

Regulated & Major Industrial Chemicals. Edited by C. D. Craver. Published by The Coblentz Society, Inc., PO Box 9952, Kirkwood, MO 63122, 1983. Price: $150.00.

This new publication includes infrared spectra of chemicals listed by the Environmental Protection Agency as Priority Pollutants, as well as other major industrial chemicals. Compounds which are closely related structurally to Priority Pollutants, or are likely to be contaminants arising from the same chemical process, are also included to help the spectroscopist with important analyses. Over 600 spectra are provided. They include multiple physical states, especially for compounds with spectra sensitive to the sampling environment. A brochure describing other reference data books in this series is available from the Society at the above address.

Toxic Oil Syndrome. Mass Food Poisoning in Spain. Edited by P. Grandjean and S. Tarkowski. Published by the World Health Organization, 1211 Geneva, Switzerland, 1984. 92 pp. Price: Sw. fr. 16–. ISBN 92-890-1021-5.

This book traces the development of the epidemic, toxic oil syndrome disease, from its first case in 1981 to the present, revealing how little is still known about how and why the disease occurred as it did. The book—compiled as a result of a meeting of experts organized by WHO in Madrid in 1983, in close cooperation with the government of Spain—gives some idea of how the catastrophe came about, what exactly the disease consisted of, and what is being done and has been done to discover its precise cause.

The United States Pharmacopeia, 21st Rev., The National Formulary, 16th Ed. Published by The United States Pharmacopeial Convention, Inc., 12601 Twinbrook Pkwy, Rockville, MD 20852, 1984. 1683 pp. Price: $155/subscription. ISSN 0195-7996; ISBN 0-913595-040-7(cloth), 0-913595-05-5(leather).

This volume contains, in addition to the official monographs ranging from acetaminophen to zomepirac, the following sections: a listing of people associated with USP, preamble, admissions, general notices and requirements applying to standards, tests, assays, and other specifications of USP; general tests and assays, including microbiological, biological, chemical, and physical; reagents, including indicators and solutions (buffer, colorimetric, test, volumetric); tables for container specifications, solubilities, and atomic weights; and an extensive index.

Environmental Sampling for Hazardous Wastes. Edited by J. A. Santolucito and G. E. Schweitzer. Published by American Chemical Society, 1155 16th St, NW, Washington, DC 20036, 1984. About 134 pp. Price: $34.95(U.S. & Canada)/$41.95(export price). ISBN 0-8412-0884-0.

This book addresses some of the important considerations in designing and implementing environmental sampling programs, particularly surface and subsurface sampling. Authors review several successful field programs, including sampling for dioxin in Missouri, for lead in Dallas, and for cyanide in Washington.

Insect Management for Food Storage and Processing. Edited by F. J. Baur. Published by the American Association of Cereal Chemists, 3340 Pilot Knob Rd, St. Paul, MN 55121, 1984. 384 pp. Price: $45.00(members)/$65.00 (nonmembers). ISBN 0-913250-38-4.

This book provides detailed information on avoiding insect problems in the food industry, detecting and controlling pests, and eliminating insect problems when they exist. Several chapters are devoted to present fumigation techniques and fumigants, and other chapters cover more novel approaches to insect control such as superheating, ionization radiation, pheromones, and electrocution light traps.

Concepts in Analytical Chemistry. By S. M. Khopkar. Published by Halsted Press, a division of John Wiley & Sons, Inc., One Wiley Dr, Somerset, NJ 08873, 1984. 368 pp. Price: $24.95. ISBN 0-470-27490-5.

This book serves as a reference for practicing analytical chemists and research and developmental workers in laboratories. It covers volumetric and gravimetric methods and newer separation methods, including solvent extraction as well as various novel chromatographic methods. In addition to normal optical methods of analysis mainly involving spectroscopy, the techniques used in elucidation of structure are included. Electroanalytical methods of analysis, along with thermal and radiochemical techniques are also presented.

Management of Hazardous Occupational Environments. By P. N. Cheremisinoff. Published by Technomic Publishing Co., Inc., 851 New Holland Ave, Lancaster, PA 17604, 1984. 202 pp. Price: $24.95. ISBN 87762-352-1.

This book was prepared for industrial safety personnel, industrial hygienists, plant engineers, and industrial managers as a guide to the identification, evaluation, and monitoring of workplace hazards. The book details effective procedures for managing a wide variety of common workplace hazards such as dust, gas, noise, biological and infectious materials, flammable materials, chemicals, and others. Included are chapters on hazard definition, analytical methods, respiratory protection, and record-keeping requirements.

Requirements of Laws and Regulations Enforced by the U.S. Food and Drug Administration. Edited by W. F. Janssen. Published by the Government Printing Office, Washington, DC 20402, 1984. 77 pp. Price: $2.50. ISBN 017-012-0031-4.

This 1984 publication is an extensive revision of the 1979 edition, which summarizes all statutes now administered

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A Chemist's Guide to Regulatory Drug Analysis.
1974. 134 pp. Hardbound. Illustrations. ISBN 0-935584-05-6. Members: $11.00 in and outside U.S.
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by FDA. It is intended mainly as an aid to understanding the *Federal Food, Drug, and Cosmetic Act* of 1938, as amended. Four new laws are summarized: the Federal Anti-Tampering Act, the Infant Formula Act, the Saccharin Study and Labeling Act, and the Orphan Drug Act. Other new material describes the cooperative federal-state program for retail food protection and the national program for sanitation in interstate travel facilities and disease prevention. Numerous cross references have been added, giving section numbers in the laws and regulations, as well as updated mail addresses of agency units for additional information, forms, etc.

Taste, Olfaction, and the Central Nervous System. Edited by D. Pfaff. Published by The Rockefeller University Press, PO Box 5108, New York, NY 10249, 1984. 368 pp. Price: $16.95. ISBN 0-87470-039-6.

This book comprises invited papers presented at a special symposium of olfaction, taste, and the central nervous system held at The Rockefeller University in honor of Carl Pfaffman, a major figure in neuroscience. The papers describe current research in 5 areas: taste receptors, neurophysiological coding of the olfactory information, olfactory determination of social behaviors, neurophysiological coding of taste information, and mechanics of behavioral responses to taste. Among topics discussed are artificial and natural sweeteners, basic theories of taste, time as a factor in perception of flavors, and effects of olfaction on behavior.

Environmental Research and Protection. Inorganic Analysis. Edited by W. Fresenius and I. Lüderwald. Published by Springer for Science, PO Box 503, 1970 AM IJmuiden, The Netherlands, 1984. 292 pp. Price: DM 42,–/approx. US $15.30. ISBN 3-540-13469-7.

This book includes chapters on sea water, analysis and quality control, atmosphere and deposition, inland waters, soils and sewage sludges, and biotic materials, which discuss inorganic chemical pollutants such as SO_2 and NO_x, and heavy metals, and metalloids. These pollutants have become significant in recent years for their toxic effects on vegetation, soil, natural waters, and air, and the threat they pose to man via the food chain.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Published by Springer for Science, PO Box 503, 1970 AM IJmuiden, The Netherlands, 1984. Vol. 91: 151 pp.; price: DM 68,–/approx. US $24.80; ISBN 3-540-90998-2. Vol. 92: 210 pp.; price: DM 88,—/approx. US $32.10. ISBN 3-540-96018-X.

Volume 91 includes the following reviews: mutagenicity in procaryotes of herbicides; temephos with particular reference to the West African Onchocerciasis Control Program, and human exposure in DEF/merphos. Volume 92 includes reviews on antibiotics in sediments and run-off waters from feedlots, and the effect of phenoxyacetic acid herbicides 2,4,5-trichlorophenoxyacetic and 2,4-dichlorophenoxyacetic acid as ascertained by direct experimentation.

FOR YOUR INFORMATION

EPA Project Summary

An interlaboratory study in which 20 laboratories participated was conducted to provide precision and accuracy statements for the proposed EPA Method 604-Phenols for measuring concentrations of the Category 8 chemicals: 2,4-dimethylphenol, 2-chlorophenol, 4-chloro-3-methylphenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol, 2-nitrophenol, 4-nitrophenol, 4,6-dinitro-2-methylphenol, and 2,4-dinitrophenol in municipal and industrial aqueous discharges.

The method involves determination of the phenols by gas chromatography (GC) with flame ionization detection (FID), or derivatization and detection by electron capture (EC).

The study design was based on Youden's plan for collaborative tests of analytical methods. Three Youden pair samples of the test compounds were spiked into 6 types of test waters and then analyzed.

The resulting data were statistically analyzed using the computer program "Interlaboratory Method Validation Study" (IMVS). Method recoveries were in the range 40–89%; overall precision, 15–37%; and single-analyst precision, 15–37%, using the GC-FID procedure. Method recoveries were in the range 32–76%; overall precision, 38–64%; and single-analyst, 29–48%, using the GC-EC procedure.

In general, mean recoveries, overall standard deviations (S), and single-analyst standard deviations (SR), were directly proportional to the true concentration levels. With the exception of the FID analysis of 2,4-dinitrophenol in 3 of the wastewaters, there were no discernible differences due to water types among mean recoveries, overall precisions, and single-analyst precisions.

Based on the results of the interlaboratory study, EPA Method 604-Phenols is a viable analytical method for measuring trace concentrations of the 11 Category 8 chemicals used in this study.

The complete report (Order No. PB 84-196 211) can be obtained for $22.00 (subject to change) from the National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161; 703/487-4650. For information, contact Edward L. Berg and Robert L. Graves, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

Quality Assurance of Supplies to Pharmaceutical Industries

The section for quality control in the pharmaceutical and cosmetic industries of the European Organization for Quality Control (EOQC) has published a manual that deals with principal requirements for suppliers to provide an effective and efficient quality assurance of supplies.

The manual's purpose is to improve communication between the pharmaceutical industry and its suppliers, giving the suppliers insight into specific requirements of the pharmaceutical industry, while providing their customers with appropriate information on the products supplied.

The manual is divided into 2 parts: Part A consists of the Guidance Notes representing a survey of quality assurance measures and precautions to be taken by suppliers; Part B contains 4 questionnaires referring to raw materials.

This 49-page publication can be ordered from the EOQC Pharmaceutical Section, PO Box 2613, CH-3001 Berne, Switzerland. Price: Sw. fr. 55.– (all charges included).

Short Courses

Massachusetts Institute of Technology will offer a one-week course, "Design and Analysis of Scientific Experiments," July 15–20, 1985. Topics covered will apply to the physical, chemical, biological, medical, engineering, and industrial sciences, and to experimentation in psychology and economics. For more information, contact: Director of Summer Session, Room E19-356, Massachusetts Institute of Technology, Cambridge, MA 02139.

Waters Associates announces a series of seminars to illustrate recent innovations in the applications of LC technology to the life sciences. These free, one-day seminars will be held in Washington, DC, during October and November 1985. Topics to be covered include (1) optimizing sample preparation and chromatographic isolation of monoclonal antibodies; (2) scale-up strategy for high resolution isolations of peptides and proteins; (3) amino acid analysis by an innovative approach; and (4) analysis of immunosuppressive polypeptides. Contact: Nick Weigner, Waters Associates, Washington, DC, 1-800-526-0771.

Standard Reference Materials

The National Bureau of Standards (NBS) Office of Standard Reference Materials announces the availability of the following Standard Reference Materials (SRM): SRM 1832 and SRM 1833 are designed for calibration of instruments used for elemental analysis of pollutants or toxic particles in waste water. Each consists of 0.55 μm thick glass film deposited on a polycarbonate filter mounted on an aluminum ring. These SRMs are certified for the following concentrations: SRM 1832, Al, Si, Ca, V, Mn, Co, and Cu; SRM 1833, Si, K, Ti, Fe, Zn, and Pb. Price: $398 each.

SRM 1618, Vanadium and Nickel in Residual Fuel Oil, contains 100 mL of No. 6 residual fuel oil as defined by the American Society for Testing and Materials (ASTM). Concentration values for vanadium and nickel are 423 and 75 μg/g, respectively. This new SRM meets the oil industry's need for an SRM with higher concentration to more realistically simulate refinery analyses. Price: $103.

SRM 1003a, Calibrated Glass Spheres (8–58 μm), consists of 25 g of material with certified values based on the microscopic measurements of nearly 8000 individual spheres taken from 10 randomly selected bottles of identical material. SRM 1003a is part of a series of small particle measurement standards that are being developed by NBS under the ASTM Research Associate Program. Price: $90.

All of the above SRMs may be purchased from: Office of Standard Reference Materials, B311 Chemistry Bldg, National Bureau of Standards, Gaithersburg, MD 20899; 301/921-2045.

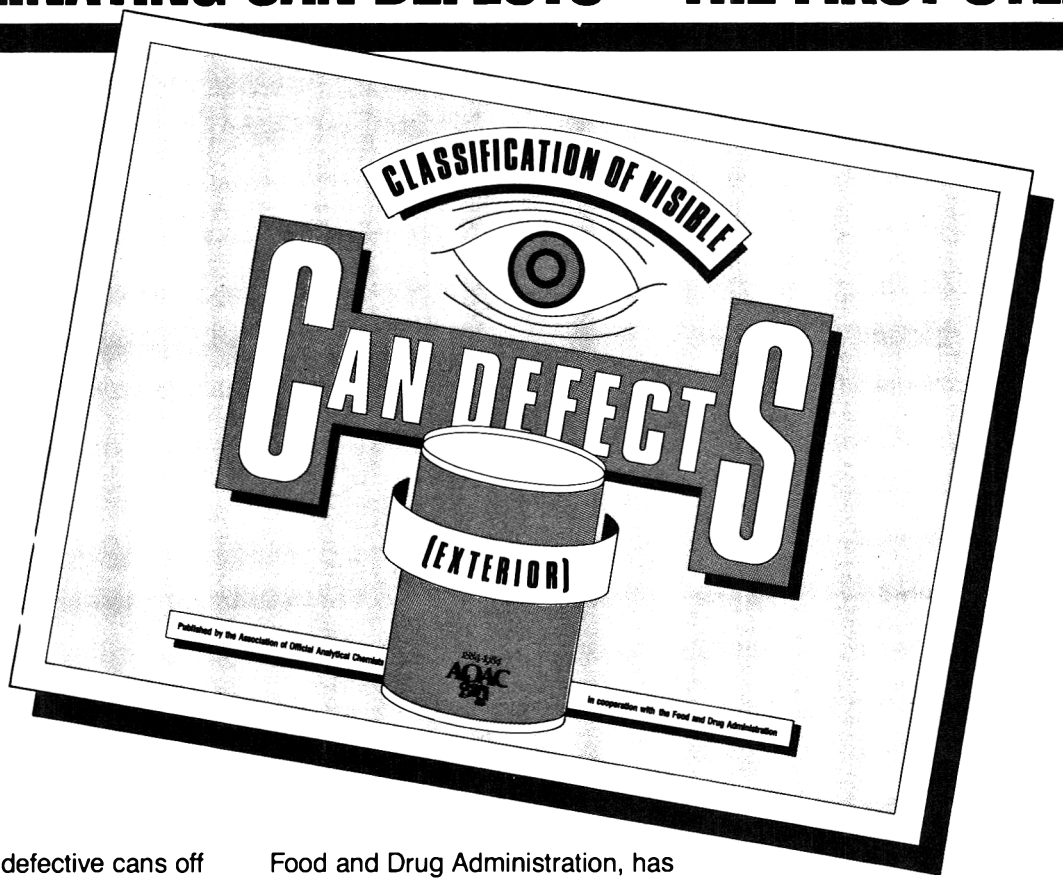
AACC Methods Supplement

The supplement to the 8th edition of the *AACC Approved Methods* includes 3 new methods: alveograph method for soft wheat flour, SDS sedimentation test for durum wheat, and macro scale pasta processing; also included are a number of consolidated and revised methods. Contact: Raymond J. Tarleton, American Association of Cereal Chemists, Headquarters, 3340 Pilot Knob Rd, St. Paul, MN 55121; 612/454-7250.

Meetings

June 12–14, 1985: Chemical Innovation Conference, sponsored by the American Association of Small Research Companies (AASRC) and the American

ELIMINATING CAN DEFECTS—THE FIRST STEP



How can we keep defective cans off the market?

Botulism and other forms of food poisoning can sometimes be traced to defective cans that have leaked and thereby allowed micro-organisms to enter food. Recognizing a can defect, doing something to correct the cause, and removing the defective cans from commerce will help prevent food poisoning outbreaks. The first step is to ensure that responsible personnel know how to identify defective cans.

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

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

For copies, write to:
Association of Official Analytical Chemists
1111 North 19th Street, Suite 210
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Chemical Society (ACS)/Small Chemical Business Division, Loews Glenpoint Hotel, Teaneck, NJ. This conference is intended for small companies to describe technology available for sale or license to more than 30 large corporations who are actively seeking products, services, processes, and technology in chemical-related areas. Registration fee for small companies is $95.00/person for AASRC or ACS/Small Chemical Business Division members, and $125.00 for non-members. Fee for large companies or brokers is $600.00 for the first person and $95.00 for each additional attendee from the same company. Contact: Joanne Martin, AASRC, Lincoln Ave, Suite 5, Prospect Park, PA 19076, USA, 215/522-1500, or Richard Varsanik, Calgon Corp., PO Box 1346, Pittsburgh, PA 15230, USA, 412/777-8866.

June 17–19, 1985: AOAC Midwest Regional Section Meeting, Holiday Inn, Hillside, IL. Contact: Devendra Trivedi, Illinois Law Enforcement Dept, Bureau of Scientific Services, 515 E Woodruff Rd, Joliet, IL 60432, USA, 815/727-5301.

June 17–19, 1985: AOAC Northeast Regional Section Meeting, University of Massachusetts at Amherst, Amherst, MA. Contact: James Fitzgerald, Food and Drug Administration, Winchester Engineering and Analysis. HFR-1390, 109 Holton Street, Winchester, MA 01890, USA, 617/729-5700.

June 20–22, 1985: AOAC Northwest Regional Section Meeting, Evergreen College, Olympia, WA. Contact: H. Michael Wehr, Oregon Dept of Agriculture, Laboratory Services Div., 635 Capitol St, NE, Salem, OR 97310-0110, USA, 503/378-3793.

June 24–27, 1985: MD & DI 85 Central, the medical device and diagnostic industry's first midwest conference/exposition, O'Hare Convention Center, Chicago, IL. Topics will include computer software use and validation, electronics and technical manufacturing issues, reimbursement and positioning strategies, management and marketing issues, drug-delivery, and technical advances. The conference also features a 40 000 + sq. ft full-service exposition with the latest product, service, and equipment options. Contact: Expocon Management Associates, 3695 Post Rd, Southport, CT 06490, USA, 203/259-5734.

August 7–8, 1985: The 12th Annual Intermountain Fertilizer and Agriculture Chemical Safety and Health School Program, Ridpath Hotel, Spokane, WA. The school is designed for fertilizer and agriculture chemical dealers, distributors, and manufacturers. The program meets criteria for the nationally accepted C.E.U. and P.C.A. credits. Contact: Maurice L. Greiner, School Director, c/o J. R. Simplot Co., PO Box 912, Pocatello, ID 83204, USA, 208/232-6620, Ext. 338.

September 29–October 4, 1985: Federation of Analytical Chemistry and Spectroscopy Societies' 12th Annual Meeting, Philadelphia, PA. Topics to be discussed include applied spectroscopy, chromatography, and allied techniques of instrumental analysis. Contact: Alan Ullman, Procter & Gamble Co., 6250 Center Hill Rd, Cincinnati, OH 45224, USA, 513/659-6445.

October 27–31, 1985: AOAC 99th Annual International Meeting, Shoreham Hotel, Washington, DC. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, USA, 703/522-3032.

May 27–30, 1986: Belgian Society of Pharmaceutical Sciences' 2nd International Symposium on Drug Analysis, Catholic University of Louvain-en-Woluwe(UCL), Brussels, Belgium. Topics will include fundamental aspects of drug analysis, quality control of natural and synthetic raw materials, analysis of pharmaceutical preparations, determination of drugs in biological media, and automation in drug analysis. Contact: C. Van Kerchove, rue Stévinstraat 137, B-1040 Brussels, Belgium; (02) 230 26 85, Ext. 33, or F. Rey, Chaussée de Waterloo 1375, B-1180 Brussels, Belgium, (02) 375 16 48.

June 1986: AOAC Midwest Regional Section Meeting, Lincoln, NE. Contact: Thomas Jensen, Nebraska Dept of Agriculture, 3703 S 14th St, Lincoln, NE 68502, USA, 402/471-2176.

July 20–26, 1986: The Analytical Division of the Royal Society of Chemistry and The Spectroscopy Group of the Institute of Physics will host the SAC 86 International Conference on Analytical Chemistry and the 3rd Biennial National Atomic Spectroscopy Symposium (BNASS), University of Bristol, Bristol, London. Invited and contributed papers will cover the entire field of analytical chemistry and all aspects of atomic spectroscopy. The program also includes workshops, update courses, exhibits, and a full range of social activities. Contact: P. E. Hutchinson, Analytical Division, Royal Society of Chemistry, Burlington House, London, W1V OBN, UK.

October 12–16, 1986: 100th Annual AOAC International Meeting, Shoreham Hotel, Washington, DC. Contact: Margaret Ridgell, AOAC, 1111 North 19th Street, Suite 210, Arlington, VA 22209, USA, 703/522-3032.

New Private Sustaining Member

AOAC welcomes a new private sustaining member to the growing list of firms aware of the need to support an independent validation association: Ross Laboratories, Columbus, OH.

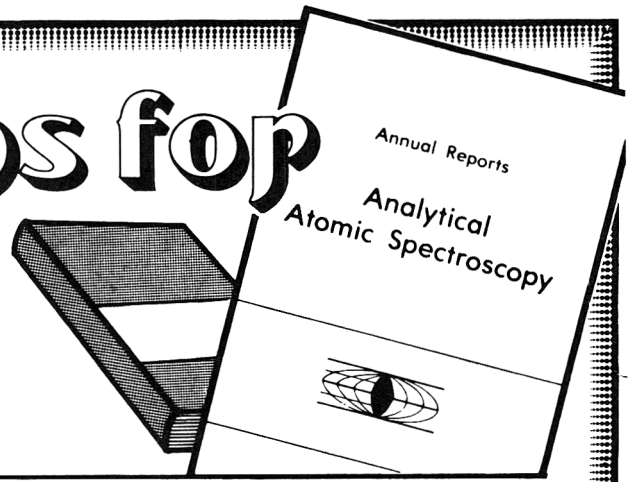
Corrections

J. Assoc. Off. Anal. Chem. (1984) **67,** 1040–1043

"Determination of Histamine in Fish by Liquid Chromatography with Post-Column Reaction and Fluorometric Detection," by Milda J. Walters, under *Experimental,* Reagent **(f),**

Replace 500 mL with 50 mL in the sentence, "Dissolve 500 mg OPA in 500 mL methanol **(a)**. . . ."

Publications for Analytical Chemists



Annual Reports on Analytical Atomic Spectroscopy Vol. 13

Edited by L.Ebdon and K.W.Jackson

This publication reports on current developments in all branches of analytical atomic emission, absorption and fluorescence spectroscopy with reference to papers published and lectures presented during 1983. Much of the information is in tabular form for ease of reference.

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

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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INSTRUCTIONS TO AUTHORS

Scope of Articles

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

Preparation of Manuscript

Authors must submit 3 copies of the complete manuscript, including tables and illustrations, to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. 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.

Style and Format

The text should be written in clear, concise, grammatical English. Unusual abbreviations should be employed as little as possible and must always be defined the first time they appear. Titles of articles should be specific and descriptive. Full first names, middle initial (if any), and last names of authors should be given. The address of the institution (including zip code) from which the paper is submitted should be given and should be in a form to which inquiries, proofs, and requests for reprints can be sent. Information supplementing the title and names and addresses should be given as footnotes.

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

Abstracts: Each manuscript should be accompanied by a concise abstract (not more than 200 words). The abstract should provide specific information rather than generalized statements.

Introduction: Each article should include a statement on why the work was done, the previous work done, and the use of the compound being studied.

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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References to journal articles must include the following information: last names and at least one initial of *all* authors (*not* just the senior author); year of publication, enclosed in parentheses; title of journal, abbreviated according to accepted *Chemical Abstracts* style; volume number; numbers of first and last pages. References to books, bulletins, pamphlets, etc. must include the following information: last names and initials of authors or editors; year of publication, enclosed in parentheses; full title of book; volume number or edition (unless it is the first edition); publisher; city of publication; numbers of pertinent pages, chapter, or section. Citation to private communications or unpublished data should be included in the text, *not* in the list of references.

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

Rev. 1/84

Comparison of Liquid Chromatographic and Bioassay Procedures for Determining Depletion of Intramuscularly Injected Tylosin

WILLIAM A. MOATS, ELMER W. HARRIS,[1] and NORMAN C. STEELE[2]
U.S. Department of Agriculture, Meat Science Research Laboratory, Animal Science Institute, Beltsville Agricultural Research Center, Beltsville, MD 20705

Crossbred pigs weighing 80–110 kg were injected intramuscularly in the ham with 8.8 mg/kg tylosin. Animals were slaughtered in groups of 3 at intervals of 4 h, and 1, 2, 4, and 8 days after injection, and samples of blood, injected muscle, uninjected muscle, liver, and kidney were analyzed by liquid chromatography (LC) and by bioassay using *Sarcina lutea* as the test organism. The LC method was far more sensitive with a detection limit of < 0.1 ppm, while the detection limit by bioassay was about 0.5 ppm in tissue. Results by bioassay and LC sometimes differed considerably for tissue samples. Residues in all tissues were below the tolerance limit of 0.2 ppm at 24 h, except in the injected muscle in one animal. Residues were not detected in any tissue of any animal at 48 h after treatment.

There is little published data on the time required for depletion of the antibacterial agent tylosin from tissues of animals treated intramuscularly with therapeutic doses. Nouws and Ziv (1) reported that tylosin residues were undetectable in meat of emergency slaughtered cattle 24 h after intramuscular treatment, but that kidney and liver samples were positive 31 h after treatment. Van Duyn and Folkerts (2) found appreciable residues in lung tissue of treated calves after 48 h, but did not report results for longer holding times. Nogawa et al. (3) found that, after intramuscular injection of 62.5 mg tylosin into broiler chickens, residues were detectable in injection site, uninjected muscle, liver, and kidney for 25, 3, 6, and 5 days, respectively. Kline and Waitt (4) found that feeding tylosin to swine did not result in detectable levels in tissues unless recommended levels were greatly exceeded. All reports used microbiological assays to determine tylosin residues.

U.S. Government regulations (5) require holding times before slaughter of 14 days for swine and 21 days for cattle after intramuscular injection of tylosin at recommended dose levels.

The standard bioassay procedures used for tylosin are barely adequate to detect tylosin at the official tolerance unit of 0.2 ppm in tissues. Moats (6) recently described a method using liquid chromatography which is more sensitive and specific than bioassays for tylosin. The present study was undertaken to obtain data on depletion of tylosin from treated animals and to compare the new LC method with bioassays for determination of incurred residues.

METHOD

Apparatus and Reagents

(a) *Glassware.*—250 mL side-arm flasks, 15 mL graduated conical centrifuge tubes, separatory funnels, 50 mL graduated cylinders and funnels. Glassware was critically cleaned in special detergents and rinsed with 1% HCl and distilled water before use.

(b) *Liquid chromatograph.*—Varian Model 5000 liquid chromatograph; Varian Automatic loop injector with 200 μL loop; Varian UV-50 variable wavelength detector set at 280 nm; Varian Micropak MCH-10-N-Cap column, 30 cm × 4.6 mm id (Varian Instrument Group, Palo Alto, CA 94303).

(c) *Bioassay medium.*—Antibiotic Medium 11 (BBL Microbiology Systems, Cockeysville, MD 21030).

(d) *Bioassay plates.*—Lab-Line bioassay plates (Lab-Line Instruments, Inc., Melrose Park, IL 60160).

(e) *Solvents.*—Acetonitrile, UV grade and residue analysis grade (Omnisolv, MCB Reagents, EM Science, Gibbstown, NJ 08027), or equivalent; methanol, petroleum ether, and methylene chloride, residue analysis grade; other chemicals, reagent grade.

(f) *Tylosin analytical standard.*—Free base (1035 μg/mg) (gift from Elanco Div., Eli Lilly and Co., Greenfield, IN 46140); tylosin tartrate (910 μg/mg) (Sigma Chemical Co., St. Louis, MO 63178).

Sample Preparation

Tissue Preparation.—Cut ca 25 g tissue into small pieces and blend 2 min with 3 volumes water for muscle or 3 volumes 0.2M, pH 2.2 phosphate buffer for liver and kidney at room temperature.

Deproteinization.—Tissue—Add slowly and with vigorous swirling 32 mL acetonitrile to 8 mL tissue homogenate in 125 mL conical flask. Let mixture stand 1 min, decant supernate through plug of glass wool in stem of funnel, and collect one-half volume (20 mL) of filtrate, equivalent to 1 g tissue.

Blood serum—Add 30 mL acetonitrile to 10 mL blood serum, as described for tissue homogenates, and collect one-half volume (20 mL) of filtrate, equivalent to 5 mL serum.

Cleanup.—Transfer each filtrate from blood serum and tissues to separatory funnel, add 20 mL water and 30 mL CH_2Cl_2, and shake mixture vigorously. Collect CH_2Cl_2 in 250 mL glass-stopper, side-arm flask. Extract aqueous layer with additional 30 mL CH_2Cl_2. Evaporate combined CH_2Cl_2 extracts to near dryness in side-arm flask, under reduced pressure, in 40–50°C water bath. Rinse residue in flask into 15 mL conical centrifuge tube, graduated to 0.1 mL, with two 3 mL portions of CH_3OH. Evaporate CH_3OH to dryness, under reduced pressure, using rotary evaporator. Take care to ensure that liquid in tube does not contact plastic tube holder to prevent contamination of sample. Take up residue in 1 mL acetonitrile with drop of water and 3 mL petroleum ether (30–60°C), and mix tube 10 s on vortex-mixer. After layers separate, carefully remove petroleum ether layer with pipet and discard. Wash acetonitrile layer again by vortex-mixing with 3 mL

[1]Food Safety and Inspection Service.
[2]Nonruminant Animal Nutrition Laboratory.
Mention of trade name, proprietary product, or vendor does not constitute a guarantee or warranty of product by USDA and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Table 1. Recoveries of tylosin, from spiked pork samples analyzed by LC[a]

Sample	Amount added, ppm	Amount found, ppm[b]			Mean % rec. ± SD (n)
Muscle	1.00	0.98	1.07	0.88	
	2.00	2.00	1.64	2.00	96.8 ± 8.1 (9)
	5.00	5.00	5.00	4.80	
Kidney	1.00	0.67	0.90	0.67	
	1.00	0.83	0.89	0.80	
	2.00	1.80	1.84	—	82.8 ± 8.5 (11)
	2.50	2.25	—	—	
	5.00	4.05	4.10	—	
Liver	0.50	0.41	0.48	0.44	
	0.50	0.51	—	—	
	1.00	0.80	0.78	0.94	88.8 ± 8.2 (9)
	1.00	0.97	0.82	—	
Blood serum	0.20	0.17	0.23	—	
	0.50	0.45	0.50	0.50	96.5 ± 9.1 (8)
	1.00	0.96	0.86	1.00	

[a]Tissue was obtained from animals with no prior history of tylosin exposure.
[b]Indicates replicate analysis of samples spiked with tylosin concentrations listed.

petroleum ether, evaporate residual petroleum ether under reduced pressure, and dilute final volume to 0.2–1.0 mL with acetonitrile.

Liquid Chromatography

Run sample blank and tylosin standard using 0.005M $NH_4H_2PO_4$–acetonitrile–CH_3OH (10 + 60 + 30) as mobile phase to determine if any interferences elute near retention time of tylosin. If so, increase the proportion of acetonitrile in mobile phase until tylosin is separated from interferences. Use the following mobile phases for different samples: tissue, 0.005M $NH_4H_2PO_4$–acetonitrile–CH_3OH (10 + 60 + 30, 5 + 65 + 30, and 5 + 70 + 25) and 0.004M $NH_4H_2PO_4$–acetonitrile–CH_3OH (8 + 72 + 20); blood serum, 0.002M $NH_4H_2PO_4$–acetonitrile–CH_3OH (5 + 75 + 20 or + 7 + 75 + 18). Flow rates are 1.5–2.0 mL/min.

Quantitation is based on peak height, which is linear with tylosin concentration up to 10 μg. Typical chromatograms are shown in Ref. 6

Bioassay

Antibiotic medium (f) is used with *Sarcina lutea* (ATCC 9341a) as test organism. Place 0.2 mL test solution in wells of bioassay plates, and incubate 16–18 h at 29°C before reading zones of inhibition.

Recovery Study

Add indicated concentration of tylosin tartrate (Table 1) to serum or tissue homogenates from stock solutions, and carry samples through LC and bioassay procedures as described above. Recovery values are based on comparison with stock solutions.

Swine Treatment Protocol

Twenty-one crossbred pigs weighing 80–110 kg were used. Fifteen of these were weighed and injected intramuscularly in the ham with 8.8 mg/kg tylosin analytical standard, the recommended therapeutic dosage. Pigs were slaughtered in groups of 3 at intervals after treatment of 4 h, and 1, 2, 4, and 8 days. Pigs were fed and watered ad libitum before and after tylosin treatment. Samples were taken for blood, injected muscle, contralateral uninjected muscle, liver, and kidney. These were immediately frozen and held frozen (−20°C) until they could be analyzed. Three control pigs were slaughtered at the beginning of the experiment and 3 more at the end. For analysis, portions of frozen tissue were removed and homogenized as indicated, and aliquots of homogenate were analyzed by both LC and bioassay.

Results and Discussion

The LC procedure developed by Moats (6) for analysis of tylosin in tissues was originally evaluated using beef muscle, kidney, and blood serum, and required some slight modifications for pork. The extraction procedure, in which tissue was blended with water and the homogenate was treated with 4 volumes of acetonitrile to precipitate proteins and lipids, was satisfactory for muscle; however, recoveries of tylosin from spiked kidney homogenates were low and erratic, and tylosin could not be recovered at all from spiked liver homogenates by this method. Recovery of tylosin from kidney and liver homogenates was improved when the pH of the homogenate was lowered. Recoveries were satisfactory after blending with pH 2.2 buffer, which gave a final pH of about 3.4 for the tissue homogenates. No significant degradation of tylosin should occur at this pH if the blended samples are not held a long time (> 1 h). Tylosin was partitioned into CH_2Cl_2 from the acetonitrile filtrates, and little further cleanup other than concentrating the filtrates was required.

An end-capped, C_{18} reverse phase column was used for analysis; both 10 μm and 5 μm particle sizes were tested. The 5 μm particle size column gave little improvement in resolution over the 10 μm particle size. The 10 μm particle size columns were generally rugged and trouble free, and high back pressures were not a problem.

The mobile phase selected was a ternary solvent system, using 0.002–0.005M $NH_4H_2PO_4$–acetonitrile–methanol. No single ratio of the 3 components was ideal for all sample types because (a) the retention time of tylosin changed slowly as the column was used, and (b) different types of samples contained different interfering peaks. The composition of the mobile phase was adjusted to compensate for these factors. The retention time of tylosin was generally affected more by changes in mobile phase composition than were the retention times of interfering peaks. Thus, depending on the type of

Table 2. Tylosin residues in muscle tissue of swine injected intramuscularly and analyzed by LC and bioassay procedures

Animal No.	Time after treatment, h	Tylosin residues, ppm			
		Sample 1		Sample 2	
		LC[a]	Bioassay[b]	LC	Bioassay
Treated Muscle					
204	4	92	24	58	26
205	4	27	17	46	9
206	4	58	32	52	32
207	24	0.27	NI[c]	0.05	NI
208	24	0	NI	0.09	NI
209	24	7.3	7.0	8.1	5.0
210	48	0	NI	—[d]	—
211	48	0	NI	—	—
212	48	0	NI	—	—
Contralateral Muscle					
204	4	1.5	NI	1.3	NI
205	4	1.3	0.92	0.65	0.68
206	4	1.3	NI	0.56	NI
207	24	0	NI	0	NI
208	24	0	NI	0	NI
209	24	0	NI	0	NI

[a]Mean of duplicate determinations.
[b]Bioassay using *Sarcina lutea*.
[c]NI = No inhibition of test organism.
[d]— = Not determined.

Table 3. Tylosin residues in liver, kidney, and blood serum of intramuscularly injected swine; samples analyzed by LC and bioassay procedures

Animal No.	Time after treatment, h	Tylosin residues, ppm					
		Blood serum		Liver[a]		Kidney[a]	
		LC[b]	Bioassay[c]	LC	Bioassay	LC	Bioassay
204	4	0.74	0.69	0.35	NI	0.53	NI
205	4	0.69	1.20	0.47	NI	0.53	NI
206	4	0.46	0.69	0.28	NI	0.59	NI
207	24	0.06	NI[d]	0	NI	0	NI
208	24	0	NI	0	NI	0	NI
209	24	0.06	NI	0	NI	0	NI
210	48	0	NI	—[e]	—	—	—
211	48	0	NI	—	—	—	—
212	48	0	NI	—	—	—	—

[a]Samples stored 2 months at −20°C before analysis.
[b]Mean of duplicate determinations.
[c]Bioassay using *Sarcina lutea*.
[d]NI = No inhibition of test organism.
[e]— = Not determined.

Table 4. Tylosin residues in liver and kidney tissue after frozen storage and determined by bioassay procedure[a]

Animal No.	Tylosin residues (ppm) after frozen storage (ca −20°C)			
	Liver		Kidney	
	7 days	60 days	45 days	75 days
204	1.6	NI[b]	3.13	NI
205	NI	NI	2.2	NI
206	NI	NI	0.92	NI

[a]All samples taken 4 h after treatment.
[b]NI = No inhibition of test organism.

sample, it was possible to select a mobile phase composition which would separate tylosin from interfering peaks in the corresponding sample blank. The retention time of tylosin could be increased by increasing the proportion of acetonitrile in the mobile phase and by decreasing the $NH_4H_2PO_4$ concentration in the aqueous phase. The retention time was shortened as the pH was increased toward 7 and was lengthened at more acid pH. The $NH_4H_2PO_2$ solution was pH 4.6. Flow rates of 2 mL/min gave sharper peaks, but slightly reduced sensitivity compared with that at lower flow rates. Sensitivity was reduced as retention time increased. It was therefore desirable to select the shortest retention time which gave adequate separation of tylosin from interferences in sample extracts. When the solvent contained more than 70% acetonitrile, it was necessary to reduce the buffer salt concentration to prevent precipitation of the buffer salt. The column showed a lag in responding to changes in solvent composition, so that solvent gradients were ineffective.

In addition to tylosin (Tylosin A), commercial tylosin contains small but significant amounts of related compounds, including desmycosin (Tylosin B), macrocin (Tylosin C), and relomycin (Tylosin D) (7). With the mobile phases used, a tylosin analytical standard will chromatograph as a single peak, sometimes with a small shoulder which eluted before or after the main tylosin peak, depending on mobile phase composition (6). Similar results were obtained with a commercial tylosin tartrate. For residue analysis, it is probably advantageous to have minor components elute with the main tylosin peak, because detection of minor components individually would be difficult. Mobile phases capable of resolving minor components from tylosin A did not give adequate separation from interferences in sample extracts. Desmycosin, formed by allowing tylosin to stand overnight under acid conditions, was separated from tylosin, but desmycosin was not detected in tylosin standards under the conditions used.

Table 1 summarizes recoveries from spiked pork samples determined by LC. Pork tissues and blood serum contained somewhat higher levels of interfering substances than beef. The recovery data were based on a commercial tylosin tartrate. Recoveries from spiked muscle (96.8%) and blood serum (96.5%) samples were somewhat better than those from liver (88.8%) and kidney (82.8%). Reproducibility was similar for all types of samples. Errors of measurement were greater for samples spiked at lower concentrations.

Tables 2 and 3 summarize results of residue analysis of treated pigs. For tissue samples, a portion of the 3:1 tissue homogenate was used for bioassay and another portion carried through the cleanup for LC to ensure that the results of the 2 procedures were based on the same samples at the same time. In injected muscle (Table 2), tylosin levels were quite high 4 h after treatment as might be expected. At 24 h after treatment, levels of tylosin in the injected muscle were quite low in 2 pigs, but were still appreciable by both bioassay and LC in one animal. No residues were detectable by either procedure in the injected muscle 48 h after treatment. In the contralateral uninjected muscle, tylosin was not detected by bioassay in muscle of 2 of 3 pigs 4 h after treatment, although residues as high as 1.5 ppm were found by the LC procedure in the muscle tissue 4 h after treatment. The uninjected muscle was negative by all procedures 24 h after treatment. Levels of tylosin found in muscle were consistently higher by LC than by bioassay.

In blood serum (Table 3), bioassay results were somewhat higher than those obtained by LC 4 h after treatment. At 24 h after treatment, traces of tylosin were found by LC in the blood serum of 2 out of 3 pigs. Tests were negative 48 h after treatment.

Results in Table 3 from liver and kidney were obtained after storing tissues frozen (−20°C) up to 2 months, while the LC procedure was modified to give consistent recoveries from spiked samples. By this time, whatever residues may have been present initially has decreased below the limit of detection by bioassay. Appreciable levels were still found by LC in samples taken 4 h after treatment, but not 24 h after treatment.

Table 4 shows results of bioassays run after frozen storage (−20°C) of these tissues. Appreciable levels were present in kidney after 45 days, but they were undetectable after 75 days. Detectable residues were found in liver of only one animal after 7 days of storage. The validity of bioassays on

water homogenates of liver is questionable. Because tylosin cannot be recovered by acetonitrile extraction from water homogenate, it may also be bound in a form which cannot be detected by bioassay. It would clearly be desirable to prepare liver and kidney extracts immediately after slaughter of the animals to obtain reliable data, unless residues in these tissues can somehow be stabilized during frozen storage. Based on the results of the present study, residue analyses on stored liver and kidney appear somewhat unreliable.

There are no published reports on depletion of tylosin injected intramuscularly in pigs. Recent regulations of the U.S. Food and Drug Administration require a holding time of 14 days before slaughter for pigs injected intramuscularly with tylosin and 21 days for cattle (5). Our results show that at recommended therapeutic dosages, holding swine for 48 h after treatment is ample to ensure that tissues are completely free of residues, and except in one case, residues were within tolerance levels after 24 h.

As compared with studies on other species, our results show more rapid depletion in swine than has been reported for cattle (1, 2) and chickens (3).

The LC procedure was shown to be more sensitive and specific for tylosin than bioassays. Recoveries of tylosin from tissue samples were usually much higher with the LC procedure following tissue extraction with acetonitrile than those obtained by direct bioassay on tissue homogenates. The sensitivity of the bioassay procedure could probably be improved by first extracting tissues with acetonitrile. Better methods of storing tissue with suspected tylosin residues before analysis are needed.

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ANTIBIOTICS

Liquid Chromatographic Determination of Narasin in Feed Premixes

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A liquid chromatographic (LC) method has been developed to determine narasin in feed premixes. Narasin is extracted from the premix with a methanol–water solvent, and the extracted solution is assayed by using LC. Recovery of narasin from a 12.5 g/lb premix is quantitative (100%), with a relative standard deviation of 1.44%. The results correlated well (coefficient 0.92) with a turbimetric bioassay method.

Narasin ($C_{43}H_{72}O_{11}$), a polyether antibiotic (1–4) produced by the organism *Streptomyces aureofaciens* NRRL 5758, is used to improve feed efficiency and control coccidiosis in poultry (5). It is incorporated into feeds at a concentration of 60–80 ppm, using a premix of 10% w/w. The compound can be determined turbimetrically (6) and, with less sensitivity, colorimetrically with a vanillin reaction. Thin layer and paper chromatographic and electrophoretic methods have already been reported (1, 6, 7). The paper chromatographic method, using bioautographic detection, can be used as a specific and sensitive test for narasin in premix and feed, but is only semiquantitative and requires a long period of time to analyze one sample. As reported previously, *S. aureofaciens* produces an antibiotic complex consisting of factors A (narasin), B, and D, with 99% of the antibiotic activity being attributed to narasin. Narasin itself has a weak nonmaximum ultraviolet (UV) absorptivity in the range of 200 to 220 nm. Thus, although some information can be derived using a UV detector at 214 nm, refractive index was finally chosen as the best means of detection when coupled with liquid chromatography. With the proper choice of column and mobile phase, there is adequate selectivity to allow a specific determination of narasin in premix samples.

METHOD

Reagents and Apparatus

(a) *Extraction solvent.*—Mix 900 mL CH_3OH, reagent grade, and 100 mL deionized, filtered water.

(b) *Methanol.*—Neutralized; to 1000 mL CH_3OH, add 1 g of $NaHCO_3$, stir well, and filter.

(c) *Mobile phase.*—Mix 850 mL CH_3CN, LC grade, and 150 mL dilute H_3PO_4 (10 mL concd H_3PO_4 added to 990 mL LC grade water). With constant stirring, slowly adjust apparent pH of mobile phase to 2.9 with concd NH_4OH. Degas solution before use.

(d) *Liquid chromatograph.*—Waters Model 6000A pump or other nearly pulse-free pump, and a Waters Model 401 refractive index detector (Waters Associates, Milford, MA 01757), or equivalent; column is Du Pont C8 30 cm × 4 mm id prepacked (Du Pont Co., Wilmington, DE 19898), or equivalent (Regis C6 25 cm × 4.6 mm id column with mobile phase of 65% CH_3CN–35% pH 2.9 dilute H_3PO_4 also provides excellent resolution); injector is Micromeritics Model 725 Autoinjector (Micromeritics Instrument Corp., Norcross, GA 30093); and recorder is Recordall Series 5000. Operating conditions: flow rate 1.5 mL/min, 50 μL loop, and ambient temperature for column.

(e) *Narasin reference standard solution.*—Accurately weigh target weight of ca 50 mg narasin reference standard (Eli Lilly and Co. Research Laboratories, Greenfield, IN 46140) into 25 mL volumetric flask. Add 6.25 mL neutralized methanol reagent and stir to dissolve. Dilute to volume with extraction solvent.

Preparation of Sample

Depending on sample activity, sample weight can be varied; however, for weakly active sample, weigh 5 g (ca 500 mg narasin) narasin premix into 100 mL volumetric flask. Add 75 mL methanol–water (90 + 10, v/v), and extract narasin by letting mixture stand 1 h. Swirl flask periodically to ensure complete extraction. Dilute to volume with pure CH_3OH. Dilute aliquot of this solution with methanol–water (90 + 10, v/v) to concentration of ca 2 mg/mL, and pour small portion through 0.45 μm filter before injection into LC system.

Determination

Use solutions of premix sample and standard for LC injections. Quantitate premix sample by comparing detector response *(PR)* of sample to that of 2.0 mg/mL standard narasin solution (e) *(PR')*. Use either peak height or peak area for quantitation. Determine premix concentration with formula:

$$\text{mg activity/g} = PR/PR' \times 2.0\ \text{mg/mL} \times 100\ \text{mL/g sample} \times AF$$

where *AF* represents aliquot factor if second dilution of sample is required, and 2.0 mg/mL represents target standard concentration or equivalent, accurately weighed.

Results and Discussion

When a narasin standard solution is diluted to 2 mg/mL in methanol–water (90 + 10, v/v), an essentially single, sharp peak with a retention time of approximately 11.5 min is obtained. When extraction is performed on a narasin premix formulation, using this same solvent mixture, the same peak is obtained with good resolution and without interference. Figure 1 shows a typical chromatogram of narasin standard and premix samples.

Instrument response was linear over the range 12.5–200 μg injected, under conditions of the method. The method was studied for premix ingredient interference, and a recovery experiment indicated 100% recovery of a spike. In addition, the methodology was studied for interference when narasin is formulated with nicarbazin. No nicarbazin peak appears when nicarbazin is injected alone, and no peak shape or recovery problems occur when nicarbazin is injected mixed with narasin. The method will also resolve salinomycin, lasalocid, and monensin (structurally similar antibiotics). Figure 2 shows a chromatogram of these 3 antibiotics mixed in solution with narasin.

Two premix samples, one with soybean mill run and the other with clay were assayed in triplicate each day for 3 days to observe sample-to-sample and day-to-day variation. The

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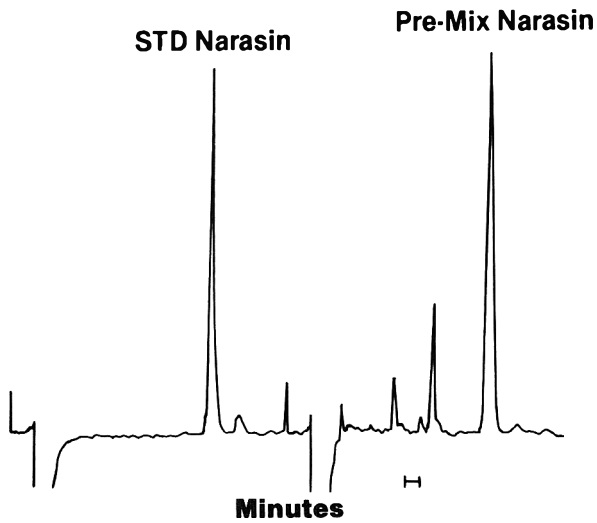

Figure 1. Typical LC chromatogram for narasin standard and premix samples; peak response is typical for 100 μg injection, and retention times are relative to bar indicating 1 min.

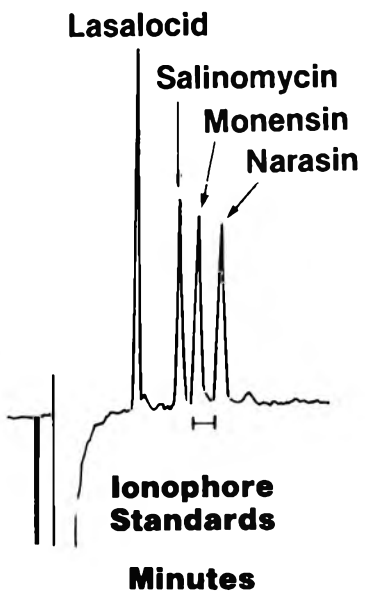

Figure 2. Chromatogram demonstrating resolution of 3 ionophores: salinomycin, monensin, and narasin; retention times are relative to bar indicating 1 min.

relative standard deviation (CV) was 1.44% on the first formulation, and 3.53% on the second formulation. The higher standard deviation obtained for the second premix formulation resulted from blending difficulties experienced at that time, not extraction difficulties. Table 1 shows data obtained for 9 sets of assays for each premix type. Premix samples chosen for evaluation were at the lowest concentration practical from a feed standpoint, 2.75% w/w. If higher premix concentrations had been chosen they would have been easier to assay because of higher response to background ratio.

Table 1. Assay reproducibility for 2 types of 12.5 g/lb narasin premix samples

Sample	Replicates of narasin, g/lb		
	Day 1	Day 2	Day 3
Soybean Mill Run			
1	12.5	12.5	13.0
2	12.4	12.4	12.6
3	12.5	12.6	12.5
Overall Av. 12.6 ± 0.18 (CV = 1.44%)			
Agsorb Clay			
1	12.9	13.1	13.8
2	13.5	12.7	13.8
3	12.5	12.9	13.2
Overall Av. 13.2 ± 0.46 (CV = 3.5%)			

Twelve different premix samples of the first formulation type, each containing different concentrations of narasin (20–42 g/lb), were assayed by using the in-house turbimetric microbiological assay procedure and by using LC. The turbimetric procedure uses the Lilly Autoturb® system, a device that automates pipetting, dilution, and measurement of the culture turbidity (8). A correlation coefficient of 0.92 was found between the LC and turbimetric results. The microbiological method can measure minor factors when present, so exact correlation was not expected. The premix samples chosen for comparative assay data were experimental samples formulated at varying concentrations.

In summary, the LC method has been shown to be simple and faster than biological methods, offers good specificity, and correlates adequately with automated bioturbimetric methods. The method is accurate (100% recovery) and precise (1.44% CV), and other polyether antibiotics do not interfere.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Gas Chromatographic Determination of Pentachlorophenol in Gelatin: Collaborative Study

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Eleven collaborators participated in this study of a gas chromatographic method for the determination of pentachlorophenol (PCP) in gelatin. Following acid hydrolysis of a 2 g sample, PCP is extracted with hexane and partitioned into KOH solution. After reacidification, PCP is again extracted with hexane for determination by electron capture gas chromatography on a 1% SP-1240DA column. Three duplicate practice samples (0.0, 0.5, and 1.5 ppm) and 5 blind duplicate collaborative samples (0.0, 0.02, 0.1, 0.5, and 2.0 ppm) were analyzed by each collaborator. Mean recoveries of PCP in the collaborative samples ranged from 88% at the 0.02 ppm fortification level to 102% at the 0.1 ppm level; the overall mean recovery was 96%. Interlaboratory coefficients of variation ranged from 16.4% for the 0.1 ppm fortification level to 22.9% for the 0.5 ppm level; the overall interlaboratory coefficient of variation was 19.5%. The method has been adopted official first action.

Pentachlorophenol (PCP) is used as a fungicide to protect animal hides from mold and fungal growth. If PCP-treated hides are diverted to gelatin production, PCP may appear as a contaminant in gelatin. Because the major use of gelatin in the United States is in food products (1), an analytical method (2) was developed to determine PCP in gelatin.

The developed method is an extension of a procedure originally intended for the determination of bioincurred residues of PCP in milk and blood of dairy cattle (3). The method uses acid and heat to hydrolyze the gelatin, base partition for separation and cleanup, and reacidification and extraction with hexane, which are followed by direct analysis using electron capture gas chromatography (GC).

On the basis of results from an intralaboratory study, the method was recommended for collaborative study. This report presents the results of the collaborative study in which 10 samples were analyzed by each of 11 collaborators.

Collaborative Study

Sample preparation.—Four packages of gelatin were purchased locally and opened, and the contents were thoroughly mixed in a large flask. The fortified practice and collaborative samples were prepared by pipetting 1 mL aliquots of hexane solutions containing known amounts of PCP into 50 mL Teflon-lined, screw-cap test tubes. After the solvent was carefully evaporated with an air jet, 2 g gelatin was added to each tube, the tubes were capped, and the contents were mixed.

Collaborative materials.—The collaborators were asked to analyze 10 samples with the method developed by Borsetti and Thurston (2). To minimize mechanical errors, much of the sample preparation was done by the Associate Referee. Collaborators were not required to weigh out standards or samples or to make fortifications. Each collaborator received the following materials: *(1) Vial containing PCP standard stock solution.*—1 μg PCP/mL hexane. The glass vial was sealed with a Teflon-lined rubber washer and crimped with an aluminum cap. The stock solution was to be diluted according to instructions for preparation of working standard solutions. The weight of each vial was recorded, and collaborators were instructed to reweigh the vials on receipt and just before use. Vials losing more than 50 mg were replaced. *(2) Six practice samples.*—Fortified in duplicate with 0.0, 0.5, and 1.5 ppm PCP. These samples were to be analyzed before the collaborative samples to provide familiarization with the method and a check on the GC system. Only if recoveries were in the 80–110% range were the collaborators to proceed with the study. *(3) Ten collaborative samples.*—Five pairs of blind duplicates fortified with 0.0, 0.02, 0.1, 0.5, and 2.0 ppm PCP. *(4) Analytical method. (5) Reporting forms.*—One for each sample for recording GC parameters, volumes and weights of samples/standards injected, and calculations of ppm PCP.

Collaborators were instructed to place all sample materials in a freezer if storage was needed, and most collaborators found it necessary to do so.

Pentachlorophenol in Gelatin Gas Chromatographic Method First Action

Principle

Sample is acid-hydrolyzed and PCP is extd with hexane, partitioned into KOH soln, acidified, and extd into hexane. Compd is sepd and detd by gas chromatgy with electron capture detection.

Apparatus

(a) *Test tubes.*—25 × 150 mm with Teflon-lined screw caps (Arthur H. Thomas Co.).

(b) *Disposable pipets.*—Pasteur type, 23 cm long (Arthur H. Thomas Co.).

(c) *Centrifuge.*—IEC Model UV with head for spinning 25 × 150 mm test tubes at 1000 × *g* (International Equipment Co., Needham Heights, MA 02194).

(d) *Shaker.*—(Optional). Shaker-in-the-Round Model S-500 (Kraft Apparatus Co., Mineola, NY 11501).

(e) *Gas chromatograph.*—Equipped with ^{63}Ni electron capture detector. Conditions: column oven 180°; injector 250°; detector 350°; 5% CH_4 in Ar carrier gas flow 60 mL/min. Adjust electrometer setting to obtain 0.5 FSD from 0.1 ng PCP (retention time ca 10 min).

(f) *Chromatographic column.*—1.8 m × 4 mm id glass column contg 1% SP-1240DA on 100–120 mesh Supelcoport (Supelco Inc.). Place small plug (2–3 mm) acid-washed glass wool in detector end of column (silanized glass wool may be substituted; however, peaks will be slightly broader). Install packed column for on-column injection. Condition by purging with carrier gas at ambient temp. for 10–15 min. Program

This report of the Associate Referee was presented at the 97th Annual International Meeting of the AOAC, October 3–6, 1983, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee E and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.

Submitted for adoption June 20, 1984.

Table 1. Collaborative results (ppm)[a] for determination of pentachlorophenol in gelatin by gas chromatography

	Fortification level, ppm									
Coll.	0.0	0.0	0.02	0.02	0.1	0.1	0.5	0.5	2.0	2.0
A	0.004	0.007	0.019 (96)	0.021 (105)	0.090 (90)	0.099 (99)	0.428 (86)	0.462 (92)	1.76 (88)	0.751[b] (38)
B	0.004	0.003	0.019 (96)	0.021 (105)	0.105 (105)	0.109 (109)	0.576 (115)	0.537 (107)	2.01 (101)	1.98 (99)
C	0.008	0.007	0.011 (55)	0.016 (80)	0.091 (91)	0.101 (101)	0.474 (95)	0.530 (106)	1.74 (87)	1.95 (98)
D	0.003	0.003	0.020 (100)	0.020 (100)	0.101 (101)	0.102 (102)	0.499 (100)	0.483 (97)	2.03 (102)	2.02 (101)
E	0.004	0.005	0.016 (80)	0.014 (70)	0.092 (92)	0.103 (103)	0.380 (76)	0.450 (90)	1.31 (66)	1.78 (89)
F	0.006	0.004	0.034[b] (170)	0.038[b] (190)	0.140 (140)	0.105 (105)	0.475 (95)	0.475 (95)	3.00[b] (150)	2.70 (135)
G	0.009	0.003	0.026[b] (130)	0.027[b] (135)	0.134 (134)	—	0.583 (117)	—	1.20 (60)	—
H	0.009	0.008	0.021 (105)	0.014 (70)	0.075 (75)	0.080 (80)	0.363 (73)	0.272 (54)	1.44 (72)	1.52 (76)
I	0.008	0.008	0.018 (90)	0.017 (85)	0.121 (121)	0.124 (124)	0.710 (142)	0.705 (141)	2.29 (115)	2.49 (125)
J	—	—	0.018 (90)	0.013 (65)	0.089 (89)	0.084 (84)	—	0.329 (66)	—	1.73 (86)
K	0	0	0.019 (95)	0.021 (105)	0.108 (108)	0.106 (106)	0.556 (111)	0.546 (109)	1.79 (90)	2.25 (113)
Av. rec., ppm			0.018		0.102		0.492		1.89	
Av. rec., %			88		102		99		95	
Repeatability										
S_o			0.0 3		0.009		0.033		0.194	
CV_o, %			14.4		8.7		6.7		10.3	
Reproducibility										
S_x			0.003		0.017		0.113		0.399	
CV_x, %			17.5		16.4		22.9		21.11	

[a]Values in parentheses are recoveries (%).
[b]Outlier by Dixon test.

from 70 to 190° at 1°/min, holding at 190° for 6–8 h. Lower temp. to 180° for PCP detn. Note: Use only recently prepd and thoroly conditioned column.

Reagents

Store all reagents in ground glass-stopper or Teflon-lined screw-cap containers.

(a) *PCP std solns.*—Dissolve 2.5 mg PCP ref. std (Standard No. 5260, Pesticide Reference Standards Section, Environmental Protection Agency, Research Triangle Park, NC 27711) in 100 mL benzene (pesticide quality). Make appropriate dilns with hexane to give std solns ranging from 0.004 to 0.4 μg PCP/mL.

(b) *Extraction solvent mixture.*—Hexane–isopropanol (pesticide quality) (4 + 1).

(c) *Acid-washed glass wool.*—Phosphoric acid-treated (Supelco).

Extraction and Cleanup

Weigh 2.0 g gelatin into 25 × 150 mm screw-cap test tube and add 10 mL 12N H_2SO_4. Tightly cap and heat 1 h in 100° H_2O bath in fume hood. Remove tube periodically during hydrolysis, wrap in cloth towel, and mix sample by carefully shaking.

Prep. reagent blank with each set of samples.

After 1 h, remove tube, let cool, add 10 mL hexane–isopropanol (4 + 1), and shake by hand or by shaker 2 min. Centrf. 2 min at 1000 × *g* and transfer upper hexane layer to second test tube with Pasteur pipet. Repeat extn and centrfgn 2 addnl times, combining all hexane exts in second test tube. To combined exts, add 5 mL 1.0N KOH, cap, shake 2 min, centrf. as before, remove upper layer with Pasteur pipet, and discard. Add 10 mL hexane, cap, shake, and centrf. as before; remove hexane wash with Pasteur pipet and discard. Add 5 mL 12N H_2SO_4, cap, and mix by carefully swirling tube. Ext 3 times by shaking 2 min each with 5, 2, and 2 mL hexane. After each extn, centrf. as before and transfer exts with Pasteur pipet to 10 mL vol. flask. Adjust to vol. for GC detn.

GC Determination

Before each injection, rinse syringe by pumping 3–5 times with soln to be injected. Inject 5 μL sample soln (equiv. to 1.0 mg sample) into gas chromatograph. Measure area or ht of PCP peak and det. amt of residue by comparison to peak area or ht obtained from injection of known amt of PCP ref. std. To ensure valid measurement of PCP residue, size of PCP peak from sample and std should be within ± 10%. Make dilns as needed. Following each injection, rinse syringe by pumping 5–10 times with hexane. After each injection of sample or std soln, inject 5 μL hexane. Appearance of ghost PCP peaks may be noted following injection of high PCP soln. Repeat injection of solv. until ghost PCP peak becomes negligible. Repeat sample and std injections until consistent responses are obtained.

Correct sample results by subtracting reagent blank. Max. acceptable reagent blank for satisfactory performance of method is 0.01 ppm.

Results and Discussion

Collaborative results are presented in Table 1 for individual analyses of samples fortified with 0.0, 0.02, 0.1, 0.5, and 2.0 ppm PCP. The results from each collaborator have been corrected for the blank value obtained by averaging the collaborator's results for the nonfortified samples. Percent recoveries are shown under the corresponding ppm values.

Collaborator G lost 3 samples because of glassware breakage.

Collaborator J had chromatography problems that included noisy baselines and negative peaks, which may have been caused by too high a sensitivity setting. Results for both unfortified samples were discarded because peak heights for PCP varied widely from injection to injection. Results for one

sample at the 0.5 ppm level and for one at the 2.0 ppm level were discarded because the collaborator stated that he might have interchanged these samples. The results for the remaining samples were corrected for background by using the average of the blank values (0.003 and 0.008 ppm) obtained in the practice set. With the practice samples, this collaborator obtained results for 2 analyses that failed the Dixon test because of low recoveries. Collaborator J also had a problem with ghost peaks. Injection of solvent alone invariably produced a peak equivalent to approximately 0.02 ng PCP.

Collaborator F did well with the practice samples; recoveries ranged from 75 to 114%. This pattern was not reflected in the results for the corresponding collaborative samples, 3 of which were discarded as outliers. Collaborator H obtained lower recoveries (54–88%) with the practice samples, as did collaborator J (46–75%), and the results of both collaborators are in line with their practice sample recoveries. Collaborator G did well with the practice samples; recoveries ranged from 91 to 126%. His collaborative sample findings contained 2 outliers, which were for the analyses of both samples fortified at the 0.02 ppm level.

Statistical findings are also shown in Table 1. Results for 6 analyses were determined to be outliers at the 90% confidence level (Dixon test). These values, representing 5.8% of the samples, were excluded in the final statistical analysis. The overall recovery is 96% at fortification levels of 0.02–2.0 ppm. Individual recoveries ranged from 54% at the 0.5 ppm fortification level to 142% at the same level. Interlaboratory standard deviations (S_x) ranged from 0.003 at the 0.02 ppm level to 0.399 at the 2.0 ppm level. Coefficients of variation for reproducibility (CV_x) ranged from 16.4% at the 0.1 ppm level to 22.9% at the 0.5 ppm level.

The most critical step in the method is the GC analysis. The column substrate, SP-1240DA, did not always provide consistent chromatographic responses for PCP in collaborating laboratories. Two potential collaborators decided that their standard responses were not sufficiently consistent for completion of the study. However, the overall reaction of the collaborators was that the GC system can be used reliably. With this method, the analyst must inject an amount of standard solution that produces a detector response that is about equal to that produced by the injected aliquot of sample extract.

The appearance of ghost peaks is especially noticeable after an injection of a large concentration of PCP onto the 1240DA column. This phenomenon, which has the effect of increasing the apparent PCP concentration in samples containing ≤0.05 ppm PCP, can be minimized by several injections of solvent.

Although GC column dimensions were included in the instructions, the collaborators used a variety of column sizes that included columns of 2 mm id instead of 4 mm id and 4 ft columns instead of 6 ft columns. Some collaborators performed the analyses with columns that had been used for earlier work. As a result, retention times and peak heights/areas varied among the collaborators. This was not considered a problem as long as the collaborators could get consistent responses and low backgrounds for PCP measurement. Retention times obtained by the collaborators ranged from 1.25 to 10.25 min.

Collaborator I was the only analyst to use a tritium detector. Collaborator I used a 4 ft × 2 mm id column, and although the baseline rose constantly after each injection, the chromatograms were suitable for determinations at all concentrations.

Comments and Recommendations

The collaborators had no trouble with the method. However, the comments and problems of the collaborators show that the critical point in the entire method is the GC analysis. To minimize GC problems, the following practices should be adopted: Use a recently prepared GC column and condition it thoroughly; clean the syringe thoroughly before each use; make several injections of sample extract or standard solution if inconsistent responses are observed.

The SP-1240DA column is usable if the analyst allows for some of the weaknesses of the substrate for PCP determination. However, it is recommended that other column substrates be investigated as future replacements.

On the basis of the overall results of the collaborative study, it is recommended that this method be adopted official first action.

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Louis Tollackson, Hazleton Raltech, Inc., Madison, WI

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Analysis of Phenols by Chemical Derivatization. IV. Rapid and Sensitive Method for Analysis of 21 Chlorophenols by Improved Chloroacetylation Procedure[1]

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A quantitative, rapid, sensitive, and isomer-specific method for the analysis of chlorophenols in natural water by in situ chloroacetylation is presented. Without pre-extraction, phenols in water are chloroacetylated by chloroacetic anhydride in the presence of K_2CO_3. Because of differences in reaction kinetics and stability, various chloroacetates were removed from the reaction mixture at different intervals. If analysis of other classes of neutral organic compounds is also required, a more elaborate procedure involving solvent extraction of organic compounds and back-extraction of phenols into K_2CO_3 solution followed by chloroacetylation is proposed. When a 12 m OV-1 fused silica capillary column was used, 22 phenol chloroacetates were easily resolved in a single run. Using distilled and natural water samples, this method has been validated and shown to be applicable over a concentration range of 0.1 to 100 μg/L of the phenols studied.

Many methods are available for the analysis of phenols in various matrices by gas chromatography (GC) (1–7) or liquid chromatography (LC) (8–10). If the analysis of phenols in water involves chemical derivatization, a solvent extraction step is usually specified to isolate phenols and other organic compounds, followed by derivatization before GC analysis. A more elaborate procedure would include base partitioning after solvent extraction to separate phenols and other acidic compounds from other neutral compounds. With such a scheme, the neutral fraction in the same sample could also be analyzed. The phenols now in the aqueous layer are re-extracted into an organic layer before they are derivatized. This extraction-partitioning-re-extraction process is considered too lengthy for routine analysis, especially because each step has to be done 2 to 3 times to ensure quantitative recovery. One approach to shorten the procedure is to derivatize phenols directly in the water sample, such as the case of in situ acetylation of phenols reported earlier (11–14). The elimination of the solvent extraction step for phenols not only saves time but also improves the recovery because the phenol acetates are easier to extract than their parent phenols. Phenol acetates are stable but such derivatives are not sensitive to nonchlorinated and monochloro phenols at low ppb levels.

In 1968, Argauer reported the aqueous chloroacetylation of microgram quantities of various phenols including 6 chlorophenols (15). The derivatives formed were then examined by packed column electron capture gas chromatography. For the analysis of phenols, the chloroacetylation technique is potentially a better method than acetylation (16–20): The chloroacetates are formed more rapidly and they elicit a more sensitive response by electron capture detection. We now wish to report an extended, quantitative, and much refined method for the analysis of 21 chlorophenols in water by the formation of the chloroacetate derivatives (Figure 1).

Experimental

Apparatus and Reagents

(a) *Gas chromatographs.—(1)* Hewlett-Packard Model 5713A equipped with ^{63}Ni electron capture detector, Model 7671A Autosampler, and Model 3390A reporting integrator.

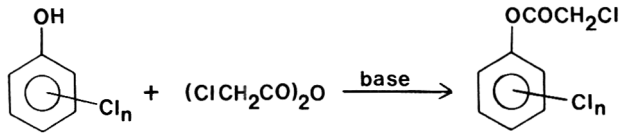

Figure 1. Chloroacetylation of chlorophenols.

Operating conditions: injection port 250°C, detector 300°C, methane–argon carrier gas (5 + 95).

(2) Hewlett-Packard Model 5880A equipped with ^{63}Ni electron-capture detector, Model 7671A Autosampler, level 4 terminals, and split-splitless capillary column injection port. Operating conditions: injection port 250°C, detector 300°C, helium carrier gas, methane–argon detector make-up gas (5 + 95) 25 mL/min.

(b) *Columns.—(1)* 1.8 m × 2 mm id glass, packed with 3% OV-1 on 100–120 mesh Gas-Chrom Q (Chromatographic Specialties Ltd, Brockville, Ontario, Canada K6V 5W1), column temperature 155°C, flow rate 26 mL/min.

(2) 12 m × 0.2 mm id fused silica capillary column, coated with cross-linked dimethyl silicone gum (0.33 μm thickness) and surface deactivated by siloxane (Part No. 19091-60312, Hewlett-Packard), linear velocity 25 cm/s. Column temperatures, initial 70°C, hold for 0.5 min, programming rate 1, 25°/min (70° to 140°C), rate 2, 2°/min (140° to 180°C), final time 5 min. Splitless valve on for 30 s.

(c) *Gas chromatograph-mass spectrometer (GC-MS).*—Finnigan Model 4000 GC-MS-DS operating in EI mode. Column, 25 m × 0.32 mm id OV-1 fused silica capillary column, 0.17 μm film thickness (Hewlett-Packard Part No. 19091-61325). Oven temperatures, initial 70°C, hold for 2 min, programming rate 1, 24°/min (70° to 140°C), rate 2, 2°/min (140° to 180°C). Helium carrier gas at 10 psi.

(d) *Chloroacetic anhydride.*—(Aldrich Chemical Co.). Dissolve anhydride in acetone and dilute to 300 mg/mL acetone. Prepare reagent fresh daily.

For other reagents and chemicals, see ref. 11.

Chloroacetylation of Phenols

Short method.—For 100 mL water sample containing phenols in 250 mL volumetric flask, test pH and adjust to neutral with acid or base if necessary. Add 1.0 g anhydrous K_2CO_3 to sample and stir until complete dissolution. Add 25 mL petroleum ether (30–60°C), pipet in 1.0 mL chloroacetic anhydride reagent, and stir exactly 1 min with top of volumetric flask removed. Caution: Prolonged stirring will cause decomposition of some derivatives (see later discussions). Immediately transfer mixture to 250 mL separatory funnel and separate layers. Drain aqueous (lower) layer to original volumetric flask and organic layer to 250 mL round-bottom flask. Add 25 mL petroleum ether and 1.0 mL chloroacetic anhydride reagent to aqueous layer and repeat extractive chloroacetylation by stirring for 1 min. Repeat layer separation as above. Perform third extractive chloroacetylation on water sample for 8 min with same amounts of reagent and solvent. After 1 min of stirring, replace top of volumetric flask. Discard water sample after third extraction. Dry combined organic layers through anhydrous sodium sulfate. To dry extract, add

[1]For previous applicable reports, see *J. Assoc. Off. Anal. Chem.* **66**, 1029 (1983); **67**, 789 (1984); and **67**, 1086 (1984), respectively.

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Table 1. Retention times, relative retention times (in brackets), and relative ECD response factors for chlorophenol chloroacetates

No. parent phenol	12 m OV-1	3% OV-1	Relative molar response	Relative ECD sensitivity, chloroacetate: acetate
1 Phenol	3.60(1.83)	0.98(0.51)	0.64	>50[a]
2 2-Chloro-	4.75(2.41)	1.62(0.84)	1.1	>50
3 3-Chloro-	5.07(2.57)	1.80(0.93)	1.8	>50
4 4-Chloro-	5.15(2.61)	1.84(0.95)	2.1	>50
5 2-Chloro-5-methyl-	6.04(3.06)	2.42(1.25)	0.70	>50
6 2,6-Dichloro-	6.36(3.23)	2.64(1.36)	3.3	7.4
7 4-Chloro-3-methyl-	6.60(3.35)	2.78(1.44)	2.0	>50
8 2,4-Dichloro-	6.74(3.42)	2.88(1.49)	4.4	9.8
9 3,5-Dichloro-	7.02(3.56)	3.09(1.60)	4.4	7.8
10 2,3-Dichloro-	7.24(3.67)	3.29(1.70)	3.8	6.3
11 3,4-Dichloro-	7.83(3.97)	3.73(1.93)	4.0	9.5
12 2,4,6-Trichloro-	8.59(4.36)	4.30(2.22)	5.8	4.5
13 2,3,6-Trichloro-	9.52(4.83)	5.09(2.63)	6.3	5.4
14 2,3,5-Trichloro-	9.81(4.98)	5.45(2.81)	5.9	5.4
15 2,4,5-Trichloro-	9.99(5.07)	5.57(2.88)	4.8	4.1
16 2-Chloro-4-tert-butyl-	10.63 (5.39)	6.05(3.12)	1.5	>50
17 2,3,4-Trichloro-	11.04(5.60)	6.57(3.39)	5.6	5.7
18 3,4,5-Trichloro-	11.58(5.88)	7.11(3.67)	4.7	3.9
19 2,3,5,6-Tetrachloro-	13.34(6.77)	9.03(4.66)	7.8	3.6
20 2,3,4,6-Tetrachloro-	13.51(6.85)	9.21(4.75)	6.9	2.8
21 2,3,4,5-Tetrachloro-	15.45(7.88)	11.92(6.15)	7.0	2.8
22 PCP	19.71(10.00)	19.37(10.00)	10.0	1.8

[a]Exact values not determined because ECD sensitivities of corresponding acetates are very low.

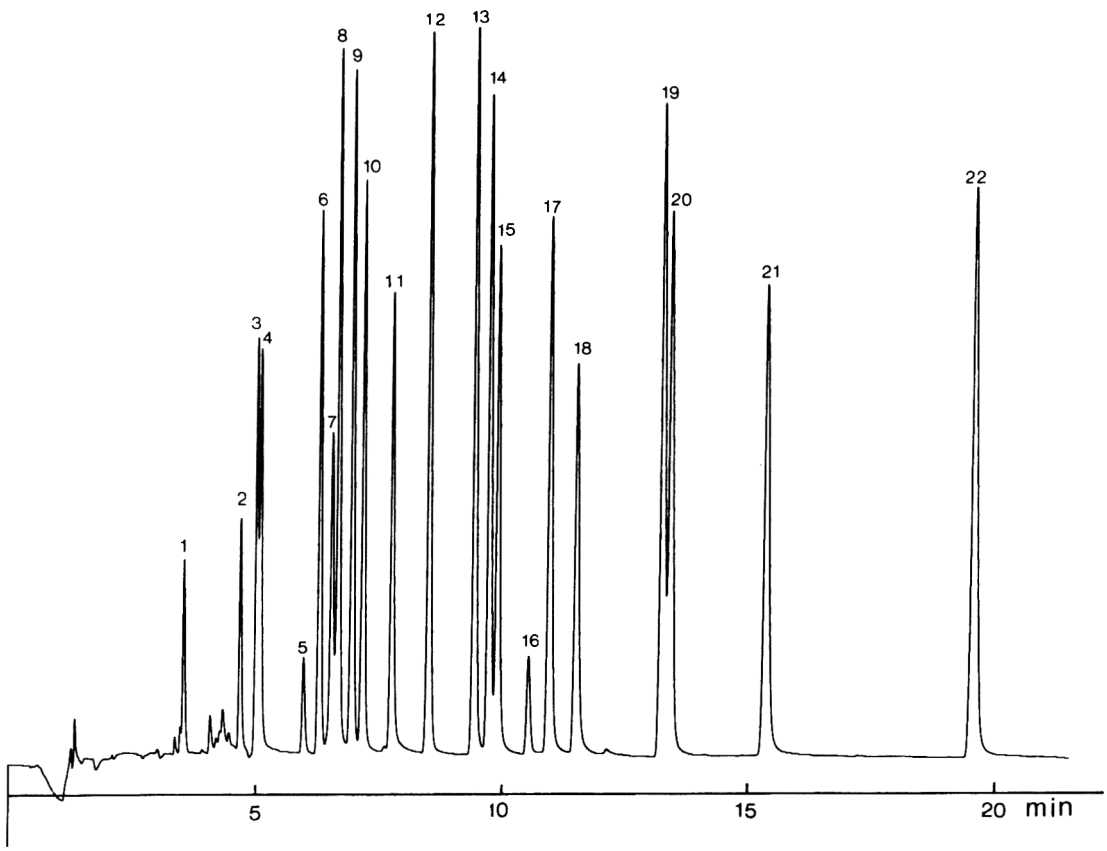

Figure 2. EC-GC chromatogram of chloroacetate derivatives of 22 phenols as resolved on 12 m × 0.2 mm id OV-1 fused silica capillary column. See Table 1 for peak identification and *Experimental* for GC conditions.

3 mL isooctane as keeper and evaporate extract to 3 mL. Dilute to 10.0 mL with isooctane and analyze by GC.

Long method.—Stir water sample (1 L) collected in long-neck whiskey bottle (1.14 L) or other suitable container, using Teflon-coated stirring bar so that vortex formed almost reaches bottom of bottle. Carefully adjust to ≤pH 2 (pH paper) with 1 + 1 (v/v) sulfuric acid and water. Add 50 mL dichloromethane and tightly cover bottle with piece of Teflon tape and cap. After stirring 30 min, transfer contents to 1 L separatory funnel. Drain organic layer into 500 mL separatory funnel. Transfer aqueous layer back to sample bottle. Rinse 1 L separatory funnel with 30 and 20 mL aliquots of dichloromethane and drain both fractions into sample bottle. Repeat above extraction twice. Discard water sample after final extraction. Add 40 mL 1% K_2CO_3 to combined organic extract in 500 mL separatory funnel and shake 2 min. Drain organic layer to second 500 mL separatory funnel and aqueous layer to 250 mL volumetric flask. Repeat back-extraction twice

with 30 mL 1% K_2CO_3. After final back-extraction, save organic layer for analysis of other classes of neutral organic compounds if necessary.

Follow the above short method for chloroacetylation of phenols. After evaporation of chloroacetate extract to 3 mL, prepare mini cleanup column by plugging long Pasteur pipet (23 × 0.5 cm id) with silanized glass wool. Fill column with 5 cm of 5% deactivated silica gel. Tap column gently and add 5 mm of 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 cleanup column. Elute column with 5 mL hexane and discard this fraction. Continue elution with 10 mL toluene. Collect this fraction and adjust volume to 10.0 mL. Analyze sample by injecting 2 μL cleanup extract splitlessly into OV-1 fused silica capillary column connected to electron-capture detector. For calibration standard, chloroacetylate known amount of phenols, using the short method.

Results and Discussion

GC Characteristics of Chlorophenol Chloroacetates

The resolution of the chloroacetates of phenol, 18 chlorophenols (Table 1), together with 2-chloro-5-methyl-, 4-chloro-3-methyl-, and 2-chloro-4-tert-butylphenols on a 12 m OV-1 capillary column is shown in Figure 2. A total of 22 well resolved peaks were observed for the above phenols. The identity of each peak in the mixture was assigned by comparison with the retention time of each chlorophenol chloroacetate obtained by reacting individual phenols under identical experimental conditions. As found in the case of chlorophenol acetates (11), the chloroacetates of 2,5- and 2,4-dichlorophenols could not be resolved on this column under various chromatographic conditions. Therefore, 2,5-dichlorophenol was omitted for all subsequent quantitative work. Not unexpectedly, the chloroacetates and acetates of chlorophenols have the same elution order on the OV-1 column and the chloroacetates exhibit much less tailing than the acetates on the capillary column. As shown in Table 1, the electron capture detector is approximately 2 to over 50 times more sensitive to the chloroacetates than the corresponding acetates. The increase in sensitivities is particularly large for monochlorophenols. Thus, the detection limits for such phenols could be greatly improved if chloroacetylation derivatization is applied. Also included in Table 1 are retention time data of the chloroacetates on a 3% OV-1 packed column operated isothermally at 155°C.

Gas Chromatography-Mass Spectrometry

Recently, GC-MS of acetates (20, 21) and some other derivatives (21) of chlorophenols has been reported. However, chloroacetate derivatives were not studied in either of these reports. The identities of chlorophenol derivatives in the present work were confirmed by combined GC-MS. In the electron impact (EI) mode, the mass spectra of each chlorophenol chloroacetate contained the following major fragments: (i) the molecular ion (M^+), (ii) the CH_2ClCO^+ ion (m/z = 77), and (iii) the parent phenoxy ion $(M\text{-}77)^-$. The expected chlorine isotopic pattern was observed in both M^+ and $(M\text{-}77)^+$ regions. Under the condtions used, the M^+ ions are about 10% or less as abundant as the $(M\text{-}77)^+$ ions.

Chloroacetylation of Chlorophenols

In Argauer's work (15), the chloroacetate derivatives were prepared by reacting a phenol solution in benzene with chloroacetic anhydride for 2 min in 15 mL 0.25N NaOH. When this procedure was applied to a mixture of chlorophenols, the chloroacetates of all phenols were formed. However, molar responses of derivatives in the same homologous series were very different, leading us to suspect that the literature condition was not optimized for all chlorophenols. Investigation was initiated to improve the yields by varying the base, the reaction time, and the amount of anhydride used. The following are some of our observations which led to the selection of the present procedure:

(1) Using 0.25N NaOH as a base, longer reaction times (e.g., 10 or 30 min) gave poorer rather than better yields of all chloroacetates.

(2) Decreasing NaOH concentration to 0.025N while maintaining reaction time at 2 min caused some extraneous peaks in the mono- and dichlorophenol region together with lower yields of all derivatives. Virtually no chloroacetates were formed when the NaOH concentration was further decreased to 0.0025N. Meanwhile, large amounts of side products of long retention times were observed.

(3) All chlorophenol chloroacetates were unstable in basic media (pH 11–13). This was confirmed by stirring a mixture of chloroacetates in petroleum ether with either an aqueous solution of 0.25N NaOH (pH ~ 13.5) or 1% K_2CO_3 (pH ~ 11.5). However, decomposition was faster in NaOH than in K_2CO_3 solution. Also, the chloroacetates of 2,6-disubstituted chlorophenols (i.e., 2,6-dichlorophenol, 2,4,6- and 2,3,6- trichlorophenols, 2,3,4,6- and 2,3,5,6- tetrachlorophenols, and PCP) were more stable under such conditions. After stirring with a 1% K_2CO_3 solution for 30 min, the 2,6-disubstituted phenol chloroacetates were 80–90% recovered while the others were only 15–50% recovered.

(4) Formation of the chloroacetates of the lower chlorophenols in 1% K_2CO_3 solution was nearly instantaneous while the formation of higher (tetrachlorophenols and PCP) chloroacetates and 2,6-disubstituted phenols was slower. The latter phenols require reaction times as long as 10 min for maximum yield.

(5) Presumably because of less decomposition, substituting 1% K_2CO_3 solution for 0.25N NaOH improved the yields of all chloroacetates. Among the various concentrations of K_2CO_3 that we have tried, 10% and 0.1% K_2CO_3 solutions gave lower (20–55%) yields for all chloroacetates while the yields were basically the same (± 10%) for K_2CO_3 concentrations of 2, 1, and 0.5%.

(6) Lower chloroacetic anhydride reagent concentrations (100 and 50 mg/mL) tended to give lower yields of derivatives. Higher chloroacetic anhydride concentrations formed large amounts of precipitate when reagent was added to sample and yields were not improved. Reactions involving 300 mg of solid chloroacetic anhydride usually gave 10–20% less yield.

Because of different reaction kinetics of chlorophenols as well as stability of various chloroacetates, it was apparent that a single reaction time (either a short one such as 1 or 2 min or a longer one such as 10 min) would not be appropriate for maximum yield for all derivatives. This problem was overcome by the incorporation of 3 consecutive extractive chloroacetylation reactions, i.e., two 1 min reactions designed for the fast-forming but unstable derivatives and one 8 min reaction for the slow-forming yet stable derivatives. Also, to ensure maximum yields, fresh chloroacetic anhydride reagent was added in the second and third extraction. No further change in yields of the 2,6-disubstituted phenols was observed by extending the reaction time for the third extraction from 8 to 28 min.

The chloroacetylation procedure was validated over a 1000-fold concentration range from 0.1 μg to 100 μg for each of

Table 2. Mean % recovery and %CV (in brackets) of phenol from fortified Lake Erie sample (No. of replicates = 6)

Vol. of sample, L:	0.1	0.1	0.1[a]	1	1[a]
[phenol], μg/L:	100	10	1	1	0.1
Parent phenol		Short method		Long method	
Phenol	103	—[b]	—[b]	—[b]	—[b]
2-Chloro-	97	107(7.6)	87	87(11.6)	118
3-Chloro-	92	104(5.0)	96	76(8.2)	79
4-Chloro-	93	115(9.1)	103	68(9.7)	92
2-Chloro-5-methyl-	89	98(8.7)	88	77(10.2)	—[b]
2,6-Dichloro-	98	103(2.1)	95	96(5.0)	112
4-Chloro-3-methyl-	91	96(3.9)	86	63(6.7)	71
2,4-Dichloro-	95	103(4.3)	105	86(5.1)	104
3,5-Dichloro-	93	104(3.6)	94	87(4.5)	103
2,3-Dichloro-	88	99(4.7)	97	90(3.2)	116
3,4-Dichloro-	90	97(2.9)	93	79(4.6)	91
2,4,6-Trichloro-	93	94(3.4)	101	95(3.8)	93
2,3,6-Trichloro-	96	96(2.8)	100	94(2.9)	93
2,3,5-Trichloro-	96	98(2.9)	87	89(4.7)	87
2,4,5-Trichloro-	99	97(3.8)	91	85(4.5)	89
2-Chloro-4-tert-butyl-	88	92(10.5)	—[b]	—[b]	—[b]
2,3,4-Trichloro-	93	96(2.0)	99	81(5.9)	88
3,4,5-Trichloro-	91	99(2.9)	83	78(6.3)	81
2,3,5,6-Tetrachloro-	94	97(1.7)	98	97(2.3)	86
2,3,4,6-Tetrachloro-	95	96(2.1)	97	95(2.0)	96
2,3,4,5-Tetrachloro	98	94(3.7)	82	90(4.4)	80
PCP	91	95(7.6)	104	91(5.8)	102

[a]Silica gel column cleanup included.
[b]Not determined because of either low sensitivity or interference.

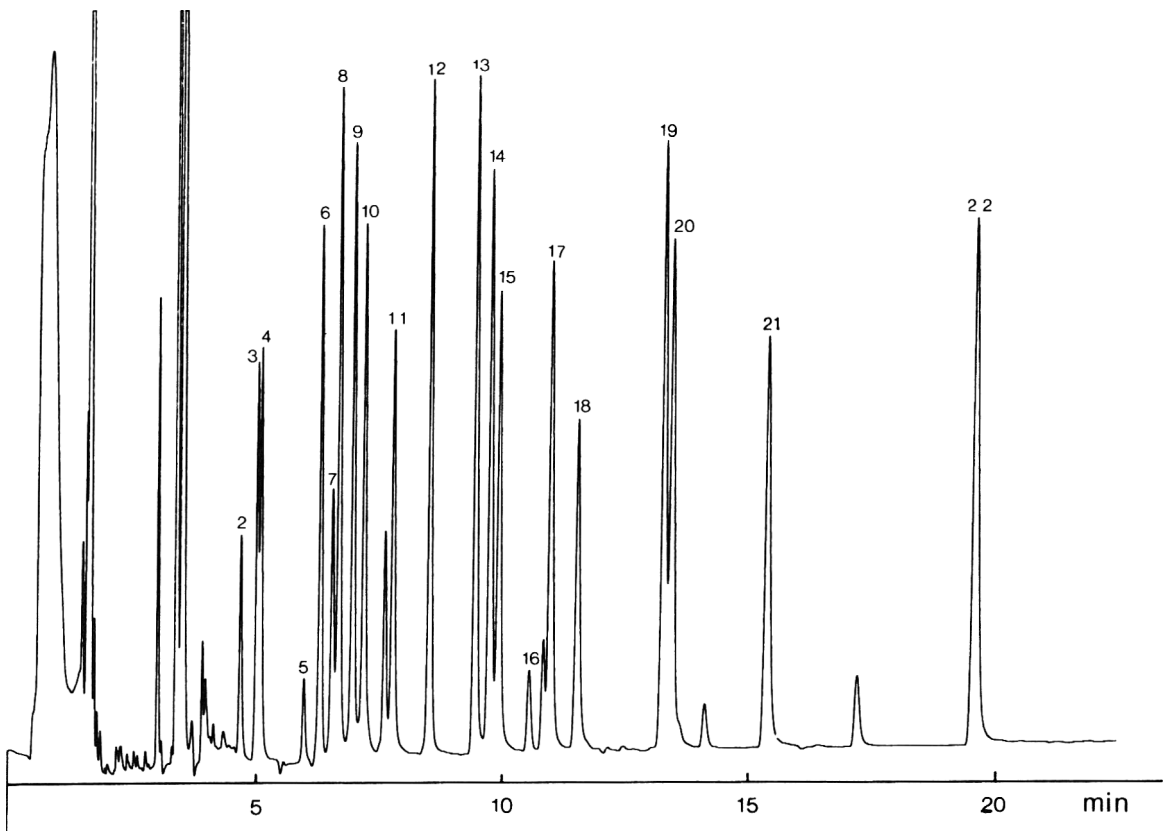

Figure 3. EC-GC chromatogram of derivatized extract from 1 L Lake Erie water fortified at 1 μg/L for each phenol.

the 22 phenols in the test mixture. Within this range, the yields of all chlorophenol chloroacetates were linear. For 6 replicate chloroacetylations of a mixture of these phenols of 1 μg each, the coefficient of variation was less than 8% for all of them.

Attempts have been made to carry out the chloroacetylation reaction in a 1 L water sample; however, yield of chloroacetates were generally low (10–40%) compared with the 100 mL reaction.

Column Cleanup

Although it may not be necessary for high phenol concentrations and clean water samples, a column cleanup step is beneficial for the determination of phenols at the detection limit of the method as well as for some highly contaminated water samples. Cleanup was initially attempted by spiking a concentrated mixture of the chloroacetates onto a 5 cm activated Florisil column in a 230 × 6 mm id Pasteur pipet. Although the recoveries of the 2,6-disubstituted derivatives

were over 90% in the toluene fraction, recoveries for others were unsatisfactory (30–80%). Using a more polar eluant such as 5% methanol in toluene resulted in even lower recoveries (5–70%) for all derivatives; again, the 2,6-disubstituted derivatives were better recovered (50–70%). On the other hand, cleanup of chloroacetate extracts on a 5% deactivated silica gel column gave quantitative recoveries for all derivatives in the toluene fraction with the exception of the derivatives of 2,3,5-, 2,4,5-, and 3,4,5-trichlorophenols and 2,3,4,5-tetrachlorophenol which were 85–90% recovered.

Application to Water Samples

Two extractive acetylation procedures were proposed in this work. The shorter one is ideal for rapid screening of chlorophenols in water because the solvent extraction of phenols is eliminated in this case. Using 100 mL of a fortified water sample from Lake Erie, which has a chlorophenol blank much lower than 0.1 μg/L, this shorter procedure was validated from 10 to 1000 μg/L (Table 2). The mean recoveries for most phenols were better than 85% and CV was less than 12%. Because of interference and lower sensitivity of their derivatives, the determination of phenol and 2-chloro-4-tert-butylphenol was not reliable at concentrations of 10 μg/L or lower.

If analysis of lower concentrations of phenols or simultaneous analysis of other classes of compounds such as PCBs, chlorobenzenes, organochlorinated insecticides, and other neutral pesticides is required, a method involving pre-extraction of organic compounds from water with subsequent back-extraction of phenols into 1% K_2CO_3 was proposed. The extraction recoveries of phenols from 1 L water by using dichloromethane have been demonstrated to be better than 80% for all chlorophenols except phenol, which was only 30% recovered (22). In the back-extraction of phenols from dichloromethane solutions, both 1% K_2CO_3 and 0.1N NaOH solutions tested gave similar results although the NaOH solution tended to produce higher recoveries (95% vs 75%) for 4-chloro-3-methyl- and 2-chloro-5-methylphenols. However, 1% K_2CO_3 was used in this work to avoid possible degradation of some labile pesticides. This longer procedure was also validated with the Lake Erie water sample fortified at 1 and 0.1 μg/L for each phenol. Figure 3 represents a typical chromatogram derived from a Lake Erie sample fortified to 1 μg/L of each phenol. The sample has been subjected to dichloromethane extraction, K_2CO_3 back-extraction, chloroacetylation, and silica gel column cleanup.

Conclusion

The method using chloroacetic anhydride to form chloroacetates of phenols has been refined to produce quantitative results for the analysis of 21 chlorophenols in water. This technique proves to be a more valuable alternative to the popular acetylation procedure because of the high sensitivities and rapid formation of the derivatives. The increase in electron-capture sensitivities of chloroacetates over the corresponding acetates extends the applicability of the method to the analysis of monochlorophenols.

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Trace Level Detection of Chlorinated Paraffins in Biological and Environmental Samples, Using Gas Chromatography/Mass Spectrometry with Negative-Ion Chemical Ionization

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A method is described for detection of chlorinated paraffins (CPs) in biological and environmental samples. Sample preparation includes sulfuric acid treatment followed by adsorption chromatography on alumina, which yields the CPs in one fraction that is almost free of interfering material. Using gas chromatography/mass spectrometry with negative-ion chemical ionization, the limit of detection is 5 ng (corresponding to the lower ppb range). CP levels of 30 ppm, 200 ppb, and 5 ppb were found in sewage sludge, human fat, and sediment, respectively.

Chlorinated paraffins (CPs) have been produced since 1930 and have found many applications, such as additives for polymers, lubricants, paints, and flame retardants (1). Annual production of CPs amounted to 300 000 tons in 1979 (1). Because of their properties (relative chemical inertness, low volatility, flame retardation, modifiable physical properties), they are suitable as substitutes in some applications for the polychlorinated biphenyls (PCBs), which have recently been restricted or prohibited for use in many countries.

Toxicological data on the CPs are sparse and limited to a few CP types. A quite comprehensive study on a widely used CP formulation was reported by Birtley et al. (2). Feeding 500 ppm CP for a 90 day period caused increases in serum glutamic pyruvate transaminase levels, in liver weight, and in the size of the smooth endoplasmatic reticulum in the rat; in the same study, similar results are given for the dog. From these data, the authors estimate a no-effect level of 250–300 ppm CPs in food. Enzyme induction by CPs is reported by Biessmann et al. (3) and Meijer et al. (4). Retention and accumulation of CPs in adipose tissue seems to depend on the type of CP: half-lives of CPs in fat in different animal species in a range from 8 days up to more than 37 days are given by several authors (2, 3, 5). The neurotoxic effects of CPs in fish are described by Bengtsson et al. (5, 6). All of these findings have to be judged with some caution. For a complete and decisive evaluation of the toxicological risk posed by the use of the CPs, the following drawbacks must be taken into account: *(1)* Due to the production processes used, CPs consist of large numbers of isomers and homologs (7). The necessary structural characterization has been lacking until now. *(2)* Cellular test systems for the assay of mutagenic properties may lead to erroneous results with compounds insoluble in water. *(3)* Specific detection of CPs at trace levels is difficult (8).

Some data on CP concentrations in environmental and biological samples have been presented by Campbell and McConnell (8). Although these authors used a rather unspecific and tedious detection procedure (9), they were able to demonstrate the presence of CPs in samples of different origin. The need for further investigations on the occurrence of CPs and their characterization led us to evaluate gas chromatography/mass spectrometry (GC/MS) as a tool for this task.

Experimental

Samples and Chemicals

Human adipose tissue from a 65-year-old male was obtained from the University Hospital of Zürich. Sewage sludge containing 5% solid matter was taken from a sewage treatment plant in an urban industrialized region known for high contamination with heavy metals and organochlorine compounds. Surface sediment from Lake Zürich was collected by a diver near the city of Zürich.

Reference chlorinated paraffins (Witaclor 149, Witaclor 352, and Witaclor 549) were generously provided by Dynamit Nobel, Rheinfelden, FRG. All solvents used were of residue analysis grade and were obtained from Burdick & Jackson (Fluka AG, Buchs, Switzerland). Cationotropic alumina (Woelm, Eschwege, FRG) was dried overnight at 130°C before adding water (5% w/w) with stirring.

Extraction and Cleanup

Adipose tissue.—Homogenize 10.8 g human adipose tissue with 50 mL methylene chloride. Percolate homogenate through chromatographic tube packed with 15 g anhydrous Na_2SO_4. After rinsing column with additional 20 mL methylene chloride, remove solvent on rotary evaporator and dissolve residue in 50 mL pentane. Transfer solution to separatory funnel and add 50 mL concentrated H_2SO_4. After thorough mixing and occasional addition of pentane, remove acid phase. Repeat this acid treatment twice with portions of 10 ml of H_2SO_4. Wash clear and colorless pentane solution with 50 mL 1M sodium bicarbonate solution, and after drying with anhydrous Na_2SO_4, concentrate to 1 mL in rotary evaporator. Sample is now ready for adsorption chromatography.

Sediment.—Extract 20 g sediment with 30 mL acetone–hexane (1 + 1 v/v). After centrifugation, wash supernatent with water; then dry and reduce in volume to 3 mL. Add 3 mL H_2SO_4, and after shaking and removing acid layer, wash clear organic phase with sodium bicarbonate solution. Dry with anhydrous H_2SO_4 and concentrate to 1 mL to produce a solution ready for adsorption chromatography, as described.

Sewage sludge.—Homogenize sewage sludge (20 g) with 20 mL acetone and extract with 20 mL pentane. After centrifugation, wash pentane phase with water, dry, and reduce in volume to 3 mL. Add 3 mL H_2SO_4, and after shaking, wash clear organic phase with sodium bicarbonate solution. Dry with anhydrous H_2SO_4 and concentrate to 1 mL to give a solution ready for adsorption chromatography.

Adsorption chromatography.—Plug Pasteur pipet with glass wool and pack with 1 g alumina containing 5% water. Apply pentane extract to top of column. Elute with 10 mL hexane to give first fraction; 10 mL hexane containing 10% (v/v) ethyl ether elutes the compounds of interest. Reduce volume to ca 100 μL, and use 2 μL aliquot for GC/MS analysis.

GC/MS Analysis

GC separation was carried out using a Fractovap 4160 gas chromatograph (Carlo Erba Strumentazione, Milano, Italy) equipped with a 13 m × 0.30 mm id glass capillary column coated with immobilized SE-54 (film thickness 0.08 μm).

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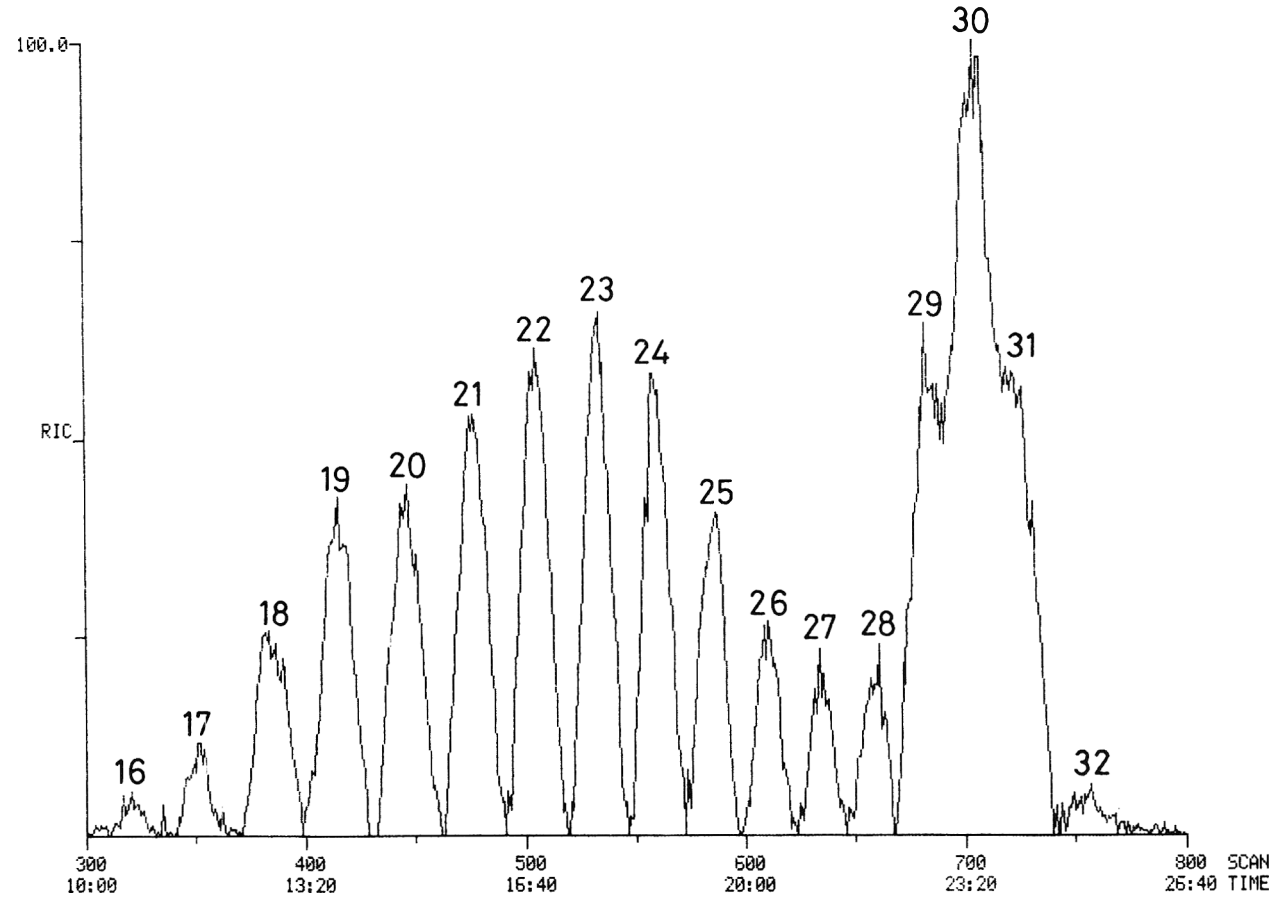

Figure 1. Reconstructed ion chromatogram of standard CP mixture (Witaclor 149, Witaclor 352, and Witaclor 549; 250 ng of each injected).

Helium at a pressure of 1.2 bar was used as carrier gas. The samples, dissolved in hexane, were injected directly on-column at an oven temperature of 60°C. After 1 min, the temperature was raised to 290°C at 10°/min (temperature programmer Carlo Erba LT Mod. 230).

A 0.15 mm id fused silica transfer line to mass spectrometer (at a temperature of 290°C) was pushed into end of glass capillary column and connection was flushed with helium. Mass spectrometer (Finnigan 4510) in NCI mode equipped with Incos data system was used. Methane reagent gas pressure was 0.6 torr; ion source was held at 190°C. Mass spectra were recorded in mass range of m/z 100 to 600 with scan time of 2 s; in MID mode, cycle time of 2 s was used.

Results and Discussion

The cleanup procedure described is based on sulfuric acid treatment to remove the bulk of coextracted compounds, as suggested for residue analysis of chlorinated hydrocarbons (10), followed by adsorption chromatography on alumina. A comparison of the chromatographic behavior of CPs on silica gel and alumina was carried out by Zitko (11, 12): CPs are separated on silica gel according to their polarity, whereas all types of CPs can be collected in the same fraction on alumina. Furthermore, chromatography on alumina allows an efficient separation of CPs from the other chlorinated hydrocarbons such as chlorinated benzenes, DDE, DDT, and the PCBs. Because alumina chromatography yields the entire CPs in one fraction, this adsorbent was used in the present study. The efficiency of this sample purification was tested using a sample of butter to which 1 ppm Witaclor 352 had been added. CP applied to the fat was recovered in the range of 80%. Stability against degradation of CPs during the sulfuric acid treatment was established using standard solutions of Witaclor 352 in hexane. Chromatography on alumina yielded 2 fractions: Hexane eluted interfering compounds, and 10% ethyl ether in hexane eluted the CPs (fraction 2). Fraction 1 contained less than 4% of the CPs detected in fraction 2.

Because of the low levels of CPs expected in biological and environmental samples (8), multiple ion detection (MID) must be used for maximum mass spectrometric sensitivity. The multitude of CPs and the complexity of their mass spectra (7, 13) require a careful evaluation of the masses detected in MID. Quadrupole mass spectrometers together with modern data systems are capable of simultaneously recording a large number of ion intensities. However, only a limited number of ions (normally up to 6) can be monitored to fully exploit the high sensitivity of mass spectrometric detection.

In the present study, the choice of the appropriate MID conditions was directed by the following criteria: *(1)* For preservation of full sensitivity only 4 masses were registered simultaneously. *(2)* The detection had to include CPs with a chain length between C_{14} and C_{30} and a chlorine content of 50%. *(3)* For the final selection of ions to be detected, a composite standard was prepared and analyzed using full scan.

Figure 1 shows the reconstructed ion chromatogram (RIC) of a mixture of 3 different types of CPs. The numbering of the chromatographic signals is described in detail elsewhere (7): The number represents the sum of the number of carbons and chlorines present in the CP compounds that make up the signal. Signals Nos. 23 and 25 were chosen for investigation. The corresponding mass spectra are shown in Figure 2. These spectra can be understood with reference to the fragmenta-

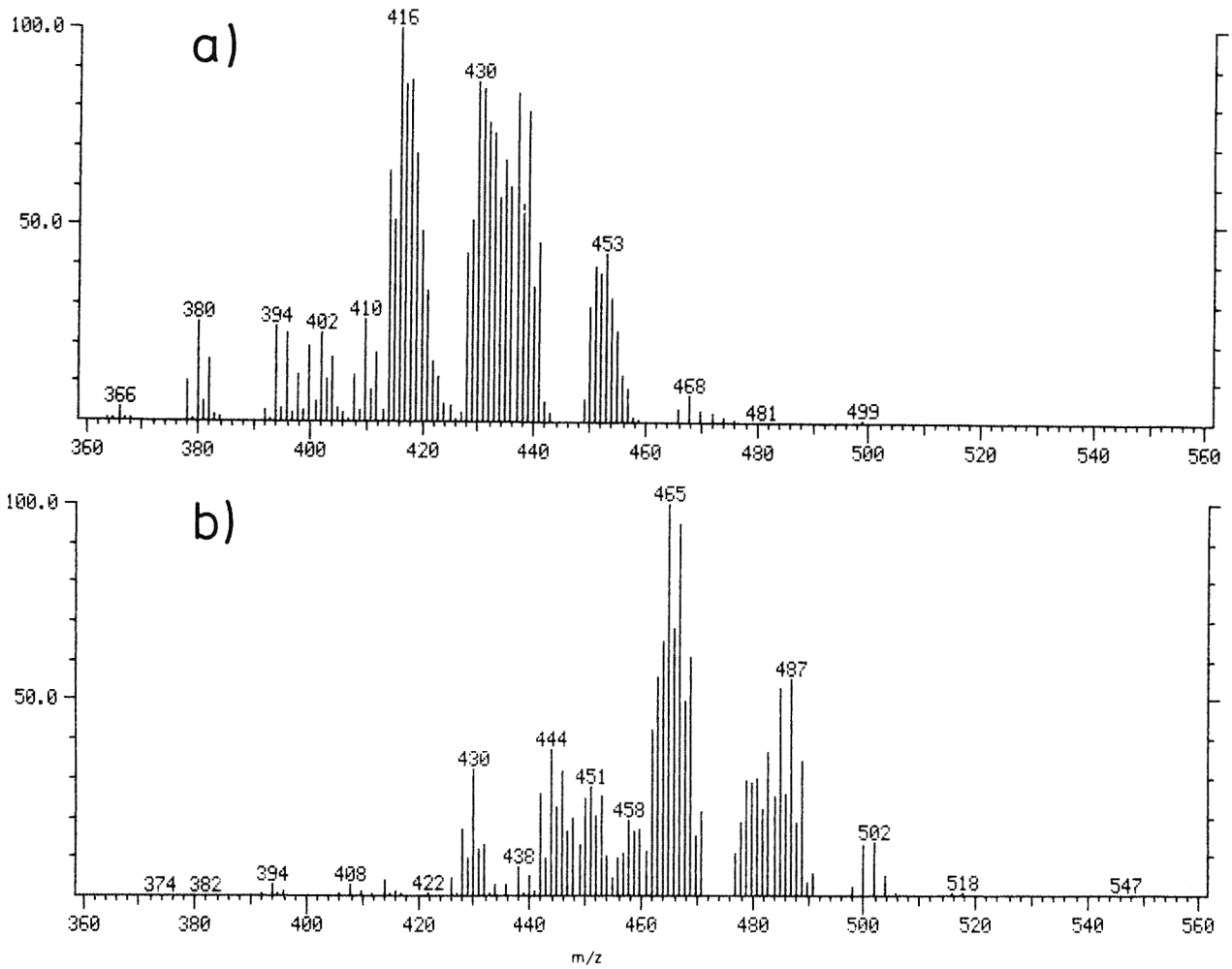

Figure 2. Negative-ion chemical ionization mass spectra of CP signals No. 23 (a) and No. 25 (b).

tion scheme described (7). The most abundant signals from these spectra were chosen for MID: m/z 416 and 430 were selected to represent signal No. 23, and for the representation of signal No. 25, m/z 465 and 487 were chosen.

We studied 3 widely different samples representative of biological and environmental exposure to CPs. The mass fragmentograms of the samples and the CP standard solution (see Figure 1) are shown in Figure 3a–c. For clarity, pairs of separately registered mass fragmentograms were summed to give a single trace, e.g., m/z 416 and 430 which are characteristic for CP No. 23 are given in one trace. The occurrence of the satellite peaks in the chromatograms can be understood by considering the corresponding mass spectra. As described earlier (7), the major fragments result from dechlorination and dehydrochlorination, respectively. As a consequence, a certain mass can belong to both a fragment and a molecular ion cluster. The mass fragmentograms clearly indicate the presence of CPs in all samples.

For quantitation, the following assumptions have to be made: *(1)* The masses chosen are representative for both the standard CP mixture and for the CP composition of the samples analyzed. *(2)* The compounds are completely recovered.

CP concentrations in the samples were calculated by comparing chromatographic peak intensities. Results are given as equivalents to a CP based on a paraffin mixture C_{14}-C_{18} with a chlorine content of 52% w/w (Witaclor 352). CP levels (w/w) found were as follows: human adipose tissue, 200 ppb; sediment from Lake Zürich, 5 ppb; and sewage sludge from an industrialized region, 30 ppm. Blank runs for all analyses showed no detectable amounts of CPs.

The accuracy of the method could be improved by using an internal standard. Unfortunately no reference material has yet been found which meets all requirements with respect to cleanup and GC/MS analysis.

The CP contents given above are comparable to the data given by Campbell and McConnell (8). Sewage sludge was found to contain the highest concentration of CPs (sum = 30 mg/kg), whereas the CP residue in sediment was lower by a factor of 6000. This may be due to the preferred adsorption of CPs in the sewage treatment plant (14). The CP residues in the samples analyzed were in the same order of magnitude as found for DDE and the PCBs (15), indicating that CP contamination is at a level that calls for further investigation.

Because of its specifity, the described procedure could help in the further investigations necessary to collect detailed information on the biological and environmental behavior of the chlorinated paraffins.

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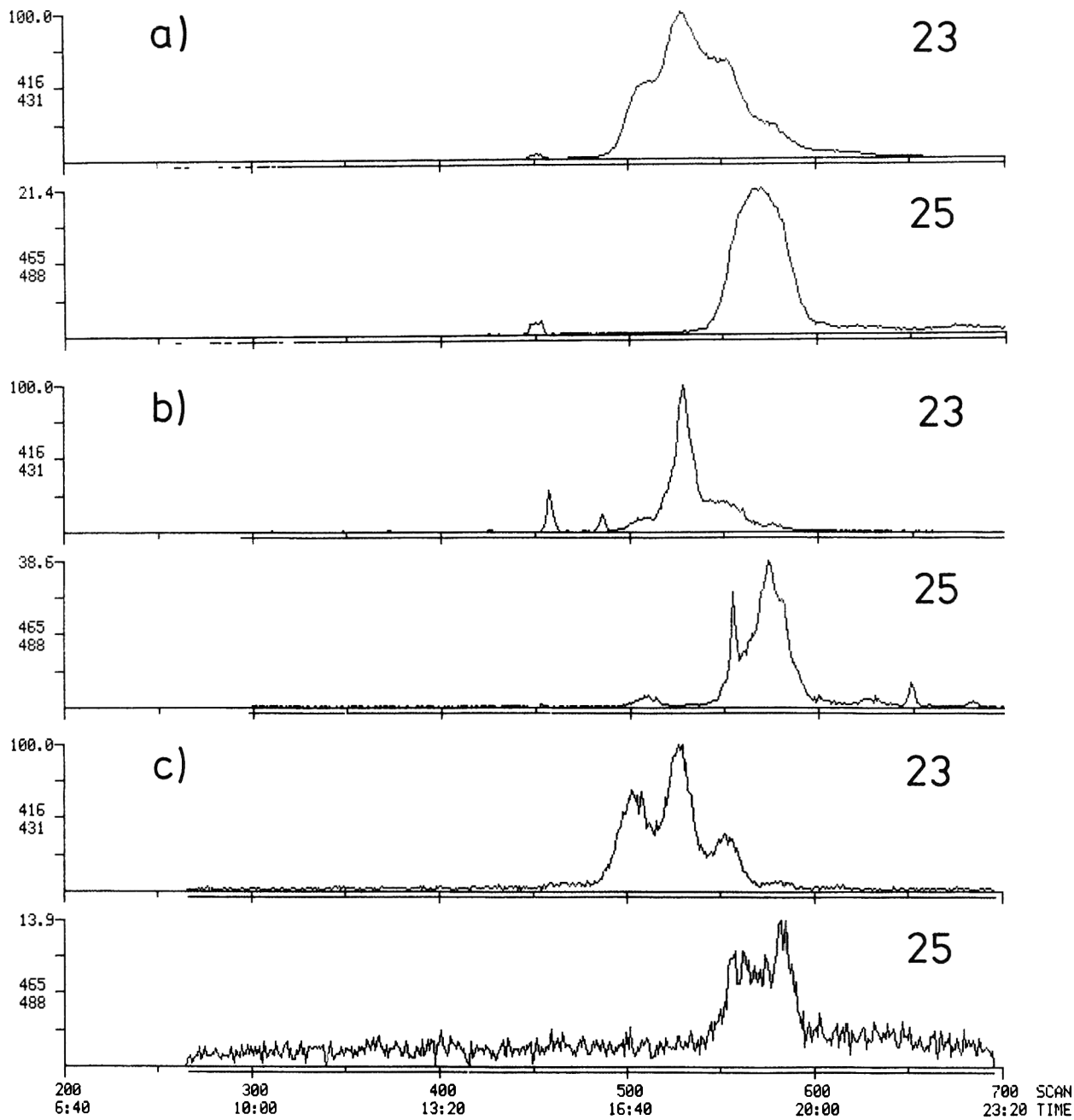

Figure 3. Ion chromatograms of (a) sewage sludge, (b) human adipose tissue, (c) CP standard mixture (Witaclor 149, Witaclor 352, and Witaclor 549; 50 ng of each injected). Upper trace of each set: summed abundances of m/z 416 and 430; lower trace: summed abundances of m/z 465 and 487.

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Purge and Trap Method for Determination of Ethylene Dibromide in Table-Ready Foods

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An improved method has been developed for the determination of ethylene dibromide (EDB, 1,2-dibromoethane) in a variety of table-ready foods. Samples are mixed with water and sparged with nitrogen for 1 h with stirring in a water bath at 100°C. The EDB collected on the adsorbent Tenax TA is eluted with hexane and determined by gas chromatography (GC) with electron capture (EC) and confirmed with Hall electrolytic conductivity (HECD) detection using a second GC column. The highest levels of EDB were also confirmed by full scan GC/mass spectrometry (GC/MS). Twenty-five table-ready foods from the Food and Drug Administration's Total Diet Study that were analyzed by this method exhibited levels up to 70 ppb (pecans). Recoveries from fortified samples ranged from 91 to 104%. Values from this procedure were compared to those obtained by a modified Rains and Holder codistillation method. In all 25 samples this purge and trap procedure showed equivalent or superior recoveries and detected levels of EDB.

The Food and Drug Administration (FDA) monitors residues of pesticides, industrial chemicals, metals, and nutrients in the nation's food supply through several studies. The Total Diet Program is one of the FDA's oldest residue surveillance studies; it has been in operation since 1964. Description of the Total Diet Program and results obtained from this study are published elsewhere (1–6).

Ethylene dibromide (EDB, 1,2-dibromoethane) has been used as a fumigant in both soil and stored food commodities (7). However, recent studies have determined that EDB causes cancer, birth defects, and genetic and reproductive disorders in test animals.

In response to, and in conjunction with, the suspension of registration of fumigation products containing EDB (8) and the establishment of new tolerances for residues of EDB in grain products and citrus fruits (9) by the Environmental Protection Agency (EPA), FDA began a limited survey of EDB in table-ready foods from its Total Diet Program.

Although several methods are available for the determination of EDB in various food products (10–16), the hexane codistillation procedure developed by Rains and Holder (16) was considered to be most appropriate for the variety of food commodities of the Total Diet Study. The table-ready food items of one market basket were analyzed by this procedure, although slight modifications were adopted during the process of examining the 234 samples. A spiked (fortified) aliquot of each food was analyzed concurrently.

Low recoveries of EDB from a number of fortified foods prompted the development of a more generally applicable method for the determination of EDB in table-ready foods.

METHOD

Principle

Samples in water are extracted (sparged) with a nitrogen stream while being stirred vigorously in a water bath at 100°C. EDB collected on the adsorbent Tenax TA and eluted with hexane is determined by gas chromatography/electron capture detector (GC/ECD) with GC/Hall electrolytic conductivity detector (HECD) and/or GC/mass spectrometry (MS) confirmation.

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Apparatus and Reagents

(a) *Gas-liquid chromatograph.*—Equipped with 180 × 0.2 cm id glass column packed with *n*-octane-Porasil C (100–120) (Alltech Associates, Inc.) and constant current ^{63}Ni ECD (Hewlett-Packard 5880 or equivalent). Conditions: injector 170°C, column 150°C, detector 325°C; carrier gas, 5% methane in argon; flow rate, 40 mL/min. Attenuate to obtain ca ½ full scale deflection for 100 pg EDB with 1 mV recorder. Second instrument equipped with 180 × 0.2 cm id glass column packed with 3% SP-1000 on Supelcoport (80–100) (Supelco, Inc.) and HECD (Tracor 560 or equivalent). Conditions: injector 225°C, column 100°C, detector 250°C; carrier gas, hydrogen; flow rate, 40 mL/min. Attenuate to obtain ca ½ full scale deflection for 100 pg EDB with 1 mV recorder.

(b) *Gas-liquid chromatograph/mass spectrometer.*—Mass spectrometer capable of obtaining full scan, EI (70 eV) spectra at 1 s/dec with sensitivity adequate to obtain 10:1 S/N for m/z 186 ($M^{+\cdot}$) for 1 ng EDB; or MID, EI (70 eV) spectra (m/z 107, 109) with sensitivity adequate to obtain 100:1 S/N for 500 pg EDB (VG 7070E or equivalent). Interfaced directly to gas chromatograph fitted with a 30 m × 0.32 mm OV-1 fused silica capillary column (Varian 3700 or equivalent). Conditions: splitless injector 150°C, column 80°C, interface line 180°C, and source 220°C; carrier gas, helium; flow rate, 2.5 mL/min. With data system capable of normalizing spectra after background subtraction (VG 11/250 or equivalent).

(c) *Flask.*—Round-bottom with 24/40 joint vertical neck and 24/40 joint angled side neck (Kontes No. K-605020).

(d) *Stirring bar.*—Teflon-coated, egg-shaped magnet ca 1.6 × 3.2 cm (Fisher No. 14-511-58A).

(e) *Tube.*—Gas dispersion with coarse porosity disc (Kontes No. K-956500).

(f) *Adapter.*—Teflon, universal with 24/40 joint to accommodate 8 mm od gas dispersion tube (Kontes No. K-179800).

(g) *Condenser.*—Liebig 20 cm with 24/40 joint at both ends (Kontes No. K-447000).

(h) *Trap tube.*—Pasteur (disposable) pipet, 6 mm id (with shortened tip) packed with 150 mg conditioning Tenax TA held in place with glass wool plugs. Trap tube is illustrated in Figure 1.

(i) *Tenax TA.*—35–60 or 60–80 mesh, washed with hexane, acetone, and methanol (50 mL/g adsorbent) in succession, drying with nitrogen between washes. Condition 2 h at 340°C under nitrogen stream (20 mL/min). Store in glass container with Teflon-lined or foil-lined screw cap (Alltech No. 04916).

(j) *Solvents.*—Hexane, acetone, and methanol; pesticide analysis quality.

(k) *Antifoam B.*—Silicone emulsion, Dow Corning (Baker No. B531-5), or equivalent.

(l) *Flow meter.*—Nitrogen, adjustable to 100 mL/min (Alltech No. 7904), or equivalent.

(m) *Nitrogen.*—Compressed gas, GC quality.

(n) *Ethylene dibromide standards.*—EPA reference standard No. 246, d 25/25 2.17, or equivalent. *(1) Stock solution:* Introduce appropriate volume from pipet or microliter syringe under a surface of hexane in volumetric flask, and dilute to volume; store in freezer. *(2) Working solution:* Dilute stock solution serially with hexane to ca 20 ng/mL. Store in freezer. *(3) Spiking solution:* Introduce appropriate volume from pipet or microliter syringe under surface of deionized water in

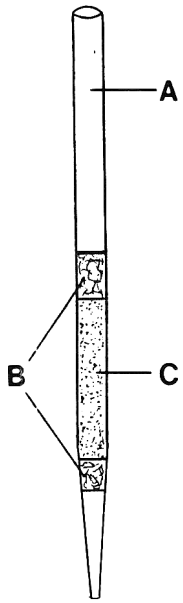

Figure 1. Tenax TA trap tube: A, Pasteur pipet with shortened tip; B, glass wool plugs; C, 150 mg Tenax TA.

volumetric flask, and dilute to volume (do not exceed 1 in 250 solubility of EDB in water). Dilute serially with deionized water to ca 10 ng/mL. Store in refrigerator.

Determination

Accurately weigh ca 10 g homogeneous, frozen or partially thawed sample into 500 mL double-neck, round-bottom flask containing 250 mL deionized water, 1 mL antifoam B, and stirring bar. Using universal adapter, place gas dispersion tube into angled side neck of flask to depth of ca 5 mm above stirring bar. Complete assembly of purge and trap apparatus by addition of Liebig condenser with second universal adapter and trap tube at top as illustrated in Figure 2.

Place assembled apparatus in boiling water bath positioned above hot plate with magnetic stirrer. Adjust nitrogen flow to 100 mL/min through gas dispersion tube and stir vigorously (maximum speed, ca 1600 rpm) to obtain strong vortex.

After 1 h, remove trap tube from universal adapter and elute with at least 1 mL hexane into suitable volumetric flask. Dilute to volume with hexane and quantitate EDB, using GC/ECD. Confirm the presence of EDB, using second GC column with HECD and/or GC/MS as described under *Apparatus and Reagents*.

Results and Discussion

The 234 food items of one market basket sample were analyzed for EDB using the codistillation procedure of Rains and Holder (16) with slight modifications. Silicone emulsion antifoam was added to some foods to retard frothing, and magnetic stirring was used during the analysis of most items. The first of 3 extractions (codistillations) with hexane was found to remove most (73–92%) of the EDB when accompanied by stirring. Samples were also examined after fortification with standard solutions of EDB in hexane. Table 1 lists EDB levels found for 25 foods analyzed by both this modified codistillation procedure and the purge and trap method described above.

All 25 samples were also examined by this purge and trap procedure after fortification with an aqueous EDB spiking solution. After 250 mL water, antifoam, and 10 g sample were added, the spiking solution was pipetted into the aqueous mixture. The apparatus was immediately assembled and placed in the boiling water bath. Table 2 lists spiking levels and recoveries of both the purge and trap and the codistillation procedures.

Aqueous spiking solutions should be analyzed daily. When equal volumes of spiking standard and hexane are shaken vigorously 1 min, 98% of EDB is extracted into the hexane layer. The hexane extract is compared by GC to an EDB standard solution prepared in hexane.

Of the food samples analyzed by this purge and trap procedure, all yielded acceptable recoveries except those that contained alcohol. Recovery of EDB from fortified samples of beer, table wine, and whisky was less than 20%. It is assumed that the distilling alcohol interfered with collection of EDB on the Tenax TA adsorbent. A simple extraction (shake-out) of fortified beer, table wine, and whisky with an equal volume of hexane yielded 75, 80 and 79% recoveries, respectively. These 3 samples were fortified at 4 ppb. Only whisky contained an incurred residue (0.94 ppb). These results compared favorably with those obtained from the modified codistillation procedure: Recovery of EDB at the 4.2 ppb level ranged from 71 to 76% for these alcoholic beverages, and only whisky was found to contain EDB (0.51 ppb).

The rate of stirring is an important parameter of this purge and trap procedure. Inserting the gas dispersion tube in the angled side neck of the round-bottom flask will assure that the nitrogen will be released just at the edge of the stirring vortex. The bubbles are pulled beneath the water surface and

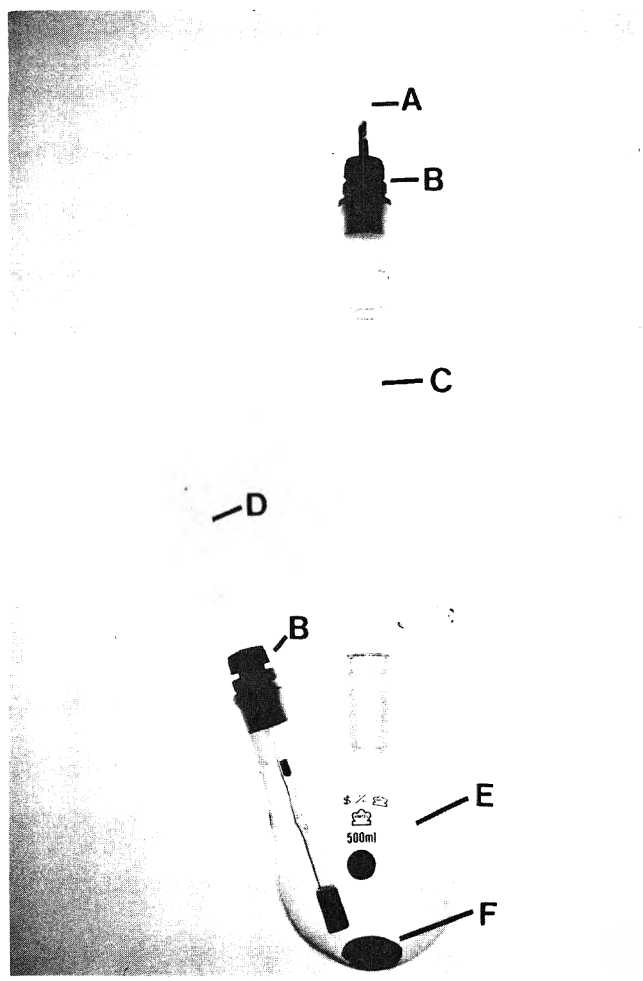

Figure 2. Purge and trap apparatus: A, Tenax TA trap tube; B, universal adapters; C, 20 cm Liebig condenser; D, gas dispersion tube; E, 500 mL round-bottom, double-neck flask; F, magnetic stirring bar.

Table 1. Comparative levels (ppb) of EDB in a variety of foods determined by codistillation and purge trap methods

Item	Modified codistillation[a]	Sparging[b]
Cabbage, boiled	0.0	0.0
Cottage cheese	0.0	0.0
Eggs, soft boiled	0.0	0.0
Pineapple, canned	0.0	0.0
Tomatoes, canned	0.0	0.0
Apple, red, raw	0.0	0.0
Avocado, raw	0.0	0.0
Soft drink, cherry	0.0	0.56, 0.69
Vegetables with bacon, ham[c]	0.0	1.0, 1.0
Round steak, stewed	0.0	4.5, 4.8
Peanut butter, creamy	0.0	5.1, 5.3
Peanuts, dry roasted	0.0	31, 32
Potato chips	0.42	1.4, 1.6
Butter, stick type	0.55	9.8, 11
Pineapple juice	0.56	3.7, 3.8
Rolls, white	0.68	10, 11
Pecans, chopped	0.80	69, 70
Farina, cooked	0.86	2.1, 2.2
Fish sticks, cooked	0.93	1.1, 1.2
Popcorn, popped	1.3, 2.8	14, 14
Honey	2.0	2.1, 2.3
Pizza, cheese, cooked	2.8	7.8, 8.0
Cookies, chocolate chip	7.3	8.1, 8.9
Cake, yellow	8.4	16, 16
Cake, chocolate, icing	12	35, 37

[a]Method used with stirring, the addition of silicone antifoam, and only one extraction.
[b]Duplicate determinations.
[c]Infant, junior food.

Table 2. Comparative recoveries from foods fortified with EDB determined by codistillation and purge and trap methods[a]

Item	Codistillation[b]	Sparging[c]
Cabbage, boiled	91 (1.7)	101 (4.0)
Cottage cheese	75 (4.2)	97 (1.9)
Eggs, soft boiled	84 (4.2)	97 (4.0)
Pineapple, canned	92 (1.7)	98 (3.8)
Tomatoes, canned	73 (1.7)	103 (1.8)
Apple, red, raw	96 (1.7)	93 (3.1)
Avocado, raw	59 (1.7)	104 (4.0)
Soft drink, cherry	86 (1.7)	102 (1.2)
Vegetables with bacon, ham[d]	83 (4.2)	95 (1.2)
Round steak, stewed	91 (4.2)	100 (3.8)
Peanut butter, creamy	82 (4.2)	104 (1.2)
Peanuts, dry roasted	12 (4.2)	97 (21)
Potato chips	46 (1.7)	95 (4.7)
Butter, stick type	30 (4.2)	95 (2.8)
Pineapple juice	44 (1.7)	98 (3.8)
Rolls, white	79 (3.6)	93 (9.4)
Pecans, chopped	62 (1.7)	99 (50)
Farina, cooked	74 (7.3)	92 (2.7)
Fish sticks, cooked	101 (4.2)	102 (1.6)
Popcorn, popped	73 (7.3)	94 (8.0)
Honey	102 (1.7)	97 (1.2)
Pizza, cheese, cooked	66 (8.0)	91 (4.6)
Cookies, chocolate chip	76 (4.2)	103 (9.4)
Cake, yellow	137 (4.2)	101 (15)
Cake, chocolate, icing	26 (4.3)	91 (30)

[a]Values expressed as percent recovery (and ppb fortified).
[b]Spiking standard in hexane; method used with stirring, addition of silicone antifoam, and only one extraction.
[c]Spiking standard in deionized water.
[d]Infant, junior food.

dispersed throughout the mixture. Very slow stirring yields significantly lower recoveries. A sample of yellow cake, shown through exhaustive sparging to contain 16 ppb, was analyzed by this purge and trap method but with stirring at about 400, 800, 1200, and 1600 rpm in separate determinations. Results obtained were 9.6, 14, 16, and 16 ppb, respectively, corresponding to 60, 88, 100, and 100% of that obtained through exhaustive sparging.

Nitrogen flow should be measured at 100 mL/min to ensure reproducibility. This same sample of yellow cake sparged with a nitrogen flow of only 50 mL/min produced results that were 94% of those obtained at 100 mL/min. A mixture of vegetable oil and water (10 + 250) was used to visualize the mixing efficiency of the apparatus. With proper stirring the mixture should appear as an emulsion with nitrogen bubbles dispersed throughout the solution.

A 1500 mL beaker containing about 500 mL water on a hot plate-stirrer was used as the boiling water bath. Water was added as necessary to maintain a level near that of the sample mixture in the round-bottom flask. Although a water bath at 100°C was used for analysis of all samples, evidence was generated to determine the effect of sparging with cooler baths. Replicate aliquots of yellow cake were analyzed by using water baths at 75, 85, and 95°C. After 1 h of sparging, values of EDB found were 87, 93, and 98%, respectively, of that found with sparging using a bath at 100°C. Furthermore, initial purge and trap tests had indicated that nearly 2 h was required to completely extract EDB from an aqueous solution with a water bath at 60°C.

Although the universal adapters make it easy to assemble and disassemble the apparatus, rubber stoppers may be substituted without affecting results or causing interfering GC peaks. In the early development of this procedure, rubber stoppers were used without any difficulties other than in adjusting the gas dispersion tube.

The adsorbed EDB is removed from the Tenax TA trap tube with 1 mL hexane. GC/MS confirmations were performed from this concentrated eluate with any necesary further dilutions being used for GC determinations. Figure 3 depicts an electron impact mass spectrum of EDB (about 700 pg) purged and trapped from chocolate cake.

Ten 10 g aliquots of yellow cake (16 ppb incurred residue) were analyzed sequentially by reusing the same Tenax TA trap tube. Each sample was sparged 1 h, the EDB was eluted with 1 mL hexane, and the tube was washed with 5 mL acetone followed by 5 mL hexane. The adsorbent was dried with nitrogen after each wash. Results ranged from 15.6 to 16.3 ppb (97.5–102%). Procedural blanks run alternately with the samples, using the same trap tube, indicated that no EDB was present.

Although incurred residues of EDB encountered in this study were limited to the ppb range, samples fortified at the ppm level were examined. Aliquots of yellow cake with an incurred residue of 16 ppb were fortified at 1, 15, and 75 ppm, respectively, by the addition of an aqueous spiking standard to the sparging flask. Analyses performed in duplicate showed 94–101% recovery for all fortified levels. A trap tube used to collect EDB at 75 ppm was washed with hexane and acetone as above, and reused to analyze an unfortified aliquot of yellow cake. An apparent carry-over of EDB from the previous analysis boosted the result to 28 ppb (175% of previous values). It is apparent, then, that while the Tenax TA trap tubes can be reused after washing, extreme care must be taken to avoid erroneous results due to carry-over of EDB. However, Tenax TA was successfully reused after analysis at the ppm level by using the conditioning procedure described under *Apparatus and Reagents*.

With 10 g sample and 1 mL hexane eluate, 0.5 ppb EDB gave about ⅛ full scale deflection (about 20 mm) with a 1 mV recorder and 5 μL injection volume. This 0.5 ppb quantitation level is acceptable for finished food products, although slightly lower levels could be quantified with attenuation adjustments. Figure 4 represents chromatograms of standard EDB, yellow cake extract (16 ppb), and fish sticks extract (1.2 ppb) with ECD. Similar chromatograms using GC/HECD are depicted in Figure 5.

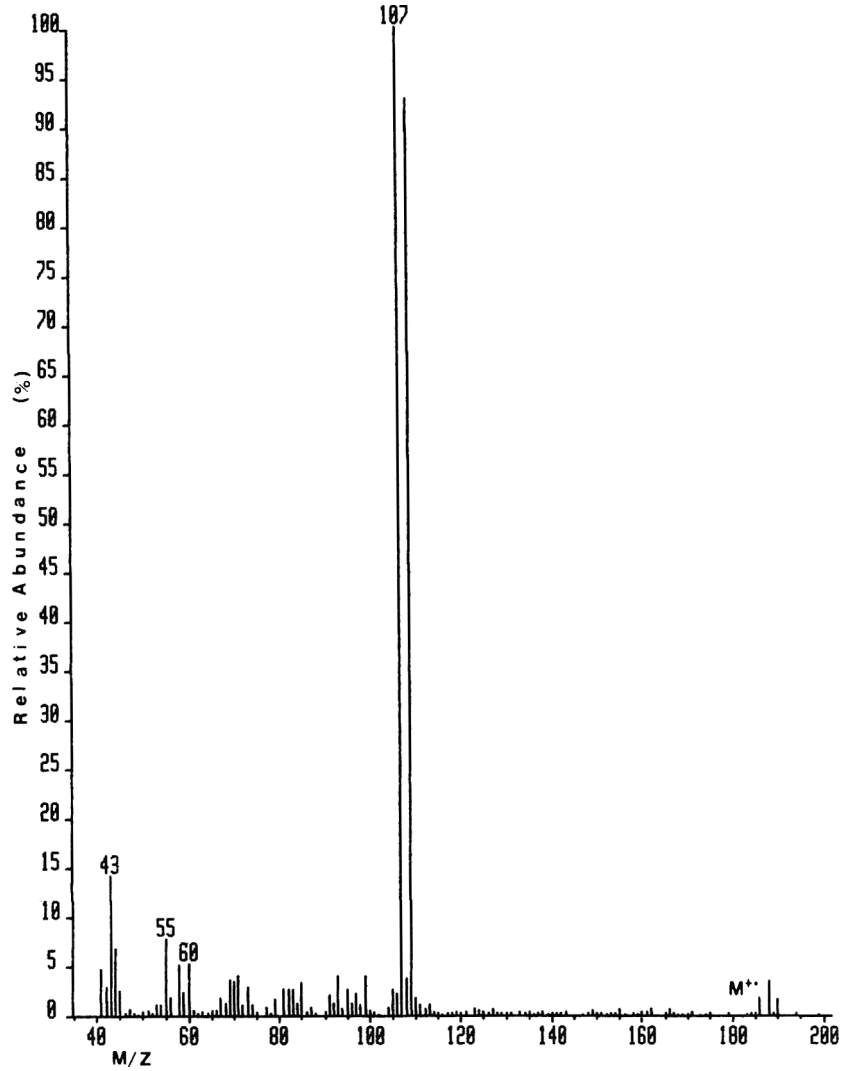

Figure 3. Electron impact mass spectrum (70 eV) of EDB (ca 700 pg) purged and trapped from chocolate cake ($M^{+\cdot}$ = m/z 186).

All analyses were performed with deionized water since tap water was found to contain several purgeable compounds which appeared as interfering GC peaks. Procedural blanks using deionized water, however, were free of GC responses with either ECD or HECD.

Several different lengths and types of water-cooled condensers were substituted in this procedure to examine extreme conditions. A 20 cm Liebig condenser without cooling water performed satisfactorily, although some condensate accumulated in the trap tube. A slight amount of condensed water in the trap tube does not affect the trapping or elution of EDB since Tenax TA has a low affinity for water. Using this condenser, temperature extremes from 0 to 28°C had no adverse effect on the recovery of EDB. However, with a 40 cm Graham (spiral) condenser cooled to 0°C, recovery of EDB was only 89% with the above procedure. This same condenser performed satisfactorily with cooling water at 27°C.

A sample of yellow cake which had been shown to have an incurred residue of 16 ppb EDB was analyzed by this purge and trap procedure as described. However, to determine the rate at which the fumigant is purged, the Tenax TA trap tube was replaced with a fresh tube at 15 min intervals. GC analysis indicated that 96% of the EDB was collected in only 15 min, with a total of 99% in 30 min, and 100% after 45 min. In this test a 20 cm Liebig condenser was used with cooling water at 27°C. The test was repeated with water at 0°C. Only 75% of the EDB was collected after 15 min, 98% after 30 min, and 100% in 45 min. It is apparent, then, that within limits, the temperature of the condenser is not critical. However, attempts to purge and trap by attaching the trap tube directly to the 500 mL flask resulted in a large volume (about 0.5 mL) of condensed water in the trap tube, and EDB could not be quantitatively eluted with 1 mL hexane.

Exhaustive sparging was used to ensure complete extraction of incurred residues of EDB. Samples of chocolate cake and chopped pecans were sparged the initial 1 h according to the above procedure. After an additional 4 h of stirring at 100°C, but without nitrogen flow, the samples were again sparged for 1 h. Finally, these samples were stirred at 100°C overnight (16 h) and then sparged again 1 h. The first hour of sparging yielded 37 and 70 ppb EDB for chocolate cake and chopped pecans, respectively. No EDB was found in the second and third sparging period for either sample. Thus, all of the EDB extracted from chocolate cake and chopped pecans was extracted in the first hour of sparging.

Mixtures of water and soybean oil were prepared to simulate fat levels of various market basket foods. Thus, 1, 4, 7, and 9 mL soybean oil taken to 10 mL with deionized water approximately corresponded to: 10% fat (ice cream, eggs, etc.); 40% fat (potato chips, cheese, etc.); 70% fat (peanut butter, bacon, etc.); and 90% fat (vegetable oil, butter, etc.). After fortification at 3.0 ppb (30 ng), the mixtures were sparged

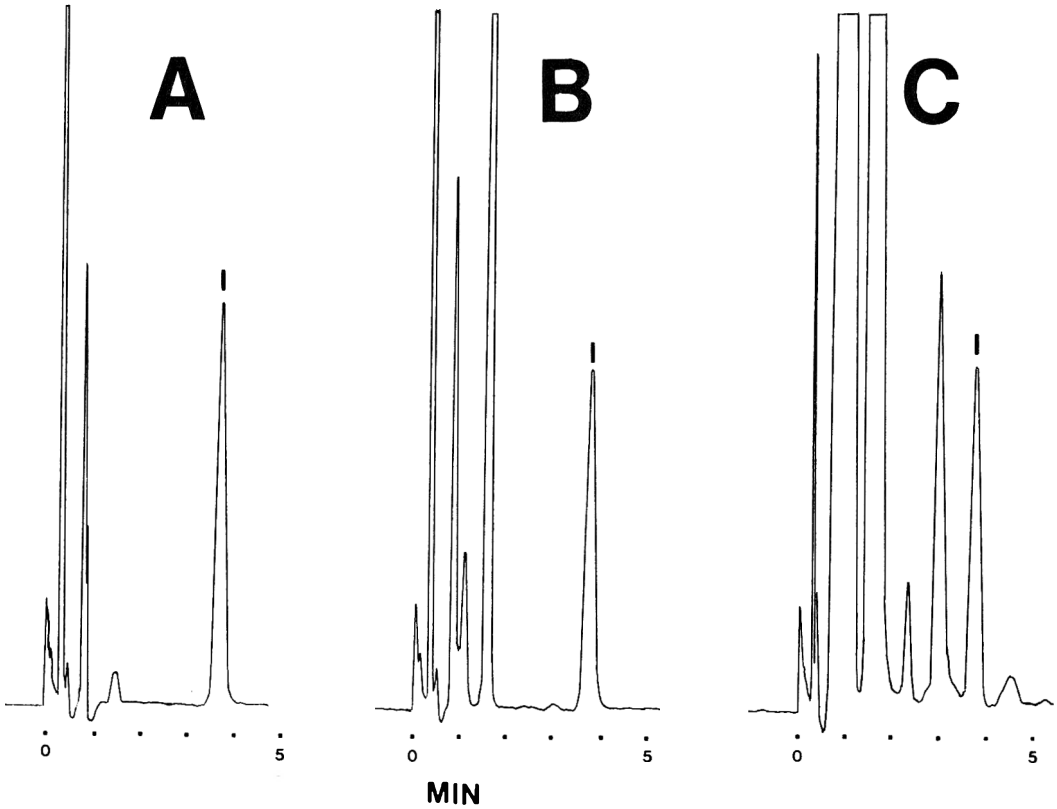

Figure 4. Chromatograms with electron capture detection (GC/ECD); *n*-octane-Porasil C (100–120), 180 × 0.2 cm, 150°C: A, EDB standard (65 pg); B, yellow cake (equivalent to 3.5 mg, 16 ppb); C, fish sticks (equivalent to 46 mg, 1.2 ppb).

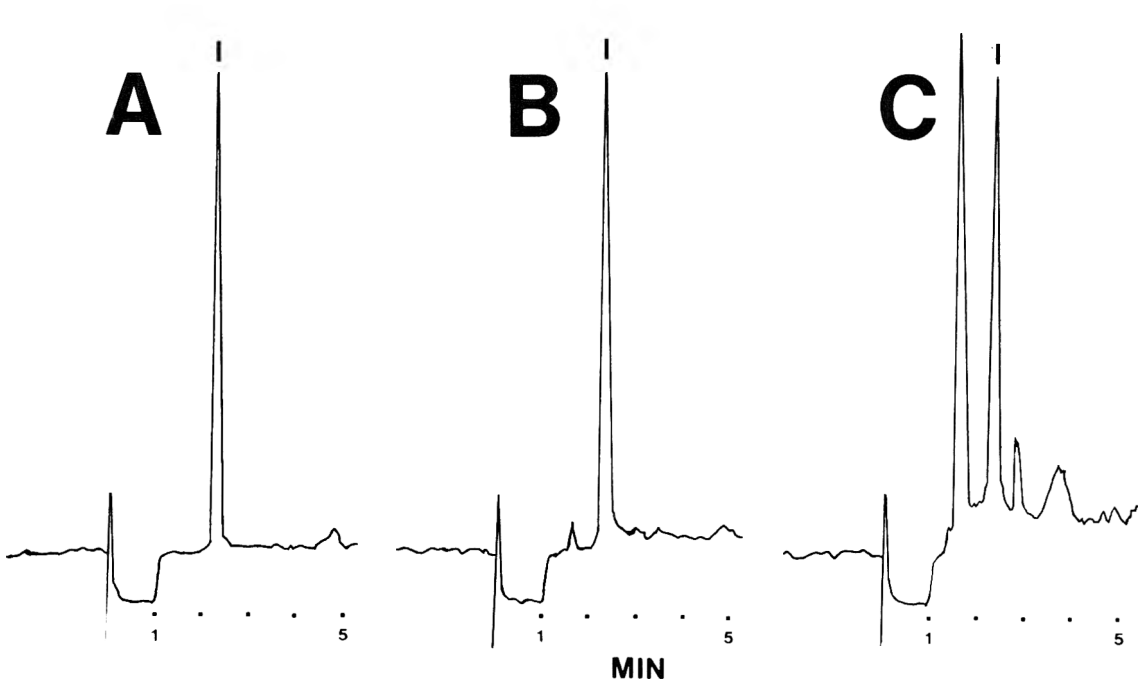

Figure 5. Chromatograms with electrolytic conductivity detection (GC/HECD); 3% SP-1000 on Supelcoport (80–100), 180 × 0.2 cm, 100°C: A, EDB standard (110 pg); B, yellow cake (equivalent to 6.8 mg, 16 ppb); C, fish sticks (equivalent to 88 mg, 1.2 ppb).

and the Tenax TA traps changed at 5 min intervals. Recovery of EDB from the samples representing 10% fat content was essentially complete after only 20 min (96%), whereas the remaining 3 mixtures were identical in that 25 min was required to recover 95–97% of the fortified EDB, indicating that fat content has little effect on extraction efficiency of sparging. A greater dependence of extraction rate appears to be due to the mixing of the sample. At 100°C, fats are liquefied and, with vigorous stirring, form emulsions with water and nitrogen. However, samples such as peanut and pecan kernels, if intact, are more difficult to extract. A sample of chopped pecan meats was divided into those pieces which passed a No. 10 (2 mm) screen and those which did not. Each sample portion was sparged and Tenax TA traps were changed at 10 min intervals. The finely chopped pecan pieces yielded 97% of the EDB found through exhaustive sparging (70 ppb) after

Table 3. GC retention times of several halogenated fumigants[a]

Fumigant	GC column	
	n-Octane[b]	SP-1000[c]
Methylene chloride	0.77	0.50
Carbon tetrachlorice	1.1	0.44
Chloroform	1.2	0.70
1,1,1-Trichloroethane	1.4	0.45
Trichloroethylene	1.4	0.65
Tetrachloroethylene	1.8	0.77
1,2-Dichloroethane (EDC)	1.9	0.87
Chloropicrin	2.3	6.4
1,2-Dibromoethane (EDB)	3.7	2.3

[a]Values expressed in minutes.
[b]*n*-Octane-Porasil C (100–120), 180 × 0.2 cm id, 150°C.
[c]3% SP-1000 on Supelcoport (80–100), 180 × 0.2 cm id, 100°C.

30 min. The larger pieces (up to 1 cm) required 50 min of sparging to extract 98% of incurred EDB.

Several halogenated fumigants were examined by using the GC columns and conditions described under *Apparatus and Reagents*. Retention data are listed in Table 3. As shown in this tabulation, none of the fumigants chromatographed had retention times similar to that of EDB.

In conclusion, this purge and trap method is suitable for the rapid determination of EDB in a variety of table-ready foods; it gives both good reproducibility and good recovery of fortified samples. It is not labor intensive, in that extraction proceeds unattended. The use of highly specialized equipment is not required. Elution of the adsorbent Tenax TA results in a relatively clean, concentrated extract suitable for both low level GC quantitation (0.5 ppb) or GC/MS confirmational analysis.

This method has also been applied to the determination of EDB in a limited number of grains and milled products and the results have been compared to those obtained from the acetone soak procedure (15). Initial results indicate agreement between the 2 methods. Consequently, a more extensive study is being undertaken to determine the feasibility of applying this purge and trap procedure to the determination of EDB in grains and intermediate grain products. Results of this study will be published in this forum.

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Formulas for Calculation of Extraction Volumes for Commonly Used Pesticide Residue Extraction Procedures

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Formulas are presented for the calculation of extraction volume for pesticide residue procedures that use a single extraction with acetone, acetonitrile, or methanol, with or without prior dilution of these solvents with water. These formulas account for the volume change on mixing and for the volume contribution from the soluble sugars sucrose, glucose, and fructose. Results using these formulas are in agreement with the observed volumes of such mixtures within 0.3% throughout the following ranges, expressed as percent water in the mixtures: 15–38% for acetone, 4.8–59% for acetonitrile, and 9.3–33% for methanol.

Pesticide residue extraction procedures that use a single extraction with a water-miscible organic solvent (1–9) depend on a calculated extraction volume. This volume is a function of the amount of solvent used, the water and soluble solids content of the sample, and the volume change that results from mixing the organic solvent with water. Some methods correct for the volume change on mixing by subtracting a constant (1, 3, 6, 7), but this type of correction is accurate only over a narrow range of water content. A correction for the volume change on mixing is not specified in other cases (2, 4, 5), and the volume contribution from soluble solids is not considered in any of these methods.

To facilitate the accurate calculation of extraction volume, we have developed general formulas that are applicable to procedures which employ a single extraction with acetone, acetonitrile, or methanol, with or without prior dilution of these solvents with water. These formulas account for the volume change on mixing and for the volume contribution from the soluble sugars sucrose, glucose, and fructose, which are present in high concentrations in some types of samples.

Experimental

Binary mixtures of water with each of the solvents acetone, acetonitrile, or methanol, with or without added sucrose, lactose, or D-glucose and D-fructose (1 + 1), were formulated. The total volume was read periodically until constant volume was attained (about 1 h). The apparatus for measurement was a volumetric flask, Class A, calibrated to contain, with neck graduated upward from 100 mL to 110 mL in 0.1 mL subdivisions.

Results and Discussion

The results are given in Tables 1–3. The formulas determined from these data are, for acetone:

$$V = 0.98\ V_o + 0.92\ V_w + 0.62\ W, \qquad (1)$$

for acetonitrile:

$$V = 0.995\ V_o + 0.96\ V_w + 0.62\ W, \qquad (2)$$

and for methanol:

$$V = 0.99\ V_o + 0.91\ V_w + 0.62\ W, \qquad (3)$$

where V = total volume, mL; V_o = volume of organic solvent, mL; V_w = volume of water, mL; and W = weight of sugar, g.

These formulas give results in agreement with the observed volumes of such mixtures within 0.24% for acetone, 0.31% for acetonitrile, and 0.25% for methanol throughout the following ranges, expressed as percent by volume of water in the mixture: 15–38% for acetone, 4.8–59% for acetonitrile, and 9.3–33% for methanol. The sugar correction is valid for sucrose, glucose, and fructose levels from zero up to the limit of solubility or phase separation for a particular mixture.

The solubility of sucrose, glucose-fructose (1 + 1), and lactose was investigated to determine the limits of applicability of the sugar correction. The exact limits for precipitation or phase separation were not determined. However, neither precipitation of dissolved sugar nor phase separation occurred for any of the combinations shown in Tables 1–3. Due to the low solubility of lactose in these solvent mixtures, routine correction for the volume contribution from lactose is not required (10, 11).

The volume expansion coefficients for water solutions of glucose, sucrose, and fructose calculated from the solution factors of Browne and Zerban (12) are 0.618, 0.614, and 0.609 mL/g, respectively. These values fall within a narrow range, and because our coefficients for sucrose in water and in water-organic solvent mixtures were not significantly different, we tested glucose and fructose in combination rather than individually. Our value for the expansion coefficient for sucrose in water for 5, 10, and 15% solutions was 0.615 mL/g, which is in good agreement with the value above.

The coefficients in Equation *1* were determined from the data given in Table 1. The water-acetone coefficients are the linear least squares parameters derived from the 11 points without added sugar in the range from 15.75 mL water mixed with 90 mL acetone to 35 mL water mixed with 70 mL acetone. This gave 0.980 mL/mL for the acetone coefficient and 0.922 mL/mL for the water coefficient. The sugar coefficient was then determined from the 13 sugar points by least squares fitting of a direct proportion of the sugar weights to the differences between the observed total volumes and the water-acetone least squares line. This gave 0.607 mL/g for sucrose and 0.620 mL/g for glucose-fructose.

The coefficients in Equation *2* and Equation *3* were determined similarly from the data given in Table 2 and Table 3, respectively. The water-acetonitrile coefficients were derived from the 27 points from 5 mL water mixed with 100 mL acetonitrile to 55 mL mixed with 55 mL. This gave 0.995 mL/mL for the acetonitrile coefficient, 0.958 mL/mL for water, 0.631 mL/g for sucrose, and 0.616 mL/g for glucose-fructose. The water-methanol coefficients were derived from the 16 points from 10 mL water mixed with 100 mL methanol to 35 mL mixed with 75 mL. This gave 0.989 mL/mL for the methanol coefficient, 0.910 mL/mL for water, 0.628 mL/g for sucrose, and 0.620 mL/g for glucose-fructose. The differences between the sugar coefficients are not significant; therefore, the mean, 0.620 mL/g (standard deviation 0.009 mL/g), is used in Equations *1–3*.

Equations *1–3* are useful for several different types of extraction procedures. A common case is that in which pure organic solvent is blended with the sample. For example, for testing frozen sweetened strawberries (75.7% water, 20% sugar), by the method of Luke et al. (3, 7), a 100 g portion of sample is blended with 200 mL acetone, and Equation *1* gives V = 278 mL. The usual calculation, which subtracts 10 mL

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Table 1. Observed and calculated volumes (mL) for mixtures of water, acetone, and various sugars (g)

Water	Acetone	Sugar	Observed volume	Calculated volume[a]	Error, %
0.00	100.00		100.00	98.00	−2.00
5.00	100.00		103.91	102.60	−1.26
10.00	100.00		108.00	107.20	−0.74
11.88	95.00		104.61	104.03	−0.55
15.75	90.00		102.94	102.69	−0.24
20.25	90.00		106.87	106.83	−0.04
20.25	90.00	1.00[b]	107.48	107.45	−0.03
20.25	90.00	3.60[b]	109.00	109.06	0.06
20.25	90.00	3.60[c]	109.16	109.06	−0.09
23.38	85.00		104.80	104.81	0.01
23.38	85.00	2.76[b]	106.40	106.52	0.11
23.38	85.00	2.76[c]	106.58	106.52	−0.06
23.38	85.00	5.52[b]	108.20	108.23	0.03
23.38	85.00	5.53[c]	108.29	108.24	−0.05
26.00	80.00		102.22	102.23	0.10
28.12	75.00	9.37[b]	105.04	105.18	0.13
28.12	75.00	9.38[c]	105.18	105.19	0.01
30.00	80.00		105.94	106.00	0.06
30.00	75.00		101.18	101.10	−0.08
30.00	75.00	5.63[b]	104.58	104.59	0.01
30.00	75.00	5.63[c]	104.61	104.59	−0.02
30.00	75.00	7.50[b]	105.81	105.75	−0.06
30.00	75.00	7.50[c]	105.81	105.75	−0.06
34.00	80.00		109.53	109.68	0.14
33.75	75.00		104.58	104.55	−0.03
35.63	75.00		106.38	106.28	−0.09
35.00	70.00		100.90	100.80	−0.10
40.00	70.00		105.60	105.40	−0.19
43.33	65.00		103.99	103.56	−0.41
55.00	55.00		105.52	104.50	−0.97
60.00	50.00		105.79	104.20	−1.50
75.00	30.00		102.14	98.40	−3.66
100.00	0.00		100.00	92.00	−8.00

[a]Using Equation *1* in text
[b]Sucrose.
[c]D-Glucose and D-fructose (1 + 1).

Table 2. Observed and calculated volumes (mL) for mixtures of water, acetonitrile, and various sugars (g)

Water	Acetonitrile	Sugar	Observed volume	Calculated volume[a]	Error, %
0.00	100.00		100.00	99.50	−0.50
1.00	100.19		101.09	100.65	−0.44
5.00	100.10		104.72	104.40	−0.31
10.00	100.19		109.30	109.29	−0.01
11.00	90.00		100.20	100.11	−0.09
15.00	90.00		103.89	103.95	0.06
20.00	90.00		108.62	108.75	0.12
22.00	80.00		100.60	100.72	0.12
27.00	80.00		105.35	105.52	0.16
31.00	80.00		109.15	109.36	0.19
31.91	75.00	1.13[b]	105.95	105.96	0.01
31.88	75.00	1.12[c]	105.82	105.92	0.10
31.91	75.00	1.88[b]	106.46	106.42	−0.03
31.88	75.00	1.87[c]	106.31	106.39	0.07
32.00	75.00		105.28	105.35	0.06
32.00	70.00		100.32	100.37	0.05
36.00	70.00		104.20	104.21	0.01
40.00	70.00		107.92	108.05	0.12
43.88	65.00		106.66	106.80	0.13
43.88	65.00	1.63[b]	107.72	107.81	0.08
43.88	65.00	1.62[c]	107.72	107.80	0.08
43.88	65.00	3.26[b]	108.77	108.82	0.05
43.88	65.00	3.25[c]	108.73	108.81	0.08
43.88	65.00	4.88[b]	109.74	109.83	0.08
43.88	65.00	4.88[c]	109.70	109.83	0.11
43.00	60.00		100.80	100.98	0.18
52.00	60.00		109.52	109.62	0.09
50.00	55.00		102.63	102.73	0.09
55.00	55.00		107.52	107.53	0.00
60.00	50.00		107.62	107.35	−0.25
65.00	47.00		109.50	109.17	−0.31
65.00	40.00		102.66	102.20	−0.45
70.00	40.00		107.65	107.00	−0.60
100.00	0.00		100.00	96.00	−4.00

[a]Using Equation *2* in text.
[b]Sucrose.
[c]D-Glucose and D-fructose (1 + 1).

Table 3. Observed and calculated volumes (mL) for mixtures of water, methanol, and various sugars (g)

Water	Methanol	Sugar	Observed volume	Calculated volume[a]	Error, %
0.00	110.00		110.00	108.90	−1.00
5.00	105.00		108.94	108.50	−0.40
10.00	100.00		108.20	108.10	−0.09
10.00	100.00	2.50[b]	109.62	109.65	0.03
10.00	100.00	2.50[c]	109.68	109.65	−0.03
14.00	96.00		107.70	107.78	0.07
17.00	93.00		107.41	107.54	0.12
20.00	90.00		107.04	107.30	0.24
20.00	90.00	4.00[b]	109.51	109.78	0.25
20.00	90.00	4.00[c]	109.60	109.78	0.16
25.00	85.00		106.75	106.90	0.14
28.00	82.00		106.59	106.66	0.07
30.00	80.00		106.41	106.50	0.08
32.00	78.00		106.41	106.34	−0.07
33.00	77.00		106.19	106.26	0.07
35.00	75.00		106.08	106.10	0.02
35.00	75.00	6.00[b]	109.95	109.82	−0.12
35.00	75.00	6.00[c]	109.78	109.82	0.04
40.00	70.00		106.12	105.70	−0.40
44.00	66.00		106.00	105.38	−0.58
50.00	60.00		106.06	104.90	−1.09
55.00	55.00		106.16	104.50	−1.56
70.00	40.00		106.82	103.30	−3.30
80.00	30.00		107.54	102.50	−4.69
90.00	20.00		108.41	101.70	−6.19
100.00	10.00		109.25	100.90	−7.64
110.00	0.00		110.00	100.10	−9.00

[a]Using Equation *3* in text.
[b]Sucrose.
[c]D-Glucose and D-fructose (1 + 1).

for volume contraction and makes no allowance for the volume contribution from soluble sugars, gives 266 mL, 4.3% lower than the volume by Equation *1*. Without the sugar correction, Equation *1* would also give 266 mL, but in general the subtraction of a constant to account for volume contraction is accurate over a narrower range of water-to-organic solvent ratios than are Equations *1–3*.

A second type of procedure is that in which a premixed water-organic solvent mixture is blended with the sample. Equations *1–3* apply, if the percent by volume of water in the premixed solvent and in the final mixture are both within the ranges previously given. The volume change has already occurred in the premixed solvent; therefore, correction coefficients are applied only to added substances. For example, for analysis of immature lima beans (67.5% water) by the method of Bertuzzi et al. (2), a 25 g portion of sample is blended with 350 mL 35% water in acetonitrile, for which Equation *2* gives V = 350 + 0.96(0.675)(25) = 366 mL. The current protocols for this procedure (6, 7) do not specify how the extraction volume is to be determined for products of water content between 10 and 75%.

A third type of procedure is exemplified by a method (9) in which, as applied to high moisture fatty products, acetonitrile is blended with a sample charge, 20 g alumina, and sufficient extra water to give 20% water in 350 mL total volume. According to these specifications the total amount of water is 0.20(350) = 70 mL, and from Equation *2* the amount of acetonitrile is (350 − 0.96(70))/0.995 = 284 mL. The only variable is the amount of extra water required, which is 70 mL minus that in the sample charge. An optimized version of this procedure (13) employs 50 g deactivated alumina containing 16–19% water. Uncertainty in the amount of water bound or released by the alumina in either version makes the volume calculation problematical.

These equations are also useful for cases in which a change is made in the specified charge size or amount of water added in a method. For example, to prevent phase separation, method **29.011(c)** and PAM 212.13d (7) specify the use of a heated water-acetonitrile mixture for extraction of products containing 15–30% sugar. A simpler approach, which avoids the use of the heated solvent, is to decrease the sample charge size to reduce the sugar below 15 g, add extra water to maintain the water-acetonitrile ratio, and use Equation *2* to calculate the extraction volume. The current formulas (6, 7) for calculation of extraction volume are not readily adaptable to such adjustments.

Conclusion

Equations *1–3* provide a means for calculating an accurate extraction volume and are applicable to a broad range of samples and methods.

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ALCOHOLIC BEVERAGES

Radiocarbon ^{14}C Differentiation of Sparkling and Carbonated Wines

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Specific ^{14}C-activities, percent of modern ^{14}C-activity, and calculated percent of fermentation CO_2 are presented for CO_2 contained in commercial sparkling wines, labeled as champagne or produced by the bulk (charmat) process. These data are given for the production years 1976–1982. The survey encompassed effervescent wines produced in Spain, Italy, West Germany, California, and New York. Addition of synthetic CO_2 to approximately 40 samples represented as sparkling wines was indicated by low ^{14}C-activities of CO_2 in these wines. Data for ^{14}C-activity were also presented for the ethanol distilled from sparkling wines for the years 1977–1980. In all cases, the ^{14}C-activity of ethanol was appropriate to the year of vintage.

The labeling of wines in the United States is regulated, and definitions clearly differentiate still wine, sparkling wine, and carbonated wine. According to regulation, a still wine cannot contain more than 0.392 g CO_2/100 mL (1, 2). Wines that contain more than this amount of CO_2, and that have an alcohol content of ≤ 14% by volume, are considered sparkling wines or artificially carbonated wines, depending on the source of the CO_2.

Effervescent wines are further subdivided into 3 types, defined by the production process: champagne, bulk (charmat) process, and artificially carbonated. Summaries of the general process of fermentation and wine production have been provided by Rose (3) and Amerine (4). The production of sparkling wine in California is described in detail by Berti (5).

Production Methods

The champagne process consists of a 2-stage fermentation. The first stage involves fermenting the grape juice to dryness: converting all the sugar to ethanol. This is followed by removal of the yeast cells and the addition of a sweetener, such as sugar or corn syrup, and yeast to the wine. The bottle is then capped, and secondary fermentation is allowed to proceed. To be classed as a champagne, the container must be a glass container of ≤ 1 gallon in volume (6).

Bulk (charmat) process is similar to the champagne process, but the container is larger than 1 gallon (7).

Carbonated wines are produced by directly pressurizing the still wine with a "food grade" CO_2, which is the cheapest method for producing effervescent wine (8).

Food grade CO_2 is usually derived from one of the following sources: (a) chemical plants producing ammonia which yields CO_2 as a byproduct; (b) lime-kiln operations in which calcium carbonate is thermally decomposed to calcium oxide and byproduct CO_2; (c) the manufacture of sodium phosphate in which phosphoric acid reacts with sodium carbonate to produce sodium phosphate and a CO_2 byproduct; and (d) CO_2 gas wells.

All 4 sources yield food grade CO_2 devoid of ^{14}C-activity because the carbon is of ancient geological origin.

For labeling purposes, wine pressurized with food grade CO_2 is artificially carbonated and must be labeled as a carbonated type of effervescent wine. The tax for artificially carbonated wine is less per gallon than that for a sparkling wine produced by the champagne or charmat process.

Dunbar (9) has studied the stable carbon isotopes of CO_2 in sparkling wines to attempt to determine its source. This approach met with good success. The ^{14}C-activity of agricultural products has been documented for the past 30 years (J. T. Swan et al., Krueger Enterprises, Inc., 1984, private communication) and has been used to determine the vintage of wines (H. W. Krueger, Krueger Enterprises, Inc., 1984, private communication). This information about ^{14}C-activity in agricultural products can be used effectively to determine if extraneous CO_2 has been added to a sparkling wine.

The radioactive isotope of carbon, ^{14}C, is continuously being produced in the earth's atmosphere. Through a series of exchange, assimilation, and other processes of carbon fixation, all living organic matter contains ^{14}C. With time, ^{14}C decays at a certain rate according to the law of radioactive decay. This allows carbonaceous compounds to be dated by measuring the remaining ^{14}C.

Krueger has demonstrated that the ^{14}C-activity of ethanol derived from the fermentation process is not much different from that of the original sugars. The CO_2 derived from fermentation also has essentially the same ^{14}C-activity as that of the agricultural product from which it was derived. Using these data from agricultural products, we conclude that the CO_2 from fermentation of grape juice or other agricultural

Table 1. Naturally carbonated sparkling wines for vintage years 1976, 1979, and 1978[a]

Sample	Total CO_2, mg/100 mL	Specific ^{14}C-activity, dpm/g C	% of modern[b]	Calcd % from fermentation[c]
1	ND[d]	17.98	132.6	97
2	ND	18.70	137.9	103
3	572	18.32	135.1	99

[a]Vintage stated on label.
[b]Relative to 95% of ^{14}C-activity of NBS Oxalic Acid Reference Standard.
[c]Based on data of Swan et al. (Krueger Enterprises, Inc., 1984, private communication)
[d]ND = Not determined.

Table 2. Naturally carbonated sparkling wine for vintage year 1980[a]

Sample	Total CO_2, mg/100 mL	Specific ^{14}C-activity, dpm/g C	% of modern[b]	Calcd % from fermentation[c]
4	ND[d]	18.59	137.1	106
5	938	17.36	128.0	99
6	ND	17.30	127.6	98
7	ND	18.46	136.1	105
8	830	17.22	127.0	98
Mean		17.79	131.2	101.2
Std dev.		0.68	5.0	4.0
3 Std dev.		2.04	15.0	11.9

[a]Vintage assumed to be year before purchase unless vintage stated on label.
[b–d]See footnotes *b–d*, Table 1.

[1]Present address: Geochron Laboratories Division, Krueger Enterprises, Inc., 24 Blackstone St, Cambridge, MA 02139.
[2]Present address: U.S. Bureau of Alcohol, Tobacco and Firearms, 1401 Research Blvd, Rockville, MD 20850.

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Table 3. Naturally carbonated sparkling wine for vintage year 1981[a]

Sample	Total CO_2, mg/100 mL	Specific ^{14}C-activity, dpm/g C	% of modern[b]	Calcd % from fermentation[c]
9	1069	17.89	131.9	104
10	938	17.10	126.1	99
11	616	17.70	130.5	102
12	1047	17.78	131.1	103
13	ND[d]	17.45	128.7	101
14	819	18.21	134.3	105
15	731	17.82	131.4	103
16	589	18.58	137.0	108
17	931	17.33	127.8	100
18	804	17.03	125.6	99
19	637	17.13	126.3	99
20	1024	17.84	131.6	103
21	ND	17.45	128.7	101
22	ND	17.38	128.2	101
23	ND	16.95	125.0	98
24	ND	17.13	126.3	99
25	ND	16.96	125.1	98
26	ND	17.49	129.0	101
27	ND	17.00	125.4	98
28	ND	16.88	124.5	98
Mean		17.45	128.7	102
Std dev.		0.46	3.67	2.78
3 std dev.		1.38	11.0	8.34

[a]See footnote a, Table 2.
[b–d]See footnotes *b–d*, Table 1.

Table 4. Naturally carbonated sparkling wines for vintage year 1982[a]

Sample	Total CO_2, mg/100 mL	Specific ^{14}C-activity, dpm/g C	% of modern[b]	Calcd % from fermentation[c]
29	978	17.03	125.3	100
30	899	16.69	123.1	98
31	945	16.65	122.8	98
32	847	17.74	130.8	100
33	903	17.37	128.1	100
34	981	16.77	123.7	99
Mean		17.04	125.6	99.2
Std dev.		0.43	3.20	0.98
3 std dev.		1.31	9.59	2.95

[a]See footnote a, Table 2.
[b,c]See footnotes *b*, c, Table 1.

sugars of a specified year should have the same ^{14}C-activities as those for agricultural products of the same year, determined by Swan et al.

Carbon dioxide recovered from champagne or from other sparkling wines which has ^{14}C activities significantly lower than the values presented by Swan et al. must be considered partly or wholly derived from a fossil fuel source.

Determination of the ^{14}C-activity of dissolved CO_2 has enabled the authors to differentiate sparkling wines produced by the champagne or bulk (charmat) process and those produced by the direct addition of food grade CO_2.

Experimental

Samples Analyzed

Bottles of sparkling wine were purchased in stores or directly from suppliers. In all instances, contents were labeled as sparkling wines, and in some, vintage was specified on the label.

Total CO_2 Determination

The sample bottle was cooled to below O°C. A needle, fitted with a valve and dispersion tube, was inserted through the cork of the bottle. The valve was opened slowly, and gas was allowed to bubble through the fritted glass tube into a flask of solution containing excess of NaOH. The bottle was gently heated in a water bath until bubbling stopped and then cooled to room temperature, and the needle was removed. Ten mL of 50% NaOH solution was injected into the bottle and was mixed well with the sample.

The contents of sample bottle and flask were combined in a graduated cylinder. Both containers were rinsed with CO_2-free water, and the rinses were added to the graduated cylinder. The volume was recorded.

Carbon dioxide was measured by following AOAC **11.066–11.067** (10). The total CO_2 in the wine sample was calculated and reported as mg/100 mL.

Sample Preparation for Isotopic Analyses

The contents of a second unopened bottle of sparkling wine were emptied into a vacuum flask, using a stainless steel needle to puncture the original closure. The total dissolved CO_2 was thus retained in the sample. The sparkling wine was acidified to completely remove the dissolved CO_2. Recovered CO_2 was purified by repetitive distillation and freezing, using liquid nitrogen. Carbon dioxide was then converted to methane, and the ^{14}C-activity was measured, using the procedures described by Swan et al. and Krueger.

Determination of ^{14}C Activity

The ^{14}C-activity of the sample was determined by low-level gas proportional counting of methane prepared from the carbon in the CO_2 sample. Massive shielding and anticoincidence

Table 5. Artificially carbonated sparkling wines for vintage year 1980[a]

Sample	Total CO_2, mg/100 mL	Specific ^{14}C-activity, dpm/g C	% of modern[b]	Calcd % from fermentation[c]
35	839	9.09	67.0	52
36	934	9.48	69.9	54
37	938	5.52	40.7	31
38	980	5.07	37.4	29
39	895	4.41	32.5	25
40	942	7.80	57.5	44
41	895	5.07	37.4	29
42	832	2.75	20.3	16
43	1004	9.59	70.7	55
44	1038	10.51	77.5	60
45	963	13.34	98.4	76

[a]See footnote a, Table 2.
[b,c]See footnotes *b*, *c*, Table 1.

Table 6. Artificially carbonated sparkling wines for vintage year 1981

Sample	Total CO_2, mg/100 mL	Specific ^{14}C-activity, dpm/g C	% of modern[b]	Calcd % from fermentation[c]
46	801	9.00	66.4	52
47	886	10.18	75.1	59
48	871	9.21	67.9	53
49	944	4.66	34.4	27
50	889	5.10	37.6	30
51	425	1.88	13.9	11
52	965	0.91	6.7	5
53	990	0.92	6.8	5
54	957	0.62	4.5	4
55	894	0.65	4.8	4
56	901	2.81	20.7	16
57	859	2.48	18.3	14
58	944	2.32	17.1	13
59	930	0.26	1.9	1
60	918	0.35	2.6	2
61	908	15.00	110.6	87
62	521	6.01	44.3	35

[a]See footnote a, Table 2.
[b,c]See footnotes *b*, c, Table 1.

Table 7. Artificially carbonated sparkling wines for vintage year 1982[a]

Sample	Total CO_2, mg/100 mL	Specific ^{14}C-activity, dpm/g C	% of modern[b]	Calcd % from fermentation[c]
63	1065	3.57	26.3	21
64	918	13.14	96.9	77
65	620	6.40	47.2	38
66	594	7.58	55.9	45
67	681	8.33	61.4	49
68	677	7.13	52.6	42
69	697	8.04	59.3	47
70	786	7.25	53.5	43
71	987	12.22	90.1	72
72	935	12.92	95.3	76

[a]See footnote a, Table 2.
[b,c]See footnotes *b*, c, Table 1.

circuitry are essential for useful results. Counter backgrounds were determined by regular analysis of methane prepared from 300 million-year-old marble which contains no ^{14}C-activity. Counter efficiencies were determined by measuring the count rate of methane prepared from the NBS (National Bureau of Standards) oxalic acid ^{14}C reference standard. With appropriate equipment and care, a ^{14}C detection limit of 0.2 disintegrations/min/g of carbon (dpm/g C) can be readily achieved. Because specific ^{14}C-activities of natural carbon are low, counting times of 1000 to 1500 min are required to achieve satisfactory precision.

Results may be reported as specific ^{14}C-activities (dpm/g C) or as percent of the "modern" ^{14}C-activity. The modern ^{14}C-activity is defined by international agreement among radiocarbon dating laboratories as the unaltered atmospheric ^{14}C-activity of the year 1950 A.D., and as 95% of the ^{14}C-activity of the NBS Oxalic Acid ^{14}C Reference Standard. Careful studies have concluded that this modern ^{14}C activity is 13.56 dpm/g C (11). Addition to the atmosphere of CO_2 from fossil fuel and nuclear weapons testing have made other modern definitions not meaningful. Detailed discussion of these problems and a summary of annual ^{14}C-activities since 1950 have been described by Swan et al.

Results and Discussion

Eighty-two samples of sparkling wines were analyzed to determine the ^{14}C-activity of CO_2 dissolved in the wine. Sparkling wines from Germany, Spain, Italy, and the United States were analyzed. For samples which had no vintage stated on the label, we assumed production occurred in the year before the date of purchase. For each sample analyzed, the ^{14}C-activity measured for dissolved CO_2 was compared to the ^{14}C-activity expected for agricultural products from that year, determined by Swan et al. In a few cases, the ^{14}C-activities were higher than expected, possibly because the sparkling wine was produced earlier than we assumed, or the sugar used in the primary or secondary fermentation was produced a year or more before the year of vintage. Total CO_2 (mg/100 mL) was determined for most of the wines which were found to be properly classified as sparkling wines.

Table 1 shows results for 3 samples of naturally carbonated sparkling wines produced in 1976, 1978, and 1979. Table 2 shows results for 5 samples of naturally carbonated sparkling wines produced in 1980. Table 3 shows results for 20 samples of naturally carbonated sparkling wines produced in 1981. Table 4 shows results for 6 samples of naturally carbonated sparkling wines produced in 1982.

Eleven sparkling wines produced in 1980 gave values of 16–76% of fermentation CO_2 (Table 5). Those wines which lie outside the 3 standard deviation (SD) limits are considered artificially carbonated, and should be labeled as carbonated wines. Seventeen sparkling wines produced in 1981 gave values of 1–87% of fermentation CO_2 (Table 6). These wines lie outside the 3 SD limits and are considered artificially carbonated; they should therefore be labeled and taxed accordingly. Ten sparkling wines produced in 1982 gave values of 21–77% of fermentation CO_2 (Table 7). These wines also lie outside the 3 SD limits and are considered artificially carbonated; they should be labeled and taxed accordingly.

To assure that ethanol in sparkling wines had ^{14}C-activity consistent with the year of production, we analyzed 10 samples. Table 8 results indicate, in all cases, that ethanol distilled from sparkling wines gives a correct modern ^{14}C-activity, and was derived from fermentation of sugars of the stated vintage. The data compare favorably with those for ethanol in sake reported by Martin et al. (12).

The dissolved CO_2 in 6 of the samples in Table 8 was also analyzed for ^{14}C activity. All sample analyses indicated major additions of fossil CO_2, despite the fact that the ethanol is completely modern. Clearly, these 6 samples are artificially carbonated "sparkling wines." It is likely that they were made by carbonating still wine, using fossil CO_2 to produce the desired effervescence. There is no question that these 6 samples as well as the other samples presented in Tables 5, 6, and 7 should be labeled carbonated wine under the terms of the applicable federal regulations.

Table 8. ^{14}C-Activities of ethanol and CO_2 from California sparkling wines

Sample	Stated or presumed vintage[a]	Specific ^{14}C-activity ethanol, dpm/g C	% of modern for ethanol[b]	% of ethanol from fermentation[c]	% of CO_2 from fermentation[c]
73	1979	17.40	128.3	98	ND[d]
74	1979	17.62	130.0	99	ND
75	1977	18.01	132.8	97	ND
76	1978	17.88	131.9	98	ND
77	1980	17.22	127.0	98	21
78	1980	18.73	138.1	107	69
79	1980	17.95	132.4	102	52
80	1979	17.38	128.2	98	49
81	1980	17.26	127.3	98	54
82	1980	17.25	127.2	98	31

[a]See footnote a, Table 2.
[b–d]See footnotes *b–d*, Table 1.

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DECOMPOSITION IN FOODS

Liquid Chromatographic Determination of Hypoxanthine Content in Fish Tissue

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A liquid chromatography (LC) method for determining the hypoxanthine content in fish tissues has been developed. Hypoxanthine is extracted with 0.6M perchloric acid, and determined by LC on a reverse phase microparticulate column with UV absorbance detection. The mobile phase is 0.01M potassium phosphate buffer (pH 4.5). The percent relative standard deviation for measurements by the recommended method was less than 7% with a detection limit of 10 ng. Recoveries of hypoxanthine added to various fish tissues were better than 90%. The operational errors, interferences, and recoveries for spiked samples have been investigated and compare favorably with an established xanthine oxidase enzyme method. The described LC method is simple, rapid, and specific for measuring hypoxanthine content in various fish tissues. Some post-mortem studies have indicated the method may also be used for the determination of adenosine monophosphate, inosine monophosphate, and inosine.

The routine monitoring of fish quality as it is received at the processing plant or marketplace would be very desirable. The development of a simple, rapid, inexpensive, and fairly reliable test on which to base quality assessments is essential for this to occur. The use of hypoxanthine in fish quality assessments provides several distinct advantages over other objective chemical tests such as trimethylamine (TMA), dimethylamine (DMA), total volatile bases (TVB), and others which essentially measure bacterial spoilage (1). The accumulation of hypoxanthine in fish tissue reflects the initial phases of autolytic deterioration (2–6) as well as later contributions through bacterial spoilage (Figure 1). Hypoxanthine concentrations are not affected by heat processing (7) or irradiation (8) and are very useful as quality indicators in certain freshwater fish which contain little or no trimethylamine oxide (TMAO) (9) and thereby render TMA analysis useless. Hypoxanthine concentrations have already proven to correlate very well with eating quality in a number of fish species (3, 4, 8, 10, 11).

The primary enzymatic method now used for the monitoring of hypoxanthine levels in fish tissue is the one recommended by the Analytical Methods Committee (12). This is essentially the same method as that described by Jones et al. (3) where xanthine oxidase (XO) is used to rapidly convert hypoxanthine to xanthine and subsequently to uric acid (Figure 1). Uric acid is then measured spectrophotometrically at 290 nm. Modifications to the method have lead to automation making use of a redox indicator dye (13), test paper strips (14), and colorimetric enzyme assay procedures. However, difficulties have been experienced because of nonspecific measurements, time-consuming procedures, and poor reproducibility.

Hypoxanthine has been isolated by ion-exchange column chromatography (15, 16) and thin layer chromatography (TLC) (17). Unfortunately, the ion-exchange method is laborious, and the TLC method is only semiquantitative. With various modifications on reverse phase LC (18–20), an LC method for determining hypoxanthine content in various fish tissues has been developed. A comparison of this method with the Analytical Methods Committee recommended method (12) is also described.

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METHOD

Apparatus

(a) *LC pump.*—Model 100A (Beckman Instruments, Inc., Berkeley, CA).

(b) *LC injector.*—Model 7161 (Rheodyne, Inc., Cotati, CA) loop injector, fitted with 20 μL loop.

(c) *Detector.*—SF 769Z variable wavelength UV detector operated at 254 nm (Kratos, Inc., Westwood, NJ).

(d) *Recorder.*—Model 7100BM strip chart recorder (Hewlett Packard, Palo Alto, CA).

(e) *LC column.*—RP-8 MPLC reverse phase analytical column, 4.6 mm id × 10 cm, 10 μm particle size, fitted with RP-8 MPLC guard column, 4.6 mm id × 3 cm, 10 μm particle size (Brownlee Labs, Inc., Santa Clara, CA).

(f) *Blender.*—Virtis Model 23 (The Virtis Co., Gardiner, NY), or equivalent.

(g) *Aqueous sample clarification kit.*—PN 26865, used to remove fine particles of 0.45 μm or greater (Waters Associates, Inc., Milford, MA).

Reagents

(a) *Extracting solvent.*—0.6M perchloric acid. Add 32.3 mL concentrated perchloric acid (60%) to 500 mL volumetric flask and dilute to volume with water.

(b) *Potassium hydroxide, phosphate buffer pH 7.6.*—Dissolve 8.16 g KH_2PO_4 in ca 60 mL water and adjust to pH 7.6 with 50% KOH. Dilute to 100 mL with water.

(c) *50% KOH.*—Dissolve 50 g KOH in 50 mL water and cool to room temperature.

(d) *LC mobile phase.*—0.01M potassium phosphate buffer pH 4.5. Dissolve 1.36 g KH_2PO_4 in ca 400 mL double-distilled water, adjust to pH 4.5 with KOH or H_3PO_4 as necessary, and dilute to 1 L.

(e) *Standards.*—Hypoxanthine (Hx), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), and inosine (INO) were purchased in purified form (Sigma Chemical Co., St. Louis, MO).

(1) Stock solutions.—Prepare individual standards for LC by dissolving 0.010 g Hx, INO, ATP, AMP, and IMP, respectively, in 40 mL water. Dilute to mark in 50 mL volumetric flasks. Keep individual standards frozen until required; thaw and dilute 1:10 before use. *(2) Mixed standards (working solutions).*—2.5, 5.0, 7.5, and 10 μg/mL of IMP and Hx and 5.0, 10, 15, and 20 μg/mL, respectively, of AMP and INO. Pipet 0.25, 0.50, 0.75, and 1.0 mL of each stock solution of IMP and Hx into separate 20 mL volumetric flasks. Add 0.50, 1.0, 1.5, and 2.0 mL, respectively, of each stock solution of AMP and INO to the flasks. Dilute to mark with water. Mixed standard solutions are stable for at least 2 weeks when stored at 0–4°C.

(f) *Interfering compounds.*—Stearic acid and TMAO (Eastman Kodak Co., Rochester, NY), lecithin (Sigma

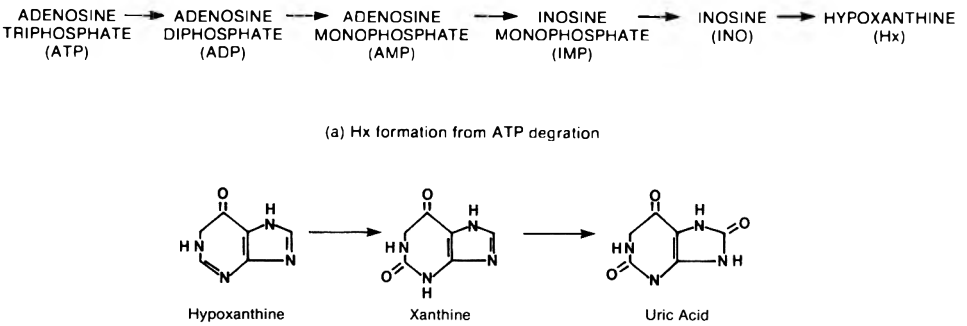

Figure 1. Reaction pathways (a) for formation of Hx from ATP degradation and (b) for XO conversion of Hx to Uric acid.

Table 1. Hypoxanthine content in various standards and fish samples as determined by recommended LC method and XO procedure

Sample	Hypoxanthine, μmoles/g ± % RSD		
	LC method (A)	XO method (B)	B/A
Standard[a] 1	0.020 ± (6.5)	—	—
2	0.043 ± (6.9)	—	—
3	0.088 ± (6.4)	—	—
4	0.126 ± (2.9)	—	—
5	0.163 ± (2.1)	—	—
6	—	0.241 ± (5.9)	—
7	—	0.459 ± (6.0)	—
8	0.588 ± (2.2)	—	—
9	—	0.941 ± (6.2)	—
10	1.50 ± (2.3)	—	—
11	—	1.93 ± (5.0)	—
12	—	2.73 ± (11)	—
		11.2 ± (11)	
14	15.6 ± (6.9)	—	—
Crabmeat 1[b]	0.072 ± (6.7)	0.124 ± (110)	1.7
2[c]	1.50 ± (7.7)	1.24 ± (7.5)	0.83
Scallop muscle 1[b]	2.16 ± (5.1)	2.07 ± (11)	0.96
2[c]	3.18 ± (2.3)	2.89 ± (1.0)	0.91
Cod fillet 1[b]	1.25 ± (6.6)	1.25 ± (11)	1.0
2[c]	1.93 ± (6.1)	2.13 ± (10)	1.1

[a]Standard concentrations are μmoles/mL; 6–9 determinations per standard.
[b]6 determinations.
[c]3 determinations. Samples analyzed after an additional 3 days at 4°C.

Chemical Co.), cystine, cysteine, *tert*-butylhydroxyanisole (TBHA), and hemoglobin (K&K Laboratory Inc., Plainview, NY), TMA (J.T. Baker Chemical Co., Phillipsburg, NJ), *tert*-butylhydroquinone (TBHQ, Pfaltz and Bauer, Inc., Stamford, CT), palmitic acid, thiourea, DMA, sorbitol, sucrose, lactic acid, $MnSO_4$, $CuSO_4$, NaCl, and $FeSO_4$ (Fisher Scientific, Fair Lawn, NJ) were used without further purification.

Fish Sampling

Atlantic cod *(Gadus morhua)*, scallop *(Placopecten magellanicus)*, and crab *(Chionecetes opilio)* were used in this investigation. Cod (average weight 1.1 ± 0.3 kg, average length 20.8 ± 2.5 cm) which had been obtained from the laboratory aquarium were killed, bled, gutted, and held on ice for 5 days before sampling. Fillets were removed (skin off) and the meat samples were homogenized in a food processor and stored at −40°C in freezer bags until analysis. Six fish were used in the study. A sample (1 kg) of "fresh" (2–3 day old) scallop adductor muscles and a sample of fresh frozen crab body and leg meat were obtained from a local fish market. Scallop and crab meat samples were homogenized and stored in a manner similar to that for cod.

Sample Preparation

Without thawing, place 5 g frozen, finely chopped fish meat sample in blender flask with 50 mL 0.6M perchloric acid. Blend 2 min at maximum speed. Suction-filter flask contents through Whatman No. 1 paper, using small amount of water to rinse blender flask. Mix filtrate well and note volume carefully (V_1). Transfer 1.0 mL aliquot of filtrate to screw-top test tube containing 1.0 mL KOH phosphate buffer (pH 7.6). Mix the solution, cool to 0–4°C, then filter through aqueous clarification kit. Inject aliquots (V_2) of neutralized filtrate directly for LC analysis. Dilute with water if necessary *(D)*.

LC Determination

Set flow rate at 1.0 mL/min and let column and detector equilibrate 20–25 min. Inject 10 μL aliquots of each mixed standard solution. Determine absorbances of various nucleotides from peak heights recorded at 254 nm. Plot peak height vs μg injected to provide standard curve. Inject 10 μL aliquots of appropriately diluted sample extracts. Prepare standard curves at least twice per day to assure accurate quantitation.

Calculations

Hypoxanthine content in fish tissue is calculated from the following equation:

$$\text{Hx content (μmoles/g)} = KPV_1D/HV_2W \qquad (1)$$

where P = peak height (mm); H = slope of standard curve (mm/μg); V_1 = total volume of perchloric extract plus wash (mL); D = dilution factor of neutralized extract before LC; V_2 = injection volume for LC (μL); W = weight of sample (g); and K = 14.71 (μL) (μmoles)/(μg)(mL), a constant which takes into account the 1:1 dilution during neutralization.

Based on our recommended procedures, the slope of the standard curve is 1435 ± 30 (mm/μg), the dilution factor *(D)* is 10, and injection volume (V_2) is 10 μL. Thus, Equation 1 can be simplified as Equation 2:

$$\text{Hx content (μmoles/g)} = (6.97 \times 10^{-4})(KPV_1/W) \qquad (2)$$

IMP, AMP, and INO content in fish tissues are calculated using Equation 1 but with the following K values: 5.75, 5.76, and 7.46 (μL) (μmoles)/(μg)(mL), respectively.

Confirmatory Test

Extract fish muscle under the conditions described and quantitate by LC. Apply xanthine oxidase treatment (12) to sample extract (incubation at 37°C for 30 min), and then reinject aliquot equivalent to original LC injection. Check for the disappearance of hypoxanthine peak.

Table 2. Percent recovery of hypoxanthine added to various fish tissue samples

Sample	Hypoxanthine added, μmoles/g	Recovery, % LC method	Recovery, % XO method
Cod fillet 1	1.47	86.0	85.1
2	12.3	93.9	82.9
Scallop muscle 1	1.47	97.7	86.0
2	12.5	91.3	84.6
Crabmeat 1	1.47	107	104
2	12.5	78.6	76.1
Spiked water[a] 1	1.47	94.7	88.2
2	0.50	93.6	85.9

[a]Indicates number of μmoles total added to 5.0 mL water.

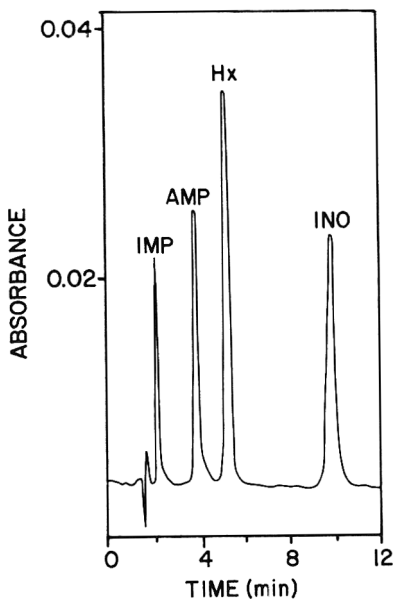

Figure 2. LC UV (254 nm) trace of separation of standard IMP, Hx (50 ng each), AMP and INO (100 ng each) run on a Brownlee MPLC RP-8 (10 μm, 4.6 mm id × 10 cm, developed with 0.01M KH_2PO_4 buffer, pH 4.5 at 1.0 mL/min) reverse phase analytical column.

Comparative Test (XO)

The method is that described by the Analytical Methods Committee (12): the sample is extracted with 0.6M perchloric acid, the extract is filtered, and a portion is neutralized. The neutralized portion is cooled to 0–4°C and centrifuged, and the pH is readjusted if necessary to pH 7–7.6. Freshly diluted (0.05M phosphate buffer pH 7.6) xanthine oxidase enzyme is added to aliquots of this neutralized fraction dissolved in phosphate buffer (0.25M, pH 7.6). Blanks consisting of aliquots of the neutralized fraction minus the enzyme, and distilled water blanks with and without the enzyme, are also analyzed. All samples and blanks are adjusted to a constant volume with water. Samples and blanks are incubated 30 min at 37°C, cooled, and read at 290 nm. Absorbances of sample solutions are compared with those of standards.

Results and Discussion

The percent relative standard deviations of hypoxanthine results determined by the recommended LC method over a range of standard and sample concentrations, compared with the XO method, are presented in Table 1. Replicate analysis of standard solutions indicated good reproducibility for both methods over the range of concentrations studied (Table 1). The LC method, however, has the advantage of being an order of magnitude more sensitive for measuring lower concentrations of hypoxanthine and appears to be slightly more reproducible overall, especially at the higher concentrations (7% vs 11% for LC and XO, respectively; Table 1).

Replicate LC analysis of homogeneous fish tissue samples gave hypoxanthine levels with means of 1.25, 2.16, and 0.072 μmoles/g for cod, scallop, and crab samples, respectively (Table 1). Percentage relative standard deviations were 6.6, 5.1, and 6.7%, respectively. Samples held an additional 3 days at 4°C gave values of 1.93, 3.18, and 1.50 μmoles/g with percent relative standard deviations of 6.1, 2.3, and 7.7% for cod, scallop, and crab, respectively (Table 1). Generally the results obtained by the XO method were in close agreement. A ratio obtained by dividing the values obtained by the XO method (B) by those obtained by the LC method (A) were in the range 0.80 to 1.1 (Table 1) with the exception of the initial testing of the crabmeat which had an observed ratio of 1.7. The initial XO value of 0.124 μmole/g for crabmeat also had a % relative standard deviation of 110%. The large variation can be explained by the fact that the observed value of 0.124 μmole/g is very near the detection limit for the XO method and thus relatively large variations can be expected. As the Hx levels increased in the crabmeat on day 3 of storage at 4°C, the B/A ratio had decreased to 0.83 and the percent relative standard deviation had decreased to 7.5%.

Table 2 gives the percentage recovery of hypoxanthine from spiked samples of cod fillet, scallop muscle, crabmeat, and water after the described extraction, cleanup, and LC measurement. The mean recoveries of added levels of 1.47 μmoles/g were 86.0, 97.7, and 107% for cod, scallop, and crab, respectively. Similarly, added levels of 12.5 μmoles/g showed mean recoveries of 93.9, 91.3, and 78.6%. Samples analyzed by the XO method showed comparable results (Table 2) but recoveries were generally a few percentage points lower. Mean recoveries of hypoxanthine from water samples spiked at levels of 0.50 and 1.47 μmoles/g showed recoveries of 93.6 and 94.7%, respectively, when analyzed by the LC method vs 85.9 and 88.2% for the XO method. The above values compare quite favorably with those reported by Worthesen et al. (19) where 0.5 μmole hypoxanthine/g added to whitefish muscle yielded recoveries of 92.5%. The slightly lower recoveries reported for the XO method, especially with the higher level spike, may be the result of incomplete conversion of hypoxanthine to uric acid during the incubation stage of the procedure.

IMP, Hx, AMP, and INO are easily separated from one another, and completely eluted in 11 min from the 10 cm RP-8 reverse phase column with 0.01M potassium phosphate buffer (pH 4.5) at 1.0 mL/min (Figure 2). ATP and ADP were not well separated from each other or the solvent front and were therefore not included in the mixed standards. In any event, ATP in many marine species is rapidly degraded to IMP during or shortly after the death struggle by the partial dephosphorylation and deamination of ATP (1, 16). The measurement of peak heights for hypoxanthine quantitation was very reproducible: 9 replicate injections for each level of 25, 50, 75, and 100 ng/injection showed a maximum variation of only 6.4%. Retention times were also very stable, varying less than 4%. Quantitation based on peak areas gave variations of less than 5% but required more sophisticated hardware. Linear responses were observed over the range of concentrations tested and the detection limit was approximately 10 ng. The RP-8 reverse phase column was remarkably stable and exhibited no changes in response or retention times over a 4-month-period of heavy usage. Other columns such as the widely used C_{18} reverse phase columns will also sep-

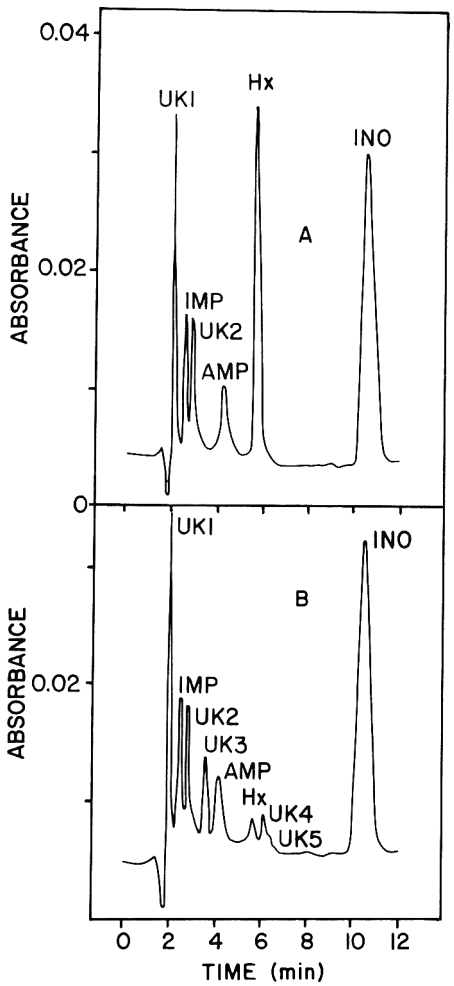

Figure 3. Chromatogram A shows scallop muscle extract elution of Hx. Chromatogram B shows same extract after treatment with XO. UK1–UK5 have not been identified. Conditions are the same as in Figure 1.

Table 3. Other nucleotides present in fish samples as detected by recommended LC method

Sample		Concentration, μmoles/g[a]			
		AMP	IMP	INO	Hx
Cod fillet	1	0.507	1.27	14.9	1.25
	2	0.151	0.560	8.80	1.93
Scallop muscle	1	2.37	0.908	4.64	2.16
	2	0.410	0.448	2.72	3.18
Crabmeat	1	14.4	0.532	0.973	0.072
	2	7.90	0.574	0.798	1.24

[a]Values are not corrected for recoveries.

arate the above compounds but require substantially longer analysis times. Presently, the use of an RP-2 analytical column (Brownlee) has led to even shorter (4–6 min) analysis times with excellent resolution at lower flow rates.

LC quantitation of scallop muscle, untreated and XO-enzyme treated extracts, is shown in Figure 3. Treatment of the extracts with XO resulted in the disappearance of the peak established as hypoxanthine by retention times and cochromatography with a standard. The XO reaction is fairly specific for the hypoxanthine degradation to uric acid, providing additional proof for the presence of the former. Also, there were no underlying or cochromatographing peaks which would complicate isolation and quantitation. The identity of IMP, AMP, and INO in all of the fish sample extracts has been established by retention times and cochromatography with authentic standards.

Tests for interferences from various biological substances possibly present in fish tissue were carried out in triplicate by adding up to 50 mg of each compound to 5 g cod homogenate. All compounds tested gave no significant interference with the described LC method. Although interference tests were not carried out as rigorously using the XO method (one analysis only), much greater variations were observed. TMAO, hemoglobin, $FeSO_4$, sorbitol, TMA, and thiourea all showed positive variations from the control in the 60–80% range. The much higher variation in Hx levels when using the XO method in the presence of interferences may be attributed either to interferences with the XO enzyme system itself or from the presence of UV-absorbing materials present in the added compounds. It would appear that these compounds are chromatographically removed in the LC method.

Overall, the results indicate that hypoxanthine measurement by the recommended LC procedure agree quite favorably with the procedure recommended by the Analytical Methods Committee (12). It has also been demonstrated that hypoxanthine content can be easily measured in representative members of vertebrate and invertebrate marine species. Although the method has been designed only for the determination of hypoxanthine in fish tissues, it may also permit measurement of other nucleotides and their breakdown products. Table 3 shows the concentrations of AMP, IMP, INO, and Hx in various commercial marine species as determined by the recommended LC method. Hypoxanthine concentrations used in conjunction with other nucleotide concentrations might form a basis for freshness evaluation criteria. Excellent correlations have already been reported between hypoxanthine levels and eating quality for such species as cod, coalfish, haddock, whiting (3), mackerel (4), irradiated ocean perch (8), scallop (11), channel catfish (10), and capelin (21).

In conclusion, the described LC measurement of hypoxanthine provides a valuable tool for the measurement of autolytic and bacterial activity in fish tissue. It is objective, simple to use, rapid, reliable, and provides a practical alternative to the XO method. It has several advantages over the XO method, such as measurement of hypoxanthine directly rather than relying on the conversion to uric acid, the ability to remove interfering compounds chromatographically, increased sensitivity, and measurement of other nucleotides. The procedure should also be quite easy to automate using an inexpensive isocratic LC system.

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FOOD ADULTERATION

Isotopic Composition of Carbon in Vinegars

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Measurements of $\delta^{13}C$ and ^{14}C-activity were performed on vinegars from various known sources. Natural vinegar can be distinguished from petrochemical acetic acid by ^{14}C-analysis: Natural vinegar currently gives values of >112% of modern activity; petrochemical acetic acid yields values of 0% of modern activity. Apple cider vinegar can be distinguished from corn-derived vinegar by $\delta^{13}C$-analysis: Cider vinegar gives $\delta^{13}C$-values near −26‰; corn-derived vinegars yield $\delta^{13}C$-values near −10‰. $\delta^{13}C$-Analysis also can be applied, with some restrictions, to wine vinegars. These techniques are applied to a series of retail vinegars.

Fraudulent mislabeling of vinegar is a major problem in the commercial market for this product. Division of the market between premium wine and cider vinegars and less expensive grain and spirit vinegars creates a financial incentive to substitute the latter for the former. Further, the availability of inexpensive acetic acid derived from petrochemical sources (oil, coal, natural gas, etc.), provides an additional financial incentive to substitute this acetic acid for authentic vinegar. Thus, for purposes of quality control and litigation, and for certain tax and duty purposes, methods for distinguishing among the various types of vinegar and acetic acid are desirable.

The success of carbon stable isotope ratio analysis (SIRA) in determining the authenticity of honey (1), fruit juices (2, 3), maple syrup (4), vanilla (5), and other foodstuffs (6, 7) led us to examine its applicability to vinegar.

Although some studies have reported a few carbon SIRA of acetic acid for various purposes (8, 9), no comprehensive baseline data exist on SIRA for various types of vinegar and synthetic acetic acid. One study reports an extensive SIRA series on calcium carbonates derived by pyrolysis of calcium acetate from vinegars and acetic acids (9). This method shows some promise for vinegar quality control, but it is inconvenient and has been shown to yield inaccurate results (10).

Petrochemically derived products are readily distinguishable from agricultural products by measurement of ^{14}C-activity of the carbon. This has been demonstrated in the literature for various products, including acetic acid (11–21). The nomenclature and data analysis in these studies, however, have often been misleading or erroneous in important respects that require clarification.

The following study presents a series of baseline data for the quality control of vinegar by SIRA and ^{14}C-techniques. A scheme of analytical methodology and data interpretation is described, and the limitations of the methods are discussed.

Experimental

Materials Analyzed

Seven 50 grain vinegars and acetic acid solutions from various known sources, as well as 6 mixtures of these samples, were obtained from the Vinegar Institute.

Seventeen authentic apple cider vinegars were obtained from H. J. Heinz, Inc.

Ten commercial vinegars were obtained from Boston retail stores.

Stable Isotope Ratio Analysis

Load 50 μL vinegar into 10 mL Pyrex tube containing 2 g CuO wire. Cool tube in dry ice/trichloroethylene, evacuate, and flame-seal. Heat tube to 500°C overnight, combusting sample to CO_2. (Caution—Substantial pressure is generated at 500°C; tubes should be adequately shielded.) Cool tube to room temperature and open under vacuum; pass combustion products through dry ice/acetone and pentane ice traps, and then freeze CO_2 into sample flask for analysis. Determine $\delta^{13}C$ of CO_2 with isotope ratio mass spectrometer in the usual manner (22).

^{14}C-Analysis

Distill 100 mL vinegar and neutralize distillate with NaOH. Add HCl until solution is just slightly acid. Evaporate this material to dryness under vacuum or in 70°C oven. Load quartz combustion tube with 2–3 g of the recovered sodium acetate. Ignite the sodium acetate in flowing O_2 by heating combustion tube with propane torch. Pass resultant gases over 800°C CuO and through traps of dry ice/trichloroethylene and liquid O_2. (Caution: Observe proper precautions in handling of liquid O_2, which is used instead of liquid N_2 to avoid O_2 condensation in gas line.) When combustion is completed, isolate liquid O_2 trap and evacuate any residual noncondensable gas. Take aliquot of CO_2 for ^{13}C-analysis. Convert remaining CO_2 to methane with tritium-free H_2 over 0.5% ruthenium on alumina at 475°C (23). Purify methane by passing through trap of dry ice/trichloroethylene, trapping on silica gel at liquid N_2 temperature, and evacuating excess H_2. Release methane by warming silica gel trap and freezing evolved methane in liquid N_2 trap. Expand into storage flask to await ^{14}C-counting. Determine ^{14}C-activity of methane by counting for 1000 min in low-level proportional gas counter with anti-coincidence circuitry. Calibrate counter by using NBS oxalic acid standard, and measure counter background by using 300-million-year-old marble with no ^{14}C-activity. Correct ^{14}C-activities for isotope fractionation by normalizing to $\delta^{13}C = -25.0‰$ (24), using the equation:

$$^{14}C\ (\text{normalized}) = {}^{14}C\ (\text{measured}) \times [1 - 2(25 + \delta^{13}C)/1000]$$

Results and Discussion

Sugars derived from corn and sugar cane differ in their ^{13}C-content from those derived from apples or grapes; corn and sugar cane sugars yield $\delta^{13}C$-values near −10‰, whereas apples and grapes give $\delta^{13}C$-values near −25‰. It is expected that vinegars derived from these various sources should yield $\delta^{13}C$-values near their starting materials.

To test this expectation, we performed SIRA on a series of vinegars derived from these sources (Table 1). The $\delta^{13}C$-values of apple cider vinegar (−24.1 to −27.1; mean = −26.0) and of wine vinegar (−27.6) agree well with the observed range of apples (mean of −25.4) (2) and grapes (mean of −26.8) (6). The corn-derived vinegar sample yielded

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Table 1. Carbon isotopes in vinegars and acetic acids of known origin

Sample		Origin	$\delta^{13}C$(PDB), ‰[a]	^{14}C-Activity,[b] % of "modern"
Acetic acid	1	petrochemical	−28.2	0
Acetic acid	2	petrochemical	−15.4	0
Acetic acid	3	sulfite waste liquor (wood)	−16.4	119
Acetic acid	4	sulfite waste liquor (wood)	−22.5	117
White distilled vinegar		corn (1982)	− 5.2	126
Wine vinegar		grapes (1982)	−27.2	120
Cider vinegar	1	apples (1982)	−25.5	125
	2		−25.1	—[c]
	3		−25.7	—
	4		−25.6	—
	5		−26.7	—
	6		−26.8	—
	7		−25.7	—
	8		−26.3	—
	9		−26.7	—
	10		−27.1	—
	11		−26.4	—
	12		−25.8	—
	13		−26.8	—
	14		−26.9	—
	15		−24.1	—
	16		−25.8	—
	17		−26.5	—
	18		−25.0	—
			mean (18) = −26.0 ±1.0	

[a]Standard deviation ± 0.1‰.
[b]Standard deviation ± 2%.
[c]Not determined.

a result close to, but somewhat more positive than the results reported for corn syrup (mean of −9.7) (1).

Further, the standard deviation of the carbon SIRA of apple cider vinegar (± 1.00) is similar to that of apples (± 1.24), showing that cider vinegars are tightly grouped in the same manner as apples (2). Thus, additions of corn-derived vinegar to cider vinegar may be detected in the same manner as corn syrup additions to apple juice.

The case of wine vinegars is similar to that of cider vinegar but is somewhat more complicated. Because many wine musts legitimately contain added sugars, some legitimate wine vinegars will be expected to yield SIRA values somewhat more positive than the pure grape values. This should be taken into account in the use of SIRA for quality assurance of wine vinegar. SIRA of residual sugars in the vinegar may yield additional information about wine vinegar adulteration, analogously to work done with sake (25), but no such analyses have been included in this study.

The addition of petrochemical acetic acid to vinegars of all types is the other major adulteration problem. We examined 2 synthetic samples of acetic acid by SIRA; as expected, the results were variable, and overlapped the range of natural vinegars. We conclude that SIRA is not a reliable method for detecting this type of adulteration.

It is further expected that SIRA will not be of use for detecting vinegars derived from feedstocks of sugar beets or grain spirits other than corn, because carbohydrates from these sources yield SIRA results similar to grapes and apples (6).

A final potential adulterant will be vinegars from feedstocks of the sulfite waste liquors of paper mills. Table 1 shows SIRA of 2 samples of acetic acid derived from sulfite liquor alcohol. The samples are somewhat different from any of the natural vinegar SIRA ranges; the obtained values are not readily explained by the usual models. SIRA may be useful for sulfite liquor detection, but more study needs to be done.

There have been several reports in the literature of ^{14}C-analysis being used to detect petrochemical products including acetic acid (11–21). Carbon from recent agricultural sources is found in equilibrium with ^{14}C-activity present in the atmosphere, whereas carbon from petrochemical sources has been fixed long enough for all of the ^{14}C-activity originally present to have decayed away. Thus, authentic vinegar will have a ^{14}C-activity near that of atmospheric CO_2, while petrochemical acetic acid will have an activity near zero.

Previous attempts to use ^{14}C-measurement for vinegar evaluation have a number of drawbacks from the standpoint of routine quality assurance. Principal among these difficulties is the failure of many authors to take adequate (or any) account of the variable nature of atmospheric ^{14}C-activity, which controls the level of ^{14}C-activity in authentic vinegars. Because these levels of activity have varied rather abruptly with atmospheric nuclear testing, and have been steadily declining in recent years, any criterion of vinegar purity must take this into account. Assertions concerning the detection limit of petrochemical acetic acid in vinegar must assume an accurate

Table 2. Known and projected ^{14}C-activities

Year	Atm. CO_2 ^{14}C-activity, % modern[a]	Year	Atm. CO_2 ^{14}C-activity, % modern[b]
1975	139	1981	127
1976	136	1982	126
1977	134	1983	124
1978	133	1984	123
1979	131	1985	121
1980	128	1986	120

[a]Calculated from Nydal and Lovseth; the standard deviation of recent atmospheric ^{14}C-measurements is approximately 2% of modern for any given year (26).
[b]Projections based on a best fit model of first order mixing of atmospheric CO_2 with a large excess of oceanic carbonate of 100% modern composition. $^{14}C_t = 100 + 49.1e^{-0.0646(t-1972)}$.

Table 3. ^{14}C-Analyses on mixtures of vinegars and acetic acids

Mixture	^{14}C-Activity predicted, % modern	^{14}C-Activity found, % modern
75% Cider vinegar/ 25% acetic acid 1	94	95
50% Cider vinegar/ 50% acetic acid 1	63	64
75% Cider vinegar/ 25% acetic acid 2	94	96
50% Cider vinegar/ 50% acetic acid 2	63	64
75% Wine vinegar/ 25% acetic acid 1	90	87
50% Wine vinegar/ 50% acetic acid 1	60	61

Table 4. Decision matrix for quality assessment of vinegars produced in 1983[a]

Product	Type of adulteration	$\delta^{13}C$, ‰	^{14}C, %
Vinegar	none	variable	>112
Vinegar	petrochemical acetic acid	variable	<112
Cider vinegar	none	< −22.0	>112
Cider vinegar	sugar cane or corn-derived vinegar	> −22.0	>112
Wine vinegar	none	< −22.0[b]	>112
Wine vinegar	sugar cane or corn-derived vinegar	> −22.0[b]	>112

[a]^{14}C-standard for future years may be established by subtracting 12% from atmospheric ^{14}C-values for that year. Estimates of atmospheric CO_2 for the next several years are listed in Table 2.
[b]Only applicable to wines, the musts of which contained no added sugar.

knowledge of the value of natural vinegar in any given year. Periodic tabulation of atmospheric ^{14}C-levels will be necessary for accurate vinegar assessment (Table 2).

Another drawback of older studies is the inadequacy of the reported standards for ^{14}C-specific activity measurement. Most accounts use a ^{14}C-enriched material as a secondary standard, with no reference to the primary standard by which it was calibrated. Radiation counters are not perfectly efficient, and efficiency will vary with the counting protocol, so a primary standard for calibration of counters is necessary. There is abundant literature on the measurement of ^{14}C-activity in association with radiocarbon age determination. A widely accepted interlaboratory standard for ^{14}C-specific activity is NBS oxalic acid: 95% of the 1950 specific activity of NBS oxalic acid (13.56 dpm/g) has been defined as the "modern" value of the specific activity of the carbon in atmospheric CO_2. This is the value the atmosphere would have had in 1950 in the absence of fossil fuel combustion and nuclear weapons testing (24). Natural ^{14}C-specific activity measurements should be reported either relative to NBS oxalic acid (i.e., as a % of "modern"), or as disintegrations per minute per gram of carbon (dpm/g carbon) in a system calibrated with NBS oxalic acid as primary standard.

Finally, most accounts neglect the effect of isotopic fractionation on ^{14}C-activity measurements. However, significant errors may be introduced when comparing samples of widely differing isotopic fractionation, as in corn vs cider vinegars. These errors can be eliminated by normalizing the measured ^{14}C-activity to that which would be obtained at constant ^{13}C-content. By convention, ^{14}C-activities are normalized to $\delta^{13}C = -25‰$ (18).

^{14}C-Analyses in this study have been reported as a percentage of modern specific activity, as defined above. Since the early 1960s, atmospheric ^{14}C-activities have been elevated because of nuclear weapons testing, reaching a peak in 1964 and 1965 and slowly declining back toward 100% since the Limited Test Ban Treaty. Table 2 lists recent ^{14}C-values for atmospheric CO_2, as well as projections for the next few years. It is expected that agricultural products grown recently will reflect these values. Table 1 shows the ^{14}C-activities of a number of samples from various sources. The 1982 agriculturally derived vinegars, as expected, give values around 124%. The 2 petrochemical acetic acids, on the other hand, yielded values of 0%. Since the analytical error of this method is about 2% of modern, and the variability of atmospheric ^{14}C-activity in any given recent year is less than 2% of modern (26), the overall standard deviation of vinegar ^{14}C-analyses for any given year will be about 3% of modern. This is confirmed by the tight grouping of the three 1982 samples in Table 1. The large difference between natural and synthetic samples, and the small analytical error thus allow detection of very small quantities of the latter in the former.

Table 3 shows the ^{14}C-activity results for several mixtures of vinegars and acetic acids. It is clear that petrochemical acetic acid can be accurately detected at levels of 50 and 25% in natural vinegar. The agreement between predicted and observed results shows the expected linear mixing behavior.

We propose the following criteria for the detection of adulterated vinegars by these methods. Using the statistical criterion proposed for apple juice, any cider vinegar with a $\delta^{13}C$-value more positive than 4 standard deviations from the mean, or −22.0‰, should be considered unquestionably adulterated. Similarly, vinegar produced in 1983 with a ^{14}C-specific activity less than 112% of modern should be considered adulterated. Atmospheric ^{14}C-values have been slowly declining in recent years, so this criterion will have to be re-evaluated periodically. For any given year, the standard may be established by subtracting 12% of modern from the mean atmospheric ^{14}C-value. These criteria are incorporated into a decision matrix for vinegar evaluation (Table 4), and will yield conclusions concerning adulteration with a very large (99.997%) degree of certainty. For many routine quality control applications, more stringent criteria of purity (values closer to the mean value), rather than impurity, will be desirable to assure sample quality.

Table 5 demonstrates the use of the decision matrix in Table 4 for the evaluation of a series of retail vinegars.

Summary

Additions of corn-derived vinegar to apple cider vinegar, and of petrochemical acetic acid to vinegar, may be reliably detected by measurement of ^{13}C and ^{14}C-activities, respectively. The matrix in Table 4 may be used as a simple tool for vinegar assessment.

Table 5. $\delta^{13}C$ and ^{14}C-activity of (1984) retail vinegars in the Boston area

Brand	Vinegar type	$\delta^{13}C$, ‰	^{14}C, %	Remarks
1	White distilled	− 8.0	125	authentic
2	White distilled	−24.1	123	authentic
3	White distilled	−29.5	6	fraudulent
4	Cider	−25.5	125	authentic
5	Cider	−25.5	119	authentic
6	Cider	−24.5	116	authentic
7	Cider	−25.7	124	authentic
8	Cider	−26.3	106	adulterated
9	Cider	−25.7	96	adulterated
10	Cider	−19.9	94	adulterated

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MYCOTOXINS

Modification of the Rapid Screening Method for Aflatoxin in Corn for Quantitative Use

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A study was made to determine if the official AOAC method for screening of aflatoxin in corn could be modified for use as a quantitative method. Several different corn products were analyzed using the modified method, with an average savings of over 1 h/sample vs the CB method. Average recoveries for aflatoxin B_1 were 94% for the low level spiked samples and 108% for the high level. Samples of corn and corn products containing naturally incurred aflatoxin were also analyzed with the modified method, and the results compared favorably with those obtained by the CB method.

Aflatoxin contamination of corn is a nationwide problem; this laboratory routinely analyzes by the CB method (**26.026–26.031**) (1) samples of corn and corn products for aflatoxin. A method for aflatoxin determination that would combine the quantitative results of the CB method with the speed and economy of the rapid corn screening method (**26.049–26.051**) (1) was desired.

Dantzman and Stoloff (2) developed a rapid screening method for aflatoxin in corn which was adopted official first action (**26.049–26.051**) (1) by AOAC. This method makes use of the fact that corn and corn products generally contain 0.5–5.0% fat or oil (3), so that when 50 g samples of corn products are extracted with 250 mL $CHCl_3$, and a 50 mL aliquot of this solution is evaporated, 50–500 μL oil remains. A portion of this oil, while still warm, is spotted on a warm 20 × 20 cm thin layer chromatographic (TLC) plate that has been scored in half horizontally. The plate is developed 30 min with anhydrous ether, dried, developed with $CHCl_3$-acetone (9 + 1) for 20 min, and then observed visually under longwave ultraviolet (UV) light for the presence of aflatoxin. In our laboratory we modified the rapid screening method as follows:

(1) Addition of 150–200 μL benzene-CH_3CN (98 + 2) to the 50–500 μL oil which remains after evaporation of the extract (representing 10 g sample) and thoroughly mixing the contents. Since the final volume for most samples is 500–650 μL, there is little, if any, loss in the amount of sample spotted (0.020–0.015 g/μL) in comparison to the CB method as used in our laboratory (500 μL added to vial instead of 200 μL) (0.020 g/μL). This method then results in screening at the 5–6.7 μg/kg level for aflatoxin B_1 when the 2 μL standard spot (**26.031(b)**) is used for comparison, which is very close to the CB screening level (5 μg/kg);

(2) Use of a whole 20 × 20 cm TLC plate instead of one-half plate, with a corresponding increase in plate development time; and

(3) Measurement of the volume of the final 500–650 μL solution used for spotting so that a quantitative result may be obtained.

Using these modifications, both spiked samples and samples containing naturally incurred aflatoxin were analyzed, and the results were compared with the results obtained by the CB method. B_1 was used for spiking because it is usually the predominant aflatoxin found in moldy corn and is also the most toxic.

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METHOD

Apparatus

(a) *Extraction equipment.*—Burrell wrist-action shaker (Burrell Corp., Pittsburgh, PA 15219).

(b) *Densitometer (optional).*—Schoeffel SD 3000 (Schoeffel Instrument Corp., Westwood, NJ 07675). Operating conditions: transmittance mode, single beam, gain 750, 1.0 absorbance, activation wavelength 360 nm with slit 0.5 mm, emission wavelength >400 nm with slit 1.0 mm, 100 mV recorder at 6 cm/min chart speed.

(c) *Integrator (optional).*—Model 3390A Hewlett-Packard (Avondale, PA 19311). Operating conditions: zero setting 5, attenuation 3, chart speed 2.0 cm/min, peak width 0.04, threshold 4, area reject 500.

(d) *Tube shaker.*—Vortex (Scientific Industries, Inc., Bohemia, NY 11716).

(e) *TLC apparatus.*—Silica gel GHR plates, 20 × 20 cm, 0.25 mm, precoated (Brinkmann Instruments, Inc., Westbury, NY 11590), activated 2 h at 80°C; viewing cabinet (Chromato-Vue Model C-70, Ultraviolet Products, Inc., San Gabriel, CA 91778); 10 μL syringe for spotting; 1.0 mL TC pipet graduated in 0.01 mL increments; unlined developing tank.

Reagents

(a) *Extraction solvents.*—$CHCl_3$ and CH_2Cl_2, reagent grade (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(b) *Filtering aid.*—Celite, analytical grade (Johns-Manville, Lompoc, CA 93436).

(c) *Dilution solvent.*—Benzene and CH_3CN, reagent grade (Mallinckrodt, Inc., Paris, KY 40361). Mix 98 + 2.

(d) *Developing solvent.*—Diethyl ether, anhydrous, reagent grade (Mallinckrodt, Inc.). $CHCl_3$ and acetone, reagent grade (J. T. Baker). Mix 9 + 1.

Table 1. Recovery (μg/kg) of aflatoxin B_1 in spiked corn meal samples, using the CB and modified rapid corn methods

Product	Found (CB)	Found (modified rapid corn)
Yellow corn meal[a]	10	9.3
	10.8	9.4
White corn meal[a]	8.9	9.1
	9.2	9.6
Mean	9.7	9.4
Std dev.	0.8	0.2
Yellow corn meal[b]	39.5	51.5
	40	54.5
	40.5	55
	45	58.5
White corn meal[b]	40.5	45
	45	47.5
	45	58
	48	63.5
Mean	37.9	54.2
Std dev.	13.7	6.1

[a]10.0 μg B_1/kg added.
[b]50.0 μg B_1/kg added.

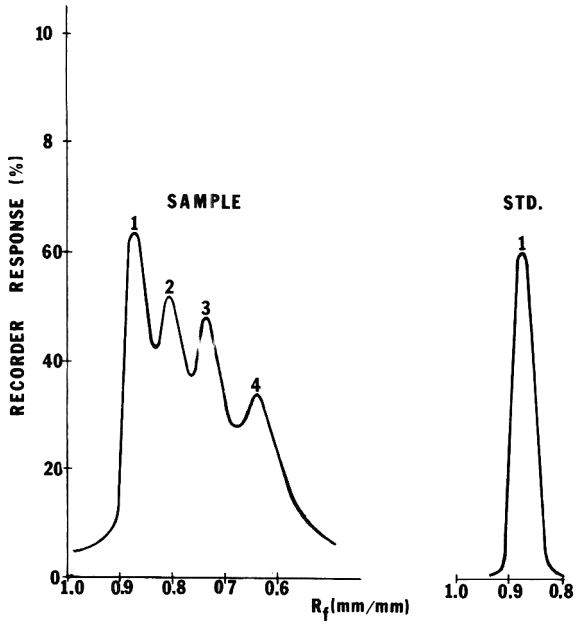

Figure 1. Densitometric trace of a spiked white corn sample of one-half plate development of aflatoxins B_1, B_2, G_1, and G_2. 1 = 2.5 ng aflatoxin B_1, 2 = 1.0 ng aflatoxin B_2, 3 = 2.5 ng aflatoxin G_1, and 4 = 1.0 ng aflatoxin G_2 (see text for instrument conditions).

(e) *Aflatoxin standards.*—Obtained as dry film from Food and Drug Administration, Washington, DC, standardized (**26.008–26.012**) (1), and diluted with dilution solvent to contain 0.5 μg B_1 and G_1/mL and 0.2 μg B_2 and G_2/mL. Store diluted standards at 0°C or lower.

Preparation of Sample

See secs **26.049–26.051** (1). Spiked samples were prepared by adding the appropriate volume of spiking standard (**26.009**) to 50 g portions of the ground product. Replicate analyses were performed on separate 50 g portions of sample. All spiked samples were initially blank.

Extraction

Prepare extract (**26.026–26.031**) (1) and transfer 50 mL $CHCl_3$ filtrate to 125 mL Phillips beaker. Evaporate on steam bath to small volume of residual oil. Transfer with $CHCl_3$ to small vial. Evaporate extract on steam bath to leave corn oil residue. Add 150–200 μL dilution solvent and *thoroughly* mix on tube shaker. (For some samples, CH_2Cl_2 was used instead of $CHCl_3$ for extraction and transfer because of lower toxicity).

Thin Layer Chromatography

Measure volume of solution with 1.0 mL pipet and pipet bulb to nearest 0.01 mL, working rapidly to minimize evaporation. Spot sample and standard solutions (**26.026–26.031(b)**) (1) on imaginary line 4 cm from bottom edge of plate. Develop plate with anhydrous ether in unlined, unequilibrated tank 40 min. Air-dry plate in hood until ether evaporates. Redevelop in unlined, unequilibrated tank with $CHCl_3$-acetone (9 + 1) 30 min. Observe developed plate for presence or absence of

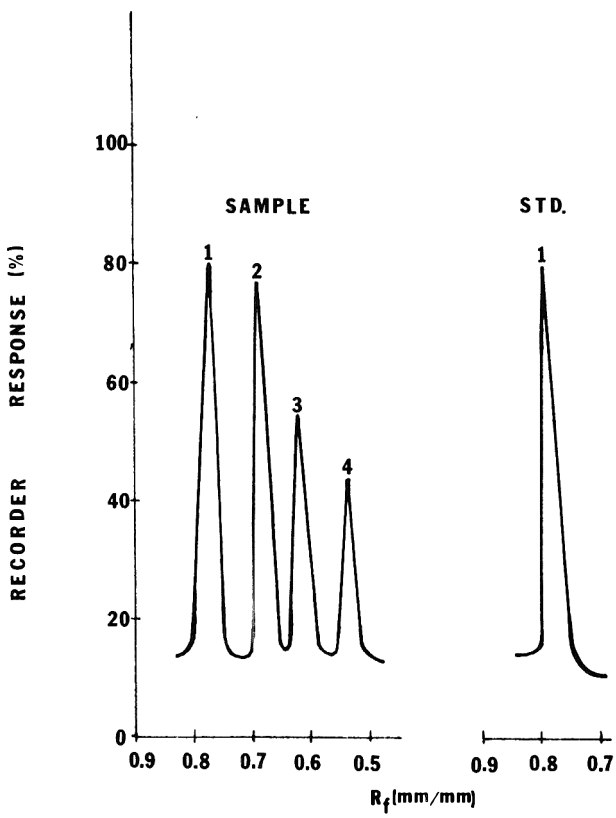

Figure 2. Densitometric trace of a spiked white corn sample of full TLC plate development of aflatoxins B_1, B_2, G_1, and G_2. 1 = 2.5 ng aflatoxin B_1, 2 = 1.0 ng aflatoxin B_2, 3 = 2.5 ng aflatoxin G_1, and 4 = 1.0 ng aflatoxin G_2 (see text for instrument conditions).

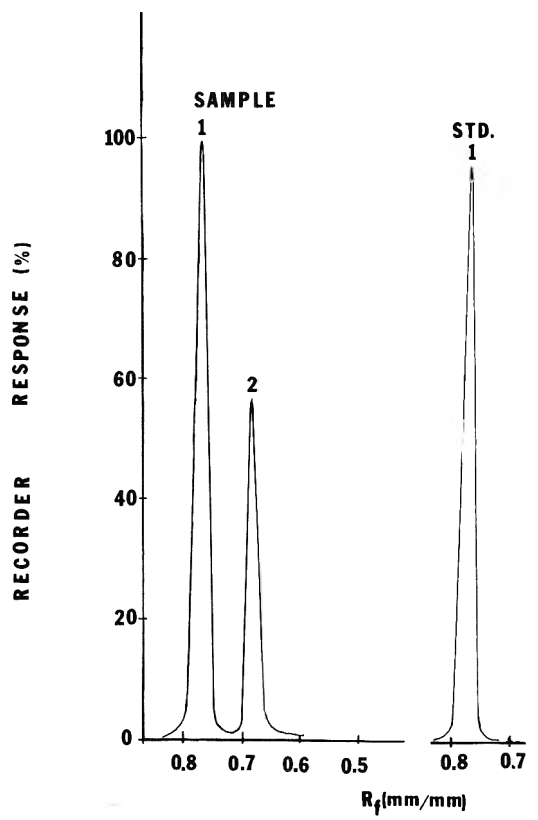

Figure 3. Densitometric trace of full TLC plate development of a yellow corn sample containing naturally incurred aflatoxins B_1 and B_2. 1 = 3.5 ng aflatoxin B_1 and 2 = 2 ng aflatoxin B_2 (see text for instrument conditions).

Table 2. Recovery of aflatoxins B_1 and B_2 in spiked samples (intralaboratory), using the modified rapid corn method

Product	Added, μg/kg		Rec., %	
	B_1	B_2	B_1	B_2
Yellow corn meal[a]	17.3[b]		108	
White corn meal	17.2[c]		104	
Shelled yellow corn[a]	10.3[c]	5.0[d]	126	102
		11.3[c]		81
	51.0[d]		76	
		15.0[d]		100
	17.0[d]		77	

[a]CH_2Cl_2 was used instead of $CHCl_3$ for sample extraction and transfer because of lower toxicity.
[b]Performed by analyst 1.
[c]Performed by analyst 2.
[d]Performed by analyst 3.

spot originating from sample and with same R_f and appearance as authentic aflatoxins. Observe internal standard spot for any change in R_f or appearance of aflatoxin caused by extract.

Determination

Measure fluorescence of sample and standard aflatoxin spots visually in viewing cabinet under longwave (366 nm) UV light or with densitometer. Confirmation of aflatoxin was by trifluoroacetic acid derivative **(26.083).**

Calculation

Calculate concentration of aflatoxin from equation:

$$\mu g \text{ aflatoxin/kg} = (S \times Y \times V) \times (A_{sample})/(A_{standard} \times W \times X)$$

where S = μL aflatoxin standard spot used for comparison with sample; Y = concentration of aflatoxin standard, μg/mL; V = μL of final dilution of sample extract spotted that gives fluorescence intensity approximately equal to fluorescence of spot S; A_{sample} and $A_{standard}$ = areas of fluorescence intensity peaks of sample and standard, respectively; W = g sample represented by extract that was evaporated (10 g if 50 mL $CHCl_3$ extract is used); and X = μL sample spot used for comparison with standard.

Results

Figure 1 shows the results of using one-half plate development of a portion of a spiked white corn sample for quantitation. The large amount of peak overlap makes one-half plate development unsuitable for quantitative use. Figure 2 shows the results of the development of another portion of the same sample, using a whole TLC plate. The almost baseline separation is a marked improvement over that obtained using the one-half plate development. Figure 3 shows the results of the development of a portion of a naturally contaminated yellow corn sample.

The recovery of B_1 obtained by using this method and the CB method on the same spiked samples of corn meal is shown in Table 1.

Shelled yellow corn was spiked with 2.0, 5.0, 10.0, and 50.0 μg B_1/kg. Using the modified rapid corn method, the recoveries were (average of 2 determinations) 1.6, 3.8, 9.7, and 47.2 μg/kg (80, 76, 97, 94%). CH_2Cl_2 was used instead of $CHCl_3$ for this experiment.

When 3 corn samples containing naturally incurred B_1 were analyzed by 2 analysts, one using the CB and the other the modified rapid corn method, the results were 24, 52, and 76 μg/kg and 27, <7, and 61 μg/kg, respectively. CH_2Cl_2 was used rather than $CHCl_3$ for these samples.

Table 2 shows the recovery of B_1 and B_2 from spiked corn and corn products, using the modified method. These results were obtained by 3 different analysts in our laboratory, using samples spiked with 10–51 μg B_1/kg and 5–15 μg B_2/kg.

Table 3 shows the recovery results of analyses of corn samples spiked with B_1 and G_1 at levels from 5 to 50 μg/kg, and spiked with B_2 and G_2 at levels from 2 to 20 μg/kg. B_1 recovery ranged from 71 to 92%. Recoveries of the other aflatoxins ranged from an undetectable amount to 110%.

Table 4 shows the results of analyses of samples containing naturally incurred aflatoxins by both the CB and the modified methods. There was good agreement between the CB method and the modified method at the 20–25 μg/kg mean B_1 level in corn (24.7 vs 20.3 μg B_1/kg, respectively). At the higher level, the modified method gave slightly lower results for B_1.

Discussion

Addition of solvent to the corn oil residue obtained by the original rapid corn method not only makes the sample easier to spot on the TLC plate by reducing the viscosity, but more liquid is available to wash down any aflatoxin still adhering to the sides of the vial.

Approximately 100 samples of corn and corn products from various sources were analyzed by this modified method, with savings of over 1 h/sample, approximately 500 mL organic solvent/sample, and 10 g silica gel/sample compared to the CB method. Thus the modified method gave results comparable to the CB method with considerable savings in time and reagents and little loss in sensitivity.

Acknowledgments

The author thanks Leo J. Lipinski, Jr for assistance in computer data retrieval and analysis of samples, Louis W. Miles, Sr and Lawrence Carter, Jr for testing the new method and running several recoveries, and Timothy J. Trepagnier, Jr for drawings.

Table 3. Recovery of aflatoxins B_1, B_2, G_1, and G_2 in spiked corn samples, using the modified rapid corn method[a,b]

Product	Added, μg/kg				Rec., %			
	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Shelled yellow corn	50	20	50	20	90	68	110	55
	10	4	10	4	92	77	71	14
	5	2	5	2	71	36	16	ND[c]

[a]CH_2Cl_2 was used instead of $CHCl_3$ for sample extraction and transfer because of lower toxicity.
[b]Average of duplicate analyses.
[c]ND = not detectable.

Table 4. Determination of aflatoxins B_1, B_2, G_1, and G_2 (μg/kg) in corn samples containing naturally incurred aflatoxins, using the CB and modified rapid corn methods

	CB				Modified rapid corn			
Product	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Shelled white corn	19.1	1.9	0	0	17.7	3.2	0	0
	24.4	2.2	0	0	19.8	3.2	0	0
	25.8	2.3	0	0	20.4	1.3[a]	0	0
	29.4	2.2	0	0	23.3	3.1	0	0
Mean	24.7	2.2			20.3	3.2		
Std dev.	4.3	0.2			2.3	0.06		
Coeff. of var., %	17.4	9.1			11.3	1.8		
Shelled white corn	202.4	23.4	40.3	trace (<1.0)	172.4	38.0	44.9	trace
	221.2	27.0	39.4	trace	181.1	36.3	37.4	trace
	222.7	29.9	53.0	trace	171.0	20.0[a]	49.8	trace
Mean	215.4	26.8	44.2		174.8	37.2	44.0	
Std dev.	11.3	3.3	7.6		5.5	1.2	6.2	
Coeff. of var., %	5.2	12.3	17.2		3.1	3.2	14.1	

[a]These points were rejected as outliers, using Dixon's criteria at the 90% significance level, and with n = 4 and 3, respectively (4).

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Fractionation of Radioactivity in the Milk of Goats Administered ^{14}C-Aflatoxin B_1

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A detailed fractionation of radioactivity in the milk of goats administered ^{14}C-aflatoxin B_1 at low doses was performed. The milk collected in the first 24 h following dosing contained radioactivity equivalent to 0.45–1.1% of the dose given. The radioactivity in each sample was partitioned into 4 fractions: ether, protein, dichloromethane, and water–alcohol. Over 80% of the radioactivity was detected in the dichloromethane fraction, of which over 95% was attributable to aflatoxin M_1. No aflatoxin B_1 or other known aflatoxin metabolites were detected in any fraction. The results indicate that the major metabolite of aflatoxin B_1 in goat milk is aflatoxin M_1 and that other metabolites, including conjugates, are of minor significance.

It is well known that aflatoxin M_1 is a major metabolite of aflatoxin B_1 in milk (1). The acute toxicity (2) and carcinogenicity (3) of M_1 as well as its frequent occurrence in dairy products (4) has been well documented. Little is known, however, about the existence of other minor aflatoxin B_1 metabolites and their conjugates in milk (5, 6). A recent improvement in the production of ring-labeled ^{14}C-aflatoxin B_1 at a high specific activity in our laboratory (7) permitted the analysis of very low levels of metabolites in milk. This paper reports the results of our studies on the transmission of radioactivity at a low dose level in lactating goats and a detailed fractionation of the radioactivity in the milk.

Experimental

Chemicals

(a) *Ring-labeled ^{14}C-aflatoxin B_1.*—Produced by feeding [1-^{14}C]acetate to cultures of *Aspergillus flavus* NRRL 3251 according to the method of Huang and Hsieh (7). ^{14}C-Aflatoxin B_1 was purified using low pressure silica gel column chromatography (7) to be chromatographically pure.

(b) *Aflatoxin M_1.*—Produced by *Aspergillus flavus* NRRL 3251 and purified by reverse phase low pressure column chromatography (8) to be chromatographically pure.

(c) *Standard aflatoxins.*—Quantitated by UV-VIS spectrophotometry (9). All other chemicals were either reagent grade or chromatographically pure.

Analysis

Aflatoxins were analyzed by thin layer chromatography (TLC) and quantitated by fluorodensitometry (Schoeffel SD3000). TLC plates (Merck silica gel 60 precoated, glass or plastic back plates) were developed with toluene–ethyl acetate–90% formic acid (6 + 3 + 1, v/v/v). Radioactivity was measured by liquid scintillation counting (Packard Model 2405).

Table 1. Summary of ^{14}C-aflatoxin B_1 administered to goats

Goat	Body weight, kg	Specific activity of ^{14}C-B_1, Ci/mol	Dose, μg/kg body wt
1	54.0	182	4.08
2	42.8	182	5.21
3	56.3	256	3.90
4	63.0	256	3.48

Table 2. Amount of milk (g) collected in first 24 h post-dosing

Post-dosing, h	Goat 1	Goat 2	Goat 3	Goat 4
6	430	485	922	960
12	300	284	532	545
18	355	226	678	367
24	360	250	708	515

[1]Visiting researcher from National Food Research Institute, Japan.

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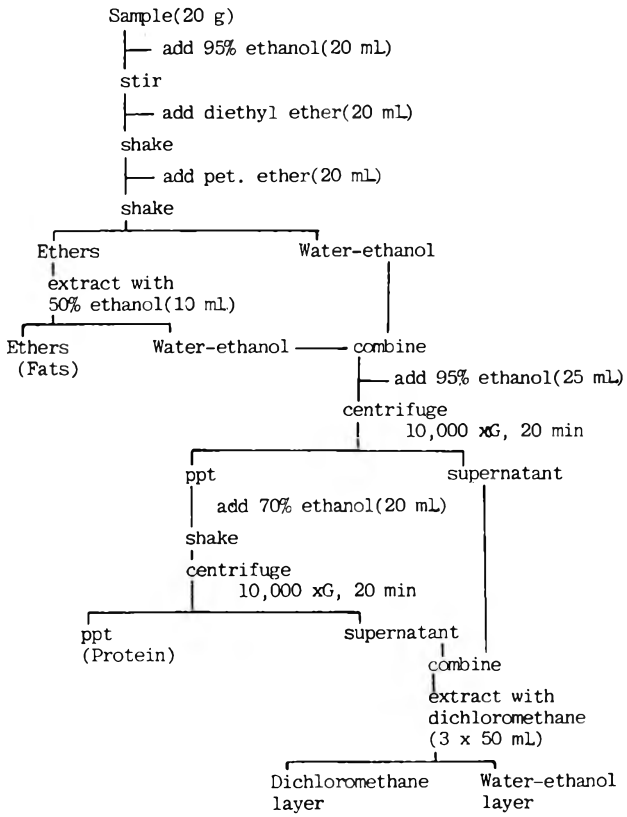

Figure 1. Fractionation of goat milk.

Administration of ^{14}C-Aflatoxin B_1 to Goats

Goats (2 or 3 months postpartum) were purchased from local herds. Experimental animals were fed aflatoxin-free commercial diet concentrate twice a day supplemented with alfalfa hay and water ad libitum. Animals were housed in metabolism cages after administration of ^{14}C-aflatoxin B_1. Goats 1 and 2 were treated with ^{14}C-aflatoxin B_1 dissolved in a vehicle of polyethoxylated vegetable oil (Emulphor EL-719, GAF Corp.)–ethanol–water (1 + 1 + 9, v/v/v) and administered intravenously. Goats 3 and 4 received ^{14}C-aflatoxin B_1 orally as a gelatin capsule with ground cottonseed meal (Tables 1 and 2). Samples of milk were collected at designated time intervals. All samples were stored at −80°C until analysis.

Fractionation of Goats' Milk

The radioactivity in each milk sample was partitioned into 4 fractions: ether extractable, protein precipitate (in 70% ethanol), dichloromethane extractable, and water-alcohol soluble, by the method outlined in Figure 1. Both the ether and dichloromethane fractions were concentrated by rotary evaporation. Protein-associated and water-soluble fractions were concentrated by lyophilization.

Results and Discussion

The milk collected in the first 24 h following dosing contained radioactivity equivalent to 0.45–1.1% of the dose (Figure 2). Of this radioactivity, 47–85% was detected in the first 6 h. The milk collected in the next 4 days contained 0.02–0.28% of the initial dose. For samples collected at 6 and 12 h, as shown in Table 3, over 74% of the radioactivity was detected in the dichloromethane-extractable fraction. This fraction contained M_1 as the only identifiable B_1 metabolite, accounting for over 95% of the radioactivity, based on TLC analysis. No other known B_1 metabolites were detectable when the sample applied to the TLC plate contained 5000 dpm in M_1. The minimum detectable radioactivity is 20 dpm, so our results indicate that no other dichloromethane-extractable B_1 metabolite was present at levels higher than 1/250 of that of M_1.

Therefore, the data indicate that aflatoxin M_1 is the major metabolite of B_1 in the milk of goats administered ^{14}C-aflatoxin B_1. Other B_1 metabolites, including conjugates, are of little significance in view of their low toxicity and limited presence in the milk.

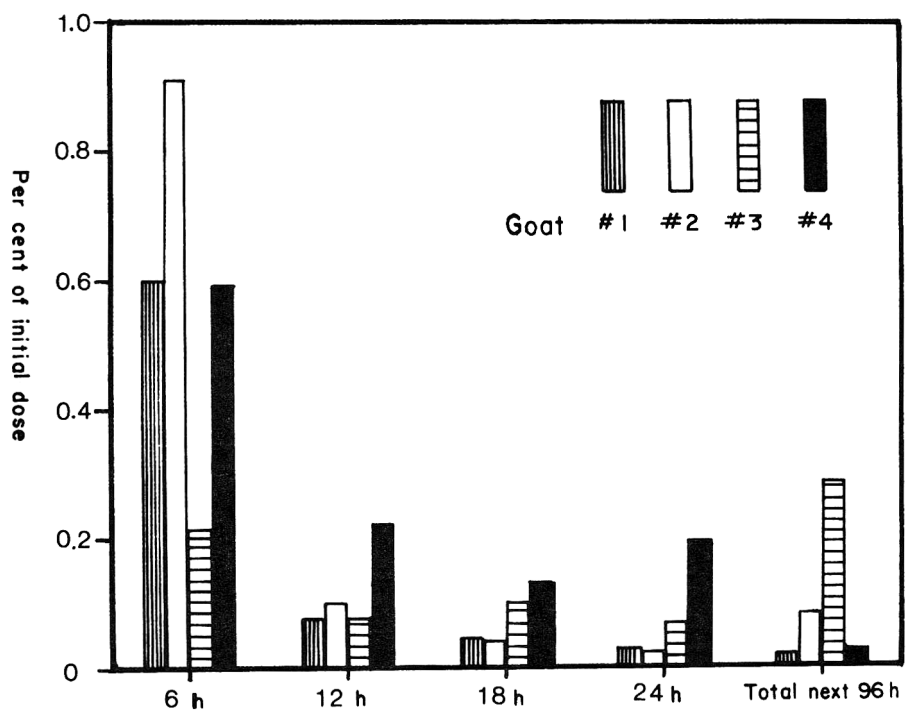

Figure 2. Radioactivity detected in milk.

Table 3. Distribution of radioactivity in milk samples collected 6 and 12 h post-dosing

Sample	Radioactivity, dpm/g milk				
	Whole milk	Ether	Protein	CH_2Cl_2	Aq. ethanol
Goat 1: 6 h	3,770	9	278	2,845	217
12 h	616	15	111	520	72
Goat 2: 6 h	5,430	63	164	4,830	308
12 h	1,127	66	109	1,020	92
Goat 3: 6 h	828	47	28	620	46
12 h	440	5	42	400	32
Goat 4: 6 h	2,230	25	71	1,960	42
12 h	1,800	13	45	1,340	80

Acknowledgments

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Simple, Rapid Cleanup Method for Analysis of Aflatoxins and Comparison with Various Methods

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A method is described for simple and rapid determination of aflatoxins in corn, buckwheat, peanuts, and cheese. Aflatoxins were extracted with chloroform–water and were purified by a Florisil column chromatographic procedure. Column eluates were concentrated and spotted on a high performance thin layer chromatographic (HPTLC) plate, which was then developed in chloroform–acetone (9 + 1) and/or ether–methanol–water (94 + 4.5 + 1.5) or chloroform–isopropanol–acetone (85 + 5 + 10). Each aflatoxin was quantitated by densitometry. The minimum detectable aflatoxin concentrations (μg/kg) in various test materials were 0.2, B_1; 0.1, B_2; 0.2, G_1; 0.1, G_2; and 0.1, M_1. Recoveries of the aflatoxins added to corn, peanut, and cheese samples at 10–30 μg/kg were > 69% (aflatoxin G_2) and averaged 91%, B_1; 89%, B_2; 91%, G_1; 78%, G_2; and 92%, M_1. The simple method described was compared with the AOAC CB method, AOAC BF method, and AOAC milk and cheese method. These methods were applied to corn, peanut, and cheese composites spiked with known amounts of aflatoxins, and to naturally contaminated buckwheat and cheese. Recoveries were much lower for the BF method compared with our simple method and the CB method.

Two official first action AOAC methods (CB and BF) have been evaluated for determination of aflatoxins in peanuts and peanut products (1). The CB method is often used as a standard of excellence against which new methods are judged (2, 3). Recently, both methods were applied to corn, wheat, pistachio nuts, and other agricultural products (4–6).

In many instances, comparisons of the CB method with the BF method showed that different results have been observed by some laboratories; results for the same sample were higher by the CB method than those obtained by the BF method (7–9). Unfortunately, the CB method requires more time and is more solvent-consuming per determination for extraction and column chromatography. The cost of reagents required in any analysis is an important consideration. Although use of commercial reagents would allow analysis without activation of silica gel and distillation of solvents, reagents must still be prepared before use in the AOAC methods. In both the AOAC CB and BF methods, for example, ether must be anhydrous and contain < 0.01% alcohol; silica gel must be activated (dry 1 h at 105°C, add 1% water by weight, seal, shake until thoroughly mixed, and store in airtight container), and other strict quality control measures must be observed. Although a method that is applicable to many agricultural commodities and dairy products is advantageous for laboratories analyzing more than one commodity, the relative effectiveness of the official methods on a variety of commodities has never been examined (1). None of these procedures, however, has the cost-effectiveness, rapidity, recovery, and general utility required for our purposes.

Therefore, we developed a method requiring less time and using commercially available reagents which require no further treatment. We describe here a method using chloroform as the extracting solvent, with purification on a Florisil column, detection by thin layer chromatography (TLC), and quantitation by densitometry.

Experimental

Apparatus

(a) *Wrist-action shaker.*—Taiyo Recipro Shaker Model SR-IIw (Taiyo Ltd, Tokyo, Japan), or equivalent.

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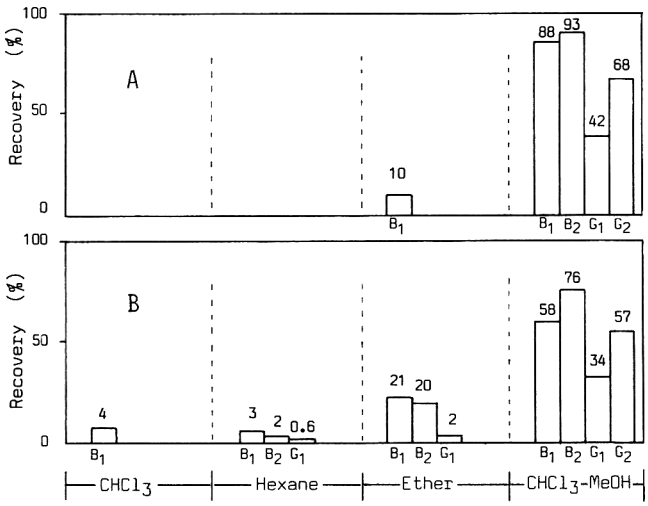

Figure 1. Recoveries of aflatoxins from a silica gel column: A, chloroform contained 0.4–0.5% ethanol; B, chloroform contained 0.5–0.9% ethanol.

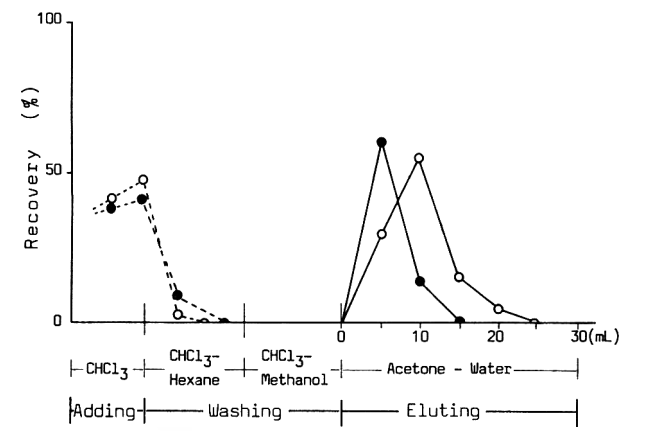

Figure 2. Elution profiles of aflatoxins from Florisil column: --○--○-- edible oil; --●--●-- tallow; --●--●-- aflatoxin M_1; --○--○-- aflatoxins.

(b) *Homogenizer.*—Nippon Seiki Model AM-7 (Nippon Seiki Ltd, Tokyo, Japan), or equivalent.

(c) *TLC densitometer.*—Shimadzu Model CS-910 dual-wavelength, TLC scanner (Shimadzu Ltd, Kyoto, Japan), or equivalent. Operating conditions: slit width 0.3 × 8 mm; linear scanning mode; scan speed 10 mm/min; excitation 365 nm, emission 450 nm.

(d) *Data processor.*—Chromatopac Shimadzu Model C-R2AX, or equivalent.

(e) *Spot viewer.*—UV Transilluminator Model TL-33 (Ultra-Violet Products, Inc.), equipped with longwave (365 nm) light.

(f) *HPTLC plate.*—10 × 10 cm (E. Merck, Art. 5631), precoated with silica gel 60 without fluorescent indicator.

(g) *Column.*—Glass, 1.0 cm id.

(h) *Filter paper.*—12.5 cm, Toyo Filter paper No. 5A (Toyo Roshi Ltd, Tokyo, Japan), or equivalent.

Reagents

(a) *Florisil.*—100–200 mesh, for column chromatography (Floridin Co.).

(b) *Trifluoroacetic anhydride (TFA).*—E. Merck, Art. 12513.

(c) *Aflatoxins.*—B_1, B_2, G_1, G_2, and M_1 were obtained from Makor Chemicals Ltd, Jerusalem, Israel.

(d) *Aflatoxins standards.—Mixture I.*—2 μg B_1, 1 μg B_2, 3 μg G_1, and 1 μg G_2/mL chloroform. *Aflatoxin M_1.*—1 μg M_1/mL chloroform.

(e) *Developing solvents.*—For aflatoxin B_1, B_2, G_1, and G_2: *(1)* chloroform–acetone (9 + 1), *(2)* ether–methanol–water (94 + 4.5 + 1.5); for aflatoxin M_1: *(3)* chloroform–isopropanol–acetone (85 + 5 + 10).

Extraction

(a) *Cereal and nut (and their products) samples.*—Weigh 20 g sample, finely ground in mill (thoroughly powder to < 20 mesh), into 200 mL glass-stopper flask, add 10 mL water and 100 mL chloroform, stopper, and shake 10 min using wrist-action shaker set at fast rate. Decant through filter paper into 50 mL glass-stopper graduated cylinders. Add ca 10 g anhydrous Na_2SO_4 to 50 mL filtrate, and hold for column chromatography.

(b) *Cheese sample.*—Weigh 20 g sample, cut into small pieces, into 500 mL blender jar, add 10 mL saturated NaCl solution and 100 mL chloroform, and blend 3 min using homogenizer set at fast rate. Decant through filter paper into 50 mL glass-stopper graduated cylinders. Add ca 10 g anhydrous Na_2SO_4 to 50 mL filtrate, and hold for column chromatography.

Column Chromatography

Prepare Florisil column for cleanup as follows: Insert glass wool in bottom of chromatographic column. Add ca 5 mL chloroform and 5 g anhydrous Na_2SO_4, and then add slurry of 0.7 g Florisil in chloroform. When gel settles, top with ca 0.5 g anhydrous Na_2SO_4, and drain solvent to top of Na_2SO_4. Transfer 50 mL filtrate to column, open stopcock, and drain solution through column by gravity. If flow rate slows, stir top of upper layer of Na_2SO_4. Rinse graduated cylinders with additional small portions of chloroform and add rinses to column. When filtrate reaches top of Na_2SO_4, rinse column sides with chloroform and drain similarly. Wash column with 30 mL chloroform–hexane (1 + 1) and 20 mL chloroform–methanol (9 + 1). Elute aflatoxins with 30 mL acetone–water (99 + 1), collecting eluate in 30 mL pear-shaped flask, and evaporate to dryness under reduced pressure on steam bath. Dissolve residue in 0.2 mL chloroform. Reserve for TLC test solution.

Thin Layer Chromatography

On imaginary or penciled line 2 cm from bottom of appropriate precoated HPTLC plate, spot 20 μL test solution and 2 μL aflatoxins mixture (B_1,2; B_2,1; G_1,3; G_2,1; and M_1,1 μg/mL) at 1.2 cm intervals. Dry spots with small fan. Develop plate in solvent *(1)* or *(2)* for extracts of cereal, nuts, and their products, or in solvent *(3)* for extracts of cheese, 9 cm from bottom edge. After development let plate dry 10 min. Observe plate under 365 nm UV light for fluorescent spot.

Quantitation by Densitometric Analysis

On HPTLC plate, spot 20 μL test solution, and 1, 2, 4 μL aflatoxins mixture at 1.5 cm intervals. Develop as described above using solvent. Scan plate on densitometer and quantitate by using data processor. If spot of sample is too intense to match standard, dilute sample, and rechromatograph. If sample extracts contain too much interfering substance on plate, experiment with 2-dimensional HPTLC plate. Spot 20 μL test solution and develop plate in solvent *(1)* in first direction. When solvent reaches score line (7 cm from bottom), remove plate, dry with small fan, spot 1, 2, and 4 μL portions of aflatoxins mixture, and redevelop in solvent *(2)* in second direction, 9 cm from bottom. Scan plate on densitometer and quantitate by using data processor.

Table 1. Comparison of methods for recovery of aflatoxins from spiked corn and peanut samples[a]

	Rec., %											
	Simple method				CB method				BF method			
Sample	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Corn	98	84	88	78	64	75	83	65	88	73	23	62
	83	90	76	69	78	82	94	78	69	70	32	75
	99	85	94	77	77	81	93	78	85	80	27	70
	88	87	92	81	72	89	82	74	81	66	34	63
	92	94	95	85	81	76	85	60	67	72	38	50
Av. rec., %	92	88	89	78	74	81	87	71	78	72	31	64
Std dev.	6.74	4.06	7.74	5.92	6.65	5.59	5.68	8.12	9.49	5.12	5.89	9.46
Coeff. of var., %	7.33	4.62	8.70	7.58	14.39	6.94	6.50	11.44	12.16	7.09	19.13	14.78
Peanut	92	89	99	84	84	81	78	74	43	72	41	66
	84	92	90	79	63	96	86	62	73	96	52	70
	98	86	93	76	70	83	71	81	52	77	46	53
	9[illegible]	101	97	85	76	85	82	65	61	83	34	51
	90	83	88	71	82	97	83	73	59	76	37	60
Av. rec., %	89	90	93	79	75	88	80	71	58	81	42	60
Std dev.	3.16	6.90	4.62	5.79	8.66	7.54	5.79	7.58	11.12	9.36	7.17	8.15
Coeff. of var., %	3.55	7.66	4.94	7.33	11.55	8.52	7.23	10.70	19.30	11.59	17.09	13.59

[a]Samples spiked with 0.2 mL of aflatoxin standard mixture: B_1, 20; B_2, 10; G_1, 30; G_2, 10 μg/kg.

Confirmation of Aflatoxin Identity

Spot 20–40 μL remaining test solution and develop plate in solvent (*1*) in first direction. After first development, remove plate from tank and dry with small fan. Carefully mark location of aflatoxin spots from sample extracts and spot from aflatoxin standard. Treat these spots with 5 μL TFA, heat 10 min at 50°C, cool plate, and redevelop in second direction in chloroform–methanol (95 + 5). Examine developed plate under 365 nm UV light and look for blue fluorescent spot representing sample extract and standard TFA derivatives. Identity of aflatoxin in sample is confirmed when R_f values of both TFA derivatives match.

Recovery Experiments

Prepare samples of ground corn and peanut by adding 0.2 mL of aflatoxin standard mixture in chloroform: B_1, 20; B_2, 10; G_1, 30; G_2, 10 μg/kg. Prepare cut cheese sample by adding 0.2 mL mixture of aflatoxin standards B_1 (20 μg/kg) and M_1 (10 μg/kg) in chloroform. Seal containers with glass stoppers and mix contents by shaking 1 min. Let stand 1 day unsealed to let solvent evaporate, and then analyze by above method and by official methods (1).

Results and Discussion

The most reliable and generally used column method for aflatoxin analysis is silica gel column chromatography, in part, because of the use of activated adsorbent and the treatment of ethanol in ether before column chromatography. Recent commercial chloroform contains 0.4–0.9% ethanol.

Figure 1 shows elution profiles from the silica gel column. Two commercial forms of chloroform were chosen: one containing 0.4–0.5% ethanol, and another containing 0.5–0.9% ethanol. Experiments were conducted to determine column retention of aflatoxins. Portions of 50 mL chloroform, extraction solvent, 150 mL hexane and 150 mL ether as the rinse solvents, and 150 mL chloroform–methanol (97 + 3) as the eluting mixture were collected. Each fraction was evaporated to dryness, dissolved with chloroform, and analyzed by TLC; toxins were quantitated by densitometry. When chloroform containing high concentrations of ethanol (0.5–0.9%) was used, aflatoxin B_1 was detected in all fractions. The results of this silica gel column chromatography strongly suggest that there is a great difference in recovery of aflatoxins, depending on the ethanol stabilizer concentration in the chloroform.

Method Development

In our experience, the extraction and cleanup method described significantly reduced analyst time and labor. By combining extraction and purification on the Florisil column, followed by TLC with quantitation by densitometry, the method can be used for both qualitative and quantitative determination in an assay of agricultural commodities and commercial foods.

Experiments were conducted using commercial supplies and reagents without further treatment, and using chloroform containing high concentrations of ethanol (0.5–0.9%). Aflatoxins were completely retained on the Florisil when the extract and rinse solvents were passed through the Florisil column. Application of aflatoxins, 10 g edible oil, and 10 g

Table 2. Comparison of methods for recovery of aflatoxins in spiked cheese[a]

	Rec., %			
	Simple method		Milk method	
Sample	B_1	M_1	B_1	M_1
Cheese	98	96	63	90
	96	101	78	91
	88	91	69	85
	90	85	84	98
	93	87	70	86
Av. rec., %	93	92	73	90
Std dev.	4.12	6.56	8.23	5.15
Coeff. of var., %	4.43	7.13	11.30	5.72

[a]Samples spiked with 0.2 mL mixture of aflatoxin standards B_1 (20 μg/kg) and M_1 (10 μg/kg).

Table 3. Comparison of methods for determination of aflatoxins in naturally contaminated buckwheat flour sample

	Aflatoxins, ng/g				
Method	B_1	B_2	G_1	G_2	Total
Simple	9.24	0.97	0.73	0.09	11.03
CB	8.08	0.72	0.70	0.04	9.54
BF	7.26	0.90	0.27	ND[a]	8.43

[a]ND = none detected.

Table 4. Collaborative results for determination of aflatoxins in naturally contaminated buckwheat flour and cheese samples

	Buckwheat flour: aflatoxins, ng/g					Cheese:
Collaborator	B_1	B_2	G_1	G_2	Total	M_1
1	10.00	0.98	1.00	0.10	12.08	0.36
2	8.35	0.76	0.77	0.09	9.97	0.34
3	7.97	0.81	0.87	0.10	9.75	0.32
4	8.82	0.84	0.80	0.06	10.52	0.30
5	8.29	0.85	0.74	0.06	9.94	0.34
Range high	10.00	0.98	1.00	0.10	12.08	0.36
low	7.97	0.76	0.74	0.06	9.75	0.30
Mean	8.69	0.85	0.84	0.08	10.45	0.33
Coeff. of var., %	9.15	9.63	12.39	24.99	9.13	6.87

tallow to the column yielded results which are presented in Figure 2. Observation of each fraction after evaporation of solvent showed that a total of 30 mL chloroform–hexane (1 + 1) is sufficient for removing oils from the column. A mixture of chloroform–methanol (9 + 1) as the rinse solution eluted fluorescent interferences and pigments without eluting aflatoxins. The Florisil column was quite retentive for aflatoxins; over 99% of the aflatoxins were retained on the Florisil column even after rinsing with the 2 solvent mixtures.

Thirty mL acetone–water (99 + 1, v/v) quantitatively eluted the aflatoxins from the column. Figure 2 presents elution profiles of the aflatoxins from the Florisil column. Aflatoxin M_1 was recovered in the first 15 mL; aflatoxin B_1, B_2, G_1, and G_2 were eluted completely in the first 25 mL. Hence, 30 mL acetone–water was used in the analytical procedure to elute all 5 aflatoxins.

Comparison with Official Methods

Results for comparing the simple, CB, BF, and milk methods for determining aflatoxins in spiked and naturally contaminated samples are presented in Tables 1–3.

When spiked corn samples were analyzed by the simple, CB, and BF methods, recoveries of aflatoxins for the simple method were considerably higher than those for the 2 official methods. Much lower recoveries of aflatoxin B_1 by the CB method and aflatoxin G_1 by the BF method were noted (Table 1). The average recoveries of aflatoxins across the 5 samples were aflatoxin B_1, 92%; B_2, 88%; G_1, 89%; G_2, 78% by the simple method; B_1, 74%; B_2, 81%; G_1, 87%; G_2, 71% by the CB method; and B_1, 78%; B_2, 72%; G_1, 31%; G_2, 64% by the BF method.

Analyses of spiked peanut samples by the same 3 methods gave results similar to those for spiked corn samples, except for the BF method. Aflatoxin recoveries of the BF method were much lower probably because peanut extracts contained excessive interferences and required silica gel column chromatographic cleanup.

When the spiked cheese samples were analyzed by the simple and milk methods, results were in good agreement, except for aflatoxin B_1 determined by the milk method. The results of this experiment are presented in Table 2. The average recoveries of aflatoxins across the 5 samples were 93% B_1, 92% M_1 by the simple method, and 73% B_1, 90% M_1 by the milk method.

When the naturally contaminated buckwheat flour samples were analyzed by the CB, BF, and simple methods, results were similar to those from spiked samples; the values obtained for aflatoxins are presented in Table 3. The CB method gave a lower value for aflatoxin B_1 than did the simple method. Moreover, the BF method gave lower values for aflatoxins than did the other 2 methods, and no aflatoxin G_2 was detected. The mean total aflatoxins value by the simple method was 11.03 μg/kg; by the CB method, 9.54 μg/kg; and by the BF method, 8.43 μg/kg.

When the naturally contaminated cheese samples were analyzed for aflatoxin M_1 by the simple and the milk methods, the 2 methods were in good agreement (simple method, 0.34 ng/g; milk method, 0.33 ng/g).

The minimum detectable aflatoxin concentrations (μg/kg) in various test materials were 0.2, B_1; 0.1, B_2; 0.2, G_1; 0.1, G_2; and 0.1, M_1.

Collaborative Study

The method described here for determination of aflatoxins was studied collaboratively by 5 laboratories to test for precision.

Samples of naturally contaminated buckwheat flour and cheese were prepared for collaborative study. Each 50 g sample was weighed into a vinyl bag container. Samples, detailed laboratory information, and the procedure were all sent to each collaborator.

Table 4 contains results from 5 collaborators for the determination of aflatoxins in naturally contaminated buckwheat flour and cheese samples. Between-collaborator results were approximately the same by the simple method. Coefficients of variation were acceptably low (B_1, 9.2%; B_2, 9.6%; G_1, 12.4%; G_2, 25.0%; M_1, 6.9%).

In conclusion, the results of this study clearly show more efficient recovery and shorter analysis time by the simple method (3 or 4 h) than the official methods (8–15 h), and indicate that the described simple method provides results equivalent to currently accepted methodology.

Acknowledgment

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Rapid Reverse Phase Liquid Chromatographic Determination of Aflatoxin M_1 in Milk

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A rapid, economical, and reliable liquid chromatographic (LC) method is described for determination of aflatoxin M_1 in milk. The method includes an improved AOAC extraction procedure, cleanup of the extract on a silica cartridge, and LC quantitation. Alternatively, a rapid column cleanup procedure can be used. Milk artificially spiked with aflatoxin M_1 at 0.05, 0.1, and 0.5 ppb was analyzed using both new approaches as well as an AOAC method coupled with LC for quantitation of the toxin. Recovery of aflatoxin M_1 by the first approach of the new method ranged between 93.4 and 99.1%, and for the alternative procedure between 92.4 and 96.8%. The AOAC method gave lower recovery (85.6–90.7%) of toxin, but the results from this method had a somewhat smaller standard deviation for replicate analyses than did results of the new method.

There is a continuing interest in developing new methods to determine aflatoxin M_1 in milk. This reflects the necessity for more reliable and sensitive methods to help ensure the safety of this food. That interest also indicates the lack of a method that meets the needs and limitations of workers in various laboratories.

A variety of methods to determine aflatoxin M_1 in milk is currently available. These methods employ either thin layer chromatography (TLC) (e.g., 1–5) or liquid chromatography (LC) (e.g., 6–11) for quantitation of the toxin. Because they are more accurate, consistent, and rapid than TLC, LC methods are currently replacing conventional TLC methods. AOAC adopted a TLC method (3) to determine aflatoxin M_1 in milk (12). More recently, the International Dairy Federation (IDF) recognized the same method as its standard method (13). The method is reasonably rapid; however, it has the disadvantages inherent in the TLC quantitation step.

Because of these limitations in TLC, we have examined available LC methods to measure aflatoxin M_1 in milk. Our experience has indicated that each of the currently available LC methods lacks one or more of such desired features as economy in usage of solvents and materials, speed, cleanliness of final extract (accordingly resolution of toxin from interfering impurities), or high recovery of aflatoxin M_1.

The method we developed was designed to use some desirable features of the AOAC method, to take advantage of the precision and accuracy of reverse phase LC techniques, and to overcome some of the drawbacks of current LC methods.

METHOD

Apparatus

(a) *Chromatographic columns.*—30 × 1.0 cm glass column (Bio-Rad, No. 737-2250) equipped with Luer nylon stopcock (Bio-Rad, No. 732-9009), and polypropylene funnel (Bio-Rad, No. 731-0001).

(b) *Butt tube.*—Pyrex, No. 3700.

(c) *Silica cartridges.*—Sep-Pak silica cartridges (Waters, No. 51900).

(d) *Rotary evaporator.*—Büchi, Rotavapor-R.

(e) *LC system.*—Equipped with pump (Waters, Model 6000A); injector (Rheodyne, Model 7120) with 10 μL sampling loop; reverse phase 5 μm C_{18} column (Waters, Nova Pak No. 86344); fluorescence detector (Waters, Model No. 420) with aflatoxin lamp (Waters, No. 78409), 365 nm excitation filter (Waters, No. 78225) and 425 nm emission filter (Waters, No. 78155); and recorder (Linear, Model 252A).

Reagents

(a) *Solvents.*—Hexane and toluene, distilled in glass (Burdick and Jackson); chloroform, AR, containing 0.75% ethanol (Mallinckrodt); acetone, AR (Mallinckrodt); dichloromethane, ChromAR (Mallinckrodt); acetonitrile and methanol, LC grade (Baker). Treat ethyl ether, AR (Mallinckrodt), as follows: Shake 75 mL ether with equal volume of water in 125 mL separatory funnel, drain water phase, and dehydrate ether layer by filtration through 20 g anhydrous granular Na_2SO_4 in Butt tube; alternatively, ChromAR grade ether (Mallinckrodt) may be used after similar treatment.

(b) *Silica gel.*—Merck No. 7754, for column chromatography. Activate 60 min at 105 ± 1°C, let cool, add 1% water, seal container, shake thoroughly, and equilibrate at least 15 h before use.

(c) *Aflatoxin M_1.*—(Sigma). Dry film was dissolved in chloroform to nominally contain 10 μg/mL (primary standard). Exact concentration of solution was measured by using a spectrophotometer (12). Vials used to prepare working standard were silylated (personal communication, R. D. Stubblefield, U.S. Dept of Agriculture, Peoria, IL) as follows: Glass vials were filled nearly full with solution of dichlorodimethylsilane in toluene (5%); then vials were heated ca 40 min at 50°C. Solution was discarded and vials were rinsed 3 times with toluene and then 3 times with methanol. Methanol was evaporated by heating vials. Working standards were prepared by transferring calculated amounts of primary standard to silylated vials, then drying under gentle stream of nitrogen. Dried film was redissolved in appropriate amount of methanol to give high (100 ng/mL) or low (10 ng/mL) concentration working standards.

Milk Samples

Raw whole milk was obtained from the University of Wisconsin dairy factory.

Recovery Samples

About half the weight of milk to be prepared was stirred by using a magnetic stirrer at medium speed. Calculated volume of working standard (100 ng/mL in methanol) was added, and then rest of milk was added. Milk was spiked to contain 0.5, 0.1, and 0.05 ppb aflatoxin.

Extraction

A modification of the AOAC extraction procedure (12) was used. Transfer 20 g milk (warmed to room temperature) to 125 mL separatory funnel. Add 50 mL dichloromethane and 5 mL saturated NaCl solution. Shake gently 2 min and then let phases separate. Stopper stem of Butt tube with glass wool, place 10 g anhydrous, granular Na_2SO_4 in tube and mount tube on 100 mL graduated cylinder. Drain lower organic phase into graduated cylinder through Butt tube. Record volume of extract collected.

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Table 1. Comparison of LC methods as applied in this study

Method	Extraction: Wt of milk, g	Extraction: Vol. of extg solv., mL	Cleanup: No. of washings	Cleanup: Total vol. of washing solv., mL	Cleanup: Vol. of eluting solv., mL	Analysis time,[a] min
I. AOAC extn and cleanup with LC	50	120 ($CHCl_3$)	3	75	60 ($CHCl_3$, acetone)	107
IIA. LC with cartridge cleanup	20	50 (CH_2Cl_2)	1	4	4 (dichloromethane, ethanol)	45
IIB. LC with column cleanup	20	50 (CH_2Cl_2)	2	40	25 (dichloromethane, ethanol)	76

[a]Analysis time is time required to extract, clean up and quantitate aflatoxin M_1 in a sample of milk. Timing did not include preparation of LC mobile phase or stabilization of LC system. Each value is the average of analysis time of 3 milk samples that were tested a single sample at a time.

Cleanup

Either of the following procedures may be used.

Silica cartridge procedure.—Quantitatively transfer contents of graduated cylinder into 250 mL round-bottom flask. Rinse cylinder with dichloromethane and transfer rinse solution to the round-bottom flask. Dry extract by using rotary evaporator. Water bath temperature should not exceed 50°C. Wrap round-bottom flask in aluminum foil (as precautionary measure, to protect dry extract from any stray light) and let cool for a few minutes. Attach long stem of silica cartridge to Luer tip of barrel of 10 mL glass syringe. To prewash cartridge, transfer 5 mL ether to syringe barrel, then install syringe plunger and push to drain ether through cartridge at flow rate of ca 3 mL/min. As soon as ether level reaches long stem of cartridge, detach cartridge, remove plunger, and then re-attach cartridge to syringe barrel. This final sequence ensures that no air is pumped into or drawn through cartridge and should be followed throughout rest of cleanup procedure. Flow rate of all solvents into cartridge was kept close to 3 mL/min.

Quantitatively transfer residue in round-bottom flask to cartridge as follows: Dissolve contents of round-bottom flask in 2 mL ether, transfer to syringe barrel by using Pasteur pipet. Repeat process using another 2 mL ether. Drain solution through cartridge. Rinse round-bottom flask with an additional 2 mL ether, transfer rinse to syringe barrel, and drain as before.

Wash cartridge with 4 mL ether–hexane mixture (3 + 1 v/v) and discard eluate. Elute toxin with 4 mL dichloromethane–ethanol mixture (95 + 5 v/v), collect eluate in 4 mL glass vial, and then cover it with Teflon-lined screw-cap.

Silica column procedure.—Fill chromatographic column half-full with dichloromethane. Drain ca 2 mL solvent and gently tap column to get rid of any air bubbles entrapped in fritted disc. Add 2 g silica to column and slurry it with a glass rod. Wash silica from interior surface of column by using 2–3 mL dichloroethane. Drain 2–3 mL solvent to speed up settling of silica. When completely settled, top silica with 2 g anhydrous granular Na_2SO_4. Wash Na_2SO_4 off column interior by using dichloromethane. Drain dichloromethane to ca 1 cm above Na_2SO_4 layer.

Attach polypropylene funnel to column. Transfer milk extract to column and let it drain to ca 1 cm above Na_2SO_4 layer. Wash graduated cylinder with dichloromethane, transfer wash to column, and drain as before. Rinse funnel and sides of column with dichloromethane and drain until solvent level reaches Na_2SO_4 layer. Wash column with 25 mL toluene–acetic acid mixture (9 + 1 v/v), followed by 15 mL hexane. Drain each solvent as indicated before and discard eluate. Elute toxin with 25 mL dichloromethane–ethanol (95 + 5 v/v), and collect eluate in 100 mL round-bottom flask. Dry contents of round-bottom flask by using rotary evaporator. Water bath temperature should not exceed 50°C. Quantitatively transfer residue to 1 mL glass vial with dichloromethane using Pasteur pipet. Cover vial with a Teflon-lined screw-cap.

Quantitation

Dry the dichloromethane solution from either cleanup procedure in water bath at ca 45–50°C under gentle stream of nitrogen. Soon after extract is dry, remove vials from water bath, dissolve residue in 100 μL methanol. Stir solution on Vortex mixer.

Prepare mobile phase consisting of mixture of water–acetonitrile (73 + 27 v/v). Deionized, glass-distilled water was used. Filter solvent mixture through 0.2 μm Durapore filter (Waters, No. GVWP-047). Sonicate under vacuum ca 7 min, just before use, to degas mobile phase. Operate LC unit with

Table 2. Recovery of aflatoxin M_1 from artificially contaminated milk samples

Method	Amt added, ppb	Rec., %, of individual samples					Av. rec., %	Std dev.
I. AOAC (1980) with LC	0.05	91.0	91.4	86.5	95.3	83.4	89.5	4.64
	0.1	87.1	86.9	82.8	85.8	85.1	85.6	1.74
	0.5	92.0	95.9	80.6	90.0	94.8	90.7	6.08
IIA. LC—cartridge cleanup	0.05	89.1	92.4	101	92.2	92.6	93.4	4.31
	0.1	101	88.3	93.1	99.6	99.9	96.5	5.60
	0.5	103	95.4	103	98.7	94.9	99.1	4.01
IIB. LC—column cleanup	0.05	92.4	81.7	96.7	105	85.7	92.4	9.34
	0.1	90.1	91.8	87.7	103	96.0	93.7	6.04
	0.5	99.9	93.4	99.5	91.0	100	96.8	4.27

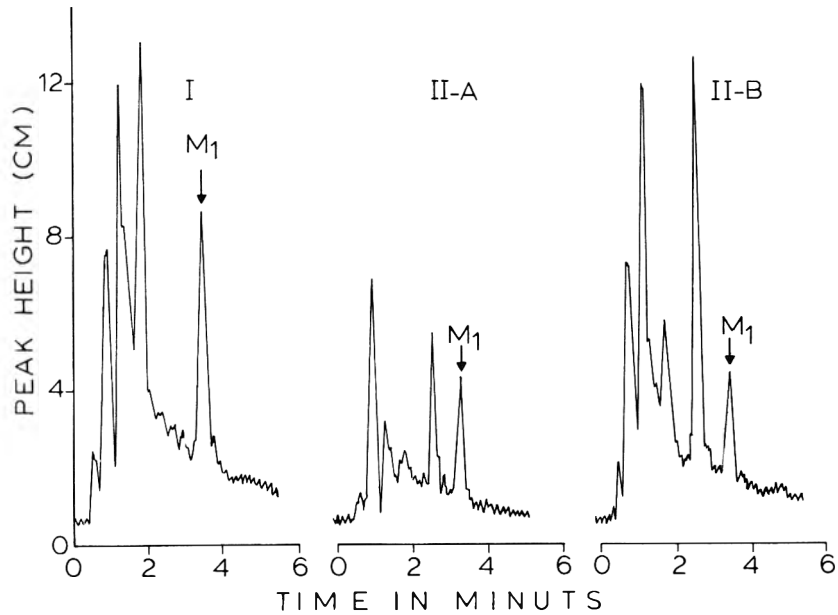

Figure 1. LC chromatogram of 50 g (I) or 20 g (II) milk samples containing 0.05 ppb aflatoxin M_1, and analyzed by the AOAC method coupled with LC quantitation (I), the cartridge cleanup version of the new method (II-A), or the column cleanup version (II-B). Detector was set at maximum gain. See text for other LC conditions.

mobile phase until system is stabilized and gives constant peak height when standard is repeatedly injected. Inject 10 μL of methanolic extract. With flow rate at 1 mL/min and using LC conditions described, aflatoxin M_1 had an average retention time of 3.5 min. Calculate concentration of toxin in milk as follows:

$$\text{ppb Aflatoxin } M_1 = (PH(M) \times C \times Vi(S) \times Vt \times Ve) / (PH(S) \times Vi(M) \times (Vr \times W)$$

where PH = peak height (mm) of standard (S) or milk sample (M); C = concentration of standard (ng/μL); Vi = injected volume (μL) of standard (S) or milk extract (M); Vt = total volume (μL) of methanolic final extract; Ve = volume of solvent used to extract milk sample (mL); Vr = volume of solvent recovered after extraction (mL); W = weight of milk sample (g).

$$\% \text{ Recovery} = \text{ppb } M_1 \text{ detected in milk} \times 100 / \text{ppb } M_1 \text{ added to milk}$$

Comparison with AOAC Method

Aflatoxin M_1-spiked milk was analyzed by using the AOAC extraction and cleanup procedure (12). Dry extract, after cleanup, was dissolved in methanol, instead of benzene–acetonitrile. Aflatoxin M_1 in the methanolic solution was measured by using the LC procedure described earlier.

Results and Discussion

Whole raw milk containing 3 levels of aflatoxin M_1 (0.5, 0.1, and 0.05 ppb) was analyzed by the 2 versions of our method as well as by the AOAC method. A comparison of the procedures is summarized in Table 1. From this comparison, it is apparent that the new method is more economical in solvent usage and faster than the AOAC method.

By using silica cartridges instead of chromatographic columns, it was possible to reduce time of analysis and use of solvents to a minimum (Table 1), and at the same time get high recovery of aflatoxin (Table 2) and a reasonably clean final extract (Figures 1 and 2). The percent of toxin recovered generally was higher when higher levels of toxin were in milk and vice versa. Several reverse phase LC methods for determination of aflatoxin M_1 in milk depend on derivatization of the toxin with trifluoroacetic acid (7, 9, 11). The derivatization product is much more polar (accordingly can be dissolved in a solvent compatible with a reverse phase system) and more fluorescent (accordingly allows detection of lower levels of toxin) than aflatoxin M_1. In spite of these characteristics of the product, the methods, in our hands, did not always perform in a satisfactory manner. Furthermore, evidence is lacking to show that derivatization of aflatoxin M_1 in milk is complete.

When we dissolved the aflatoxin standard and milk extract in methanol we obtained consistent results, and working standards were stable for at least a week when stored in the freezer. Furthermore, it is faster and more economical to dissolve the toxin in methanol and thus to avoid the derivatization process. There was speculation about the possibility of incomplete solubilization of pure aflatoxin from dry films, using solvents other than chloroform (14). To exclude this possibility, glass vials used for our working standards were silylated to prevent possible adsorption of toxin to glass surfaces. Using the highest setting of gain on the detector, it was possible to detect levels of toxin as low as 50 ppt (0.05 ppb) (Table 2 and Figure 1).

The alternative cleanup procedure (IIB) is a modification of the AOAC method where the volume of solvents to clean up the extract and elute the toxin was considerably reduced. This method gave higher recovery of toxin than the AOAC method as used in our study, but lower than the cartridge procedure (IIA). Washing the silica column with a hexane–ether–acetonitrile mixture in the AOAC method was eliminated in our method, because that step tended to reduce recovery of toxin. The need for a cleaner extract in the AOAC method than in our procedure is understandable because the AOAC method was originally designed to be used with TLC quantitation. Using LC for quantitation in our modified procedure (IIB), toxin was well resolved from impurities in the final extract (Figures 1 and 2). The problem in the AOAC extraction procedure of a tendency to form an emulsion which is hard to break into 2 phases (4, 9) was minimized in our method by using the less polar dichloromethane (instead of

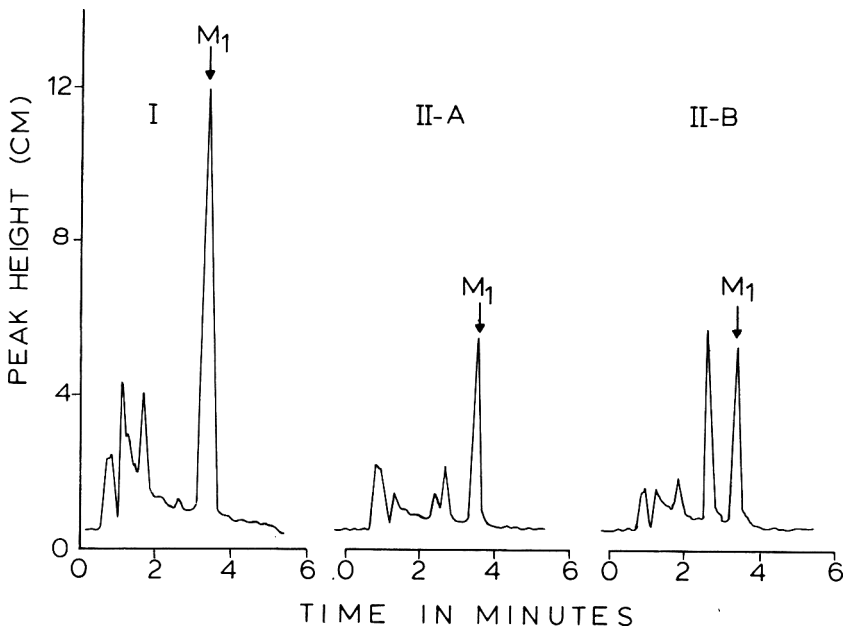

Figure 2. LC chromatograms for same analytical conditions as in Figure 1 except milk contained 0.5 ppb aflatoxin M_1, and detector was set at 25% of its maximum gain.

chloroform) and shaking gently for 2 min (instead of shaking vigorously for 1 min as in the AOAC method).

Although it gave lower recovery values for toxin than did the new method, the AOAC method (with LC quantitation) in this study gave higher recovery values than those reported in other studies where the toxin was quantitated with TLC (3, 4, 9). Variability between replicate samples (as measured by standard deviation) was comparable (IIA) or somewhat higher (IIB) for our method than for the AOAC method as applied in this study. The larger standard deviation for our method probably resulted from reducing the size of the milk sample to be analyzed. Variability between replicates tested by the new method, however, was much less than that reported in other studies in which the same levels of aflatoxin we used were measured by the conventional AOAC method (3, 4, 9).

In conclusion, the method we have described is sufficiently rapid, economical, and precise to meet the needs of laboratories where an assay for aflatoxin M_1 in milk is done routinely. Either of the 2 approaches for sample cleanup can be followed, depending on the particular needs of the laboratory, costs, and availability of materials.

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FERTILIZERS

Performance Criteria Proposed for Automated Determination of Direct Available P_2O_5 in Fertilizers

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Recent efforts to promote the performance style of writing AOAC methods has successfully produced a performance-oriented official flame photometric method for K_2O in fertilizers. This official K_2O method specifies a fixed chemistry which may be implemented by whatever instrumental or manual design the user chooses so long as the performance criteria are met. It is hoped that this could be done for the determination of available P_2O_5 in fertilizers. This report is intended to serve as a first step toward that objective by giving a detailed description of the current AOAC official automated method for available P_2O_5 in fertilizer which will provide the necessary understanding of the complexity involved in writing the P_2O_5 method in terms of performance. Included in this report is a proposed generalized method written in a performance-oriented fashion with few restrictions and without endorsing a particular manufacturer's equipment. Our laboratory has contributed much to the original development of the official P_2O_5 method and has acquired considerable knowledge about this method through the many years it has been used in our fertilizer control program. We feel a more generalized treatment of this method would be worthwhile and benefit users by giving them more flexibility.

Recently a collaborative study was done on the flame photometric determination of K_2O in fertilizers. The method given to the collaborators was written in a general performance-oriented fashion, and included a detailed automated instrumental system which served as an example procedure that met the performance requirements outlined. The collaborators were instructed to use either the example system or any analysis scheme that met the performance criteria given to them. Within this framework, the K_2O method was successfully collaborated and is now an official first action method. It is hoped that this could also be done for the determination of available P_2O_5 in fertilizer. This report is intended to serve as a first step toward that objective.

There is a growing concern about the difficulties that arise when official methods specify a particular manufacturer's equipment or rigid design. When new or improved instrumentation become available, which is occurring with greater frequency, users apparently cannot take advantage of the new designs and still produce an analysis that is considered official, or worse, their current equipment becomes obsolete or discontinued and there is no source for replacement parts. Many laboratories may avoid making major investments in expensive equipment which may only be useful for a short time. If an official method were described in a general fashion which included criteria for performance to ensure its proper use, then users would have the flexibility to take advantage of improvements or adapt their current instrument model to a new better suited configuration.

The current automated method for available P_2O_5 in fertilizer is written specifically for continuous segmented flow application in terms so rigid that only one analytical range and one sampling rate may be used. It is also tied, practically speaking, to Technicon Corp. AutoAnalyzer equipment even though the term "or equivalent" is used when referring to this equipment in the official method. Certainly it would be worthwhile to have this method described in a general, performance-oriented fashion to allow users greater flexibility.

It was decided to begin this report with a detailed description of the principles underlining the AOAC official automated method for available P_2O_5 in fertilizers. This description would provide the necessary understanding of the complexity involved in writing this method in terms of performance.

It is difficult, at present, to perceive adapting this method to other continuous flow principles such as flow injection. But this method certainly could be adapted to automatic discrete analyzer systems, manual or mechanized systems and it is hoped that enough general information about the method will be provided within this report to allow these applications to be considered.

Also included in this report is a proposed generalized method written in a performance-oriented fashion with few restrictions and without endorsing a particular manufacturer's equipment. This generalized method serves as an example of how performance criteria might be described for an automated method.

General Principles of the Method, Applicability and Limitations

The official automated method (1) measures P_2O_5 as orthophosphate from a direct available extract by the colorimetric vanadomolybdate method. All steps necessary for measuring P_2O_5 in the extract are incorporated in the automated system. These steps are hydrolysis of non-orthophosphate, destruction of coloring matter, digestion of citrate, and final colorimetric measurement. Hydrolysis of non-orthophosphate and destruction of coloring matter are accomplished by controlled heating at 95°C with 4N $HClO_4$. This step will also digest citrate, but it is believed that some undigested citrate remains which interferes slightly with color development. This interference must be masked, and masking of residual citrate must be considered an important step in the procedure. This masking is accomplished in the system by controlling the concentrations of molybdate, vanadate, and acid at color development. Users unaware of this potential interference can experience problems. The effect of citrate can cause either a positive or negative interference, especially if the reagent ion concentrations are nearly, but not exactly correct. Much of the discussion about this method in this report will center around the citrate effect.

One limitation of the method is its inability to measure some extractable organic phosphorus forms found in a few specialty fertilizers. If available P_2O_5 is defined as total phosphorus measured in an aliquot from the direct available extract, then it appears that the digestion conditions in the automated system are not vigorous enough to handle some soluble organic forms. There have been a few cases in this laboratory where higher results were obtained after the extract was treated for organic phosphorus as compared with results by the automated method. Practically speaking, only a few commercial

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grades present a problem, but users should be made aware of this limitation.

Another limiting factor is a result of the direct available sample preparation procedure which dictates that a specific sample size be extracted to a specific volume whether the sample contains 1 or 50% P_2O_5. The result is a wide range of phosphorus in the extracts to be analyzed. Those samples outside the 7.5 to 17.5% P_2O_5 range must be diluted or spiked prior to analysis. Users do not have the option of selecting sample size as a means of optimizing the method. A more dynamic analysis range would benefit those laboratories, especially control laboratories, that must deal with this wide range of phosphorus levels. Also, systems could be designed for high precision and high accuracy analysis of the high level phosphate fertilizers, such as triple superphosphate and di- and monoammonium phosphate. These customized systems would be helpful to both control and industry laboratories.

The current automated system, without any modification, will also measure P_2O_5 in an extract prepared for total P_2O_5. Measuring total P_2O_5 this way is more a convenience than a necessity because the hydrolysis step is not required. A much shorter and therefore faster system could be designed for measuring total P_2O_5 in a HNO_3–$HClO_4$ wet-ash whose only requirement is color development.

Major Chemistry Considerations of the Official Method

Hydrolysis of non-orthophosphate requires time, temperature, and acid conditions. The hydrolysis apparatus of the official method is composed of two 10.6 mL coils, positioned closely together but each set in separately controlled heating blocks maintained at 95°C. Residence time through both coils at 95°C is about 15 min at flow rates of 0.60 mL/min of acid, 0.23 mL/min of sample, and 0.32 mL/min of air. As an alternative, one may use a 40 ft × 1.6 mm id coil set in an AutoAnalyzer-style heating oil bath. Both set-ups accomplish the conversion of non-orthosphosphate, destruction of coloring matter, etc; however, the oil bath is now a discontinued item.

Perchloric acid was chosen as the hydrolysis acid because it was most effective at digesting citrate and most compatible with the color reaction when citrate was present. Hambleton (2) experimented with sulfuric acid and found it to be quite effective at hydrolyzing non-orthophosphate as expected, but ineffective at destroying the citrate, which seriously affects the color reaction. An $HClO_4$ strength of 4N will achieve hydrolysis with little difficulty, as will lesser amounts such as 3N, which was determined by Hambleton (2). The 4N strength was chosen over 3N for the official method because Wall and Gehrke (3) found it to be more effective at digesting citrate. However, they reported that concentrations above 4N or longer digestion times proved ineffective at removing the traces of citrate that remained and interfered with the color reaction. Only changing the composition of the color reagent proved successful in controlling the interference from the remaining citrate.

The concentrations of molybdate and vanadate required for adequate color development and masking of citrate interference are not easily determined. Several solutions of varying molybdate, vanadate, and acid composition must be made and tested with different phosphate and citrate levels. This is how the reagent ion concentrations were originally determined, mainly through the work of Wall and Gehrke (3). The maximum possible amount of ammonium citrate in the sample extract that needs to be considered is 20 v/v% and some $HClO_4$ must be added to the combined molybdovanadate reagent to stabilize it. The current official reagent contains 8.25 g ammonium molybdate·$4H_2O$, 0.30 g ammonium metavanadate, and 30 mL 70% $HClO_4$/L. It is pumped in at a rate of 1.0 mL/min and combined with digested sample pumped at a rate of 0.23 mL/min. This color reagent composition produces adequate color for the 150–350 ppm P_2O_5 range where the response is linear, or nearly so, when measured at 420 nm in a 1.5 × 15 mm flowcell. When the waste from the flowcell was collected and the absorption was measured in 1.0 cm cells, the absorbance values ranged from 0.14 for the low 150 ppm P_2O_5 to 0.34 for the high 350 ppm P_2O_5.

Wall and Gehrke's (3) working color reagent contained 6.25 ± 0.25 g ammonium molybdate·$4H_2O$, 0.325 ± 0.025 g ammonium metavanadate, and 27.5 ± 2.5 mL $HClO_4$ acid/L. Their system used the same sample and reagent flow volumes throughout as are now used in the official method, but the phosphorus level was somewhat different, 100–250 ppm P_2O_5. In their study, they varied the color reagent composition from their working range and found that the residual citrate from the maximum 20 v/v% in the sample caused a decrease in response when the color reagent concentration was too low (5.0 g MO; 0.25 g VAN; 32.5 mL acid/L) and an increase in response when the reagent concentration was too excessive (20 g MO; 1.0 g VAN; 125 mL acid/L). Although they express some flexibility in their working reagent composition, they did make the following cautionary statement regarding the citrate interference:

"The ratio of ammonium molybdate and ammonium vanadate to P_2O_5 in the final reaction mixture is apparently rather critical to avoid citrate interference. It is therefore advisable to evaluate the effect of citrate on the P_2O_5 response initially when setting up the automated system. The citrate effect should be checked periodically (weekly) during routine use of the method."

It appears that this masking of the citrate interference is considered critical on the one hand, yet can be handled with some flexibility. This is somewhat contradictory and is probably not completely understood. What is important to bear in mind is that it can cause either a positive or negative interference.

The hydrolyzed stream is resampled before color development because it is too acidic (ca 2.9N); added color reagent will not produce any color. Hambleton (2) studied the effect of final $HClO_4$ concentration on the P_2O_5 response in an automated system very similar in design to the official one, which included a hydrolysis bath and resample arrangement. Final $HClO_4$ concentration is the result of the combined contributions from both the color reagent and hydrolysis bath. Hambleton found that final $HClO_4$ concentrations ranging from 0.4N to 0.79N did not change P_2O_5 response. Levels above 0.79N caused a decrease in response. The final acid concentration at color development in the official method, calculating from the flow volumes, is 0.83N. This value appears to exceed, or at least be very close to Hambleton's maximum. However, in manual wet chemistry versions of the molybdenum yellow method applied to various sample matrices, the amount of acid required for the formation of the phosphomolybdovanadate complex is not considered critical (4, 5). A final acid concentration of ca 0.5N is usually recommended and concentrations above 1.0N are known to slow color formation and produce less color. To obtain full color development in ca 5 min, the acidity should be less than 1N.

Table 1 is a summary comparing the final acid, molybdate, and vanadate concentrations at color development among the 3 method configurations discussed, the official, the Wall and Gehrke, and the Hambleton configurations. All final concentrations among the 3 configurations are reasonably similar;

Table 1. Conditions at color development for 3 configurations of automated method for available P_2O_5 in fertilizers

Configuration	Normality, $HClO_4$	Ammonium molybdate·$4H_2O$, mg/mL	Ammonium metavanadate, mg/mL	Range of final P_2O_5, ppm
Official	0.83	6.7	0.24	7.8–18.1[b]
Wall and Gehrke	0.78–0.83[a]	4.9–5.3[a]	0.24–0.28[a]	5.2–13.0[c]
Hambleton	0.68	5.5	0.28	6.9–16.1[d]

[a]Workable range.
[b]Initial range 150–350 ppm P_2O_5.
[c]Initial range 100–250 ppm P_2O_5.
[d]Initial range 150–350 ppm P_2O_5.

this is understandable because the official method is a composite of the other two. Of interest is that vanadate is the least varied constituent of the color reagent and that the Wall and Gehrke workable range covers the entire range of the 3 configurations. This may be a coincidence or it may be that vanadate is a more critical component of the color reagent.

System Design of the Official Method

The design of the hydrolysis section in the official method deserves particular attention because it can be a source of problems and frustration. In this section the sample travels a long distance through high temperature baths to allow time to complete hydrolysis. The recommended wetting agent (dilute Ultrawet 60L) which is added to the hydrolysis acid is not effective enough by itself to even the flow and eliminate surging in the heat baths. This combination of system length, high temperature, and ineffective wetting agent has caused some flow problems which can upset the overall performance of the system. The hydrolysis waste arrangement is designed to provide a slight back pressure to help stabilize the flow through the heat baths. Most of the waste, including all of the air bubbles, is pulled back through the pump; the excess is forcefully directed through 6 ft of 0.030 in. id polyvinyl chloride back pressure line. This small diameter line tends to clog easily because the wetting agent is unstable in $HClO_4$ and eventually coats the inner walls of all PVC tubing. We chose to abandon this arrangement entirely and simply direct all waste through glass to the sink. The wetting agent apparently does not coat glass. One 10T coil is placed immediately following the resample fitting, then lengths of 1.6 mm id glass cover the remaining distance. All waste, including air bubbles, is carried to the sink and none is pulled back through the pump. This all-glass arrangement has proved both workable in controlling the surging through the heat baths and reliable over long term use.

Also, partly because of the wetting agent coating problem, but also because the Ultrawet 60L clouds the 4N $HClO_4$ reagent upon setting, we introduce the wetting agent into the system through a separate line. A water–Ultrawet 60L solution made from 10% Ultrawet 60L in water and diluted again 1 mL per 100 mL is introduced into the system at a rate of 0.10 mL/min directly following the sample injection fitting, but before mixing, through an A-10 fitting.

These changes may not be major system improvements, but they have proved useful in providing better long-term performance. Finding a more suitable wetting agent would improve the flow stability through the hydrolysis section of the official method.

Generalized Method

With all of the method complexity in mind, the following is an attempt to generalize the conditions of the method as much as possible, making no specific endorsement as to what type of analysis scheme or manufacturer's equipment might be used to accomplish the analysis. The conditions outlined for the generalized method encompass and expand on those summarized in Table 1. The current official method stands as an example method which meets the performance and chemistry requirements set forth.

Detector

Any colorimeter or spectrophotometer may be used which is capable of making absorbance measurements at a wavelength setting between 400 and 490 nm in a cell, fixed or flow-through, whose path length may be varied between 0.5 and 2.0 cm, depending on the sensitivity needed for measurement.

Calibrations

The standard curve shall consist of at least 6 points evenly distributed over the entire concentration range. Standard points shall be made from NBS or primary standard KH_2PO_4, previously dried 2 h at 105°C. No greater than a 3rd order least square fit may be used to define the standard curve. Calculated standard values may not differ from known values by more than ±1% in any one instance. The average of the absolute values of the calculated percent differences may not exceed 0.5% (optimum calibration of the official method is 0.37 and 0.14, respectively).

Hydrolysis

The sample–$HClO_4$ hydrolysis mixture shall have a final $HClO_4$ concentration between 2N and 4N. The mixture shall be heated at 90–98°C for the time required to completely convert all non-orthophosphate to the ortho form for the maximum possible concentration of non-orthophosphate that will be measured, and also destroy any interfering coloring matter.

To check for complete hydrolysis and destruction of coloring matter, analyze, in duplicate, water solutions of ferric pyrophosphate and sodium metaphosphate which have been previously assayed (triplicate assay on each) for available P_2O_5 by the gravimetric quimociac method, AOAC **2.053.** Prepare the non-orthophosphate solutions to read within the upper ⅓ of a 6 point calibration curve prepared as outlined in the calibration section. Calculate percent recovery for each material by using the average value determined gravimetrically as theory. Recovery on each material shall be within 98–102%. If hydrolysis is incomplete, or too much coloring matter remains, increase the strength of the $HClO_4$ reagent, increase the time allotted for hydrolysis, or decrease the amount of non-orthophosphate to be hydrolyzed.

Color Development

Final concentrations of reagents and analyte at color development shall be within the following ranges: 0.5–1.0N $HClO_4$; 4–8 mg/mL of ammonium molybdate·$4H_2O$; 0.15–0.35 mg/mL of ammonium metavanadate; 1–20 μg/mL of phosphorus as P.

Table 2. Instrument performance of official automated direct available P_2O_5 analyzer

40/h, 4:1 Sample-to-wash ratio
Low: 150 ppm P_2O_5
Middle: 250 ppm P_2O_5
High: 350 ppm P_2O_5

	Random readings			Mid-point readings		
Date	P_2O_5, $\bar{x}$ ppm[a]	SD	% RSD	P_2O_5, $\bar{x}$ ppm[b]	SD	% RSD
2/6/81	150.7	0.68	0.45	250.6	0.41	0.16
	250.3	1.15	0.46			
	349.3	1.31	0.38			
1/29/82	150.2	0.98	0.65	250.0	0.52	0.21
	249.4	1.68	0.67			
	349.2	1.41	0.40			
7/28/82	149.7	0.40	0.27	250.1	0.51	0.20
	249.6	0.63	0.27			
	349.1	0.76	0.22			
1/28/83	151.0	0.45	0.30	250.0	0.16	0.06
	250.1	0.65	0.26			
	349.1	0.72	0.21			

[a]Average of 10 or more readings.
[b]Average of 30 or more readings.

To check for stable color development, system drift, noise, and reading precision, analyze the high standard 30 times in succession with system fully equilibrated and detector response adjusted to 90% full scale. For stable color development and minimum drift, change in response may not exceed 1% full scale for any 10 readings in succession. For reading precision, the range of response over the entire 30 readings may not exceed 2% full scale. Noise, considered to be the difference between adjacent readings or peaks, may not exceed 2% peak to peak.

If complete color development is desirable, this may be checked by analyzing the high standard continuously. Once the high standard has fully reached the flowcell with the system fully equilibrated, stop the flow and observe any change in response for 5 min. Complete color development will be indicated by a nearly perfect steady response.

To measure the effect of citrate on the P_2O_5 response, analyze solutions containing equal amounts of phosphorus but different amounts of neutral ammonium citrate as follows: Prepare 2 low phosphorus standards, with one to contain 20 v/v% of citrate reagent and prepare 2 high phosphorus standards in the same manner. Adjust the detector response to ca 10% full scale for the low phosphorus standards and ca 90% full scale for the high phosphorus standards. Read the low phosphorus standards in order of their citrate content as follows: 2 readings 0% cit; 4 readings 20% cit; 2 readings 0% cit. Read the high phosphorus standards in the same manner. Average the readings on each of the 4 standards and calculate the effect of citrate as a percentage of the total analysis range measured at both the low and high ends. The formulas are as follows:

$$\% \text{ Effect at high} = \frac{[(\text{av. high P, 20\% cit} - \text{av. high P, 0\% cit}) \times 100]}{(\text{av. high P, 0\% cit} - \text{av. low P, 0\% cit})}$$

$$\% \text{ Effect at low} = \frac{[(\text{av. low P, 20\% cit} - \text{av. low P, 0\% cit}) \times 100]}{(\text{av. high P, 0\% cit} - \text{av. low P, 0\% cit})}$$

The citrate effect may not exceed ±1% at either the high or low phosphorus response.

If the analysis scheme is such that maximum amount of citrate in the solution presented to the instrument is less than 20 v/v%, then use that maximum place of the 20% level and determine the citrate effect as outlined.

The citrate effect may be compensated for by adding a nominal amount of citrate to the standard curve. If that is the case, substitute the nominal level for the zero citrate level and determine the citrate effect relative to the highest possible citrate level, and also to the lowest possible level, if lower than the nominal. These checks shall be performed as outlined for the high and low phosphorus responses and shall not exceed ±1%.

Overall System or Instrument Stability Check

To determine overall system stability, calibrate the instrument for about 10% and 90% response for the low and high standards, respectively; then read the low, middle, and high standard curve points in random order and collect at least 10 readings on each point. Calculate percent relative standard deviation, (std dev./mean) × 100, for each of the 3 standards. Percent RSD may not exceed 1% in any case. Also read the middle standard at least 30 times in succession. Calculated %RSD for the midpoint read in succession may not exceed 0.5%. Examples of system stability of the official method are shown in Table 2.

Overall Performance Check

Extract and analyze 10 different Magruder samples (single determinations on each), or other similar performance check samples which have been assayed by interlaboratory study. Also extract and analyze 5 separate 1 g portions of NBS or primary standard KH_2PO_4. Random-order the Magruder and KH_2PO_4 samples. Analyze and calculate $\%P_2O_5$. The average bias of the Magruder results, (Magruder grand average − calculated $\%P_2O_5$)/10, must be less than ±0.1. The average absolute value of the differences must be less than 0.4. For the 5 analyses of standard KH_2PO_4, the difference between the mean $\%P_2O_5$ of the test results and the known $\%P_2O_5$ must not be more than ±0.3 with a standard deviation not greater than 0.25.

These guidelines for establishing overall performance have been taken directly from the new AOAC official flame photometric determination for K_2O in fertilizer (6), and adjusted for P_2O_5.

Conclusions

The generalized P_2O_5 method in this report is strictly a proposal of how a generalized method might be written. It should not be taken as the definitive answer. Because of the masking of the citrate interference, the performance criteria must be regarded as method performance as opposed to simply instrument performance surrounding a straightforward fixed chemistry.

The chemistry of the current official method is delicate and much of the success of this method is due to the methodical sample treatment inherently provided in an automated system. It is doubtful whether a generalized method for this particular chemistry could be written to stand alone without the support of a detailed descriptive method included as an example procedure. Including a detailed descriptive method within a generalized method format or surrounding an existing method with performance criteria may well be the best approach to take. This serves in 2 ways: It encourages improvements and expansion outside the slow collaborative process, which will help AOAC keep pace with developments, while it still provides users with the detailed descriptive method which has been the backbone of AOAC.

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COLOR ADDITIVES

Liquid Chromatographic Determination of Intermediates in D&C Yellow No. 7 and D&C Yellow No. 8

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A sensitive, reproducible method that uses liquid chromatography in the reverse phase mode has been developed for the determination of phthalic acid, resorcinol, and 2-(2',4'-dihydroxybenzoyl)benzoic acid in D&C Yellow No. 7 and D&C Yellow No. 8. The method uses a 10 μm, C-8 column, a 1% acetic acid-methanol gradient, and UV absorption detection at 280 nm. Average recoveries of phthalic acid, resorcinol, and 2-(2',4'-dihydroxybenzoyl)benzoic acid were 100, 98, and 102%, respectively, from fluorescein standard (certifiable as D&C Yellow No. 7) spiked with each compound at levels ranging from 0.13 to 1.3%.

D&C Yellow No. 7 (Colour Index No. 45350:1) and D&C Yellow No. 8 (Colour Index No. 45350) are synthetic color additives that are prepared by the fusion of resorcinol and phthalic anhydride (1). D&C Yellow No. 7 is the free acid, fluorescein, and D&C Yellow No. 8 is the disodium salt, uranine. Every batch of certifiable D&C Yellow No. 7 and D&C Yellow No. 8 that is manufactured must first be chemically analyzed and approved by the Certification Branch, Division of Color Technology, Food and Drug Administration, before it may be used in externally applied drugs and/or cosmetics in the United States. The *Code of Federal Regulations* (CFR) (2) limits the amounts of phthalic acid, resorcinol, and 2-(2',4'-dihydroxybenzoyl)benzoic acid to 0.5% each by weight of D&C Yellow No. 7. For D&C Yellow No. 8, the CFR (3) places a 1.0% limit on the amount of phthalic acid permitted, while limiting the amounts of the other 2 compounds to 0.5% each.

At the present time, these contaminants are determined by a cellulose column/ammonium sulfate eluant procedure (4). Although this method gives reproducible results, it is time consuming and cannot be used to accurately determine phthalic acid and resorcinol when they are both present because they co-elute and have similar UV spectra. A reverse phase liquid chromatographic (LC) method using a C-8 column was developed to separate phthalic acid, resorcinol, and 2-(2',4'-dihydroxybenzoyl)benzoic acid from D&C Yellow No. 7 and D&C Yellow No. 8. This method is faster than the currently used method, is automated, and permits quantitation of each of the 3 compounds in the presence of the others.

METHOD

Apparatus

(a) *Liquid chromatograph.*—With gradient elution capability. Varian VISTA 5060 and VISTA 401 controller/data system and Series 8000 autosampler with 20 μL injection loop (Varian Associates, Inc., Palo Alto, CA 94303), or equivalent instrumentation. Operating conditions: chart speed 0.6 cm/min; attenuation 128; flow rate 1.2 mL/min; column temperature 35°C. Optimize signal-to-noise, peak width, and peak reject values for each column. *Gradient program:* initial composition 0% B; at t = 0, change linearly from 0 to 75% B in 25 min; at t = 25, change linearly from 75 to 100% B in 0.5 min; hold at 100% B for 5.5 min; at t = 31, change linearly from 100 to 0% B in 1 min; hold at 0% B for 7 min. The time needed at 100% B to wash any late eluting material from the column or the time needed at 0% B for equilibration may be adjusted to fit the needs of different LC equipment or columns.

(b) *Detector.*—Waters Model 440 dual wavelength detector (Waters Associates, Inc., Milford, MA 01757) operated at 280 nm and 0.064 AUFS.

(c) *Chromatographic column.*—Rainin Microsorb C-8 column, 5 μm spherical particles, 25 cm × 4.6 mm id (Rainin Instrument Co., Inc., Woburn, MA 01801), or equivalent.

Reagents

(a) *Water.*—Deionized and passed through Milli-Q water purification system (Millipore Corp., Bedford, MA 01730).

(b) *Primary solvent A.*—1.0% (v/v) glacial acetic acid. Measure 10 mL glacial acetic acid, transfer to 1 L volumetric flask containing ca 800 mL water, dilute to volume, and filter through 0.2 μm filter.

(c) *Secondary solvent B.*—Methanol, LC grade.

(d) *Stock solutions for calibration.—(1) Phthalic acid.*—Aldrich, 99% (Aldrich Chemical Co., Inc., Milwaukee, WI 53233). Dissolve 200–210 mg in water in 200 mL volumetric flask and dilute to volume. Determine concentration of phthalic acid stock solution from UV absorbance of dilute solution prepared as follows: Pipet 1 mL aliquot of stock solution into 100 mL volumetric flask containing 25 g ammonium sulfate, 1 mL HCl, and 70 mL water; dilute to volume with water. The approximate absorptivity is 0.045 L/(mg × cm) at 228 nm (5). *(2) Resorcinol.*—Fisher certified (Fisher Scientific Co., Pittsburgh, PA 15235). Dissolve 80–100 mg in water in 200 mL volumetric flask and dilute to volume. Determine concentration of resorcinol stock solution from UV absorbance of dilute solution prepared as follows: Pipet 2 mL aliquot of stock solution into 100 mL volumetric flask and dilute to volume with methanol. The approximate absorptivity is 19.18 L/(g × cm) at 275 nm (6). *(3) 2-(2',4'-Dihydroxybenzoyl)benzoic acid.*—Prepare as described by Graichen in an internal progress report (C. Graichen, Division of Color Technology, Aug. 7, 1961).[1] Dissolve 80–100 mg in water in 200 mL volumetric flask, add a few drops NH_4OH, and dilute to volume with water. Determine concentration of 2-(2',4'-dihydroxybenzoyl)benzoic acid stock solution from UV absorbance of dilute solution prepared in the same manner as for phthalic acid. The approximate absorptivity is 0.053 L/(mg × cm) at 286 nm (5).

(e) *Fluorescein standard.*—Free of intermediates and side reaction products and prepared according to method described by Dolinsky and Jones (7).

Calibration

Construct calibration curves for phthalic acid, resorcinol, and 2-(2',4'-dihydroxybenzoyl)benzoic acid by plotting

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[1] A copy of the procedure for the preparation of 2-(2',4'-dihydroxybenzoyl)benzoic acid can be obtained from the Division of Color Technology, HFF-434, 200 C St, SW, Washington, DC 20204.

Table 1. Recovery data and paired-difference 99% confidence interval results

	Phthalic acid, %			Resorcinol, %			2-(2′,4′-Dihydroxybenzoyl) benzoic acid, %		
Detn	Added	Found	Rec.	Added	Found	Rec.	Added	Found	Rec.
1	0.13	0.13	100	0.16	0.16	100	0.87	0.86	99
2	0.37	0.37	100	0.27	0.24	89	0.52	0.53	102
3	0.62	0.62	100	0.38	0.35	92	0.41	0.42	102
4	0.87	0.88	101	0.48	0.46	96	0.17	0.18	106
5	1.12	1.12	100	0.80	0.73	91	0.29	0.30	103
6	0.25	0.25	100	0.21	0.24	114	1.16	1.18	102
7	0.50	0.50	100	0.43	0.40	93	0.9	0.94	101
8	0.75	0.75	100	0.64	0.67	105	0.7	0.70	100
9	1.25	1.24	99	1.07	1.11	104	0.23	0.23	100
Av.			100			98			102
Paired-difference 99% confidence interval:									
		0.00 ± 0.006			−0.01 ± 0.04			0.007 ± 0.010	

detector response (peak area) vs % by weight of the corresponding compound relative to the amount of fluorescein standard in the solution.

Prepare solutions for calibration as follows: Dissolve 750 mg purified fluorescein standard in 2 mL ethanol and 0.5 mL NH_4OH, and transfer to 100 mL volumetric flask. Add appropriate aliquots (0.5–10.0 mL) of each stock solution to volumetric flask and dilute to volume with water. Analyze 6 calibration solutions containing fairly evenly spaced concentrations of each compound.

For each compound in the solution, calculate C, % by weight relative to the amount of fluorescein standard in solution:

$$C = V \times C' \times 100\% \times (1/750 \text{ mg})$$

where V = volume of stock solution aliquot (mL), and C' = concentration of stock solution (mg/mL) determined spectrophotometrically.

On the basis of current allowable limits, the concentration of phthalic acid, expressed as % by weight of fluorescein standard, should cover the range 0.1–1.2%; the ranges for the resorcinol and 2-(2′,4′-dihydroxybenzoyl)benzoic acid concentrations should be 0.1–0.6%.

Mathematically choose the best fitting straight line for the calibration data by the method of least squares. For each compound calculate the regression line, $y = bx + a$, by using the following equations:

$$b = [\Sigma(x - \bar{x})(y - \bar{y})]/[\Sigma(x - \bar{x})^2]$$
$$= [\Sigma(xy) - [(\Sigma x \Sigma y)/n]]/[\Sigma x^2 - [(\Sigma x)^2/n]]$$
$$a = \bar{y} - b\bar{x}$$

where x = concentration of calibration standard; y = peak area response to the calibration standard for component of interest; n = number of calibration solutions analyzed; b = slope of regression line; and a = y intercept of regression line.

Determine the linear correlation between peak area and concentration of the standards by calculating the correlation coefficient r:

$$r = [\Sigma(x - \bar{x})(y - \bar{y})]/\sqrt{[\Sigma(x - \bar{x})^2][\Sigma(y - \bar{y})^2]}$$

The value of r should be between 0.98 and 1.00.

Determination

D&C Yellow No. 8.—Dissolve 750 mg sample in 60 mL water. Transfer quantitatively to 100 mL volumetric flask. Dilute to volume and mix well. *D&C Yellow No. 7.*—Dissolve 750 mg sample in 2 mL ethanol and 0.5 mL NH_4OH. Transfer quantitatively to 100 mL volumetric flask. Dilute to volume with water and mix well.

As described under *Calibration*, prepare a fluorescein standard solution containing all of the components of interest. Load autosampler in following way: first position, a blank gradient vial which contains water; second position, the fluorescein standard vial; from this position on, all positions contain sample vials. The last position is the fluorescein standard vial. After all samples are loaded in the autosampler, start the instrument. Place inlet tubes from pump into appropriate solvents, which have been freshly prepared. Pump solvent through lines with column bypass valve opened. Pump long enough to ensure that all lines contain fresh solvent. Set pump to 0% B at 2 mL/min for 10 min; then set flow to 0 and close bypass valve. Set flow to 1.2 mL/min and % B to 0; then start injection program. From this point on, the chromatograph will operate automatically. After all samples have been run, rinse column with 100% B to remove all acetic acid; then set flow to 0 and %B to 0; then open bypass valve. Remove inlet tube from primary solvent and place in water. Rinse primary pump with water to remove all acetic acid from pump and lines. After pump and lines have been rinsed, set flow to 0 and close bypass valve.

From regression line equation, $y = bx + a$, calculate x, % of compound of interest in D&C Yellow No. 7 or D&C Yellow No. 8 sample, by substituting value of y (peak area for compound of interest) and solving for x.

Results and Discussion

For the development of the method, we used concentrations of phthalic acid, resorcinol, and 2-(2′,4′-dihydroxybenzoyl)benzoic acid in 11 calibration solutions that covered the following ranges expressed as % by weight of fluorescein standard: phthalic acid, 0.13–1.25%; resorcinol, 0.16–1.07%; and 2-(2′,4′-dihydroxybenzoyl)benzoic acid, 0.17–1.16%.

The following limits of determination were calculated according to the method of statistical analysis described by Bailey et al. (8): phthalic acid, 0.02%; resorcinol, 0.16%; and 2-(2′,4′-dihydroxybenzoyl)benzoic acid, 0.04%.

Recovery studies with this method gave the following recovery ranges and averages for each compound of interest: phthalic acid, 99–101%, 100%; resorcinol, 89–114%, 98%; and 2-(2′,4′-dihydroxybenzoyl)benzoic acid, 99–106%, 102%. Recovery data for the individual determinations are given in Table 1. The paired-difference 99% confidence intervals all included zero.

A new standard solution should be analyzed every day that D&C Yellow No. 7 or D&C Yellow No. 8 samples are run. Each set of acceptable calibration data is added to the preceding data and a new regression line and coefficient of cor-

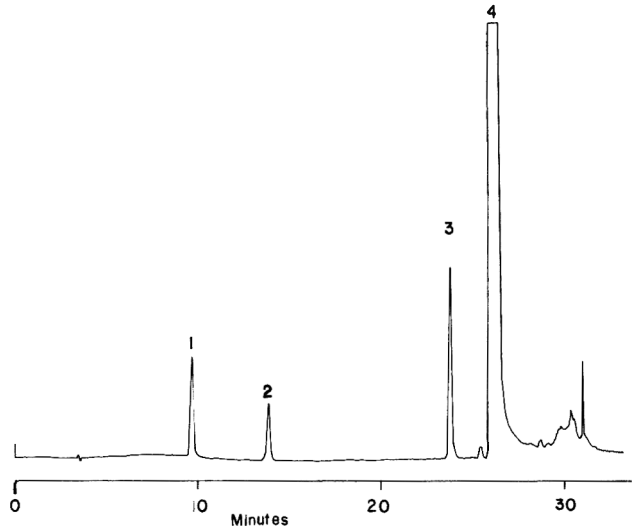

Figure 1. LC chromatogram of purified fluorescein standard (peak 4) with added intermediates. 1 = resorcinol; 2 = phthalic acid; 3 = 2-(2',4'-dihydroxybenzoyl)benzoic acid.

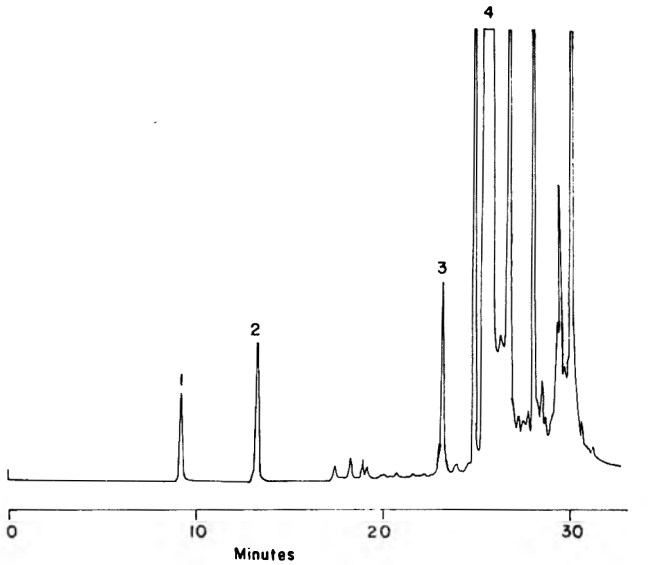

Figure 2. LC chromatogram of the pharmacology sample of D&C Yellow No. 7 (peak 4). 1 = resorcinol, 0.2%; 2 = phthalic acid, 0.5%; 3 = 2-(2',4'-dihydroxybenzoyl)benzoic acid, 0.1%.

relation are calculated. The correlation coefficient should be between 0.98 and 1.00. A column with a corresponding correlation coefficient of less than 0.98 should be restandardized with new standards. If restandardization does not bring the correlation coefficient to within the acceptable range, the column should not be used.

Figure 1 shows the separation of phthalic acid, resorcinol, and 2-(2',4'-dihydroxybenzoyl)benzoic acid from fluorescein standard. Because C-8 columns vary from company to company, modifications in the gradient may be needed to achieve similar results.

Isophthalic acid and terephthalic acid were examined as possible interferences. They eluted 5 and 4 min, respectively, after phthalic acid and did not interfere with the quantitation of any of the components of interest.

Seventeen samples of commercial D&C Yellow No. 8 and 2 samples of commercial D&C Yellow No. 7 were analyzed by the proposed method. The concentrations of the components of interest found in these samples were as follows: phthalic acid, 0.04–1.1%; resorcinol, <0.16–0.37%; and 2-(2',4'-dihydroxybenzoyl)benzoic acid, 0.07–1.06%. For most of the samples that contained both phthalic acid and resorcinol, both of these compounds were reported as phthalic acid when the cellulose column/ammonium sulfate eluant procedure was used.

Fluorescein does not exhibit good stability to light (1,7), but is reasonably stable if kept in the dark (7). When analyzed by LC, standard solutions of fluorescein show considerable decomposition in 24 h if exposed to light, but very little decomposition in 48 h if kept in the dark. Care must be exercised in using base and/or heat to dissolve the sample in order to avoid the formation of 2-(2',4'-dihydroxybenzoyl)benzoic acid or the decomposition of resorcinol.

Chromatograms of most commercial samples showed a large number of unidentified peaks (Figure 2). A similar pattern of unidentified peaks was produced in the chromatogram of a standard solution of fluorescein that had been allowed to decompose in the presence of excess NH_4OH.

Separation of the components of interest can be achieved at an initial composition of 25% B. However, if the sample solutions are basic, the peak shapes of phthalic acid and resorcinol are skewed. Starting at an initial composition of 0% B helps to overcome this problem.

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Liquid Chromatographic Determination of 2,4-Dinitroaniline and 2-Naphthol in D&C Orange No. 17

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A method is described for the determination of the intermediates in D&C Orange No. 17 by reverse phase liquid chromatography. The pigment is dissolved in boiling dioxane and then precipitated. The filtrate is chromatographed by isocratic elution, which is followed by a wash and equilibration. Peak area calibrations were linear. At the provisional specification levels, 99% prediction limits were 0.200 ± 0.0012% 2,4-dinitroaniline (2,4-DNA) and 0.200 ± 0.006% 2-naphthol. The limits of determination were 0.0023% for 2,4-DNA and 0.013% for 2-naphthol at the 99.5% confidence level. Recoveries were 98–100% for 2,4-DNA added at the 0.005–2% level, and 93–103% for 2-naphthol added at the 0.025–2% level. A survey of certified D&C Orange No. 17 samples showed that the lots contained higher levels of the intermediates than were determined previously by a cellulose column method, in which the pigment is not dissolved.

D&C Orange No. 17 (Figure 1, Colour Index No. 12075, 1-[(2,4-dinitrophenyl)azo]-2-naphthalenol) is an azo pigment that is manufactured by diazotization of 2,4-dinitroaniline (2,4-dinitrobenzenamine, 2,4-DNA) and coupling with 2-naphthol (2-naphthalenol) (1). The color may be used in the United States in externally applied cosmetics after the Food and Drug Administration (FDA) certifies that each lot of the color additive meets published provisional specifications, including a limit of 0.2% for each of these intermediates (2).

The intermediates in D&C Orange No. 17 were previously determined by FDA with a method that is a modification[1] of a general procedure (3) for the determination of intermediates in FD&C colors. The pigment is mixed with 95% ethanol, water, and cellulose, and the mixture is transferred to a cellulose column. The intermediates are eluted from the column with ammonium sulfate in aqueous ethanol and are determined from the absorption spectra of eluate fractions. In the development of the modified method, the possibility was raised that the intermediates are not completely extracted from the color.

Consequently, a reverse phase liquid chromatographic (LC) method for determination of the intermediates in D&C Orange No. 17 was developed and is reported here. A mixture of the color or laked color containing 25 mg pigment and 8 mL dioxane is boiled in a 15 mL beaker to dissolve the color and then is cooled in ice. The mixture is transferred to a 10 mL volumetric flask and diluted to volume with dioxane. The mixture is filtered through a membrane filter. Dioxane blanks, standard dioxane solutions of the intermediates, and filtrates of the color are chromatographed. Calibration lines are calculated by least squares linear regression from the peak areas of the blank and standard solutions. The concentrations of the intermediates in the color are calculated from the calibration lines and the peak areas of filtrates of D&C Orange No. 17. Because specifications could be changed, several calibration ranges were used.

Experimental

Apparatus

(a) *Syringe.*—10 mL polyethylene and polypropylene; Aldrich "all-poly" (Aldrich Chemical Co., Inc., Milwaukee, WI 53233).

(b) *Syringe filter unit.*—25 mm diameter, 0.5 μm pore, polytetrafluoroethylene membrane filter with polyethylene housing; Millipore Millex-SR (Millipore Corp., Bedford, MA 01730).

(c) *Liquid chromatograph.*—With gradient capability. (A system with eluant change capability may be used, but minor changes in eluant strength will be difficult.)

(d) *Pumps and flow controller.*—Waters Model 720 system controller with 2 Waters 6000A pumps (Waters Associates, Milford, MA 01757). Elution program beginning at injection at 2 mL/min: 45% eluant B (remainder eluant A) for 8 min, to 100% eluant B in 0.1 min, 100% eluant B (wash) for 3.9 min, return to 45% eluant B in 0.1 min, and equilibrate at least 5.9 min. Collect data for 10 min (run time = 10 min), and initiate next injection at 18 min (equilibration delay = 8 min). The effective wash-equilibration delay is 12 min because automatic injection takes 2 min. (*Note:*—Two min is saved by initiating the wash at 8 min; however, a single isocratic pump and manual switching of eluant can be used by eluting with 45% eluant B for 10 min, switching to 100% eluant B for a 4 min wash, and switching back to 45% eluant B for an 8 min equilibration.)

(e) *Injector.*—Waters Model 710B WISP Autoinjector set at 25 μL.

(f) *LC column.*—Waters RCM-C18, 10 μm particle size, 100 × 8 mm id, in RCM 100 compression unit. Variations in retention times of 2-naphthol at ambient temperature suggest controlling column temperature.

(g) *Detector.*—Waters Model 440 dual wavelength UV-Vis detector set at 254 nm and 0.005–0.2 AUFS. Qualitative detection at 340 nm and 0.01–0.5 AUFS aids in distinguishing the intermediate peaks from those of other impurities.

(h) *Data system.*—Waters Model 730 data module.

Reagents

(a) *Water for LC eluants.*—Distilled and passed through a Millipore Milli-Q water purifier.

(b) *Acetonitrile.*—Distilled-in-glass (Burdick & Jackson Laboratories Inc., Muskegon, MI 49442).

(c) *Ammonium acetate.*—"Baker Analyzed" reagent crystals (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

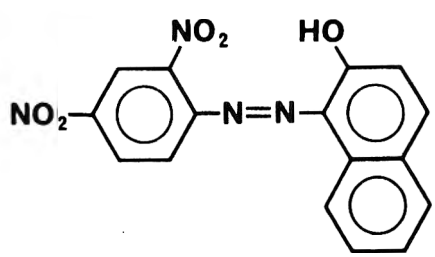

Figure 1. D&C Orange No. 17.

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[1] A copy of the modified method can be obtained from the Division of Color Technology, HFF-436, 200 C St, SW, Washington, DC, 20204.

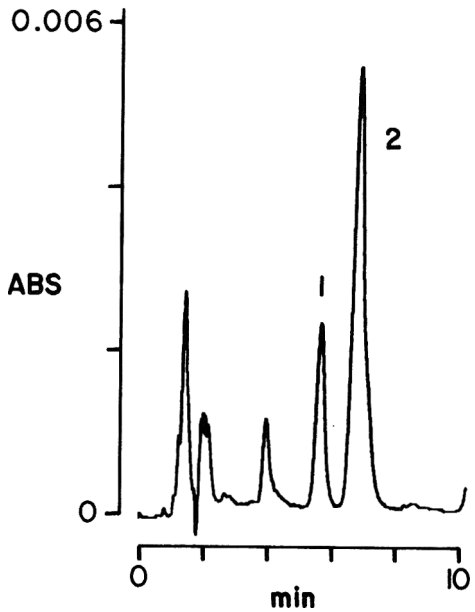

Figure 2. Chromatogram of filtrate of D&C Orange No. 17 (sample 4, Table 4). 1 = 2,4-DNA; 2 = 2-naphthol.

Table 1. Preparation of recovery mixtures for LC determination of intermediates in D&C Orange No. 17

		Spiking level, %	
Spiking soln	Aliquot, mL	2,4-DNA	2-Naphthol
1a	4	2	
	1	0.5	
1b	3	0.075	
	1	0.025	
1c	1	0.005	
2a	2		2
2b	3		0.75
	1		0.25
2c	4		0.1
	1		0.025

(d) *2,4-DNA.*—Recrystallized from aqueous alcohol (4).

(e) *2-Naphthol.*—Fisher certified recrystallized reagent (Fisher Scientific Co., Pittsburgh, PA 15219).

(f) *1,4-Dioxane.*—Distilled-in-glass (Burdick & Jackson Laboratories Inc.). Use only in hood; this compound is a suspected carcinogen. Test for peroxides with peroxide test strips (Cat. No. 10011-1, EM Science, Gibbstown, NJ 08027).

Solutions

(a) *LC eluants.—(1) Eluant A.*—Aqueous 0.1M ammonium acetate. Dissolve 7.708 g ammonium acetate in water and dilute to 1 L. *(2) Eluant B.*—Acetonitrile.

(b) *2,4-DNA standard solutions.—(1) Stock solution.*—Accurately weigh ca 0.125 g 2,4-DNA and transfer to 100 mL volumetric flask. Dissolve in dioxane and dilute solution to 100 mL. *(2) Intermediate solutions.—(2a)* Dilute 5 mL stock solution to 100 mL with dioxane. *(2b)* Dilute 10 mL solution *2a* to 100 mL with dioxane. *(2c)* Dilute 20 mL solution *2b* to 100 mL with dioxane. *(3) Working solutions.*—Dilute 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 mL aliquots of each intermediate solution to 10 mL with dioxane. Use 5 evenly spaced calibration points in each range plus a dioxane blank for screening. The working and intermediate solutions corresponding to solutions *2c, 2b,* and *2a* contain the equivalent of 0.0025–0.05, 0.0125–0.25, and 0.125–2.5% 2,4-DNA, respectively, relative to 25 mg D&C Orange No. 17/10 mL.

(c) *2-Naphthol standard solutions.*—Follow procedure for 2,4-DNA standard solutions, but use 2-naphthol in place of 2,4-DNA and omit *(2c)*. The working and intermediate solutions corresponding to solutions *2b* and *2a* contain the equivalent of 0.0125–0.25 and 0.125–2.5% 2-naphthol, respectively, relative to 25 mg D&C Orange No. 17/10 mL.

Isolation of Intermediates

Accurately weigh ca 25 mg color or laked color containing ca 25 mg pigment into 15 mL beaker. Add small Teflon-coated stirring magnet (0.5 × 5/16 in.) and 8–9.5 mL dioxane, and cover beaker with watch glass. Boil gently with stirring 5–7 min on preadjusted stirrer-hot plate. Cool 5–10 min in ice water with stirring as color and dioxane precipitate. Stir 5 min in room temperature bath; then transfer mixture, using Pasteur pipet, into 10 mL volumetric flask. Wash watch glass, magnet, beaker, and pipet with four 0.5 mL portions of dioxane. Transfer washings to flask and dilute to volume with dioxane. Attach barrel of syringe to filter unit and place outlet in sample vial. Pour mixture from volumetric flask into filter unit, insert plunger, and apply moderate pressure to filter.

Liquid Chromatography

Sparge eluants with helium, and set up gradient and detector. Prime and purge pumps and injection system. Wash column at 2 mL/min with water, then 100% eluant B for 15 min, and then 45% eluant B for 6 min. Chromatograph 2 preliminary blanks of dioxane.

For calibration, chromatograph, in random order, at least 5 evenly spaced levels of working standard solutions of each intermediate and at least 1 dioxane blank. Plot peak area vs equivalent percent for each intermediate from chromatograms of standards and blank, and calculate slopes and intercepts of calibration lines by least squares linear regression. The intercepts should be indistinguishable from zero.

For analysis of intermediates, chromatograph color filtrate, as shown in Figure 2, and dioxane blank. If analysis is not concurrent with calibration, prepare and chromatograph working standard solution of each intermediate or 1 solution containing both intermediates at the specification levels or other level(s) of interest. The data from chromatography of the confirmatory standard solution(s) should be within the prediction intervals of the calibration lines. From calibration lines and peak areas of color filtrate, calculate percent of each intermediate in sample.

If this method was not used previously, chromatograph mixture of most concentrated intermediate solutions followed by dioxane blank. Confirm that peaks are well separated and appear within reasonable time (k' = 3–4). Confirm that carryover is negligible and that transient gradient induced by dioxane does not interfere with peaks. If necessary, increase or decrease % eluant B to decrease or increase retention time of intermediates. (An increase of 3% produced a decrease of 0.8 min in retention time.) Chromatograph mixture of least concentrated working solutions. Adjust detector attenuation for proper display at level of interest and adjust integration parameters for proper quantitation at lowest level.

Recovery Studies

D&C Orange No. 17 that contained 0.069% 2,4-DNA and 0.54% 2-naphthol was recrystallized once from dioxane (5 g/1800 mL). Twenty-five recovery mixtures, prepared with recrystallized D&C Orange No. 17, were fortified with 5 levels of 2,4-DNA and 5 levels of 2-naphthol. Three unfortified D&C Orange No. 17 mixtures were also prepared. Fil-

Table 2. Least squares linear regression analysis of calibration data for intermediates in D&C Orange No. 17[a]

Intermediate	Slope ± conf. int., kct/%[b]	Intercept ± conf. int., kct[b]	Corr. coeff.	Upper limit of blank, kct[c]	c_L, %[c]	Area corresponding to c_L, kct[c]	Prediction limits, %[b]
2,4-DNA							
0.005–0.05%	4467.4 ± 112.0	−2.138 ± 2.949	0.99973	4.385	0.0029	10.8	—
0.025–0.25%	4576.5 ± 18.3	−2.809 ± 2.319	0.99999	2.564	0.0023	7.9	0.200 ± 0.0012
0.25–2.5%	4619.2 ± 22.0	−2.067 ± 27.826	0.99999	63.83	0.028	130	—
2-Naphthol							
0.0125–0.25%	1687.8 ± 37.5	−1.203 ± 4.925	0.99979	9.691	0.013	20	0.200 ± 0.006
0.25–2.5%	1598.2 ± 63.6	−9.138 ± 80.075	0.99921	180.5	0.24	366	—

[a]Abbreviations: conf. int. = confidence interval; kct = 1000 integrator area counts; c_L = limit of determination.
[b]Confidence level 99%.
[c]Confidence level 99.5%.

trates from these mixtures were chromatographed in random order.

Spiking solutions.—2,4-DNA.—(1a) Ca 12.5 mg 2,4-DNA was accurately weighed and transferred to a 100 mL volumetric flask. The 2,4-DNA was dissolved in dioxane, and the solution was diluted to 100 mL. *(1b)* A 5 mL aliquot of solution *1a* was diluted to 100 mL with dioxane. *(1c)* A 5 mL aliquot of solution *1b* was diluted to 25 mL with dioxane. *2-Naphthol.—(2a)* Ca 25 mg 2-naphthol was accurately weighed and transferred to a 100 mL volumetric flask. The 2-naphthol was dissolved in dioxane, and the solution was diluted to 100 mL. *(2b)* A 25 mL aliquot of solution *2a* was diluted to 100 mL with dioxane. *(2c)* A 5 mL aliquot of solution *2b* was diluted to 50 mL with dioxane.

Recovery mixtures.—For each recovery mixture, ca 25 mg recrystallized D&C Orange No. 17 was accurately weighed in a 15 mL beaker. An aliquot of the appropriate spiking solution of each intermediate was added as shown in Table 1 (all 25 combinations). The volume was adjusted to 8–9.5 mL with dioxane.

Results and Discussion

Calibration

Within each set, the calibration solutions and blanks were chromatographed in random order. The peak areas were plotted vs the equivalent percentages of intermediate for each set, and the data were analyzed by least squares linear regression (Table 2). Plots of the data were linear, and the intercepts could not be distinguished from zero, except for the middle range calibration line for 2,4-DNA. The probability that this significant −2809 area intercept is real is reinforced by the nonsignificant −2067 and −2138 area intercepts at the other two 2,4-DNA levels. Integration parameters may have been slightly misset. The lowest limits of determination were 0.0023% for 2,4-DNA and 0.013% for 2-naphthol (5). The 99% prediction limits at the provisional specification levels were 0.200 ± 0.0012% 2,4-DNA and 0.200 ± 0.006% 2-naphthol.

Recovery Studies

Recoveries (Table 3) from the quintuplicate analyses at each level shown in Table 2 were 98–100% for 0.005–2% 2,4-DNA and 93–103% for 0.025–2% 2-naphthol.

From the standard deviations of the determinations at the lowest fortification levels, the limits of determination were 0.002% for 2,4-DNA and 0.006% for 2-naphthol. These values were calculated from

$$c_L = 4.604 \times SD_{blank}/\text{slope } \%$$

in which a 99.5% one-sided t-value was used instead of $k = 3$ (6). These limits are comparable to the corresponding limits of determination of 0.0023 and 0.013% that were calculated from calibration data.

Survey

Twenty samples of certified D&C Orange No. 17, consisting of colors and laked colors that included 2 pharmacology samples, were analyzed by the LC method. The results of these analyses and those obtained during certification analysis by the cellulose column method are summarized in Table 4. All lots of D&C Orange No. 17 were found to contain higher levels of intermediates by the LC method than by the cellulose column method. Because analyses of D&C Orange No. 17 spiked with solutions of intermediates by the cellulose column method had demonstrated satisfactory recoveries, the low results in this survey by the cellulose column method are attributed to incomplete extraction of the intermediates from the color.

The levels of intermediates found by the LC method in virtually all lots surveyed, including the pharmacology samples, are greater than the provisional specification limits.

Conclusions

This LC method is a significant improvement over the previously used cellulose column method. If specifications

Table 3. Recovery data for LC determination of intermediates in D&C Orange No. 17[a]

2,4-DNA				2-Naphthol			
Added, %	Found ± SD, %	Rec., %[b]	CV, %	Added, %	Found ± SD, %	Rec., %[b]	CV, %
0	0.00106 ± 0.00059[c]	—	—	0	0[c]	—	—
0.00516	0.00615 ± 0.00040	99	7.9	0.0253	0.0236 ± 0.0014	93	5.7
0.0258	0.0259 ± 0.0009	100	3.5	0.1012	0.0945 ± 0.0064	93	6.8
0.0774	0.0759 ± 0.0005	98	0.6	0.253	0.244 ± 0.007	97	3.0
0.516	0.512 ± 0.005	99	1.0	0.759	0.779 ± 0.005	103	0.6
2.064	2.019 ± 0.013	98	0.7	2.024	2.085 ± 0.026	103	1.2

[a]Quintuplicate analyses.
[b]Corrected for blank.
[c]Triplicate analyses.

Table 4. Survey of certified D&C Orange No. 17 for intermediates

Sample	Color, %	2,4-DNA, %[a,b]	2-Naphthol, %[a]	
			LC	Cellulose column
1	97[c,d]	0.31	0.80	0.16
2	97[c,d]	0.29	0.70	0.15
3	98	0.125	0.62	0.11
4	96	0.068	0.58	0.17
5	96	0.034	0.74	0.18
6	94[c]	0.048	0.72	0.11
7	98	0.056	0.85	0.12
8	98	0.051	0.80	0.16
9	98	0.047	0.79	0.14
10	98	0.031	0.93	0.13
11	19[c]	0.041 (0.22)	0.17 (0.89)	0.05 (0.26)
12	97	0.059	0.89	0.15
13	98	0.028	0.87	0.19
14	80[c]	0.61 (0.76)	1.15 (1.44)	0.15 (0.19)
15	18[c]	0.25 (1.41)	0.34 (1.90)	0.14 (0.78)
16	21[c]	0.0043 (0.020)	0.18 (0.84)	0.09 (0.43)
17	22[c]	0.0051 (0.023)	0.18 (0.80)	0.11 (0.50)
18	21[c]	0.0118 (0.056)	0.23 (1.08)	0.12 (0.57)
19	21[c]	0.0111 (0.053)	0.25 (1.21)	0.11 (0.52)
20	98	0.043	0.81	0.09

[a]As % of sample. Numbers in parentheses are % of color.
[b]Results shown are for LC method; results for cellulose column method were 0% for all 20 samples surveyed.
[c]$BaSO_4$ lake.
[d]Pharmacology sample.

are raised, a smaller sample can be used. If specifications are lowered, electrochemical detection may be needed.

The discrepancy between the cellulose column method and this LC method illustrates that recovery studies are not valid if the spiked samples are heterogeneous. To ensure validity, proof is needed that no analyte remains in the undissolved portion. The validity of analysis of laked colors by this LC method needs to be confirmed; proof is needed that laked intermediates are quantitatively extracted with boiling dioxane.

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Liquid Chromatographic Determination of 2-(2-Quinolinyl)-1*H*-indene-1,3-[2*H*]-dione and Other Organic-Soluble Matter in D&C Yellow No. 10

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A sensitive, reproducible method that uses an Extrelut QE column and liquid chromatography (LC) in the reverse phase mode is described for the determination of 2-(2-quinolinyl)-1*H*-indene-1,3-[2*H*]-dione and other organic-soluble matter found in D&C Yellow No. 10. With this method the organic-soluble matter is extracted from D&C Yellow No. 10 on an Extrelut QE column, and the extract is concentrated and analyzed by LC. Recoveries averaged 104% for 2-(2-quinolinyl)-1*H*-indene-1,3-[2*H*]-dione added to purified D&C Yellow No. 10 at levels ranging from 0.50 to 5.96 ppm.

D&C Yellow No. 10 (CAS Reg. No. 8004-92-0), Colour Index No. 47005, is a mixture of the sodium salts of the mono- and disulfonic acids of 2-(2-quinolinyl)-1*H*-indene-1,3-[2*H*]-dione. The principal components are the monosodium salts of 2-(2,3-dihydro-1,3-dioxo-1*H*-indene-2-yl)-6-quinolinesulfonic acid and 2-(2,3-dihydro-1,3-dioxo-1*H*-indene-2-yl)-8-quinolinesulfonic acid, with smaller amounts of the disodium salts of the disulfonic acids.

D&C Yellow No. 10 is prepared by the condensation of quinaldine with phthalic anhydride to yield 2-(2-quinolinyl)-1*H*-indene-1,3-[2*H*]-dione (unsulfonated quinoline yellow), Colour Index No. 47000, which is then sulfonated with fuming sulfuric acid. Every batch of certifiable D&C Yellow No. 10 that is manufactured must first be chemically analyzed and approved by the Certification Branch, Division of Color Technology, Food and Drug Administration, before it can be used in drugs or cosmetics in the United States. The *Code of Federal Regulations* (1) limits the amounts of 2-(2-quinolinyl)-1*H*-indene-1,3-[2*H*]-dione and other diethyl ether-soluble matter, calculated as 2-(2-quinolinyl)-1*H*-indene-1,3-[2*H*]-dione, to not more than 4 and 2 ppm, respectively. The diethyl ether-soluble material has been characterized as composed mostly of chlorinated 2-(2-quinolinyl)-1*H*-indene-1,3-[2*H*]-dione by high resolution, electron ionization, and chemical ionization mass spectrometry as well as Fourier transform proton NMR (S. J. Bell, Division of Color Technology, 1983).

A method (S. J. Bell, Division of Color Technology, 1983), using ether shake-out extraction and reverse phase liquid chromatography (LC), was developed to enable the Certification Branch to analyze batches of certifiable D&C Yellow No. 10 for unsulfonated quinoline yellow and other ether-soluble matter at the ppm level. Although this unpublished method gives reproducible results, it is time-consuming and limited by an average recovery of 71% of unsulfonated quinoline yellow. A new method using extraction on an Extrelut QE column (2), followed by a concentration step and reverse phase LC analysis, has been developed. The new method is faster, gives reproducible results, and has a recovery average of 104%. This method is being used by the Certification Branch to analyze D&C Yellow No. 10.

METHOD

Apparatus

(a) *Liquid chromatograph.*—With gradient elution capability. Varian Vista 5060 and Vista 401 controller/data system

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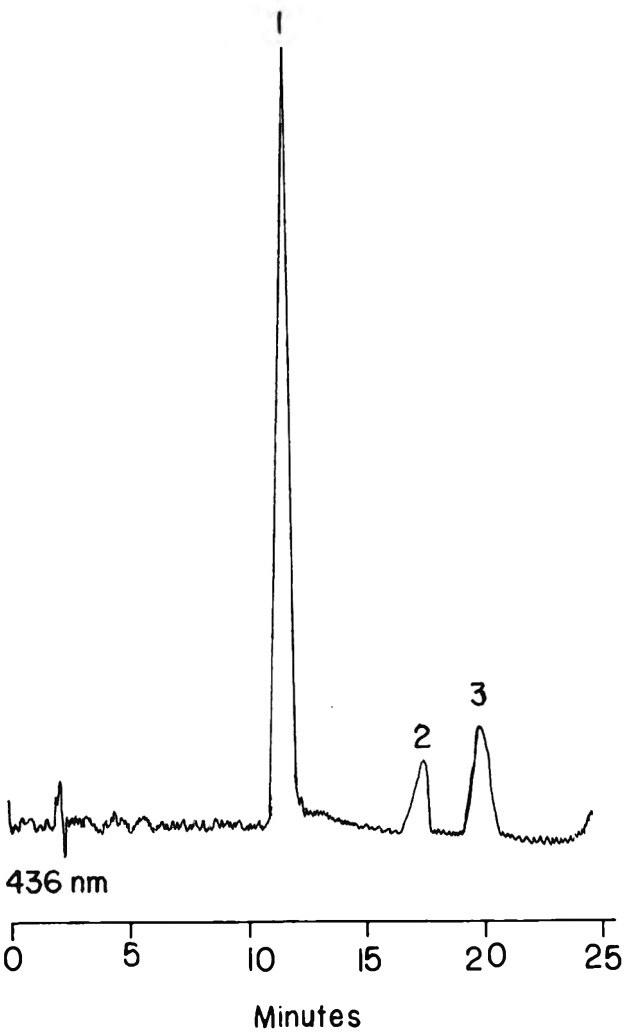

Figure 1. LC chromatogram of extract from D&C Yellow No. 10. 1 = 2-(2-quinolinyl)-1*H*-indene-1,3-[2*H*]-dione; 2 and 3 = other organic-soluble matter, characterized by mass spectrometric and NMR analyses as mostly chlorinated 2-(2-quinolinyl)-1*H*-indene-1,3-[2*H*]-dione (S. J. Bell, Division of Color Technology, 1983).

and Series 8000 autosampler with 100 μL injection loop (Varian Associates, Inc., Palo Alto, CA 94303), or equivalent instrumentation.

(b) *Detector.*—Waters Model 440 dual wavelength detector (Waters Associates, Inc., Milford, MA 01757), or equivalent.

(c) *Guard column.*—RP-18 Spheri-5 Brownlee cartridge system, 3 cm × 4.6 mm (Rainin Instrument Co., Inc., Woburn, MA 01801), or equivalent.

(d) *Chromatographic column.*—Varian Micro-Pak MCH-10 C-18 column, 30 cm × 4 mm id (Varian Associates, Inc., Palo Alto, CA 94303), or equivalent.

(e) *Spectrophotometer.*—Lambda 5 (Perkin-Elmer Corp., Norwalk, CT 06856), or equivalent.

(f) *Extraction column.*—Extrelut QE, 20 mL capacity (EM Science, Gibbstown, NJ 08027).

(g) *Rotary evaporator.*—Rotavapor R110 (Brinkmann Instruments Co., Westbury, NY 11590), or equivalent.

Reagents

(a) *Extraction solvent.*—Chloroform, reagent grade (J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(b) *Water.*—Deionized and passed through Milli-Q water purification system equipped with 0.22 μm filter (Millipore Corp., Bedford, MA 01730), or equivalent.

(c) *Primary solvent A.*—0.05M ammonium acetate.

(d) *Secondary solvent B.*—Acetonitrile, LC grade (Burdick & Jackson Laboratories Inc., Muskegon, MI 49442).

(e) *Unsulfonated quinoline yellow standard.*—Available as certified D&C Yellow No. 11 from H. Kohnstamm & Co., Inc. (Camden, NJ 08105) or Pylam Products Co., Inc. (Garden City, NY 11530).

Preparation of Stock Solution

Dissolve 0.09–0.10 mg unsulfonated quinoline yellow standard in chloroform in 50 mL beaker. Transfer compound to 100 mL volumetric flask and dilute to volume with chloroform. Determine exact concentration of stock solution from its visible spectrum. This solution can be run without dilution. The absorptivity of this compound is 0.135 L/(mg × cm) at 420 nm (3).

Parameters for Liquid Chromatograph

Operating conditions.—Chart speed 0.4 cm/min, flow rate 1 mL/min, column temperature ambient, detector set at 436 nm, attenuation 1.0 V/AUFS.

Gradient program.—Hold at 60% B for 19.9 min; go from 60 to 100% B in 0.1 min; hold at 100% B for 5 min; go from 100 to 60% B in 0.1 min; hold at 60% B for 5 min. The time needed at 100% B to wash the column of any late eluting material or the time needed at initial composition during equilibration may be adjusted to fit the needs of different LC columns or equipment. Because C-18 columns vary from company to company, modifications in the gradient may be needed with new columns to achieve a chromatogram similar to that shown in Figure 1.

Calibration

Prepare series of 6 standard solutions containing 0.25 to 6 mL stock solution in evenly spaced concentrations as follows. Pipet aliquot of stock solution into 30 mL beaker. Place beaker on steam bath to remove the chloroform. After all chloroform has been evaporated, let beaker cool to room temperature. Pipet 5 mL acetonitrile into beaker, stir to dissolve unsulfonated quinoline yellow, and place solution in sample vial. Analyze 6 standard solutions of unsulfonated quinoline yellow. For each standard solution, calculate C, ppm unsulfonated quinoline yellow relative to the amount of D&C Yellow No. 10 that has been extracted:

$$C = V \times C' \times (1000/W)$$

where V = volume of stock solution aliquot (mL), C' = concentration of stock solution (mg/mL) determined spectrophotometrically, and W = weight of D&C Yellow No. 10 (g). On the basis of current allowable limits (1), the concentration of unsulfonated quinoline yellow should cover the range 0.25–6 ppm, relative to the weight of D&C Yellow No. 10.

Construct calibration plot for unsulfonated quinoline yellow from integrated area response of detector. By method of least squares, calculate the regression line, $y = bx + a$, by using the following equations:

$$b = [\Sigma (x - \bar{x})(y - \bar{y})]/[\Sigma(x - \bar{x})^2]$$
$$= [\Sigma(xy) - [(\Sigma x \Sigma y)/n]]/[\Sigma x^2 - [(\Sigma x)^2/n]]$$
$$a = \bar{y} - b\bar{x}$$

where x = concentration of calibration standard; $\bar{x}$ = mean concentration from calibration data; y = peak area response for unsulfonated quinoline yellow; $\bar{y}$ = mean peak area response from calibration data; n = number of calibration solutions; b = slope of regression line; and a = y intercept

Table 1. Recovery data and paired-difference 99% confidence interval results for unsulfonated quinoline yellow

Detn	Added, ppm	Found, ppm	Rec., %
1	2.98	3.01	101
2	5.96	5.88	99
3	0.50	0.55	110
4	0.99	1.01	102
5	1.99	1.98	100
6	2.98	3.04	102
7	5.96	6.13	103
8	1.99	2.04	102
9	0.99	1.09	110
10	0.50	0.54	108
Av.			104

Paired-difference 99% confidence interval 0.04 ± 0.07

Table 2. Amounts of unsulfonated quinoline yellow found by Extrelut QE column and ether shake-out methods

	Extrelut QE column method		Ether shake-out method[b]	
Sample[a]	Unsulfonated quinoline yellow, ppm	Other organic-soluble matter, ppm	Unsulfonated quinoline yellow, ppm	Other ether-soluble matter, ppm
9	2.5	105.6	1.7	80
10	1.5	58.9	1.0	38
13	0.8	20.8	0.55	16.5
Pharm 205	1.9	0.89	1.6	0.78
Pharm 238	2.1	0.18	1.4	0.20
Pharm 269	0.66	0.67	0.55	0.56

[a]Sample numbers as reported in an internal progress report (S. J. Bell, Division of Color Technology, 1983).
[b]Data from internal progress report (S. J. Bell, Division of Color Technology, 1983). Values for samples 9 and 10 are results of individual analyses; all other values are averages of the 2 analyses reported.

of regression line. Determine linear correlation between peak area and concentration of standard by calculating correlation coefficient *r*:

$$r = [\Sigma(x - \bar{x})(y - \bar{y})]/\sqrt{[\Sigma(x - \bar{x})^2][\Sigma(y - \bar{y})^2]}$$

Analyze a new standard solution every day that D&C Yellow No. 10 samples are run. Add each new point to the preceding data and calculate a new regression line and correlation coefficient. The correlation coefficient *(r)* should be between 0.98 and 1.00. If *r* is less than 0.98, restandardize the C-18 column and obtain a new calibration plot. If restandardizing and recalibrating do not bring the recalculated correlation coefficient within the proper range, the column should not be used.

Determination

Weigh 1000 ± 0.1 mg D&C Yellow No. 10 into 30 mL beaker. Add 10 mL water to beaker and place on steam bath. Stir sample with glass rod until all dye is in solution. Remove beaker from steam bath and let cool to room temperature. Pour solution onto Extrelut QE column. Rinse beaker with 1 mL portion of water and pour onto Extrelut QE column. Continue rinsing beaker with 1 mL portions of water until water is colorless. Do not use more than a total of 10 mL to rinse beaker because the Extrelut QE column can absorb only 20 mL water. After final rinse has been added to Extrelut QE column, let column stand for 5 min. Extract column with 20 ± 1 mL chloroform. Wait 2–5 min, and then extract with another 20 mL portion of chloroform. Extract column as stated above with a total of five 20 mL portions of chloroform. Collect extract from column in 250 mL round-bottom flask. Evaporate chloroform with rotary evaporator. After all chloroform is removed, pipet 5 ml acetonitrile into flask to dissolve residue. Transfer some of this solution to autosampler vial. Prepare standard solution of unsulfonated quinoline yellow as described under *Calibration*. Load autosampler in the following way: first position, a blank gradient vial containing acetonitrile; second position, an unsulfonated quinoline yellow standard vial; from this position on, all positions contain extracted sample vials. The last position is an unsulfonated quinoline yellow standard vial. Start the liquid chromatograph after all samples are loaded in the autosampler.

From regression line equation, $y = bx + a$, calculate x, ppm unsulfonated quinoline yellow in the D&C Yellow No. 10 sample, by substituting value of y (peak area response for unsulfonated quinoline yellow) and solving for x. Use the same regression line equation to calculate the ppm level of the other organic-soluble contaminants appearing in the chromatogram after the unsulfonated quinoline yellow peak by substituting corresponding value of y (peak area response for other organic-soluble material) and solving for x.

Results and Discussion

A recovery study was undertaken, using diethyl ether on an Extrelut QE column, but only 70–90% of the unsulfonated quinoline yellow was recovered. Chloroform extraction on an Extrelut QE column was then tried, and excellent recoveries were obtained.

For the development of the method, the concentrations of unsulfonated quinoline yellow in the 12 calibration solutions ranged from 0.25 to 5.96 ppm, relative to the weight of D&C Yellow No. 10. The limit of determination for unsulfonated quinoline yellow is 0.32 ppm, calculated according to the method of statistical analysis described by Bailey et al. (4).

Recoveries of unsulfonated quinoline yellow with this method ranged from 99 to 110% and averaged 104%. Recovery data for the individual determinations are given in Table 1. The paired-difference 99% confidence interval included zero.

Table 2 compares results obtained by the method containing the shake-out extraction with those obtained by the method described here.

Figure 1 shows the separation of unsulfonated quinoline yellow and 2 other contaminants found in samples of D&C Yellow No. 10.

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MEAT AND MEAT PRODUCTS

Comparison of the Volhard and Potentiometric Methods for the Determination of Chloride in Meat Products: Collaborative Study

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A collaborative study of the determination of chloride in meat products was conducted by the International Organization for Standardization (ISO) to compare the ISO 1841 method (Volhard titration) with the FAO/WHO Codex Alimentarius Committee method (potentiometric titration). Five canned luncheon meat products containing 0.25–2.0% sodium chloride at 4 different spiking levels were analyzed by 11 laboratories. The data were analyzed by ISO statistics (ISO 5725) and by AOAC statistics (Youden-Steiner), the major differences being in the rejection of outliers and in the statement of precision parameters. Good agreement was found between the mean chloride contents of the products as determined by both methods and with the added amounts, although statistically significantly higher sodium chloride recoveries were obtained with the potentiometric method. The within-laboratory variability (repeatability) is greater for the Volhard method, especially for chloride levels below 1.0%. Therefore it is proposed to set the lowest level of determination for the Volhard method at about 1.0% sodium chloride. The among-laboratories variability (reproducibility) of the potentiometric method was comparable with the results from the collaborative studies for chloride in cheese, giving acceptable values for relative standard deviations of 1.5–3.0% for meat products with 0.3–2.0% added sodium chloride. It is recommended that further work be conducted to reduce or eliminate the systematic error present with the potentiometric method as applied to meat and meat products.

As a result of a resolution of the 12th meeting of ISO/TC 34/SC6 Meat and Meat Products, held at Balatonfüred, Hungary, on September 23 and 27, 1982 (1), it was decided on the basis of a preliminary investigation (2) that The Netherlands would conduct a second collaborative study comparing the ISO 1841 method (Volhard titration) (3) with the Codex Alimentarius Committee method (potentiometric titration) (4) for chloride in meat and meat products. The purpose of this study was to compare these analytical methods and to establish the lowest practical level of determination for chloride in these foods.

The collaborative study was conducted by The Netherlands and US laboratories. The results without the statistical calculations and conclusions were reported in a first draft (5). This publication describes the results of the statistical analyses by ISO statistics (6) and by AOAC statistics (7) together with the conclusions and recommendations.

Collaborative Materials

Four luncheon meats were especially manufactured for this study from the following formula: 25% lean beef, 38% lean pork, 20% shredded bacon, 12% water, 4.0% starch, 0.3% phosphate starch, and 0.045% spices. Four levels (about 0.25, 0.50, 1.0, and 2.0%) of sodium chloride were added. These 4 products together with an additional sample of one of them as a blind duplicate (for a total of 5 samples) were analyzed by the participants during the period from April to November 1983. No loss of chloride from the collaborative materials was observed during this period.

Graphical Evaluation of the Results

The reported data from the collaborators are tabulated in Table 1. Laboratories 4 and 11 did not conduct potentiometric titrations. Additional pairs of duplicate materials were also distributed to determine the homogeneity of the collaborative materials. The values from these duplicate materials, given in Table 2, were close to those of the original materials. To avoid complicating the calculations and the interpretation, these data were not included in the statistical analysis, but when present, are plotted in Figure 1 with the original data of Table 1.

Before the statistical analysis was performed, the results were plotted in 2 graphs, the first containing the Volhard points and the second containing the potentiometric points. The plotted data are given in Figure 1. The absolute sodium chloride content is the vertical axis and the laboratory number is on the horizontal axis. When the data are considered as a whole, the following points are apparent: *(1)* The Volhard method is much more variable among laboratories than the potentiometric method. Except for laboratory 5, which was flagged as an outlier by the statistical analysis, and which was found to have had an electrode problem, the potentiometric method gives consistent results among 8 laboratories. *(2)* The results by the potentiometric method are slightly higher than the results by the Volhard method.

Statistical Evaluation of the Results

The statistical parameters of the data of Table 1 were calculated by the ISO directions (6) and by the computer program FDACHEMIST described by Horwitz and Albert (8), which is based on the Youden-Steiner directions (7). The computer program was subsequently enlarged to encompass the ISO 2-tail Dixon test for outliers and applied to the data of Table 1. Consequently calculations by both sets of statistical directions can provide identical parameters and outlier indications. Values flagged by both sets of directions by the 2-tail Dixon test are indicated in Table 1 by single asterisks at the confidence level (CL) of 95–99% (designated as stragglers but included in the calculations of the statistical parameters). Those flagged at the 99 ± % CL are indicated by double asterisks (designated as outliers and omitted from the statistical calculations). Similarly, Cochran outliers are designated by single and double plus signs at the corresponding CLs and are treated in the calculations in the same manner.

The potentiometeric results of laboratory 5 were excluded because of a problem caused by fat deposits on the electrode surface. The data from this collaborator by this method are enclosed in parentheses in Table 1. The data from the blind duplicates were also excluded. To have included them would have given an unbalanced design, complicating the interpretation of the results, and would have hindered the application of the Cochran test for outliers.

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Table 1. Collaborative results for sodium chloride (%) in luncheon meat by Volhard and potentiometric methods[a]

Added NaCl, %	Method	Laboratory No. 1	2	3	4	5	6	7	8	9	10	11
0.27	Volhard	0.25	0.30	0.36	0.43	0.28	0.38++	0.26	0.07	0.29	0.13	0.18
		0.25	0.30	0.36	0.39	0.28	0.24++	0.27	0.09	0.26	0.12	0.21
	Potentiometric	0.37	0.35	0.28**		(0.24)	0.38	0.36	0.36	0.36	0.37	
		0.38	0.35	0.32**		(0.22)	0.37	0.36	0.35	0.36	0.36	
0.52	Volhard	0.52	0.50	0.61	0.45	0.56	0.59++	0.49	0.34	0.47	0.28	0.41
		0.52	0.55	0.61	0.48	0.57	0.87++	0.51	0.36	0.53	0.27	0.42
	Potentiometric	0.60	0.61	0.53**		(0.40)	0.59	0.61	0.59	0.60	0.59	
		0.61	0.60	0.52**		(0.39)	0.61	0.61	0.61	0.59	0.59	
1.01	Volhard	1.04	1.00+	1.07	0.99	0.99	1.20	1.04	0.90	0.87	0.71	0.92
		1.10	0.85+	1.10	0.96	1.03	1.19	0.99	0.89	0.90	0.78	0.92
	Potentiometric	1.07	1.07	1.03		(0.68)	1.11	1.09	1.08	1.10	1.08	
		1.10	1.06	1.05		(0.67)	1.10	1.11	1.09	1.10	1.06	
1.99	Volhard	1.27++	1.90	2.02	1.95	2.02	2.17	2.03	1.90	2.00	1.48**	1.86
		1.47++	1.95	2.05	1.90	2.00	2.21	2.00	1.92	1.95	1.53**	1.85
	Potentiometric	2.00	2.07	2.03		(1.15)	2.07	2.05	2.02	2.10	2.01	
		2.08	2.07	2.08		(1.15)	2.09	2.07	2.00	2.12	1.98	

[a]Outlier indications: Dixon extreme value (2-tail) test, applied to laboratory means, * = 95–99% CL, ** = 99+% CL. Cochran maximum variance test, + = 95–99% CL, ++ = 99+% CL. Values designated with a * or + (stragglers) are included when parameters are calculated without outliers. The results from laboratory 5 were omitted from the calculations of the potentiometric method because of an electrode problem.

Table 3 expresses the statistical analysis in both AOAC and ISO terminology. In this table the 4 chloride levels are separated by horizontal lines; within each of the 4 sets the Volhard data are given first and the potentiometric data second. The first line for each product-method combination gives results obtained by using all accepted values (only excluding laboratory 5, potentiometric method). Subsequent lines give the results with the indicated outliers removed.

The data of Tables 1 and 2 are visualized in Figure 1. The filled circles indicate the outliers removed (99+% CL). Outliers at the 1–5% level (stragglers) for both the Dixon and Cochran tests are not indicated because they are not excluded in calculating the precision parameters. The solid horizontal lines in Figure 1 for each level represent the added amount of sodium chloride for each product-method combination. The dotted horizontal lines for each product-method combination are the means calculated from the final accepted data from that combination (excluding outliers). The values for the individual data points and means are read on the "absolute % sodium chloride" scale on the left.

Statistical Interpretation of Results

Outliers—The data of Table 1 were first examined for outliers by the Dixon test for extreme values and by the Cochran test for extreme range of duplicates. The Grubbs test used in the computer program did not supply any additional information.

At the 0.25% NaCl level, laboratory 6 is an outlier by the Cochran test at the 99+% CL in the Volhard method; laboratory 3 is an outlier by the Dixon 2-tail test at the 99% CL in the potentiometric method.

At the 0.5% NaCl level, laboratory 6 is again an outlier by the Cochran test at the 99+% CL in the Volhard method; laboratory 3 is again an outlier by the Dixon 2-tail test at the 99% CL in the potentiometric method.

At the 1.0% level, laboratory 2 is a Cochran straggler at the 96% CL whose removal does not affect RSD_r because it appears in the middle of the data set. No outliers were flagged for the potentiometric method.

At the 2.0% level in the Volhard method, laboratory 10 masks laboratory 1 for the Dixon test. The 2-tail Dixon test does not even indicate these values as stragglers, yet as Figure 1 shows, both of these values are obviously outliers, not flagged because of the masking effect of two adjacent values. However, laboratory 1 is also a Cochran outlier at the 99+% CL, and its removal releases laboratory 10 to be flagged as an outlier by the Dixon 2-tail test. Both laboratories 1 and 10 were removed as outliers. The results by the potentiometric method were very consistent and no outliers or stragglers were flagged by either statistical procedure.

Means—From the results in Table 3 it is apparent that the mean sodium chloride content by the potentiometric method is statistically significantly higher than the mean by the Volhard method. Recoveries higher than 100% (see Table 4) are found with the potentiometric method and are illustrated in Figure 1 by the dotted lines representing the recovery values. The mean recoveries by the Volhard procedure are satisfactory and in agreement with the formulated values.

The significantly higher recoveries obtained with the potentiometric method, especially for the low chloride products, may be attributed to the presence of meat proteins in the solution during the titration with silver nitrate. Brammell (9, 10) showed that titrating solutions prepared from protein-rich food products would tend to give high results due to the reaction of silver nitrate with proteins. Further work is contemplated to reduce or eliminate this systematic error.

In the collaborative study of the determination of chloride in cheese, Poortvliet et al. (11) found the largest difference

Table 2. Collaborative results for sodium chloride (%) in blind duplicates of the same materials of Table 1

Added NaCl, %	Laboratory Number	Volhard Method		Potentiometric Method	
0.27	10	0.10	0.09	0.35	0.37
0.52	1	0.30	0.47	0.62	0.67
	8	0.35	0.34	0.59	0.60
1.01	3	1.08	1.07	0.98	0.96
1.99	2	1.95	1.95	2.05	2.06
	4	2.02	2.00		
	5	1.94	1.98	(0.91)	(1.09)
	6	2.18	2.16	2.06	2.05
	7	2.01	2.02	2.07	2.08
	9	1.92	1.92	2.09	2.10
	11	1.79	1.88		

Table 3. Statistical parameters as calculated by AOAC and ISO procedures[a]

Added NaCl,%	Method	Data used	N	m	s_r	s_R	RSD_r	RSD_R	r	R	r/R
0.27	Volhard	All values	22	0.259	0.0328	0.0988	12.64	38.12	0.0927	0.02795	0.33
		Omit Lab 6	20	0.254	0.0141	0.1001	5.57	39.42	0.0400	0.02834	0.14
	Potentiometric	All values	16	0.354	0.0132	0.0248	3.74	7.01	0.0374	0.0701	0.53
		Omit Lab 3	14	0.361	0.0093	0.0104	2.56	2.86	0.0262	0.0293	0.89
0.52	Volhard	All values	22	0.496	0.0627	0.1309	12.64	26.39	0.1775	0.3704	0.48
		Omit Lab 6	20	0.472	0.0201	0.1018	4.26	21.54	0.0570	0.2880	0.20
	Potentiometric	All values	16	0.591	0.0087	0.0282	1.46	4.76	0.0245	0.0797	0.31
		Omit Lab 3	14	0.601	0.0089	0.0092	1.48	1.53	0.0251	0.0260	0.96
1.01	Volhard	All values	22	0.974	0.0416	0.1241	4.26	12.73	0.1176	0.3511	0.34
	Potentiometric	All values	16	1.081	0.0122	0.0236	1.13	2.19	0.0347	0.0669	0.52
1.99	Volhard	All values	22	1.883	0.0497	0.2414	2.64	12.82	0.1406	0.6832	0.21
		Omit Labs 1, 10	18	1.982	0.0256	0.0990	1.29	5.00	0.0725	0.2802	0.26
	Potentiometric	All values	16	2.052	0.0267	0.0417	1.30	2.03	0.0755	0.1181	0.64

[a]Symbols: N = number of observations (= number of analysts × 2); m = mean; s_r = standard deviation (absolute), within-laboratory; s_R = standard deviation (absolute) among-laboratories (including within-); RSD_r = relative standard deviation, within-laboratory; RSD_R = relative standard deviation, among-laboratories; $r = 2\sqrt{2} \times s_r$ (repeatability); $R = 2\sqrt{2} \times s_R$ (reproducibility). Units: m, s_r, s_R, r, and R are % NaCl; N, RSD_r, and RSD_R are dimensionless.

(0.05% chloride) between the potentiometric and the Volhard methods at the 1.8% level. Statistically significantly higher results were also obtained with the potentiometric method.

In the present study, linear regression analysis for the means of the test materials showed that the 2 methods are in satisfactory agreement. The regression equations of the means for both methods, together with their correlation coefficients, $r_{correl.}$, are:

Potentiometric method: $Y = 0.091 + 0.984\,X$; $r_{correl.} = 1.000$;

Volhard method: $Y = -0.039 + 1.013\,X$; $r_{correl.} = 0.998$,

where Y is the % NaCl found and X is the % NaCl added. The absolute value for overestimating the sodium chloride mean for the potentiometric method is 0.065%, if the inherent sodium chloride content of the ingredients used in preparing the test products (about 0.01%) is ignored. The influence of this value on the analytical result is greatest for low chloride products, but despite this fact the values by the potentiometric method are still practically acceptable for meat products.

Repeatability (within-laboratory variability).—The repeatability (r) values of Table 3 demonstrate the agreement of replicates by the same operator. Figure 1 illustrates the high degree of repeatability of duplicates for both methods, indicating the skill of the analysts and their acquaintance with the analytical procedures. The repeatability of the Volhard method in this study is better than the 0.2 g per 100 g claimed in section 9.2 of the ISO 1841 method (3). The repeatability of the potentiometric method is comparable to that of the Volhard method for the 2% sodium chloride levels and better for the lower salt concentrations. In general, both methods demonstrate satisfactory repeatabilities.

Reproducibility (among-laboratories variability).—The reproducibility values (R) of Table 3 demonstrate the agreement of the results by different operators in different laboratories.

The results of this study indicate that the Volhard method is not applicable to the determination of the 0–1.0% concentration levels of NaCl. This is also apparent from a visual examination of the results in Figure 1. The R value of 0.28% for the 0.27% NaCl level is as great as the amount of salt added, m. Since R>m, it is concluded, according to the ISO statistics, that the Volhard method is not applicable to this level. Removal of outlying laboratory 6 hardly improves the situation because it leaves laboratories 10, 4, and 8 as extremes.

The situation is the same for the 0.52% added salt level. The only outlier comes from laboratory 6, but removal of this laboratory again does not improve the situation. The RSD_R values changes little, from 26 to 22%, or in terms of % NaCl, the reproducibility decreases from 0.37% to 0.29%. A reproducibility of 0.3% (RSD_R = 22%) is still not satisfactory when dealing with 0.5% NaCl. With the 1.0% level a RSD_R value of 13% is rather poor, as is the high reproducibility value of R = 0.35% NaCl.

An acceptable RSD_R = 5.0% and a reproducibility R = 0.28% are calculated for the 2.0% added salt level in the Volhard method, if laboratories 1 and 10 are ignored as outliers.

Satisfactory RSD_R values of 1.53–2.86% with corresponding R values of 0.03–0.12% NaCl were calculated for the 4 materials analyzed by the potentiometric method. For the 0.27% and 0.52% added salt products laboratory 3 is regarded as an outlier at the 99% CL according to the ISO 2-tail Dixon test and by the Grubbs test. This laboratory has a consistent low bias with 3 of the 4 products. Its removal from the 2 lowest levels would give RSD_R values more in line with those of the other materials and more consistent with the statistical data obtained from other studies of this method (10, 11).

Blind duplicate samples were analyzed during this study by both procedures. The results of these assays listed in Table 2 prove that the collaborative materials are homogeneously spiked with sodium chloride. The blind duplicates are ignored in the statistics since their values are close to the values from the original material.

Level of determination.—The results of this study indicate that the Volhard method is applicable to meat samples with sodium chloride concentrations higher than about 1.0%. The lowest determination level of this method for reliable NaCl measurements is about 1.0%. The determination level of the potentiometric method is set at 0.25% because of the rather inaccurate inflection point of the titration curve below this level.

Comparison with other studies.—The overall results obtained in this study are roughly comparable to the collaborative data and statistics for cheese published by Brzenk et al. (12) and by Poortvliet et al. (11). In these studies the direct Volhard method has a RSD_R value of 3% at NaCl levels of 1.4–2.2%, whereas the indirect Volhard method with filtration and the potentiometric method showed corresponding values of about 2.0 and 1.4%, respectively.

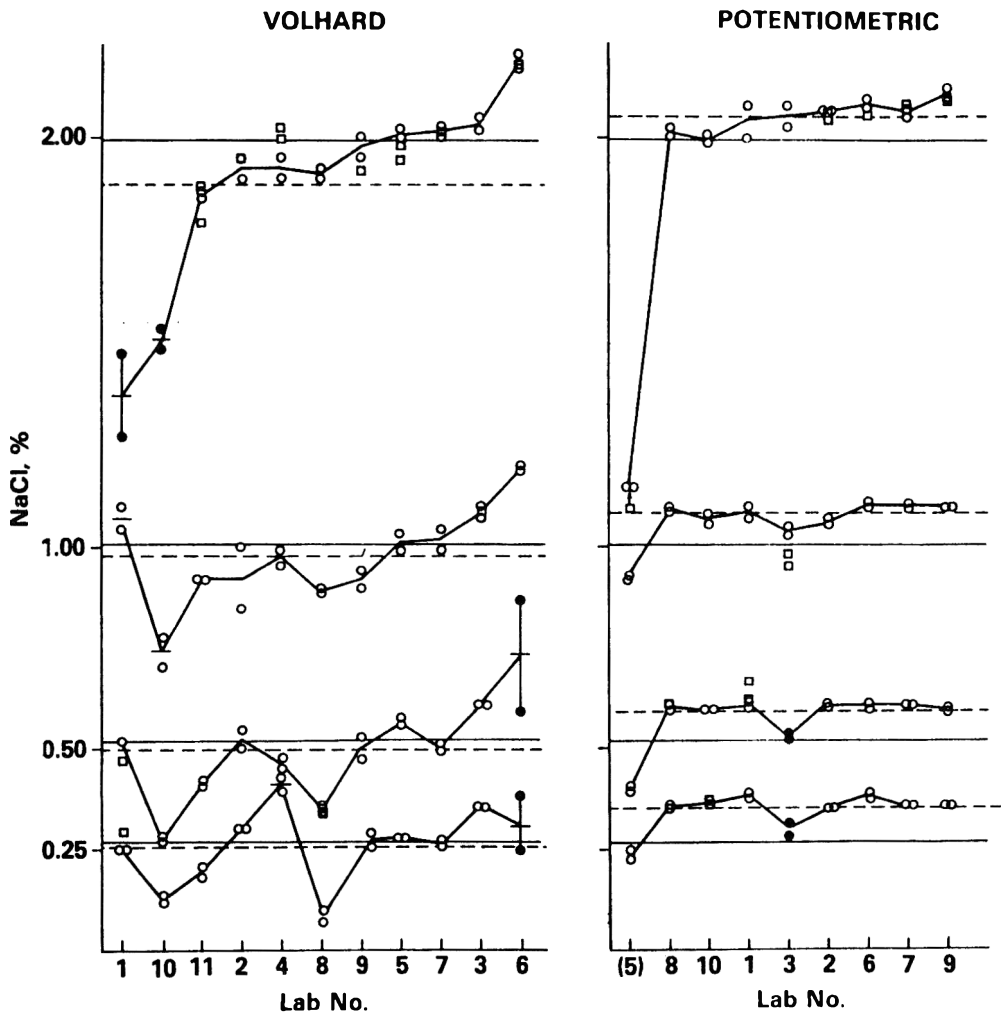

Figure 1. Collaborative data for percent sodium chloride in luncheon meat from 4 materials at 4 different spiking levels from 0.25 to 2.00% NaCl; 2 different methods were used by 11 laboratories (Volhard) and 8 laboratories (potentiometric). Solid circles indicate outliers by the 2-tail Dixon test or by the Cochran test, both at the 99% CL. The solid horizontal lines are the spike levels; the dotted horizontal lines are the mean levels found, using all the data. The square points represent the values for the blind duplicates.

The RSD_R values of 5% for the Volhard method and 2% for the potentiometric method calculated from the present study for the 2% added salt products are comparable to RSD_R values reported in the previous studies. Since the concentration range in this study is considerably greater than the ranges given in other publications, no comparisons can be made to the RSD_R values for lower levels (< 1.5% NaCl).

Volhard vs potentiometric method.—The reproducibility (R) of the Volhard method (ISO 1841) for 2% NaCl level is about 0.3%, which is acceptable for the determination of NaCl in meat products. The reproducibility of the potentiometric method is about 0.1% NaCl, which is much better than that of the Volhard method at the same level. The R values of the potentiometric method decrease somewhat for lower levels: about 0.07% at 1%, 0.03% at 0.5%, and 0.03% at 0.27% added salt levels. Across the board, a single value of 2.2% is calculated for the RSD_R value in this concentration range of 0.25–2.00% NaCl, demonstrating the applicability of the potentiometric method for low concentration levels.

The potentiometric method was easy to perform and more feasible for routine determinations than the ISO method. The method is also suitable for automation. The disadvantage of using a silver electrode is of minor importance when compared with the disadvantages of the ISO method such as a time-consuming deproteination step, cumbersome visual observation of the titration end point, and use of nitrobenzene in the titration.

Conclusions

The overall results obtained in this study lead to the following conclusions:

(a) The aim of this study has been accomplished.

(b) Both the AOAC and ISO statistics are consistent with one another, resulting in the same conclusions.

(c) Significantly higher results are obtained with the potentiometric method than with the Volhard method. The absolute value for overestimating the average NaCl means for the potentiometric method is 0.065%, possibly due to an effect from meat proteins. Additional research on this subject is envisaged.

(d) The mean NaCl contents determined in 4 meat products representing 4 different salt levels are in satisfactory agreement for both methods as shown by the regression equations and correlation coefficients.

(e) The within-laboratory variability (repeatability) of both methods is comparable even at lower salt levels.

(f) The among-laboratories variability (reproducibility) of the potentiometric method is better than that of the Volhard method; RSD_R values of 1.5–3.0% are calculated for the potentiometric method (added salt levels 0.3–2.0%), whereas the lowest RSD_R value of 5.0% is established at the 2.0% level for the Volhard procedure. RSD_R values of 12.7, 26.4, and 39.4% for spiking levels of 1.0, 0.5, and 0.3%, respectively, indicate the poor reproducibility of the Volhard method at the lower levels.

Table 4. Summary of the mean sodium chloride (%, absolute) values and recoveries (%, relative) calculated from the collaborative data for each spiking level by both methods.

	Found NaCl, %		Recovery, %	
Added NaCl, %	Volhard	Potentiometric	Volhard	Potentiometric
0.27	0.254	0.361	94	134
0.52	0.472	0.601	91	116
1.01	0.974	1.081	96	107
1.99	1.984	2.052	100	103

(g) The lowest level of determination yielding reliable results is about 1% NaCl for the Volhard method and 0.25% for the potentiometric method.

(h) The potentiometric method is preferred for assaying meat products for sodium chloride because of its simplicity of performance, its feasibility for routine determinations, and its production of reliable results even for the lower concentration levels (< 1.0% NaCl).

Recommendation

It is recommended that the potentiometric method for the determination of chloride in meat and meat products be investigated further to reduce or eliminate the small systematic error which is present. It can then be considered for adoption as the ISO reference standard to replace the current ISO 1841 (Volhard) reference method. AOAC may also wish to consider accepting this method for meat and meat products, as the official method (13) is also a Volhard method.

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Effect of pH on Absorbance of Azo Dye Formed by Reaction Between Nitrite and Sulfanilamide/*N*-(1-Naphthyl)ethylenediamine in Residual Nitrite Methods for Foods

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Current methods used for determining residual nitrite concentration in foods involve forming an azo dye that is measured spectrophotometrically. Conventional procedures do not specify control of pH for the final colored solution. Because many indicator dyes are pH-dependent, absorbance of the azo dye may vary not only because of nitrite concentration differences, but also because of pH variation. Thus, erroneous results could arise from a standard curve developed at one pH and used for a test sample at a different pH. This would cause an inaccurate concentration conversion from the standard curve. In this study, absorbances at λ_{max} wavelengths were recorded at 28 pH values (range 1.0–4.5) for each of 5 nitrite concentrations. A mathematical equation was fitted to these data and its plot gives a 3-dimensional response surface showing the relationships between pH, nitrite concentration, and absorbance. A modification for the spectrophotometric measurement of nitrite is proposed.

Certain procedures to determine residual nitrite in meat products specify the diazotization of an aromatic amine followed by a coupling reaction with an aromatic compound to form an azo dye that is quantitated by absorbance spectrophotometry. Both of these reactions require a mildly acidic medium for optimum reaction (1). However, pH of the color development stage in many nitrite methods, including the AOAC official method (2), is not specifically controlled. Pioneering work by Ilosvay (3) and by Rider and Mellon (4) showed that both the rate of azo compound formation and absorbance of the azo dye were affected by pH. Work by Bendschneider and Robinson (5) suggests that a pH $\leq$ 2.0 is required to produce maximum color intensity for determining nitrite in sea water with 1.0% sulfanilamide reagent and 1 min diazotization.

The purpose of this study was to determine the effect of pH on the final absorbance of the azo compound formed at differing nitrite concentrations and to compare results with those from the present method (2) used for residual nitrite estimation in meat products.

Experimental

Reagents

Use deionized, distilled water throughout.

(a) *Sulfanilamide (SFA) reagent.*—Dissolve 0.5 g sulfanilamide in 150 mL 15% (v/v) acetic acid.

(b) *N-(1-Naphthyl)ethylenediamine dihydrochloride (NED) reagent.*—Dissolve 0.2 g NED in 150 mL 15% (v/v) acetic acid.

(c) *Hydrochloric acid solution.*—Dilute concentrated HCl to 2N with water.

(d) *Sodium hydroxide solution.*—Prepare 2N solution with water.

(e) *Nitrite solution.*—Dissolve 0.2 g sodium nitrite ($NaNO_2$) in 1 L water (stock solution). Dilute 5 mL stock solution to 1 L with water (working solution). Each mL working solution contains 1 μg $NaNO_2$.

Procedure

Five standard concentrations of nitrite (0.1, 0.2, 0.3, 0.4, and 0.5 μg/mL) were prepared by pipetting (volumetric) 5, 10, 15, 20, and 25 mL working solution into separate 50 mL volumetric flasks. Water was added to each flask to ca 30 mL. After diazotization was initiated by adding 2.5 mL SFA reagent, flasks were swirled and allowed to stand 5 min. Formation of the colored azo compound was completed by adding 2.5 mL NED reagent, after which flasks were again swirled and allowed to stand for 15 min. The pH of this reaction mixture was ca 2.6. Following color development, pH of the colored reaction mixture was adjusted by adding measured amounts of HCl or NaOH to the flask to achieve a targeted pH value, ranging from pH 1.0 to 4.5. Contents were then diluted to volume (50 mL) with water. Absorbance of the azo dye was measured at room temperature (22°C) on a Beckman Model 35 dual beam, scanning, recording spectrophotometer. Maximum absorbance was read from the digital display as the instrument reached peak wavelength of the azo dye scanning from 750 nm down to 350 nm. A reagent blank minus nitrite was used as the reference. After absorbance measurement, pH of the remaining dye solution in the volumetric flask was determined and recorded. This pH value along with absorbance was used to plot Figure 1. A Radiometer expanded scale pH meter with a Radiometer combination glass electrode standardized with commercial buffers at pH 1, 2, 4, and 7 was used to determine pH values.

Results and Discussion

The absorbance for this system is characterized by an asymmetric peak for which the maximum shifts from 540 nm at pH 1.0 to less than 500 nm for pH $\geq$ 4.2 (Figure 1). An isosbestic point occurs at 490 nm at each concentration. These observations are adequately accounted for by assuming the presence of 2 overlapping peaks, one due to the ionized form of the indicator and the other to the protonated form. For a cell of unit thickness, the absorbance of this system is given by

$$A = a_{G^-}c_{G^-} + a_{GH}c_{GH}$$

where a is a pH-independent, wavelength-dependent absorptivity, and c is a pH-dependent concentration. G^- refers to the ionized form and GH to the protonated form of the azo compound. The pH-dependence of c_{G^-} and c_{GH} can be found from the definition of the dissociation constant:

$$K_a = \frac{[H^+]c_{G^-}}{c_{GH}}$$

and the equation: $c_{G^-} + c_{GH} = c$ (total azo reaction product concentration. Thus,

$$c_{G^-} = \frac{K_a c}{[H^-] + K_a} \text{ and } c_{GH} = \frac{[H^+]c}{[H^+] + K_a}$$

and absorbance is expressed as

$$A = \left[a_{G^-} \frac{K_a}{[H^+] + K_a} + a_{GH} \frac{[H^+]}{[H^+] + K_a} \right] c = a_e c$$

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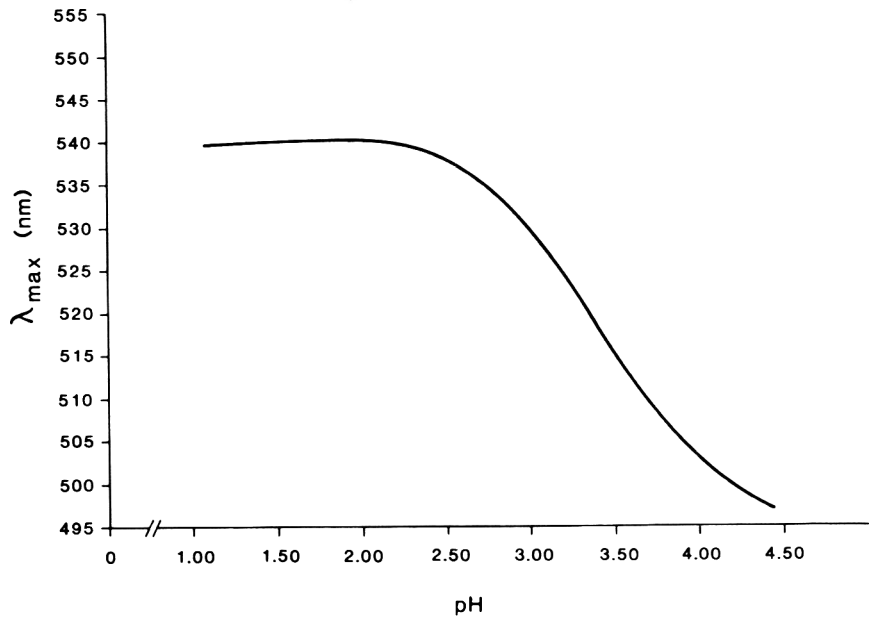

Figure 1. Plot of wavelength for maximum absorbance (λ_{max}) of azo dye formed from nitrite with SFA/NED over pH range 1.0–4.4.

The expression in brackets, a_ϵ, becomes an effective absorptivity which is pH-dependent. It can be written as

$$a_\epsilon = \frac{a_{GH} + a_G \cdot 10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}} \qquad (1)$$

It should be noted that the absorbance is linear with concentration for any fixed values of wavelength and pH. But, if the wavelength chosen is not that corresponding to the combined peak maximum, the sensitivity will be lower than it could be. Thus, the optical density data were all taken at the wavelength corresponding to maximum absorbance for the pH involved.

Equation *1* has the form

$$a_\epsilon = \frac{B + C\ 10^{(pH - D)}}{1 + 10^{(pH - D)}} \qquad (2)$$

where B, C, and D are constants for a given wavelength. The envelope of all curves of the type described by Equation *2* corresponding to all wavelengths in the region of interest gives the maximum effective absorptivity as a function of pH and has a similar, if not identical, shape to Equation *2*. Since the purpose of this paper is to establish a highly accurate means for predicting concentrations from the measured absorbance and pH, the best possible equation for the relationship between maximum effective absorptivity and pH is desired. Thus, the values of the constants in an equation like Equation *2* which give the best fit are desired. It was found that a better fit would be achieved through the use of a fourth parameter. If activity coefficients had been included in this development, analogously to Flexner et al. (6), it would have led to the form:

$$a_\epsilon = \frac{B' + C'E'\ 10^{(pH - D')}}{1 + E'\ 10^{(pH - D')}} \qquad (3)$$

An alternative form:

$$a_\epsilon = \frac{B'' + C''\ 10^{E''(pH - D'')}}{1 + 10^{E''(pH - D'')}} \qquad (4)$$

where B″ = 0.7818, C″ = 0.3568, D″ = 2.713, and E″ = 1.164, was found to give a better fit than Equation *3*. Its error sum of squares was half that found for the fit for Equation *3*. While no theoretical basis is known for the use of Equation *4*, the improved fit justifies its use in constructing Table 1. This table provides a simple means of arriving at maximum effective absorptivity for pH 1.0 to 4.4. Figure 2 shows the fit of Equations *3* and *4* to the maximum effective absorptivity data using a grid search method. Each of the data points in Figure 2 has an ordinate determined by the slope of absorbance (λ_{max}) vs concentration curve. Figure 3 pictures the relationships between concentration, absorbance, and pH.

We thus arrive at a general procedure for the colorimetric determination of nitrite concentration when pH is an included

Table 1. Tabulation of maximum effective absorptivity, a_ϵ (pH), from Equation *4* for pH 1.0–4.4

pH	a_ϵ	pH	a_ϵ	pH	a_ϵ	pH	a_ϵ	pH	a_ϵ
1.0	0.7775	1.7	0.7554	2.4	0.6535	3.1	0.4680	3.8	0.3787
1.1	0.7762	1.8	0.7479	2.5	0.6284	3.2	0.4474	3.9	0.3737
1.2	0.7746	1.9	0.7386	2.6	0.6012	3.3	0.4298	4.0	0.3699
1.3	0.7724	2.0	0.7270	2.7	0.5730	3.4	0.4150	4.1	0.3669
1.4	0.7696	2.1	0.7129	2.8	0.5446	3.5	0.4028	4.2	0.3646
1.5	0.7660	2.2	0.6960	2.9	0.5171	3.6	0.3929	4.3	0.3628
1.6	0.7613	2.3	0.6762	3.0	0.4914	3.7	0.3850	4.4	0.3614

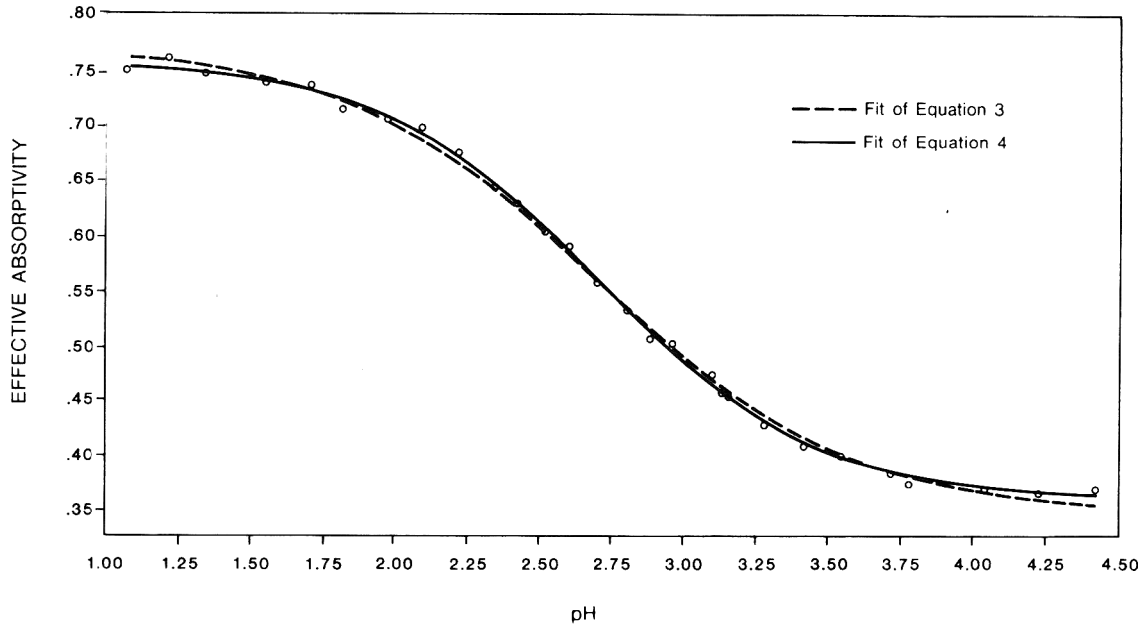

Figure 2. Fit of effective absorptivity data to Equations *3* and *4* over pH range 1.0–4.4.

variable: *(1)* Measure and/or adjust the pH; *(2)* Find the wavelength for maximum sensitivity corresponding to that pH from Figure 1; *(3)* Set the spectrophotometer to that wavelength; *(4)* Determine the maximum effective absorptivity for that pH from Equation *4* or Table 1; *(5)* Determine concentration in the usual manner.

Conclusion

The current procedure for measuring residual nitrite in meat products (2) does not require pH adjustment to a specific value prior to absorbance measurement. Thus, in the final color solution, errors in estimates of residual nitrite concentrations may occur due to variation in the absorbance spectrum of the azo reaction product with pH. To eliminate this source of error, the pH of the final color solution must be taken into account. This can be done in one of 2 ways: *(1)* Measure the pH of the final color solution and use the appropriate a_ϵ (pH) to calculate nitrite concentration. *(2)* Adjust the pH of the final color solution to the range 1.2–1.3 (Figure 1) for maximum sensitivity. This will permit the use of a fixed value of a_ϵ (pH). This would be accomplished by adding 3.5

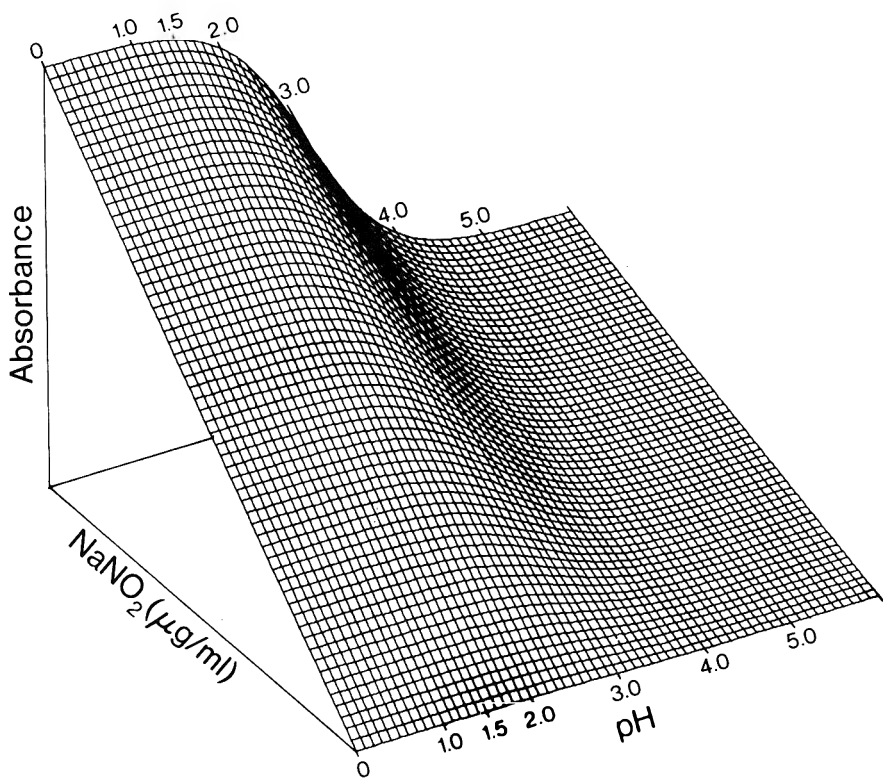

Figure 3. Response surface plot of Equation *4* for absorbance vs nitrite concentration and pH.

mL 2N HCl to the colored reaction mixture just before diluting to 50 mL for the absorbance measurement.

The second alternative has the advantages of being simpler and more like the current standard method than the first. Therefore, the authors recommend that alternative 2 be considered as the basis for a modification of the standard method for nitrite determination (2).

Acknowledgment

The authors thank Jay Fox, Agricultural Research Service, Wyndmor, PA, for his very helpful suggestions.

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Rapid Quantitative Method for Simultaneous Determination of Benzoic Acid, Sorbic Acid, and Four Parabens in Meat and Nonmeat Products by Liquid Chromatography

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A liquid chromatographic (LC) method for the simultaneous determinations of benzoic acid, sorbic acid, and methyl, ethyl, propyl, and butyl parabens (methyl, ethyl, propyl, and butyl-*p*-hydroxybenzoates) in meat and nonmeat products was developed. Benzoic acid, sorbic acid, and parabens were extracted from meat and nonmeat products with 70% ethanol. After filtration, extracts were analyzed by reverse phase liquid chromatography. Homogeneously ground samples of fresh sausage and hamburger were fortified with benzoic acid, sorbic acid, and each paraben at 5 different concentrations. Average recovery (after discarding outliers) for each preservative at all 5 levels was greater than 95% with a coefficient of variation less than 5%.

Requests for analyses of benzoic acid, sorbic acid, and parabens in meat products and in seasoning mixes are common in the Eastern Laboratory. These antimicrobial preservatives are not permitted in fresh meat products or in seasoning mixes used in the formulation of these products. Part 318.7 of USDA Meat and Poultry Inspection (MPI) Regulation states, "No substance may be used in or on the product if it conceals inferiority or makes the product appear better than it is." Our goal was to develop a simple and rapid method for the simultaneous quantitative determinations of all 6 antimicrobial preservatives to assure that this regulation was not being abused by use of these compounds.

Several gas chromatographic (GC) and liquid chromatographic (LC) methods have been published to determine these preservatives in various food products, beverages, and cosmetics: sorbic acid and benzoic acid in rye bread and margarine (1); sorbic acid and sodium benzoate in table syrup (2); methyl, ethyl, and propyl parabens in salad dressings, sauce mixes containing oil, ketchup and pickles (3); benzoic acid and sorbic acid in fish homogenate, apple juice, and almond paste (4); benzoic acid and sorbic acid in beverages (5); methyl and propyl *p*-hydroxybenzoates in comminuted meats (6); methyl and propyl parabens in cosmetics (7); sorbic acid and benzoic acid in jam, jellies, and prepared meats (E. Neidert (1983) Laboratory Services Division, Ministry of Agriculture, Canada, unpublished data); and potassium sorbate in meat (R. B. Ashworth (1979) USDA, FSIS, Science, Chemistry Division, unpublished data). None of these methods was capable of providing simultaneous determination of 6 preservatives. Moreover, with few exceptions, the extraction procedures used in these methods were complicated and lengthy.

Because of the steadily increasing number of samples of meat and spice mixes in need of analyses for antimicrobial preservatives, the need for a faster assay method became evident. An LC method with UV detection offered the advantages of simplicity, speed, and reliability. Because LC methods are not specific, however, we felt that positive results should be confirmed by another technique. Accordingly, further purification steps were added to this method for GC/MS (mass spectrometry) confirmation.

This paper describes a reverse phase LC method for quantitative determination of benzoic acid, sorbic acid, and methyl, ethyl, propyl, and butyl parabens in comminuted meats. Recoveries from meat samples fortified with 200, 400, 600, 800, and 1000 ppm benzoic acid; 8, 16, 24, 32, and 40 ppm sorbic acid; and 20, 40, 60, 80, and 100 ppm of each paraben were determined. These levels were selected because minimum inhibitory concentrations of benzoic acid, sorbic acid, and parabens on various spoilage microorganisms, including the toxin-producing organisms (8–11), are considerably higher than the lowest fortification levels used in this experiment. After LC analysis, the extracts were further purified for GC/MS confirmation according to the procedure of Sun (12).

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Experimental

Apparatus

(a) *Magnetic stirrer.*—Variable speed, Nuova 7 Thermolyne (Sybron Corp., Dubuque, IA 52001), or equivalent.

(b) *Stirring bar.*—Plastic coated, magnetic, 1 in. long.

(c) *Filter paper.*—Reeve Angel Grade 802 (Sargent-Welch Scientific Co., Skokie, IL 60077), fast filtering and medium porosity (18.5 cm), or equivalent.

(d) *Solvent clarification kit.*—With Durapore filters (0.45 μm, 47 mm), but without pump and filtering flask (Waters Associates, Milford, MA 01757).

(e) *Evaporator.*—N-Evap Model III (Organomation Associates, Inc., South Berlin, MA 01549), or equivalent.

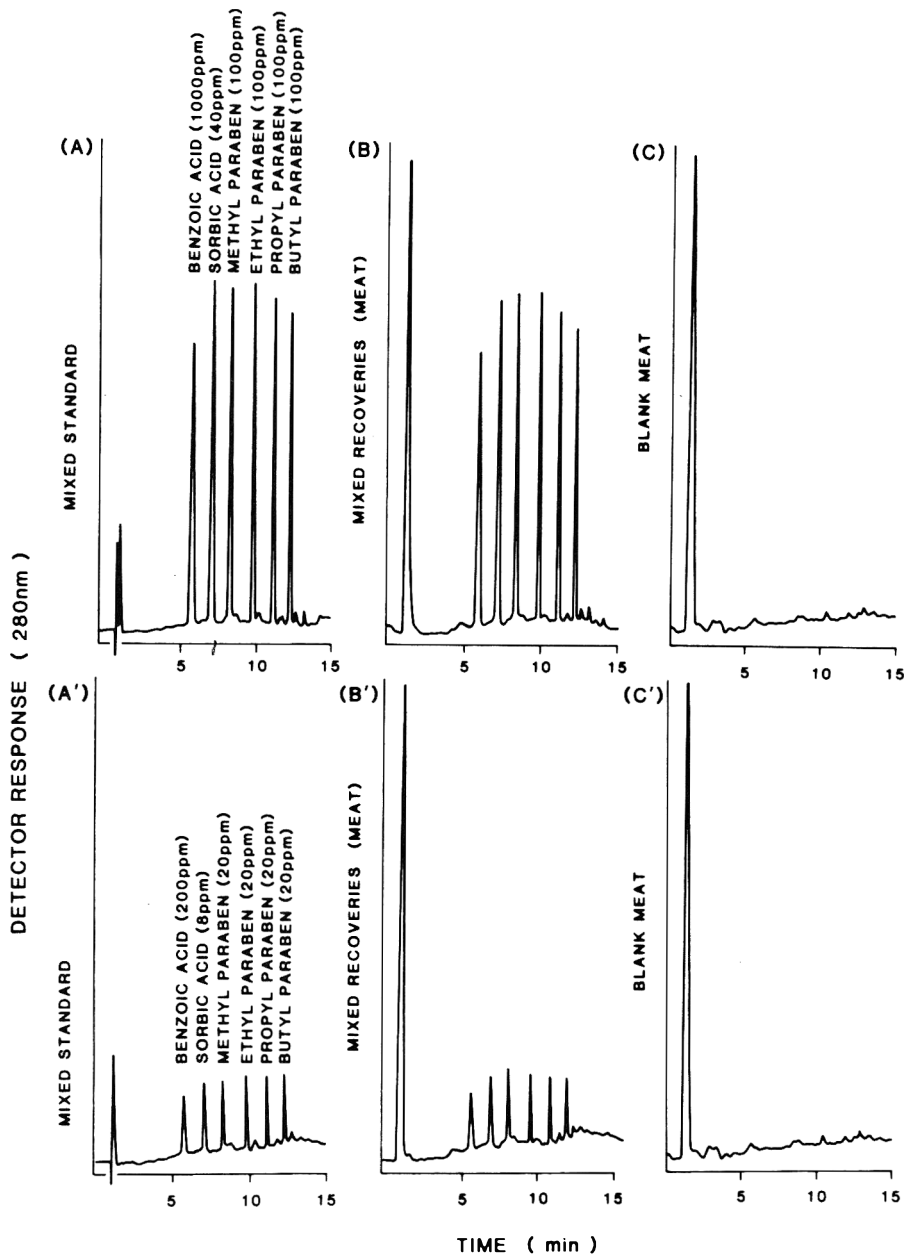

Figure 1. LC chromatograms of (A) mixed standards and (B) mixed recoveries of benzoic acid, sorbic acid, and 4 parabens at the highest level used in the method and (C) unfortified meat; (A') mixed standard and (B') mixed recoveries of benzoic acid, sorbic acid, and 4 parabens at the lowest level used in the method and (C') unfortified meat. UV detector, wavelength 280 nm, sensitivity 0.05 AUFS.

(f) *Sample filtration apparatus.*—Stainless steel Swinney filter holder, used with 0.45 μm Fluoropore filters (Millipore Corp., Bedford, MA 01730).

(g) *Liquid chromatograph.*—Waters Associates, Model 244 liquid chromatograph, equipped with 2 pumps, Model M-6000A (one designated as pump A and the other as pump B), Model 440 absorbance detector, Wisp 710B sample processor, and Model 730 data module, or equivalent. Strip chart recorder, OmniScribe Recorder Model 35217-1 (Houston Instrument, Austin, TX 77041), or equivalent, is optional.

(h) *Column.*—15 cm × 3.9 mm id, Nova-pak C18 reverse phase packing, 5 μm spherical silica (Waters Associates), or equivalent.

(i) *pH meter.*—Fisher Accumet, Model 210 (Fisher Scientific Co., Pittsburgh, PA 15219), or equivalent.

Reagents

(a) *Absolute ethanol.*—200 proof (U.S. Industrial Chemicals, Inc., Division of National Distillers Products Corp., New Orleans, LA).

(b) *Methanol.*—Distilled in glass, UV grade (Burdick and Jackson Laboratories Inc., Muskegon, MI 49442), or equivalent.

(c) *Glacial acetic acid.*—ACS grade.

(d) *Water.*—Low organic content and specific resistance ≥ 10 megaohms/cm to avoid problems with extraneous interferences, Milli-Q Water Purification System (Millipore Corp.).

(e) *Ammonium acetate.*—LC grade (Fisher Scientific Co.).

(f) *Hydrochloric acid.*—ACS grade.

(g) *Benzene.*—Thiophene-free, ACS grade (Mallinckrodt, Inc., St. Louis, MO 63134).

(h) *LC mobile phase.—(1) Buffer solution.*—1.5% (w/v) aqueous ammonium acetate and 1.5% (v/v) aqueous acetic acid. Into 1 L graduated cylinder, add 15 g ammonium acetate and 15 mL glacial acetic acid. Dilute to volume and mix well to dissolve ammonium acetate. Filter resultant aqueous acidic solvent (pH 4.50) through Durapore filter into 2 L filtering flask. Degas solvent under vacuum. Use solvent for pump A of the liquid chromatograph. *(2) Methanol.*—LC grade, filter and degas. Use solvent for pump B of liquid chromatograph.

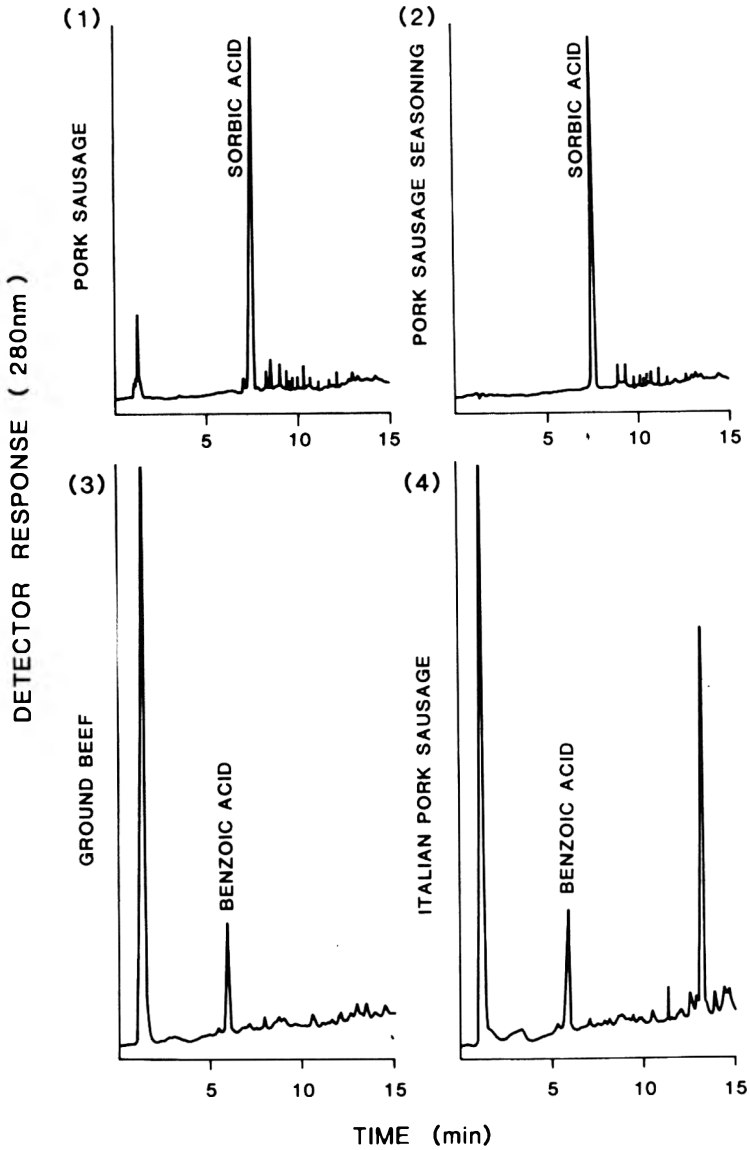

Figure 2. LC chromatograms of official samples: sorbic acid (807 ppm) found in pork sausage (extract diluted); sorbic acid (3646 ppm) found in pork sausage seasoning (extract diluted); benzoic acid (350 ppm) found in ground beef; and benzoic acid (444 ppm) found in Italian pork sausage. UV detector, wavelength 280 nm, sensitivity 0.05 AUFS.

Linear gradient.—Use linear gradient, programmed from 10–70% methanol in 1.5% aqueous ammonium acetate and 1.5% aqueous acetic acid over 10 min, with 10 min hold and 5 min equilibration delay at 1.5 mL/min. Equilibrate entire system with gradient elution of mobile phase until stable baseline is obtained (ca 30–45 min) at flow rate of 1.5 mL/min. Under conditions used, retention times (min) of all 6 preservatives were as follows: benzoic acid, 5.80; sorbic acid, 7.05; methyl paraben, 8.23; ethyl paraben, 9.70; propyl paraben, 10.99; and butyl paraben, 12.14.

(i) *Mixed standard solution.*—2.0 mg/mL benzoic acid, 0.08 mg/mL sorbic acid, and 0.20 mg/mL each of methyl, ethyl, propyl, and butyl parabens. Weigh exactly 400.0 mg benzoic acid, 16.0 mg sorbic acid, and 20 mg each of methyl, ethyl, propyl, and butyl parabens into 200 mL volumetric flask. Add ca 50 mL 70% ethanol to dissolve, and dilute to volume with 70% ethanol. For the standard curve, dilute 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL of mixed standard to 100 mL with 70% ethanol.

Sample Extraction

Weigh 10 g thoroughly comminuted meat product or 2 g seasoning mix into 250 mL Erlenmeyer flask. Select "blank" meat sample as control and for spiking. (Our familiarization protocol required fortifying with 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL of mixed standard per 10 g of sample.)

Add 70 mL ethanol to sample in flask; add milli-Q water to make a final liquid volume of 100 mL. (Moisture content of meat product should be used in determining liquid volume.) Break up lumps of meat with clean spatula, add magnetic stirring bar, stopper, and mix on magnetic stirrer 10 min at medium speed. Filter ca 45 mL extract into 50 mL stoppered test tube using filter paper (c). Refilter ca 2 mL aliquot of filtrate through 0.45 μm filter (f) into glass vial for LC analysis. Save remaining filtrate for GC/MS confirmatory analysis. With each set of official samples to be analyzed, process one regeant blank, one control, and one sample fortified with medium level (3.0 mL) mixed standard: 600 ppm benzoic acid, 24 ppm sorbic acid, and 60 ppm of each paraben.

Determination

Using Wisp 710B sample processor, inject (in duplicate) 20 μL portions each of sample extracts and mixed standards, and program solvent as described. Set range to 0.05 AUFS. Calculate concentration of each preservative in sample as follows: Using peak areas or peak heights and concentrations

Table 1. Statistical data of recoveries for each concentration of benzoic acid, sorbic acid, and 4 parabens[a]

Compound	Concn, ppm	No. of recoveries	Mean rec., %[b]	SD, %	CV, %
Benzoic acid	200	22	103.2	3.82	3.70
	400	27	100.7	2.44	2.43
	600	28	99.9	4.36	4.36
	800	30	99.5	4.11	4.13
	1000	30	97.3	4.15	4.26
Sorbic acid	8	29	100.5	3.95	3.93
	16	29	98.8	2.59	2.62
	24	30	99.1	2.81	2.83
	32	30	99.2	3.10	3.12
	40	30	98.6	4.51	4.58
Methyl paraben	20	28	101.4	4.74	4.68
	40	29	101.4	4.14	4.09
	60	29	100.5	3.07	3.05
	80	30	100.5	3.50	3.49
	100	30	100.4	4.75	4.73
Ethyl paraben	20	29	101.2	4.15	4.10
	40	28	100.8	3.25	3.22
	60	30	100.4	3.56	3.55
	80	30	100.6	4.27	4.24
	100	29	100.4	4.13	4.11
Propyl paraben	20	28	101.0	3.92	3.87
	40	28	100.2	3.42	3.41
	60	29	99.8	3.14	3.15
	80	29	100.3	4.08	4.07
	100	30	100.0	4.33	4.33
Butyl paraben	20	28	102.8	3.99	3.88
	40	27	100.5	3.51	3.49
	60	29	99.6	3.43	3.44
	80	29	100.3	4.38	4.37
	100	30	99.9	4.66	4.66

[a]Dixon test outliers were excluded from this table.
[b]Expected recovery for each compound at each concentration was 90–110%.

of standards, construct linear standard curve for each compound based on formula $y = mx + b$, where x is peak area or height, y is concentration (ppm), m is slope, and b is y-intercept. The correlation coefficient (r) should be ≥ 0.9980. Calculate recovery of spiked sample (included with every set), and correct sample results for recovery.

Extraction of Benzoic Acid, Sorbic Acid, and Parabens for GC/MS Confirmation

Transfer remaining filtrate (see *Sample Extraction*) to 125 mL separatory funnel. Acidify with dilute HCl (1 + 1) to ca pH 3.0. Remainder of steps should be carried out in fume hood. Add 20 mL benzene, shake 1 min, and allow phases to separate by letting stand 10 min. Discard lower aqueous layer and filter benzene layer through glass funnel containing ca 5 g of anhydrous Na_2SO_4 into 25 mL test tube. Evaporate extract to dryness on evaporator at 60°C under nitrogen stream. Derivatize residue for GC/MS confirmation following method of Sun (12). Inject residue in acetone with constant time delay (TD), using *N, O*-bis(trimethylsilyl)-acetamide (BSA), into gas chromatograph equipped with either OV-3 or OV-7 liquid phase column for retention times of the molecular ions $(M)^+$ and other important characteristic ions, e.g., $(M\text{-}CH_3)^+$, $(M\text{-}CH_3CO_2)^+$, $(M\text{-}OSiMe_3)^+$, $(M\text{-}COOSiMe_3)^+$. Also measure accurate masses of full mass spectra by comparison with reference standards.

Results and Discussion

The minimum concentrations of benzoic acid, sorbic acid, and parabens required to inhibit the growth of spoilage microorganisms including toxin-producing organisms vary from organism to organism. These effects have been studied extensively (8–11). The lowest inhibitory levels were found to be 400 ppm benzoic acid, 50 ppm sorbic acid, 160 ppm methyl paraben, 80 ppm ethyl paraben, 40 ppm propyl paraben, and 20 ppm butyl paraben. In this method, 200 ppm benzoic acid, 8 ppm sorbic acid, and 20 ppm of each paraben were selected to obtain comparable peak heights for all compounds.

The separation mode used in this method was ion suppression reverse phase. In choosing the extraction solvents, 3 objectives were considered: (a) solvent should be inexpensive and nontoxic with no unpleasant odor; (b) solvent should eliminate as much soluble protein as possible from the final extract, because accumulated proteins will eventually contaminate the C18 column and cause loss of efficiency; and (c) solvent should extract free benzoic acid and sorbic acid, as well as their salts. Solubilities of sodium benzoate are 55.5% in water and 1.3% in alcohol. Solubilities of potassium sorbate are 58.2% in water and 6.5% in alcohol. All the above requirements were satisfied with 70% aqueous ethanol as the extraction solvent. In addition, an extraction time of 10 min was selected for this method.

Gradient elution was used for several reasons: to lessen the retention time of the compounds, to reduce total analysis time, to increase overall resolution per unit time, to sharpen peak shape, and to increase sensitivity. LC analysis was performed in approximately 25 min with baseline separation of all 6 compounds. Figure 1 shows chromatograms of benzoic acid, sorbic acid, and methyl, ethyl, propyl and butyl parabens at the highest and lowest levels of interest. Figure 2 shows chromatograms of official samples collected by USDA inspectors and submitted for analysis. The samples were found to be adulterated with sorbic acid and benzoic acid. Interferences were observed in the region of ethyl, propyl, and butyl parabens (9–13 min) only when the sample contained soy products, and in the butyl paraben region when the sample contained antioxidants (propyl gallate, BHA, and BHT).

Recovery data were based on meat fortified with each preservative at 5 different levels. The ranges of concentrations used in this study were 200–1000 ppm for benzoic acid, 8–40 ppm for sorbic acid, and 20–100 ppm for each paraben. The average recovery (after discarding Dixon Test outliers) of benzoic acid, sorbic acid, and parabens at all levels was greater than 95% with a coefficient of variation less than 5%, as shown in Table 1.

Because LC methods are not specific, we felt that our results should be confirmed by another technique. The extracts after LC analysis were further purified and derivatized for GC/MS confirmation. GC/MS analyses confirmed the presence of all 6 preservatives. The compounds were identified by relative retention times and relative intensities of characteristic fragment ions, as described previously by Sun (12) for these 6 preservatives.

The present method is expedient (total analysis time is about 40 min), relatively inexpensive, and sensitive. It is reliable for routine, simultaneous determinations of benzoic acid, sorbic acid, and parabens in meat and seasonings, and could be applicable to a variety of other products.

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FOOD ADDITIVES

Liquid Chromatographic Determination of Barbaloin (Aloin) in Foods

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A simple and rapid liquid chromatographic method is described for the determination of barbaloin (aloin, 10-D-glucopyranosyl-1,8-dihydroxy-3-(hydroxymethyl)-9(10*H*)-anthracenone) in foods. Barbaloin is extracted with water from foods containing aloe and the extract is cleaned up on a disposable cartridge by using methanol–water (55 + 45) as eluant. The eluted barbaloin is separated by liquid chromatography on a YMC A-302 column with methanol–water (50 + 50) mobile phase, and detected at 293 nm. Recoveries of barbaloin added to foods at the levels of 0.05 and 0.50 mg/g were 94.4–100%. Assay results for commercial food samples indicated that the present method is applicable to a variety of foods supplemented with aloe.

Aloe arborescens Mill. contains various physiologically active compounds, and has been traditionally used as an aliment and domestic medicine for the treatment of gastrointestinal disturbances, burns, and insect bites. Recently, in Japan, aloe has been added to foods such as candy, jelly, juice, and grains. Although aloe has been widely used in the food, pharmaceutical, and cosmetic industry, its use in foods has not been regulated. Therefore, we needed a method for the determination of the major component barbaloin (aloin) (1, 2) in foods.

Techniques to separate barbaloin and isobarbaloin in aloe extract by liquid chromatography (LC) have been reported (3–5), but determination of barbaloin in foods by LC has not. The proposed method involves extraction, cleanup, and LC separation on a YMC A-302 column with methanol–water (50 + 50) as the mobile phase.

METHOD

Reagents

Use analytical reagent grade chemicals and deionized water unless otherwise specified.

(a) *Solvents.*—Methanol, *n*-butanol, and chloroform (Wako Pure Chemical Industry, Ltd, Osaka, Japan).

(b) *Disposable cartridge.*—Sep-Pak C_{18}, Waters Associates, Inc., Milford, MA 01757. Before use, prepare cartridge by flushing with 20 mL methanol, followed with 10 mL water.

(c) *Mobile phase for LC.*—Combine 500 mL water and 500 mL methanol in 1 L volumetric flask. Mix thoroughly and degas in ultrasonic bath 1 min.

(d) *Solvent for cartridge.*—55% (v/v) methanol in water.

(e) *Standard barbaloin.*—Prepare as follows: Extract barbaloin from 100 g condensed *Aloe arborescens* Mill. (kindly supplied from Aloe Foods Co., Fujieda, Japan) with 100 mL *n*-butanol. Use Sephadex LH-20 column, 1.5 id × 60 cm (Pharmacia Fine Chemicals Co., Ltd), and Wakogel C-200 column, 1.5 id × 12 cm (Wako Pure Chemical Industry, Ltd), for cleanup of extracted portions. Inject aliquot into preparative LC column, YMC A-324 (Shimakyu Chemicals Co., Ltd, Osaka, Japan) for further cleanup. Evaporate eluate to dryness in rotary evaporator. Crystallize twice from methanol and identify by comparison with authentic barbaloin (kindly supplied from Aloe Pharmaceutical Co., Shizuoka, Japan).

(f) *Standard barbaloin solution.*—*(1) Stock solution.*—Weigh exactly 10.0 mg barbaloin and dissolve in 100 mL water. *(2) Working solution.*—2, 5, 10, 15, and 20 μg barbaloin/mL. Dilute 2, 5, 10, 15, and 20 mL stock solution to 100 mL with mobile phase, just before use.

Apparatus

(a) *Liquid chromatograph.*—Shimadzu Model LC-5A (Shimadzu Seisakusho Ltd, Kyoto, Japan), or equivalent. Operating conditions: flow rate, 0.9 mL/min; column temperature, ambient; chart speed, 5 mm/min.

(b) *Detector.*—Shimadzu Model SPD-2A UV detector (Shimadzu Seisakusho Ltd) operated at 293 nm with attenuation set at 0.01 AUFS, or equivalent.

(c) *Injector.*—Rheodyne Model 7125 sample injection valve fitted with 20 μL sample loop (Rheodyne, Inc., Berkeley, CA 94710). Inject 10 μL onto column.

(d) *Recorder.*—Hitachi Model 056 (Hitachi Ltd, Tokyo, Japan), or equivalent.

(e) *Chromatographic column.*—YMC A-302 column, 4.6 × 150 mm, 5 μm (Shimakyu Chemicals Co., Ltd, Osaka, Japan), or equivalent.

(f) *Centrifuge.*—Hitachi Centrifuge Model SCR20BA (Hitachi Koki Co., Ltd, Tokyo, Japan), or equivalent.

(g) *Ultrasonic bath.*—Model UT-6 (Kokusan Electric Co., Ltd, Tokyo, Japan), or equivalent.

(h) *Rotary evaporator.*—Model N-2 (Tokyo Rikakikai Co., Ltd, Tokyo, Japan), or equivalent; water bath temperature at 35°C.

Preparation of Sample

(a) *Semisolids or solids.*—Weigh 1.0 g well mixed solid samples into 25.0 mL volumetric flask. Add 10 mL water and place in ultrasonic bath 5 min. Dilute to 25 mL with water. Centrifuge mixture at 5000 rpm (3000 × *g*) 5 min. After centrifugation, pass 10 mL aliquot of clear supernate through Sep-Pak C_{18} cartridge and rinse cartridge with 20 mL water. Elute barbaloin with 10 mL methanol–water (55 + 45). Dilute an aliquot of eluate with mobile phase for barbaloin concentration of 2–20 μg/mL before LC analysis.

Table 1. Recoveries of barbaloin from candy and jelly by proposed method[a]

Sample	Added barbaloin, mg/g	Recovery, %[b]
Candy	0.05	96.3 ± 1.5
	0.50	100.0 ± 0.8
Jelly	0.05	94.4 ± 1.3
	0.50	99.0 ± 0.8

[a]Lower detection limit for barbaloin, 20 ng.
[b]Mean ± standard deviation, n = 5.

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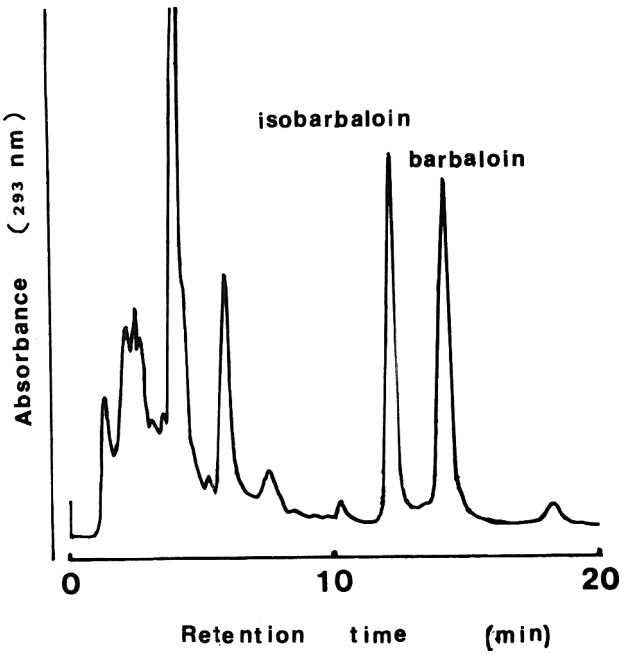

Figure 1. Typical chromatogram of candy extract.

Table 2. Analysis of commercial foods containing aloe by proposed method

Sample		Concn, mg/g	Content, mg/piece
Candy	1	0.11	0.40
	2	0.12	0.39
	3	0.12	0.42
	4	0.08	0.25
	5	0.09	0.33
	6	0.11	0.34
	7	0.08	0.27
	8	0.09	0.46
	9	0.19	0.80
	10	0.04	0.10
	11	0.12	0.35
	12	0.10	0.32
	13	0.09	0.49
	14	0.07	0.22
	15	0.16	0.69
	16	0.09	0.25
	17	0.10	0.31
Jelly		0.06	0.49
Juice	1	<0.01	—
	2	<0.01	—
	3	<0.01	—
Grain	1	1.41	0.14
	2	6.98	0.75
Dried leaf	1	4.05	—
	2	4.43	—

(b) *Liquids.*—Weigh 20 g and pass through Sep-Pak C_{18} cartridge. Proceed as for solid sample.

Determination

Equilibrate column with mobile phase set at 0.9 mL/min. Inject 10 μL sample solution and 5 working solutions, and measure peak heights. Calculate amount of barbaloin by using calibration plot. Dilute sample solution, if the sample has high concentration of barbaloin.

Results and Discussion

Pure barbaloin (0.12 g) for standard was obtained from a condensed solution (100 g) of *Aloe arborescens* Mill. This barbaloin was compared with authentic barbaloin by mixed melting point, LC analysis, and IR, NMR, and MS spectrometry, and was used in subsequent experiments.

The use of a reverse phase column to separate barbaloin from isobarbaloin in aloe extract has been reported (3–5). For chromatographic separation of barbaloin in foods containing aloe, we used the YMC A-302 column. Baseline separation of barbaloin and isobarbaloin was obtained with a mobile phase of methanol–water (50 + 50) at a flow rate of 0.9 mL/min. The resolution (R_s) between barbaloin and isobarbaloin under these conditions was 2.6 with a capacity factor (k') of 7.6 for barbaloin. A study of the effect of pH on chromatographic behavior of barbaloin showed constant values for peak height and capacity factor for phosphate buffer pH 3–8.

The cartridge was used to remove co-extractives that might irreversibly adsorb on the analytical column and to reduce analysis time by removing some late-eluting substances. Preliminary experiments showed that more than 97% barbaloin was retained on the cartridge, when 100 mL sample solution was eluted. Interferences were removed with 20 mL water, and barbaloin was quantitatively eluted with 10 mL methanol–water (55 + 45). After a 10 mL methanol wash, the cartridge was ready for reuse.

UV detector response at 293 nm was linear for 20–200 ng barbaloin injected in 10 μL eluant (r = 0.9998); 20 ng was the lower detection limit. A typical chromatogram obtained from a candy extract is shown in Figure 1. There were no interfering extraneous peaks after cleanup, and similar chromatograms were obtained from jelly, grains, and dried aloe leaves.

Table 1 shows the recoveries from candy and jelly fortified at 0.05 and 0.50 mg barbaloin/g. Recoveries of barbaloin were reproducible and estimated to be 94.4–100%. The amounts of barbaloin in several commercial foods and dried aloe leaves were analyzed by the present method. The results are given in Table 2.

This method presents a simple, rapid, and accurate procedure for determining barbaloin in foods containing aloe.

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PLANTS

Electrothermal Atomic Absorption Spectroscopic Determination of Chromium in Plant Tissues: Interlaboratory Study

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An electrothermal atomic absorption (ETAA) method for determining chromium in plant tissues was collaboratively studied by 6 laboratories. The sample is digested in nitric, perchloric, and sulfuric acids. Chromium is reduced by sodium sulfite and then separated from the digest matrix by coprecipitation with ferric hydroxide. Iron is removed by dissolution and extraction. The remaining silica is dissolved with hydrofluoric acid, and chromium is then determined. After unusually high and low data sets and remaining outliers were rejected, the CV for samples containing 3 μg chromium was 2% but increased to 84% as the chromium concentration in tissue decreased to 0.02 μg/g. The method is recommended for further study.

There is no official method for determining chromium in plants. Some published methods work well when chromium levels exceed several micrograms per gram of tissue. Others may work well in the submicrogram range but require specialized equipment that would not be available to many laboratories. Sample contamination and erroneous signal due to matrix have been critical problems to overcome. The method of Cary and Rutzke (1) should be within the capability of many laboratories, and is free of matrix interference.

Collaborative Study

The 7 collaborators were supplied with 11 samples. Sample 11, a grass, was to be used in practice runs to familiarize analysts with the procedure. Each collaborator was asked to report any deviation from the method supplied, to make only 1 determination per sample, to dry a separate subsample of each sample at 80°C overnight for moisture determination, and to report all results. The report form also asked for the following: final dilution, injected volume, 3 replicate values for each sample, 2 reagent blanks, 1 standard blank, and 4 standards.

Samples

The study samples were paired as to type of biological material. Each pair was a subsample of a large stock sample that had not been recently mixed. After the stock was sampled for 2 portions of 500 g each, wheat seed was washed with deionized water and dried 1 week at 70°C. Wheat flour was used as received. Grass and alfalfa stock samples had been ground to pass a 1.5 mm stainless screen. Two 500 g subsamples of each material were passed through a 0.5 mm Dacron sieve. The tree leaf sample appeared to be uniform and no sieving was attempted. These materials were dried 8 h at 70°C. Then each material was mixed, subsampled, and packaged in a random order. A sample weight range to be used for analysis was listed on each container.

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Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be available.

METHOD

The method follows closely the method of Cary and Rutzke (1).

Principle

The sample is digested in a HNO_3–$HClO_4$–H_2SO_4 mixture. Chromium is reduced with Na_2SO_3 and separated from the digest matrix by coprecipitation with iron. Iron is then extracted into 4-methyl-2-pentanone from 9.6M HCl and discarded. Chromium and solids remain in the aqueous portion. Silica is then dissolved with HF. Chromium is determined by electrothermal atomic absorption spectroscopy (ETAA).

Apparatus

(a) *Atomic absorption spectrometer.*—With digital readout and graphite furnace accessory.

(b) *Pyrolytic coated graphite tube.*

(c) *Pipet.*—With disposable tip, up to 3 mL capacity.

(d) *Digestion tubes.*—Pyrex or Kimax, approximately 20 × 175 mm.

(e) *Pyrex boiling beads.*

(f) *Polypropylene centrifuge tubes.*—Calibrated to 15 mL.

Reagents

Use distilled or deionized water and AR grade or better reagents.

(a) *Argon.*

(b) *Sulfuric acid.*—Ultrex, or equivalent.

(c) *Nitric acid.*—Redistilled in glass. Distill ca 90% of total.

(d) *Perchloric acid.*—70% double distilled from Vicor (G. Frederic Smith Chemical Co., No. 230, or equivalent).

(e) *Sodium sulfite.*—1% (w/v). Prepare fresh each day.

(f) *4-Methyl-2-pentanone (MIBK).*—Eastman No. 416, or equivalent.

(g) *Chloroform.*

(h) *Methyl red.*—0.02 g in 60 mL ethanol. Dilute to 100 mL with water.

(i) *Chromium standard solution.*—Dissolve 0.3736 g K_2CrO_4 in water and dilute to 1 L. Dilute this solution to working concentration of 0.1 μg Cr/mL. Commercial atomic absorption standards are also satisfactory solutions from which to make appropriate dilutions.

(j) *Iron solution.*—Dissolve 10 g $FeCl_3 \cdot 6H_2O$ in 10 mL HCl. Add 0.5 mL 1% Na_2SO_3 and mix. Extract 3 times with 20 mL MIBK, combining organic phases after each extraction. Wash combined organic phase with 3 mL 6M HCl. Extract organic phase twice with 50 mL portions of water. Save aqueous phase and dilute to 500 mL with water.

Glassware

Clean digestion tubes and glass boiling beads by heating beyond fuming with H_2SO_4 and $HClO_4$, 100 + 1 (v/v), for 15 min the first time they are used. Soak polyethylene materials for 24 h in 10% HNO_3–H_2SO_4. Rinse everything with 10%

HCl followed by water. Use for chromium determinations only.

Standards

Carry 2 reagent blanks through entire procedure. If extra HNO_3 is needed during digestion, one of these blanks should also receive an equivalent portion of HNO_3.

Standards for a 5-point standard curve are carried through method except for digestion. These standards consist of 0, 0.1, 0.3, 0.5, and 0.7 μg Cr added and include all reagents except HNO_3. Identify reagent blanks as digested reagent blank, digested reagent blank plus extra HNO_3, and undigested reagent blank.

Determination

Weigh sample to nearest mg and transfer it to digestion tube containing 2 glass beads. Add 0.5 mL H_2SO_4, 5 mL HNO_3, and 0.5 mL $HClO_4$. Add $HClO_4$ with or after HNO_3. Heat slowly until brown fumes begin to clear. Then heat can be increased. Continue digestion until all HNO_3 has distilled. *Note:* If digest turns brown or black at this time, immediately add 0.25 mL HNO_3 and continue heating. Repeat if necessary. If extra HNO_3 was added, add similar amount of HNO_3 to one digested blank. After $HClO_4$ fumes appear and digest remains clear, continue boiling digestate for at least 10 min. Cover mouth of digestion tube with inverted hollow stopper and let contents cool to about room temperature.

Transfer contents into 15 mL polypropylene centrifuge tube with three 1 mL water rinses. Include boiling beads. Add 0.05 mL fresh 1% Na_2SO_3 and mix. Add 1.0 mL Fe solution and mix. Add 2.5 mL NH_4OH and mix. Age precipitate at least 5 min. Centrifuge 2 min in clinical centrifuge at full speed, then decant carefully. Resuspend precipitate in ca 6 mL water. Centrifuge and decant. Add 1 mL 9.6M HCl and 1 drop of methyl red (for a phase marker), and let precipitate dissolve. Extract twice, first with 5 mL, then with 3 mL portions of MIBK. Discard MIBK phase. Carry out extraction by using pipet with disposable tip. Draw organic phase and small portion of aqueous (red) phase into pipet tip. Then expel red aqueous portion back into centrifuge tube. Add 1 mL $CHCl_3$ and mix; then add 0.1 mL HF. Do not mix. Let solids at $CHCl_3$–water interface dissolve. If they do not dissolve in 2 h, continue with next step. Dilute aqueous phase to 8 mL with water. Mix well, cap tube, and centrifuge for 1 min. Inject 10 μL aliquots of this aqueous phase into pyrolitic HGA tube. Use the following spectrometer settings: wavelength, 357.9 nm; drying, 110°C for 20 s; charring, 1000°C for 10 s; atomization, 2700°C for 9 s; mode, peak height; signal, concentration; gas flow, normal; gas flow rate, 40 through Brooks tube size R-2-15A(CO); background correction, none.

Calculations

A second order polynomial equation in the form $Y = a + bA + cA^2$ where a = intercept, b = slope, c = curvature, A = absorbance reading corrected for the proper reagent blank, and Y = μg Cr, is generated using the Gaussian elimination technique for solving the polynomial regression (2). Then

$$\text{Cr, } \mu\text{g/g} = (1/\text{sample wt (g)}) \times Y$$

Discussion

Comments of Collaborators

Collaborator 1: Sample preparation—drying before weighing, if necessary, should be noted. Rinsing of digestion flask—with the amount of silica present after digestion an increase in wash volumes seems to be necessary for most samples. Addition of iron solution—it would clarify the method to state why it is necessary to add iron. Extraction—it should be stated that the MIBK is added into the 15 mL centrifuge tube. Determination by AA—with the conditions noted (atomization at 2600°) carry over was very prominent after each atomization and a burn off cycle had to be implemented. General comment—a more detailed description of the method would be helpful.

Collaborator 2: In some samples, a small amount of solid material remained after treatment with HF for 2 h. It was noted that in 3 samples, stirring before taking the 5 μL sample for analysis sometimes increased the reading.

Collaborator 5: The greatest advantage of the proposed method is the apparent freedom of matrix interferences for older instruments which do not have visual background correction capability and are not equipped with a graphite furnace having an optical temperature control and a maximum power heating system. Glassware cleaning—from considerable personal experience, we suggest the use of 10% (v/v) H_2SO_4. Heat for a short time and soak overnight. Atomic absorption spectrometer—in the newer instruments, the atomization step can be shortened to 3 s and the temperature can be decreased to 2600 or 2650°C with equally good results

Table 1. Collaborative results for Cr (μg/g) (closely matched pairs)

	Wheat		Alfalfa		Grass		Wheat flour[a]		Tree leaf		Total
Coll.	1	5	2	4	3	7	6	9	8	10	rank
1	0.1045	ND[b]	0.6310	0.5189	0.5385	0.4210	0.1595	0.0706	4.012[c]	2.421[c]	33
2[d]	0.1389	0.1295	0.8517	0.8682	0.6354	0.8349	0.4904	0.4269	3.051	3.365	61
3	0.0233	0.0193	0.6586	0.7782	0.4589	0.3804	0.2156	0.2426	3.364	3.080	38
4	0.0310	0.0057	1.3528	0.6852	0.4062	0.3974	0.2224	0.2035	3.460	2.971	41
5	0.0588	0.0431	0.9268	0.8837	0.5792	0.5468	0.2523	0.2669	3.332	3.005	54
6[d]	ND	ND	0.4220	0.4095	0.2594	0.2486	0.1854	0.1820	2.401	2.255	15
7	0.0628	ND	1.2345	0.8518	0.5212	0.5412	0.0810	0.1325	2.765[c]	3.619[c]	39
Av.[e]	0.0599	0.0282	0.8682	0.7136	0.4855	0.4815	0.2295	0.2179	3.198	2.959	
SD[e]	0.048	0.047	0.335	0.186	0.125	0.186	0.061	0.113	0.519	0.483	
CV,%[e]	80.13	166.66	38.59	26.06	25.75	38.63	26.58	51.86	16.23	16.32	
Av.[f]	0.0561	0.0227	0.9607	0.7436	0.5008	0.4574	0.1862	0.1832	3.385	3.019	
SD[f]	0.032	0.019	0.328	0.147	0.068	0.080	0.068	0.081	0.067	0.056	
CV,%[f]	57.04	83.70	34.14	19.77	13.58	17.49	36.52	44.21	1.98	1.85	

[a]National Bureau of Standards sample No. 1567.
[b]ND = none detected.
[c]Rejected as outlier, using Dixon test at 95% confidence limit.
[d]Data sets rejected for unusually high (2) and unusually low (6) scores, Youden, 5% 2-tail limits are 23 and 57.
[e]All data used.
[f]Rejected data not used.

Table 2. Instruments and programs used

Coll.	Instrument, spectrometer/furnace	Program Step	Ramp	Hold, s	Temp.,°C
1	Varian 775 Perkin-Elmer HGA400	1	1	10	250
		2	2	12	1100
		3	0	5	2600
		4	1	20	40
		5	1	5	2700
2	Varian AA6 CRA 90	used manufacturer's recommendations: ramp rate of 400°C/s and 2 s hold at 2300°C			
3	Perkin-Elmer 603 HGA 2100	1		20	110
		2		10	1000
		3		9	2700
4	Perkin-Elmer 603 HGA 2100	1		20	110
		2		10	1000
		3		9	2700
5	Perkin-Elmer 5000 HGA 500	1	5	20	110
		2	10	10	1000
		3	0	3	2700
6[a]	Perkin-Elmer 5000 HGA 500	1	40	20	110
		2	50	10	1000
		3	0	9	2600
		4	1	3	2700
7[b]	Perkin-Elmer 5000 HGA 500	1	5	20	100
		2	5	10	200
		3	5	10	1000
		4	1	9	2700

[a]Used tantalum-coated tubes.
[b]Used nitrogen gas.

and an appreciably extended tube lifetime. The drawbacks of the proposed method are a slow sample throughput and inadequate sensitivity for many plant samples, such as grains.

Collaborator 6: No problems were encountered with the proposed method.

Collaborator 7: Sample digestion was carried out with HNO_3 and H_2SO_4 acids until the solution was clear, then $HClO_4$ was added. For some samples five 0.25 mL portions of extra HNO_3 were needed so all samples received 7 mL of HNO_3. Also nitrogen gas was used instead of argon. Suprapur nitric acid from Merck was used without further purification.

Results

The data reported by each collaborator were converted to μg Cr/g sample based on the undried weight of sample taken for analysis (Table 1). Percent moisture of each sample was determined by each collaborator but the variation was large. Because all samples had been predried at 70°C immediately before sealing in polyethylene vials for distribution, no moisture correction was used in making final calculations. Statistical analyses were carried out using procedures described by Youden and Steiner (3) for a set of closely matched sample pairs. Laboratories giving outlying data sets were identified using Youden's ranking test (3). Individual outliers from the remaining data sets were rejected using Dixon's test (3). Results reported by Collaborator 2 were most often higher than those reported by the other collaborators. Table 2 lists the various instruments and programs used by the collaborators. Undoubtedly, the furnace and graphite tube used by Collaborator 2 was much different than those used by the other collaborators; however, a reason for consistently high results is not apparent. Results reported by Collaborator 6 were consistently lower than those from other collaborators. This collaborator used a Ta-treated graphite tube and atomized at 2600°C rather than at the requested 2700°C temperature. The Ta-treated graphite tube may not be appropriate for Cr analysis. On the other hand, these data were very consistent between similar pairs (Table 1). Absorbance values for the various blanks are given in Table 3. An average reagent blank value was used when more than one value was supplied.

Data from Collaborator 1 were retained even though the standard deviation, ± 0.072, of the mean absorbance value (0.415) for the digested blanks was an order of magnitude greater than the absorbance for the undigested blank (Table 3). Collaborators 3 and 4 were in the same laboratory; Collaborator 3 was experienced with the method. The first of these 2 data sets was obtained 5 months after the second. It appears that a reagent blank of about 0.030 absorbance unit should be obtained using the stated method before sample analysis is attempted. Obviously, a lower reagent blank absorbance is obtainable and very desirable.

Pair statistics for the 5 different plant tissues are given in Table 4. Analysis of all values shows significant systematic error in 3 of 5 sets. When only those values that were not

Table 3. Absorbance values of reagent and standard blanks

Coll.	Absorbance[a] Reagent	Reagent plus extra HNO_3	Standard	Volume Final dilution, mL	Injected, μL
1	0.416	none	0.005	10	10
	0.438				
	0.504				
	0.303				
2	0.099	0.122 (0.25 mL)	0.084	4	5
3	0.025	none	0.016	8	10
	0.028				
4	0.028	0.035 (0.25 mL)	0.033	8	10
5	0.003	none	0.004	8	10
	0.005				
6	0.043	none	0.000	8	20
	0.037				
7	0.140	0.217 (2.00 mL)	0.033	8	10

[a]Reagent is a blank that was carried through the entire procedure. Reagent plus HNO_3 is same as above but reflects extra HNO_3 that was used for digestion of some or all samples. Standard is a blank that had no HNO_3 and was not digested.

Table 4. Statistical analysis of sample pairs

Material	Pair	S_c	S_r	S_b	F	DF	S_R	Means
				Selected Values[a]				
Wheat seed	1,5	0.0219	0.0282	—[b]	0.60	4	0.0282	0.0394
Alfalfa	2,4	0.2845	0.2199	0.1277	1.67	4	0.2543	0.8522
Grass	3,7	0.0982	0.0388	0.0637	6.4**	4	0.0746	0.4791
Wheat flour	6,9	0.0983	0.0369	0.0643	7.08**	4	0.0741	0.1847
Tree leaf	8,10	0.0413	0.1976	—[b]	0.04	2	0.1976	3.202
				All Values				
Wheat seed	1,5	0.0619	0.0271	0.0394	5.22**	6	0.0479	0.0440
Alfalfa	2,4	0.3297	0.1945	0.1882	2.87	6	0.2706	0.7909
Grass	3,7	0.2090	0.0711	0.1389	8.64***	6	0.1560	0.4835
Wheat flour	6,9	0.1670	0.0352	0.1154	22.14***	6	0.1206	0.2237
Tree leaf	8,10	0.4687	0.5329	—[b]	0.77	6	0.5329	3.0785

[a]Those values that were not rejected on the basis of Dixon's or Youden's tests.
[b]Square root of negative number.
**5% significance.
***1% significance.

rejected are analyzed, there is evidence of systematic error for the grass and wheat flour sets based on the F-test. S_d is smaller than S_r for the wheat seed and tree leaf sets, which indicates no evidence for systematic error. Because the number of degrees of freedom is low, this test is not very sensitive. Reproducibility, S_R, where $S_R = (S_r^2 + S_b^2)^{1/2}$ is shown in the second to last column of Table 4. This is a measure of variation arising from different laboratories.

Examination of CV for those analyses not rejected (Table 1) shows that the precision for the tree leaf sample was excellent, about 2%, but decreased rapidly as the Cr concentration decreased, reaching almost 84% for sample 5. The precision attained for the tree leaf sample is excellent and for the grass sample is probably acceptable based on the complexity of this method. The low and variable precision for the other sample pairs could be due to several unidentifiable factors. Nonhomogenicity of sample is possible but would seem to be less likely for the wheat pair and the NBS Wheat Flour pair than for the alfalfa sample pair. For the wheat pair, a small error in the reagent blank value will have a relatively large effect on the precision. In theory, the Cr concentration in the reagents should be constant and not affect the precision. However, other sources of Cr contamination during analysis such as airborne dust contribute to loss of precision. Better housekeeping and larger samples are reasonable ways to reduce this problem if the sample is homogenous with respect to Cr distribution.

Because there is no official method for Cr determination in biological materials, the Cr concentration of these materials is not absolutely known. However, analysis of NBS standard samples 1569, 1570, 1571, and 1575 by this method agreed well with certified and suggested values (1).

Recommendations

This method overcomes matrix-related problems and offers an easy way to concentrate Cr; however, the precision needs to be increased on samples containing nanogram concentrations of Cr. Even though the results are highly encouraging, it is recommended that the method be subjected to further study.

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Elemental Analysis of Plant Tissue by Plasma Emission Spectroscopy: Collaborative Study

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Fourteen laboratories participated in a collaborative study of 6 homogeneous plant tissue samples to determine the elements P, K, Ca, Mg, Mn, Fe, Al, B, Cu, Zn, and Na by plasma emission spectroscopy. Samples were dry ashed using AOAC method 3.007(a) (13th Ed.). An NBS citrus leaf standard was prepared and a portion of the resulting solution was sent to each collaborator to evaluate sample preparation errors independent of instrument error. Coefficients of variation were better than those obtained in an earlier collaborative study on multielement analysis by spark emission spectroscopy. The plasma emission method has been adopted official first action for determination of P, K, Ca, Mg, Mn, B, Cu, and Zn in plant tissues.

The evolution of analytical techniques for the analysis of macro and micro elements in plant tissue began with a method using arc excitation emission spectroscopy, which was adopted official first action in 1953 (1). The method involved photographic plates and comparator readings against standards. The advent of atomic absorption put relatively low cost, precise equipment within the reach of most analytical laboratories. Microprocessor technology has enabled the development of direct reading spark emission spectrographs for multielement analysis of plant tissue. Atomic absorption (2) and spark emission spectroscopic (3) methods were adopted in 1975.

Recently, plasma emission spectroscopy has come to the forefront as an analytical tool for multielement determination in plant tissue. A spark emission direct reader is somewhat limited in the type of samples (solid or liquid) and the concentration of various elements that can be handled. The plasma system is much more adaptable to matrix changes and sample type. Its dynamic linear range enables analysis of low and high concentrations of the same element. A collaborative study was carried out to evaluate use of plasma emission spectroscopy for determination of macro and micro elements in plant tissue.

Collaborative Study

Fourteen laboratories participated in the collaborative study. Six plant tissue samples and one liquid extract were sent to each collaborator. The plant tissues selected for this study included 2 corn leaf samples and one sample each of corn stalk, peach leaves, pecan leaves, and bermuda grass. The pair selections (Table 1) were chosen such that the values were fairly close in amounts for the elements P, K, Ca, and Mg (those that were reported on a percentage basis). The sample materials were to be dry ashed by the official method, **3.007(a)** (4), and analyzed by plasma emission spectroscopy for P, K, Ca, Mg, Mn, Fe, Al, B, Cu, Zn, Na, and any other elements normally included in their suite of analysis. Collaborators were instructed to make appropriate dilutions such that the concentrations measured would be bracketed by their standards. The liquid extract was a 10 g sample of NBS Citrus Leaves 1572, digested according to **3.007(a)** and diluted to 500 mL; each collaborator was given approximately 30 mL of the extract.

System performance criteria were included in the instructions to each collaborator. Collaborators were asked to analyze the samples immediately after standardizing their instrument and to analyze their standards immediately after completion of the sample analysis. If a drift of more than 5% occurred for any element, they were requested to restandardize the instrument and run the analysis again. Included in the system performance criteria was a list of suggested standard concentrations which were optional for the collaborators to use in standardizing their instruments.

Evaluation of Data

The collaborative study data (Table 2) were subjected to Youden's laboratory ranking test (5) and Dixon's outlier test (6) to identify and delete spurious results. The remaining data were then used to calculate the average elemental value, standard deviation, and coefficient of variation for each sample (Table 3). Youden's technique of matched pairs (7) was used to examine the data for information on precision (Table 4) by determining the standard deviations for the estimate of random error (S_r), the estimate of interlaboratory bias (S_b), and the estimate of overall precision (S_d).

The *t*-test (8) was applied to the NBS citrus leaves liquid extract data to determine any inherent systematic error in the method. A comparison was made between the collaborative results and the NBS values for the citrus leaves (Table 5).

Metals and Other Elements in Plants

Inductively Coupled Plasma Spectroscopic Method

First Action

(Applicable to B, Ca, Cu, K, Mg, Mn, P, and Zn)

3.A01 ***Principle***

Sample is dry-ashed, treated with HNO_3, and dissolved in HCl; elements are detd by ICP emission spectroscopy.

Table 1. Sample identification and pair selection of collaborative study samples

Sample	Material	Pair
1	Corn leaves	1
2	Pecan leaves	2
3	Corn stalk	3
4	Peach leaves	2
5	Corn leaves	1
6	Bermuda grass	3

Submitted for publication September 20, 1984.
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.* (1985) **68**, 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.

Table 2. Collaborative results for multielement analysis of plant tissues[a]

Coll.	1	2	3	4	5	6	LE
				Phosphorus, %			
1	0.28	0.12	0.20	0.17	0.27	0.15	0.14
2	0.27	0.12	0.16	0.17	0.26	0.14	0.11
3	0.24	0.10	0.14	0.15	0.13[b]	0.13	0.11
4	0.31	0.13	0.18	0.19	0.29	0.15	0.13
5	0.30	0.14	0.18	0.21	0.30	0.16	0.15
6	0.29	0.12	0.16	0.17	0.26	0.14	0.13
7	0.29	0.13	0.17	0.17	0.28	0.16	0.13
8	0.28	0.13	0.16	0.16	0.24	0.13	0.13
9	0.27	0.09	0.14	0.11[b]	0.28	0.14	0.14
10	0.29	0.13	0.17	0.18	0.27	0.15	0.14
11	0.24	0.11	0.13	0.17	0.27	0.14	0.12
12	0.28	0.15	0.15	0.16	0.26	0.14	0.13
13	0.29	0.12	0.17	0.16	0.27	0.15	0.13
				Potassium, %			
1	2.10	0.71	1.13	2.03	2.02	0.80	1.83
2	2.25	0.93	1.14	2.29	2.22	0.90	1.77
3	1.50	0.49	0.79[b]	1.37[b]	1.24[b]	0.43[b]	1.32[b]
4	2.03	1.23	1.15	2.01	2.08	0.92	2.09
5	2.15	0.83	1.14	2.15	2.10	0.84	1.84
6	2.17	0.67	1.26	2.21	2.54[b]	0.78	1.82
7[c,d]	2.25	0.71	1.13	2.10	2.12	0.85	1.84
8	1.80	1.27	1.21	1.93	1.85	0.80	1.86
9	1.91	0.51	0.91[b]	1.30[b]	2.19	0.74	1.84
10[d]	1.57	0.26	0.55	1.38	1.42	0.33	1.82
12	1.96	1.00	1.12	1.91	1.94	0.96	1.63
13	2.34	0.77	1.19	2.12	2.20	0.91	1.88
				Calcium, %			
1	0.56	2.00	0.22	1.10	0.65	0.28	3.36
2	0.53	1.86	0.20	1.14	0.64	0.28	2.68
3	0.52	1.85	0.18	1.09	0.64	0.27	3.35
4	0.60	1.99	0.22	1.15	0.70	0.29	3.10
5	0.56	1.99	0.22	1.13	0.67	0.28	3.12
6	0.57	1.85	0.22	1.11	0.67	0.28	3.31
7	0.57	1.99	0.21	1.12	0.67	0.30	3.30
8	0.55	1.26[b]	0.19	1.03	0.63	0.27	2.67
9	0.49	1.42[b]	0.18	0.73[b]	0.66	0.28	3.18
10	0.59	1.96	0.23	1.16	0.67	0.27	3.16
12	0.59	2.22[b]	0.21	1.05	0.65	0.26	2.90
13	0.60	2.09	0.23	1.13	0.69	0.31	3.33
				Magnesium, %			
1	0.38	0.24	0.25	0.40	0.12	0.14	0.59
2	0.41	0.27	0.27	0.43	0.16	0.18	0.48
3	0.38	0.24	0.23	0.38	0.13	0.14	0.45
4	0.46	0.29	0.31	0.46	0.19	0.20	0.56
5	0.40	0.29	0.28	0.46	0.16	0.17	0.59
6	0.34	0.20	0.22	0.35	0.12	0.13	0.47
7	0.42	0.27	0.27	0.42	0.16	0.18	0.59
8	0.41	0.28	0.27	0.41	0.15	0.16	0.57
9	0.38	0.20	0.24	0.26[b]	0.16	0.16	0.57
10	0.41	0.27	0.28	0.42	0.15	0.16	0.54
12	0.49	0.49[b]	0.38[b]	0.51	0.26[b]	0.29[b]	0.65
13	0.44	0.29	0.29	0.44	0.17	0.18	0.60
				Manganese, ppm			
1	57	612	44	69	142	38	21
2	62	663	49	76	151	42	20
3	58	670	42	66	141	33	16
4	68	665	47	76	161	42	24
5	62	666	49	77	149	38	25
6	66	665	47	75	159	41	23
7	63	571	47	71	155	43	23
8	60	632	41	65	135	35	21
9	56	510[b]	40	46[b]	155	37	23
10	62	675	48	75	147	34	23
12	47	161[b]	47	56	55[b]	20[b]	27
13	61	669	46	71	149	40	22
				Iron, ppm			
1	167	118	977	99	119	29	97
2	176	111	903	106	135	29	95
3	151	101	1010	100	106	43	113
4	233[b]	144	1269	130[b]	146[b]	38	164[b]
5	247[b]	170[b]	1128	188[b]	285	76[b]	120
6	174	117	1008	102	117	28	105
7	185	121	1030	107	136	34	109
8	190	119	781	106	113	36	115
9	164	102	729	68[b]	118	35	188[b]
10	188	121	706	133[b]	252[b]	29	111
12	183	114	866	98	110	29	114
13	190	121	1064	104	152	30	108

Table 2. (continued)

				Aluminum, ppm			
1	199	1094	512	125	86	15	69
2	2·9	1100	458	126	96	20	67
3	187	651	432	162	220[b]	52	61
4	277	1445	623	160	122	35	109
5	237	1139	536	156	99	20	73
6	243	1268	638	155	100	21	81
7	231	1130	527	133	105	22	77
8	236	881	509	156	120	32	79
9	171	767	322	77[b]	76	19	88
10	551[b]	1320	519	852[b]	373[b]	35	75
12	233	1517	482	135	102	20	87
13	249	1181	604	128	99	16	78
				Boron, ppm			
1	3.9	31	2.8	22	2.4	2.4	59
2	4.0	29	3.5	26	3.2	2.2	57
3	6	31	2	21	3	1	63
4	6	41	6	23	6	2	51
5	4 0	36	3.3	24	3.5	1.7	65
6	4	31	4	23	3	1	63
8	4 0	34	4.0	23	2.3	0.50	65
9	6 1	33	5.0	16[b]	4.9	3.4	73
10	6 5	43	5.5	25	5.1	2.3	66
12	5	40	4	24	3	2	61
13	6.3	39	4.9	25	5.2	3.3	66
				Copper, ppm			
1	10	6.8	6.2	4.5	9.7	1.2	13
2	10	6.9	6.0	5.2	9.0	2.0	15
3	15[b]	13	14[b]	9	20[b]	5	12
4	11	7	6	5	10	2	24[b]
5	13	9.8	8.2[b]	7.5	14	3.3	19
6	12	8.0	6	6	12	3	15
7	12	6.6	6.6	4.7	11	1.8	16
8	12	7.7	6.7	5.3	13	3.0	16
9	10	5.8	5.8	2.7	12	0.4	15
10	20[b]	22[b]	16[b]	27[b]	38[b]	12[b]	16
12	12	14	7	7	13	2	14
13	11	5.9	6.0	4.2	11	2.0	14
				Zinc, ppm			
1	29	163	54	15	15	6.0	25
2	32	174	58	19	17	8.2	25
3	28	162	52	16	16	6	22
4	33	193	57	18	17	8	34
5	44	205	79[b]	33[b]	29[b]	16[b]	36
6	32	167	56	17	16	7	27
7	34	183	61	19	18	8.1	30
8	33	171	53	18	19	9.0	28
9	33	143	52	22	22	12	29
10	32	178	59	20	17	4.3	29
12	36	237[b]	59	20	19	8	35
13	37	184	58	17	12	4.8	24
				Sodium, ppm			
1	159	138	147	152	193	404	187
2	76	35	27	27	33	300	144
3	143	121	133	133	161	301	—[e]
5	143	94	173	82	140	299	172
6	210	167	227	163	220	423	174
7	40	10	10	10	10	320	152
8	181	129	222	168	189	427	173
9	68	52	39	19	29	286	160
10	1860	1060	1600	2060	2200	1260	147
12	147	154	171	181	198	391	148

[a]See Table 1 for sample identification and pairs; LE = liquid extract of NBS Citrus Leaves 1572. LE results reported as moisture corrected values.
[b]Dixon outliers at the 95% confidence level.
[c]Flame emission results.
[d]Results not included in application of the Dixon outlier test.
[e]Value not reported.

Table 3. Averages, standard deviations, and coefficients of variation for collaborative study results

Sample	Av. ± SD	CV,%	Sample	Av. ± SD	CV,%
	Phosphorus, %			Iron, ppm	
1	0.28 ± 0.02	7.38	1	167 ± 32	19.2
2	0.12 ± 0.02	13.0	2	117 ± 11	9.76
3	0.16 ± 0.02	11.8	3	956 ± 167	17.5
4	0.17 ± 0.01	6.41	4	103 ± 3	3.40
5	0.27 ± 0.02	5.78	5	125 ± 16	12.7
6	0.14 ± 0.01	6.69	6	33 ± 5	14.9
LE	0.13 ± 0.01	9.62	LE	109 ± 8	7.37
	Potassium, %			Aluminum, ppm	
1	2.10 ± 0.17	8.10	1	226 ± 30	13.3
2	0.86 ± 0.24	28.5	2	1124 ± 258	23.0
3	1.16 ± 0.05	4.04	3	514 ± 87	17.0
4	2.08 ± 0.13	6.06	4	144 ± 15	10.7
5	2.08 ± 0.12	6.01	5	101 ± 14	13.6
6	0.85 ± 0.07	8.34	6	26 ± 11	42.2
LE	1.84 ± 0.11	6.08	LE	78 ± 12	15.6
	Calcium, %			Boron, ppm	
1	0.56 ± 0.03	5.92	1	5.1 ± 1.1	21.9
2	1.95 ± 0.08	4.26	2	35 ± 4.8	13.6
3	0.21 ± 0.02	8.61	3	4.1 ± 1.2	29.1
4	1.11 ± 0.04	3.60	4	24 ± 1.5	6.38
5	0.66 ± 0.02	3.17	5	3.8 ± 1.3	33.7
6	0.28 ± 0.01	4.98	6	2.0 ± 0.91	45.9
LE	3.12 ± 0.25	8.01	LE	63 ± 5.8	9.22
	Magnesium, %			Copper, ppm	
1	0.40 ± 0.03	8.09	1	11.3 ± 1.1	9.37
2	0.26 ± 0.03	13.1	2	8.3 ± 2.8	33.6
3	0.27 ± 0.03	10.2	3	6.3 ± 0.4	6.54
4	0.43 ± 0.04	10.1	4	5.6 ± 1.7	31.3
5	0.15 ± 0.02	14.1	5	11.5 ± 1.6	14.1
6	0.16 ± 0.02	12.9	6	2.3 ± 1.2	51.9
LE	0.56 ± 0.06	10.7	LE	15.0 ± 1.8	12.0
	Manganese, ppm			Zinc, ppm	
1	60 ± 5	8.95	1	33 ± 3	8.02
2	649 ± 34	5.20	2	171 ± 13	7.90
3	46 ± 3	6.78	3	56 ± 3	5.51
4	71 ± 6	9.04	4	18 ± 2	11.0
5	149 ± 8	5.33	5	17 ± 3	14.9
6	39 ± 3	8.96	6	7.4 ± 2.1	29.0
LE	22 ± 3	12.4	LE	29 ± 4	15.6

3.A02 *Reagents and Apparatus*

(a) *Stock solns.*—1000 μg/mL. Weigh designated reagent into sep. 1 L vol. flasks, dissolve in min. amt of dissolving reagent, and dil. to vol. with H_2O.

Element	Reagent	g	Dissolving Reagent
B	H_3BO_3	5.7192	H_2O
Ca	$CaCO_3$	2.4973	6N HCl
Cu	pure metal	1.0000	HNO_3
K	KC	1.9067	H_2O
Mg	$MgSO_4 \cdot 7H_2O$	10.1382	H_2O
Mn	MnC_2	1.5825	6N HCl
P	$NH_4H_2PO_4$	3.7138	H_2O
Zn	pure metal	1.0000	6N HCl

(b) *Std solns.*—Pipet following vols of stock soln into 1 L vol. flasks. Add 100 mL HCl and dil. to vol. with H_2O.

	Std Soln 1		Std Soln 2	
Element	Stock Soln, mL	Final Concn, μg/mL	Stock Soln, mL	Final Concn, μg/mL
B	0	0	10	10
Ca	5	5	60	60
Cu	0	0	1	1
K	5	5	60	60
Mg	1	1	20	20
Mn	0	0	10	10
P	5	5	60	60
Zn	0	0	10	10

Make any needed subsequent dilns with 10% HCl (1 + 9).

(c) *ICP emission spectrometer.*—Suggested operating parameters: forward power, 1.1 kilowatts; reflected power, <10 watts; aspiration rate, 0.85–3.5 mL/min; flush between samples, 15–45 s; integration time, 1–10 s.

Element	Wavelength, Å
B (CAS-7440-42-8)	2496
Ca (CAS-7440-70-2)	3179
Cu (CAS-7440-50-8)	3247
K (CAS-7440-09-7)	7665
Mg (CAS-7439-95-4)	2795
Mn (CAS-7439-96-5)	2576
P (CAS-7723-14-0)	2149
Zn (CAS-7440-66-6)	2138

3.A03 *Dry Ashing*

Accurately weigh 1 g sample, dried and ground as in **3.002(a)**, into glazed, high-form porcelain crucible. Ash 2 h at 500°, and let cool. Wet ash with 10 drops of H_2O, and carefully add 3–4 mL HNO_3 (1 + 1). Evap. excess HNO_3 on hot plate set at 100–120°. Return crucible to furnace and ash addnl 1 h at 500°. Cool crucible, dissolve ash in 10 mL HCl (1 + 1), and transfer quant. to 50 mL vol. flask. Dil. to vol. with H_2O.

3.A04 *Determination*

Elemental detn is accomplished by inductively coupled plasma emission spectroscopy. Calibration of instrument is done thru use

Table 4. Precision data for collaborative study results

Element	Pair	S_r	S_d	S_b
P	1	0.0131	0.0198	0.0148
	2	0.0119	0.0176	0.0130
	3	0.0106	0.0189	0.0156
K	1	0.0899	0.204	0.183
	2	0.220	0.135	—[a]
	3	0.0681	0.0392	—[a]
Ca	1	0.0173	0.0333	0.0284
	2	0.0561	0.0661	0.0350
	3	0.0126	0.0195	0.0149
Mg	1	0.0283	0.0371	0.0240
	2	0.0091	0.0442	0.0433
	3	0.0100	0.0308	0.0291
Mn	1	4.48	7.49	6.00
	2	22.9	25.1	10.3
	3	2.26	4.13	3.46
Fe	1	9.73	16.7	13.6
	2	4.42	6.13	4.25
	3	116	113	—[a]
Al	1	13.0	29.0	25.9
	2	134	175	113
	3	62.9	61.5	—[a]
B	1	0.562	1.59	1.49
	2	3.27	3.99	2.29
	3	0.809	1.26	0.966
Cu	1	0.682	1.73	1.59
	2	1.10	3.14	2.94
	3	0.545	0.982	0.817
Zn	1	2.58	2.62	0.456
	2	10.4	9.37	—[a]
	3	3.14	2.23	—[a]

[a] S_b^2 is a negative number.

of known calibration stds. After calibration is complete, samples can be analyzed. Check calibration after every 10 samples. If instrument has drifted out of calibration (>3% of original values), recalibrate.

Calc. concn for each element of each dild sample as μg/mL.

Results and Discussion

One collaborator reported results using a DC instrument. This is an ICP study, so those values are not included.

Thirteen collaborators reported results for P determinations; 12 collaborators for K, Ca, Mg, Mn, Fe, Al, Cu, and Zn; 11 collaborators for B; 10 collaborators for Na; 4 collaborators for Ba, Cr, Ni, Cd, and Pb; 3 collaborators for Sr and Mo; 2 collaborators for Co and Si; and 1 collaborator for Be, Sb, Sn, Ti, and V.

Ten collaborators reported using simultaneous analysis, and 3 reported using sequential analysis. Collaborator 2 used sequential analysis for Na and K, and simultaneous analysis for all other elements.

Sample aspiration rates varied from 0.85 to 3.5 mL/min. Flush times varied from 15 to 45 s. Sample integration times varied from 1 to 10 s; 10 s was used for all of the simultaneous-type instruments. Collaborator 2 reported results on the basis of two 10 s integrations. Collaborator 4 reported results on the basis of three 10 s integrations. Of the sequential-type instruments, Collaborator 3 reported an integration time of 1 s; Collaborator 11 reported 3 s and Collaborator 8 reported "variable."

Instruments from 6 manufacturers were used by the 13 collaborators (Table 6).

The statistical treatment described in *Evaluation of Data* was applied to P, K, Ca, Mg, Mn, Fe, Al, B, Cu, and Zn values. Sodium data were not included because of the poor results obtained on the plant tissue samples. It is interesting to note, however, that the average Na value obtained on the NBS citrus leaves liquid extract (162 ± 15 ppm) agrees very closely with the stated NBS value (160 ± 20 ppm). The remaining elements were excluded from statistical treatment because of the small number of collaborators reporting results for those elements.

Collaborator 7 reported K values by flame emission rather than ICP emission.

Collaborator 10 reported difficulty in maintaining his oven temperature. He reported a glowing red condition which is indicative of much higher temperatures than 500°C. Kometani (9) and Grove et al. (10) report losses of K under these conditions. Inspection of the K results from Collaborator 10 indicates that this is indeed the case.

Therefore, the K results for Collaborators 7 and 10 were omitted before application of the Dixon outlier test.

Processing the data by the Dixon criteria resulted in 58 outliers representing 7.0% of the data. This is roughly equivalent to the 6.9% outliers found in the dry-ash portion of the Isaac and Johnson study (2), which was adopted by AOAC for the elemental analysis of plant tissue by atomic absorption spectrophotometry. The dry-ash procedure used in the Isaac and Johnson study was the same as the one described for this study. Five of the collaborators were responsible for 90% of the outliers.

All Dixon outliers were omitted from further statistical consideration. In addition, if a collaborator failed the laboratory ranking test for an element and at least 4 of 6 plant tissue values were outliers, then the remainder of results for that element were omitted from further statistical consideration. As a consequence of the above treatment, K values for Collaborator 3, Fe and Zn values for Collaborator 5, Cu values for Collaborator 10, and Mg values for Collaborator 12 were omitted. This resulted in 7 additional values being omitted from the data.

Table 5. Liquid extract of NBS Citrus Leaves 1572: NBS value vs average for this study and *t*-test results

Element	NBS value	Average[a]	*t*-Test
P, %	0.13 ± 0.02	0.13 ± 0.01	0.000
K, %	1.82 ± 0.06	1.84 ± 0.11	0.603
Ca, %	3.15 ± 0.10	3.12 ± 0.25	0.693
Mg, %	0.58 ± 0.03	0.56 ± 0.06	1.386
Mn, ppm	23 ± 2	22 ± 3	1.154
Fe, ppm	90 ± 10	109 ± 8	8.227[b]
Al, ppm	92 ± 15	78 ± 12	4.041[b]
Cu, ppm	16.5 ± 1.0	15.0 ± 1.8	2.887[c]
Zn, ppm	29 ± 2	29 ± 4	0.000
Na, ppm	160 ± 20	162 ± 15	0.400

[a] Moisture corrected.
[b] Significant at the 99% confidence level.
[c] Significant at the 95% confidence level.

Table 6. Instruments used for analysis

Coll.	Manufacturer
1	Jarrell-Ash
2	Instruments S A
3	Perkin-Elmer[a]
4	Applied Research Laboratories
5	Bausch and Lomb
6	Jarrell-Ash
7	Jarrell-Ash
8	Perkin-Elmer[a]
9	Applied Research Laboratories
10	Jarrell-Ash
11	Leeman Labs[a]
12	Jarrell-Ash
13	Jarrell-Ash

[a] Sequential instrument.

Table 7. Correlation coefficients of the excluded data[a]

Coll.	Element	Corr. coeff.
3	K	0.996
7[b]	K	0.994
10	K	0.978
10	Cu	0.651
5	Fe	0.992
5	Zn	0.998
12	Mg	0.941

[a]Data for which all 6 plant tissue values were deleted from further statistical considerat on.
[b]Flame emission resu ts.

Table 8. Comparison of this study with earlier studies that resulted in official methods

	Average % coefficient of variation		
Element	This study	Jones study (3)	Isaac and Johnson study (2)
P	8.51	12.7	—
K	10.2 (6.51)[a]	15.8	2.40
Ca	5.09	11.2	5.90
Mg	11.4	11.1	5.06
Mn	7.38	26.4	3.35
Fe	12.9	30.4	6.82
Al	20.0	23.8	—
B	25.1	16.4	—
Cu	24.5	23.8	20.4
Zn	12.7	27.3	12.7

[a]Deleting the value for Sample 2.

Correlation coefficients were computed on the excluded data (Table 7), using the omitted values vs the average value, to see if anything can be said about these results. With the exception of the Cu results for Collaborator 10, the correlations are very good. Possible causes for this large systematic error include differences between the sample and standard matrices and/or incorrect standard concentrations.

The coefficients of variation for each element were generally in the same range for the 6 samples. There is no apparent reason to explain the poor K results for Sample 2. Results on the other elements for this sample indicate that sample homogeneity was not the problem.

The average of the coefficients of variation for each element compares very favorably with those obtained in the Jones study (3) which was adopted by AOAC for the elemental analysis of plant tissue by emission spectroscopy using the rotating disc arc-spark technique (Table 8). For P, K, Ca, Mn, Fe, Al, and Zn, the average coefficient of variation was better than the ones in the Jones study. For Mg, B, and Cu, they were slightly worse. The poorer coefficients of variation for Mg, B, and Cu are probably due to differences in the levels measured in the 2 collaborative studies. The average elemental values for the 5 samples in the Jones study were 0.48% for Mg, 38 ppm for B, and 12 ppm for Cu. The average elemental values for the 6 samples in this study are 0.27% for Mg, 12 ppm for B, and 7.4 ppm for Cu. However, the averages of the coefficients of variation do not compare as well with those obtained in the Isaac and Johnson study (Table 8). P, B, and Al were not included in the Isaac and Johnson study.

The standard deviations S_r and S_d were used to calculate F values for each of the pairs to check for the presence of systematic error (Table 9). Eleven of the 30 F values showed significance at the 95% confidence level, indicating that systematic error was the major contributor to the total error. Calculations for the S_r component showed that random error contributed only a small portion to the total error. For these calculations, the pairs selected should be similar and have values that are fairly close in amounts. Unfortunately, this ideal state is difficult to attain in multielement analysis unless blind duplicates are used. Of particular problem in this study were pairs 2 and 3 for Al, pair 2 for Mn, pair 3 for Fe, and pair 2 for Zn.

Because the NBS citrus leaves were not dried before weighing, a moisture factor was used to correct the results to a dry weight basis. Results of the t-test on the NBS citrus leaves liquid extract showed no inherent systematic error in the method.

Conclusions and Recommendations

The proposed method performed well on a variety of plant tissues for several elements. The percentage of outliers was only slightly higher than those reported by Isaac and Johnson for the dry-ash portion of their collaborative study. The coefficients of variation were better than those reported by Jones in his collaborative study. With the exception of Fe and Al, the collaborative results on the NBS citrus leaves liquid extract were very good. The systematic error component of the standard deviations would probably decrease if instrument standardization is done with standards containing the same acid strength as the sample solutions.

It is recommended that the ICP emission spectroscopy method be adopted official first action for the elements P, K, Ca, Mg, Mn, B, Cu, and Zn; that it be stipulated as part of the method that standards contain 10% HCl; that dilutions, if needed, be made with 10% HCl solution.

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Table 9. *F*-Test for each pair of collaborative study samples

Element	Pair 1	Pair 2	Pair 3
P	2.28	2.19	3.18[a]
K	5.15[a]	0.365	0.331
Ca	3.71[a]	1.39	2.40
Mg	1.72	23.6[b]	10.9[b]
Mn	2.80	1.20	3.34[a]
Fe	2.95	1.92	0.949
Al	4.98[b]	1.71	0.956
B	8.00[b]	1.49	2.43
Cu	6.43[b]	8.15[b]	3.25[a]
Zn	1.03	0.812	0.504

[a]Significant at the 95% confidence level.
[b]Significant at the 99% confidence level.

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MEDICAL DEVICES

Rapid Gas Chromatographic Determination of Ethylene Oxide, Ethylene Chlorohydrin, and Ethylene Glycol Residues in Rubber Catheters

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Isothermal gas chromatography with flame ionization detection was used to determine residual ethylene oxide (EtO), ethylene chlorohydrin, and ethylene glycol in soft rubber catheters that had been sterilized with EtO. Catheter samples were extracted by shaking with carbon disulfide, and the extract was analyzed on a 3% Carbowax 20M on 80–100 mesh Chromosorb 101 column, using nitrogen as the carrier gas. Ten replicate injections of a mixed standards solution gave coefficients of variation of 1.91, 1.23, and 4.74% for EtO, ethylene chlorohydrin, and ethylene glycol, respectively. A linear response was obtained with concentrations ranging from 1.0 to 7.9 μg EtO, 14.0 to 88.0 μg ethylene chlorohydrin, and 31.0 to 98.5 μg ethylene glycol. The proposed method detected as little as 0.5, 5.0, and 16.5 ng EtO, ethylene chlorohydrin, and ethylene glycol, respectively.

Ethylene oxide (EtO) is a colorless gas used to sterilize many drugs, biological products, and medical devices for human use. This gas is used when other sterilants such as heat, steam, radiation, or liquid chemicals are either impractical or detrimental to the product (1). Sterilization with EtO includes a final aeration step to remove the sterilant from the product. If this step is carried out improperly or for too short a time, residues of EtO will remain on the product. These residues can combine with chloride ions, present in many plastic products and in human skin, to form ethylene chlorohydrin, or more often, with water to form ethylene glycol (1).

EtO and its 2 main reaction products are toxic to both humans and laboratory animals. In humans, chronic exposure to EtO increases the incidence of chromosomal aberrations and the risk of spontaneous abortions and death from leukemia and other forms of cancer (1–3). In laboratory animals, EtO and its derivatives are carcinogenic, mutagenic, and teratogenic (1, 2). Their presence in drug products and medical devices for human use may cause toxic reactions in the users. In any event, maximum residue limits and exposure levels, which take into account the lowest possible limits achievable under current good manufacturing practices, have been suggested for all 3 contaminants. In the United States, the Food and Drug Administration has proposed maximum limits for the content of these residues in medical devices that range from 5 to 250 ppm for EtO, 10 to 250 ppm for ethylene chlorohydrin, and 10 to 5000 ppm for ethylene glycol, depending on the application of the device (1). However, much controversy still exists as to what the "toxic" and "no effect" levels are.

The permeation of EtO and its reaction products from or into a medical device is influenced by several factors that are inherent in the device, namely, the physical dimensions, exposed surfaces, packing, type of material, etc. (4–6). The greater the density or thickness of the product material, the more EtO will tend to remain in the device. The chemical makeup of the material can either aid or hinder this progress. Therefore, one of the problems encountered during the testing of medical devices for residues of EtO and its 2 main reaction products is that they may be found within the device as well as on it. Another difficulty is the physical nature of the residues. At room temperature, EtO is a gas, bp 10.7°C, whereas ethylene chlorohydrin and ethylene glycol are viscous liquids, bp 219 and 198°C, respectively. These properties are of concern when one uses gas chromatographic (GC) techniques for determining these residues. The significant differences in volatility among the 3 compounds would dictate the need for temperature programming despite the possibility of baseline problems. Alternatively, all 3 residues have been determined using either 2 different chromatographic columns coupled to flame ionization detectors (FID) (7, 8), or a single chromatographic column that had to be set at 2 temperatures (9). Other published GC methods will only determine EtO (10, 11), ethylene glycol (12,) or ethylene chlorohydrin (13), or mixtures of EtO with either ethylene chlorohydrin (14, 15) or ethylene glycol (16).

This paper describes a GC method for the simultaneous determination of EtO and its 2 main reaction products in commercial rubber catheters under isothermal conditions. The proposed method was quantitative for EtO and ethylene chlorohydrin and semiquantitative for ethylene glycol in the samples tested.

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Experimental

Reagents and Apparatus

(a) *Ethylene oxide (EtO).*—Purity 99.7% (Matheson Gas, East Rutherford, NJ). Use within 90 days from filling date stated on cylinder. Keep refrigerated. *Caution:* Toxic and explosive.

(b) *Ethylene chlorohydrin.*—Analytical grade 2-chloroethanol (Fisher Scientific Co., Springfield, NJ). *Caution:* Toxic.

(c) *Ethylene glycol.*—Analytical grade (Fisher Scientific Co.).

(d) *Carbon disulfide.*—Spectroscopic grade (Fisher Scientific Co.).

(e) *Gas chromatograph.*—Tracor Model 550 fitted with FID and 1.83 m × 4 mm id column packed with 3% Carbowax 20M on 80–100 mesh Chromosorb 101 (Tracor Inc., Austin, TX). Column was conditioned overnight at 190°C while passing nitrogen at flow rate of 80 mL/min.

Operating conditions: Temperatures (°C) – column 140, injector 190, detector 165; carrier gas nitrogen, flow rate 40 mL/min; detector, FID operated at air flow rate of 300 mL/min and hydrogen flow rate of 25 mL/min; detector sensitivity 4×10^{-12} full-scale deflection (FSD).

Standard Solutions

Ethylene oxide standard solution.—Condense ca 1.1 mL EtO in graduated cylinder that has been immersed in mixture of ice and sodium chloride. Transfer EtO to 100 mL volumetric flask which has been tared with 50 mL carbon disulfide. Stopper flask immediately and let solution equilibrate to room temperature. Reweigh flask and its contents, add carbon disulfide to volume, and mix. Quantitatively dilute further with carbon disulfide to obtain EtO concentration of 100 μg/mL. Use this solution within 3 days and store in refrigerator in Teflon-capped vial.

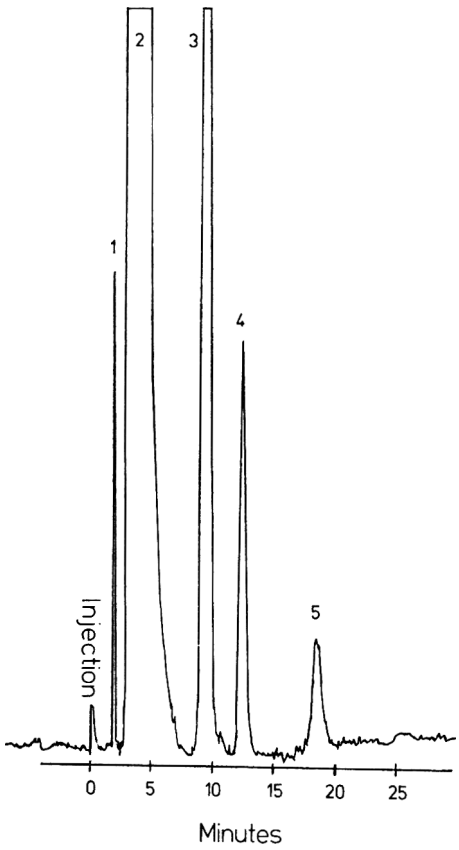

Figure 1. Gas chromatogram of 1, ethylene oxide (7.9 ng); 2, carbon disulfide; 3, solvent preservative; 4, ethylene chlorohydrin (80.6 ng); and 5, ethylene glycol (74.9 ng).

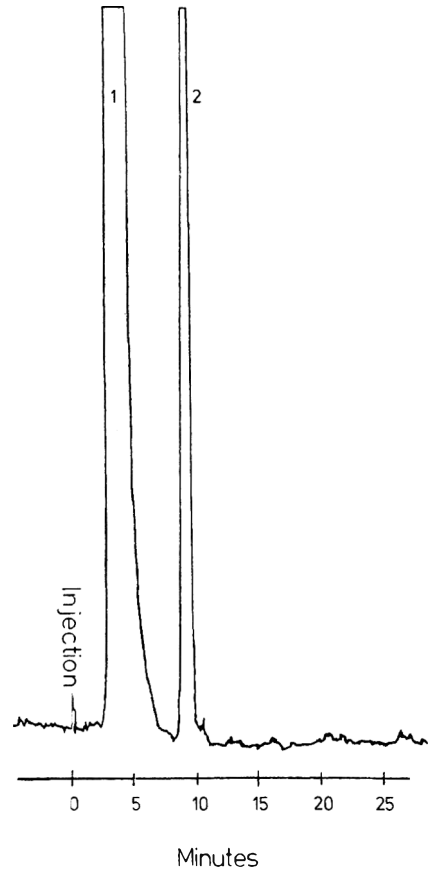

Figure 2. Gas chromatogram of 1, carbon disulfide and 2, solvent preservative.

Ethylene chlorohydrin standard solution.—Accurately weigh ca 100 mg ethylene chlorohydrin, transfer to 100 mL volumetric flask, dissolve in and dilute to volume with carbon disulfide, and mix.

Ethylene glycol standard solution.—Accurately weigh ca 10 mg ethylene glycol on tared ¾ in. square piece of glassine paper. Transfer paper and its contents to 1000 mL volumetric flask. Add 500 mL carbon disulfide, sonicate 1 h, cool to room temperature, dilute to volume with carbon disulfide, and mix.

Mixed standards solution.—Pipet 1.0 mL each of EtO standard solution and ethylene chlorohydrin standard solution into 100 mL volumetric flask. Dissolve in and dilute to volume with ethylene glycol standard solution, and mix.

Sample Preparation

Robinson soft rubber catheters (American Hospital Supply, McGaw Park, IL, Part No. 20856-008) ca 38.5 cm long, were cut into equal 8 cm portions after discarding the lower (2 cm) and upper (4.5 cm) end portions. Each section of catheter was placed in a tared 8 dram vial fitted with a Teflon-lined cap, and the vial and its contents were weighed. After 20.0 mL carbon disulfide was added to each vial, the vials were capped and mechanically shaken 1 h. After the catheter sections were removed and discarded, the vials were immediately capped and set aside for GC analysis.

Gas Chromatography

Using 10 μL syringe, inject 5 μL carbon disulfide into GC column. Then inject 5 μL each of individual standard solutions, mixed standards solution, and sample preparation. Calculate approximate quantities of each residue using either peak heights or peak areas from:

$$C = (Pu/Ps) \times Cs$$

where C = concentration of residue, Pu = peak area or peak height of sample preparation, Ps = peak area or peak height of standard preparation, and Cs = concentration of standard preparation, mg/mL.

Results and Discussion

The chromatographic column used in this study has been previously applied to analysis of ethylene glycol (9) and of combinations of ethylene glycol with ethylene chlorohydrin (7). This column is commercially available on a made-to-order basis; however, we found that our laboratory-made column yielded somewhat better resolution. A typical chromatogram of a mixture of EtO and its main reaction products is shown in Figure 1. The approximate retention times for EtO, ethylene chlorohydrin, and ethylene glycol were 2, 12.5, and 19 min, respectively. The solvent peak appeared at about 4 min, and the solvent preservative at about 9.5 min. A catheter extracted with carbon disulfide before sterilization with EtO did not show any discernible chromatographic peaks that corresponded to any of these residues (Figure 2). In its pure form EtO is highly flammable and explosive. For this reason it is often combined with other gases, freon for example, to form a mixture that has a low flammability and negligible explosive hazard (17). Freon eluted at about 1 min under the described experimental conditions.

Two attempts were made to recover EtO from commercial rubber catheters. Initially, samples for GC were prepared from catheters that were sterilized in a commercial EtO sterilizer, aerated for intervals ranging from 0 to 24 h, and extracted with carbon disulfide. Although a close control was maintained over the sterilization conditions, recovery values were

erratic. For example, for 10 catheters divided into sections of equal size, recovery values ranged from 0 to 45.6 μg (coefficient of variation, CV, 91.5%). A different approach consisted of adding known amounts of each residue to extraction vials, each containing a section of catheter. In this case recoveries ranged from 93.0 to 99.7% (CV 1.92%, $n = 9$) for EtO, 57.8 to 60.6% (CV 2.25%, $n = 4$) for ethylene glycol, and 100.6 to 103.2% (CV 1.30%, $n = 4$) for ethylene chlorohydrin. The low recoveries noted for ethylene glycol are probably the result of its more extensive retention by, or to its condensation on the chromatographic column, which was being heated to a temperature that was about 52°C lower than the boiling point of this residue. Raising the column temperature to offset this problem results in a loss of the resolution between the EtO and carbon disulfide peaks, and between the ethylene chlorohydrin and the solvent preservative peaks. For a more accurate determination of ethylene glycol, one must first determine EtO and ethylene chlorohydrin, using the recommended chromatographic conditions, and subsequently determine ethylene glycol by a second injection but at a higher column temperature.

Different extracting solvents were tested during the study. Solvents such as water, acetone, and ethanol were unsuitable because they either saturated the detector or gave peak responses that interfered with one of the peaks of interest. In contrast, carbon disulfide was devoid of all these problems, and furthermore, by causing the swelling of the sample matrix, it facilitated the dissolution of any trapped residue. Mechanical agitation of the sample with the extracting solvent improved the recovery of residues from the catheters.

On the basis of peak height measurements for 10 replicate injections of a mixed standards solution, the proposed GC method showed good precision for EtO (CV 1.91%) and ethylene chlorohydrin (CV 1.23%). Greater variability was noted for ethylene glycol (CV 4.74%), probably because of the same factors that were mentioned earlier to account for the lower recovery values for ethylene glycol.

Detector responses were linearly related to the concentrations of residue, ranging from 1.0 to 7.9 μg ($r = 0.999$) for EtO, 14.0 to 88.0 μg ($r = 0.977$) for ethylene chlorohydrin, and 31.0 to 98.5 μg ($r = 0.969$) for ethylene glycol. At higher concentrations, ethylene glycol will deviate from linearity due to its low solubility in the extracting solvent. At a detector sensitivity setting of 2×10^{-12} FSD, which gave a peak response about 5% of full scale, the limits of detection for EtO, ethylene chlorohydrin, and ethylene glycol were 0.5, 5.0, and 16.5 ng, respectively.

In summary, the proposed GC method will be useful for the rapid and simultaneous determination of EtO and its 2 main reaction products under isothermal conditions. To improve the accuracy and precision of the method for the determination of ethylene glycol, a higher column temperature should be used after EtO and ethylene chlorohydrin have been determined by using the chromatographic conditions given here.

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VITAMINS AND OTHER NUTRIENTS

Vitamin A and Vitamin E Content of Infant Formulas Produced in the United States

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Vitamin A (vitamin A palmitate) and vitamin E (α-tocopheryl acetate) levels were determined in 77 samples of fortified infant formulas manufactured by 4 firms in the United States from 1981 to 1983 and were compared by formulation base (soy, milk) and manufacturing firm. For vitamin A and vitamin E, the mean values (IU/100 kcal) were 454 ± 95 (range 248–614) and 2.0 ± 0.7 (range 1.1–5.0), respectively. No significant differences (α = 0.05) were found in levels (IU/100 kcal) of vitamin A and vitamin E between milk- and soy-based formulas. When the mean vitamin A and vitamin E levels of formulas produced by the various firms were compared on an IU/100 kcal or percent of label declaration basis, significant differences (α = 0.05) were found among firms. Mean vitamin A levels for the various products compared to label declarations ranged from 126% of declared for the ready-to-use formulas to 139% of declared for the powders. Mean vitamin E levels ranged from 97% of declared for ready-to-use formulas to 118% of declared for concentrates. Except for one sample that contained 248 IU vitamin A/100 kcal, the formulas met the requirements of the 1980 Infant Formula Act.

Modern infant formulas consist of a wide array of products formulated to meet the needs of healthy full-term infants as well as infants with special dietary requirements. Formulas for the normal full-term infant are usually milk- or soy-based, with nutrient levels specified by the Infant Formula Act of 1980 (1). These nutrient levels are primarily based on the recommendations of the Committee on Nutrition of the American Academy of Pediatrics (2). Since human milk represents the pattern of nutrients most suitable to meet the physiological requirements of the infant, many of the recommendations are based on the composition of mature human milk.

Since promulgation of the Infant Formula Act of 1980, the Food and Drug Administration (FDA) has been increasingly involved in analysis of infant formula products to ensure accuracy of label statements and compliance with the Act. This paper presents data on the vitamin A and vitamin E content of a broad spectrum of infant formulas produced in the United States and collected under the infant formula survey program.

METHOD

Samples

Samples were collected from the manufacturer as part of routine FDA survey programs. The 77 infant formulas were manufactured by 4 U.S. firms from 1981 to 1983. The soy- and milk-based product types consisted of 32 ready-to-use samples, 28 concentrate samples, and 17 powder samples and represented 12 brands. Aseptic techniques were used to prepare liquid (secs **16.002(b)**, **16.187** (3)) or powder (secs **16.002(b)**, **16.211** (3)) sample composites by combining and mixing the contents of 12 units in a nitrogen-purged container. Composited samples were held refrigerated and under a nitrogen atmosphere. Analyses were normally initiated within 24 h of compositing.

Procedure

A liquid chromatographic (LC) method (4) was used for determining vitamin A as retinyl palmitate and vitamin E activity as the supplemental form, α-tocopheryl acetate. Natural tocopherols were not quantitated. The lipid-soluble components were extracted from the aqueous phase by homogenizing in a solvent mixture of isopropanol and methylene chloride with magnesium sulfate added to remove water. The vitamins were fractionated from the lipid material by gel permeation chromatography followed by quantitation using nonaqueous reverse phase LC on a Zorbax ODS column.

Recovery data for the method and comparison of results to official AOAC methodology have been reported (4). Additional validity studies were completed during routine laboratory quality assurance studies. For vitamin A, 14 replicate analyses of one formula performed by 2 analysts on 4 separate dates gave a mean of 3339 ± 206 IU/L (coefficient of variation (CV) 6.2%). For vitamin E, 16 replicate analyses of one formula performed by 2 analysts on 4 separate dates gave a mean of 6.4 ± 0.51 IU/L (CV 7.9%).

Statistical analyses were performed using the general linear models procedure and Duncan's multiple range test in the Statistical Analysis System (5) package.

Results and Discussion

Mean vitamin A and vitamin E contents of the 77 infant formula samples were 454 ± 95 and 2.0 ± 0.7 IU/100 kcal, respectively. Vitamin A levels ranged from 248 to 614 IU/100 kcal. Vitamin E levels ranged from 1.1 to 5.0 IU/100 kcal. All samples met the vitamin E requirement of 0.7 IU/100 kcal with 0.7 IU/g linoleic acid as specified by the Infant Formula Act (1). Only one sample contained less (248 IU/100 kcal) than the 250 IU/100 kcal minimum level of vitamin A specified by the Act. None were above the maximum allowable vitamin A level of 750 IU/100 kcal.

No statistical differences (α = 0.05) in vitamin A and vitamin E contents were found between milk- and soy-based formulas (Table 1).

The mean vitamin A and vitamin E values for the formulas produced by the 4 manufacturers are given in Table 2. On the basis of IU/100 kcal, the firms fell into 2 statistically similar (α = 0.05) groups for vitamin A content (Firms A and B vs Firms C and D), which indicates differences in vitamin A formulation philosophies of the various firms. The mean vitamin A content of each firm's product was well within the range of from 250 to 750 IU/100 kcal required by the Infant Formula Act (1).

For vitamin E, the mean values of formulas produced by the 4 firms ranged from 1.4 to 3.0 IU/100 kcal (Table 2), a significant (α = 0.05) variation among manufacturers on an IU/100 kcal basis. Since the Infant Formula Act of 1980 requires 0.7 IU vitamin E/g linoleic acid, differences in the vitamin E content of formulas produced by several manufacturers would be expected due to variation in the linoleic acid content of the vegetable oils used to prepare the formulations.

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Table 1. Vitamin A and vitamin E content of milk- and soy-based infant formulas

Formula	No. samples	IU/100 kcal[a] Vitamin A	IU/100 kcal[a] Vitamin E	% of label declaration[a] Vitamin A	% of label declaration[a] Vitamin E
Milk-based	46	462 +90[b]	2.0 ±0.8[b]	136 ±26[b]	105 ±21[b]
Soy-based	31	442 ±103[b]	2.0 ±0.6[b]	125 ±24[b]	115 ±53[b]

[a]Mean ± SD; means with the same letter in the same column are not significantly different ($\alpha = 0.05$).

Table 2. Vitamin A and vitamin E content of infant formulas produced by different manufacturers

Mfr	No. samples	IU/100 kcal[a] Vitamin A	IU/100 kcal[a] Vitamin E	% of label declaration[a] Vitamin A	% of label declaration[a] Vitamin E
A	14	366 ±76[b]	2.3 ±0.2[b]	117 ±24[b]	134 ±72[b]
B	17	386 ±101[b]	1.8 ±0.2[c]	143 ±40[c]	102 ±7[c]
C	17	532 ±43[c]	3.0 ±0.7[d]	144 ±12[c]	110 ±21[c]
D	29	490 ±57[c]	1.4 ±0.4[a]	125 ±14[b]	101 ±27[c]

[a]Mean ± SD; means with the same letter in the same column are not significantly different ($\alpha = 0.05$).

The mean percent of label declaration for the 77 formulas was 132 ± 26 for vitamin A and 109 ± 37 for vitamin E. Label declaration values ranged from 85 to 242% for vitamin A and from 79 to 272% for vitamin E. Six of the 77 formulas contained less than the declared level of vitamin A; however, 4 of these samples contained more than 96% of the declared vitamin A level. For vitamin E levels, a wider variation existed. The samples contained from 719 to 159% of the minimum level of vitamin E required by the Infant Formula Act (1). Thirteen of the 77 samples contained less than 90% of the label declaration, and one sample contained less (78%) than 80% of the declared vitamin E. However, naturally occurring vitamin E forms that might originate with the vegetable oils in the formulation were not measured. Inclusion of these forms would probably raise the vitamin E activity to levels above label declarations in all samples.

Using percent of label declaration for vitamin A and vitamin E, no statistical differences ($\alpha = 0.05$) were noted between milk- and soy-based formulas (Table 1). In terms of different types of products, mean vitamin A levels ranged from 126% of declared for ready-to-use products to 139% of declared for powders. Mean vitamin E levels ranged from 97% of declared for ready-to-use formulas to 118% of declared for concentrates.

Statistical differences ($\alpha = 0.05$) were apparent when the mean vitamin A and vitamin E levels of the formulas produced by the 4 manufacturers were compared using percent of label declaration values (Table 2). For vitamin A, formulas produced by Firms A and D were statistically similar (117 and 125% of label declaration) as were formulas produced by Firms B and C (143 and 144% of label declaration). The values provide insight into the amount of overage that the industry feels is necessary for a micronutrient subject to processing and storage losses.

For vitamin E, percent of label declaration values were statistically similar ($\alpha = 0.05$) for 3 of the manufacturers (Firms B, C, and D). For these firms, the mean percent of label declaration ranged from 101 to 110, indicating little or no addition above that necessary to meet declared values. For Firm A, the percent of label declaration of 134 ± 72 shows either a higher planned overage or lack of quality control. The vitamin E content of the 14 formulas from Firm A ranged from 90 to 272% of declared.

Grouping of products by individual brand (Table 3) gives a better indication of a manufacturer's quality control. The variation within a specific brand is usually small. However, Brand 1, produced by Firm A, shows greater variation than the other formulas included in the survey.

To relate the vitamin A and vitamin E levels reported in this study to infant requirements, comparisons were made with the Recommended Dietary Allowances (RDA) for infants to 6 months of age (6). The RDA of vitamin A for infants in this age category is 420 retinol equivalents (RE), which is based on the consumption of 850 mL human milk with a retinol content of 49 μg/100 mL (6). The average infant formula assayed in this survey would provide 2959 IU/serving (one serving is defined as 1 qt or L as specified by the manufacturer) or 889 RE/serving (1 RE = 3.33 IU). The lowest level of vitamin A found in an infant formula was 1588 IU/L, which would supply 477 RE to the infant consuming 1 L of that formula per day, a vitamin level which is still well above the RDA.

As with vitamin A, the RDA for vitamin E is based on the level of vitamin E activity normally found in human milk, and for infants up to 6 months of age is 3 α-tocopherol equivalents (α-TE) or 4.5 IU, where 1 mg *d*-α-tocopherol is equal to 1 α-TE or 1.5 IU. Based on an average vitamin E content of 13.1 IU/serving, determined in this study as α-tocopheryl acetate and calculated as *dl*-α-tocopheryl acetate (1 mg = 1 IU = 0.67 α-TE), the average infant formula surveyed would provide 8.78 α-TE/serving (7). The lowest level of vitamin E found in this study was 7.1 IU/L or 4.76 α-TE/L.

Summary

Except for one sample that contained 248 IU vitamin A/100 kcal (99.2% of the minimum requirement), the formulas met the requirements of the 1980 Infant Formula Act. Mean levels, expressed as IU vitamin A/100 kcal, were similar in soy- and milk-based formulas. Significant variations were noted in the vitamin A and vitamin E levels of formulas produced by the various manufacturers. More variation was noted in vitamin E levels, although overages were higher for vitamin A than for vitamin E on a percent of label declaration

Table 3. Vitamin A and vitamin E content of individual brands of formulas

Mfr	Brand	No. samples	Vitamin A[a] IU/100 kcal	Vitamin A[a] % of label declaration	Vitamin E[a] IU/100 kcal	Vitamin E[a] % of label declaration
A	1	11	355 ± 72	114 ± 23	2.2 ± 0.2	143 ± 79
	2	3	405 ± 92	130 ± 29	2.4 ± 0.2	101 ± 8
B	3	9	379 ± 102	141 ± 41	1.8 ± 0.3	101 ± 7
	4	7	404 ± 110	151 ± 42	1.9 ± 0.2	106 ± 7
C	5	5	561 ± 63	152 ± 17	2.8 ± 0.3	98 ± 9
	6	5	535 ± 27	145 ± 7	2.8 ± 0.5	109 ± 8
	7	3	493 ± 12	133 ± 3	3.9 ± 1.4	142 ± 33
	8	4	521 ± 16	141 ± 4	2.7 ± 0.2	101 ± 12
D	9	12	482 ± 71	123 ± 18	1.4 ± 0.4	101 ± 28
	10	2	491 ± 13	126 ± 3	1.3 ± 0.1	94 ± 5
	11	15	497 ± 49	127 ± 12	1.4 ± 0.4	102 ± 28

[a]Mean ± SD.

basis. The vitamin A and vitamin E content per serving of each formula examined in the study satisfies the RDAs for infants to 6 months of age.

Acknowledgments

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Atomic Absorption Spectrophotometric Determination of Cobalt in Foods

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A method is described for the determination of cobalt in foods. After wet digestion, iron in the sample is removed by liquid-liquid extraction, and cobalt is isolated and extracted. Final determination is done by flame atomic absorption spectrophotometry. Analysis of NBS reference materials by this procedure gives results in close agreement with certified values. The limit of quantitation is 4.3 ng/mL. Recovery studies and analysis of standard materials show that this method is reliable.

Cobalt is an essential element for humans. It is ubiquitous and, therefore, is present in foods, water, and beverages (1). In addition to some beneficial effects, toxic side effects are observed in humans (most notably polycythemia and vasodilation), so it is very important to know the source and amount of cobalt oral intake. It is evident, therefore, that a method is needed for determination of cobalt at the low levels, in the nanogram range, present in foods.

The methods proposed for cobalt determination in foods (2-5) involve isolation and previous concentration of cobalt by liquid-liquid extraction with 4-methyl-2-pentanone (MIBK) after formation of a chelate with ammonium pyrrolidine dithiocarbamate (APDC) or diethylammonium diethyldithiocarbamate (DDDC). The possible interference of iron in the formation of cobalt chelates with APDC in products from animal sources has been reported (2); therefore, eliminating iron is recommended as a first step. This interference has also been reported when the iron content in foods is higher than 70 mg/kg (3).

When we determined cobalt in foods by means of the APDC-MIBK system, we noted interference by the usual levels of iron in foods of both animal and plant sources, and in many cases presence of less than 70 mg iron/kg interfered in the analysis.

To solve this problem, we tried several procedures to mineralize the organic matter in the samples. As a result of these assays, we developed a method for determination of cobalt in foods, which comprises mineralization of the organic matter by repeated attacks with HNO_3, formation of cobalt chelates with APDC-MIBK after eliminating iron by means of a cupferron-chloroform system, and determination of cobalt by flame atomic absorption spectrophotometry.

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METHOD

Apparatus

All glassware was soaked in 10% (v/v) HNO_3 for 24 h and rinsed with deionized water before use.

(a) *Atomic absorption spectrophotometer.*—Perkin-Elmer Model 703. Operating conditions—impact ball: wavelength 240.7 nm; slit width 0.2 nm; lamp current 30 mA; acetylene pressure 12 psig, flow rate 0.6 L/min; air pressure 40 psig, flow rate 15.5 L/min; aspiration rate 4 L/min; burner height, adjust for optimum reading. Use a background correction at 238.4 nm from the same lamp.

(b) *pH meter.*—Digital (CRISON-Digilab 517).

Reagents

All reagents were reagent grade unless otherwise stated. Distilled and deionized water was used.

(a) *Ammonium pyrrolidine dithiocarbamate (APDC).*—(E. Merck). 1% APDC solution. Dissolve 1 g APDC in 100 mL water and extract for 1 min with 100 mL MIBK. Filter aqueous phase through Whatman No. 2 paper and store it in a precleaned polyethylene bottle at 4°C. Solution was stable for at least 1 week.

(b) *Cupferron.*—(E. Merck). 6% (w/v).

(c) *4-Methyl-2-pentanone (MIBK).*—Saturate with water by shaking MIBK and water, 2 + 1, v/v. After phases separate, filter organic layer through Whatman 1PS paper.

(d) *Sodium citrate.*—40%, w/v.

(e) *Cobalt standard solution.*—Dissolve 4.9379 g $Co(NO_2)_2.6H_2O$ in water and dilute to 1 L. Dilute this solution as needed to working concentration of 1 μg Co/mL.

Determination

Wet digestion.—Weigh 10 g (wet weight) sample in 250 mL beaker. Add 10 mL HNO_3 (sp. gr. 1.33) and 15 mL water. Place beaker in sand bath at 210°C until solution has evaporated. Remove beaker, let cool, and then add 10 mL HNO_3. Cover with watch glass and place beaker in sand bath until

Table 1. Comparison of Co in blanks treated by different mineralization methods, reported as ng Co/mL

Statistic	HNO_3	HNO_3–H_2SO_4	HNO_3–H_2SO_4–$HClO_4$	HNO_3–H_2O_2
Co concn ($\bar{x}_b$)(n = 12)	1.40	4.36	4.05	3.41
Std dev. (σ_b)	0.29	1.10	1.25	1.10
Detection limit ($\bar{x}_b + 3\sigma_b$)	2.27	7.67	7.81	6.71
Quantitation limit ($\bar{x}_b + 10\sigma_b$)	4.3	15.36	19.6	14.41

solution has evaporated. Repeat this operation as many times as necessary to obtain a white residue. Dissolve residue in 1 mL HNO_3 and dilute to 25 mL with water.

Elimination of iron.—Adjust pH of solution to 1.3 with sodium citrate. Transfer solution to separatory funnel. Add 1 mL cupferron and 10 mL $CHCl_3$. Shake 1 min. Draw off aqueous phase and discard $CHCl_3$ phase. Repeat $CHCl_3$ extraction. Draw off aqueous phase into a 50 mL beaker and warm to remove $CHCl_3$.

Extraction.—Adjust pH of aqueous solution to 4–4.5 with sodium citrate. Dilute solution to 50 mL with water and transfer to separatory funnel. Add 2 mL APDC and 5 mL MIBK. Shake vigorously 1 min. Let solution stand until phases separate (about 6 min). Centrifuge the organic layer to eliminate excess water traces.

Aspirate into atomic absorption instrument reading at 240.7 nm line, with flow rate of 3–4 mL MIBK/min.

Preparation of Standard Curve

Prepare cobalt standards to bracket cobalt concentrations in samples in range from 2 to 100 ng Co/mL. Carry 2 reagent blanks through extraction procedure. Standard and blanks need not be digested but should contain 1 mL HNO_3.

Construct calibration curve for each set of simultaneous determinations.

Results and Discussion

Sample Digestion

In order to mineralize organic matter, dry mineralization was carried out, using HNO_3, as well as wet mineralization with the following oxidants: HNO_3–H_2SO_4 (6); HNO_3–H_2SO_4–$HClO_4$ (6); HNO_3–H_2O_2 (7); and HNO_3 (8).

Table 1 summarizes data comparing the blank levels and the variability for the different mineralization methods as well as the detection limit and the quantitation limit obtained for each. From all these methods, the one consisting of several attacks with HNO_3 was chosen, because it provides good recoveries and little variability of the blanks, and allows the use of analytical grade reagents, as well as the simultaneous treatment of a great number of samples without excessive surveillance for the operator. These features are not accomplished by any of the other methods.

A study of the influence of the HNO_3 used in the mineralization process on the readings of blanks was carried out. Fourteen blanks treated in a similar manner as the samples and 14 blanks that had not been so treated were assayed and the following results (average ± standard deviation) were obtained: blanks treated in a similar manner, 1.40 ng Co/mL ± 0.29; blanks not mineralized, 1.42 ng Co/mL ± 0.33.

These data show that the difference in HNO_3 content between blanks for samples and for standards has no influence at the levels used.

Interferences

In the presence of iron (at the usual levels in foods) and on adding APDC, a precipitate is formed which remains even after adding MIBK and which interferes in the cobalt determination. Elimination of this interference was studied by using 4 standard aqueous solutions containing 5, 10, 20, or 40 ng/mL of cobalt and 500 μg/mL of iron each. The iron content corresponded to 50 mg/kg of fresh product. This value of 50 mg/kg was chosen because it is one of the highest iron contents in plants, although less than the levels present in many products of animal source.

We tried to retain the iron in solution by masking it with a 60% w/v solution of critic acid and with 4% w/v NaF, which failed to prevent the formation of a precipitate when APDC was added. We then decided that iron should be eliminated, and tried 2 methods to achieve this: (*1*) extraction of iron with MIBK in the presence of 6–8M HCl; (*2*) extraction of iron with the cupferron-chloroform system.

In the first method, an important background absorption was observed, which reduced sensitivity of the instrumental measurement. This was not observed in the second method, which determined our choice of the first one.

The water remaining in the organic phases interfered in the atomic absorption measurements, so it was necessary to prevent any trace of water. Due to practical reasons, Whatman 1PS filter paper was used for solvent clarification and centrifugation for cobalt standard solutions and samples.

In the method proposed, the absence of interferences was checked by the addition method, which was applied to 2 standard matrixes, plant (spinach) and animal (calf kidney).

The stability of the APDC–Co complex was checked with standard cobalt solutions (2, 5, 10, 20, 40, 60, 80 and 100 ng Co/mL). Absorbances were read 2, 7, 16, 22, 29, and 91 h after extraction against freshly prepared standards. During this time the solutions are kept at 5°C. The APDC–Co complex is stable at least 91 h.

Quantitation limit

The quantitation limit (LOQ) is calculated according to the definition (9): $\bar{x}_b + 10\sigma_b$, where $\bar{x}_b$ is the field blank and σ_b is the variability in the field blank: LOQ = 4.3 ng/mL.

To determine the number of blanks, the following formula (10) is applied:

$$n = \left(\frac{2S_{(n-1)}}{0.001}\right)^2$$

In this formula, $S_{(n-1)}$ corresponds to a tentative group of 6 blanks, and, considering that the standard deviation does not vary with the number of blanks, this dispersion is used to calculate n. The value 0.001 is the precision wanted and corresponds to 10% of the mean value of the blank.

A total of 14 blanks was obtained. The value for the 14 blanks was used to determine the quantitation limit, although this does not mean that every time that samples are analyzed this number of blanks is necessary.

Precision

The precision of the method was determined from the "variation coefficient" (relative standard deviation). This was calculated from the analysis of 12 homogeneous samples of

Table 2. Results of cobalt recovery from foods and standard materials[a]

Sample	No. of samples	Cobalt, ng/g			Recovery, %
		Present	Added	Found	
Spinach	9	32.8 ± 3.47	25	52 ± 2.0	90 ± 1.93
Apples	9	37.5 ± 2.8	25	57.5 ± 1.0	92 ± 0.94
Bread	9	17.5 ± 2.1	10	28.1 ± 1.42	102 ± 4.8
Milk[b]	9	3.8 ± 1.01	5	7.4 ± 0.48	84 ± 3.9
Veal chop	9	13.1 ± 1.51	10	23.7 ± 1.97	103 ± 4.14
Calf kidney	9	33.5 ± 0.47	50	84.1 ± 1.37	101 ± 1.13
NBS Bovine Liver	3	210 ± 0.05		210 ± 0.07	
NBS Tomato Leaves[c]	3	600		571 ± 1.2	

[a]Each product was analyzed 9 times at the unspiked level and an additional 9 analyses were carried out on the spiked samples. Results are the confidence intervals at a 95% confidence level.
[b]Sample weight = 25 g.
[c]Uncertified value.

2 standard matrixes, plant (spinach) and animal (calf kidney). The values found were 8.7 and 10.7%, respectively.

Accuracy

The accuracy of the method was verified by means of recovery assays applied to one food (9 samples) representative of each of the 6 groups of foods, in all 54 samples. An amount of cobalt equivalent to the average content was added.

Accuracy was also verified by analyzing 2 biological standards, a plant source (Tomato Leaves NBS SRM 1573) and an animal source (Bovine Liver NBS SRM 1577a). The results are summarized in Table 2.

Conclusions

The results obtained for the quantitation limit (4.3 ng/mL), the recovery values (84–104%), the contents found in the NBS samples, as well as the absence of interferences, prove that this method is precise and accurate enough to be applied to the determination of cobalt in foods. Among the advantages of the proposed method are its ability to determine cobalt in the nanogram range by FAAS (flame atomic absorption spectrophotometry) and the use of low cost, analytical grade reagents.

Because of the relatively long time needed for extraction (4–5 h) due to the complexity of the procedure, some efforts have been made to simplify this method; however, at present, they have been unsuccessful. Therefore, to obtain satisfactory results, the method described should be applied.

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Methods of Analysis for Infant Formula: Food and Drug Administration and Infant Formula Council Collaborative Study

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Because the U.S. Infant Formula Act of 1980 requires manufacturers to produce formula containing a specific minimum amount of nutrients, it became necessary to establish analytical methods for the nutrients listed in the Act. The Food and Drug Administration, the Infant Formula Council, its member companies, contract laboratories, and other government laboratories undertook a collaborative study of available methods; the specific nutrients studied to date include vitamins A, B_6, C, riboflavin, and niacin, and the elements calcium, magnesium, iron, zinc, copper, manganese, sodium, and potassium. The coefficients of variation in most cases have been as good as those that could be predicted from other collaborative studies. The methods studied for these nutrients have been adopted official first action except the method for vitamin A.

Infant formula is the most thoroughly regulated consumer food product on the market today. A past episode of deficient chloride content in one infant formula led to metabolic alkalosis in some children who received only formula for their total nutritional needs. In 1980, the U.S. Infant Formula Act (P.L. 96-359) was signed into law; this Act places on the manufacturers the responsibility for producing formula containing a specific minimum amount of nutrients and also charges them with maintaining records to support this action. In addition to minimum amounts, in some cases the Act specifies maximum amounts of listed nutrients; requires that the Food and Drug Administration (FDA) be notified when a manufacturer determines that an infant formula may be adulterated or misbranded; authorizes FDA to set requirements for voluntary recalls initiated by firms; and increases the inspection authority of the U.S. Department of Health and Human Services. FDA has established a series of quality control procedures for the manufacture of infant formulas to assure that the formulas contain the necessary nutrients at levels required by the Act.

In April 1982, the Commissioner of Food and Drugs met with executives of the infant formula industry on the subject of analytical variability in methodologies used by industry laboratories and those used by FDA laboratories. In May and June 1982, members of FDA's Center for Food Safety and Applied Nutrition, staff of the infant formula manufacturers, the Infant Formula Council (IFC) staff, representatives of AOAC, and other interested parties met to develop a plan to conduct a collaborative study of methods of analysis for all 24 nutrients required by the Act to be present in milk-based infant formula. Government laboratories in Canada and England also participated in the study under a tripartite agreement. The purposes of this collaborative study were *(1)* to undertake an interlaboratory comparison of methods of analysis for nutrients in infant formula, and *(2)* to identify reference methods of analysis for infant formula for inclusion in *Official Methods of Analysis*.

Because of the large number (24) of nutrient methods to be studied, the collaborative study was divided into 4 phases: Phase I consisted of the analysis of vitamin A, vitamin B_6, calcium, magnesium, potassium, and sodium. Phase II consisted of the analysis of vitamin C, riboflavin, niacin, manganese, copper, iron, and zinc. Phase III included proximates, chloride, phosphorus, total pantothenates, thiamin, vitamin A, vitamin B_{12}, and vitamin E. Phase IV is planned to include the remainder of the nutrients specified by the Act for inclusion in milk-based formulas. The present report will address the first 2 phases of the study, which have been completed.

Collaborative Study

FDA and Infant Formula Council members agreed that a milk-based formula with iron was the most commonly used formula and therefore would be the first product matrix studied. A ready-to-feed (RTF) formula was agreed on to ensure homogeneity of the samples. Three companies, Mead Johnson and Co., Ross Laboratories, and Wyeth Laboratories, currently manufacture the majority of RTF milk-based formula in the United States. These companies agreed to produce sample formulas similar to their usual product for the collaborative study.

Robert Gelardi of the Infant Formula Council, representatives from the production departments of the 3 companies, and Ray Hedblad of the FDA Detroit District Office formed a subcommittee to produce and distribute sample formulas in such a manner that participating laboratories could not identify their manufacturer. All samples were manufactured, then packaged in unlabeled, identical 32 oz cans. Approximately 1000 cans were produced by each manufacturer. Shortly before each phase began, sufficient formula from each company was sent to Mr. Hedblad for coding and distribution to participating collaborators.

For each phase, the FDA Detroit District office sent each collaborator 24 cans of infant formula produced by each of 3 manufacturers. A separate 12-can composite was prepared from each of the 3 sample formulas, and each composite sample was analyzed for the specified nutrients. The remaining 12 cans from each manufacturer for each phase were to be used as back-up samples, if needed. The samples were analyzed as directed within the 60-day time frame per phase as agreed to by the collaborators.

Collaborators were instructed to assay each nutrient on day 1 and repeat the assay on day 2, unless otherwise noted in the method, to give the within-laboratory variability. Immediately before compositing the sample, laboratories were

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The recommendations of the Associate Referee were approved by Committee D and adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.

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Table 1. Collaborative results (mg/L) for calcium in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	457	443	579	587	595	597
2	496.9	446.0	629.2	581.7	680.4	611.1
3	473	465	588	582	606	594
4	498	503	647	655	659	672
5	460	450	590	590	600	600
6	454	445	566	564	575	575
7	445	448	573	581	584	585
8	457	482	592	603	586	631
9	432	431	552	536	563	546
Mean, mg/L[a]	460.3		588.7		603.3	
S_o	14.3		12.6		20.3	
CV_o, %	3.1		2.1		3.4	
S_x	22.3		30.9		37.0	
CV_x, %	4.8		5.2		6.1	

[a]Nine laboratories.

Table 2. Collaborative results (mg/L) for magnesium in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	76.4	76.9	65.0	65.2	60.7	63.2
2	72.2	70.2	63.7	62.1	75.5	73.0
3	82.0	83.0	69.0	70.0	67.0	67.0
4	71.9	73.1	60.8	62.4	58.2	59.5
5	73.0	75.0	61.0	61.0	59.0	60.0
6	75.8	73.7	64.5	62.2	61.4	61.4
7	69.4	68.1	57.4	58.1	55.6	57.4
8	70.0	75.5	57.7	61.2	55.4	60.8
9	69.0	70.0	59.0	59.0	57.0	56.0
Mean, mg/L[a]	73.6		62.2		61.6	
S_o	1.63		1.16		1.64	
CV_o, %	2.2		1.9		2.7	
S_x	4.31		3.68		5.88	
CV_x, %	5.9		5.9		9.5	

[a]Nine laboratories.

Table 3. Collaborative results (mg/L) for sodium in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	181	180	257	254	244	247
2	205.8	218.0	200.5	214.0	203.0	203.8
3	187	193	269	267	264	262
4	173	168	245	256	234	230
5	168	171	250	250	240	230
6	142	155	222	225	207	215
7	162	159	206	239	188	234
8	192	159	258	239	226	242
9	168	172	243	249	233	236
Mean, mg/L[a]	175.2		241.3		230.0	
S_o	9.14		10.0		12.0	
CV_o, %	5.2		4.1		5.2	
S_x	19.2		20.6		20.6	
CV_x, %	11.0		8.5		9.0	

[a]Nine laboratories.

instructed to shake, then warm individual cans for long enough to achieve uniformity, not to exceed 2 h at 60°C. For each phase, a composite sample was prepared from 12 cans by transferring equal aliquots from each can under nitrogen and subdued light to a flask thoroughly cleaned or sterilized according to AOAC method **16.002(b)**(*1*) (1). Composite samples were thoroughly mixed under nitrogen by stirring gently to avoid foaming or excessive inclusion of air. Portions of the composite sample were poured into thoroughly cleaned or sterile 500 mL polyethylene bottles and designated for mineral analysis. All other nutrients were to be analyzed from the remainder of the composite, which was poured into clean or sterile containers with air-tight caps; the containers were closed securely, identified, and refrigerated at 4°C. Labile nutrients were assayed within 4 h after the sample was composited. Results of the analyses were reported to AOAC for statistical review.

Basic references for the methods collaboratively studied are given below. Most of the methods were modified slightly or substantially (2–4); the modified versions are the methods that were recommended for adoption. Several of the methods are microbiological assays, and although these are older methods that use very little instrumentation, they are still the most effective methods for the analysis of certain nutrients.

Table 4. Collaborative results (mg/L) for potassium in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	767	743	764	742	955	947
2	702.2	707.9	661.9	676.9	901.7	903.7
3	878	866	833	803	1084	1078
4	820	774	814	767	994	948
5	740	750	750	760	920	930
6	733	757	730	744	901	982
7	735	695	754	734	897	903
8	793	725	789	665	965	911
9	757	765	764	766	940	930
Mean, mg/L[a]	761.6		751		949.5	
S_o	23.4		33.2		25.8	
CV_o, %	3.1		4.4		2.7	
S_x	52.0		47.7		57.1	
CV_x, %	6.8		6.4		6.0	

[a]Nine laboratories.

Table 5. Collaborative results (IU/L) for vitamin A in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	3545.7	3519.3	2421.1	2280.6	4079.4	3837.8
2	5028	5919	2835	2811	5041	4473
4	3700	3700	2200	2300	4200	3800
5	7300[a]	9700[a]	3100	3200	6400	7200
6	3520	3370	2540	2270	3890	4180
7	4500	4400	2000	2000	3500	3400
8	3314	3550	1952	2147	3721	3371
9	4120	4460	2650	2650	4280	4270
Mean, mg/L[b]	4046.1		2459.8		4352.7	
S_o	267		97.2		295.6	
CV_o, %	6.6		4.0		6.8	
S_x	774.2		399.3		1089.6	
CV_x, %	19.1		16.2		25.0	

[a]Rejected as outliers by Youden test.
[b]Sample A: 7 labs; Sample B: 8 labs; Sample C: 8 labs.

Table 6. Collaborative results (mg/L) for vitamin B_6 in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	0.74	0.73	0.64	0.64	0.64	0.63
2	0.66	0.67	0.54	0.51	0.52	0.53
4	0.682	0.661	0.518	0.555	0.544	0.569
5	0.61	0.60	0.54	0.52	0.51	0.50
6	0.531	0.541	0.475	0.362	0.359	0.413
7	0.763	0.743	0.623	0.616	0.639	0.644
8	0.81	0.76	0.69	0.69	0.67	0.66
Mean, mg/L[a]	0.679		0.566		0.559	
S_o	0.0163		0.0333		0.0168	
CV_o, %	2.4		5.9		3.0	
S_x	0.0893		0.0937		0.0989	
CV_x, %	13.0		16.6		17.7	

[a]Seven laboratories.

Methods

Vitamin A.—Carr-Price colorimetric methods, **43.008–43.013** (1).

Riboflavin.—Fluorometric method, **43.039–43.042** (1).

Vitamin B_6.—Microbiological method, **43.229–43.234** (1), with modifications (5).

Vitamin C.—Method **43.064–43.068** (1), with modifications.

Niacin.—Microbiological method, **43.196–43.199** (1), with modifications.

Minerals.—Composite of several AOAC methods.

Nutrients in Ready-to-Feed Milk-Based Infant Formula

First Action

43.A21 ***Compositing of Sample***

To prep. composite, select 12 cans of ready-to-feed formula, 1 from each of 12 different randomly chosen shipping cases which are representative of a lot. (Lot comprises cans with common container code or marking, or a day's production.)

Warm previously shaken individual cans at 60°, sufficient time to achieve uniformity but <2 h, shaking occasionally. Transfer equal aliquots under N and subdued light from each can to flask thoroly cleaned or sterilized by **16.002(b)(1)**. Thoroly mix combined liq.

Table 7. Collaborative results (mg/L) for zinc in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	5.42	5.10	6.75	6.48	7.94	7.92
2	4.34	4.34	5.85	5.86	7.18	7.25
3	4.5	4.6	6.0	6.1	7.5	7.6
4	4.4	4.5	6.2	6.0	7.4	7.3
5	4.5	4.6	6.2	6.1	7.5	7.5
6	4.76	4.46	6.28	6.04	7.60	7.46
7	4.31	4.36	5.79	5.84	7.52	7.37
8	4.78	4.01	6.15	5.23	7.40	6.51
9	4.7	4.6	6.3	6.0	7.9	8.1
Mean, mg/L[a]	4.57		6.06		7.50	
S_o	0.21		0.25		0.22	
CV_o, %	4.6		4.1		2.9	
S_x	0.32		0.32		0.36	
CV_x, %	7.0		5.3		4.8	

[a]Nine laboratories.

Table 8. Collaborative results (mg/L) for iron in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	15.9	15.2	17.1	16.7	18.4	17.7
2	12.8	12.8	14.4	14.4	14.9	15.0
3	13.8	13.8	16.2	15.4	17.2	16.6
4	16.0	15.6	18.3	17.7	19.5	18.4
5	15.0	15.1	17.0	16.8	17.6	18.2
6	13.8	13.3	15.5	15.1	16.5	16.0
7	21.3	21.3	23.9	25.7	24.7	25.6
8	14.4	14.5	15.4	15.7	16.2	17.1
9	15.3	14.0	17.3	15.2	17.8	17.6
Mean, mg/L[a]	15.2		17.1		18.1	
S_o	0.38		0.71		0.49	
CV_o, %	2.5		4.2		2.7	
S_x	2.5		3.1		2.9	
CV_x, %	16.4		18.1		16.0	

[a]Nine laboratories.

Table 9. Collaborative results (mg/L) for copper in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
2	0.507	0.511	0.648	0.651	0.768	0.748
3	0.45	0.45	0.70	0.69	0.75	0.75
4	0.44	0.42	0.78	0.79	0.78	0.79
5	0.57	0.55	0.84	0.90	0.90	0.99
6	0.521	0.523	0.822	0.799	0.842	0.840
7	0.64	0.64	0.98	0.96	0.92	0.96
8	0.526	0.518	0.796	0.797	0.808	0.860
9	0.51	0.49	0.86	0.75	0.89	0.83
Mean, mg/L[a]	0.52		0.80		0.84	
S_o	0.0009		0.032		0.032	
CV_o, %	1.7		4.0		3.8	
S_x	0.065		0.101		0.078	
CV_x, %	12.5		12.6		9.3	

[a]Eight laboratories.

under N by gently stirring so as to avoid foaming or excessive inclusion of air. Pour portion of mixed sample into 500 mL thoroly cleaned or sterile polyethylene bottle and designate for mineral analysis. Pour aliquots of remaining composite into clean or sterile containers with air-tight caps and close securely. Identify samples. Refrigerate at 4°. Assay labile nutrients, vitamins A and C, immediately after compositing sample; assay other nutrients as soon as possible.

43.A22 *Sampling Liquids for Analysis*

Let composite subsample come to room temp. in area protected from direct light. Gently mix and transfer portion of subsample to small vessel from which an aliquot for analysis is withdrawn.

Maintain clean or sterile conditions in subsample by not introducing unclean pipets, stirrers, etc., into liq.

Riboflavin

Fluorometric Method

43.A23 See **43.039–43.042.**

Vitamin B_6 (Pyridoxine, Pyridoxal, Pyridoxamine)

Microbiological Method

(Caution: Loosen caps or stoppers on tubes when liqs are being autoclaved.)

Table 10. Collaborative results (μg/L) for manganese in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	196	201	184	187	64.8	64.9
2	224	205	227	207	109	84.7
3	210	220	200	190	70	70
4	190	200	200	190	80	70
5	240	240	240	220	107	94
6	179	183	170	167	67.4	71.3
7	200	200	200	200	60	60
8	223	224	204	209	96	81
9	200	200	180	170	70	90
Mean, mg/L[a]	207.5		197		73.8	
S_o	5.8		8.0		9.1	
CV_o, %	2.8		4.1		11.6	
S_x	18.0		20.2		15.6	
CV_x, %	8.7		10.3		19.9	

[a]Nine laboratories.

Table 11. Collaborative results (mg/L) for riboflavin in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	2.42	2.40	1.06	1.06	1.66	1.72
2	2.34	2.41	1.10	1.10	1.63	1.69
4	2.42	2.52	1.08	1.08	1.75	1.80
5	2.00	2.47	1.06	1.38	1.67	1.60
6	2.33	2.32	0.877	1.00	1.63	1.83
7	2.3	2.2	0.98	0.97	1.6	1.5
8	2.21	2.29	0.96	1.03	1.60	1.64
9	2.31	2.43	1.08	1.10	1.66	1.63
Mean, mg/L[a]	2.34		1.06		1.66	
S_o	0.13		0.09		0.07	
CV_o, %	5.6		8.5		4.2	
S_x	0.13		0.11		0.08	
CV_x, %	5.6		10.4		4.8	

[a]Eight laboratories.

43.A24 ***Reagents***

(Work in subdued light with all solns contg vitamin B_6.)

(a) *Vitamin soln I.*—Dissolve 10 mg thiamine and 1 g inositol in ca 200 mL H_2O and dil to 1 L. Store in refrigerator. (1 mL = 10 μg thiamine and 1 mg inositol.)

(b) *Vitamin soln II.*—Dissolve 10 mg biotin in 100 mL 50% alcohol. Store in refrigerator. (1 mL = 100 μg biotin.) Dissolve 200 mg Ca pantothenate and 200 mg niacin in ca 200 mL H_2O; add 8 mL biotin soln and dil. to 1 L with H_2O. Store in refrigerator. (1 mL = 200 μg each Ca pantothenate and niacin and 0.8 μg biotin.)

(c) *Salt soln I.*—Dissolve 17 g KCl, 10.3 g $MgSO_4.7H_2O$, 100 mg $FeCl_3.6H_2O$, and 100 mg $MnSO_4.H_2O$ in ca 800 mL H_2O. Add 2 mL HCl. Dissolve 5 g $CaCl_2.2H_2O$ in ca 100 mL H_2O, add to first soln, and dil. to 1 L with H_2O. Store in refrigerator. (1 mL = 17 mg KCl, 10.3 mg $MgSO_4.7H_2O$, 100 μg $FeCl_3.6H_2O$, 100 μg $MnSO_4.H_2O$, and 5 mg $CaCl_2.2H_2O$.)

(d) *Salt soln II.*—Dissolve 22 g KH_2PO_4 and 40 g $(NH_4)_2HPO_4$ in H_2O and dil. to 1 L. Store in refrigerator. (1 mL = 22 mg KH_2PO_4 and 40 mg $(NH_4)_2HPO_4$.)

(e) *Polysorbate 80 soln.*—Weigh 2.5 g polysorbate 80 (Tween 80) in small beaker. Transfer with warm (45°) H_2O and dil. to 500 mL. Store in refrigerator. (1 mL = 5 mg polysorbate 80.)

(f) *Citric acid soln.*—(1 + 1). Dissolve 50 g citric acid in 50 mL H_2O. Store at room temp. in bottle with plastic stopper.

(g) *Ammonium phosphate soln.*—(1 + 2). Dissolve 25 g $(NH_4)_2HPO_4$ in 50 mL H_2O. Store at room temp. in bottle with plastic stopper.

(h) *Pyridoxine std solns.*—(*1*) *Stock soln.*—10.0 μg/mL. Dissolve 12.16 mg pyridoxine.HCl in 1N HCl and dil. to 1 L with 1N HCl. Store in g-s bottle in refrigerator. (*2*) *Intermediate soln.*—100 ng/mL. Dil. 5 mL stock soln to 500 mL with H_2O. Prep. fresh for each assay. (*3*) *Working soln.*—1.0 ng/mL. Dil. 5 mL intermediate soln to 500 mL with H_2O and mix. Prep. fresh for each assay.

(i) *Citrate buffer soln.*—Dissolve 65 g KOH and 82 g citric acid in H_2O and dil. to 1 L with H_2O.

(j) *Basal medium stock soln (for 200 tubes).*—To make 1 L medium, add to ca 400 mL H_2O: 100 mL citrate buffer, 2 g asparagine, 50 mL vitamin soln I, 50 mL vitamin soln II, 50 mL salt soln I, and 50 mL salt soln II. Dissolve 100 g glucose in this soln. Dissolve 22 mg DL-tryptophan, 27 mg L-histidine.HCl, 100 mg DL-methionine, 216 mg DL-isoleucine, and 256 mg DL-valine in 10 mL HCl (1 + 9) in small beaker and add to above. Add 20 mL polysorbate 80 soln. Adjust to pH 4.5 with citric acid (1 + 1) or $(NH_4)_2HPO_4$ (1 + 2) solns. Dil. to 1 L with H_2O. Store in Pyrex bottle plugged with cotton in refrigerator. Prep. ≤ 24 h before use.

(k) *Test organism.*—*Saccharomyces uvarum* (ATCC No. 9080). Maintain by weekly transfers on wort agar slants (l). Incubate these freshly seeded agar slants 24 h at 30° and refrigerate.

(l) *Agar culture medium.*—Suspend 25 g Bactowort agar in ca 400 mL H_2O in marked 500 mL wide-mouth erlenmeyer. Cover to prevent contamination, steam ca 10 min to dissolve agar, and adjust vol. to 500 mL. Pipet hot agar in ca 7 mL portions into 20 × 150 mm test tubes, plug with absorbent cotton, and autoclave 15 min at 121°. Since this medium has acid reaction, avoid overheating, which results in softer medium. Tilt hot agar tubes to form slants and cool in this position.

(m) *Liquid culture medium.*—Dil. 20 mL intermediate soln, (h)(*2*), to 1 L with H_2O in vol. flask. Pipet 5 mL of this soln and 5 mL basal medium stock soln, (j), into 16 × 150 mm screw-cap tubes contg two 4 mm glass beads. Autoclave 10 min at 121°. Cap, and store tubes in refrigerator.

(n) *Inoculum rinse.*—Pipet 5 mL H_2O and 5 mL basal medium stock soln, (j), into stainless steel-cap or screw-cap 16 × 150 mm test tubes. Autoclave 10 min at 121°.

Table 12. Collaborative results (mg/L) for vitamin C in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	100.45	86.78	77.21	53.50	126.31	114.42
2	96.1	92.0	62.9	58.8	121.0	117.0
4	99.0	85.0	74.0	62.0	120.0	108.0
5	98.0	98.0	67.0	65.0	123.0	124.0
6	101.0	101.0	73.9	70.1	125.0	126.0
7	94.6	87.8	69.8	66.4	121.4	111.5
8	103.2	101.7	77.9	76.1	128.4	123.3
9	102.6	101.4	72.08	69.98	121.1	115.2
Mean, mg/L[a]	96.8		68.5		120.4	
S_o	5.3		6.9		5.4	
CV_o, %	5.5		10.1		4.5	
S_x	6.0		6.9		5.7	
CV_x, %	6.2		10.1		4.7	

[a]Eight laboratories.

Table 13. Collaborative results (mg/L) for niacin in infant formula

	Sample A		Sample B		Sample C	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	5.86	5.76	8.74	8.53	8.02	7.61
2	7.53	7.84	11.6	11.9	10.2	10.7
4	6.9	6.4	9.7	9.7	8.8	8.7
5	6.6	5.9	9.9	9.2	8.5	8.5
6	6.63	6.92	10.4	10.1	9.34	8.62
7	6.53	7.27	10.33	10.40	9.24	8.81
8	6.52	5.84	10.16	9.42	9.06	8.20
9	6.15	5.85	8.2	8.8	9.55	9.75
Mean, mg/L[a]	6.53		9.82		8.98	
S_o	0.36		0.32		0.35	
CV_o, %	5.5		3.3		3.9	
S_x	0.65		1.06		0.83	
CV_x, %	10.0		10.8		9.2	

[a]Eight laboratories.

43.A25 ***Assay Inoculum***

(Caution: See **51.005**.)

Incubate cells for inoculum on agar 24 h at 30° before use. Transfer these cells under aseptic conditions to liq. broth culture tubes. Place tubes on shaker 20 h in 30° room or in mech. shaker H_2O bath 20 h at 30°. Centrf. 1.5 min at 2500 rpm. Decant liq. and resuspend in 10 mL inoculum rinse. Sep. by centrfg 1.5 min at 2500 rpm. Decant liq., resuspend in second 10 mL sterile inoculum rinse, centrf. 1.5 min, and decant. Resuspend in third 10 mL sterile inoculum rinse. Add 1 mL of third suspension to 10 mL inoculum rinse. This is assay inoculum.

43.A26 ***Preparation of Sample***

Weigh 10.0 g composite into 500 mL erlenmeyer. Add 200 mL 0.05N HCl. Autoclave 5 h at 121°. Cool to room temp., and adjust to pH 4.5 with 6N KOH. Quant. transfer soln to 250 mL vol. flask, and dil. to vol. with H_2O. Filter thru Whatman No. 40 paper. Dil. 1 mL of this soln to 20 mL with H_2O. This is assay soln.

43.A27 ***Assay***

Heat 16 × 150 mm screw-cap tubes contg two 4 mm glass beads 2 h at 260°. For std curve, pipet into triplicate tubes, 0.0, 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL pyridoxine working soln, (**h**)(*3*). Similarly pipet 1.0, 2.0, 3.0, and 4.0 mL assay soln into duplicate tubes. Pipet H_2O into all tubes to bring vol. to 5 mL/tube. Cap all tubes and autoclave 10 min at 121°. Cool to room temp. using H_2O bath or refrigerator. Aseptically inoculate, except first set of 0.0 level for std curve, with 1 drop assay inoculum. Incubate tubes on const reciprocal shaker 22 h in temp.-regulated room (30°) or in mech. shaker H_2O bath 22 h at 30°. Steam tubes in autoclave 5 min. Cool and read $\%T$ at 550 nm on spectrophtr. Set 100% T with H_2O to read uninoculated blanks. Set 100% T with uninoculated blank to read inoculated blank. Mix the 3 inoculated blank tubes, and with this mixture set at 100% T, read all remaining tubes.

Av. readings for std curve triplicate tubes and plot $\%T$ against ng pyridoxine for each level of std soln on semilog paper. Det. amt of pyridoxine/sample tube by interpolation. Calc. μg pyridoxine/g sample. (Calcn may be performed using computer program as in JAOAC **56**, 754(1973).)

CAS-65-23-6 (pyridoxine)

Vitamin C (Reduced Ascorbic Acid)

2,6-Dichloroindophenol Titrimetric Method

43.A28 ***Principle***

Ascorbic acid is estd by titrn with colored oxidation-reduction indicator, 2,6-dichloroindophenol. EDTA is added as chelating agent to remove Fe and Cu interferences.

43.A29 ***Reagents***

(a) *Precipitant soln.*—Dissolve with shaking 15 g glacial HPO_3 pellets in 40 mL glacial HOAc and 150 mL H_2O. Dil. to 250 mL with H_2O and filter rapidly thru folded qual. paper (rapid, 18.5 cm, Whatman No. 541 or equiv.) into 500 mL beaker.

Dissolve with shaking 0.9 g EDTA in 200 mL H_2O and dil. to 250 mL. Mix equal vols of HPO_3 and EDTA solns immediately before use.

(b) *Ascorbic acid std soln.*—1 mg/mL. Accurately weigh 50 mg USP Ascorbic Acid Ref. Std (stored in desiccator away from sunlight). Transfer to 50 mL vol. flask. Dil. to vol. with precipitant

Table 14. Revised mean and other related measures when outliers are omitted

Nutrient	Sample	No. labs	Mean	S_o	CV_o, %	S_x	CV_x, %	Rejected as outlier, Coll. No.	Outlier test
Calcium (Table 1)	A	7	458.7	16.18	3.5	16.18	3.5	4,9	Youden
	B	7	586.1	13.48	2.3	16.35	2.8	4,9	Youden
	C	7	601.4	22.32	3.7	27.47	4.6	4,9	Youden
Sodium (Table 3)	C	8	232.3	5.35	2.3	18.98	8.2	7	Cochran
Potassium (Table 4)	A	8	747.8	24.61	3.3	33.40	4.5	3	Youden
	B	7	744.8	16.08	2.2	39.14	5.2	3,8[a]	Youden
	C	8	933.0	27.27	2.9	30.57	3.3	3	Youden
Vitamin A (Table 5)	A	7	4046.1	267.04	6.6	774.17	19.1	5	Youden
	B	7	2361.2	100.44	4.2	307.94	13.0	5	Youden
	C	7	4003.1	232.64	5.8	469.49	11.7	5	Youden
Vitamin B_6 (Table 6)	A	5	0.69	0.0107	1.6	0.0599	8.7	6,8	Youden
	B	5	0.57	0.0165	2.9	0.0562	9.9	6,8	Youden
	C	5	0.57	0.0097	1.7	0.0631	11.0	6,8	Youden
Zinc (Table 7)	A	8	4.48	0.213	4.8	0.213	4.8	1	Dixon
	B	8	6.00	0.257	4.3	0.257	4.3	1	Dixon
	C	8	7.56	0.082	1.1	0.276	3.6	8	Cochran
Iron (Table 8)	A	7	14.7	0.43	2.9	0.90	6.1	2,7	Youden
	B	7	16.4	0.65	4.0	1.05	6.4	2,7	Youden
	C	7	17.5	0.50	2.9	1.00	5.7	2,7	Youden
Manganese (Table 10)	A	8	203.4	6.14	3.0	14.21	7.0	5	Youden
	B	8	192.8	6.81	3.5	16.72	8.7	5	Youden
	C	8	75.6	9.12	12.1	13.93	18.4	5	Youden
Riboflavin (Table 11)	A	7	2.35	0.057	2.4	0.091	3.9	5	Cochran
	B	7	1.03	0.038	3.7	0.069	6.7	5	Cochran
Vitamin C (Table 12)	A	7	96.0	5.65	5.9	5.91	6.2	8	Youden
	B	7	67.3	7.36	10.9	7.36	10.9	8	Youden
	C	7	119.6	5.58	4.7	5.59	4.7	8	Youden
Niacin (Table 13)	A	7	6.37	0.372	5.8	0.490	7.7	2	Youden
	B	7	9.54	0.331	3.5	0.764	8.0	2	Youden
	C	7	8.76	0.344	3.9	0.612	7.0	2	Youden

[a]Coll. 8 was rejected by Cochran's test at $P < 0.01$.

soln, (a). Prep. *immediately* before use in stdzn of indophenol std soln.

(c) *Indophenol std soln.*—Dissolve 0.0625 g 2,6-dichloroindophenol Na salt (stored in desiccator over soda lime) in 50 mL H_2O in 250 mL vol. flask to which has been added 0.0525 g reagent grade $NaHCO_3$. Shake vigorously; when dye dissolves, dil. to 250 mL with H_2O. Filter thru rapid-flow folded paper into amber glass bottle. Store in refrigerator.

Transfer three 2.0 mL aliquots of ascorbic acid std soln, (b), into each of three 50 mL erlenmeyers contg 5 mL precipitant soln, (a). Using 25 mL buret calibrated in 0.05 mL and with glass or Teflon stopcock, titr. rapidly with indophenol std soln until light but distinct rose-pink persists for 5 s. (Each titrn should require ca 15 mL and titrns should check ±0.1 mL.) Titr. 3 blanks composed of 7 mL precipitant soln plus 15 mL H_2O. Av. blank is 0.1 mL. Calc. dye equivs: ascorbic acid equiv. to 1 mL indophenol std soln = mg ascorbic acid/(mL dye − blank titrn) = 2 mg/(mL dye − blank titrn).

43.A30 *Preparation of Sample Assay Solution*

Pipet 25–30 mL composite and equal vol. of precipitant soln, (a), into 125 mL beaker. Designate total vol. as V mL and vol. of composite aliquoted as E mL. Filter thru folded rapid qual. paper, 18.5 cm (Whatman No. 54, or equiv.). Designate filtrate as assay soln.

43.A31 *Determination*

Pipet three 10 mL aliquots of assay soln each into sep. 50 mL erlenmeyers and titr. with indophenol std soln. Similarly titr. 2 blanks composed of mL precipitant soln and H_2O equiv. to their resp. vols in assay soln aliquot titrd. Add vol. of H_2O equiv. to mL indophenol std soln used in titrn of assay soln. Titr. with indophenol std soln to same color end point observed in titrn of std aliquot.

mg ascorbic acid/L ready-to-feed formula

$$= (X - B) \times (F/E) \times (V/Y) \times 1000$$

where X = av. mL for assay soln titrn; B = av. mL for assay soln blank titrn; F = mg ascorbic acid equiv. to 1.0 mL indophenol std soln; E = vol. composite aliquot; V = assay soln vol.; Y = vol. assay soln titrd = 10 mL; 1000 = conversion of mL to L.

CAS-50-81-7 (ascorbic acid)

Niacin and Niacinamide (Nicotinic Acid and Nicotinamide)

Microbiological-Turbidimetric Method

43.A32 *Basal Medium Stock Solution*

Using solns prepd as in **43.167**, combine, with mixing, and in following order: 25 mL cystine–tryptophan soln, (e); 5 mL adenine–guanine–uracil soln, (b); 5 mL vitamin soln IV, (o); 5 mL vitamin soln V, (p); 5 mL salt soln A, (i); and 5 mL salt soln B, (j). Add ca 100 mL H_2O, and add, with mixing, 2.5 g vitamin-free casein, hydrolyzed, (a); 10 g anhyd. glucose; and 5 g NaOAc, anhyd. When soln is complete, adjust to pH 6.8 with NaOH soln, and dil. with H_2O to 250 mL. (Some comm. sources of basal media have been found satisfactory, e.g., Difco niacin assay medium (0322-15).)

43.A33 *Niacin Standard Solutions*

(a) *Stock soln.*—100 μg/mL. Accurately weigh, in closed system, 50–60 mg USP Niacin Ref. Std that has been dried to const wt and stored in dark over P_2O_5 in desiccator. Dissolve in 25% alcohol, and dil. with addnl 25% alcohol to make niacin concn exactly 100 μg/mL. Store in dark at ca 10°.

(b) *Intermediate soln.*—1 μg/mL. Dil. 5 mL stock soln, (a), with 25% alcohol to 500 mL.

(c) *Working soln.*—0.014 μg/mL. Dil. 7 mL intermediate soln, (b), with H_2O to 500 mL. Also prep. secondary working solns, one higher and one lower in niacin concn (usually 0.01 and 0.02 μg/mL)

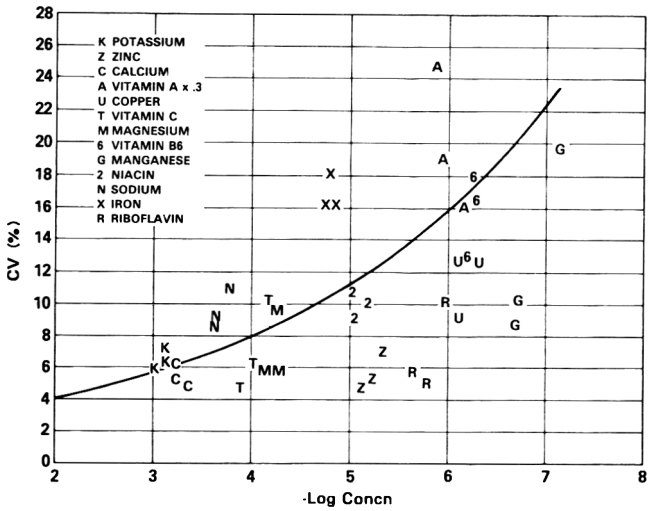

Figure 1. Infant formula collaborative study results, Phases I and II. Plot of coefficient of variation (CV) vs concentration.

than primary working soln. This is done in case sample niacin concn is too high or too low for primary working soln to be used.

43.A34 *Inoculum*

(a) *Liquid culture medium.*—Dil. measured vol. basal medium stock soln, **43.A32**, with equal vol. aq. soln contg 0.2 μg niacin/mL. Add 10 mL portions dild medium to test tubes, cover to prevent contamination, sterilize 15 min in autoclave at 121–123°, and cool tubes as rapidly as practicable to avoid color formation from overheating. Store in dark at ca 10°.

(b) *Inoculum.*—Make transfer of cells from stock culture of *Lactobacillus plantarum*, **43.169(c)**, to sterile tube contg 10 mL liq. culture medium, (**a**). Incubate 6–24 h in const temp. bath at 37°. Under aseptic conditions, centrf. culture and decant supernate. Wash cells with 3 ca 10 mL portions sterile 0.9% NaCl soln. Resuspend cells in 10 mL sterile 0.9% NaCl soln. Cell suspension so obtained is inoculum.

43.A35 *Assay Solution*

Place measured amt of composite contg 10–30 μg niacin in flask and add vol. 1N H_2SO_4 equal in mL to ≥10 times dry wt sample in g. Then agitate vigorously and wash down sides of flask with 1N H_2SO_4.

Autoclave mixt. 30 min at 121–123° and cool. If lumping occurs, agitate mixt. until particles are evenly dispersed.

If dissolved protein is present, adjust mixt., with vigorous agitation, to pH 6.0–6.5 with NaOH soln; then immediately add dil. HCl until no further pptn occurs (usually ca pH 4.5, isoelec. point of many proteins). Dil. mixt. to measured vol. with H_2O, and filter. (In case of mixt. difficult to filter, centrfg and/or filtering thru fritted glass, using suitable anal. filter-aid, may often be substituted for, or may precede, filtering thru paper. Ash-free filter paper pulp and Celite Analytical Filter-Aid have been found satisfactory.) Take aliquot of clear filtrate and check for dissolved protein by adding dropwise, first dil. HCl, and if no ppt forms, then, with vigorous agitation, NaOH soln, and proceed as follows with this aliquot:

(*1*) If no further pptn occurs, add, with vigorous agitation, NaOH soln to pH 6.8, and dil. with H_2O to final measured vol. contg, per mL, niacin ca equiv. to that of std soln, **43.A33(c)**. If cloudiness occurs, refilter.

(*2*) If further pptn occurs, adjust mixt. again to point of max. pptn, dil. with H_2O to measured vol., and then filter. Take aliquot of clear filtrate and proceed as in (*1*).

Designate final measured vol. obtained as assay soln.

43.A36 *Assay*

Using std soln, **43.A33(c)**, assay soln, **43.A35**, basal medium stock soln, **43.A32**, and inoculum, **43.A34(b)**, proceed as in **43.170**, **43.173**, and **43.174**. Value so obtained is potency of sample expressed as niacin. Multiply this value by 0.992 if potency is to be expressed as niacinamide.

CAS-59-67-6 (niacin)
CAS-98-92-0 (niacinamide)

Minerals

Atomic Absorption Spectrophotometric Method

(Caution: See **51.006**.)

(Applicable to Ca, Mg, Fe, Zn, Cu, Mn, Na, and K)

43.A37 *Apparatus*

(a) *Glassware.*—Thoroly clean all glassware by soaking overnight in 20% HNO_3. Rinse all glassware 3 times with distd-deionized or 18 M Ω resistance H_2O.

(b) *Evaporating dish.*—100 mL unetched Vycor (or Pt), flat-bottom, with pour spout, which withstands temps to 600°.

(c) *Atomic absorption spectrophotometer.*—Perkin-Elmer 503, or equiv.

(d) *Furnace.*—With pyrometer to control temp. range of 250–600° ± 10°.

43.A38 *Reagents*

(a) *Water.*—Distd, deionized, or 18 M Ω resistance (DD), for prepn of std or sample solns.

(b) *Std stock solns.*—Com. prepd, certified AA stds, or prepd in laboratory by one of the following methods: Na, **18.038(c)**; K, from Spex Industries, Inc., Box 798, Metuchen, NJ 08840. Mn, **2.127(f)**; Zn, **2.127(g)**.

(c) *Nitric acid.*—Unless specified otherwise, use redistd or ultrapure.

(d) *Lanthanum oxide.*—La_2O_3, 99.99%; AAS quality. (Alfa Division, Ventron Corp., Box 299, 152 Andover St, Danvers, MA 01923, Stock No. 87808).

(e) *Lanthanum chloride soln.*—$LaCl_3$, 1% w/v. Weigh 11.7 g (± 100 mg) La_2O_3 and transfer to 1 L vol. flask. Add sufficient DD H_2O to wet powder and then slowly add 50 mL concd HCl (Caution: Exothermic reaction). Let powder dissolve and then dil. to vol. with DD H_2O and mix. Make fresh every 6 months or purchase from Spex Industries, Inc., Box 798, Metuchen, NJ 08840.

(f) *Cesium chloride soln.*—CsCl, 10% w/v. Weigh 12.7 g (± 100 mg) CsCl (Spex Industries Cat. No. Cs-35) and transfer to 100 mL vol. flask. Dil. to vol. with DD H_2O and mix. Make fresh every 6 months.

(g) *Filter pulp* —Anal. ash-free (American Scientific Products, 1430 Waukegan Rd, McGaw Park, IL 60085, or equiv.).

43.A39 *Ashing Procedure*

Place composite aliquot in previously cleaned Vycor evap. dish (which may contain 5 g filter pulp for ease of handling). Exact amt of composite required will depend on concn of minerals present. In general, 25 mL will be adequate. If some minerals, in particular Fe, Cu, or Mn, are at very low levels, larger sample size (up to 50 mL) may be necessary.

Dry aliquot in 100° oven overnight or in microwave oven (programmed over ca 30 min). After sample is dry, heat on hot plate until smoking ceases. Place sample in 525° furnace (carefully avoiding ignition) for min time necessary to obtain ash that is white and free from C, normally 3–5 h, but <8 h. Remove sample from furnace and let cool. Ash should be white and free from C. If ash contains C particles (i.e., it is gray), wet with DD H_2O and add 0.5–3 mL HNO_3. Dry on hot plate or steam bath and return sample to 525° furnace 1–2 h. Repeat until ash is white. (This step should be avoided if possible because K may be lost.)

Dissolve in 5 mL 1N HNO_3, warming on steam bath or hot plate 2–3 min to aid in soln. Add soln to 50 mL vol. flask and repeat with 2 addnl portions of 1N HNO_3. Dil. to 50 mL with 1N HNO_3.

43.A40 ***Determination***

Add $LaCl_3$ soln to each std and sample final diln to make 0.1% w/v La for detn of Ca and Mg only. Add CsCl soln to each std and sample final diln to make 0.5% w/v Cs (0.04M) for detn of Na and K only.

Prep. blanks representing all reagents and glassware, and carry thru entire procedure.

Prep. calibration curve (concn vs *A*) for each mineral to be detd,

Element	Wavelength, nm (instrument setting-UV/vis range)	Flame
Ca (CAS-7440-70-2)	422.7 (211-vis)	reducing air–C_2H_2
Cu (CAS-7440-50-8)	324.7 (325-UV)	oxidizing air–C_2H_2
Fe (CAS-7439-89-6)	248.3 (248-vis)	oxidizing air–C_2H_2
K (CAS-7440-09-7)	766.5 or 769.9 (383-vis)	oxidizing air–C_2H_2
Na (CAS-7440-23-5)	589.0 (295-vis)	oxidizing air–C_2H_2
Mg (CAS-7439-95-4)	285.2 (285-UV)	oxidizing air–C_2H_2
Mn (CAS-7439-96-5)	279.5 (279-UV)	oxidizing air–C_2H_2
Zn (CAS-7440-66-6)	213.9 (214-UV)	oxidizing air–C_2H_2

using wavelength and flame specified in table. Optimize flame parameters in accordance with instrument manufacturer's instructions. Prep. solns for calibration of instrument to cover linear range of calibration curve. See instrument instruction manual.

Assay samples in similar manner. Det. concn of each mineral from its calibration curve, and calc. concn in sample, taking into account sample size and dilns.

Results and Discussion

Results of the study are given in Tables 1–14. Figure 1 shows a plot of the coefficients of variation against the concentration (log scale). A value of 7 for the negative log of the concentration corresponds to a concentration of 0.1 ppm, 6 corresponds to 1 ppm, 5 corresponds to 10 ppm, and so on. The solid line represents the average CV for a large number of collaborative studies at various concentrations. A complete discussion of this curve is given by Horwitz et al. (6). It is used here to represent the "expected" coefficient of variation at the various concentrations. As shown here, the precision corresponds to the "expected" curve with few exceptions.

After the complete set of methods for nutrient analysis of milk-based infant formulas has been established, the collaborators are expected to begin the study of methodology for soy-based, powdered, and whey-based infant formulas.

Recommendations

On the basis of the results of this collaborative study, it is recommended that the methods of analysis for vitamin C, riboflavin, vitamin B_6, niacin, calcium, magnesium, iron, zinc, copper, manganese, sodium, and potassium in milk-based infant formula be adopted official first action.

The method for vitamin A described in this study is not recommended for adoption because of the high coefficient of variation. However, until more suitable methodology is developed, it may be used for analysis of milk-based infant formula provided the proper error limits are applied.

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Wyeth Laboratories, Mason, MI

Food Directorate, Sir Frederick G. Banting Research Center, Tunney's Pasture, Ottawa, Ontario, Canada

Hazleton Americana, Inc., Madison, WI

Ministry of Agriculture, Fisheries and Food, London, UK

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DRUGS

Liquid Chromatographic Determination of Morphine Sulfate and Some Contaminants in Injections and Bulk Drug Material

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A liquid chromatographic (LC) procedure is described for the assay of morphine sulfate in bulk drug material and injection solutions. The bulk drug and injection samples are prepared by direct dilution with LC mobile solvent. The average bulk drug purity (5 manufacturers) determined by the LC method was 99.9% with a difference of 0.1% from the average purity (anhydrous) found by the official USP XX procedure. The average LC recovery (19 studies) of morphine sulfate added to injection samples was 99.4% with a coefficient of variation (CV) of 1.14%. Morphine sulfate content was determined in triplicate for 53 injection samples (1–15 mg morphine sulfate/mL) formulated by 6 manufacturers, using the proposed LC procedure. Individual sample CV (n = 3) averaged 1.14%. The LC method is simple and specific for morphine sulfate. Major degradation products, preservatives, and some contaminants and related compounds are separated during LC.

The official USP XX method (1) of analysis for morphine sulfate in injections employs a slight modification of the procedure of Holcomb et al. (2). In the USP procedure, morphine is separated from preservatives, pseudomorphine, and other degradation products by a column chromatographic-extraction technique. However, the method cannot be automated for a large number of assays, nor does it give information on the type of preservative, degradation products, or other contaminants in the injection.

Several investigators have used liquid chromatography (LC) to determine morphine in injections (3–5) and in illicit street heroin (6–12). In this paper we report an LC method that is not only useful for determining morphine sulfate in bulk drug substances and injections, but is also capable of separating and, if desired, quantitating the degradation product pseudomorphine (13), the contaminant 2-mercaptobenzothiazole (2-MCBT) (14), the injection preservatives, and the sodium bisulfite interaction product, 8-sulfonic acid dihydromorphinone (8-SDHM) (15). The proposed method is a modified version of a procedure (6) for determining morphine, codeine, heroin, and other related narcotic compounds in illicit street drugs.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Modular unit, or equivalent, consisting of Waters Model M-45 4000 PSI pump, Waters Model WISP 710A automatic injector, Waters Model 730 recording integrator (Waters Associates, Milford, MA 01757), variable wavelength ultraviolet (UV) detector with sensitivity range from 0.01 to 2.0 absorbance units full scale at 284 and 323 nm (Model SF 769Z, Kratos, Inc., Pasadena, CA 91109), and injection valve (Model 7120, Rheodyne Inc., Berkeley, CA 94710).

(b) *Reverse phase LC column.*—4.6 mm × 30 cm 10 μm C18 octadecylsilane (Waters Associates).

(c) *Recording spectrophotometer.*—HP 8450A UV/VIS (Hewlett-Packard Co., Palo Alto, CA 94303).

(d) *Filters.*—Nylon 66, 0.45 μm pore size (D&L Filter Corp., Woburn, MA 01801), or equivalent.

Reagents

(a) *Methanol.*—LC or distilled-in-glass grade.

(b) *1-Heptanesulfonic acid sodium salt.*—Eastman Kodak Co., Rochester, NY 14650; Fisher Scientific Co., Fair Lawn, NJ 07410; Aldrich Chemical Co., Inc., Milwaukee, WI 53233.

(c) *Mobile solvent.*—Mix 280 mL methanol with 720 mL 0.005M 1-heptanesulfonic acid sodium salt in water and 10 mL acetic acid. Pass through 0.45 μm filter before use. Adjust methanol or 1-heptanesulfonic acid sodium salt solution content so that LC system meets system suitability test.

(d) *Standards.*—Morphine sulfate, codeine phosphate (USP Reference Standard); pseudomorphine (synthesized by method of ref. 13); 2-MCBT (Food and Drug Administration (FDA), St. Louis, MO); phenol (Fisher Scientific Co.).

(e) *Morphine sulfate standard.*—Dissolve accurately weighed amount USP Reference Standard morphine sulfate in mobile solvent to yield ca 0.24 mg/mL solution. Prepare solution fresh daily.

(f) *Morphine sulfate-phenol standard solution.*—Prepare standard solution with mobile solvent to yield ca 0.24 mg morphine sulfate/mL and ca 0.15 mg phenol/mL.

Sample Preparation

Dilute accurately measured volume of morphine sulfate injection with mobile solvent to yield ca 0.24 mg morphine sulfate/mL.

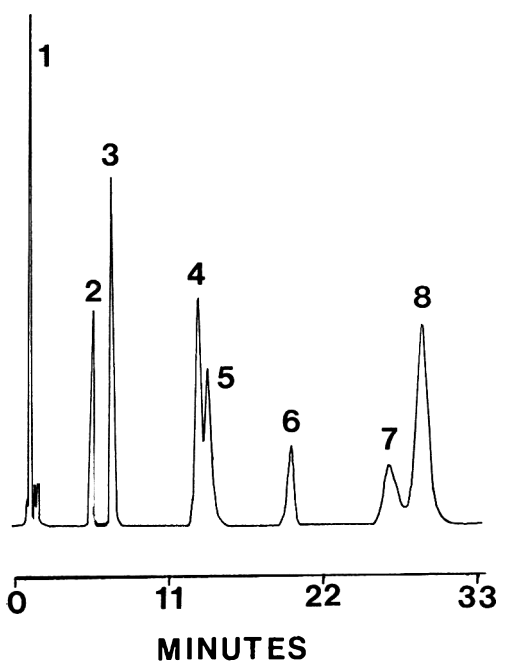

Figure 1. Representative chromatogram: 1, 8-SDHM; 2, phenol; 3, morphine (as sulfate); 4, methyl paraben; 5, codeine (as phosphate); 6, 2-MCBT; 7, pseudomorphine; 8, ethyl paraben.

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Table 1. Retention time (RT) of selected solutes on C18 μBondapak columns

Solute	Column 1[a] RT[d]	Column 1[a] Rel. RT	Column 2[a] RT	Column 2[a] Rel. RT	Column 3[b] RT	Column 3[b] Rel. RT	Column 4[c] RT	Column 4[c] Rel. RT
8-SDHM	1.99	0.330	1.94	0.246	1.44	0.209	1.41	0.196
Phenol	4.31	0.718	5.57	0.705	5.32	0.771	5.79	0.804
Morphine	6.00	1.000	7.90	1.000	6.90	1.000	7.20	1.000
Methyl paraben	ND[e]	—	12.0	1.52	ND	—	13.39	1.86
Codeine	10.16	1.69	14.58	1.85	12.66	1.83	14.04	1.95
2-MCBT	11.71	1.95	17.81	2.25	17.53	2.54	20.14	2.80
Ethyl paraben	ND	—	25.38	3.21	ND	—	29.48	4.09
Pseudomorphine	17.85	2.98	30.00	3.80	24.71	3.58	27.13	3.77
Propyl paraben	ND	—	>40	—	ND	—	>50	—

[a]Mobile solvent: methanol–acetic acid–0.005M 1-heptanesulfonic acid (25 + 1 + 75); flow rate 1.3 mL/min.
[b]Mobile solvent: methanol–acetic acid–0.005M 1-heptanesulfonic acid (28 + 1 + 72); flow rate 1.6 mL/min.
[c]Mobile solvent: methanol–acetic acid–0.005M 1-heptanesulfonic acid (27 + 1 + 73); flow rate 1.6 mL/min.
[d]Expressed in minutes.
[e]ND = not determined.

Table 2. Comparison of morphine sulfate purity (%) of bulk drug substance determined by using LC and USP XX methods

Mfr[a]	LC[b] Detn 1	LC[b] Detn 2	LC[b] Av.	USP XX[c]
A	100.8	100.8	100.8	100.8
B	98.6	99.8	99.2	99.6
C	99.6	99.2	99.4	99.8
E	100.4	99.8	100.1	99.5
F	100.5	99.8	100.2	100.5
Av.			99.9	100.0

[a]Manufacturers of morphine sulfate injection.
[b]USP morphine sulfate pentahydrate used as external standard and calculated as if 100% pure.
[c]Calculated as anhydrous morphine sulfate.

System Suitability Test

Let chromatographic system equilibrate with mobile solvent ca 1 h; then inject 25 μL aliquots of standard (**f**). Retention time for morphine sulfate should be between 5 and 8 min. Coefficient of variation (CV) of 5 replicate injections of standards should be < 2%. Resolution factor should be > 2 for morphine sulfate and phenol. Tailing factor for morphine sulfate peak should be < 2.

LC Determination

Let chromatographic system equilibrate with mobile solvent ca 1 h. Chromatograph equal volumes (typically 25 μL) of samples and standards (**e**), using mobile solvent flow rate of ca 1.5 mL/min and detection at 284 nm. Use average of duplicate injections of each determination for calculation.

Linearity—Morphine Sulfate

The peak areas per microgram morphine sulfate determined by LC were linear over the working concentration range of 3.2–12.2 μg/injection. The average peak area per microgram morphine sulfate was 79 031 with a CV of 1.7% for 7 different concentrations, each injected in duplicate. The proposed LC procedure specifies a concentration of 0.24 mg/mL or 6 μg morphine sulfate/25 μL injected.

Recovery of Morphine Sulfate from Sample Injections

For each concentration range (1–15 mg/mL) of morphine sulfate injections from 6 manufacturers, standard morphine sulfate was added equal to one-half the amount of morphine sulfate sampled for analysis. Each standard recovery solution was analyzed simultaneously by LC with each corresponding sample. The average recovery of morphine sulfate standard added to the injection samples in 19 studies run over a period of 3 months was 99.4% with a range of 97.2–101.4% and a CV of 1.14%.

Chromatographic Column Variation

Five C18 μBondapak columns in general use in our laboratory were tested for morphine sulfate-phenol resolution and morphine sulfate tailing factor. The morphine sulfate peak eluted between 5 and 8 min, with a resolution factor for the phenol and morphine sulfate peaks ranging from 2.3 to 4.2. The tailing factor for morphine sulfate varied from 1.2 to 1.4. The methanol in the mobile solvent ranged from 25 to 28% and the flow rate varied from 1.3 to 1.6 mL/min. Column pressure varied from 1500 to 2200 psi.

Retention Times of Selected Compounds

To demonstrate the LC separation of morphine sulfate from preservatives, degradation products, and some impurities that may be present in injections, a standard mixture of morphine sulfate, phenol, codeine phosphate, 2-MCBT, 8-SDHM, and pseudomorphine was chromatographed on 4 C18 μBondapak columns. This mixture plus ethyl, methyl, and propyl paraben was chromatographed on 2 of the columns to show that these preservatives were also separated from morphine (Figure 1). The acid/base UV spectra of both the purified pseudomorphine substance and the LC eluate of 8-SDHM corresponded to the UV spectra reported in the literature. Pseudomorphine is the oxidation breakdown product of morphine in aqueous solutions (13). Stability studies on morphine in aqueous solutions containing the antioxidant sodium bisulfite have shown that the 2 compounds interact to form 8-SDHM (15). All of the morphine sulfate injection solutions assayed in this study contained bisulfite ion as an antioxidant. Codeine was included

Table 3. Relative[a] retention time[b] of impurities in bulk drug morphine sulfate determined by using LC

Mfr[c]	Impurity 1	Impurity 2	Impurity 3	Impurity 4
USP	0.78(T)[d]	—	1.81(0.71)	—
A	—	—	1.81(T)	3.27(T)
B	0.80(0.11)	—	1.80(0.34)	—
C	0.80(T)	1.59(0.93)	1.81(0.31)	—
D	ND[e]			
E	0.78(T)	—	—	3.36(T)
F	0.80(T)	—	1.83(T)	—

[a]Relative to morphine sulfate peak.
[b]Percent in parentheses calculated relative to morphine sulfate peak response.
[c]Manufacturers of morphine sulfate injections.
[d]T = trace = <0.1%.
[e]ND = not determined.

Table 4. Representative average values of assays of morphine sulfate injection samples by proposed LC method

Sample	Mfr	Morphine sulfate declared, mg/mL	Morphine sulfate found, % Av.[a]	CV
1	A	15	98.0	1.04
2	B	8	98.1	2.77
3	B	10	96.6	1.31
4	B	15	98.4	2.01
5	C	2	97.2	0.88
6	C	4	100.0	1.36
7	C	8	99.0	1.43
8	C	10	100.7	0.74
9	D	8	99.2	0.47
10	D	10	100.1	0.75
11	D	10	97.9	4.10
12	D	15	100.9	0.66
13	E	1	99.4	0.56
14	E	2	106.2	0.98
15	F	2	99.6	0.82
16	F	4	99.0	0.42
17	F	8	98.0	0.57
18	F	10	99.4	0.23
19	F	10	96.7	3.53
20	F	15	97.9	0.75

[a] $n = 3$.

Table 5. 2-MCBT found in morphine sulfate injection (Manufacturer F) by LC method

Sample	Exp. date	2-MCBT found[a], ppm
40	3/84	ND[b]
41	3/84	ND
42	12/83	40
43	11/83	53
44	12/83	syringe 1: none detected syringe 2: none detected
45	11/83	ND
46	11/83	49
47	1/84	ND
48	12/83	syringe 1: 2.9 syringe 2: 3.6
50	8/83	23
51	10/83	14
52	10/83	38
53	10/83	49

[a] Determined at 323 nm.
[b] ND = not detected.

because it is chemically similar to morphine. 2-MCBT has been recently shown (14) to be a contaminant in injections, leached from the rubber closures of syringe-type containers.

The relative retention times of these compounds on the 4 chromatographic columns are reported in Table 1. As seen in Figure 1, the compounds are separated from morphine sulfate by the proposed LC procedure and may be identified and quantitated if necessary.

Results and Discussion

The purity of bulk drug morphine sulfate samples from 5 manufacturers of morphine sulfate injection was determined in duplicate by the proposed LC procedure and singly by the official USP XX method (1) (Table 2). The average bulk drug purity determined by the LC procedure was 99.9% with a difference of 0.1% from the average purity (anhydrous) found by the USP XX method. The difference in the duplicate determinations for each sample by the LC method ranged from 0 to 1.2%.

The official method of analysis (1) for morphine sulfate is a nonspecific nonaqueous titration for the purity content and a nonspecific chloroform extraction, acid-base titration for total foreign alkaloids. The proposed LC procedure gives a

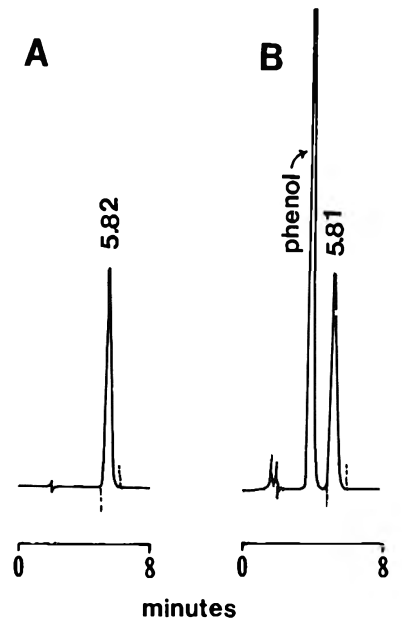

Figure 2. Typical chromatograms: A, morphine sulfate standard; B, 2 mg/mL morphine sulfate injection sample containing 5 mg/mL phenol.

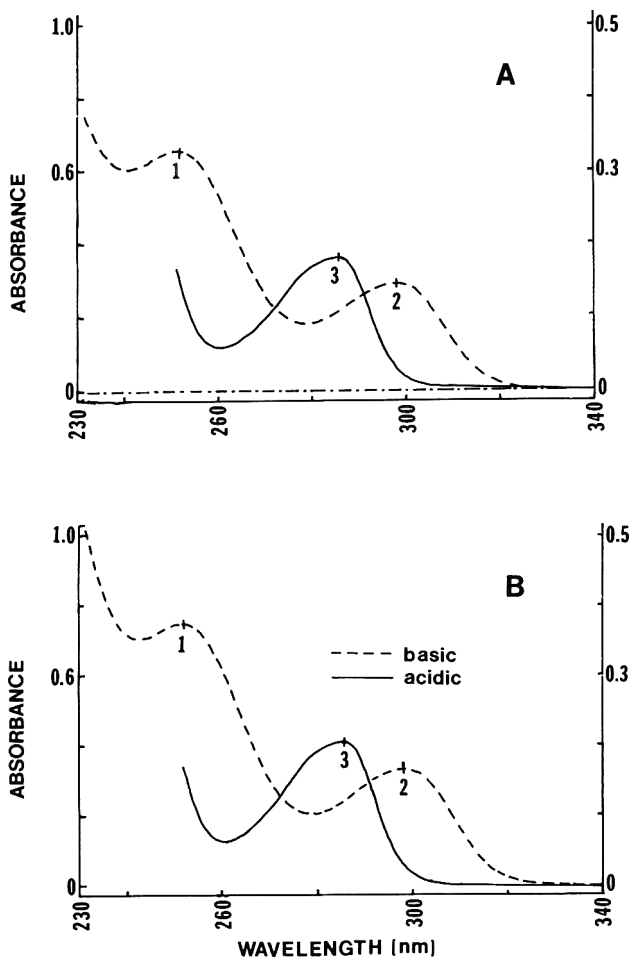

Figure 3. Acidic/basic UV spectra: absorbance scale, 0 to 0.5 for acidic spectra, 0 to 1 for basic spectra; A, 0.043 mg/mL morphine sulfate standard; B, pooled LC morphine sulfate peak eluates from an 8 mg/mL morphine sulfate injection sample containing 5 mg/mL pheonl; 1, 251 nm; 2, 298 nm; 3, 285 nm.

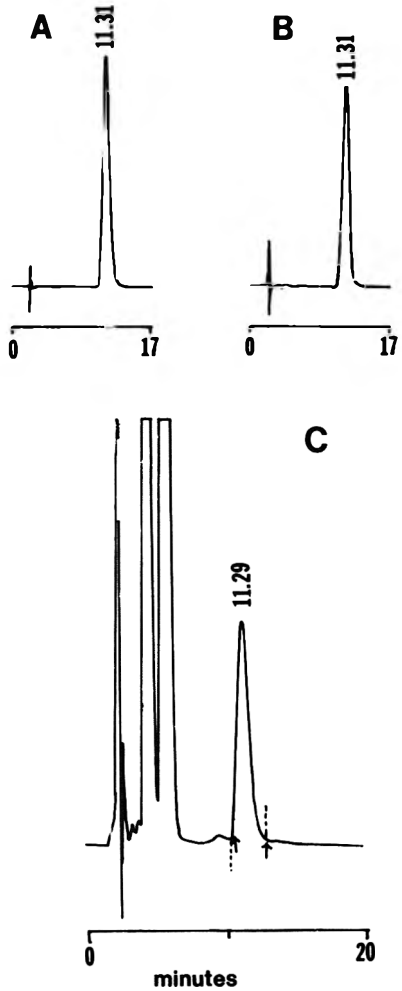

Figure 4. Representative LC chromatograms: A, 2-MCBT standard detected at 323 nm; B, 2 mg/mL morphine injection sample from manufacturer F detected at 323 nm; C, chromatographic purity of the 2 mg/mL morphine sulfate injection sample detected at 284 nm.

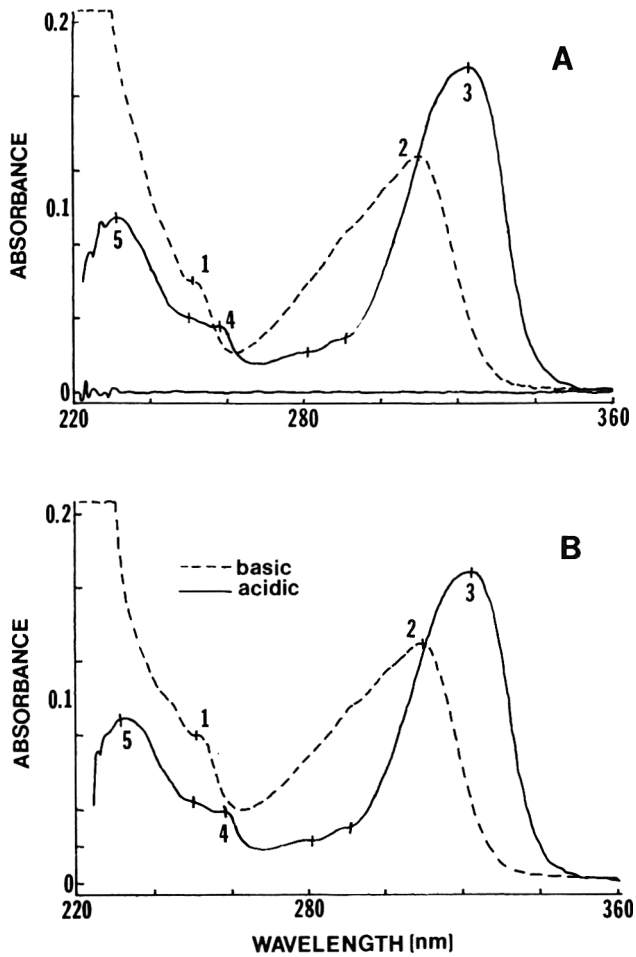

Figure 5. Acidic/basic spectra of 2-MCBT: A, standard; B, LC eluate of contaminant peak (RT = 11.29 min) shown in Figure 4C; 1, 251 nm; 2, 310 nm; 3, 323 nm; 4, 258 nm; 5, 231 nm.

profile of extraneous compounds that may be in the bulk drug substance. Extraneous peaks found in the LC chromatograms of USP morphine sulfate and the 5 bulk drug materials are listed in Table 3. One impurity was common to the USP Reference Standard and to 4 of the 5 bulk drugs. The retention time was similar to that for codeine; however, this was not verified because of lack of time.

Representative average (n = 3) values of morphine sulfate determined by the proposed LC method for 53 samples of morphine sulfate injections from 6 manufacturers are shown in Table 4. The concentration of morphine sulfate in the samples ranged from 1 to 15 mg/mL. Phenol was the preservative in all but 13 samples. Figure 2 represents typical LC chromatograms of standard morphine sulfate and a 2 mg/mL morphine sulfate injection sample containing 5 mg/mL phenol. The preservative chlorobutanol, found in 2 samples, did not interfere in the analysis. The average morphine sulfate found in the 53 samples ranged from 95.6 to 106.2% with an overall average sample (n = 3) CV of 1.14%. The high CVs shown in Table 4 for samples 11 and 19 were the result of a low value found for one of the 3 subsamples assayed for each of the 2 samples. Not enough material was available to verify those results.

Chromatographic purity of the injection samples and identification of the morphine peak by UV spectroscopy were simultaneously determined by chromatographing about 200 μg morphine sulfate/two 100 μL injections, collecting the 2 eluates corresponding to the morphine peaks, and scanning the collected solution on a UV/VIS diode spectrophotometer. The resulting acidic/basic spectra from each sample corresponded to the acidic/basic spectra of USP morphine sulfate. A representative acidic/basic UV spectrum for an 8 mg/mL morphine sulfate injection sample and the USP standard is shown in Figure 3. The corresponding chromatograms from the injection samples in general displayed the chromatographic profile of the bulk drug material in addition to the preservative peak, if present in the sample. However, the chromatograms of 8 samples from manufacturer F displayed an extraneous peak. The LC retention time of the peak determined at 284 and 323 nm, and the acidic/basic UV spectra of the eluate from the LC peak corresponded to 2-MCBT. Figure 4 is an example of LC chromatograms of the 2-MCBT contaminant peak found at 284 nm and quantitated at 323 nm from one sample from manufacturer F. Figure 5 shows the acidic/basic UV spectra of both the above sample LC contaminant peak and the 2-MCBT standard. As shown in Table 5, the amount of 2-MCBT detected in the 14 injection samples ranged from 0 to 53 μg/mL.

The proposed LC procedure is simple and specific for morphine sulfate. Major degradation products, preservatives, and some contaminants and related compounds are separated during the chromatography.

Acknowledgments

The authors thank Edward Smith for his valuable suggestions during this study and the St. Louis FDA Science Branch for supplying the 2-MCBT standard material.

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Rapid Microchemical Identification of Four Phenothiazine Antiemetics with Gold Bromide and Iodine–Potassium Iodide Reagents: Collaborative Study

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A microchemical method was developed for the rapid identification of 4 phenothiazine antiemetics. Perphenazine, promethazine, thiethylperazine, and triflupromazine were positively identified and differentiated with the aid of a gold bromide reagent and an iodine–potassium iodide reagent. Only promethazine and triflupromazine yielded microcrystalline derivatives with gold bromide; only perphenazine and thiethylperazine reacted with iodine–potassium iodide. For each pair of positive reactions, the crystalline products were morphologically distinguishable under a microscope. The 2 tests were collaboratively studied by 7 independent laboratories and found to be simple, rapid, and effective for identifying the phenothiazines of interest. The method has been adopted official first action.

Several reports (1–3) have appeared on the use of microchemical tests for the identification of phenothiazines. Clarke (1) described crystal tests for identifying more than 30 phenothiazines, Andres (2) tested 12 phenothiazines with 6 different microchemical reagents, and Fulton (3) presented data on the behavior of 30 phenothiazines with as many as 9 reagents. The AOAC methods (4) do not include any microchemical tests for these drugs. The purpose of this study was to expand on the drugs studied by Andres (2) and to supplement the microchemical tests listed by Clarke (1) and Fulton (3). Although a large number of reagents were investigated for their ability to form microcrystalline products with a group of 14 different phenothiazine compounds, only the gold bromide and iodine–potassium iodide reagents yielded derivatives suitable for identification purposes. This report deals with the results obtained by this laboratory and by 6 outside collaborators when these 2 reagents were used on perphenazine, promethazine, thiethylperazine, and triflupromazine. Future work will include dosage forms and additional drugs.

This report of the Associate Referee was presented in part 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 B and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) **68,** March issue.

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Collaborative Study

Each of 7 collaborators was given 4 vials, labeled A to D, which contained samples of perphenazine, promethazine, thiethylperazine, and triflupromazine, respectively. The actual identity of each vial remained unknown to the collaborator. Instructions were provided for the performance of each test and for the preparation of the reagent and sample solutions. Collaborators were asked to record their findings and comments on a report form provided and to submit photomicrographs of each crystalline product. To aid in the interpretation of the test results, a copy of the author's results was included with the instructions.

Phenothiazine Drugs

Microcrystalline Identification

First Action

(Applicable to perphenazine, promethazine, thiethylperazine, and triflupromazine pure drug substances)

36.A07 ***Reagents***

(a) *Gold bromide soln.*—Dissolve 1 g $HAuCl_4.3H_2O$ and 1 g NaBr in 30 mL H_2SO_4 (2 + 3). Before use, mix 3 vols of this soln with 1 vol. of glacial HOAc. Store in dark bottle.

(b) *Iodine-potassium iodide acidic soln.*—Dissolve 5 g I and 30 g KI in 100 mL H_2O. Mix 1 vol. of this soln with 1.5 vols of HCl and 1.5 vols of H_3PO_4. Store in dark bottle.

36.A08 ***Standard Solutions***

Prep. individual solns of perphenazine, promethazine, thiethylperazine, and triflupromazine by dissolving in HOAc (2 + 1) to final concn of ca 2 μg/μL. If necessary, dil. further with HOAc (2 + 1) for optimum results. Exact concn depends on compd being tested.

36.A09 ***Sample Solutions***

Dissolve sample in HOAc (2 + 1) to final concn of ca 2 μg/μL. If necessary, dil. further with HOAc (2 + 1) to obtain solns with strength equiv. to std solns.

Table 36:A1 Characteristics of Microchemical Tests for Synthetics

Synthetic	Solvent	Concn of Synthetic	Reagent	Description of Test and Crystals
Perphenazine	HOAc (2 + 1)	1:500	Iodine-potassium iodide acidic	X-shape serrated blades. Pos. elongation
Thiethylperazine	HOAc (2 + 1)	1:500	Iodine-potassium iodide acidic	Branching clusters. Neg. elongation
Promethazine	HOAc (2 + 1)	1:500	Gold bromide in H_2SO_4/HOAc	Small rods; clusters of rods
Triflupromazine	HOAc (2 + 1)	1:500	Gold bromide in H_2SO_4/HOAc	Large X-shape segmented plates; small swirling tufts

Table 1. Collaborative results of microcrystalline test for 4 antiemetic phenothiazines with I_2-KI reagent

Coll.	Promethazine (μg/μL)	Triflupromazine (μg/μL)	Perphenazine (μg/μL)	Thiethylperazine (μg/μL)
A	oily droplets (2)	oily droplets (2)	X-shaped serrated blades; serrated blades (2)	small Xs; hairy clusters (2)
B	no reactior	no reaction	X-shaped plates; aggregates (≥0.5)	serrated blades in Xs; needles[a](≥0.5)
C	no reactior (1)	no reaction (1)	blades; X-shaped blades[b] with negative elongation (1)	branching clusters with negative elongation (1)
D[c]	no reactior	no reaction	irregular large segmented plates	needles in tufts
E	oily droplets (2)	oily droplets (2)	serrated X-shaped (2)	X-shaped; clusters of branched crystals (2)
F	oily droplets (2)	oily droplets (2)	X-shaped blades; blades (≥0.5)	small, branched (≥0.5)
G	oily droplets (2)	oily droplets (2)	serrated, X-shaped blades; serrated blades (0.5)	branching clusters (0.125)

[a]After 5 min.
[b]After 2 min.
[c]No concentrations reported.

Table 2. Collaborative results of microcrystalline test for 4 antiemetic phenothiazines with gold bromide reagent

Coll.	Promethazine (μg/μL)	Triflupromazine (μg/μL)	Perphenazine (μg/μL)	Thiethylperazine (μg/μL)
A	rods; clusters of rods some in rosettes (2)	S-shaped, tufts; X-shaped segmented plates (0.5)	small grains (2)	small grains (2)
B	feathery rosettes[a] (≥0.5)	swirling feathery tufts (≥0.5)	fine grains (≥0.5)	fine grains (≥0.5)
C	clusters of small rods (1)	swirling tufts (1)	small grains (1)	small grains (1)
D[b]	rods; clusters of rods	no reaction	small grains	no reaction
E	burrs; some tufts (2)	X-shaped; branched; tufts (2)	small grains (2)	grains (2)
F	rosettes of rods (1); small oily droplets (0.25)	swirling tufts; some X-shaped (1); small oily droplets (0.25)	oily droplets (2)	small oily grains (2)
G	clusters of small rods (0.25)	X-shaped (0.5); swirling tufts (2)	small grains (2)	small grains (2)

[a]After 5 min.
[b]No concentrations reported.

36.A10 ***Procedure***

Place small drop (ca 10 μL) of sample soln on each of 2 clean microscope slides. Place small drop (ca 10 μL) of reagent soln (a) or (b) on 2 cover slips, resp.; invert cover slips and place over sample solns on slides Let stand until crystn occurs (immediately with some compds; 1–20 min with others). Examine slide under polarizing microscope at ca 100–300×. If microcrystn product is formed, note shape and rate of formation of crystals. See Table **36:A1**. Repeat tests with corresponding std soln of comparable strength, and compare product with that obtained with sample soln.

Results and Discussion

Of the 4 phenothiazines studied, only triflupromazine was tested by Clarke (1) with a reagent included in the present study. Andres (2) reported the reaction between promethazine and triflupromazine with gold chloride solution, and Fulton (3) tested all 4 compounds but with reagents that differed from those described here.

The results submitted by the 7 collaborators are shown in Tables 1 and 2. Three collaborators included drawings and one submitted photomicrographs. Both types of evidence were comparable to the descriptions and photomicrographs provided to the collaborators.

One collaborator (D) did not report the amount of drug used for both tests. The same collaborator reported a negative test for triflupromazine with gold bromide solution. No attempts were made to obtain a clarification of these discrepancies.

In spite of the subjectivity in interpreting the positive tests, agreement was good among the majority of the collaborators. Notable exceptions were the reaction of thiethylperazine with I_2-KI solution reported by Collaborator F (Table 2) and the reaction between promethazine and gold bromide reported by Collaborator E (Table 1).

Most collaborators worked with sample solutions in the concentration range 0.5–2.0 μg/μL. One collaborator (E) reported results for perphenazine and thiethylperazine with

I_2-KI solution in the concentration range 0.125–0.25 μg/μL. In general, drug concentrations below 0.5 μg/μL usually resulted in the formation of either scanty microcrystals or oily droplets.

It is recommended that this method be adopted official first action.

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Reverse Phase Liquid Chromatographic Determination of Bisacodyl in Dosage Forms

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A method is described for the determination of bisacodyl in enteric-coated tablets and suppositories by liquid chromatography (LC). The method will also determine the hydrolysis degradation products monoacetylbisacodyl and desacetylbisacodyl. The sample is dissolved in 2-propanol, and the extract is diluted with the mobile phase and injected into a liquid chromatograph fitted with a μBondapak C_{18} column and an ultraviolet detector set at 254 nm. The column is eluted with methanol-acetonitrile-0.01M citric acid (25 + 25 + 50). The pooled mean recovery value for bisacodyl from commercial enteric-coated tablets and suppositories was 99.7% with a pooled coefficient of variation (CV) of 0.72%. For content uniformity assays, the CVs were 0.7 and 1.0% for groups of 10 individual commercial suppositories and tablets, respectively. Differences between assay values by the LC and USP XX methods were 0.2% of declared for enteric-coated tablets (n = 5) and 1.0% of declared for suppositories (n = 2). The LC method can determine as little as 0.015 μg of the monoacetyl or desacetyl degradation product.

Bisacodyl, 4,4′-(2-pyridylmethylene)diphenol diacetate, is a synthetic contact laxative that acts directly on the colonic mucosa to produce normal peristalsis throughout the large intestine (1). Its unique mode of action permits either oral or rectal administration whenever constipation is a problem. In dosage forms, bisacodyl may be accompanied by its hydrolysis degradation products, monoacetylbisacodyl and desacetylbisacodyl (2).

Methods for the determination of the parent drug and its degradates in pharmaceutical and biological samples have included the use of gas chromatography (GC) (3), GC-mass spectrometry (3, 4), thin layer chromatography (TLC) with densitometry (3, 5), column chromatography (6), and liquid chromatography (LC) (2, 7, 8). In terms of experimental flexibility, simplicity, and instrumental requirements, LC offers a number of advantages over the other chromatographic techniques. It does not, for example, require the derivatization step used in the GC methods (3, 4), and it is less susceptible to the analytical variability often encountered with TLC methods. Ali (2) determined bisacodyl and its degradation products in dosage forms on a reverse phase C_{18} column, using a mobile phase of water-acetonitrile-sodium acetate and detection at 254 nm. The coefficients of variation (CVs) for bisacodyl and the degradates were reported as 1.44 and 2.40%, respectively. No data were presented for the accuracy of the method. A similar LC method using acetonitrile-pH 6 acetate buffer was described by Salvesen et al. (7). That method, however, necessitates a lengthy sample preparation and shows poor accuracy.

This paper reports an LC method for the assay of bisacodyl and its 2 hydrolysis products in enteric-coated tablets and suppositories. The method is rapid, simple, accurate, and precise and gives analytical results that are in excellent agreement with those obtained by the spectrophotometric method of USP XX (9).

METHOD

Apparatus

(a) *Liquid chromatograph.*—Tracor Model 950 pump with Model 970A variable wavelength detector and TS-10 recorder (Tracor, Inc., Instrument Group, Austin, TX 78721), or equivalent. Operating conditions: flow rate 1.5 mL/min; detector wavelength 254 nm, attenuation 0.08 AUFS; column temperature ambient; chart speed 0.5 cm/min; injection volume 20 μL.

(b) *Injector*—Rheodyne Model 7125 with 20 μL loop (Rheodyne, Inc., Cotati, CA 94928), or equivalent.

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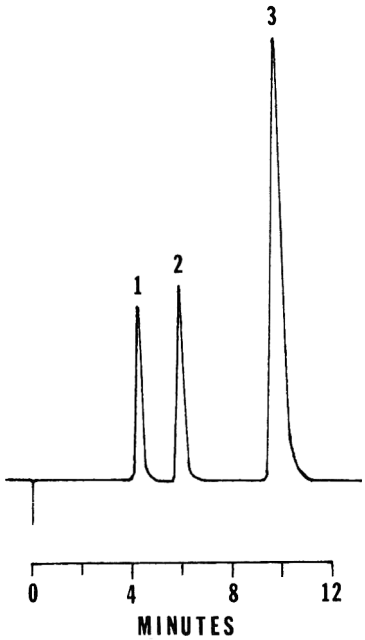

Figure 1. Liquid chromatogram of a mixture of 1, desacetylbisacodyl; 2, monoacetylbisacodyl; and 3, bisacodyl.

(c) *Chromatographic column.*—Stainless steel, 30 cm × 3.9 mm id, packed with μBondapak C_{18}, 10 μm particle size (Waters Associates, Milford, MA 01757), or equivalent.

Reagents

(a) *Solvents.*—Analytical reagent 2-propanol (Fisher Scientific Co., Pittsburgh, PA 15219); LC grade acetonitrile and methanol (Fisher Scientific Co.); double distilled-in-glass water.

(b) *Citric acid.*—Analytical reagent, monohydrate, powder (Fisher Scientific Co.).

(c) *Mobile phase.*—In suitable flask, combine 250 mL acetonitrile with 250 mL methanol and 500 mL 0.01M citric acid. Filter solution through 0.8 μm membrane filter, and place in ultrasonic bath for 10 min to deaerate.

(d) *Standard preparation.*—Dissolve USP Bisacodyl Reference Standard in mobile phase with sonication to obtain solution containing 200 μg/mL. Transfer 5.0 mL solution to 10 mL volumetric flask, dilute to volume with mobile phase, and mix.

System Suitability

Equilibrate system with mobile phase flow rate set at 1.5 mL/min. Inject 20 μL standard preparation and adjust sensitivity of detector so that peak response is ca 60% full scale. In a suitable system, the CV of the peak responses of 5 replicate injections is ≤2.0%, and the resolution factor is ≥2.0 for the parent compound and its degradate.

Sample Preparation

(a) *Tablets.*—Weigh ≥ 20 tablets and grind to powder that will pass through No. 60 sieve. Transfer accurately weighed portion of powder, equivalent to ca 5 mg bisacodyl, to 25 mL volumetric flask. Add 10–15 mL 2-propanol, and sonicate solution for 15 min. Let cool to room temperature. Dilute solution to volume with 2-propanol, mix, and centrifuge for 5 min. Filter supernate through 0.8 μm membrane filter. Transfer 5.0 mL filtrate to 10 mL volumetric flask, and evaporate solvent inside hood with aid of gentle jet of air. Dissolve residue in mobile phase. Dilute solution to volume with mobile phase and mix.

(b) *Suppositories.*—Place several suppositories, equivalent to ca 50 mg bisacodyl, in 150 mL beaker. Dissolve suppositories in suitable volume of warm (60°C) 2-propanol. Quantitatively transfer solution to 250 mL volumetric flask. Cool to ca 15°C in water bath, dilute to volume with 2-propanol, and mix. Transfer 25–35 mL solution to glass-stopper 50 mL centrifuge tube, and centrifuge for 5 min at 2000 rpm. Transfer 5.0 mL supernate to 10 mL volumetric flask. Evaporate solvent inside hood with aid of gentle jet of air. Dissolve residue in mobile phase. Dilute solution to volume with mobile phase and mix. Centrifuge for 5 min at 2000 rpm. Using syringe and avoiding supernate, withdraw 20 μL aliquot of aqueous layer for injection.

Determination

Inject 20 μL each of standard and sample preparations, and measure peak response (peak height or peak area) for each. Calculate amount of bisacodyl in each tablet or suppository as follows:

$$\text{mg/tablet} = (R/R') \times C \times D \times (A/W)$$

$$\text{mg/suppository} = (R/R') \times C \times (D/N)$$

where R and R' are peak responses of sample and standard preparations, respectively; C is concentration of bisacodyl in standard preparation in mg/mL; D is sample dilution factor; A is average tablet weight in mg; W is sample weight taken in mg; N is number of suppositories taken, equivalent to 50 mg bisacodyl.

Results and Discussion

The use of mobile phases containing an acetate buffer (2, 7) was found to cause some degradation of bisacodyl during LC analysis. In addition, these mobile phases yield optimum separations only within a narrow pH range. The chromatographic separations reported here (Figures 1 and 2) were

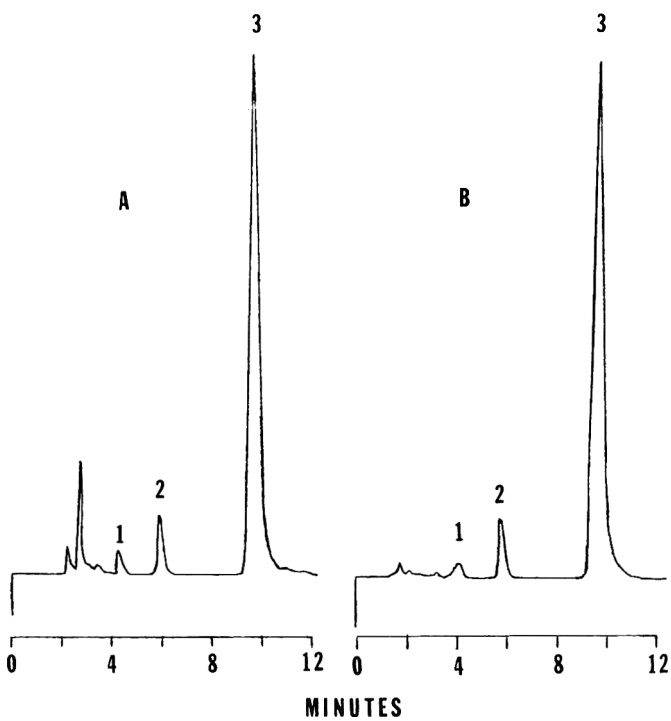

Figure 2. Liquid chromatograms of (A) bisacodyl tablet and (B) bisacodyl suppository: 1, desacetylbisacodyl; 2, monoacetylbisacodyl; and 3, bisacodyl.

Table 1. Recovery of bisacodyl from commercial dosage forms by LC method

Sample	Found[a] before spiking, mg	Added, mg	Recd by addn method mg	Recd by addn method %
Tablet, 5 mg declared				
A	5.02	2.521	2.513	99.7
B	5.24	2.656	2.633	99.1
C	5.15	2.512	2.537	101.0
D	5.21	2.627	2.614	99.5
E	5.07	2.568	2.547	99.2
Mean				99.7
SD				0.76
CV, %				0.76
Suppository, 10 mg declared				
F	10.17	5.070	5.080	100.2
G	9.80	4.973	4.923	99.0
Mean				99.6
Pooled mean rec., %				99.7
Pooled SD				0.72
Pooled CV, %				0.72

[a]Assay values by USP XX spectrophotometric method.

Table 2. Determination of bisacodyl in commercial dosage forms by USP XX and LC methods

Sample	Found: USP XX mg	USP XX % of decld	LC mg	LC % of decld
Tablet, 5 mg declared				
A	5.09	101.8	5.06	101.2
B	4.81	96.2	4.89	97.8
C	4.74	94.8	4.80	96.0
D	5.13	102.6	5.12	102.4
E	5.05	101.0	4.91	98.2
Mean	4.964	99.28	4.956	99.12
SD		3.53		2.62
CV, %		3.56		2.64
Suppository, 10 mg declared				
F	10.07	100.7	9.95	99.5
G	10.09	100.9	10.01	100.1
Mean	10.08	100.8	9.98	99.8
Pooled mean found, %				99.5
Pooled SD				2.6
Pooled CV, %				2.6

accomplished with a mobile phase in which the acetate buffer was replaced by a citric acid solution to avoid these problems. The retention times for bisacodyl, monoacetylbisacodyl, and desacetylbisacodyl were approximately 10.0, 6.0, and 4.5 min, respectively (Figures 1 and 2). A study was conducted to determine the effect of varying the proportions of each of the components of the recommended mobile phase. Retention times for bisacodyl were shortened with increasing concentrations of either 0.01M citric acid or acetonitrile, with a loss of resolution between the degradation products. Increasing the proportion of methanol slowed the elution of bisacodyl but brought the degradates closer to the solvent front.

Serial dilutions of a standard preparation of bisacodyl that were carried through the entire procedure exhibited a linear relationship between peak responses and concentration over the range 2.5–300 μg/mL (correlation coefficient, $r = 0.9998$).

Mean recovery values for bisacodyl added to samples of commercial enteric-coated tablets ($n = 5$) and suppositories ($n = 2$) by the method of standard addition were 99.7 and 99.6%, respectively (Table 1).

Three solvents were evaluated for the extraction of bisacodyl from dosage forms. Ali (2) recommended the use of methanol for extracting this drug and its hydrolysis products from enteric-coated dragees and suppositories. We found that this solvent also extracted tablet components such as coloring agents and binders, which interfered with the LC analysis. Salvesen et al. (7) extracted dosage forms with the chromatographic mobile phase. The use of this approach for the suppositories resulted in a cloudy solution which could not be cleared by centrifugation or filtration. These problems were not encountered with 2-propanol as the extracting solvent. Recoveries obtained by sonication of the sample with the extracting medium were higher than those obtained by mechanical shaking.

Five samples of tablets and 2 samples of suppositories were assayed by the LC and USP XX methods. Table 2 shows the close agreement between each pair of assay results. Two tablet samples and 2 suppository samples were found to contain traces of desacetylbisacodyl (Figure 2). All samples contained the monoacetyl derivative (Figure 2) at a mean concentration of about 2.6% relative to bisacodyl. The USP Reference Standard contained neither derivative. The approximate limit of determination for each degradate is 0.015 μg.

Tables 3 and 4 show the suitability of the proposed LC method for content uniformity testing of bisacodyl in solid dosage forms. For groups of 10 individual suppositories and tablets, the CVs were 0.7 and 1.0%, respectively.

In summary, the method presented here represents an improvement over other published LC methods for the determination of bisacodyl in tablets and suppositories. It is simple, rapid, and sensitive enough for content uniformity testing and the simultaneous determination of degradation products in solid dosage forms.

Table 3. Content uniformity assay of commercial bisacodyl suppositories (10 mg declared) by LC method

Sample	Found mg	Found % of decld
1	9.92	99.2
2	10.02	100.2
3	10.03	100.3
4	10.06	100.6
5	10.16	101.6
6	10.08	100.8
7	10.02	100.2
8	10.03	100.3
9	10.08	100.8
10	10.13	101.3
Mean		100.5
SD		0.7
CV, %		0.7

Table 4. Content uniformity assay of commercial bisacodyl tablets (5 mg declared) by LC method

Sample	Found mg	Found % of decld
1	4.99	99.8
2	5.00	100.0
3	4.95	99.0
4	5.03	100.6
5	4.93	98.6
6	4.90	98.0
7	4.93	98.6
8	5.06	101.2
9	5.00	100.0
10	4.96	99.2
Mean		99.5
SD		1.0
CV, %		1.0

Acknowledgments

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DC Polarographic Assay of Piperazines

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Piperazine, piperazine salts, and piperazine-containing compounds, e.g., diethylcarbamazine and piperazine theophylline, are determined by direct current polarography of the corresponding nitroso-derivative. The nitroso-derivative exhibits a well defined cathodic wave at −0.8 V vs SCE. The plot of peak height vs concentration is rectilinear between 1.6×10^{-5} and 19.2×10^{-5}M. Recoveries from bulk drugs were 97.7–101.12% with a standard deviation of 2.14–3.44. The procedure was successfully applied to analysis of drug content in representative dosage forms with good accuracy.

Piperazine, its salts, and derivatives, have long been used in pharmaceutical preparations. They are dispensed as an anthelmintic for humans and in animal feeds, and also for treatment of arthritis and nephritis. Piperazine is incorporated in some drugs as acefylline (piperazine theophylline) because it is more soluble and causes less nausea and gastric irritation than aminophylline (theophylline ethylenediamine). The widespread use of this compound requires the availability of a rapid and reliable method for its assay.

Piperazine and its salts (phosphate, adipate, and citrate) has been assayed by numerous methods, e.g., gravimetric (1–3), nonaqueous titrimetric (4), chelatometric (5), colorimetric (6, 7), spectrophotometric (8), near infrared spectroscopic (9), or polarographic (10). Diethylcarbamazine (1-diethylcarbamoyl-4-methylpiperazine) has been determined titrimetrically (11), by gas chromatography (12), colorimetrically with phenol (13) reagent and picric acid (14), by using an ion-responsive electrode (15), and spectrophotometrically with picrolonic acid (16). Piperazine theophylline (acefylline) is a nonofficial drug and could be assayed gravimetrically as the picrate (1–3) after pretreatment to separate the theophylline part of the molecule, or by UV absorption spectroscopy ($A^{1\%}_{1cm}$ at 274 nm is 329) (17).

Until now, the utility of conventional or recent polarographic methods for specific assay has not been reported, although these procedures can offer a selective microdetermination of piperazine compounds in the presence of some additives or excipients; other methods such as spectrophotometry, colorimetry, or gravimetry are subject to certain interferences. Van Kerchove and Schoenmakers (10) recently published dc and normal pulse polarographic methods for determination of piperazine and piperazine salts. The polarographic procedures are based on reaction of piperazine with formaldehyde and are rather unselective because the presence of other compounds, especially primary amines, seriously affects the polarographic wave height. Moreover, the cathodic wave depends on the formaldehyde concentration.

The present report describes a dc polarographic procedure for microdetermination of piperazine compounds alone or in dosage forms. The method is based on the formation of polarographically active nitroso-derivatives, which produce a well defined cathodic wave at pH 2.3. The nitroso-derivatives of secondary amines exhibit a well defined cathodic wave (18) which has been used for their differentiation from primary or tertiary amines. This is the basis of the present study. The developed procedure was successfully applied to the microdetermination of the drugs in their pharmaceutical preparations.

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METHOD

Apparatus and Reagents

(a) *Polarograph.*—All polarograms were recorded on a Tacussel assembly, fitted with 3 electrodes: saturated calomel electrode (SCE), platinium wire as counter electrode, and dropping mercury electrode. Potential was scanned between 0.0 and −1.2 V vs SCE. Operating conditions: droptime 3.15 s, voltage range 1.0 V, current range 25 μamp full-scale, room temperature 20°C, capillary characteristics: $m^{2/3} t^{1/6} = 2.34$ (m in mg/s, t in s) in nitrogen-saturated solution.

(b) *Britton-Robinson buffer (19).*

(c) *Sodium nitrite solution.*—1%, aqueous.

(d) *Ammonium sulfamate solution.*—5%, aqueous.

(e) *Standard piperazine solution.*—Hexahydrate, phosphate, adipate, citrate, diethylcarbamazine citrate, and piperazine theophylline (100% purity calculated on dried basis). Prepare a solution 4×10^{-4}M in each compound in water.

Piperazine or Its Salts

Take 1.0–12 mL aliquots of piperazine or its salt from 4×10^{-4}M solution, transfer to separate 25 mL volumetric flasks, add 1.0 mL buffer solution and 0.5 mL sodium nitrite, and

Table 1. Regression equation and correlation coefficient for piperazine salts and compounds with nitrous acid[a]

Compound	Regression equation	CC
Piperazine hexahydrate	$id = 0.895 \times 10^5C + 0.53$	0.99942
Piperazine phosphate	$id = 0.83 \times 10^5C + 0.46$	0.99985
Piperazine adipate	$id = 0.946 \times 10^5C + 10.33$	0.99998
Piperazine citrate	$id = 2.47 \times 10^5C - 0.29$	0.99892
Diethylcarbamazine citrate	$id = 0.366 \times 10^5C - 0.31$	0.99955
Piperazine theophylline	$id = 0.538 \times 10^5C - 0.4$	0.99995

[a] id = diffusion current
C = concentration in mmole/L
CC = correlation coefficient

heat in 80°C water bath 15 min. Cool, add 1 mL ammonium sulfamate solution, 10 mL 0.1M KCl, and dilute to volume with water. Transfer entire solution to polarographic cell, bubble purified nitrogen through solution 5 min, and record polarograms in potential range 0.0 to −1.2 V vs SCE.

Diethylcarbamazine Citrate

Accurately weigh 1.565 g diethylcarbamazine citrate, and heat in long-neck flask with 10 mL nitrogen-free H_2SO_4 (50% v/v) for 2 h. Cool, dilute with water, transfer to 1 L volumetric flask, and dilute to volume with water. Dilute 10 mL of this solution to 100 mL with water in volumetric flask (4×10^{-4}M). Transfer aliquots to separate 25 mL flasks and treat as above, beginning with "add 1.0 mL buffer solution. . . ."

Piperazine Theophylline

Accurately weigh 2.25 g piperazine theophylline salt, transfer to 100 mL volumetric flask, dissolve in and dilute to volume with water. Accurately transfer 10 mL to separatory funnel, acidify with 5 mL dilute H_2SO_4, and extract with 3 successive 15 mL volumes of chloroform–isopropanol (10 + 5), washing each extract with the same two quantities each of 20 mL water. Combine aqueous layer and washings in 100 mL volumetric flask and dilute to volume. Dilute 10 mL of this solution to 100 mL with water in 100 mL volumetric flask (4×10^{-4}M).

Transfer aliquots to 25 mL volumetric flask and treat as above, beginning with "add 1.0 mL buffer solution. . . ."

Dosage Forms

Direct measurement.—Accurately weigh suitable quantity of powdered tablets or effervescent granules, or volume of syrup or ampule equivalent to 40 mg. Dissolve and dilute to 100 mL with water in volumetric flask. Measure 1.0 mL of this solution into 25 mL volumetric flask and treat as above, beginning with "add 1.0 mL buffer solution. . . ."

Obtain concentration of piperazine salt or compound in preparation from the following equation: $y = bx + a$, where b = slope, a = intercept (Table 1).

Standard addition method.—Transfer portion of prepared solutions (1 mL) containing 0.4 mg to 25 mL volumetric flask and treat as above. Then add volume of standard solution (containing 0.3 mg) (after reaction with sodium nitrite) to cell and re-record polarogram. Determine concentration of piperazine or its salt in formulation by the following equation:

$$C_x = \frac{-v\,C_s h}{h\,V - H(V + v)}$$

where C_x = concentration of unknown sample; V = volume of unknown sample; h = wave height of unknown sample; C_s = concentration of standard; H = total wave height; v = volume of standard.

Results and Discussion

Piperazine is inactive polarographically, but as a secondary amine it can be transformed to a polarographically active species by nitrosation. This procedure has been described for secondary amines in general (18).

In a previous study in our laboratory (8), piperazine was assayed as this nitroso-derivative by UV spectrophotometry. The reaction was complete and stoichiometric. This encouraged us to try a dc polarographic method for piperazine, using this reaction.

The nitroso-derivative under the established conditions of the reaction gives a well-defined, diffusion-controlled cathodic wave with $E_{1/2} = -0.8$ V of a reasonable height that enables us to determine an amount as low as 1.6×10^{-5}M with good precision (Figure 1).

The average recoveries for bulk drugs were 97.67, 99.53, 99.19, 99.3, 101.12, and 99.99% (SD = 3.44, 2.14, 2.47, 2.47, 2.78, and 2.3) for piperazine hexahydrate, phosphate, adipate, citrate, diethylcarbamazine citrate, and piperazine theophylline, respectively (Table 2). These results generally compare well with those obtained by the standard method (gravimetric method (1)) for piperazine or its salts, in which diethylcarbamazine is assayed titrimetrically by standard perchloric acid in nonaqueous medium.

Polarographic Parameters

Effect of buffer and pH.—The following buffers were examined: Britton-Robinson (19) and phthalate-hydrochloric acid (19) at various pH values. Solutions of piperazine, diethylcarbamazine, and piperazine theophylline (pretreated as in the procedure) were prepared in the individual buffer solutions, and a polarogram was recorded. The optimum buffer system was selected, and the influence of pH was studied at intervals of 0.1 pH unit (between pH 2 and 11 for Britton-Robinson buffer). The pH was measured and adjusted for all solutions before electrolysis. The study revealed that Britton-Robinson buffer at pH 2.3 is optimum for a complete reaction and well-defined peak. This pH (2.3) was also reported in the previous study on nitroso formation for UV spectrophotometry (8). $E_{1/2}$ varied with pH changes ($\Delta E_{1/2}/\Delta$ pH = −0.0648 V).

Effect of reagents and interferences.—Excess nitrous acid is completely eliminated by addition of ammonium sulfamate; this had been checked by the color with potassium iodide-starch paper.

Possible interfering compounds usually incorporated with piperazine, e.g., atropine sulfate, colchicine, sodium citrate,

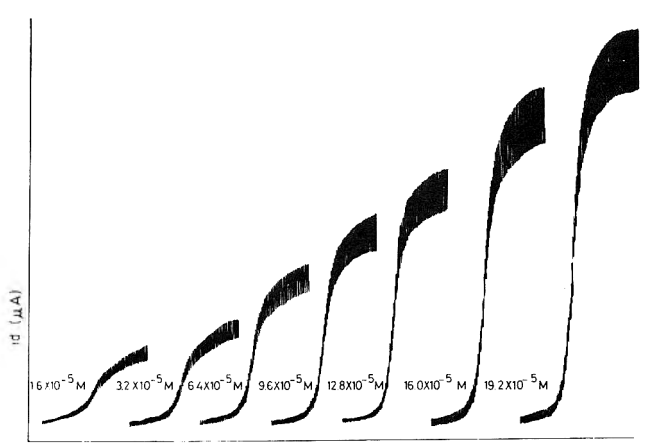

Figure 1. Effect of concentration on wave height. id = f (c) of piperazine hexahydrate.

Table 2. Assay of piperazine salts and compounds alone and in pharmaceutical preparations

Sample	Declared piperazine content, g	Proposed method: Amt found, g	Proposed method: Recovery, %[a]	Official method
Piperazine hexahydrate			97.67 ± 3.44	100.05
Effervescent granules A	0.05/5 g	0.0577	115.34 ± 1.55	92.33 ± 0.65
Effervescent granules B	0.128/5 g	0.1366	106.70 ± 1.8	94.15 ± 0.57
Piperazine granules	3.5/100 g	3.5011	100.03 ± 0.75	95.09 ± 0.83
Piperazine phosphate			99.53 ± 2.14	99.63
Tablets	0.3/tab.	0.2997	99.91 ± 0.9	78.5 ± 0.36
Piperazine adipate			99.19 ± 2.47	99.79
Piperazine citrate			99.3 ± 2.47	99.39
Syrup A	0.5/5 mL	0.5766	115.32 ± 0.93	111.7 ± 1.98
Syrup B	0.7/5 mL	0.7850	112.2 ± 0.87	125 ± 1.83
Eff. granules	0.18/60 g	0.2126	118.12 ± 0.96	119.3 ± 2.86
Diethylcarbamazine citrate			101.12 ± 2.78	99.78 ± 0.18
Tablet	0.5/tab.	0.4460	89.27 ± 0.61	99.47 ± 0.66
Piperazine theophylline			99.99 ± 2.30	99.73 ± 2.96[b]
Ampule	0.5/5 mL	0.5757	115.13 ± 0.83	103.08 ± 2.17

[a]The average of 3 determinations.
[b]Spectrophotometric assay (17).

sodium bicarbonate, Tr. ammivisnaga, diluents, and additives, e.g., sugar and flavor, have been investigated. Solutions containing one or more of these compounds were treated without piperazine and scanned polarographically. No sign of polarographic activity was observed under the conditions of the reaction.

Concentration effect.—Peak height increases linearly with increasing concentration. The graph of the peak height measured at −0.8 V vs SCE is rectilinear for the concentration range 1.6×10^{-5}M to 19.2×10^{-5}M (3.1–37.2 μg/mL). This indicates that the wave is controlled by diffusion.

Logarithmic analysis (20) of the cathodic wave resulted in a reversible characterization with nearly 4 electron transfer, which is in good agreement with previously reported data (21).

The described method was applied to assay the content of representative dosage forms. The results were higher than the labeled amount, but corresponded to those obtained by a spectrophotometric method with chloranilic acid (22).

The developed procedure can be used to assay the content of piperazines with satisfactory precision. The method also has the advantage of being less affected by interferences which are often present in pharmaceutical preparations.

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Quantitation of Indomethacin, Naproxen, and Ibuprofen in Pharmaceutical Dosage Forms by First and Second Derivative Ultraviolet Spectrometry

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First and second derivative ultraviolet spectrometric methods are described for the estimation of indomethacin, naproxen, and ibuprofen in pharmaceutical dosage forms. The proposed methods permit the rapid, precise, and accurate determination of indomethacin capsules BP, naproxen capsules, and ibuprofen tablets BP. Matrix interference is successfully corrected. The results obtained by first and second derivative techniques are in accord with those obtained by the official assay method.

Indomethacin, naproxen, and ibuprofen are widely used as analgesics and anti-inflammatory agents. The official compendium describes alkalimetric titration for indomethacin (1), naproxen (2), and ibuprofen (3) in pure form, spectrophotometric assay for indomethacin capsules (4), and alkalimetric titration for ibuprofen tablets (5). Several methods have been reported for the estimation of indomethacin, naproxen, and ibuprofen. These include colorimetric (6, 7), spectrophotometric (8, 9), fluorometric (10, 11), gas chromatographic (12, 13), liquid chromatographic (14–16), and nonaqueous titration (17, 18) methods.

Derivative ultraviolet spectrometry has been proved to be a valuable technique for the identification and quantitation of several organic compounds and drugs (19–21). This technique is particularly useful in pharmaceutical analysis to assay drugs with low absorptivity (a) values or when the drug in formulated products is susceptible to interference from excipients (20, 22).

This paper describes the application of a first derivative method (D_1) to the estimation of indomethacin in 0.1N H_2SO_4 and a second derivative method (D_2) to the determination of naproxen and ibuprofen in 0.1N NaOH. The proposed methods offer the advantages of rapidity, simplicity, specificity, and accuracy.

Experimental

Reagents

(a) *Drugs.*—Pharmaceutical grade indomethacin (MSD), naproxen (Gruenenthal), and ibuprofen (Kahira). All drugs were obtained as gifts from the manufacturers.

(b) *Sulfuric acid solution.*—0.1N aqueous.

(c) *Sodium hydroxide solution.*—0.1N aqueous.

(d) *Methanol.*—Analytical grade (BDH).

Apparatus

Spectral measurements were made with a Perkin-Elmer, Model 550 S recording UV-VIS spectrophotometer with first and second derivative accessory. Quartz cells of 1 cm path length were used. The first derivative spectrum (indomethacin) was obtained with the following instrumental parameters: scan speed 120 nm/min; chart speed 30 mm/min; response time 6 s; recorder range −0.08 to +0.08. The second derivative spectra were recorded with the following instrumental parameters: scan speed 120 nm/min (naproxen, ibuprofen); chart speed 30 mm/min (naproxen), 60 mm/min (ibuprofen); response time 8 s (naproxen, ibuprofen); recorder range −0.04 to +0.04 (naproxen), −0.08 to +0.08 (ibuprofen).

Preparation of Standards and Calibration Graphs

First derivative method.—Accurately weigh ca 50 mg indomethacin into 50 mL volumetric flask. Dissolve and dilute to volume with methanol. Transfer various aliquots containing 0.2 to 1.0 mg indomethacin into 100 mL volumetric flasks and dilute to volume with 0.1N H_2SO_4. Record first derivative absorption spectrum against corresponding reagent blank, and measure deflection from largest peak at 248 nm to largest trough at 232 nm (D_1 value) (Figure 1).

Second derivative method.—Accurately weigh ca 125 mg naproxen or 250 mg ibuprofen into 50 mL volumetric flask. Dissolve and dilute to volume with methanol. Transfer various aliquots containing 4–10 mg naproxen or 10–50 mg ibuprofen into 100 mL volumetric flasks and dilute to volume with 0.1N NaOH. Record second derivative absorption spectra against corresponding reagent blank, and measure deflection from largest peak at 336 nm to largest trough at 328 nm

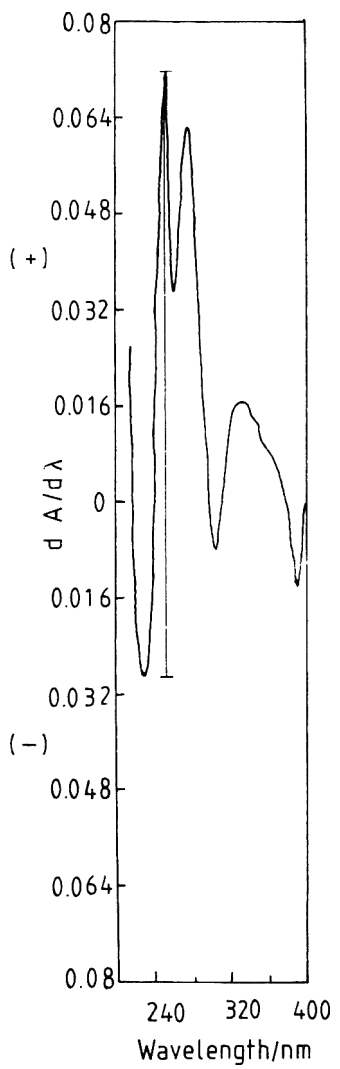

Figure 1. First derivative UV spectrum of 1 mg% Indomethacin in 0.1N H_2SO_4, showing method of measurement.

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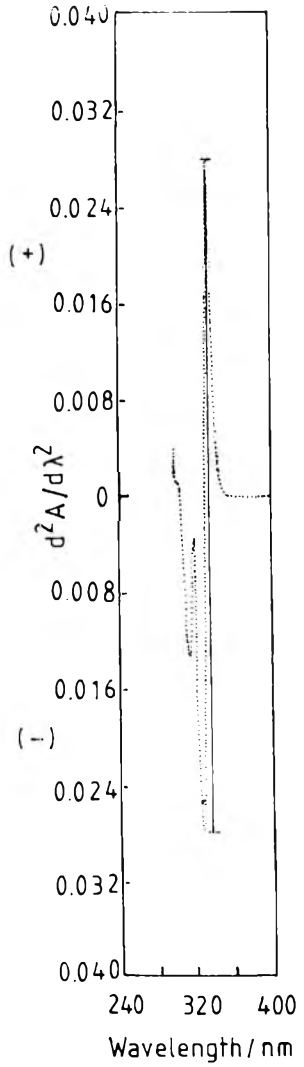

Figure 2. Second derivative UV spectrum of 10 mg% naproxen in 0.1N NaOH.

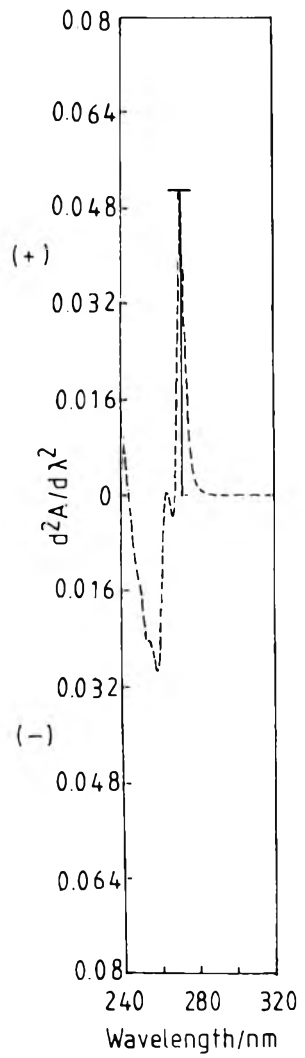

Figure 3. Second derivative UV spectrum of 50 mg% ibuprofen in 0.1N NaOH.

for naproxen or from baseline to peak at 272 nm for ibuprofen (D_2 value) (Figures 2, 3).

Conventional UV spectrometric method.—For the above prepared working solutions, measure absorbance at 245 nm for indomethacin, at 330 nm for naproxen, and at 263 nm for ibuprofen against corresponding reagent blank.

Preparation of Samples

Capsules.—Weigh and mix contents of 20 capsules. Accurately weigh an amount of powder equivalent to ca 50 mg indomethacin or 125 mg naproxen into 50 mL volumetric flask. Add ca 25 mL methanol, shake well 15 min, and dilute to volume with the same solvent. Filter and discard first 10 mL filtrate. Pipet 1 mL indomethacin solution or 4 mL naproxen solution into 100 mL volumetric flask. Dilute to volume with 0.1N H_2SO_4 (indomethacin) or 0.1N NaOH (naproxen). Record first and second derivative spectra and measure D_1 and D_2 values as mentioned above. Measure absorbance at corresponding λ_{max} according to directions given for conventional UV spectrometric method.

Tablets.—Weigh and finely powder 20 tablets. Accurately weigh portion of powdered sample, equivalent to ca 250 mg ibuprofen into 50 mL volumetric flask. Shake well with methanol, dilute to volume with the same solvent, filter, pipet 10 mL clear filtrate into 100 mL volumetric flask, and dilute to volume with 0.1N NaOH. Record second derivative spectrum and measure D_2 value as mentioned above. Measure absorbance at 263 nm against reagent blank.

Results and Discussion

The UV absorption spectra (zero order) of indomethacin, naproxen, and ibuprofen are shown in Figure 4. Indomethacin in 0.1N H_2SO_4 displays 2 absorption maxima at 245 nm (*a*: 490) and at 318 nm (*a*: 177). Although the absorbance at 318 nm is one-fifth as strong as that at 245 nm, indomethacin capsules are quantitated spectrophotometrically at λ_{max} 318 nm (4) to avoid interference from absorbing excipients in the region 230–300 nm. This interference was eliminated by using the first derivative technique (peak-trough amplitude at 248–232 nm). A mixture of excipients alone gave only a baseline spectrum. Furthermore, the determination was possible with lower concentrations of indomethacin. Naproxen contains a naphthalene moiety and the weak UV spectrum has the typical fine structure at 280–340 nm (*a* in 0.1N NaOH at 316 nm: 52; at 330 nm: 70). Ibuprofen in 0.1N NaOH possesses the typical benzenoid fine structure in the region 240–280 nm (*a* at 258 nm: 15; at 263 nm: 18; at 272 nm: 15). The low absorptivity values of naproxen and ibuprofen indicate that conventional UV spectrometric assays of these drugs in pharmaceutical formulations are susceptible to matrix interference. First and second derivative spectrometric assay procedures

Table 1. D_1/D_2 values for indomethacin, naproxen, and ibuprofen standard solutions[a]

Indomethacin		Naproxen		Ibuprofen	
Concn, mg/100 mL	D_1 (248–232 nm)	Concn, mg/100 mL	D_2 (336–328 nm)	Concn, mg/100 mL	D_2 (272 nm)
0.2	0.0198	4.0	0.0220	10.0	0.0100
0.4	0.0400	5.0	0.0272	20.0	0.0200
0.6	0.0590	6.0	0.0331	30.0	0.0300
0.8	0.0816	8.0	0.0442	40.0	0.0400
1.0	0.1008	10.0	0.0552	50.0	0.0498
RSD, %	1.38		0.59		0.78

[a]Average of 6 separate determinations.

Table 2. Assay results (%)[a] for indomethacin in powder form and in capsules by 3 methods

	Method				
	D_1 (248–232 nm)		Conventional UV (245 nm)		Official
Drug form	Taken, mg/100 mL	Recovery,%	Taken, mg/100 mL	Recovery,%	Recovery,%
Powder	0.5	100.6	0.5	99.6	100.3
	0.6	99.3	0.6	99.0	100.5
	0.7	100.0	0.7	98.7	98.8
	0.8	99.4	0.8	99.5	99.1
	0.9	100.9	0.9	100.2	99.8
	1.0	100.0	1.0	99.5	100.4
Mean ± std dev.		100.1 ± 0.7		99.4 ± 0.5	99.8 ± 0.7
Capsules[b]	1.0	102.4	1.0	106.5	103.0
	1.0	102.6	1.0	106.0	105.8
	1.0	102.8	1.0	105.2	103.9
	1.0	102.4	1.0	104.0	102.9
	1.0	102.9	1.0	104.2	103.0
	1.0	102.6	1.0	104.0	104.8
Mean ± std dev.		102.6 ± 0.2		105.2 ± 1.1	103.9 ± 0.9

[a]Average of 6 separate determinations.
[b]Labeled to contain 25 mg indomethacin per capsule.

Table 3. Assay results (%)[a] for naproxen in powder form and in capsules by 3 methods

	Method				
	D_2 (336–328 nm)		Conventional UV(330 nm)		Official
Drug form	Taken, mg/100 mL	Recovery,%	Taken, mg/100 mL	Recovery,%	Recovery,%
Powder	4.0	99.3	4.0	101.0	99.3
	5.0	98.2	5.0	99.4	101.0
	6.0	99.8	6.0	99.3	97.3
	8.0	99.7	8.0	99.5	97.5
	9.0	100.1	9.0	100.2	101.2
	10.0	98.2	10.0	101.2	99.3
Mean ± std dev.		99.2 ± 0.9		101.1 ± 0.8	99.3 ± 1.7
Capsules[b]	10.0	100.8	10.0	104.5	
	10.0	101.7	10.0	104.7	
	10.0	100.8	10.0	103.5	
	10.0	100.7	10.0	102.4	
	10.0	99.7	10.0	104.5	
	10.0	100.9	10.0	102.2	
Mean ± std dev.		100.7 ± 0.8		103.4 ± 1.2	

[a]Average of 6 separate determinations.
[b]Labeled to contain 250 mg naproxen per capsule.

described here allow simple, direct, and accurate determination of indomethacin, naproxen, and ibuprofen.

The specificity of the described procedures was assessed by recovery experiments for the examined compounds. These were carried out by adding the drug in solution to mixtures of the following excipients in the appropriate quantities used in capsules and tablets: talc, lactose, starch, and magnesium stearate. Percent recoveries (n = 6) were 101.0, 99.8, and 99.9 for indomethacin, naproxen, and ibuprofen, respectively.

A linear correlation was obtained between the amplitudes in the first and second derivative spectra and the concentration, C, of indomethacin, naproxen, and ibuprofen over the range 0.2–1.0 mg%, 4–10 mg%, and 10–50 mg%, respectively

Table 4. Assay results (%)[a] for ibuprofen in powder form and in tablets by 3 methods

	Method				
	D_2 (272 nm)		Conventional UV (263 nm)		Official
Drug form	Taken, mg/100 mL	Recovery,%	Taken, mg/100 mL	Recovery,%	Recovery,%
Powder	25.0	98.4	25.0	100.2	99.7
	30.0	98.8	30.0	100.7	100.1
	35.0	99.4	35.0	99.6	99.5
	40.0	98.5	40.0	101.2	101.3
	45.0	100.5	45.0	100.3	100.3
	50.0	100.7	50.0	99.2	101.0
Mean ± std dev.		99.4 ± 1.0		100.2 ± 0.5	100.3 ± 0.5
Tablets[b]	50.0	102.6	50.0	107.9	105.0
	50.0	102.0	50.0	106.7	104.2
	50.0	101.7	50.0	105.0	103.7
	50.0	101.6	50.0	108.0	103.6
	50.0	102.5	50.0	106.8	104.9
	50.0	101.8	50.0	105.3	103.8
Mean ± std dev.		102.0 ± 0.4		106.6 ± 1.4	104.2 ± 0.6

[a]Average of 6 separate determinations.
[b]Labeled to contain 200 mg ibuprofen per tablet.

(Table 1). The correlation coefficients were between 0.9996 and 0.9999. The regression equations derived using the method of least squares (23) are:

$$D_1 = 0.0004 + 0.1000C \quad \text{for indomethacin}$$
$$D_2 = -0.0005 + 0.0056C \quad \text{for naproxen}$$
$$D_2 = 0.0007 + 0.0010C \quad \text{for ibuprofen}$$

where C is mg%.

The precision of the proposed assay procedures was evaluated by determining indomethacin capsules BP, naproxen capsules, and ibuprofen tablets BP. Results obtained are the mean of 6 separate assays. The coefficients of variation of the results obtained for indomethacin, naproxen, and ibuprofen, were 0.19, 0.78, and 0.39%, respectively, indicating satisfactory precision.

The accuracy of the described D_1/D_2 methods was assessed by comparing their results with those obtained by the conventional UV spectrometric method and the official assay methods (1–5). Results recorded in Tables 2–4 indicate that recoveries obtained by the proposed methods for indomethacin, naproxen, and ibuprofen in pure form are in good agreement with those obtained by 2 other analytical procedures. Applying the conventional UV spectrometric method and the official assay methods to the determination of capsules and tablets, resulted in moderately high recoveries. This is attributed to the interference from formulation excipients.

Conclusions

The reported methods give results of high precision and accuracy for the entire range of concentrations tested. Compared with the official BP method which involves several extraction steps (ibuprofen tablets) or the conventional UV spectrometric method which is susceptible to interference from excipients (indomethacin capsules, naproxen capsules, and ibuprofen tablets), the developed derivative techniques offer distinct advantages in simplicity, rapidity, and specificity over other reported methods.

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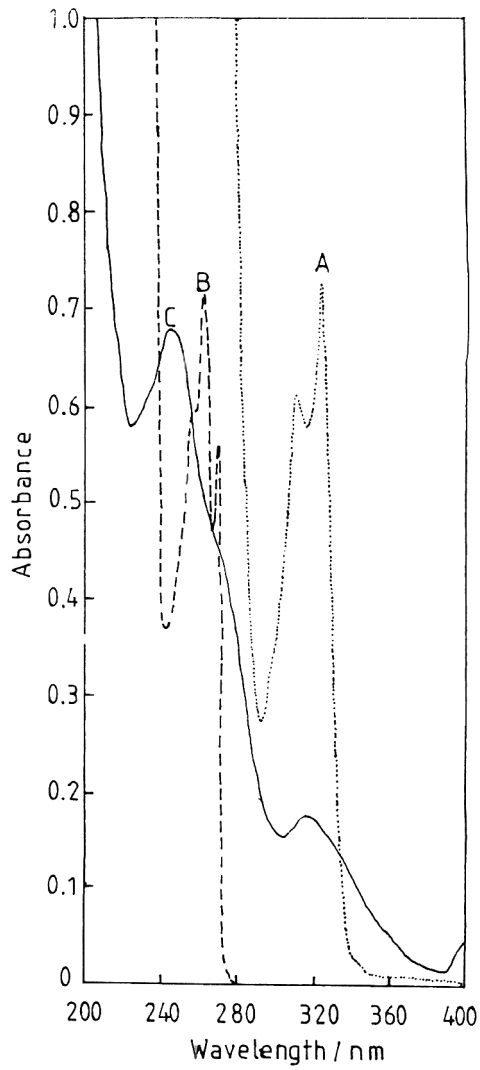

Figure 4. UV absorption spectra of A, 10 mg% naproxen in 0.1N NaOH; B, 50 mg% ibuprofen in 0.1N NaOH; and C, 1 mg% indomethacin in 0.1N H_2SO_4.

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Liquid Chromatographic Determination of Organic Nitrogenous Bases in Dosage Forms: A Progress Report

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A liquid chromatographic (LC) method has been developed as a general procedure for the assay of the salts of organic nitrogenous bases in a variety of dosage forms. The method uses a nitrile-bonded reverse phase column, a methanol-0.003M ammonium acetate (90 + 10) mobile phase, and photometric detection at 254 nm. The sample is dissolved in the mobile phase and an aliquot is injected through a 20 μL injection loop. Average recovery values for duplicate assays were chlorpheniramine maleate injection 97.8%, chlorpheniramine maleate tablets 99.1%, cyclizine hydrochloride tablets 100.0%, doxylamine succinate tablets 103.3%, mesoridazine besylate tablets 100.4%, pentazocine hydrochloride tablets 103.0%, promethazine hydrochloride injection 98.4%, protriptyline hydrochloride tablets 101.2%, pyrilamine maleate tablets 97.8%, pyrimethamine tablets 100.0%, tripelennamine citrate elixir 100.0%, and tripelennamine hydrochloride tablets 97.2%. Results by this method were in good agreement with those obtained by the USP XX method. This study, which is being continued, will be expanded to include additional drugs.

The USP XX (1) classifies a large number of drugs under the general title of organic nitrogenous bases. These drugs, in the form of salts or corresponding free bases, are commercially available in a variety of dosage forms containing from 1 to several active ingredients (2).

The compendial assay of each drug substance entails a nonaqueous titration with a particular combination of solvent and end point indicator (3). The assay method for the dosage forms is a general one that requires a lengthy back-extraction procedure followed by a spectrophotometric determinative step at an appropriate wavelength (3). In general, these methods are time consuming and prone to interference by other components of the dosage forms.

Liquid chromatography (LC) has been successfully used for the analysis of basic drugs in synthetic mixtures (4, 5) and commercial dosage forms (6, 7). None of these methods, however, possesses the general applicability needed for the assay of the official organic nitrogenous bases.

The objective of this study was to develop a rapid and straightforward LC method that could be used to determine organic nitrogenous bases in dosage forms and to analyze for degradation products and other contaminants. This paper reports an LC method that uses a μBondapak CN column, a methanol-0.003M ammonium acetate mobile phase, and photometric detection at 254 nm. Eleven organic nitrogenous bases in solid and liquid pharmaceutical formulations were assayed by this method, and the results were compared with those obtained by the compendial method.

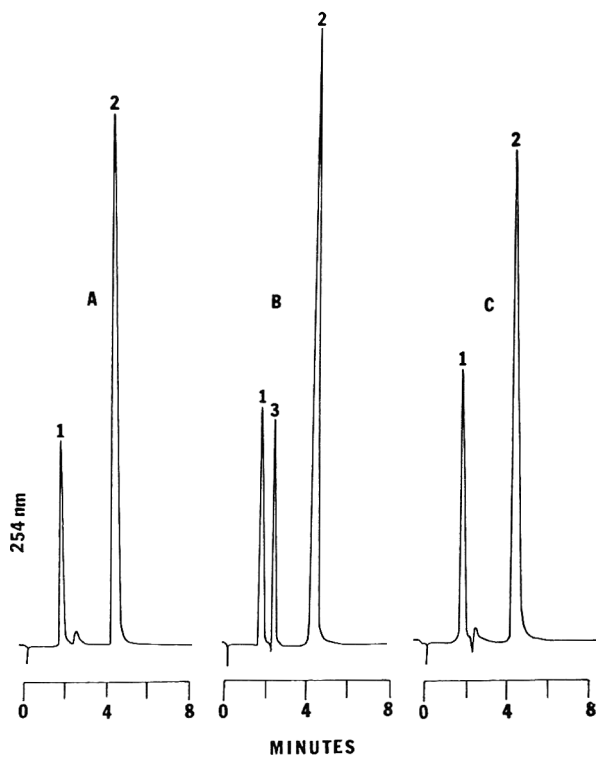

Figure 1. Liquid chromatograms of chlorpheniramine maleate in (A) standard preparation, (B) injectable sample, and (C) tablet sample: 1, solvent front; 2, chlorpheniramine maleate; 3, preservative.

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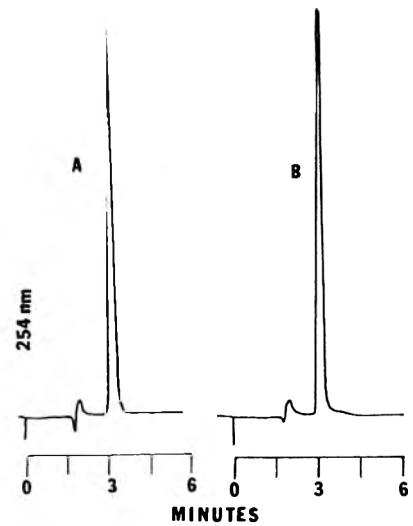

Figure 2. Liquid chromatograms of promethazine hydrochloride in (A) standard preparation and (B) tablet sample.

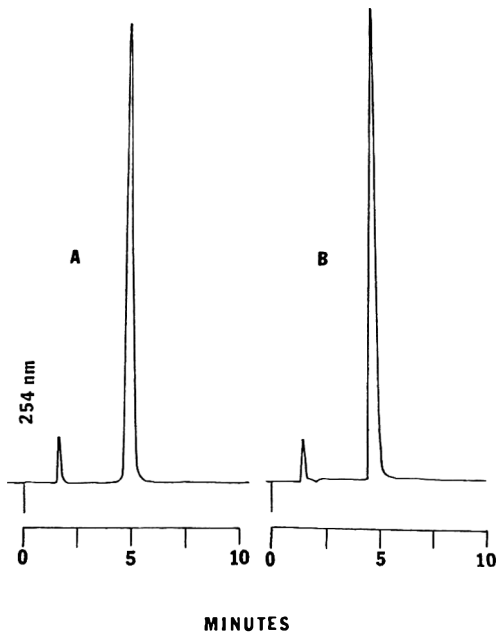

Figure 3. Liquid chromatograms of pyrilamine maleate in (A) standard preparation and (B) tablet sample.

METHOD

Reagents

(a) *Solvents.*—Methanol, analytical reagent grade (Fisher Scientific Co., Pittsburgh, PA 15219); water, distilled in glass.

(b) *Standards.*—All nitrogenous bases were USP Reference Standards (USP Pharmacopeial Convention, Inc., Rockville, MD 20852).

(c) *Standard preparation.*—Accurately weigh ca 10 mg (ca 50 mg for cyclizine hydrochloride) organic nitrogenous base and transfer to 100 mL volumetric flask. Add mobile phase and sonicate to dissolve standard. Dilute to volume with mobile phase and mix. Dilute further with mobile phase if necessary.

(d) *Mobile phase.*—Methanol-0.003M ammonium acetate (90 + 10).

Apparatus

(a) *Liquid chromatograph.*—Tracor Model 950 solvent pump, Model 970A variable wavelength detector, Model 26325 strip chart recorder (Tracor Instruments, Austin, TX 78721), and Rheodyne Model 7120 injector with 20 μL loop (Rheodyne Inc., Cotati, CA 94928), or equivalent. Operating conditions: column temperature, ambient; mobile phase flow rate, 1.3 mL/min; detection wavelength, 254 nm; detector attenuation, adjusted as needed to obtain peak responses of ca 60% FSD; recorder, 1 mV; chart speed, 0.25 in./min.

(b) *Chromatographic column.*—Stainless steel tube, 30 cm × 3.9 mm id, packed with μBondapak CN, 10 μm (Waters Associates Inc., Milford, MA 01757), or equivalent.

Sample Preparation

(a) *Tablets.*—Weigh and finely powder ≥20 tablets. Accurately weigh quantity of powder equivalent to ca 10 mg drug (ca 50 mg for cyclizine hydrochloride) and transfer to 100 mL volumetric flask. Add ca 75 mL mobile phase, and sonicate for ca 20 min. Let solution cool to room temperature, dilute to volume with mobile phase, and mix. Filter solution through 0.45 μm membrane filter, discarding first 10 mL filtrate. If necessary, dilute filtrate further with mobile phase.

(b) *Syrups, elixirs, and injectables.*—Accurately pipet volume of sample equivalent to ca 10 mg drug (ca 50 mg cyclizine hydrochloride) into 100 mL volumetric flask. Add ca 75 mL mobile phase, and sonicate for ca 20 min. Let solution cool to room temperature, dilute to volume with mobile phase, and mix. If necessary, dilute solution further with mobile phase.

System Suitability Check

Equilibrate chromatographic system with mobile phase at 1.3 mL/min until steady baseline is obtained. Inject 20 μL standard preparation into chromatograph with chromatographic conditions adjusted to give peak responses of ca 60% FSD. Under these conditions, 5 replicate injections of stan-

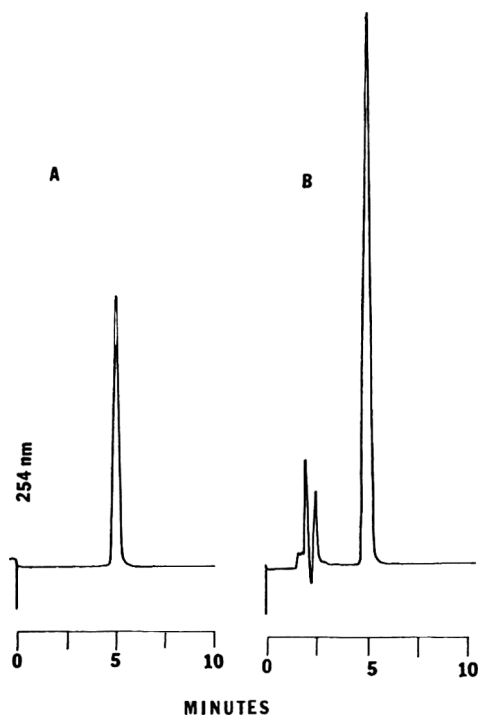

Figure 4. Liquid chromatograms of tripelennamine citrate in (A) standard preparation and (B) elixir sample.

Table 1. Recovery of organic nitrogenous bases from commercial dosage forms by LC method

Nitrogenous base[a]	Dosage form	Declared	Added, mg	Rec.,[b] mg	Rec.,[b] %	Av., %
CPM	injection	10 mg/mL	0.441	0.430, 0.432	97.5, 98.0	97.8
CPM	tablet	4 mg/tab	0.441	0.437, 0.437	99.1, 99.1	99.1
CZH	tablet	50 mg/tab	0.520	0.540, 0.500	103.8, 96.2	100.0
DXS	tablet	12.5 mg/tab	0.213	0.220, 0.220	103.3, 103.3	103.3
MDM	tablet	10 mg/tab	0.1006	0.101, 0.101	100.4, 100.4	100.4
PZH	tablet	50 mg/tab[c]	0.505	0.512, 0.528	101.4, 104.6	103.0
PMH	injection	25 mg/mL	0.124	0.122, 0.122	98.4, 98.4	98.4
PTH	tablet	5 mg/tab	0.0568	0.0575, 0.0575	101.2, 101.2	101.2
PAM	tablet	25 mg/tab	0.2098	0.2045, 0.2055	97.5, 98.0	97.8
PMA	tablet	25 mg/tab	0.060	0.060, 0.060	100.0, 100.0	100.0
TPAC	elixir	37.5 mg/5 mL	0.015	0.015	100.0	—
TPAH	tablet	25 mg/tab	0.250	0.243	97.2	—

[a]CPM = chlorpheniramine maleate; CZH = cyclizine hydrochloride; DXS = doxylamine succinate; MDM = mesoridazine besylate; PZH = pentazocine hydrochloride; PMH = promethazine hydrochloride; PTH = protriptyline hydrochloride; PAM = pyrilamine maleate; PMA = pyrimethamine; TPAC = tripelennamine citrate; TPAH = tripelennamine hydrochloride.
[b]Pairs represent duplicate injections of the sample.
[c]Calculated as the base.

dard preparation should give peak heights with a coefficient of variation (CV) that is ≤3%.

Determination

Make duplicate 20 μL injections of sample and standard preparations. Calculate amount of drug in portion of tablets taken or in each mL of liquid taken as follows:

$$\text{mg} = CD(R_U/R_S)$$

$$\text{mg/mL} = (CD/V)(R_U/R_S)$$

where C is concentration of standard drug in standard preparation in mg/mL; D is dilution factor; V is volume of liquid dosage form taken in mL; and R_U and R_S are peak responses obtained from sample and standard preparations, respectively.

Results and Discussion

Eleven organic nitrogenous bases included in USP XX were analyzed by the proposed LC method. Chromatograms of standard and sample preparations of chlorpheniramine maleate, promethazine hydrochloride, pyrilamine maleate, and tripelennamine citrate are shown in Figures 1–4. Linearity studies conducted with 4 of the 11 drugs indicated that the method is linear for the following ranges of each injected drug: pyrilamine maleate, 0.38–1.32 μg; pyrimethamine, 0.2–2.0 μg; tripelennamine citrate, 0.10–0.59 μg; and tripelennamine hydrochloride, 0.40–1.68 μg.

The accuracy of the proposed method was tested by adding known quantities of each organic nitrogenous base to the corresponding dosage form(s). Table 1 summarizes the results of this study.

CVs calculated from peak height measurements for 5 consecutive injections of each standard preparation were <2% for all drugs studied by the proposed method.

Table 2 shows the results for 12 commercial preparations of organic nitrogenous bases that were assayed by the LC and USP methods. Assay values obtained by the 2 methods agreed to within 2% for 5 samples; assay values for the other 7 samples showed differences that ranged from 2.4 to 4.9%. Results by the LC method were higher for 9 of the 11 drugs; only chlorpheniramine maleate and protriptyline hydrochloride gave higher assay values by the USP spectrophotometric method. The difference appears to be significant for 7 of these drugs. These differences and the apparent bias may be due to losses of analyte incurred with the compendial method during the multiple back-extraction steps required for sample preparation. Losses were minimal with the LC method, however, because of the direct dissolution of the sample in the mobile phase.

Attempts using the described chromatographic conditions failed to resolve all 11 organic nitrogenous bases in a mixed standard preparation because several of the compounds exhibited similar elution times (Table 3). Results were no better with the various combinations of column and mobile phase that are described in the literature for basic drugs

Table 2. Determination of organic nitrogenous bases in commercial dosage forms by LC and USP XX methods

Nitrogenous base[a]	Dosage form	Declared	Found, % of decld: USP XX	Found, % of decld: LC
CPM	injection	10 mg/mL	103.5[b]	102.0
CPM	tablet	4 mg/tab	101.5[b]	99.6
CZH	tablet	50 mg/tab	95.5[b]	100.4
DXS	tablet	12.5 mg/tab	98.0[b]	101.7
MDM	tablet	10 mg/tab	102.0	104.4
PZH	tablet	50 mg/tab[c]	97.1[b]	101.4
PMH	injection	25 mg/mL	96.2	99.9
PTH	tablet	5 mg/tab	90.0	90.0
PAM	tablet	25 mg/tab	94.5[b]	98.9
PMA	tablet	25 mg/tab	97.4[b]	102.1
TPAC	elixir	37.5 mg/5 mL	101.1	101.2
TPAH	tablet	25 mg/tab	96.0[b]	96.4

[a]See Table 1 for identification of samples.
[b]Average of 2 determinations.
[c]Calculated as the base.

Table 3. Retention times of organic nitrogenous bases on μBondapak CN column[a]

Nitrogenous base[b]	Retention time, min
CPM	4.3
CZH	3.3
DXS	4.9
MDM	5.2
PZH	5.3
PMH	3.3
PTH	6.6
PAM	5.0
PMA	2.7
TPAC	4.9
TPAH	4.9

[a]Mobile phase, methanol-0.003M ammonium acetate (90 + 10); flow rate, 1.3 mL/min; detector wavelength, 254 nm.
[b]See Table 1 for identification of samples.

(4–7). Future work will attempt to completely resolve these compounds in mixtures by using the LC method as part of the identification test, and suitable chromatographic systems will be developed for the detection and measurement of degradation products.

Acknowledgment

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Determination of Reserpine in Commercial Tablets by Liquid Chromatography with Fluorescence Detection

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A procedure is presented for the determination of reserpine in commercial tablets by liquid chromatography (LC). The sample is extracted with methanol if only reserpine is present. If the sample contains other ingredients, $CHCl_3$ is used for extraction from aqueous suspension; the $CHCl_3$ is subsequently completely evaporated in the presence of methanol. For LC, a normal phase column, methanol as the eluting solvent, and a fluorometric detector are used. A recovery study indicated that no measurable degradation of reserpine occurs during evaporation of the $CHCl_3$ extract. Several commercial tablets containing reserpine alone or in combination with other ingredients were analyzed by the proposed method, and the results were compared with those obtained by the current official USP methods for reserpine.

Reserpine, a mild hypotensive and sedative agent, has been isolated from *Rauwolfia serpentina* (1), and has also been obtained synthetically (2). Experiments in this laboratory have indicated that reserpine contains trace quantities of other *Rauwolfia* alkaloids, and it has been reported that oxidation may readily occur (3). The current official methods for the determination of reserpine in finished products (4, 5) convert the intact reserpine to its degradation products with nitrous acid, a procedure originally proposed by Szalkowski and Mader (6) and subsequently slightly modified by Banes et al. (7). These procedures require a blank determination to correct for other alkaloids and for degradation products present in the sample before the assay. The main product obtained when reserpine is oxidized by the official methods (4, 5) is 3,4-dehydroreserpine, but smaller quantities of other degradation products are also formed (3, 8). For the determination of reserpine in individual tablets, vanadium pentoxide is used as the oxidizing agent instead of nitrous acid, but the products of oxidation are apparently the same (9).

Some recently published methods use liquid chromatography (LC) with UV absorption detection for the determination of reserpine in finished products (10–12). The procedure presented in this report also uses LC, but with a fluorometric detector, as in the previously reported analysis of *Rauwolfia serpentina* preparations (13). This type of detection permits greater sensitivity and decreases possibilities of interference.

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METHOD

Apparatus and Reagents

(a) *Reserpine.*—USP; available from U.S. Pharmacopeial Convention, Rockville, MD 20852.

(b) *Methanol.*—LC grade (Burdick & Jackson Laboratories Inc., Muskegon, MI 49442), or equivalent.

(c) *Liquid chromatograph.*—Model U6K universal injector and Model 6000A solvent delivery system (Waters Associates, Inc., Milford, MA 01757) connected to FS-970 fluorescence detector (Kratos Analytical Instruments, Westwood, NJ 07675). A fluorescence detector with settings for both excitation and emission wavelengths can also be used.

(d) *LC column.*—μPorasil, 30 cm × 3.9 mm id (Waters Associates, Inc.). Any other normal phase column can be used, provided that it successfully separates reserpine from degradation products (Figure 1, No. 2).

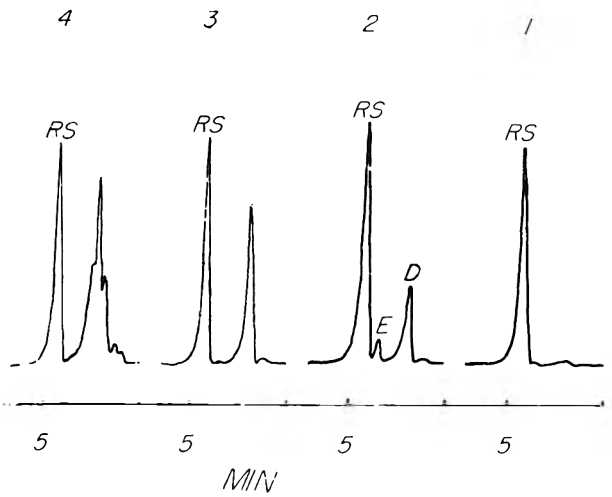

Figure 1. LC chromatograms: 1, reserpine (RS) reference solution (b); 2, oxidation mixture of reserpine: D = 3,4-dehydroreserpine (tentative identification), E = 3-isoreserpine (tentative identification); 3, methanol extract of reserpine tablet, sample solution (d) *(1)*; 4, extract of tablets containing reserpine in combination with hydrochlorothiazide and hydralazine, sample solution (d) *(2)*.

Table 1. Recovery of reserpine by the proposed LC method[a]

Detn	Added, mg	Found, mg	Rec., %
1	0.100	0.101	101
2	0.100	0.0991	99.1
3	0.100	0.0990	99.0
4	0.100	0.101	101

[a]Extraction procedure used is described under sample solution **(d)** *(2)*: An aliquot of $CHCl_3$ solution of reserpine was transferred to separator containing 5 mL 0.1N H_2SO_4.

Solutions

(a) *Reserpine stock solution.*—Store in dark and discard after 4 weeks. Accurately weigh ca 50 mg reserpine and transfer to 100 mL volumetric flask. Add 1 mL $CHCl_3$, swirl to dissolve, dilute to volume with methanol, and mix.

(b) *Reserpine reference solution.*—Dilute reserpine stock solution with methanol to prepare solution containing 0.1 mg (± 5%) reserpine/50.0 mL.

(c) *System suitability solution.*—Dissolve 5 mg reserpine in 10 mL $CHCl_3$. Transfer 0.50 mL aliquot to small beaker and irradiate 10 min under longwave UV light. Evaporate $CHCl_3$ on steam bath. Dissolve residue in methanol, and transfer to 50 mL volumetric flask. Rinse beaker with methanol, and add rinsings to flask. Dilute to volume with methanol and mix.

(d) *Sample solution.—(1) Tablets containing only reserpine:* Transfer weighed sample containing ca 0.1 mg reserpine to 30 mL beaker. Add 10 mL methanol and warm gently on steam bath 5 min. Cool to room temperature, and transfer to 50 mL volumetric flask. Rinse beaker with small portions of methanol, and add rinsings to flask. Dilute to volume with methanol, mix, and filter through paper.

(2) Tablets containing reserpine in combination with one or more ingredients such as hydrochlorothiazide, chlorothiazide, or hydralazine: Transfer weighed sample equivalent to ca 0.1 mg reserpine to separator containing 5 mL 0.1N H_2SO_4. Extract with 25 mL $CHCl_3$, wash in second separator containing 5 mL 0.1N NaOH, filter washed extract through cotton, and receive in 250 mL beaker containing 50 mL methanol. Extract with 3 additional 25 mL portions of $CHCl_3$, and wash each portion in second separator. Filter and receive in same 250 mL beaker. Evaporate to ca 25 mL on steam bath with aid of air current. Rinse walls of beaker with 20 mL methanol. Continue heating on steam bath until volume is reduced to 20 mL. Transfer to 50 mL volumetric flask. Rinse beaker with small portions of methanol, and add rinsings to flask. Dilute to volume with methanol and mix.

LC Determination

Set excitation at 280 nm, and insert 370 nm filter (or set emission at 360 nm if detector can be adjusted). Elute methanol through column at flow of 1.5 mL/min. Inject 50 μL reserpine reference solution **(b)**. Adjust flow rate and sensitivity so that reserpine peak appears after 4–5 min, and height of reserpine peak is ca 60% of chart height (Figure 1, No. 1). Inject 50 μL system suitability solution **(c)**. In addition to the reserpine peak, the chromatogram should have 2 distinct peaks, one immediately before the reserpine peak and the other ca 2 min earlier (Figure 1, No. 2).

After satisfying above conditions, proceed as follows: Inject three 50.0 μL aliquots each of reserpine reference solution **(b)** and sample solution **(d)** *(1)* or **(d)** *(2)*. Average 3 peak heights for each solution. Designate average peak height of reserpine reference solution and average peak height of reserpine in sample chromatograms as *Hr* and *Hs*, respectively. Determine amount of reserpine in sample solution as follows:

$$\text{Reserpine (mg)} = (Hs)(Wr)/(Hr)$$

where *Wr* is mg reserpine in 50.0 mL reserpine reference solution **(b)**. Calculate amount of reserpine per tablet.

Results and Discussion

A satisfactory LC procedure for the determination of reserpine must show that the alkaloid is adequately separated from degradation products that may be present in the commercial products. The conditions used in this investigation are essentially the same as those described in a previous report (13). The column contains silica gel, and the eluting solvent is methanol. The main degradation products, tentatively identified as 3,4-dehydroreserpine and 3-isoreserpine, are eluted earlier than reserpine (Figure 1, No. 2). Using a normal phase column but a different solvent, Butterfield et al. (11) found that isoreserpine is eluted just before reserpine, and that the 2 other degradation products are retained on the column. Vincent and Awang (12), using a reverse phase column and a buffered solvent, reported that all 3 degradation products are eluted later than reserpine. Honigberg et al. (10) do not indicate the positions of the degradation products in their LC analysis.

Fluorometric detection is preferable to UV detection for the determination of reserpine because it increases the sensitivity of detection by a factor of ≥ 50 and lessens the possibility of interferences. Direct extraction with methanol is satisfactory for samples containing only reserpine. The chromatograms of such samples contain a foreign peak that results from tablet excipients and appears at approximately the same retention time as the first degradation peak (Figure 1, No. 3). Hydrochlorothiazide, chlorothiazide, and hydralazine, if present, are also eluted at the approximate retention time of the first degradation product and the inert excipients; consequently, they do not interfere with the determination of reserpine. If methanol extraction is used, however, the peak resulting from the other ingredient(s) is often very large and may distort the shape of the reserpine peak. With samples of this type, the $CHCl_3$ extraction described for sample solution

Table 2. Analysis of commercial tablets by current USP methods and by the proposed LC method

Label, mg/tablet	Current USP method[a]		Proposed method[a]	
	Reserpine found, mg/tablet	Percent of label	Reserpine found, mg/tablet	Percent of label
Reserpine 0.1 mg	0.101	101.0	0.103	103.0
Reserpine 0.25 mg	0.238	95.2	0.238	95.2
Chlorothiazide 250 mg, reserpine 0.125 mg	0.128	102.4	0.130	104.0
Hydrochlorothiazide 25 mg, reserpine 0.125 mg	0.124	99.2	0.123	98.4
Hydrochlorothiazide 25 mg, hydralazine HCl 25 mg, reserpine 0.1 mg	0.0985	98.5	0.0993	99.3
Hydralazine HCl 25 mg, reserpine 0.1 mg	0.0956	95.6	0.0962	96.2

[a]Ref. (4), p. 706, for reserpine alone, and p. 708, for reserpine in combination with other ingredients.

(d) *(2)* is preferred; the $CHCl_3$ extract is completely evaporated on the steam bath in the presence of methanol. Small quantities of other ingredients are still present, but the peak produced by them is small and does not affect the shape of the reserpine peak (Figure 1, No. 4). Recovery studies indicated that no measurable degradation of reserpine occurs during the evaporation of the $CHCl_3$ on the steam bath, provided that some methanol is always present and the volume of liquid does not go below 20 mL (Table 1).

The results of the analyses of some commercial tablets are reported in Table 2. The reserpine content of these samples determined by the proposed method is very close to that obtained by the current USP official methods (4). The simultaneous determination of reserpine and another ingredient was not considered in this investigation, although such analyses have occasionally been performed by LC under different conditions (10, 11). The method presented here is for the determination of reserpine only; another method would be needed to determine any of the other ingredients used in combination with reserpine.

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Liquid Chromatographic Determination of Diazepam in Tablets: Collaborative Study

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Collaborators: N. Falcone; M. Lookabaugh; G. D. Reed; S. E. Roberts; M. Smela, Jr; R. E. Sutton

A stability indicating liquid chromatographic method for the determination of diazepam in tablets was collaboratively studied by 6 laboratories. The method uses a C_{18} reverse phase column, a methanol–water mobile phase, *p*-tolualdehyde as the internal standard, and photometric detection at 254 nm. The collaborators were supplied with a synthetic tablet powder and 3 commercial tablet samples. The mean recovery of diazepam from the synthetic tablet powder was 100.2%. For all samples analyzed, the coefficient of variation was < 1.5%. The method has been adopted official first action.

A method for the determination of the tranquilizer diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one, was developed as the result of a study conducted under the Compendial Monographs Evaluation and Development program of the Food and Drug Administration (FDA). The purpose of the study was to evaluate and revise the existing official assay methods for the various dosage forms of diazepam. The current official methods for diazepam tablets, capsules, and injections are similar liquid chromatographic (LC) procedures; however, they differ in the internal standards used and in the ratio of components in the mobile phase (1, 2). To obviate these variations, a published stability indicating LC method for the assay of diazepam tablets (3) was modified to incorporate the internal standard used in the USP XX method for diazepam injection (2). Thus the different dosage forms will be assayed under the same chromatographic conditions.

Collaborative Study

Collaborators from 6 laboratories received 3 samples of commercial tablets and 1 synthetic tablet powder as blind duplicates. The commercial tablet samples consisted of 3 dosage levels from 1 manufacturer. The collaborators were also furnished an instruction sheet, a copy of the method, and a reporting form.

Diazepam in Drug Tablets
Liquid Chromatographic Method
First Action

Principle

Diazepam content of tablets is detd by reverse phase liq. chromatgy using MeOH–H_2O mobile phase, UV detection at 254 nm, and *p*-tolualdehyde as internal std.

Apparatus

(a) *Liquid chromatograph.*—Tracor Model 950 solv. pump with Model 970A variable wavelength detector capable of monitoring elution at 254 nm (Tracor Instruments Inc.), injection valve with 20 μL sample loop (Valco Instruments, Inc., Houston, TX 77055), and suitable strip chart recorder, or equiv. LC system. Operating conditions: mobile phase flow rate ca 1.2 mL/min, temp. ambient, sensitivity adjusted to give 60–90%. FSD for sample and std injections. Retention time for *p*-tolualdehyde and diazepam ca 5 and 10 min, resp.

(b) *Chromatographic column.*—Stainless steel, 30 cm × 3.9 mm id, packed with C_{18} μBondapak, 10 μm (Waters Associates, Inc.) or equiv. column meeting LC system suitability requirements.

Reagents

(a) *p-Tolualdehyde.*—98% (Fluka Chemical Corp., Hauppauge, NY 11787, or equiv.).

(b) *Solvents.*—LC grade MeOH (J. T. Baker Chemical Co., or equiv.).

(c) *Mobile phase.*—MeOH–H_2O (65 + 35), degassed before use.

(d) *Internal std soln.*—Prep. fresh daily as follows: Pipet 1 mL *p*-tolualdehyde into 50 mL vol. flask, dil. to vol. with MeOH, and mix. Pipet 4 mL of this soln into 250 mL vol. flask, dil. to vol. with MeOH, and mix.

(e) *Diazepam std soln.*—USP Diazepam Ref. Std (RS), previously dried in vac. over P_2O_5 4 h at 60°. Dissolve accurately weighed amt in MeOH, and dil. quant. with MeOH to ca 1 mg/mL. Pipet 5.0 mL of this soln and 5.0 mL internal std soln into 25 mL vol. flask, dil. to vol. with MeOH, and mix.

LC System Suitability Test

Make 5 replicate injections of std soln and record peak ht or peak area responses. System is suitable if relative std dev. (S_r) is ≤ 2.0%, using the equation:

$$S_r, \% = \frac{100}{\bar{x}}\left[\frac{\sum_{i=1}^{n}(x_i-\bar{x})^2}{n-1}\right]^{1/2}$$

where $\bar{x}$ = mean of set of n measurements, and x = individual measurement.

Resolution factor, R, between *p*-tolualdehyde and diazepam should be ≥3.5, using the equation:

$$R = [2(t' - t))]/(PW + PW')$$

where t and t' = mm retention of diazepam and *p*-tolualdehyde, resp.; and PW and PW' = mm peak widths measured at baseline of diazepam and *p*-tolualdehyde, resp.

Tailing factor ratio, T, should be ≤2.5, using the equation:

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

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

Sample Preparation

Weigh and finely powder ≥20 tablets. Transfer accurately weighed portion of powder, equiv. to ca 10 mg diazepam, into 50 mL vol. flask. Pipet 10 mL internal std soln into flask, add ca 25 mL MeOH, mech. shake 30 min, dil. to vol. with MeOH, and mix. Filter soln thru 0.5 μm membrane filter, discarding first 10 mL filtrate.

This report of the Associate Referee was presented in part at the 97th Annual International Meeting of AOAC, Oct. 3–6, 1983, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee B and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.

Submitted for adoption July 30, 1984.

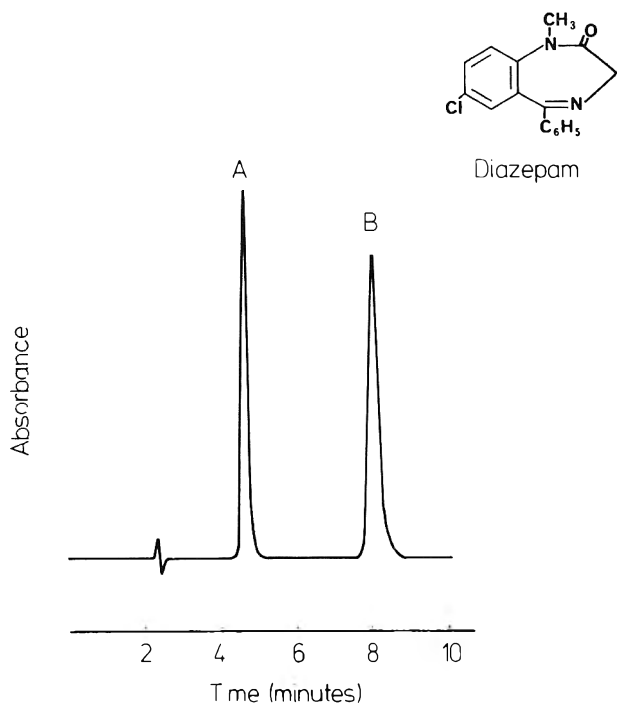

Figure 1. LC separation of A, *p*-tolualdehyde (0.064 μL/mL), the internal standard; and B, diazepam (0.2 mg/mL). Chromatographic conditions: column, μBondapak C_{18}; mobile phase, methanol–water (65 + 35); flow rate, 1.2 mL/min; detection, 254 nm; 0.64 AUFS; temperature, ambient.

Determination

Introduce equal vols (10–20 μL) of sample prepn and std soln into liq. chromatograph by means of suitable syringe or sampling device. Measure responses and det. response ratios (diazepam/internal std peaks) for sample and std solns.

$$\text{Diazepam, mg/tablet} = 50C \times (R/R') \times (T/W)$$

where C = concn diazepam in std soln, mg/mL; R and R' = ratios of peak responses of diazepam to *p*-tolualdehyde for sample prepn and std soln, resp.; T = av. tablet wt, mg; and W = sample wt taken for assay, mg.

Results and Discussion

The collaborative results and statistical evaluations are presented in Table 1. The commercial tablets showed a repeatability coefficient of variation (CV_0) of less than 0.90% and a reproducibility coefficient of variation (CV_x) of less than 1.00%. The corresponding values for the synthetic formulations were 0.96% and 1.14%.

Although 6 laboratories participated in the study, the results from one of the collaborators were not included in Table 1 because this collaborator did not follow the method as written. Collaborator 3 used a 25 cm × 4.6 mm chromatographic column whereas the other collaborators used 30 cm × 3.9 mm columns. Collaborators 1 and 2 ran the assays at a flow rate of 1.0 mL/min; the other collaborators used a flow rate of 1.2 mL/min. Collaborators 4 and 5 measured peak areas; the other collaborators based their calculations on peak height measurements. Either approach yielded satisfactory results.

Table 1. Collaborative assay results (blind duplicates) for LC determination of diazepam

	Commercial tablets, % of decl.			
Coll.	2 mg/tab	5 mg/tab	10 mg/tab	Synthetic tablets, % rec.[a]
1	101.6	99.2	102.2	99.3
	101.1	99.8	102.8	99.8
2	102.5	100.8	104.0	99.7
	101.8	100.8	105.1	102.5
3	103.9	101.2	104.1	101.0
	101.5	101.0	103.4	101.3
4	102.0	100.4	102.1	99.5
	101.4	101.2	102.2	100.5
5	102.1	99.4	102.9	99.2
	103.1	99.6	103.3	99.0
Mean	102.1	100.3	103.2	100.2
S_o[b]	0.89	0.33	0.47	0.96
S_x[b]	0.89	0.81	1.02	1.14
CV_o[c]	0.87	0.33	0.46	0.96
CV_x[c]	0.87	0.81	0.99	1.14

[a]Formulated to contain 6.04 mg/100 mg.
[b]S_o and S_x are the repeatability SD and reproducibility SD, respectively.
[c]CV_o and CV_x are the repeatability CV and reproducibility CV, respectively.

None of the collaborators reported any problems with the proposed method. However, at the suggestion of one of the collaborators, the instructions for the preparation of the sample were modified to specify the porosity of the membrane filter used to filter the sample.

A typical chromatogram of diazepam and *p*-tolualdehyde, the internal standard, is shown in Figure 1. A resolution factor of about 3.5 was specified as the minimum necessary to separate diazepam from its related compounds. Collaborators reported an average resolution factor of 5.9 (range 4.2–7.8).

Recommendation

It is recommended that the liquid chromatographic method for diazepam tablets, described in this report, be adopted official first action.

Acknowledgments

The Associate Referee thanks William M. Plank, Research Coordinator, FDA New York Regional Laboratory, Brooklyn, NY, and Cesar A. Lau-Cam, Science Advisor, FDA New York Regional Laboratory, and Professor of Pharmacognosy, St. John's University, College of Pharmacy and Allied Health Professions, Jamaica, NY, for their assistance in the preparation of this paper; and the following collaborators from the Food and Drug Administration who participated in the study: N. Falcone, Philadelphia, PA; M. Lookabaugh, Boston, MA; G. D. Reed, Kansas City, MO; S. E. Roberts, Winchester, MA; M. Smela, Jr, Brooklyn, NY; and R. E. Sutton, Detroit, MI.

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Thin Layer Chromatographic Identification and Quantitation of Niacin and Niacinamide in Pharmaceutical Preparations

BANDITA SARANGI, SANAT K. CHATTERJEE, KAJAL DUTTA, and SAROJ K. DAS[1]
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A safe and simple thin layer chromatographic method has been developed for the estimation of niacin and niacinamide in pharmaceutical preparations containing other vitamins, enzymes, herbs, antiamoebic drugs, etc. The method involves removal of excipients from the preparation by ethanol precipitation and isolation of niacin or niacinamide from other ingredients by TLC on silica gel with water as developing solvent, followed by extraction in 0.1N HCl, and spectrophotometric estimation of the vitamin at 262 nm. The percent recoveries for niacin and niacinamide were 100.1 ± 1.9 and 100.2 ± 1.5, respectively.

The methods available for the quantitation of niacin or niacinamide involve either Konig's reaction (1), or the use of physicochemical properties of the intact molecule, viz, measurement of UV absorption (2), color change of bromothymol blue (1), nonaqueous titration (3), etc. These vitamins may also be estimated by reaction with *N*-phenylnaphthylamine (1) or sodium-1,2-naphthaquinone-4-sulfonate (4), or by a microbiological method (5). When present in dosage forms in combination with other drugs or vitamins, niacin or niacinamide is generally assayed by the first method (1), which involves opening up the pyridine ring by cyanogen bromide, followed by coupling the resulting compound with a suitable organic base. The other methods are handicapped by low specificity and by general interference from various ingredients present in commercial vitamin mixtures. The cyanogen bromide method also has some serious limitations: Pyridoxine, thiamine, and some pyridine derivatives interfere (1,5); interferences are also encountered when the original drug is colored; and this method does not differentiate niacin and niacinamide (1, 5).

The most serious problem, however, is that cyanogen bromide and potassium cyanide, which is used to prepare the former, are extremely poisonous compounds and are unpleasant to handle. Because it is difficult to observe the proper precautions when handling a large number of samples, a fatal accident may result. Thus, we tried to develop a simple, specific, economic, and safe method for the assay of niacin and niacinamide in various formulation drugs. This paper describes the method we have developed for the assay of niacin and niacinamide by thin layer chromatography followed by UV spectroscopy.

METHOD

Apparatus

(a) *Spectrophotometer.*—Perkin-Elmer double beam spectrophotometer, Model Hitachi 200: slit width, 2 nm; recorder range, 0–1 OD.

(b) *UV lamp.*—Shortwave, Hanovia Chromatolight.

(c) *TLC plates.*—20 × 20 cm, 0.4 mm thick silica gel plate; 39 g Kieselgel 60 G and 13 g Kieselgel 60 HF_{254} (E. Merck) were mixed with 120 mL water by shaking vigorously 45 s and were used to coat 8 plates (6).

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Reagents

(a) *Standard solution.*—Niacin and niacinamide (USP RS), 1 mg/mL in ethanol.

(b) *Ethanol.*—Distilled, dehydrated ethanol (USP grade).

(c) *Hydrochloric acid.*—0.1N prepared from HCl AR (BDH Chemicals Ltd, England)

(d) *TLC developing solvent.*—Distilled water.

Preparation of Sample

(a) *Tablets.*—Select 20 intact tablets, weigh accurately, and grind to smooth powder with mortar and pestle. Accurately weigh powder equivalent to ca 50 mg niacin or niacinamide, and quantitatively transfer to 50 mL volumetric flask, followed by 20 mL distilled, dehydrated ethanol. Shake thoroughly on vortex-mixer 2 min, and dilute to volume with ethanol. Mix again and let excipients settle. Use clear supernate for TLC.

(b) *Capsules.*—Accurately weigh contents of 20 capsules and mix thoroughly with mortar and pestle. Accurately weigh amount powder equivalent ca 50 mg niacin or niacinamide and treat as for tablets.

(c) *Liquid preparations other than syrups.*—Dilute pharmaceutical preparations like vitamin injection, pediatric drops, etc., with dehydrated ethanol to produce 1 mg/mL of niacin or niacinamide. If any precipitate appears, remove it by centrifugation, and use clear supernatant alcoholic solutions for TLC. Liquids containing < 2 mg/mL of the vitamin are treated as syrups as described below.

(d) *Syrups.*—Treat samples containing > 3 mg/mL of niacin or niacinamide like liquid preparations other than syrups, described in (c). For more dilute samples, transfer amount equivalent to 5 mg niacin or niacinamide to glass planchet and dry overnight, positioned over P_2O_5, under vacuum. Dissolve residue in 1 mL water and transfer to 5 mL volumetric flask. Wash planchet repeatedly with four 1 mL portions of dehydrated ethanol, and add washings to flask. Thoroughly mix contents and allow any precipitates to settle. Use clear supernate for TLC. Using dehydrated ethanol helps precipitate out TLC-interfering sugars, minerals, proteins, etc., from liquid and syrup preparations.

Thin Layer Chromatography

Apply 50 μL of each alcohol extract of samples or standard as 1 cm band on silica gel plate, 2 cm from bottom. Plates are developed to 12 cm height with distilled water. After development, view plates under shortwave UV lamp, and mark spots for standard niacin or niacinamide.

Table 1. Recovery of niacin and niacinamide from thin layer chromatogram

Sample	Amt sample applied, μg	Amt sample, recd, μg: Mean (n = 10) ± SD	Rec., %: Mean ± SD
Niacin	50.0	50.1 ± 1.0	100.1 ± 1.9
Niacinamide	50.0	50.1 ± 0.7	100.2 ± 1.5

Table 2. Recovery of niacin and niacinamide added to commercial vitamin B complex or multivitamin preparations and determined by proposed method

Sample[a]	Vitamin form	Amount (mg) Declared[b] mean ± SD	Added	Total mean ± SD	Found mean ± SD	% Rec.
A	Niacin	—	70	70.22 ± 0.88	70.22 ± 0.88	100.32 ± 1.26
	Niacinamide	73.95 ± 1.80	70	143.89 ± 3.64	69.94 ± 2.80	99.92 ± 4.00
B	Niacin	—	15	15.21 ± 0.35	15.21 ± 0.35	101.38 ± 2.34
	Niacinamide	11.80 ± 0.14	15	26.94 ± 0.33	15.05 ± 0.19	100.32 ± 1.28
C	Niacin	—	50	50.29 ± 0.61	50.29 ± 0.61	100.58 ± 1.22
	Niacinamide	49.24 ± 0.76	50	101.36 ± 2.47	48.61 ± 0.83	97.22 ± 1.66
Overall rec.	Niacin					100.76 ± 1.53
	Niacinamide					99.15 ± 2.69

[a]Sample composition : (A) niacinamide 70 mg, vitamin B_1 15 mg, B_6 5 mg, folic acid 2 mg, B_2 15 mg, B_{12} 20 μg, and C 300 mg (capsule)
(B) niacinamide 15 mg, Diastase 250 mg, Pepsin 15 mg, vitamin B_1 2 mg, B_2 2 mg, B_6 0.5 mg (per 10.0 mL liquid syrup)
(C) niacinamide 50 mg, vitamin B_1 10 mg, B_6 3 mg, A 1000 I.U., B_2 10 mg, D 200 I.U., (sugar coated tablet)
[b]Average of 6 independent determinations.

Determination

Scrape each silica gel spot containing desired material into separate 15 mL centrifuge tube and extract twice (3 mL + 2 mL) with 0.1N HCl by vortex-mixing (30 s) and then centrifuging at 1000 × *g* for 10 min. Measure optical density of each combined clear extract and of appropriate silica gel blank at 262 nm using 1 cm cell. The silica gel blank obtained by extraction of silica gel from an area equivalent to sample spots gives very low absorption value at 262 nm and may be replaced by 0.1N HCl if desired.

For recovery assay, mix each sample with known amount of niacin or niacinamide and assay as described above.

Results and Discussion

Table 1 gives the recovery of niacin and niacinamide from thin layer chromatography. A known amount of niacin or niacinamide was applied to the TLC plate, and assayed after chromatography as described. The recoveries (compared with standards prepared in 0.1N HCl) of niacin and niacinamide were excellent and were found to be 100.09 and 100.23%, respectively.

Table 2 shows the recoveries of niacin and niacinamide added to different commercially available samples. The average recovery obtained was 100.76% for niacin and 99.15% for niacinamide. Because the assay is performed after isolating the vitamin by TLC, interferences from other vitamins, coloring materials, excipients, etc., are not encountered.

The method described here is extremely simple, safe, and inexpensive. A large number of samples can easily be handled in any laboratory. We have already assayed more than 1000 samples of syrup tonics, injections, and capsules by the proposed method without any problem. Because niacin and niacinamide have different R_f values in distilled water, these may both be assayed when present together without duplicate determination. In addition, other water-soluble vitamins like riboflavin, thiamin, and ascorbic acid may be identified without extra cost or labor, because these are well separated under the experimental conditions described (7).

Acknowledgment

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FISH AND OTHER MARINE PRODUCTS

Detection of Parasites in Fish Muscle by Candling Technique

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This study determined optimum conditions for detecting codworms in skinned cod fillets by using candling tables under commercial conditions. The best balance of factors was sought for obtaining maximum lighting conditions, reducing operator fatigue, retaining natural fillet color, and having a high contrast between parasites and fish flesh. Based on the results obtained, a recommended procedure has been adopted by AOAC.

When parasites are found in fishery products, their presence can cause a financial loss to a fish processor who should remove them even though this reduces processing yield and capacity. If they are not removed, complaints from consumers are likely. A considerable amount of time and money has been spent in searches for ways or means to reduce parasite occurrence or to remove them automatically from fish muscle; studies so far have met with little success. The only practical method to date is personal observation of fish flesh and hand-removal of any parasites found. Chemical methods for their extraction are available (1), but these methods destroy the fish flesh.

A common parasite is the codworm (*Phocanema decipiens*, also known as *Porrocaecum decipiens* or *Terranova decipiens*). It is not a worm but a nematode with a rather complicated life history. In nature, an adult nematode lays eggs in the intestinal tract of a harbor seal or a gray seal. The eggs are passed by the seal and hatch in sea water into minute larvae. These small larvae are eaten by larger invertebrates such as shrimp and other small crustaceans which, in turn, are eaten by vertebrate fish. This larval form may be passed from small fish to larger fish by predation. While in the larval form, it increases in size but it cannot mature. When a seal preys on fish that contain the larval form, the larvae then mature in the seal and start the cycle all over again.

Another worm-like parasite is the nematode *Anisakis species*, sometimes called the herring worm or *Anasakis simplex*. It, the codworm, and other nematodes have been found on occasion in most species of commercially important fish. The frequency of their occurrence is difficult to estimate accurately but it is related to the abundance of seals or whales in nearby waters. In the trade, these nematodes are most frequently associated with gadoid species such as cod, pollock, haddock, and hake.

These nematodes inhabit the intestinal tract; however, if a fish is heavily infested or if it is kept on ice for a few days before gutting, a few of the larvae may migrate into the flesh. If they are not removed by a processor, a consumer complaint is likely. Even though nematodes may survive in raw fish fillets for several days, they are killed by heating to 70°C for 7 min (2) or by freezing at −20°C for 24 h (3). Thus, if present in cooked or frozen fish flesh, they are not a public health problem (4–6); their presence is simply but definitely a matter of esthetic downgrading of quality in fish flesh.

A different kind of parasite is *Sphyrion lumpi* which is a copepod ectoparasite frequently called "buttons." It attaches itself to the skin of fish such as ocean perch (redfish) and buries its head in the flesh. It is harmless to humans but is esthetically objectionable. In the United States, the regulatory tolerance (defect action level) is not more than 3% of the fillets examined contain one or more copepods accompanied by pus pockets.

Other parasites form cysts in fish flesh, for example, in freshwater fish. The United States defect action level is 60 cysts per 100 fish (fish 1 lb or less) or per 100 lb of fish (fish over 1 lb) provided that 20% of the fish examined are infested if the fish are blue fin and other freshwater herring. For tullibees, ciscoes, inconnus, chubs, and white fish, the defect action level is 50 parasitic cysts per 100 lb (whole or fillets) provided that 20% of the fish examined are infested. Even though parasites such as these are not considered to be a public health problem if present in cooked or frozen fish flesh, their appearance is detrimental to the overall quality of edible fish.

Superficial inspection does not reveal nematode parasites imbedded in the flesh. Candling (showing a bright light through the flesh) reveals most of these parasites if present. Slicing cod fillets longitudinally into pieces that are ½ in. (13 mm) thick can increase the efficiency of detection (7). This technique may be used for fish fillets that are frozen into fish blocks but it destroys the natural appearance of fish fillets sold as such. However, candling and removal of the parasites, individually, by hand is the only method which has so far shown itself to be commercially feasible.

In an earlier study, Power (8) tested the efficiency of candling cod fillets with various values and colors of light transmitted through the cod flesh and various values of incident light. He recommended white light for transmission through a fillet and a low level of incident light to maximize detection of parasites.

In Iceland, considerable effort has been directed toward improving the efficiency of detecting and removing codworms from fillets (9). The methods presently used in Iceland for candling skinned fillets are based on a series of experiments carried out for the Icelandic Freezing Plants Corp. by Agustsson (10). The objective of these experiments was to determine the optimum lighting conditions for detecting codworms in skinned cod fillets on candling tables under commercial conditions of use, to obtain the best balance of transmitted and incident light, to keep operator fatigue to a minimum, to give as natural a color of the fillet as possible, and to give the highest possible contrast between parasites and fish flesh.

Parasites in Fish Muscle

Candling Procedure

18.A01 ***Apparatus***

(a) *Candling table.*—Rigid framework to hold light source below rigid working surface of white, translucent acrylic plastic or other suitable material with 45–60% translucency. Length and width of

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Table 1. Detection of codworms in parasitized cod fillets by candling under different lighting conditions

Overhead illumination, lux	Total number of codworms detected at various candling light intensities (lux)			Number of fillets
	710	1800	13 000	
140	6	8	8	1
280	6	8	8	1
760	30	33	33	8

working surface should be large enough to examine entire fillet, e.g., 30 × 60 cm sheet, 5–6 mm thick.

(b) *Light source.*—"Cool white" with color temp. of 4200°K. At least two 20 watt fluorescent tubes are recommended. Tubes and their electrical connections should be constructed to prevent overheating of light source.

Av. light intensity above working surface should be 1500–1800 lux as measured 30 cm above center of acrylic sheet. Distribution of illumination should be in ratio of 3:1:0.1, i.e., brightness directly above light source should be 3 times greater than that of outer field and brightness of outer limit of visual field should be not more than 0.1 that of inner field.

Overhead illumination (indirect light) in vicinity of candling table should be ≥500 lux.

18.A02 ***Procedure***

Place skinned fish fillets in single layer on lighted working surface and examine visually for parasites. Normally, fillets are not cut into pieces before examination, but if presence of parasites is suspected, thick fillets may be cut for further examination.

Results and Discussion

During the initial testing of light sources in the candling table, 9 differently colored fluorescent tubes were tried as well as an ordinary incandescent (tungsten) light bulb. After examining several infested fillets, the inspectors concluded that a "cool white" fluorescent tube (4200°K) gave the best overall result. This conclusion is similar to that of Power (8) except that using a fluorescent tube instead of an incandescent bulb allows a larger area of uniform illumination and reduces heat output from the light source. In addition, when a candling table was fitted with two 20 watt fluorescent tubes instead of two 40 watt fluorescent tubes, the light ratio of brightness was an improvement in reducing operator fatigue.

A comparison was made of 3 different candling light intensities with 3 different conditions of overhead illumination (incident light). The results (Table 1) indicate that varying the intensity of overhead illumination between 140 and 760 lux had no effect on the ease of detection. They also suggest that a high candling light intensity of 13 000 lux does not increase the number of parasites detected compared with an intensity of 1800 lux. The fillets used were 10–20 mm thick.

Similar results were obtained upon examination of several other parasite-infested cod fillets. Although different numbers of parasites were found in different fillets, the same trends were observed in number detected in relation to different lighting conditions.

Codworms placed in fillets to a depth of 6 mm or more could not be detected regardless of candling light intensity. Power (7) found it necessary to slice large cod fillets longitudinally into slices ½ in. (13 mm) thick to increase the efficiency of candling heavily infested fillets to over 95%. Under commercial conditions, it is not practical to slice large fillets in this manner because it destroys their natural appearance and adds to the processing cost. Instead, we recommend that large fish be eviscerated as soon as possible (preferably at sea), well iced during transport, and filleted as soon as practical to reduce the potential for parasites migrating from the intestinal tract into the edible flesh. In addition, if heavily infested fish are found during processing into fillets, they are normally diverted to nonfood uses because the time and cost of removing these parasites by hand becomes uneconomical.

After the completion of the experimental work in this study, the Codex Committee on Fish and Fishery Products of the Codex Alimentarius Commission proposed draft standards for fish fillets, for fish blocks, and eventually for all fishery products in which parasites may be found (11): "Parasites or parasitic infestation detected by the candling procedure. Any parasite or parasitic infestation detectable on a 5 mm thick acrylic sheet with 45% translucency, and candled with a light source giving 1500 lux 30 cm above the sheet."

The thickness of the acrylic sheet, its translucency, and the intensity of the candling light in the Codex proposal differ slightly, but not significantly, from the conditions used in the present investigation. Recognizing that tolerances for normal production variations between different manufacturers do exist, we have added them to our proposed procedure.

On the basis of these results and observations, we recommend that the following specifications be considered to increase operator efficiency after repeated observations with optimal detection of parasites (Figure 1):

(1) The color of the light source should be "cool white" with a color temperature of 4200°K. At least two 20 watt fluorescent tubes are recommended in a candling table.

(2) The working surface on top of a candling table should be an acrylic sheet or other suitable material 5–6 mm thick with 45–60% translucency. The length and width of this sheet depend on the size of the fillets being examined and the size of the light source. A 30 × 60 cm sheet is suggested for most applications.

(3) The average light intensity above a candling table should be 1500–1800 lux as measured 30 cm above the center of the acrylic sheet.

(4) The light source should be arranged to give a distribution of illumination in a ratio of 3:1:0.1 through the acrylic sheet. In other words, the brightness directly above the light source should be 3 times greater than that of the outer field and the brightness of the outer limit of the visual field should not be more than one-tenth that of the inner field.

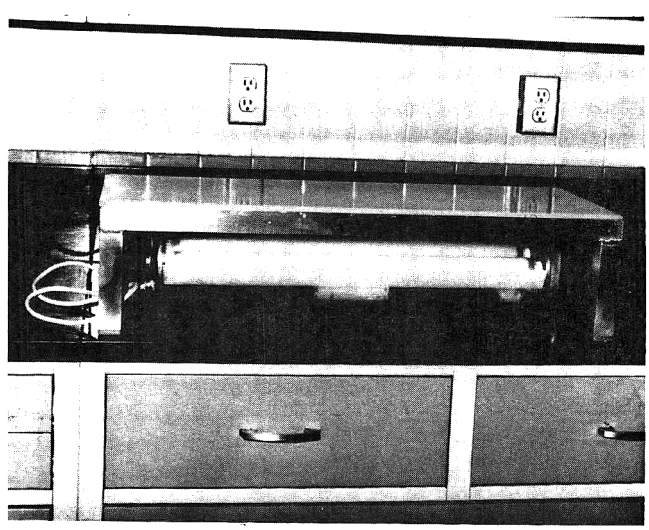

Figure 1. Candling table.

(5) The overhead illumination (incident light) should be at least 500 lux.

Our proposed procedure is based on detection of nematode parasites in fillets obtained from gadoid species of fish. These parasites and these fish species include the majority of applications for which this procedure is intended. However, comments are welcomed from interested persons who use it to detect other kinds of parasites and in other species of fish.

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MICROBIOLOGICAL METHODS

Improved Enrichment for Recovery of *Shigella sonnei* from Foods

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Shigella **species were recovered from foods by the procedure described in the *Bacteriological Analytical Manual*, 5th Ed. The method is effective if *Shigella* species are present at about 10^6 cells/g. A 25 g food portion was incubated in Gram-negative (GN) and selenite cystine broths for 16 h at 35°C and streaked onto MacConkey, Levine's eosin methylene blue, desoxycholate citrate, and xylose lysine desoxycholate agars. *S. sonnei* cells were recovered quantitatively at 44.5°C, and along with other *Shigella* species, were grown with *Escherichia coli* in a tryptone broth under anaerobic conditions. *Shigella* species were also grown in a mixed microflora from foods. *S. sonnei* cells were inoculated into an enrichment broth containing 20 g tryptone, 2 g K_2HPO_4, 2 g KH_2PO_4, 1 g glucose, 5 g NaCl, 1.5 mL Tween 80, and 0.5 mg novobiocin/L (pH 7.0) and incubated for 20 h at 44°C. Enrichments were streaked onto MacConkey agar and the plates were incubated 20 h at 35°C. Suspect *Shigella* colonies were screened in glucose, tryptone, and lysine broths and in triple sugar iron and motility agars. The sensitivity varied from 0.3 to 1000 bacteria/g. The method has been examined with artificially inoculated lettuce, celery, brussels sprouts, mushrooms, and hamburger. It is also applicable to *S. flexneri* if incubation is conducted at 42°C.**

Shigella species are recognized foodborne pathogens (1). *S. sonnei* is encountered in industrialized nations, whereas *S. dysenteriae, S. flexneri,* and *S. boydii* are largely confined to developing countries. However, the incidence of shigellosis, regardless of source, is unknown because of insensitive methodology for retrieval and deficiencies in the reporting system. For feces, both direct streaking and selective enrichment are recommended (2). The former entails streaking to 2 selective agars, e.g., MacConkey or eosin methylene blue (EMB) and xylose lysine desoxycholate (XLD), Hektoen enteric (HE), or Salmonella-Shigella (SS). The indirect approach involves transfer of 1 g specimen to 9 mL Gram-negative (GN) or selenite cystine broths and incubation for 6–18 h at 35°C, followed by streaking to the above-mentioned agars.

In water analysis, concentration of the bacteria from a 1–10 L sample on a membrane is recommended (3). The membrane is placed in 1 of 2 enrichment media, e.g., nutrient broth (adjusted to pH 8.0) or lactose broth containing an autocytotoxic agent, 1 mM 4-chloro-2-cyclopentylphenyl β-D-galactopyranoside, buffered to pH 6.5 with 0.05 M citric acid-sodium citrate. The former enrichment is incubated for 6–18 h at 35°C, the latter for 24 h at 35°C. Both enrichments are streaked to XLD agar after incubation.

Two enrichments have been proposed for food. The *Bacteriological Analytical Manual* (4) suggests incubation of 25 g portions in GN and selenite cystine broths without homogenization, and incubation for 16 h at 35°C followed by steaking onto MacConkey, EMB, desoxycholate citrate, and SS agars. The American Public Health Association method (3) recommends enrichment in GN broth for 18 h at 35–37°C, followed by streaking to 3 agars: Tergitol 7 or MacConkey XLD, and either SS, HE, or desoxycholate citrate (5). In all methods, cultures are identified by the reactions proposed in ref. 6.

The major problems in the recovery of *Shigella* are low numbers in the specimen and the deleterious effect of metabolic products of other members of the indigenous microflora, such as coliforms and *Escherichia coli*. Hentges (7–9) and Fishbein et al. (10) showed that the volatile aliphatic acids, formic and acetic, formed during the fermentation of carbohydrates (and possibly amino acids) were toxic for *Shigella*. Thus, if a *Shigella* strain and an *E. coli* strain without the capacity to catabolize formic and acetic acids were inoculated into trypticase soy broth to the levels of 10^6 and 10^2 cells/mL, respectively, the *Shigella* species could not be detected after overnight incubation at 35°C (11). If, however, the *Shigella* species were inoculated together with a coliform with the capacity to catabolize these acids, the *Shigella* species would resume growth after 48 h, i.e., when the pH increased. These observations corroborate the use of enrichment media which contain little or no carbohydrate. However, even with this improvement, *Shigella* species can only be recovered from foods if their initial number approximates 10^6 cells/g. To compensate for the inadequacy of the enrichment, multiple isolatory plates to increase the amount plated and a series of media of varying selectivity have been proposed in most methods (2, 4, 5). We have found that some media, e.g., SS and desoxycholate citrate, are too toxic for some *Shigella* strains.

In our recent studies (12) to improve recovery methodology for *E. coli,* and specifically the biotypes resembling *Shigella* strains, we found that consideration of ecological factors was essential. It is known that *Shigella* organisms multiply and produce lesions of the colon, conjunctiva, and membranes of the urogenital tract (13–16). These habitats are characterized by anaerobiosis and/or low concentrations of small molecular weight compounds. The purpose of this paper is to present initial results regarding the recovery of *Shigella* species from foods, using an enrichment based on anaerobiosis and an improved medium containing novobiocin.

Experimental

Anaerobiosis

To achieve anaerobiosis, tubes, bottles, and flasks were incubated in standard anaerobic jars (BBL Model 60465) with GasPak™ and catalyst (17). Media and diluents were not steamed before use. Jars were incubated at 35 ± 0.5, 42 ± 0.3, or 44.0 ± 0.3°C. Temperature control was obtained by the installation, in a standard incubator, of a Model 70-115/208/115 proportional controller with potentiometer and thermistor sensor (RFL Industries, Boonton, NJ).

Media

Initial studies were performed with 1% tryptone, the medium recommended for indole production. In later experiments, we examined a recently developed medium, TP broth, proposed for the enrichment of *E. coli* in food (12). The medium was composed of 20 g tryptone, 2 g K_2HPO_4, 2 g KH_2PO_4, 5 g NaCl, and 1 L distilled water (pH 7.0 ± 0.2) and was autoclaved for 15 min at 121°C. This medium was further supplemented with glucose, yeast extract, and potassium

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Table 1. Recovery of *Shigella* species from *E. coli* interaction[a] after anaerobic incubation for 20 h at 35°C

Shigella species	*Shigella* spp. colonies (%)[b]	
	E. coli K12	*E. coli* LY186
S. sonnei M186-1	67	1.5
S. sonnei 2084	75	6.0
S. boydii 2	1.5	1.3
S. flexneri 2a	ND[c]	ND
S. dysenteriae 2	ND	ND

[a]The *Shigella* species strain and *E. coli* were inoculated to a level of 100 cells/mL in 2% tryptone, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.1% glucose, 0.5% NaCl. Suspensions were streaked to MacConkey agar and plates were incubated 20 h at 35°C.
[b]Percentage of non-lactose fermenting colonies (*Shigella* species) was used for enumeration of *Shigella* species.
[c]ND, not detected.

Table 2. Anaerobic tolerance of *Shigella* species for novobiocin[a]

Shigella species	Maximum tolerated[b], μg/mL		
	35°C	42°C	44°C
S. dysenteriae	20	1	—[c]
S. flexneri 2a	10	10	—
S. boydii 3	10	10	—
S. sonnei M186-1	5	5	0.5

[a]Serial dilutions of novobiocin were added to 2% tryptone, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.5% NaCl, 0.1% glucose.
[b]Cultures were grown in veal infusion, diluted in 0.5% saline and incubated to a level of 100 cells/mL.
[c]—No tolerance.

malate (adjusted to pH 7.0) to the concentrations described. The Shigella broth contained 20 g tryptone, K_2HPO_4, 2 g KH_2PO_4, 5 g NaCl, 1 g glucose, 1.5 mL Tween 80, and 1 L distilled water (pH 7.0 ± 0.2) and was autoclaved for 15 min at 121°C.

Selective Factors

The effects of 3 surface-active agents (bile salts No. 3, lauryl sulfate, and Tween 80) were tested in the concentrations routinely used for enrichment of enteric bacteria. In TP broth, 0.05% bile salts No. 3 and 0.01% lauryl sulfate were distinctly toxic for *Shigella* species. A 0.15% concentration of Tween 80 was tolerated by most strains and was used at this level when specified. Aliquots of novobiocin, sterilized by filtration through a 0.45 μm membrane, were added to Shigella broth to the levels specified.

Preparation of Cultures

Cultures of *Shigella* and *E. coli* were grown in veal infusion for 20 h at 35°C under aerobic conditions without agitation. Cultures were diluted in 0.5% saline and added to test media. The indigenous microflora of brussels sprouts, mushrooms, lettuce, celery, bean sprouts, and ground beef was obtained by suspending 25 g of sliced vegetable or meat in 225 mL Shigella broth in a 500 mL Erlenmeyer flask. The mixture was shaken intermittently during a 10 min period and the eluate was decanted for use.

Enumeration

Pure cultures of *E. coli* and *Shigella* were enumerated by the pour plate method, using plate count agar (PCA) and incubation for 48 h at 35°C. Total counts of food eluates were determined on PCA, but plates were incubated 72 h at 35°C. For estimation of the number of *Shigella* species in the presence of *E. coli*, mixed cultures were streaked to MacConkey agar and incubated for 20 h at 35°C. The percentage of *Shigella* species was calculated by determining the ratio of non-lactose fermenters to total count and multiplying by 100. Although the technique would miss *Shigella* species present at less than 1% of the population mixture, this problem was considered insignificant from a practical point of view.

Results and Discussion

During recent studies of *E. coli* retrieval, the cultures grew well in veal infusion broth cultured aerobically, but poorly in 1% tryptone under anaerobic conditions. However, addition of glucose, KNO_3, and malate to the 1% tryptone anaerobic culture resulted in growth comparable to that obtained aerobically in veal infusion.

When a strain of *S. sonnei* and one of *E. coli* were selected and inoculated to a level of 100 cells/mL in tryptone and tryptone-supplemented broths, *E. coli* overgrew *Shigella* aerobically; anaerobically, however, both cultures grew concomitantly in tryptone. Addition of glucose or malate favored the dominance of *S. sonnei*. Bile salts No. 3, tested at a level less than that recommended for most enteric bacteria, inhibited the growth of *Shigella*. Addition of glucose, malate, and yeast extract suppressed the inhibitory action of bile salts. Other strains of *S. sonnei*, *E. coli*, and *S. boydii* (Table 1) behaved similarly, although quantitative differences were observed in these cultural interactions. *S. sonnei* strains tended to overgrow the K12 strain of *E. coli* but not the LY186 strain. Differences in relative growth rates or production of colicins (bactericidal toxins liberated by *E. coli*) may explain these variations as well as the failure to retrieve strains of *S. flexneri* and *S. dysenteriae* in culture with *E. coli*.

To study the retrieval of *Shigella* species from the natural microflora of foods, we selected two media: 2% tryptone, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.5% NaCl, 0.1% glucose and the previous medium supplemented with 0.1% malate. The natural microflora of lettuce was eluted in both media. *S. sonnei* was added in graded concentrations, and the enrichments were incubated at 35 and 44 °C. The elevated temperature was used because our previous data demonstrated growth of *S. sonnei* at 44.5–45°C (18). In the base without malate, *S. sonnei* was consistently recovered at 35 and 44°C when the initial inoculum was 1000 cells/g. Inclusion of malate led to sporadic recovery, suggesting the enrichment of another population.

These data supported the feasibility of using a tryptone-phosphate broth with a small quantity of glucose for the recovery of *Shigella*. However, this base lacks specificity. To minimize the emergence of Gram-positive bacteria, a surface-active agent is generally added. Because of the unsuitability of bile salts No. 3 under anaerobic conditions and the toxicity of lauryl sulfate under these conditions, Tween 80 was used. It was not detectably toxic at a concentration of 0.15%. Another selective factor for *Salmonella* and *Shigella* is novobiocin (19). Data concerning the tolerance of *Shigella* species for this agent as a function of temperature are shown in Table 2. On the basis of these results, we suggest that this antibiotic be incorporated at the levels of 5 and 3 μg/mL at 35 and 42°C, respectively. For *S. sonnei*, 0.5 μg novobiocin/mL at 44°C is suggested. The third selective factor is temperature; for recovery of *S. sonnei*, we recommend 44°C rather than the 42°C used for the other species.

The results of experiments to recover *S. sonnei* and *S. flexneri* from artificially contaminated foods are summarized in Table 3. Using novobiocin and 44°C, the sensitivity of the procedure for *S. sonnei* in celery and mushrooms was about 1 cell/g or 1 cell in a total microflora of 2.0–5.0 × 10^8 cells/g. Without novobiocin the sensitivity ranged from 100 to 1000

Table 3. Recovery of *Shigella* species[a] from food

Food	*Shigella sonnei*			*Shigella flexneri* 2a		
	Incubation temp.[b]	Sensitivity, cells/g	Total microflora, cells/g[c]	Incubation temp.[b]	Sensitivity, cells/g	Total microflora, cells/g[c]
Celery	44°C[d]	0.34	1.7×10^8			
Mushrooms	44°C[d]	1.0	4.7×10^8			
Ground beef	35 and 44°C	100	1.0×10^5	35°C[e]	100	4.5×10^6
				42°C	10	4.5×10^6
Brussels sprouts	44°C	94	3.7×10^8			
Bean sprouts	44°C	940	4.8×10^8			
Lettuce	35 and 44°C	1000	2.7×10^7	35°C	200	5.2×10^4

[a] *Shigella* cultures were grown for 24 h at 35°C in veal infusion, diluted, and added to eluate.
[b] Base 2% tryptone, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.5% NaCl, 0.1% glucose, 0.15% Tween 80.
[c] Plate Count Agar, 72 h at 35°C.
[d] 0.5 μg novobiocin/L added.
[e] 0.15% yeast extract added.

cells/g. *S. flexneri* strain 2a was also retrieved from hamburger (35 and 42°C) and lettuce (35°C). An interesting observation was made concerning the requirement for yeast extract at 35°C, but not at 42°C. At the latter temperature, supplementation with yeast extract inhibited recovery, suggesting overgrowth by another population.

In summary, anaerobiosis along with the addition of Tween 80 and novobiocin to the suggested medium, and incubation at 42 or 44°C, constitutes a useful stage in the development of an improved enrichment for *Shigella*. Future studies should consider the following factors:

Recovery of stressed cells.—Incubation for 2 h at 35°C under aerobic conditions in the basal medium without antibiotic and surface-active agent should be sufficient for repair.

Food.—Enrichment conditions are partly dependent upon the nature of the food. A key factor in recovery of *Shigella* is maintenance of nutrients, primarily carbohydrate, at relatively low concentrations to minimize the accumulation of volatile acids. The effect of yeast extract also deserves consideration.

Rapidity.—Cultures giving positive reactions for *Shigella* in primary screening reactions can be obtained within 72 h, a period comparable to that required for *Salmonella*. Use of a fluorescent antibody could reduce the period to 20 h.

Sensitivity.—For *S. sonnei*, the sensitivity in mushrooms and celery has been reduced to about 1 cell/g or 1 cell in a total microflora approximating 10^8 bacteria. For *S. flexneri*, the sensitivity varies from 10 to 200 cells/g. Adjustment of nutrients or development of additional selective agents is required to make the procedure applicable to *S. dysenteriae* and *S. boydii*. The use of an additional selective agar, e.g., XLD, may also increase sensitivity.

Specificity.—The procedure also retrieves strains of anaerogenic, nonmotile, non-lactose-fermenting *E. coli* which possess serological affinities with *Shigella* species and the classical Alkalescens-Dispar strains. Cultures giving *Shigella*-like behavior in biochemical but not serological tests should be sent to institutes such as the Centers for Disease Control because of the discovery of provisional serotypes of *Shigella* species (5, 20). For differentiation from *E. coli*, additional tests (e.g., acetate oxidation, Christensen's citrate, and acid production from mucate) are required.

Number of positive cultures and specimens.—In our preliminary investigation, we recovered *S. dysenteriae* strain 3 from lettuce. (Data not shown.) Additional surveys will be required.

Practicality.—Because of the size and number of samples, it probably will be necessary to use Case jars instead of the standard anaerobic jar. The effect of prior heating of the medium to minimize dissolved oxygen should also be studied.

Effect upon pathogenicity.—If virulence of isolates is to be confirmed, the effect of enrichment on pathogenicity attributes, such as plasmids, must be evaluated.

Suitability for quantitation.—Additional effort is needed to modify the procedure for quantitation.

To expedite the identification of suspicious isolates, colonies should be picked to motility agar, TSI agar slants, glucose broth with Durham tube, lysine broth, and 1% tryptone for indole. The characteristic reactions of *S. sonnei*, *S. dysenteriae*, *S. flexneri*, and *S. boydii* are as follows: motility (−); TSI (K/A); indole (+ or −, except for *S. sonnei*, which is −); acid from glucose (+); and lysine decarboxylase (−). Reactions should be observed for 4 days to differentiate *Shigella* species from *E. coli* strains; some *Shigella* strains ferment lactose slowly and produce an acid reaction on the second or third day of incubation. If indole is negative at 20 h, another tube should be inoculated and examined at 48 h. In addition to the production of acid from glucose, some members of *S. flexneri* biotype 6 may also produce gas. Subsequent to the testing procedure, the TSI slant may be used for serological analysis.

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Rapid Hydrophobic Grid Membrane Filter Method for *Salmonella* Detection in Selected Foods: Collaborative Study

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A collaborative study was carried out in 36 laboratories to validate a hydrophobic grid membrane filter method for rapid (2–3 days) *Salmonella* detection by comparing its performance against the AOAC/BAM reference method. Six products were included in the study: semi-sweet chocolate, raw ground poultry meat, ground black pepper, cheese powder, egg powder, and nonfat dry milk. With the exception of the naturally contaminated poultry, all products were inoculated in advance with low concentrations of a variety of *Salmonella* serotypes. The hydrophobic grid membrane filter method detected the following numbers of positive samples (results of the AOAC/BAM reference method are shown in parentheses): chocolate, 67(68); raw ground poultry, 133(131); ground black pepper, 57(56); cheese powder, 81(80); egg powder, 51(45); and nonfat dry milk, 68(72). The 2 methods did not differ significantly at the 95% confidence level in any of the 6 foods. The hydrophobic grid membrane filter method has been adopted official first action for detection of *Salmonella* in selected foods.

In 1982, Entis et al. (1) described a rapid, automated method for *Salmonella* detection in a variety of foods. This method, which used a hydrophobic grid membrane filter (HGMF), was demonstrated to be equivalent in sensitivity to the method recommended by the Canadian Health Protection Branch, while producing results in only 48–72 h.

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Because the apparatus required for the automated method was not available commercially, we recently developed a manual version of the HGMF *Salmonella* method and compared its performance against the method currently listed by AOAC (2) and the Food and Drug Administration (3) (Entis, unpublished data). Based on the results of that evaluation, a collaborative study was carried out to validate the HGMF method for rapid *Salmonella* detection against the AOAC official final action method, **46.115–46.128.**

Collaborative Study

Thirty-six collaborators took part in this study. Six food products were included: semi-sweet chocolate, raw ground poultry meat, ground black pepper, cheese powder, whole egg powder, and nonfat dry milk. Collaborators were permitted to choose which products they would test (Table 1).

Each collaborator received a replicate set of 10 samples of a given product. Except for the poultry meat, which consisted entirely of naturally contaminated material, 2 samples in each series were uninoculated controls and the remaining 8 samples were each inoculated with a low concentration of individual *Salmonella* serotypes. Table 2 summarizes the inoculation pattern for all 5 inoculated product categories.

The chocolate samples were prepared and inoculated as follows: 100 g aliquots of semi-sweet chocolate chips were weighed into individual sterile polyethylene bags and melted by holding in a circulating air incubator at 43°C. Each bag

Table 1. Products tested by each collaborator

Coll.	Semi-sweet chocolate	Poultry	Pepper	Cheese powder	Egg powder	Nonfat dry milk
1	Y[a]	Y	Y	Y	—[b]	Y
2	Y	—	—	Y	—	—
3	—	Y	—	Y	Y	Y
4	—	Y	—	Y	Y	Y
5	Y	Y	—	—	—	—
6	—	Y	Y	Y	—	—
7	—	Y	Y	—	—	—
8	Y	—	Y	Y	Y	—
9	—	—	—	—	—	Y
10	—	Y	—	—	Y	Y
11	—	—	Y	Y	—	Y
12	—	Y	—	—	—	—
13	—	—	—	Y	—	Y
14	Y	Y	Y	Y	—	—
15	Y	—	—	Y	—	Y
16	Y	—	Y	Y	Y	Y
17	Y	—	Y	Y	—	Y
18	—	—	—	—	—	Y
19	—	Y	—	—	Y	—
20	Y	Y	Y	—	—	Y
21	Y	Y	Y	Y	Y	Y
22	—	—	—	—	—	Y
23	—	Y	—	—	—	—
24	—	Y	—	Y	Y	—
25	Y	Y	Y	Y	—	—
26	—	—	—	Y	—	Y
27	—	—	—	Y	—	—
28	Y	—	Y	—	—	—
29	Y	—	Y	Y	Y	Y
30	Y	—	Y	—	Y	—
31	—	—	—	—	Y	Y
32	—	Y	—	—	—	—
33	—	—	—	Y	Y	Y
34	—	—	—	—	Y	—
35	—	Y	—	—	—	—
36	—	—	—	—	Y	Y

[a]Collaborator participated.
[b]Collaborator did not participate.

Table 2. *Salmonella* serotypes used to inoculate collaborative study samples

Sample	Serotype				
	Chocolate	Pepper	Cheese powder	Egg powder	Nonfat dry milk
1	*S. infantis*	*S. st. paul*	control[a]	*S. give*[b]	*S. montevideo*
2	*S. aastbourne*	*S. infantis*	*S. thompson*	*S. infantis*	*S. kentucky*[c]
3	control	control	*S. typhimurium*	*S. typhimurium*	control
4	*S. infantis*	*S. typhimurium*	*S. enteritidis*	*S. hvittingfoss*	*S. typhimurium*
5	*S. chester*	*S. blockley*	*S. infantis*	control	*S. infantis*
6	*S. typhimurium*	control	*S. infantis*	*S. typhimurium*	control
7	*S. haardt*	*S. cubana*	control	*S. bareilly*	*S. infantis*
8	*S. typhimurium*	*S. typhimurium*	*S. arizonae*	*S. infantis*	*S. tennessee*
9	control	*S. agona*	*S. typhimurium*	control	*S. newington*
10	*S. indiana*	*S. infantis*	*S. senftenberg*	*S. heidelberg*	*S. typhimurium*

[a]Uninoculated control sample.
[b]H_2S-negative strain; did not survive.
[c]Lactose-positive strain.

was inoculated with 1.0 mL suspension of the appropriate *Salmonella* serotype and sealed; the contents were kneaded to disperse the inoculum uniformly through the molten chocolate. The chocolate was then flattened in the bag and allowed to solidify. The four powdered products were inoculated with freeze-dried *Salmonella* cultures as previously described (4) except that cultures were lyophilized in 12.5% (w/v) reconstituted nonfat dry milk. Raw poultry samples were prepared by pooling raw ground poultry in 3 kg quantities in large sterile polyethylene bags, kneading the bags to obtain a homogeneous sample, and subdividing the sample into 100 g aliquots. The individual poultry samples were held and shipped in a frozen state. The other 5 products were held and shipped at ambient temperature.

Collaborators received all samples at least one week in advance of the scheduled initiation dates and were instructed to store the poultry at −20°C and the other products at ambient temperature, protected from light. The initiation dates for each product were specified. Analysis of samples 1–5 in each series was initiated on the first working day of the week and analysis of samples 6–10 started on the second working day. Uninoculated controls were positioned within the sample series to provide one control sample for each day's analysis.

Collaborators were instructed to analyze each sample by the HGMF method and by the AOAC reference method, **46.115–46.128** (2), supplemented where necessary by the BAM method (3). Separate 25 g sample portions were used for each

Table 3. Concentration of *Salmonella* in collaborative study samples on day of test

Sample	Enrichment[a]	MPN/g[b] Chocolate	Poultry[c]	Pepper	Cheese powder	Egg powder	NFDM[d]
1	TBG	4.60	0.93	0.43	<0.03	<0.03	<0.03
	SC	4.60	4.60	0.43	<0.03	<0.03	<0.03
2	TBG	2.40	4.60	0.93	0.09	0.04	0.23
	SC	2.40	4.60	0.93	0.09	0.04	0.23
3	TBG	<0.03	1.50	<0.03	<0.03	0.15	<0.03
	SC	<0.03	0.93	<0.03	<0.03	0.15	<0.03
4	TBG	2.40	1.50	<0.03	<0.03	<0.03	<0.03
	SC	2.40	2.40	<0.03	<0.03	<0.03	<0.03
5	TBG	>11.00	0.23	0.39	0.43	<0.03	0.09
	SC	>11.00	0.03	0.23	0.43	<0.03	0.09
6	TBG	11.00	>11.00	<0.03	4.60	0.09	<0.03
	SC	11.00	2.90	<0.03	4.60	0.09	<0.03
7	TBG	4.60	0.15	0.04	<0.03	0.04	0.23
	SC	4.60	0.15	0.04	<0.03	0.04	0.23
8	TBG	>11.00	0.15	<0.03	0.07	0.23	0.04
	SC	>11.00	0.07	<0.03	0.07	0.23	0.04
9	TBG	<0.03	0.28	0.64	0.04	<0.03	0.04
	SC	<0.03	0.93	0.64	0.04	<0.03	0.04
10	TBG	>11.00	0.29	0.09	0.09	<0.03	0.09
	SC	>11.00	0.93	0.09	0.09	<0.03	0.09

[a]TBG: Tetrathionate brilliant green broth.
SC: Selenite-cystine broth.
[b]3-tube most probable number, AOAC/BAM method.
[c]Naturally contaminated product.
[d]Nonfat dry milk.

Table 4. Detection of Salmonella in chocolate by AOAC/BAM and HGMF methods

Coll.	Results of Salmonella analysis									
	AOAC/BAM									
	1	2	3[c]	4	5	6	7	8	9[c]	10
1	+	+	-	+	+	+	+	+	-	+
2[d]	+	+	-	+	+	+	+	+	-	+
5[d]	+	+	-	+	+	+	+	+	-	+
8	+	+	-	+	+	+	+	+	-	+
14	+	+	-	+	+	+	+	+	-	+
15	+	+	-	+	+	+	+	+	-	+
16	+	+	-	+	+	+	+	+	-	+
17	+	+	-	+	+	+	+	+	-	+
20[e]	+	+	-	+	+	+	+	+	-	+
21	+	+	-	+	+	+	+	+	-	+
25[e]	+	+	-	+	+	+	+	+	-	+
28[e,g]	+	+	-	+	+	+	+	+	-	+
29	+	+	-	+	+	+	+	+	-	+
30[d]	+	+	-	+	+	+	+	+	-	+

Coll.	Results of Salmonella analysis									
	HGMF - SLA[a]									
	1	2	3[c]	4	5	6	7	8	9[c]	10
1	+	-	-	+	+	+	+	+	-	+
2[d]	+	+	-	+	+	-	-	-	+	+
5[d]	+	+	+	+	+	+	+	+	+	+
8	+	+	-	+	+	+	+	-	-	+
14	+	+	-	+	+	+	+	+	-	+
15	+	+	-	+	+	+	+	+	-	+
16	+	+	-	+	+	+	+	+	-	+
17	+	+	-	+	+	+	+	+	-	+
20[e]	A[f]	A	A	+	+	+	+	+	-	+
21	+	+	-	+	+	+	+	+	-	+
25[e]	+	+	-	+	+	+	+	+	+	+
28[e,g]	+	+	-	+	+	+	+	+	+	+
29	+	+	-	+	+	+	+	+	-	+
30[d]	+	+	+	+	+	+	+	+	+	+

Coll.	Results of Salmonella analysis									
	HGMF - HE[b]									
	1	2	3[c]	4	5	6	7	8	9[c]	10
1	+	-	-	+	+	+	+	+	-	+
2[d]	+	+	+	+	+	+	+	+	+	+
5[d]	+	+	+	+	+	+	+	+	+	+
8	+	+	-	+	+	+	+	+	-	+
14	+	+	-	+	+	+	+	+	-	+
15	+	+	-	+	+	+	+	+	-	+
16	+	+	-	+	+	+	+	+	-	+
17	+	+	-	+	+	+	+	+	-	+
20[e]	A	A	A	+	+	+	+	+	+	+
21	+	+	-	+	+	+	+	+	-	+
25[e]	+	+	-	+	+	+	+	+	-	+
28[e,g]	+	+	+	+	+	+	+	+	+	+
29	+	+	-	+	+	+	+	+	-	+
30[d]	+	+	+	+	+	+	+	+	+	+

[a] HGMF results obtained using Selective Lysine Agar (SLA) filter.
[b] HGMF results obtained using Hektoen Enteric Agar (HE) filter.
[c] Uninoculated control sample.
[d] Salmonella isolation reported by HGMF from both control samples. Results from all 10 samples excluded from statistical analysis.
[e] Salmonella isolation reported by HGMF from control sample for samples #6-10. Results from samples #6-10 excluded from statistical analysis.
[f] Data lost due to lab accident. Remaining results from samples #1-5 excluded from statistical analysis.
[g] Method deviation. Common non-selective enrichment broth used for both methods. Results from all samples excluded from statistical analysis.

method. Concurrently, each sample series was analyzed by the coordinating laboratory to determine the concentration of *Salmonella* inoculum present in each sample on the dates specified for initiating analyses. This work was carried out using a 3-tube 3-dilution most probable number method in conjunction with the AOAC reference method. As described by Andrews et al. (5), some minor deviations from the reference method were necessary due to the logistics of the MPN determination. The HGMF method was carried out as described below, with the following exceptions: (a) instructions to collaborators did not specify that brilliant green dye water was to be prepared by adding brilliant green dye solu-

Table 5. Detection of *Salmonella* in raw poultry meat by AOAC/BAM and HGMF methods

Coll.	Results of *Salmonella* analysis																															
	AOAC/BAM										HGMF - SLA[a]										HGMF - HE[b]									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
1	M[c]	M	M	M	M	+	+	+	-	-	M	M	M	M	M	+	M	+	+	+	M	M	M	M	M	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4[d]	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-
10[e]	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	+	+	-	+	+	+	+	+
14	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	+	-	+	+
19	+	+	-	+	-	+	+	+	-	+	-	+	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	-	+	+	+
23	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-	+	+	-	-	-	+
25	M	M	M	M	M	+	-	-	+	+	M	M	M	M	M	+	-	+	+	+	M	M	M	M	M	-	+	M	M	M
32	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-

[a,b] See footnotes to Table 4.

[c] Missing data. Analysis not done or incomplete.

[d] Method deviation. SLA incubated at incorrect temperature. SLA results from samples #6-10 excluded from statistical analysis.

[e] Method deviation. Iodine not added to tetrathionate broth. Results from samples #6-10 excluded from statistical analysis.

Table 6. Detection of *Salmonella* in ground black pepper by AOAC/BAM and HGMF methods

Coll.	Results of *Salmonella* analysis																															
	AOAC/BAM										HGMF - SLA[a]										HGMF - HE[b]									
	1	2	3[c]	4	5	6[c]	7	8	9	10	1	2	3[c]	4	5	6[c]	7	8	9	10	1	2	3[c]	4	5	6[c]	7	8	9	10
1[d]	+	+	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
6	+	+	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	-	-	-
7	+	+	-	+	+	-	-	+	+	+	+	+	-	+	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+
8	+	+	-	-	+	-	-	-	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	+	-	-	+	+	+
11	+	+	-	-	+	-	-	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	+	-	-	+	+	+
14	+	+	-	-	+	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-
16[d]	+	+	-	-	+	-	-	+	+	+	+	+	-	-	+	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-
17	+	+	-	-	+	-	-	-	+	+	+	+	-	-	+	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-
20[e]	+	+	-	-	+	+	-	-	+	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	+	-	-	+	+	+
21	+	+	-	-	+	-	-	-	+	+	+	+	-	+	+	-	-	-	+	+	+	+	-	+	+	-	-	-	+	+
25	+	+	-	+	+	-	-	-	+	+	M[f]	M	-	-	+	-	-	-	M	+	+	+	-	-	-	-	-	-	+	-
28	+	+	-	-	+	-	-	-	+	+	+	+	-	+	+	-	-	+	+	+	+	+	-	+	+	-	-	+	+	+
29[g]	+	+	-	-	+	-	+	-	-	+	+	+	-	-	+	-	+	-	-	+	+	+	-	-	+	-	+	-	-	+
30	+	+	-	-	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+	-	-	+	-	-	+	+	+

[a,b,c] See footnotes to Table 4.

[d] Method deviation. Did not use waterbath to incubate tetrathionate for HGMF method. Results from samples #1-10 excluded from statistical analysis.

[e] *Salmonella* isolation reported by AOAC/BAM from control sample for samples #6-10. Results from samples #6-10 excluded from statistical analysis.

[f] Missing data. Analysis not done or incomplete.

[g] Method deviation. Did not carry out any biochemical or serological confirmation of isolates for either AOAC/BAM or HGMF. Results from samples #1-10 excluded from statistical analysis.

tion to sterile water; (b) collaborators used both selective lysine agar (SLA) and Hektoen enteric agar (HE) for all samples; (c) MacConkey agar (MAC) or HE purity plates were not inoculated simultaneously with triple sugar iron agar (TSI) and lysine iron agar (LIA); and (d) LIA reactions were read and recorded after 24 h and 48 h at 35°C.

Salmonella Detection in Foods

Hydrophobic Grid Membrane Filter Method

First Action

(Applicable to detection of *Salmonella* from chocolate, raw poultry meat, pepper, cheese powders, powdered egg, and nonfat dry milk)

46.A06 *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.

46.A07 *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.

(d) *Blender.*—Waring, or equiv. with high-speed operation at 20,000 rpm, and 500 mL glass or metal blender jars with covers. One jar is required for each sample.

(e) *Vacuum pump.*—H_2O aspirator vac. source is satisfactory.

(f) *Manifold or vacuum flask.*

(g) *Peptone diluent.*—Dissolve 1.0 g peptone (Difco 0118) in 1 L H_2O. Dispense enough vol. into diln bottles to give 99 ± 1 mL after autoclaving 15 min at 121°.

(h) *Lactose broth.*—See **46.005(f)**. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust if necessary to 225 mL. Final pH 6.7 ± 0.2.

(i) *Trypticase (tryptic) soy broth.*—Suspend 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose in 1 L H_2O. Heat gently to dissolve completely. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust if necessary to 225 mL. Final pH 7.3 ± 0.2.

(j) *Reconstituted nonfat dry milk with brilliant green dye (NFDM-BG).*—Suspend 100 g dehydrated NFDM in 1 L H_2O; mix by swirling until dissolved. Autoclave 15 min at 121°. Add brilliant green dye soln after blending sample/broth mixt. as described below.

(k) *Tetrathionate broth (with iodine and brilliant green).*—Suspend 5.0 g polypeptone, 1.0 g bile salts, 10 g $CaCO_3$, and 30 g $Na_2S_2O_3.5H_2O$ in 1 L H_2O, mix thoroly, and heat to bp. (Ppt will not dissolve completely.) Cool to <45° and store at 5–8°. Prep. I-KI soln by dissolving 5 g KI in 5 mL sterile H_2O, adding 6 g resublimed I, dissolving, and dilg to 20 mL with sterile H_2O. Prep. brilliant green soln by dissolving 0.1 g dye in sterile H_2O and dilg to 100 mL. On day medium is used, add 20 mL I-KI soln and 10 mL brilliant green soln per 1 L basal broth. Resuspend ppt by gentle agitation and aseptically dispense 10 mL portions in 16 × 150 mm

Table 7. Detection of Salmonella in cheese powder by AOAC/BAM and HGMF methods

Coll.	Results of Salmonella analysis																															
	AOAC/BAM										HGMF - SLA[a]										HGMF - HE[b]									
	1[c]	2	3	4	5	6	7[c]	8	9	10	1[c]	2	3	4	5	6	7[c]	8	9	10	1[c]	2	3	4	5	6	7[c]	8	9	10
1	-	-	-	-	+	+	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-
2	-	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+	-	-	-	+
3	-	+	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-
4	-	-	-	-	+	+	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	-	+	+	+	+	-	-	+	-
6	-	+	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-	-	+	+	-	+	-	-	+	+	-	-	+	+
8	-	-	+	-	+	+	-	-	+	+	-	-	+	-	+	+	-	-	+	+	-	-	+	-	+	+	-	-	+	+
11	-	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+	-	-	+	+	-	+	+	+	+	+	-	-	+	+
13	-	+	+	+	+	+	-	+	-	-	-	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+	-	-	-	+
14	-	-	+	-	+	+	-	-	-	-	-	-	+	-	+	+	-	-	-	+	-	-	+	-	-	+	-	-	-	+
15[d]	-	+	-	-	+	+	-	+	+	-	-	+	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	-
16	-	+	+	-	+	+	-	+	-	+	-	-	+	+	+	+	-	+	-	+	-	-	+	+	+	+	-	+	-	+
17	-	-	+	-	+	+	-	+	-	+	-	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+	-	-	-	+
21	-	+	+	-	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+	-	+	+	-	+	+	-	-	+	+
24[d]	-	-	+	-	+	+	-	-	+	-	-	-	+	-	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+
25	-	+	+	-	+	+	-	+	-	+	-	-	+	-	+	+	-	+	+	+	-	-	M[e]	-	M	M	-	M	M	M
26	-	-	-	-	+	+	-	+	+	+	-	-	-	-	+	+	-	+	+	+	-	-	-	-	+	+	-	+	+	+
27	-	-	+	-	+	+	-	+	-	+	-	-	+	-	+	+	-	+	-	+	-	-	+	-	+	+	-	+	-	+
29[f]	-	+	+	-	+	M	M	M	M	M	-	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+	-	-	-	+
33	-	+	-	+	+	+	-	-	-	+	-	M	-	M	M	M	-	-	-	M	-	+	-	+	+	+	-	-	-	+

[a],[b],[c] See footnotes to Table 4.
[d] Salmonella isolation reported by HGMF from control sample for samples #6-10.
[e] Results from samples #6-10 excluded from statistical analysis.
[f] Missing data. Analysis not done or incomplete.
Method deviation. Did not carry out any biochemical or serological confirmation of isolates for either AOAC/BAM or HGMF. Results from samples #1-10 excluded from statistical analysis.

Table 8. Detection of Salmonella in whole egg powder by AOAC/BAM and HGMF methods

Coll.	Results of Salmonella analysis																															
	AOAC/BAM										HGMF - SLA[a]										HGMF - HE[b]									
	1	2	3	4	5[c]	6	7	8	9[c]	10	1	2	3	4	5[c]	6	7	8	9[c]	10	1	2	3	4	5[c]	6	7	8	9[c]	10
3	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
4	-	+	+	+	-	-	+	+	-	-	-	+	+	+	-	-	+	-	-	-	-	+	+	+	-	-	+	-	-	-
8	-	+	+	-	-	+	+	+	-	-	-	+	+	+	-	-	+	+	-	+	-	+	+	+	-	-	+	+	-	+
10	-	-	-	-	-	+	+	+	-	-	-	+	-	+	-	+	+	+	-	-	-	+	-	+	-	+	+	+	-	-
16	-	+	+	-	-	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
19	-	+	+	-	-	-	+	+	-	-	-	+	-	M[d]	-	M	-	-	-	-	-	M	+	+	-	+	+	+	-	+
21	-	+	+	-	-	+	+	-	-	+	-	+	+	-	-	+	+	-	-	+	-	+	+	-	-	+	+	-	-	+
24	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-
29[e]	-	+	+	-	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	+	-	-
30	-	+	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
31	-	+	-	-	-	+	-	+	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-
33	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-
34	-	+	+	-	-	-	+	+	-	-	-	+	+	+	-	+	+	-	-	-	-	+	+	+	-	+	+	+	-	-
36	-	+	+	+	-	+	+	-	-	-	-	+	-	+	-	-	+	+	-	-	-	+	-	+	-	-	+	+	-	-

a,b,c, See footnotes to Table 4.
d Missing data. Analysis not done or incomplete.
e Method deviation. Did not carry out any biochemical or serological confirmation of isolates for either AOAC/BAM or HGMF. Results from samples #1-10 excluded from statistical analysis.

sterile tubes. Do not heat medium after addn of I-KI and dye solns. Temper to 25–35° before use.

(l) *Selective lysine agar (SLA).*—Suspend 5.0 g proteose peptone No. 3, 3.0 g yeast ext, 10.0 g L-lysine-HCl, 3.5 g glucose, 1.5 g bile salts No. 3, 0.001 g crystal violet (1.0 mL of 0.1% (w/v) aq. soln), 0.03 g bromocresol purple, 0.3 g sulfapyridine, and 15.0 g agar in 1 L H_2O and heat to bp with stirring to dissolve completely. Autoclave 15 min at 121°. Cool to 45–50°. Dispense 20 mL vol. in 15 × 100 mm petri dishes. Final pH, 6.8 ± 0.1.

(m) *Hektoen enteric agar (HE).*—Suspend ingredients (*1*) or (*2*) (varies with manufacturer of formula) in 1 L H_2O and mix thoroly. Heat to boiling with frequent agitation and let boil few moments. Do not overheat. Cool in H_2O bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 7.6 ± 0.2. Do not autoclave.

(*1*) 12.0 g thiotone peptone, 3.0 g yeast ext, 9.0 g bile salts, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.064 g bromothymol blue, 0.1 g acid fuchsin, and 13.5 g agar.

(*2*) 12.0 g proteose peptone, 3.0 g yeast ext, 9.0 g bile salts No. 3, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.065 g thymol blue, 0.1 g acid fuchsin, and 14.0 g agar.

(n) *Triple sugar iron agar (TSI agar).*—Suspend ingredients (*1*) or (*2*) in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil ca 1 min until ingredients dissolve. Fill 16 × 150 mm tubes ⅓ full and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 3 cm) and adequate slants (ca 5 cm) are formed on solidification.

(*1*) 20 g polypeptone, 5.0 g NaCl, 10 g lactose, 10 g sucrose, 1 g glucose, 0.2 g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.2 g $Na_2S_2O_3$, 0.025 g phenol red, and 13 g agar. Final pH, 7.3 ± 0.2.

(*2*) 3.0 g beef ext, 3.0 g yeast ext, 15 g peptone, 5.0 g proteose-peptone, 1.0 g glucose, 10 g lactose, 10 g sucrose, 0.2 g $FeSO_4$, 5.0 g NaCl, 0.3 g $Na_2S_2O_3$, 0.024 g phenol red, and 12 g agar. Final pH, 7.4 ± 0.2.

(o) *Lysine iron agar (LIA) (Edwards and Fife).*—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext, 1.0 g glucose, 10 g L-lysine, 0.5 g ferric ammonium citrate, 0.04 g anhyd. $Na_2S_2O_3$, 0.02 g bromocresol purple, and 15 g agar in 1 L H_2O, heating until dissolved. Dispense 4 mL portions into 13 × 100 mm test tubes and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that 4 cm butts and 2.5 cm slants are formed on solidification. Final pH 6.7 ± 0.2.

(p) *MacConkey agar (MAC).*—Suspend 3.0 g proteose peptone or polypeptone, 17 g peptone or gelysate, 10 g lactose, 1.5 g bile salts No. 3 or bile salts mixt., 5.0 g NaCl, 3.0 mL 1% neutral red (30 mg) soln, 1 mL 0.1% crystal violet (1.0 mg) soln, and 13.5 g agar in 1 L H_2O and mix thoroly until homogeneous. Heat, with occasional agitation, and boil 1-2 min until ingredients dissolve. Autoclave 15 min at 121°. Cool to 45–50° and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with plates covered. Do not use wet plates. Final pH 7.1 ± 0.2.

(q) *Sodium hydroxide soln.*—1N. Dissolve 42.11 g 95% reagent NaOH in sterile H_2O and dil. to 1 L.

(r) *Hydrochloric acid soln.*—1N. Dil. 89 mL to 1 L with sterile H_2O.

(s) *pH Test paper.*—Min. range 6.0–7.6 with max. gradations of 0.4 pH unit per color change.

(t) *Sterile distilled water.*—Dispense 1 L H_2O into 2 L wide-mouth flask or wide-mouth jar; plug or cap loosely. Autoclave 20 min at 121°.

(u) *Brilliant green dye soln.*—1%. Dissolve 1 g in sterile H_2O and dil. to 100 mL. (Since some batches of dye are unusually toxic, test all batches of dye before use, and use only those producing satisfactory results when tested with known pos. and neg. test organisms.)

(v) *Brilliant green dye water.*—Prep. sterile H_2O, (t), and add 2 mL of 1% aq. brilliant green dye, (u), per L sterile H_2O and mix well.

46.A08 *Preparation of Sample*

(a) *Powdered egg.*—Aseptically open sample container and aseptically weigh 25 g sample into sterile, empty, wide-mouth, screw-cap pt (500 mL) jar. Add ca 15 mL sterile lactose broth. Stir with sterile glass rod, sterile spoon, or sterile tongue depressor to smooth suspension. Add 3 addnl portions lactose broth, 10, 10, and 190 mL

Table 9. Detection of Salmonella in nonfat dry milk by AOAC/BAM and HGMF methods

Coll.	Results of Salmonella analysis																															
	AOAC/BAM										HGMF - SLA[a]										HGMF - HE[b]									
	1	2	3[c]	4	5	6[c]	7	8	9	10	1	2	3[c]	4	5	6[c]	7	8	9	10	1	2	3[c]	4	5	6[c]	7	8	9	10		
1	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+		
3[d]	-	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-		
4	-	+	-	+	+	-	+	-	-	+	-	-	-	+	+	-	+	-	+	+	-	-	-	-	+	-	+	-	+	+		
9	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	+	-		
10	-	+	-	+	-	-	+	-	+	-	-	+	-	+	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-	+		
11	+	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	+	-	+		
13	-	+	-	-	M[e]	-	+	+	+	M	-	-	-	+	M	-	+	-	+	M	-	-	-	+	M	-	+	-	+	M		
15	-	-	-	+	+	-	+	+	+	+	-	-	-	-	+	-	+	-	+	+	-	-	-	-	+	-	+	-	+	+		
16	-	-	-	+	+	-	+	-	-	+	-	+	-	+	-	-	+	+	+	-	-	-	-	+	-	-	+	+	+	-		
17	-	-	-	-	+	-	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	+	+	-		
18[f]	-	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	+	+		
20	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+		
21	-	-	-	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+		
22	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-		
26	-	-	-	+	+	-	+	-	-	+	-	-	-	-	+	-	+	-	-	+	-	-	-	-	+	-	+	-	-	+		
29[g]	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
31	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+	-	-	+	-	+		
33	-	-	-	-	+	-	+	+	-	+	-	-	-	-	M	-	M	M	-	+	-	-	-	-	+	-	+	+	-	M		
36	-	+	-	-	+	-	+	+	+	M	-	+	-	-	+	-	+	+	+	-	-	-	-	-	+	-	+	+	+	-		

[a],[b],[c] See footnotes to Table 4.

[d] Method deviation. Disinfected filtration units with quaternary ammonium compound and did not rinse adequately. Results from samples #1-10 excluded from statistical analysis.

[e] Missing data. Analysis not done or incomplete.

[f] Method deviation. HGMF tetrathionate broths for samples #1-5 incubated at 25°C. Results from samples #1-5 excluded from statistical analysis.

[g] See corresponding footnote to Table 6.

for total of 225 mL. Stir after each addn until sample is suspended without lumps. Cap jar securely and let stand at room temp. 60 min. Mix well by shaking, and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Loosen jar cap ca ¼ turn and incubate 18–24 h at 35°.

(b) *Chocolate.*—Aseptically weigh 25 g sample into sterile blender jar. Add 255 mL sterile reconstituted NFDM-BG. Blend 2 min at high speed and decant blended homogenate into sterile 500 mL jar. Cap jar securely and let stand 60 min at room temp. Mix well by shaking, and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Add 0.45 mL 1% aq. brilliant green dye and mix well. Loosen jar cap ¼ turn and incubate 18–24 h at 35°.

(c) *Raw meat.*—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth and blend 2 min at high speed. Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Aseptically transfer sample to sterile 500 mL wide-mouth screw-cap jar. Loosen jar cap ¼ turn and incubate 18–24 h at 35°.

(d) *Cheese powder.*—Aseptically weigh 25 g sample into sterile 500 mL wide-mouth screw-cap jar. Add 225 mL sterile lactose broth and mix well. Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Loosen jar cap ¼ turn and incubate 18–24 h at 35°.

(e) *Pepper.*—Aseptically weigh 25 g sample into sterile 500 mL wide-mouth screw-cap jar. Add 225 mL sterile trypticase soy broth and mix well. Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Loosen jar cap ¼ turn and incubate 18–24 h at 35°.

(f) *Powdered milk.*—Use sterile funnel to aseptically add 25 g sample slowly and gently to 225 mL sterile brilliant green dye water in 500 mL wide-mouth screw-cap jar. Do not mix. Allow to soak undisturbed 60 min at room temp. Do not mix or adjust pH. Loosen jar cap ¼ turn and incubate 18–24 h at 35°.

46.A09 *Isolation*

(a) *Selective enrichment.*—Gently shake incubated sample mixt. and transfer 0.1 mL to 10 mL tempered (25–35°) tetrathionate broth. Mix inoculated broth on vortex mixer or by hand to disperse inoculum. Incubate in H_2O bath 6–8 h at 35 ± 0.5°.

(b) *Filtration and selective isolation.*—Mix incubated tetrathionate broth by hand or vortex-mixer to resuspend. For raw meats, prep. 10^{-2} diln by transferring 1.0 mL into 99 mL sterile peptone diluent. Mix by shaking. For all other products, use undild tetrathionate.

(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 until shut by pressing with thumb on latch B.

Aseptically add ca 15–20 mL sterile H_2O to funnel. Pipet 1.0 mL of required tetrathionate diln 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.

Table 10. Statistical comparison of *Salmonella* detection by AOAC/BAM and HGMF[a] methods

Product	No. of samples[b]	No. of positive samples			No. of disagreements[d]		Z-value[e]
		AOAC/BAM	HGMF	Combined[c]	AOAC/BAM	HGMF	
Chocolate	68	68	67	68	0	1	1.0000
Raw poultry meat	150	131	133	138	7	5	−0.5801
Ground black pepper	84	56	57	61	5	4	−0.3315
Cheese powder	136	80	81	89	9	8	−0.2434
Whole egg powder	104	45	51	59	14	8	−1.2822
Nonfat dry milk	129	72	68	87	15	19	0.6843

[a]HGMF results reflect combined performance of SLA and HE filters.
[b]Negative control samples excluded from statistical analysis.
[c]Reflects combined results of AOAC/BAM and HGMF methods (i.e., number of samples positive by at least one method).
[d]No. of false negative samples compared to combined results of AOAC/BAM and HGMF methods.
[e]As determined by 2-sided statistical pair-wise comparison of recovery of *Salmonella* and rate of false negative reactions (5).

Open clamp A. Release latch B, and rotate back funnel C. Aseptically remove HGMF and place on surface of pre-dried SLA. Avoid trapping air bubbles between filter and agar. For nonfat dry milk samples, insert second sterile HGMF into same filtration unit, repeat filtering procedure and place second HGMF on surface of pre-dried HE. Incubate SLA 24 ± 2 h at 43 ± 0.5°, and HE 24 ± 2 h at 35°. If HGMFs do not have typical or suspicious colonies or do not contain growth, record as neg. test result.

(c) *Appearance of typical Salmonella colonies.*—(*1*) *On SLA.*—Blue-green, blue, or purple colonies (lysine-pos. reaction). Typically, *Salmonella* produces relatively flat colonies which are neither watery nor mucoid. Lysine-neg. colonies are typically yellow or yellow-green. However, this can be masked if large no. of lysine-pos. colonies are present on HGMF.

(*2*) *On HE.*—Black or green with black centers. Some *Salmonella* will produce yellow colonies with black centers or green colonies with no blackening. H_2S reaction can be partially suppressed if very heavy growth is present on HGMF.

46.A10 *Treatment of Typical or Suspicious Colonies*

(a) *Inoculation of TSI, LIA, and MAC or HE.*—(*1*) *Raw meats.*—Select 5 typical or suspicious colonies from each HGMF.

(*2*) *All other products.*—Select 3 typical or suspicious colonies from each HGMF.

Using sep. sterile, completely cooled needle for each colony, pick each selected colony and inoculate TSI slant with portion of colony by stabbing butt and streaking slant. Without heating needle or obtaining more inoculum, inoculate LIA with portion of colony by stabbing butt in 2 places and streaking slant. Without heating needle or obtaining more inoculum, streak remainder of inoculum to MAC or HE. Incubate TSI, LIA, and MAC or HE 24 ± 2 h at 35°. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H_2S production.

(b) *Presumptive positive reactions.*—*Salmonella* cultures typically have alk. (red) slant and acid (yellow) butt, with or without H_2S (blackening of agar) in TSI agar. In LIA, *Salmonella* cultures typically have alk. (purple) reaction in butt. Consider only distinct yellow coloration in butt of LIA tube as acidic (neg.) reaction. Do not eliminate cultures that produce discoloration in butt solely on this basis. Most *Salmonella* cultures produce H_2S in LIA. Retain all presumptive pos. *Salmonella* cultures on TSI agar (alk. slant and acid butt) for biochem. and serological tests whether or not corresponding LIA reaction is pos. (alk. butt) or neg. (acid butt). Do not exclude TSI culture that appears to be non-*Salmonella* if reaction in LIA is typical (alk. butt) for *Salmonella*. Treat these cultures as presumptive pos. and submit them to further examination. LIA is useful in detection of *S. arizonae* and atypical *Salmonella* strains that utilize lactose and/or sucrose. Discard only apparent non-*Salmonella* TSI agar cultures (acid slant and acid butt) if corresponding LIA reactions are not typical (acid butt) for *Salmonella*.

46.A11 *Purification and Identification*

(a) *Appearance of Salmonella colonies.*—(*1*) *On MAC.*—Typical colonies appear transparent and colorless, sometimes with dark centers. *Salmonella* will clear areas of pptd bile caused by other organisms sometimes present in medium.

(*2*) *On HE.*—Blue-green to blue colonies with or without black centers. Many *Salmonella* cultures may have large glossy black centers or may appear as almost completely black colonies.

(b) *Purification of mixed cultures.*—Examine MAC or HE. If pure, proceed with identification. If mixed culture, pick with needle ≥2 well isolated typical or suspicious colonies and inoculate TSI, LIA, and MAC or HE as described above. Incubate and examine for presumptive pos. reactions.

(c) *Identification.*—Carry out biochem. and serological identification procedures on 3 presumptive pos. TSI cultures from each HGMF as described in **46.121–46.128**. As alternative to conventional tube system for *Salmonella*, any one of 3 commercial biochem. systems (API, Enterotube, or Minitek) may be used for presumptive generic identification of foodborne *Salmonella*. See **46.133–46.135**.

Results and Discussion

The results of quantitative *Salmonella* analyses carried out by the coordinating laboratory are shown in Table 3. Samples (except for raw poultry) had been inoculated at levels designed to yield concentrations in the range of 1–2 viable *Salmonella* cells per 25 g sample (0.04–0.08/g) by the dates specified for initiating analyses. Preliminary experiments had been carried

Table 11. Statistical comparison of *Salmonella* detection by AOAC/BAM and HGMF-SLA methods

Product	No. of samples[a]	No. of positive samples			No. of disagreements[c]		Z-value[d]
		AOAC/BAM	HGMF-SLA	Combined[b]	AOAC/BAM	HGMF-SLA	
Chocolate	68	68	66	68	0	2	1.4272
Raw poultry meat	140	120	115	126	6	11	1.2143
Ground black pepper	81	53	53	58	5	5	0.0000
Cheese powder	131	75	72	83	8	11	0.6877
Whole egg powder	102	45	40	56	11	16	0.9608
Nonfat dry milk	127	69	56	81	12	25	2.1695[e]

[a]Negative control samples excluded from statistical analysis.
[b]Reflects combined result of AOAC/BAM and HGMF-SLA methods (i.e., number of samples positive by at least one method).
[c]No. of false negative samples compared to combined results of AOAC/BAM and HGMF-SLA methods.
[d]As determined by 2-sided statistical pair-wise comparison of recovery of *Salmonella* and rate of false negative reactions (5).
[e]Statistically significant ($P<0.05$).

Table 12. Statistical comparison of *Salmonella* detection by AOAC/BAM and HGMF-HE methods

Product	No. of samples[a]	No. of positive samples			No. of disagreements[c]		Z-value[d]
		AOAC/BAM	HGMF-HE	Combined[b]	AOAC/BAM	HGMF-HE	
Chocolate	68	68	67	68	0	1	1.0000
Raw poultry meat	147	129	106	134	5	28	4.2297[e]
Ground black pepper	84	56	49	60	4	11	1.8330
Cheese powder	130	75	74	83	8	9	0.2421
Whole egg powder	103	44	50	58	14	8	−1.2819
Nonfat dry milk	128	71	60	83	12	23	1.8755

[a]See corresponding footnote to Table 11.
[b]Reflects combined result of AOAC/BAM and HGMF-HE methods (i.e., number of samples positive by at least one method).
[c]No. of false negative samples compared to combined results of AOAC/BAM and HGMF-HE methods.
[d]See corresponding footnote to Table 11.
[e]Statistically significant ($P<0.05$).

out to enable the probable extent of inoculum die-off to be established for each product. This resulted in inocula very close to the targeted levels for most of the powdered food samples. In the chocolate, less die-off occurred than had been predicted and, as a result, inocula were higher than the desired levels. Nevertheless, a sufficient number of samples overall contained inocula at or below the targeted levels to permit an appraisal of the sensitivity of the HGMF method relative to the reference method.

The data reported by collaborators are presented in Tables 4–9, inclusive. It was necessary to exclude some data from statistical analysis due to significant deviations or to *Salmonella* having been isolated from an uninoculated control sample. These cases are indicated by superscripts in Tables 4–9 and explained in the corresponding footnotes. Uninoculated control samples from the 5 artificially contaminated product series were also excluded from statistical analysis.

The remaining data were evaluated by a 2-sided statistical pair-wise comparison of the recovery of *Salmonella* and rate of false negative reactions (5), using 3 different pairings. First, the combined HGMF results using SLA and HE were compared to the reference method results. Then, the results obtained using SLA alone and HE alone were each compared to the reference method. The numbers of *Salmonella*-positive samples and the results of the statistical evaluations are shown in Tables 10–12, inclusive. The HGMF and reference methods were statistically equivalent in all but 2 cases, specifically, SLA used alone for nonfat dry milk and HE alone for raw poultry meat. Also, the performance of HE alone was only marginal in pepper and nonfat dry milk sample series.

At least 8 of the individual samples for which SLA alone gave a false negative reaction could be traced to inappropriate subculturing technique. In one sample each of chocolate and cheese powder and in 6 egg powder samples, collaborators reported typical colonies on SLA, but no growth on subculture to TSI and LIA. Discussion with these collaborators established that inoculating needles had been inadequately cooled before use. *Salmonella* produces a very thin flat colony on the HGMF on SLA, compared with a much taller colony on HE. It is possible that these flatter colonies were more susceptible to incineration by a hot needle than the more robust HE colonies. When this information was received soon enough to allow the subculture technique to be adjusted for the remaining sample series, the problem did not recur.

An important factor in evaluating a *Salmonella* method is the ability to recover *Salmonella* from samples containing no more than 1–2 cells/25 g. Sixteen of the inoculated powders were in this range or even lower (see Table 3). An additional 6 samples were only slightly higher (2.24 cells/25 g). In all of these cases, the HGMF method performed equivalently to the reference method.

Some less common or atypical serotypes were also included in the study: *S. arizonae*, *S. kentucky* (lactose-positive), and *S. give* (H_2S-negative) (see Table 2). The *S. give* inoculum did not survive; however, the other 2 serotypes were recovered equivalently by both the HGMF and the reference methods. The lactose-positive strain, *S. kentucky*, appeared to be slightly more readily recovered on SLA than on HE, although too few samples were positive to allow any firm conclusions. HE relies on lactose fermentation and H_2S production as the primary screening characteristics, while SLA uses lysine decarboxylation.

The 13th edition of *Official Methods of Analysis* (6) requires a single stab of the LIA butt and a 48 h incubation period at 35°C. In the 14th edition, this has been changed to a double stab, although the incubation period remains unchanged. Collaborators were requested to examine their LIA subcultures from HGMF at 24 and 48 h and to record the reactions at both times. In almost every instance, no change was reported during the second 24 h except for an intensification of the H_2S reaction and, sometimes, the production of small amounts of gas in the butt. The 48 h incubation only rarely altered or intensified the lysine decarboxylase reaction beyond that observed at 24 h.

The HGMF *Salmonella* method can produce a reliable negative result in as little as 48 h from initiation of the analysis, compared with 96 h by the AOAC reference method. A preliminary biochemical screen and purity plate (TSI, LIA, and MAC) is available after an additional 24 h, allowing the clearance of a large percentage of samples within 72 h of initiation of the analysis. Samples appearing to be positive at this stage should, of course, be carried through the complete biochemical and serological identification protocol. As shown by the results of this collaborative study, the isolation/detection time is shortened without sacrificing sensitivity of method.

Recommendation

It is recommended that the rapid HGMF method for *Salmonella* detection in foods described herein be adopted official first action.

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PESTICIDE FORMULATIONS

Proton Magnetic Resonance Spectroscopic Assay of Propoxur

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A procedure is described for the assay of propoxur (*o*-isopropoxyphenyl *N*-methylcarbamate) by nuclear magnetic resonance (NMR) spectroscopy. Methanol was used as internal standard and chloroform as solvent. Known standard and technical materials were analyzed and results of NMR and liquid chromatographic methods were compared. The NMR method is straightforward, with a precision of ± 1.6% (95% confidence limits) for 2 sample weighings.

Propoxur (*o*-isopropoxyphenyl *N*-methylcarbamate) is a broad spectrum insecticide. Analytical procedures are available for the technical material and formulations, and for residue analysis. Measurement of the infrared absorbance of the carbonyl peak at 1750 cm^{-1} is a widely used method that is applicable to technical material and formulations (1). Colorimetry as well as thin layer chromatography have been in general use for carbamate analysis (2–5). Gas chromatographic methods for *N*-methylcarbamates generally require hydrolysis and/or derivatization steps before analysis, because of the thermal sensitivity of the carbamates (6, 7). A liquid chromatographic (LC) method for the analysis of a 2% propoxur formulation has been reported; however, special precautions may be required to prevent column damage by formulation insolubles (8).

Nuclear magnetic resonance (NMR) spectroscopy is finding increasing use in quantitative analysis (9–16). Biros reported the semiquantitative NMR analysis of *p,p'*-DDT and *p,p'*-DDE (17), and Dorsey et al. described the determination of the epoxide equivalent weight of glycidal ethers (18).

Because of the difficulties in the GC method, we considered it worthwhile to develop an NMR method for determination of propoxur. Also, if formulation impurities are present in sufficient quantities, NMR analysis normally provides an indication of their nature, and, in some instances, a semiquantitative estimation of the impurities is possible.

Experimental

Apparatus and Reagents

(a) *Spectrometer.*—Varian EM-360 NMR spectrometer equipped with EM-3630 spin decoupler.

(b) *Liquid chromatograph.*—Varian 5020 equipped with Model UV-1 single wavelength (254 nm) ultraviolet detector.

(c) *Calorimeter.*—Perkin-Elmer DSC-2 differential scanning.

(d) *Internal standard.*—Methanol (analytical grade, Frutarom Chemicals, Haifa, Israel) was used without further purification.

(e) *Chloroform.*—Purify as follows: Place 250 mL $CHCl_3$ in 500 mL separatory funnel and wash with two 25 mL portions of concentrated H_2SO_4. After careful separation, wash $CHCl_3$ layer with water until neutral. Dry over anhydrous Na_2SO_4 and filter to obtain ethanol-free $CHCl_3$ (16).

(f) *Internal standard solution.*—Place 1.0 mL analytical grade methanol in 25 mL volumetric flask and add $CHCl_3$ to mark.

(g) *Propoxur.*—Propoxur standard and samples were supplied by Makhteshim Chemical Works Ltd.

Procedure

Accurately weigh in duplicate ca 150 mg propoxur and add 1.0 mL internal standard solution to each portion. Transfer ca 0.6 mL of the solution to NMR sample tube and place in instrument probe. Adjust spin rate to ca 40 cps. Integrate areas of interest in an unbroken scan 7 times and discard highest and lowest integral values.

Calculations

Calculate amount of propoxur as follows:

$$Wt_u = (Wt_s \times P_s \times MW_u \times I_u)/(P_u \times MW_s \times I_s)$$

where Wt_u = calculated weight of sample; Wt_s = weight of internal standard; P_u and P_s = number of protons integrated in unknown and internal standard, respectively; MW_u and MW_s = molecular weight of unknown and standard, respectively; I_u and I_s = value of integral (average of 5 integrations) for unknown and standard, respectively;

$$\% \text{ Propoxur in sample} = (Wt_u/Wt_a) \times 100$$

where Wt_a = actual weight of sample.

Results and Discussion

Carbon tetrachloride appeared to be the solvent of choice for this study. However, because of the relative insolubility of the carbamate in carbon tetrachloride use of this solvent was abandoned, and chloroform was chosen in its place. To remove traces of ethanol, the stabilizer present in the solvent, it was necessary to wash the chloroform twice with concentrated sulfuric acid before washing with water. Repeated

Table 1. Linearity of response as a function of sample weight

Propoxur, mg	% Found	Variance	95% Confidence range
20–51	102.9	2.226	100.4–105.5
100–152	101.1	1.106	99.3–102.9
201–205	99.4	3.089	96.4–102.4

Table 2. Consecutive analysis of 2 propoxur samples

Sample	% Propoxur found[a]	Std dev.	Range, 95% CL[b]
Standard	98.7	0.52	97.6–99.7
	98.8	0.59	97.6–100.0
	98.6	0.79	97.0–100.2
Mean	98.69	0.60	97.6–99.8
N-6	101.6	0.74	100.1–103.1
	101.1	1.25	98.6–103.6
	101.1	1.08	99.0–103.3
	100.5	1.14	98.2–102.8
Mean	101.0	1.05	99.3–102.9

[a]Five integrations for each value reported: total of 15 for standard sample, and 20 for N-6.
[b]CL = confidence limits.

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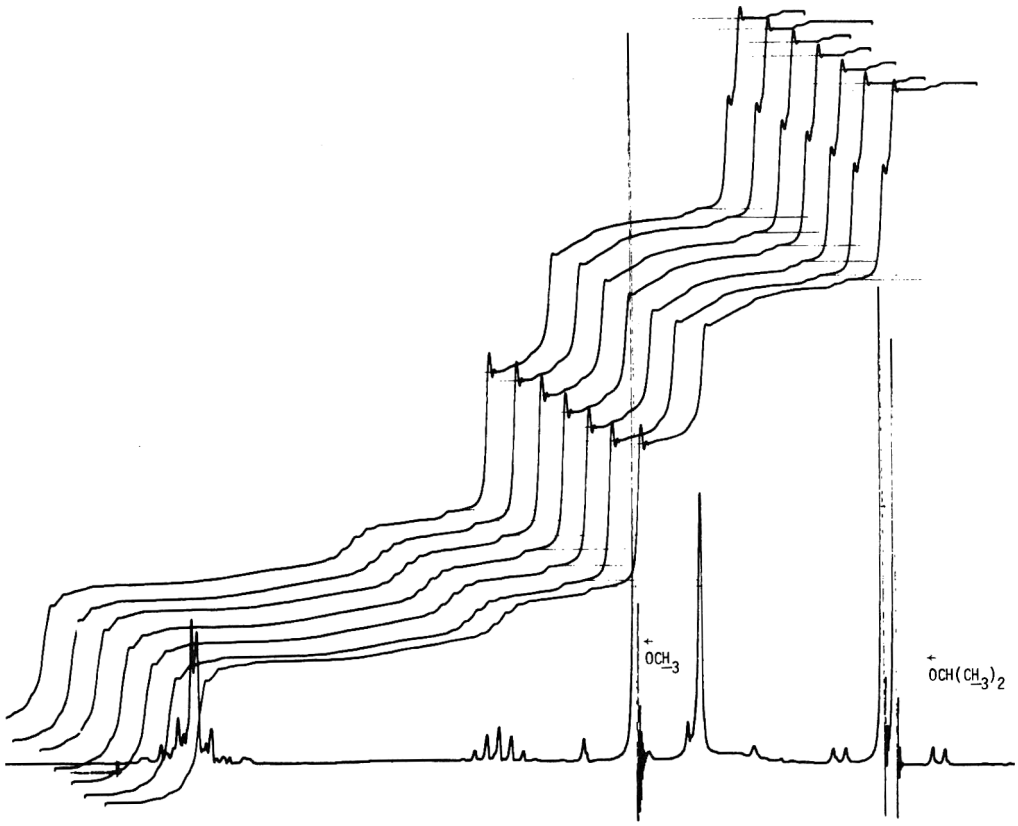

Figure 1. NMR spectrum of propoxur containing methanol internal standard.

Table 3. Comparison of results of LC and NMR analyses of propoxur

	Propoxur, %	
Sample No.	LC	NMR
1	98.3	98.3
2	98.3	99.8
3	97.5	98.0
4	97.5	97.4
5	98.4	98.8
6	98.3	98.6
7	98.5	98.9
8	99.3	99.5
9	98.7	96.8
10	99.0	99.3

water washings without acid treatment failed to remove the last traces of ethanol which were apparent in the solvent NMR spectra. Spinning sidebands and ^{13}C resonances were checked to assure that they did not appear in an area of interest. To control this situation, the sample spinning rate was maintained at 40 ± 5 cps throughout the investigation.

Methanol was satisfactory as the internal standard despite the fact that the CH_3OH proton absorption fell in the $C(O)NHCH_3$ doublet region (2.78δ). It was necessary to store the methanol–chloroform standard solution in a tightly stoppered vial to prevent composition changes due to evaporation. It was convenient to prepare 25 or 50 mL of standard solution at one time, and store the solution as 5 or 10 mL aliquots in septum-cap vials. These total amounts were sufficient for about 20 or 40 analyses, respectively, performed in replicate.

The signals at ~3.38 (CH_3OH) and 1.38 [$OCH(CH_3)_2$] were used for the analysis. A typical spectrum for propoxur analysis is shown in Figure 1. In each instance, the spectrum of the material without internal standard was run to assure that no impurity absorption fell in the methanol standard range (~3.38). An unknown impurity peak appeared as a multiplet centered at 2.358 and presented no problems. The aromatic protons occur as a complex multiplet centered at 7.08 and the N-H absorption as a broad singlet at 5.158; a 3H doublet (2.788) and a 6H doublet (1.38) appear for the $NH\text{-}CH_3$ and $OCH(CH_3)_2$, respectively.

o-Isopropoxyphenol is a potential impurity that could interfere with the analysis. Comparison of the $NH\text{-}CH_3$ integral value with $OCH(CH_3)_2$ integral value indicated that this impurity was not present in the samples under investigation. LC analysis confirmed that *o*-isopropoxyphenol was present below 0.1%.

Linearity of response as a function of sample weight was determined at constant internal standard weight. Results (Table 1) show a dependence on the size of the propoxur sample. Smaller variances were obtained with samples of 100–150 mg, which makes this the area of greatest precision. All further analyses reported herein are limited to samples in the 100–150 mg weight range.

The relationship between the number of replicates and the precision were determined. For this study, a sample of standard propoxur (standard in Table 2) and a sample of technical material (N-6 in Table 2) were analyzed with 2 and 3 replicates, respectively. The pooled estimates of the variance using both samples is 0.899. The probable error of a single determination is 2% at the 95% confidence level (CL). Precision with duplicate samples (i.e., analyses + replicate) is ±1.35% (90% CL), ±1.575% (95% CL), and 1.80 (99% CL). LC results are compared with the NMR results in Table 3.

Results of the hypothesis test that was run on the data, as a set of matched pairs, indicated that the LC results and the NMR results were from the same distribution at 90% confidence. A very close correspondence of 98.3, 98.5, and 98.7% was obtained when one sample was analyzed by LC, NMR, and differential scanning calorimetry, respectively.

In summary, the NMR method for the analysis of propoxur is applicable to the technical material. The accuracy and precision of the method have been investigated. Comparison of the LC and NMR method indicate that comparable results were obtained.

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Liquid Chromatographic Determination of Aminocarb in Technical and Formulated Products: Collaborative Study

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An LC method for determination of aminocarb (Matacil™) in aminocarb technical and formulated products has been developed and subjected to a collaborative study with 10 participating collaborators. Formulations are extracted with tetrahydrofuran–buffer (60 + 40) and analyzed by reverse phase chromatography, with *n*-butyrophenone as internal standard. Collaborators were furnished with standards and samples of technical and 180 oil flowable products for analysis. Coefficients of variation obtained on these samples were 0.72 and 1.7%, respectively. The method has been adopted official first action.

Aminocarb, 4-(dimethylamino)-3-methylphenol methylcarbamate (ester), is available as a 180 g/L active ingredient, oil flowable (180 OF) formulation. Aminocarb is a carbamate insecticide which is highly effective against pests in deciduous and coniferous forests. Direct gas chromatography (GC) of aminocarb has been reported for several residue methods (1–3). This technique is frequently variable in results because of the thermal instability of carbamates (4, 5). Recognition of this instability has resulted in many derivative GC methods for aminocarb (6–10). Liquid chromatography (LC) eliminates the need for derivatization, and several LC methods have been reported for aminocarb (11–14). Lanouette and Pike reported an external standard LC method for aminocarb in formulations (15).

Collaborative Study

A reverse phase LC method, with *n*-butyrophenone as an internal standard, was developed and submitted to 10 collaborating laboratories. Collaborators were also furnished duplicate subsamples of the aminocarb technical material and formulation, a reference standard, and the internal standard. The study was designed according to suggestions given by Youden and Steiner (16). This paper reports the results of the collaborative study on the method.

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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.* (1985) **68**, March issue.

This report of the Associate Referee was presented at the 98th Annual International Meeting of the AOAC, Oct. 28–Nov. 2, 1984, at Washington, DC.

Aminocarb Technical and Pesticide Formulations

Liquid Chromatographic Method

First Action

CIPAC–AOAC Method

(Method is suitable for tech. aminocarb and formulations with aminocarb as only active ingredient.)

6.A25 ***Principle***

Aminocarb is detd by liq. chromatgy, using *n*-butyrophenone as internal std.

6.A26 ***Apparatus***

(a) *Liquid chromatograph.*—Able to generate >17.5 MPa (> 2500 psi) and measure *A* at 246 nm.

(b) *Chromatographic column.*—250 × 4.6 mm id packed with ≤10 μm C18 bonded silica gel (Partisil-10 ODS-3, Whatman Chemical Separations, Inc.; MicroPak MCH-10, Varian Instrument Group; Ultrapack-ODS, Altex Scientific; Zorbax Sil, Du Pont Co.; or equiv. is suitable). Operating conditions: column temp. ambient; mobile phase flow rate 1.5 mL/min (ca 2000 psi); chart speed 0.5 cm/min; injection vol. 10 μL; *A* range 0.320 AUFS; retention times: aminocarb ca 2.65 min, internal std ca 3.80 min. Pump LC mobile phase thru column until system is equilibrated (flat baseline). Allow ca 6 min after each injection.

(c) *Filters.*—0.45 μm porosity (Gelman Acrodisc-CR or equiv.).

Table 1. Collaborative results of the LC analysis of aminocarb technical and formulation

	Technical		180 OF	
Collab.	A	B	A	B
1	97.96	97.68	20.89[a]	21.32[a]
2	97.89[a]	95.71[a]	20.10	20.23
3	97.24	97.18	20.37	20.34
4	91.67[a]	93.46[a]	19.79	19.79
5	98.62	98.63	20.89	20.93
6	96.40	97.37	20.66	20.57
7	97.27	96.91	20.77	20.65
8	97.72	97.02	20.44	20.42
9	96.62	96.63	20.14	20.23
10	96.76	96.72	20.72	20.72
Mean	97.30		20.43	
S_x	0.700		0.351	
S_o	0.340		0.056	
S_L	0.613		0.346	
CV_x, %	0.719		1.72	
N	16		18	

[a]Values > 2 S_o from average are omitted from calculations (17).

6.A27 *Reagents*

(a) *n-Butyrophenone internal std soln.*—3 g/100 mL tetrahydrofuran.

(b) *Tetrahydrofuran.*—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).

(c) *Buffer soln.*—Dissolve 1.36 g KH_2PO_4 and 2.68 g $Na_2HPO_4.7H_2O$ in 1 L H_2O.

(d) *Water.*—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).

(e) *Mobile phase.*—Tetrahydrofuran–buffer soln (60 + 40).

(f) *Aminocarb reference std soln.*—Accurately weigh ca 250 mg ref. std (Mobay Chemical Corp., Agricultural Chemicals Div., PO Box 4913, Kansas City, MO 64120) into 100 mL vol. flask. Pipet 5.0 mL internal std soln into flask, dil. to vol. with tetrahydrofuran, and mix well. Pipet 5.0 mL this soln into 100 mL vol. flask, dil. to vol. with mobile phase, and mix well. Filter portion of soln and hold for LC analysis.

6.A28 *Preparation of Sample*

Accurately weigh amt sample contg ca 250 mg aminocarb into 100 mL vol. flask. Pipet 5.0 mL internal std soln into flask, dil. to vol. with tetrahydrofuran, and shake 1 min. Pipet 5.0 mL this soln into 100 mL vol. flask, dil. to vol. with mobile phase, and mix well. Filter portion of soln and hold for LC analysis.

6.A29 *Determination*

Adjust operating parameters to cause aminocarb to elute in 2.6–3.1 min. Adjust injection size and attenuation to give largest possible on-scale peaks. Using same injection vol. for samples and stds, make repetitive injections of std soln and calc. response ratios by dividing peak ht of aminocarb by that of internal std peak. (Note: Peak area measurements are unacceptable.) Response ratios must agree within ± 1%. Average duplicate response ratios obtained with std solns.

Inject duplicate aliquots of each sample soln. Average duplicate response ratios for each sample soln. Response ratios must agree within ± 1%. If not, repeat detn, starting with std injections.

Re-inject std soln twice. Average response ratios of stds immediately preceding and following sample injections. These must agree within ± 1%. If not, repeat detn.

6.A30 *Calculation*

$$\text{Aminocarb, wt\%} = (R/R') \times (W'/W) \times P$$

where R and R' = av. response ratios for sample and std solns, resp.; W' and W = wt (mg) of aminocarb std and sample solns, resp.; and P = purity of aminocarb std (%).

Results and Discussion

A complete set of results was received from each of 10 collaborators (Table 1). Excellent agreement was obtained among collaborating laboratories, especially for the formulation, and no severe problems were encountered.

Collaborators used 5 brands of pumps, 5 brands of detectors, 6 types of columns, and 4 brands of injectors for the analyses. Sample volumes injected varied from 10 to 20 μL, pressures obtained varied from 5.2 to 22.4 MPa, and flow rates varied from 1.0 to 2.2 mL/min. Four collaborators determined peak heights with data systems, while 6 collaborators measured them manually.

This method was originally developed using methanol–water as the column solvent and was used successfully for several years. At the beginning of the collaborative study, however, it soon became apparent that the aminocarb retention time was quite variable depending on the column packing used. This fluctuation may have resulted from various pH values of silica used in producing these packings. Further research indicated that the addition of a phosphate buffer eliminated these retention time fluctuations, while simultaneously slightly decreasing peak tailing. The substitution of tetrahydrofuran in the mobile phase solvent resulted in complete dissolution of the formulation and produced better chromatographic peak shapes.

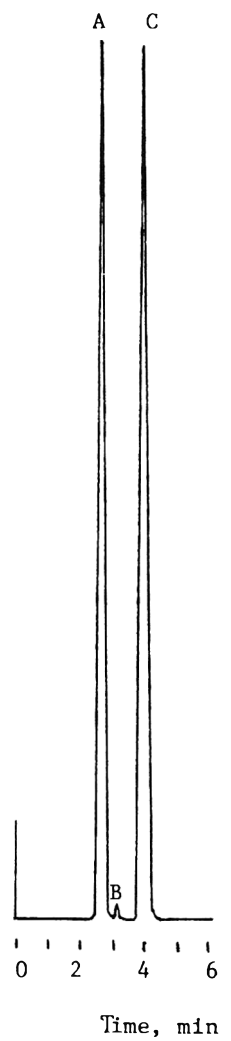

Figure 1. LC chromatogram of aminocarb (A), aminocarb phenol (B), and butyrophenone internal standard (C).

Although this method is amenable to peak area measurements, peak heights were chosen to eliminate difficulties in data processing of the aminocarb phenol impurity peak (B) shown in Figure 1. A difficult problem to address affecting reproducibility is the wide variety of data systems used by collaborators to interpret integration events (e.g., slope sensitivities, tangent skimming, forced baselines, vertical valley definition). One collaborator experienced problems in reproducibility resulting from the use of a data system for peak height measurement; however, a manual measurement of these peaks showed excellent reproducibility of injections. The incorporation of peak height measurements for this study eliminated the possibility of summing the aminocarb peak with its phenol, especially where resolution was less than desirable. This impurity is usually insignificant, but should be recognized as a major decomposition product in degraded materials.

Recommendation

It is recommended that the LC method be adopted as an official first action CIPAC-AOAC method.

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John E. Forrette, Velsicol Chemical Corp., Chicago, IL

W. Elwood Hodgins and James P. Minyard, Jr, Mississippi State Chemical Laboratory, Mississippi State, MS

S. Wendy King, Florida Dept of Agriculture, Tallahassee, FL

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Hurbert Tengler and H. Krüger, Bayer AG, Leverkusen, GFR

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Gas Chromatographic Determination of Metolachlor in Pesticide Formulations: Collaborative Study

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A gas chromatographic (GC) procedure for the determination of metolachlor in emulsifiable concentrate formulations containing about 76% active ingredient was collaboratively studied using the matched pair scheme. Metolachlor was extracted from the formulation with acetone containing dipentyl phthalate as the internal standard, chromatographed on OV-101, and detected by flame ionization. Determinations on the 4 samples by 21 government, university, and industrial collaborators using peak area measurements showed within-laboratory repeatability of better than 0.5%. Reproducibility was better than 1% for the formulation. The method has been adopted official first action.

Metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide] is a selective herbicide recommended as a preplant-incorporated or preemergence surface-applied treatment in water or fluid fertilizer for control of most annual grasses and certain broadleaf weeds in corn, cotton, peanuts, pod crops, potatoes, safflowers grain or forage sorghum, soybeans, and woody ornamentals. Metolachlor is the active ingredient in the product Dual® (registered trademark of Ciba-Geigy Corp., Agricultural Div., Ciba-Geigy Ltd, Canada, and Ciba-Geigy Ltd, Switzerland).

Collaborative Study

Two sets of matched pair samples of metolachlor, Dual® 8E and 6E formulations, together with standard, practice sample, internal standard, column packing, and detailed guidelines were sent to 24 collaborators in Europe and the United States. All collaborators were asked to pack and condition a column and to make at least one practice run for familiarization. The collaborators were asked to make a single GC determination for each sample from duplicate injections, and to report area integration and peak height measurements. The collaborators were also requested to submit the raw data and the chromatograms.

Metolachlor in Pesticide Formulations

Gas Chromatographic Method

First Action

AOAC–CIPAC Method

(Method is suitable for formulations where metolachlor is only active ingredient.)

6.A14 ***Principle***

Metolachlor is extd with acetone and detd by flame ionization gas chromatgy, using dipentyl phthalate as internal std. Identity is verified simultaneously by comparing retention times with std.

6.A15 ***Apparatus***

(a) *Gas chromatograph.*—Equipped with flame ionization detector.

(b) *Chromatographic column.*—2 mm id × 1.83 m (6 ft) glass column packed with 3% OV-101 on 80–100 mesh Gas-Chrom Q, or equiv. Condition column ≥24 h at 240°, using carrier gas at ca 20 mL/min. Operating conditions: injector 250°, detector 250°, column 180° ± 10°, He carrier gas flow ca 25 mL/min. Retention times for metolachlor and internal std are ca 8.8 and 15.6 min, resp.

6.A16 ***Reagents***

(a) *Internal std soln.*—4 mg/mL. Dissolve 4.0 g dipentyl phthalate in acetone and dil. to 1 L with acetone. Check internal std for interfering components by injecting aliquot into chromatograph.

(b) *Metolachlor std soln.*—Accurately weigh 200 mg metolachlor std of known purity (Ciba-Geigy Corp., Production Technical Dept, PO Box 18300, Greensboro, NC 27419) into 4 oz bottle. Pipet 50.0 mL internal std soln into bottle, cap, and shake 10 min.

6.A17 ***Preparation of Sample***

Accurately weigh amt sample contg ca 200 mg metolachlor into 4 oz bottle. Pipet 50.0 mL internal std soln into bottle, cap, and shake 10 min to ext.

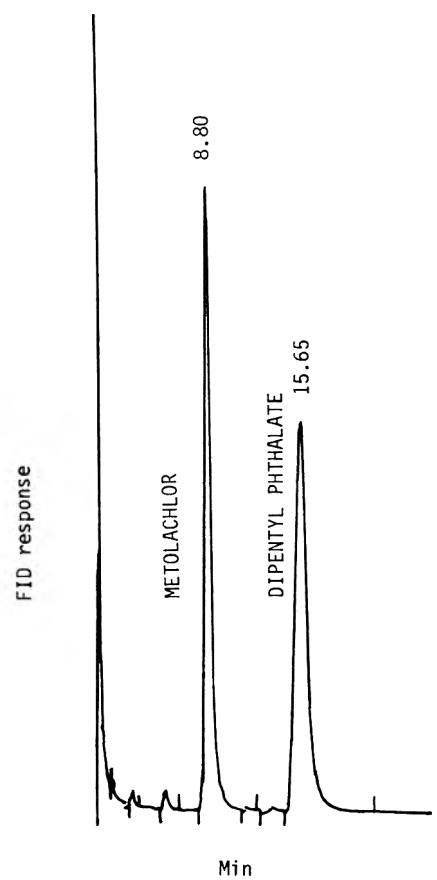

Figure 1. Typical chromatogram of metolachlor formulation.

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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 *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.

Table 1. Percent metolachlor calculated by comparison with internal standard area (closely matched pairs)

Calculation	Laboratory																					Theory
	1	2	3	4	5	8	11	12	13	14	17	18	19	20	22	23	25	26	27	28	29	
Metolachlor—SPL1	86.73	86.12	86.74	85.68	87.37	86.62	86.77	86.11	86.18	88.60	87.15	86.21	86.40	86.67	86.76	86.27	86.85	86.85	85.18	85.45	87.20	87.20
Metolachlor—SPL2	85.12	83.95	84.38	84.84	85.34	84.56	83.98	83.97	84.69	86.41	84.98	84.59	83.89	84.98	84.85	84.75	84.81	85.20	83.63	82.90	83.50	85.10
Differences, SPL1 − SPL2	1.61	2.17	2.36	0.84	2.03	2.06	2.79	2.14	1.49	2.19	2.17	1.62	2.51	1.69	1.91	1.52	2.04	1.65	1.55	2.55	3.70	2.10
Totals, SPL1 + SPL2	171.85	170.07	171.12	170.52	172.71	171.18	170.75	170.08	170.87	175.01	172.13	170.80	170.29	171.65	171.61	171.02	171.66	172.05	168.81	168.35	170.70	172.3
$(T_i - \bar{T})^2$	0.4900	1.1664	0.0009	0.3969	1.0404	0.0009	0.1600	1.1449	0.0784	14.8996	0.0004	0.1225	0.7396	0.2500	0.2116	0.0169	0.2601	0.8100	2.3400	7.8400	0.2025	—
Metolachlor—SPL3	69.69	66.77	69.60	69.54	70.16	69.50	69.80	68.75	68.87	68.71	69.68	69.18	68.87	69.05	69.38	69.16	69.56	70.11	69.11	68.97	69.53	69.30
Metolachlor—SPL4	70.53	67.63	70.01	70.41	70.96	70.44	70.70	69.33	70.10	69.87	70.89	70.46	69.97	70.10	70.51	70.21	70.40	71.05	69.85	69.75	70.57	70.30
Differences, SPL4 − SPL3	0.84	0.86	0.41	0.87	0.80	0.94	0.90	0.58	1.23	1.16	1.21	1.28	1.10	1.05	1.13	1.05	0.84	0.94	0.74	0.78	1.04	1.00
Totals, SPL3 + SPL4	140.22	134.40	139.61	139.95	141.12	139.94	140.50	138.08	138.97	138.58	140.57	139.64	138.84	139.15	139.89	139.37	139.96	141.16	139.06	138.72	140.10	139.6
$(T_i - \bar{T})^2$	0.8000	25.2004	0.0361	0.2809	2.8900	0.2704	1.1664	1.7956	0.2025	0.7056	1.3225	0.0484	0.3364	0.0729	0.2209	0.0025	0.2916	3.0276	0.1296	0.4900	0.4624	—

6.A18 ***Determination***

Set integration parameters and stabilize instrument by injecting 1–3 μL aliquots of std soln until area ratios of metolachlor to internal std vary <2% for successive injections. Using same established injection vol. as for std, inject sample. Sample area ratio should be ±10% of std area ratio. Inject 2 aliquots of std and 2 aliquots of sample followed by 2 aliquots of second sample and 2 aliquots of std. Repeat sequence until all samples are analyzed. Calc. response factor, R, for each injection:

$$R = \text{peak area (or ht) metolachlor/peak area (or ht) internal std}$$

$$\text{Metolachlor, \%} = (R/R') \times (W'/W) \times P$$

where R and R' = av. response factor for sample and std solns, resp.; W and W' = mg sample and std, resp.; and P = purity (%) of std.

Results

Twenty-two collaborators analyzed 4 samples. These included a matched pair of an 8E emulsifiable concentrate differing by 2.1% and a matched pair of a 6E emulsifiable concentrate differing by 1.0%. All of the participants performed the desired number of analyses. Twenty-one of the participants reported peak area data, and only 14 reported peak height data.

Results were reviewed, tabulated, and statistically analyzed (Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Arlington, VA, pp. 13–42). Outliers were detected using 99% confidence limits. Statistical analysis was performed under the guidance of Edwin Glocker, Committee A statistician. Peak area, peak height, and statistics are given in Tables 1, 2, and 3.

The differences in calculated means for area data between sample pairs are 2.03 and 0.94. These data compare well with the theoretical differences of 2.10 and 1.00 and provide validation for accuracy.

A wide variety of gas chromatographs, integrators, and data systems as well as both automated and hand injection were used. Helium and nitrogen carrier gases and columns of 2, 3, and 4 mm id and 1.2 m (4 ft) to 2 m (6.6 ft) length were used. Most collaborators performed analyses on a single day, but a few used 2 days. Minor modifications and variation of available instrumentation were expected. A typical chromatogram of the sample is presented in Figure 1.

Collaborator comments on the method were favorable. Some would like to shorten the 10 min extraction time and reduce active ingredient and internal standard concentration, and others noted that active ingredient/internal standard peak height response was not correlated.

Conclusion

As the results reveal, the analytical method tested is rugged and accurate and yields reproducible results. No collaborator had difficulty with the method. Peak height analysis is being replaced by modern integrators of peak area. This study shows essentially no difference between values and statistics between the 2 data forms.

Recommendation

The Associate Referee recommends that this gas chromatographic method for the determination of metolachlor in formulations be adopted official first action. The lowest concentration tested was 69.3% and the highest concentration tested was 87.2%. The preferred method of quantitation is peak area measurement.

Table 2. Percent metolachlor calculated by comparison with internal standard peak height (closely matched pairs)

Calculation	Laboratory														Theory
	1	2	8	11	12	14	16	17	19	20	22	23	25	29	
Metachlor—SPL1	86.58	84.86	86.45	86.47	86.39	87.81	86.86	85.80	85.68	85.95	86.48	86.27	86.06	86.55[a]	87.20
Metolachlor—SPL2	84.97	83.58	84.53	83.90	84.60	86.10	85.35	84.67	83.80	84.54	84.93	84.75	84.94	79.32	85.10
Differences, SPL1 − SFL2	1.61	1.28	1.92	2.57	1.79	1.71	1.51	1.13	1.88	1.41	1.55	1.52	1.12	7.23	2.10
Totals, SPL1 + SPL2	171.55	168.44	170.98	170.37	170.99	173.91	172.21	170.47	169.48	170.49	171.41	171.02	171.00	165.87	172.3
$(T_i - \bar{T})^2$	0.3600	6.3001	0.0009	0.3364	0.0016	8.7616	1.5876	0.2304	2.1609	0.2116	0.2116	0.0049	0.0025	—	—
Metolachlor—SPL3	69.34	72.96	69.06	69.70	69.10	69.58	69.64	69.22	69.02	68.81	69.10	69.16	70.23	69.02	69.30
Metolachlor—SPL4	70.16	74.82	70.20	70.16	70.17	70.65	70.33	70.71	69.71	69.52	70.49	70.21	71.12	70.06	70.30
Differences, SPL4 − SPL3	0.82	1.86	1.14	0.46	1.07	1.07	0.69	1.49	0.69	0.71	1.39	1.05	0.89	1.04	1.00
Totals, SPL3 + SPL4	139.50	147.78	139.26	139.86	139.27	140.23	139.97	139.93	138.73	138.33	139.59	139.37	141.35	139.08	139.6
$(T_i - \bar{T})^2$	0.4356	58.06	0.8100	0.0900	0.7921	0.0049	0.0361	0.0529	2.0449	3.3489	0.3249	0.6241	1.4161	1.1664	—

[a]Lab. 29 eliminated as an outlier.

Table 3. Statistical summary for metolachlor collaborative study

Sample	N	Mean	Statistic				
			$S_x(=S_d)$	$S_L(=S_b)$	$S_o(=S_r)$	CV_x	CV_o
Peak area:							
Pr 1, 2	42	85.6 (86.6,84.5)	0.746	0.620	0.414	0.87	0.48
Pr 3, 4	42	69.7 (69.2,70.2)	0.713	0.696	0.153	1.02	0.22
Peak height:							
Pr 1, 2	26	85.5 (86.3,84.7)	0.676	0.619	0.272	0.79	0.32
Pr 3, 4	28	70.1 (69.6,70.6)	1.168	1.139	0.261	1.65	0.37

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Gas Chromatographic Determination of Alachlor in Formulations: Collaborative Study

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An isothermal gas chromatographic method for measuring alachlor in formulations was tested by 26 collaborators. The samples were prepared in acetone and determined with a gas chromatographic column of 10% SP 2250 on 100–120 mesh Supelcoport and with di-*n*-pentyl phthalate as internal standard. Collaborators made single determinations on each of 2 similar materials (matched pairs) on 2 days for 3 pairs of samples. The study generated 44 matched pairs which were used in Youden's matched-pair calculations. The coefficients of variation (CV_x) for liquid formulations were 1.23% and 0.94%, while the CV_x for the granular formulation was 1.60%. The method was simple to use and did not reveal any interferences in the samples tested. The method has been adopted official first action.

Alachlor (2-chloro-2′-6′-diethyl-*N*-(methoxymethyl) acetanilide) is the active ingredient in Lasso®, a herbicide for weed control in corn, soybeans, peanuts, dry beans, grain sorghum (milo), lima beans, red kidney beans, potatoes, and other listed crops. It is used worldwide before planting as a surface-applied or shallowly incorporated herbicide to control yellow nutsedge, annual grasses, and broadleaf weeds (1).

Methods of analysis based on gas chromatography (GC) have been used by Monsanto Co. for over 15 years. This collaborative study evaluated a GC method developed by D. F. Tomkins and B. W. Mueller for alachlor formulated materials (2).

Collaborative Study

Three matched pairs of alachlor formulations (2 liquid and one granular), together with standards, practice samples, and detailed guidelines were sent to 28 collaborators in Europe and the United States. Collaborators were requested to pack a column, stabilize their equipment, and establish good resolution and quantitation during injections of practice sample solutions. The collaborators were asked to make a single determination of alachlor on each of 6 samples (3 pairs) on 2 days. The participants were also requested to provide the raw data and sample chromatograms along with the data report sheet. The method was tested for interferences by obtaining all known impurities over 0.1%. The method successfully separated all components and these were completely resolved from the alachlor and internal standard peaks at up to 10 times the expected levels.

Alachlor in Pesticide Formulations

Gas Chromatographic Method

First Action

(Method is suitable for formulated products, including emulsifiable concs and granulated formulations.)

6.A05 ***Principle***

Sample is dissolved in acetone contg di-*n*-pentyl phthalate as internal std, analyzed by gas chromatgy with flame ionization detector, and quantd by comparison with internal std.

6.A06 ***Apparatus***

(a) *Gas chromatograph.*—With flame ionization detector and on-column injection ports. Temps (°)—column oven 230, injection port 250, detector 260; gas flows (mL/min)—He carrier gas 35, H 30; air 250; sample size 1.0 μL; run time 15 min.

(b) *Column.*—6 ft × 2 mm (id) glass column (on-column configuration) packed with 10% SP-2250 on 100–200 mesh Supelcoport (Supelco, Inc., Cat. No. 1-2132), or equiv. SP-2250 is methyl–phenyl silicone (50 + 50). Precondition overnight at 250° before use. Retention times for alachlor and internal std are ca 5.5 and 11.2 min, resp.

6.A07 ***Reagents***

(a) *Acetone.*—Pesticide grade, Fisher, or equiv.

(b) *Di-n-pentyl phthalate internal std.*—CTC Organics, PO Box 6933, Atlanta, GA 30315. Weigh 5.3 g di-*n*-pentyl phthalate into 1 L vol. flask. Dissolve in acetone and dil. to vol. with acetone.

(c) *Alachlor.*—Recrystallized from MeOH (Monsanto Co., Muscatine, IA 52761). Accurately weigh 0.2 g alachlor into small flask. Add by pipet 30.0 mL internal std soln and shake to dissolve.

6.A08 ***Determination***

Accurately weigh sample contg ca 0.2 g alachlor into small flask. Add by pipet 30.0 mL internal std soln and shake well to ext alachlor. For granular formulation, mix ≥5 min on mech. shaker.

Make replicate 1 μL injections of alachlor std soln and measure response ratios, R (area alachlor peak/area internal std peak) for each injection. Repeat until consecutive response ratios R agree within 0.5%.

Make duplicate injections of sample soln and det. av. R. Follow with injection of alachlor std soln; det. av. R' for std before and after sample injection.

$$\% \text{ Alachlor} = (R/R') \times (W'/W) \times P$$

where R and R' = av. response ratios for sample and std, resp; W and W' = wt (g) of sample and std, resp.; P = % purity of std.

Results and Discussion

Results were reviewed, tabulated, and sent for statistical analysis to Edwin Glocker, Committee A statistician. Youden's matched-pair calculations with data from 26 laboratories with 44 matched pairs were used for evaluation and are presented in Table 1 (3). Using 99% confidence limits, results from Collaborators 11 and 17 were omitted as outliers. Also, results from Collaborator 9 were omitted on matched-pair 1. Matched-pair data from Collaborators 1 and 14, along with data from matched-pair 3 from Collaborator 21 were identified as outliers. The outliers are all marked in Table 1. Results from Collaborators 26 and 27 arrived after the statistical report was prepared; therefore, they are not included in Table 2. Collaborators 2, 5, 9, 11, 16, 19, and 27 used in aliquot of internal standard solution rather than direct weighing. Col-

This report of the Associate Referee was presented at the 98th Annual International Meeting of the AOAC, Oct. 28–Nov. 2, 1984, 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.* (1985) **68**, March issue.

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Table 1. Results from collaborative study of alachlor (%) by using an isothermal GC method

		Matched-Pair 1		Matched-Pair 2		Matched-Pair 3	
Coll.	Day	A	B	A	B	A	B
1[a]	1	45.4	43.6	45.3	43.7	15.4	15.4
	2	45.3	43.2	48.2*	43.0*	15.5	15.5
2	1	46.15	44.42	45.60	44.16	15.74	16.04
	2	45.50	43.68	45.73	43.37	15.52	15.98
3[b]	1	43.58	41.43	44.22	44.28	16.48	15.05
	2	44.00	42.83	44.67	44.67	15.60	15.96
4	1	45.2	42.7	45.2	43.8	15.7	15.8
	2	45.0	42.4	45.3	43.3	15.3	16.0
5	1	44.95	43.99	44.78	42.47	15.22	14.89
	2	45.05	44.30	45.07	43.08	15.49	15.52
6	1	45.29	43.69	45.57	43.33	15.51	15.72
	2	45.05	43.13	45.51	43.55	15.65	15.74
7	1	45.21	43.45	45.39	43.07	15.64	15.73
	2	45.75	43.04	45.80	43.26	15.71	15.85
8	1	45.34	43.28	45.39	43.34	15.46	15.38
	2	45.65	43.43	45.57	43.47	15.50	15.38
9	1	45.11*	45.46*	45.10	43.11	15.50	15.50
	2	45.23*	44.89*	45.23	43.09	15.54	15.41
10	1	45.41	42.81	46.02	42.84	15.43	15.35
	2	45.11	42.87	46.05	43.37	15.38	15.34
11	1	42.1*	42.5*	42.1*	42.5*	15.1*	15.1*
	2	43.1*	43.9*	44.9*	44.5*	15.6*	15.6*
12	1	44.79	43.05	45.10	43.47	15.70	15.80
	2	45.43	42.61	45.46	43.19	15.47	15.60
13	1	45.29	43.08	45.33	43.15	15.35	15.46
	2	45.21	43.03	45.24	43.01	15.47	15.45
14	1	45.67	44.08	46.65	44.40	15.99	15.63
	2	44.95	45.18	45.30	45.30	15.23	15.60
15	1	45.17	43.03	44.96	43.10	15.78	15.24
	2	45.07	43.37	44.95	43.28	15.62	15.98
16	1	44.97	43.01	45.21	42.97	15.37	15.34
	2	45.08	43.16	45.03	43.04	15.43	15.35
17[c]	1	50.12*	48.06*	49.70*	47.20*	16.92*	16.32*
	2	48.88*	46.40*	48.77*	46.29*	16.63*	16.65*
18	1	45.2	43.7	45.7	43.4	15.5	15.5
	2	44.6	42.8	45.1	42.9	15.5	15.6
19	1	44.88	43.87	45.4	43.26	15.6	15.52
	2	45.60	43.01	44.93	43.59	15.74	15.81
20[d]	1	45.01	43.19	45.20	43.01	15.36	15.49
	2	45.02	43.01	45.04	42.99	15.34	15.52
21[e]	1	45.58	42.08	44.93	42.99	16.99	15.71
	2	45.64	43.33	45.92	43.95	17.66	16.01
22[f]	1	44.80	42.80	44.74	42.71	15.19	15.52
	2	45.34	43.13	45.70	43.26	15.55	15.54
23[g]	1	45.00	42.98	44.98	43.79	15.56	15.24
	2	44.73	43.80	45.54	43.62	15.75	15.27
24[h]	1	45.11	42.78	44.70	42.66	15.34	14.87
	2	44.97	43.02	—	—	15.50	15.49
25[i]	1	44.62	43.51	45.52	42.86	15.91	15.56
	2	45.41	43.78	45.67	43.33	15.49	15.27
26[j]	1	45.47	43.32	45.36	43.19	15.59	15.68
	2	44.71	42.74	44.74	42.70	15.45	15.33
27[j]	1	44.98	43.40	45.88	43.64	15.48	15.42
	2	45.15	43.29	45.62	43.21	15.20	15.33

*Results were identified as outliers.
[a]Used N_2 as carrier gas.
[b]Injection port temp. 280°C.
[c]Used same standard solution both days.
[d]20 mL/min carrier flow and 240°C oven temp.
[e]Used 2 μL injection.
[f]Oven temp. 240°C.
[g]Used OV-17 column.
[h]Omitted 2A, 2B because ran out of internal standard.
[i]Used 1.4 m × 3 mm id column.
[j]Results arrived too late to be included in statistical summary.

Table 2. Statistical summary of collaborative results for GC analysis of alachlor in Lasso

Statistic	Matched-Pair 1	2	3
No. of pairs	44	41	44
Mean, %	44.19	44.29	15.54
S_o	0.447	0.418	0.237
S_L	0.307	—[a]	0.076
S_x	0.542	0.418	0.249
CV_x, %	1.23	0.94	1.60

[a]See *AOAC Statistical Manual*, p. 24, "Sampling phenomenon" (3).

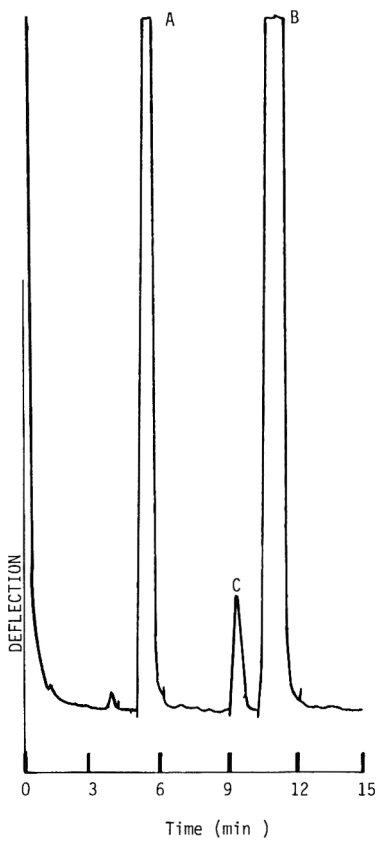

Figure 1. GC chromatogram of alachlor standard (A), internal standard dipentyl phthalate (B), and unknown from internal standard (C).

laborators 2, 12, and 21 had difficulty obtaining 0.5% consecutive response factors for standard solutions. Other comments are included in Table 1.

Although a few results have been rejected as outliers, a more than adequate number of collaborators achieved satisfactory performance from the method to recommend it for official first action. The statistics are given in Table 2. The results gave a S_x: 44% liquid = 0.542, 15% granular = 0.249 and S_L: 44% liquid = 0.307, 15% granular = 0.076. Standard deviation among labs (S_L) (laboratory bias) has a negative value for matched-pair 2. Statistically, this means that the laboratories when taken as a group had statistically less variation than the individual laboratories. This could indicate that the variations result more from instrumental variation than from analyst or method variations. The coefficients of variation for liquid formulations were somewhat better than those for granular formulations. The increase in the coefficient of variation is believed to result from nonhomogeneity of the samples and from variations in extraction efficiency from the sample matrix. Figure 1 is a typical chromatogram of the standard solution.

Recommendation

It is recommended that the gas chromatographic method for determination of alachlor in Lasso formulations be adopted official first action, as a CIPAC-AOAC method.

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Gas Chromatographic Method for Determination of Fenitrothion in Technical and in Emulsifiable Concentrate and Water-Dispersible Powder Formulations: Collaborative Study

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A variety of column packings and internal standards were evaluated to determine the most satisfactory system to use in a gas chromatograhpic (GC) method for analysis of fenitrothion, technical and formulations. Fenitrothion and the most closely related isomer, *O,O*-dimethyl *O*-(4-methyl-3-nitrophenyl) phosphorothioate, were resolved on columns packed with OV-210 and with polyphenyl ether, 6-ring (PPE-6R). A method based on the separation of fenitrothion on a PPE-6R column with fluoranthene as internal standard was selected for use in a limited collaborative trial and later for use in a full-scale collaborative trial with 21 collaborators participating. Each collaborator was furnished matched pairs of samples of technical fenitrothion, emulsifiable concentrate, and water-dispersible powder. The coefficients of variation (CV) for the paired samples were 1.02, 1.11, and 1.01, respectively, for technical fenitrothion, emulsifiable concentrates, and water-dispersible powders. Data are also presented for an alternative method in which compounds are separated on an OV-210 column with dibutyl sebacate as the internal standard. The method has been adopted official first action.

Specifications for technical fenitrothion and its water-dispersible powder formulations were first included in the World Health Organization (WHO) manual of specifications in 1973 (1). The active ingredient was analyzed by a titrimetric method based on reducing the fenitrothion with zinc in acid solution, and titrating the resulting amine with sodium nitrite. This method is nonspecific and time-consuming. We presented a paper (2) on the analysis of fenitrothion by gas chromatography (GC) at the 1977 meeting of the WHO Expert Committee on Chemistry and Specifications of Pesticides. The method was a modification of that of Takimoto et al. (3), in which separation was made on a QF-1 column with dibutyl sebacate as internal standard. Although the data showed that excellent precision could be obtained by the method, the committee decided to defer adoption of the method until it could be collaboratively tested (4). In 1978, the U.S. Agency for International Development (AID) published specifications for technical fenitrothion and water-dispersible powder, which used this GC method. Subsequently, WHO published interim specifications for technical fenitrothion, water-dispersible powder, and emulsion concentrate, which also used this GC method (5).

In 1982, the Centers for Disease Control (CDC), Atlanta, GA, and the Sumitomo Chemical Co., Osaka, Japan, undertook independent studies to find the most satisfactory GC method for analyzing fenitrothion. Sumitomo recommended using polyphenyl ether, 6-ring, (PPE-6R) as the stationary phase and fluoranthene internal standard for the analysis. We evaluated this system and 8 other GC systems for separating and quantifying fenitrothion (Table 1). The impurities most difficult to separate from fenitrothion are 3 isomers of fenitrothion: *O,O*-dimethyl *O*-(3-methyl-2-nitrophenyl) phosphorothioate; *O,O*-dimethyl *O*-(2-methyl-5-nitrophenyl) phosphorothioate; and *O,O*-dimethyl *O*-(4-methyl-3-nitrophenyl) phosphorothioate.

Each of these isomers was synthesized by refluxing the corresponding phenol with *O,O*-dimethyl phosphorothiochloridate in methyl ethyl ketone in the presence of potassium carbonate. They were added to fenitrothion and chromatographed on various columns to determine if resolution was achieved.

Of the systems tested, the last 3 shown in Table 1 proved to be satisfactory. The method with PPE-6R as the GC stationary phase and bis(2-methoxyethyl) phthalate as internal standard has the advantage of the shortest run time; however, this system was abandoned when a late-emerging peak was discovered in bis(2-methoxyethyl) phthalate. Chromatograms demonstrated the resolution of fenitrothion from impurities and the selected internal standards (Figure 1). In each case, the small peak immediately preceding the fenitro-

Table 1. Results obtained by using various columns for GC analysis of fenitrothion formulations

Column	Int. std	Results
4% OV-101	—	Inadequate resolution from isomers
5% OV-225	—	Inadequate resolution between fenitrothion and late-emerging peaks
5% OV-275	—	Polarity too high; fenitrothion did not emerge
7.5% XE-60	—	Inadequate resolution from isomers
3.0% XF-1150 (4 ft. column)	—	Inadequate resolution between fenitrothion and late-emerging peaks
3.0% EGSS-X	dipropyl phthalate	Reproducibility of relative factors a function of time column held at elevated temperature after analysis
7.5% OV-210	2-naphthyl benzoate	Resolution good; precision good; run time, 35 min
3.0% PPE-6R*	fluoranthene	Resolut on good; precision good; run time, 35 min
3.0% PPE-6R	bis(2-methoxyethyl) phthalate	Resolution good; precision good; run time, 25 min

*Method recommended by Sumitomo Chemical Co.

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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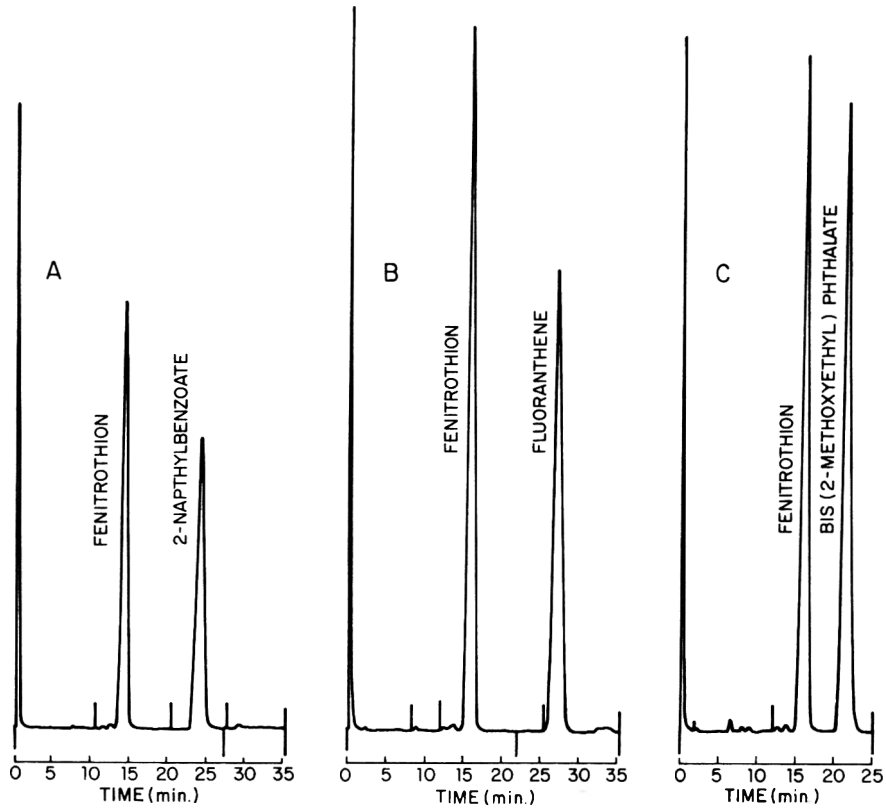

Figure 1. Chromatograms of technical fenitrothion obtained on OV-210 (A) and PPE-6R (B and C) columns.

Table 2. Collaborative results for GC analysis of fenitrothion (%) in matched pairs of technical fenitrothion samples

	Technical			
Coll.	A	B	Diff.	Total
1	93.72	95.22	−1.50	188.94
2	94.18	95.36	−1.18	189.54
3	94.63	95.64	−1.01	190.27
4	94.7	94.8	−0.1	189.5
5	95.1	95.1	0.0	190.2
6	94.67	95.55	−0.88	190.22
7	95.92	96.22	−0.30	192.14
8	94.73	96.12	−1.39	190.85
9	94.35	95.19	−0.84	189.54
10	95.58	98.18	−2.60	193.76
11	95.02	96.29	−1.27	191.31
12	94.56	95.90	−1.34	190.46
13	94.26	95.06	−0.80	189.32
14	92.42	93.26	−0.84	185.68
15	95.1	95.2	−0.1	190.30
16	95.08	97.04	−1.96	192.12
17	93.7	94.7	−1.0	188.4
18	94.74	95.83	−1.09	190.57
19	94.78	94.47	+0.31	189.25
20	94.52	95.37	−0.85	189.89
21	92.60	93.14	−0.54	185.74
Av.	94.49	95.41	−0.92	189.90
S_r			0.48	
S_b			0.86	
S_d			0.97	
CV_d	94.49	95.41	1.02	

thion peak is *O,O*-dimethyl *O*-(4-methyl-3-nitrophenyl) phosphorothioate, the most difficult isomer to resolve.

After carefully considering the advantages and disadvantages of all of the systems tested, we initiated a mini-collaborative trial of a method using a GC column packed with PPE-6R on Chromosorb W-HP, 100–120 mesh, with fluoranthene as internal standard. Three laboratories participated in the study, in which samples of technical fenitrothion, water-dispersible powders, and emulsifiable concentrates were analyzed. Excellent results were obtained in the mini-collaborative trial, and the method was then subjected to a large, international collaborative trial.

Collaborative Study

To reduce the workload for the collaborators and to reduce bias, we used the method of measurement of precision without duplicates, suggested by Youden (6), for the large collaborative trial. Each participant was furnished 3 pairs of samples. Members of each pair were similar, but not identical. These included 2 samples of technical fenitrothion from different sources; 2 samples of water-dispersible powder from different sources; and 2 samples of emulsifiable concentrate from the same source, but in different formulations. Participants were requested to weigh each sample only once and make only one injection of each. A sample of technical grade material from a third source was furnished to allow the participants to practice with the method before running the unknown samples. Participants were also furnished a supply of column packing, internal standard, and fenitrothion primary standard.

Fenitrothion Technical and Pesticide Formulations
Gas Chromatographic Method
First Action

6.A19 ***Principle***

Samples of fenitrothion tech. and formulations are dissolved in $CHCl_3$ with fluoranthene added as internal std. Fenitrothion content is detd by gas chromatgy with flame ionization detection.

6.A20 ***Apparatus and Reagents***

(a) *Gas chromatograph.*—Suitable for on-column injection and equipped with flame ionization detector.

Table 3. Collaborative results for GC analysis of fenitrothion (%) in matched pairs of emulsifiable concentrate (EC) fenitrothion samples

	EC			
Coll.	A	B	Diff.	Total
1	50.22	51.08	−0.86	101.30
2	50.62	51.31	−0.49	102.13
3	50.72	50.71	+0.01	101.43
4	50.7	50.3	+0.4	101.0
5	50.6	50.3	+0.3	100.9
6	50.95	50.69	+0.26	101.64
7	51.46	51.28	+0.18	102.74
8	50.41	50.82	−0.41	101.23
9	49.60	50.92	−1.32	100.52
10	50.92	50.09	+0.83	101.01
11	51.29	50.81	+0.48	102.10
12	51.76	51.09	+0.67	102.85
13	50.64	50.81	−0.17	101.45
14	52.06	51.21	+0.85	103.27
15	50.5	50.1	+0.4	100.6
16	51.00	51.12	−0.12	102.12
17	50.5	49.7	+0.8	100.2
18	51.10	51.58	−0.48	102.68
19	50.56	50.89	−0.31	101.47
20	50.83	50.84	−0.01	101.67
21	52.16	51.95	+0.21	104.11
Av.	50.90	50.84	+0.06	101.73
S_r			0.40	
S_b			0.40	
S_d			0.57	
CV_d			1.11	

Table 4. Collaborative results for GC analysis of fenitrothion (%) in matched pairs of water-dispersible powder (WDP) fenitrothion samples

	WDP			
Coll.	A	B	Diff.	Total
1	39.33	40.84	−1.51	80.17
2	39.38	39.66	−0.28	79.04
3	38.94	40.29	−1.35	79.23
4	38.6	40.1	−1.5	78.7
5	38.7	39.9	−1.2	78.8
6	39.20	40.31	−1.11	79.51
7	39.34	40.28	−0.94	79.62
8	38.98	39.28	−0.30	78.26
9	38.16	39.77	−1.61	77.93
10	38.58	39.72	−1.14	78.30
11	39.13	39.92	−0.79	79.05
12	39.47	40.58	−1.11	80.05
13	38.65	39.91	−1.26	78.56
14	39.68	40.74	−1.06	80.42
15	39.9	40.2	−0.3	80.1
16	39.16	40.19	−1.03	79.35
17	38.9	39.8	−0.9	78.7
18	39.58	40.45	−0.87	80.03
19	38.77	39.94	−1.17	78.71
20	38.92	40.09	−1.17	79.01
21	38.91	39.86	−0.95	78.77
Av.	39.06	40.09	−1.03	79.15
S_r			0.26	
S_b			0.30	
S_d			0.40	
CV_d			1.01	

(b) *Gas chromatographic column.*—2 mm id × 1.83 m glass column packed with 3.0% PPE-6R (polyphenylether, Alltech Associates, Inc., 2051 Waukegan Rd, Deerfield, IL 60015) on 100–120 mesh Chromosorb W-HP. Operating conditions: temps: injector, 200°; detector, 250°; column, 195°; N carrier gas flow ca 30 mL/min.

Approximate retention times for fenitrothion and internal std are 16 and 26 min, resp.

(c) *Internal std soln.*—Accurately weigh ca 1.5 g fluoranthene into 500 mL vol. flask, dil. to vol. with $CHCl_3$, and mix.

(d) *Fenitrothion std soln.*—Accurately weigh amt of std fenitrothion (Sumitomo Chemical Co., Ltd, Osaka, Japan) contg ca 200 mg active ingredient into 50 mL screw-cap bottle. Add by pipet 25.0 mL internal std soln and mix to dissolve fenitrothion.

6.A21 *Preparation of Chromatographic Column*

Clean glass column by passing H_2SO_4 thru column, and rinse with H_2O. Draw ca 50 mL acetone thru column followed by 50 mL MeOH. Pass N thru column until it is dry. Treat column with 5% soln of dichlorodimethylsilane in toluene; rinse with toluene followed by MeOH. Pass N thru column to dry.

Attach 7.6 cm funnel to exit end of column. While tapping column with glass rod fitted with short length of heavy rubber tubing, add prepd packing in small quant. until exit end of column is filled to ca 0.5 cm from end of tube. Move funnel to entrance of column. Insert pledget of silane-treated glass wool in exit end of column, and attach a source of moderate vac. to exit end. Continue to add packing slowly with vigorous tapping until tube is filled to ca 0.5 cm from entrance end. Insert pledget of glass wool in entrance end; compress glass wool only enough to hold packing in place.

Condition column overnight at 230°. This step should be conducted with exit end of column disconnected from detector but with carrier gas flowing at recommended rate.

6.A22 *Preparation of Standard and Sample Solutions*

Accurately weigh samples of fenitrothion tech., emulsifiable conc., and H_2O-dispersible powder, each contg ca 200 mg active ingredient, into sep. 50 mL screw-cap bottles. To each bottle add by pipet 25.0 mL internal std soln and shake 30 s. Filter or centrf. H_2O-dispersible powder to remove particulates.

6.A23 *Determination*

Inject 2 μL portions of std soln until response ratios (area or peak ht) of fenitrothion to internal std agree ±2%. Make duplicate injections of std soln, followed by duplicate injections of sample solns (see Note 1). Recalibrate after not more than 4 injections of sample solns. (Note 1: To avoid interference from late-emerging impurity (retention time, ca 45 min), subsequent samples must be injected not earlier than 7 min after elution of internal std. Thus, total analysis time for each sample is ca 35 min.)

6.A24 *Calculation*

For each injection, response ratio (R) = area (or ht) of fenitrothion peak/area (or ht) of internal std peak.

$$\text{Fenitrothion, wt \%} = (R/R') \times (W'/W) \times P$$

where R' and R = av. response ratio for std and sample solns, resp.; W' and W = wt (mg) of fenitrothion std and sample, resp.; and P = purity (%) of fenitrothion std.

Results and Discussion

Data from the 21 participating collaborators are presented in Tables 2–4. Sixteen of the collaborators followed the prescribed procedure without deviation. Four collaborators used columns shorter than the prescribed 1.83 m. These ranged in length from 0.91 to 1.6 m. A study of the chromatograms showed that all the isomers of fenitrothion were resolved on each of these columns. Three collaborators experienced difficulty in pipetting chloroform; 2 suggested that the internal standard be prepared in a more dilute solution and that 25 mL of this solution be added to the sample. Four collaborators believed that the long retention time of the internal standard was a disadvantage. One operator increased the injection block temperature to 220°C, and another to 250°C.

The precision achieved with all 3 pairs of samples was excellent, as demonstrated by the statistical data (Tables 2–4). None of the values reported could be rejected as outliers according to the Dixon test (7). The standard deviation, S_b,

Table 5. Comparison of data on samples of technical fenitrothion obtained on OV-210 column with those on PPE-6R column[a]

	Fenitrothion, %					
	3% PPE-6R			7.5% OV-210		
Sample	Run 1	Run 2	Av.	Run 1	Run 2	Av.
A	94.6	95.0	94.8	94.6	94.6	94.6
B	93.5	93.9	93.7	93.5	93.7	93.6
C	94.0	94.6	94.3	94.1	94.5	94.3
D	94.2	94.6	94.4	94.3	94.5	94.4
E	92.9	93.5	93.2	93.0	93.4	93.2
F	93.6	94.2	93.9	93.3	93.9	93.6

[a]GC parameters furnished with above data from Sumitomo Chemical Co.:

	PPE-6R	*OV-210*
Column	Glass, 3 mm id, 1 m; 3% PPE-6R on Chromosorb W (AW, DMCS, 60–80 mesh)	Glass, 3 mm id, 1.5 m; 7.5% OV-210 on Chromosorb W (AW, DMCS, 100–120 mesh)
Temperatures; °C		
Column	180	170
Injection port	220	200
Detector	220	200
Carrier gas		
N_2, mL/min	50	50
Retention time, min		
Fenitrothion	16	20
Fluoranthene	27	—
Dibutyl sebacate	—	25

the distribution of systematic error, was calculated from the equation $(S_d)^2 = 2(S_b)^2 + (S_r)^2$, where S_d, the total standard deviation, was calculated from the sum of the values of the paired samples and S_r, the random standard deviation, was calculated from the differences (6). The coefficients of variation for the paired samples (21 collaborators) were 1.02, 1.11, and 1.01, respectively, for technical fenitrothion, emulsifiable concentrate, and water-dispersible powder samples.

Alternative Procedure

Although excellent results were obtained in the collaborative study, the operational guidelines suggested by AOAC (8) had not been strictly followed; the liquid phase used in the PPE-6R column packing is not one of those recommended by AOAC. The Sumitomo Co., therefore, undertook an experiment to compare data obtained with a PPE-6R column with those obtained using one of the recommended columns, OV-210 (Table 5). Different chromatographic conditions are required when the alternative column packing is used. The concentration of the internal standard, dibutyl sebacate, is twice that required for fluoranthene.

Recommendations

We recommend that the GC analytical method described for fenitrothion in technical, emulsifiable concentrate, and water-dispersible powder formulations be adopted official first action. We also recommend that columns packed with 7.5% OV-210 be allowed as alternatives to those packed with 3% PPE-6R, provided that dibutyl sebacate is used as internal standard.

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Simultaneous Liquid Chromatographic Determination of Rotenone and Pyrethrins in Formulations

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This paper describes a reverse phase liquid chromatographic (LC) method to simultaneously determine rotenone and pyrethrins in pesticide formulations. The mixed standards along with the formulations were accurately weighed to contain approximately 200 μg rotenone and 150 μg pyrethrins/mL. Stabilized tetrahydrofuran was used to dissolve all substances. An aliquot was injected into the LC system equipped with a Zorbax ODS column, and chromatographed with a mobile phase of acetonitrile–water (70 + 30). Rotenone and the pyrethrins were monitored at 240 nm and 0.4 AUFS. Retention times for rotenone, pyrethrin II, and pyrethrin I were approximately 7, 11.5, and 25.5 min, respectively. For 3 different formulations analyzed 6 times each, the percent coefficients of variation were all < 3. This method is also applicable to products containing either rotenone or pyrethrins. No significant interferences were observed from the inactive ingredients of the formulations at the concentrations added.

Rotenone and pyrethrins are naturally occurring insecticides. Rotenone is found in many leguminous species of the genera *Derris, Lonchocarpus, Amorpha,* and *Tephrosia* (1), while pyrethrins are obtained from the plant *Chrysanthemum cinerariaefolium* (2).

Analysis of pesticide formulations containing either rotenone or pyrethrins is quite difficult because of the number of compounds involved. For example, the technical rotenone added to formulations contains at least 8 similar co-extractants or rotenoids while the pyrethrins comprise 6 constituents.

Methods thus far have been useful only for determining either rotenone or pyrethrins, but not both in the same formulation. For rotenone, the most successful procedures have used liquid chromatography (LC) (1) while gas chromatographic (3) and LC (2, 4) methods have been used with success for pyrethrins.

This paper describes a reverse phase LC method that can simultaneously, rapidly, and accurately determine rotenone and pyrethrins in pesticide formulations. Total analysis time is approximately 28 min.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Waters Associates (Milford, MA) 6000A pump and U6K septumless injector, Schoeffel variable wavelength UV detector (Westwood, NJ), and Omni-Scribe recorder (Houston Instrument, Austin, TX). Operating conditions: injection volume, 5 μL; flow rate, 1.0 mL/min; wavelength, 240 nm; absorbance range, 0.4 AUFS; recorder setting, 10 mV; chart speed, 1.0 cm/min.

(b) *Chromatographic column.*—25 cm × 4.6 mm id stainless steel column containing Zorbax ODS (HPLC Technology Ltd, Palos Verdes Estates, CA).

(c) *Mobile phase.*—Acetonitrile–water (70 + 30). All solvents LC grade (Fisher Scientific, Fair Lawn, NJ).

(d) *Sample extraction solvent.*—Stabilized certified tetrahydrofuran (THF) (Fisher Scientific).

(e) *Standard solutions.—Stock rotenone standard solution.*—Weigh 25 mg 97.3% rotenone standard (Environmental Protection Agency, Research Triangle Park, NC) into 25 mL actinic volumetric flask and dilute to volume with THF. *Stock pyrethrin standard solution.*—Weigh 96 mg 20% technical pyrethrin (EPA) into actinic 10 mL volumetric flask and dilute to volume with certified THF. *Mixed standard solution.*—Take 5 mL rotenone standard solution along with 2 mL pyrethrin standard solution and place in 25 mL actinic volumetric flask. Dilute to volume with THF. Actinic glassware is necessary because rotenone and pyrethrins degrade readily in most types of light.

Preparation of Sample

(a) *Liquid.*—Weigh sample equivalent to 200 μg rotenone and 150 μg pyrethrins/mL into 25 mL actinic volumetric flask. Dissolve sample in THF. Inject 5 μL into LC system.

(b) *Solids.*—Weigh sample equivalent to 200 μg rotenone/mL into 50 mL actinic Erlenmeyer flask. Add 25.0 mL THF and sonicate 5 min. Inject 5 μL aliquot.

Determination

Inject standard, followed by 2 injections of sample, and finally another standard. Measure peak heights, average, and substitute into formula below:

$$\% \text{ Rotenone or } \% \text{ pyrethrins} = (H/H') \times (W'/W) \times \% \text{ purity std}$$

where H and H' = average peak heights of sample and standard, respectively; W' = g rotenone or pyrethrin standard/25 mL; and W = g sample extracted. The % pyrethrins is calculated on the basis of one of the major peaks (pyrethrin I) of the pyrethrin extract.

Results and Discussion

Three different liquid formulations containing rotenone (1% level) and pyrethrins (0.8% level) were analyzed. The analysis

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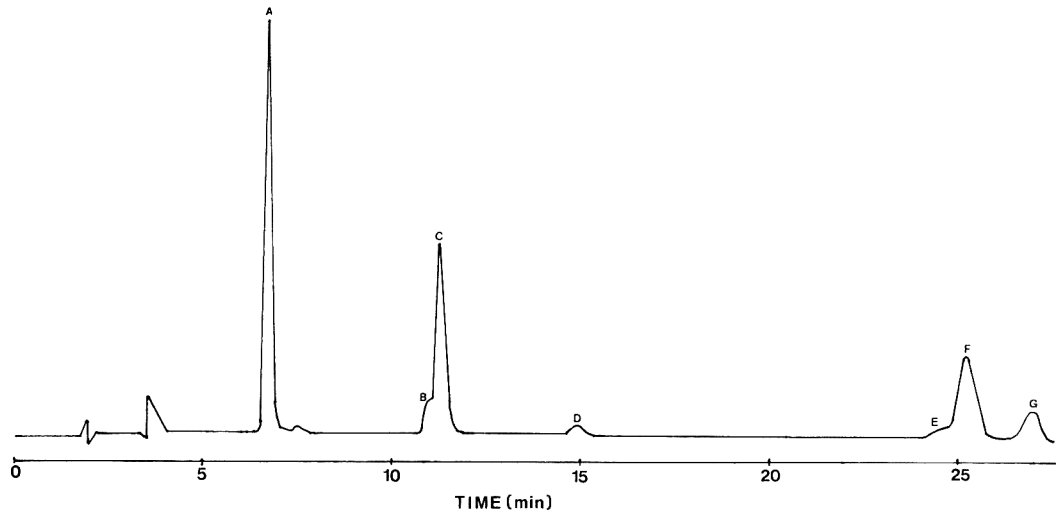

Figure 1. Liquid chromatogram of 5 μL mixed standard: A, rotenone; B, unknown pyrethrin; C, pyrethrin II; D, unknown pyrethrin; E, unknown pyrethrin; F, pyrethrin I; G, BHT.

is more difficult with liquids because of the emulsifiers and solvents used in the formulation. This is demonstrated by chromatograms of a mixed standard (Figure 1) and a formulation (Figure 2). As can be seen, numerous constituents present in the formulation do not occur in the standard. These nonpesticide components along with the numerous rotenoids and pyrethrins present increased the difficulty of developing a simultaneous analysis. However, at 240 nm and with a Zorbax ODS column and acetonitrile–water (70 + 30) mobile phase, these pesticides could be quantitated. Rotenone eluted in approximately 7 min while pyrethrins II and I eluted at approximately 11.5 and 25.5 min, respectively.

Of the 6 active components of pyrethrins (jasmolins I and II, cinerins I and II, pyrethrins I and II), only pyrethrin I was used for measuring the pyrethrins because no interferences were present except for a slight shoulder at the beginning from one of the other minor pyrethrins. Pyrethrin II, the other major pyrethrin, was partially separated from the other material in the formulations. One might be able to use peak height measurement of pyrethrin II for calculating the concentration of pyrethrins, but further study is needed to validate this.

There are no analytical standards available for individual pyrethrins, so quantitation must be based on a technical mixture, the purity of which must be accurately known. Furthermore, concentrations of the 6 active constituents of pyrethrin can vary in mixtures from different sources. However, preliminary results for pyrethrin from different sources indicate that pyrethrin I may not vary significantly. In fact, in this study, concentrations of pyrethrin I in the EPA and Penick 20% material were identical. Thus, if pyrethrin I cannot be isolated for quantitation, then the technical standard should come from the technical material used in the formulation.

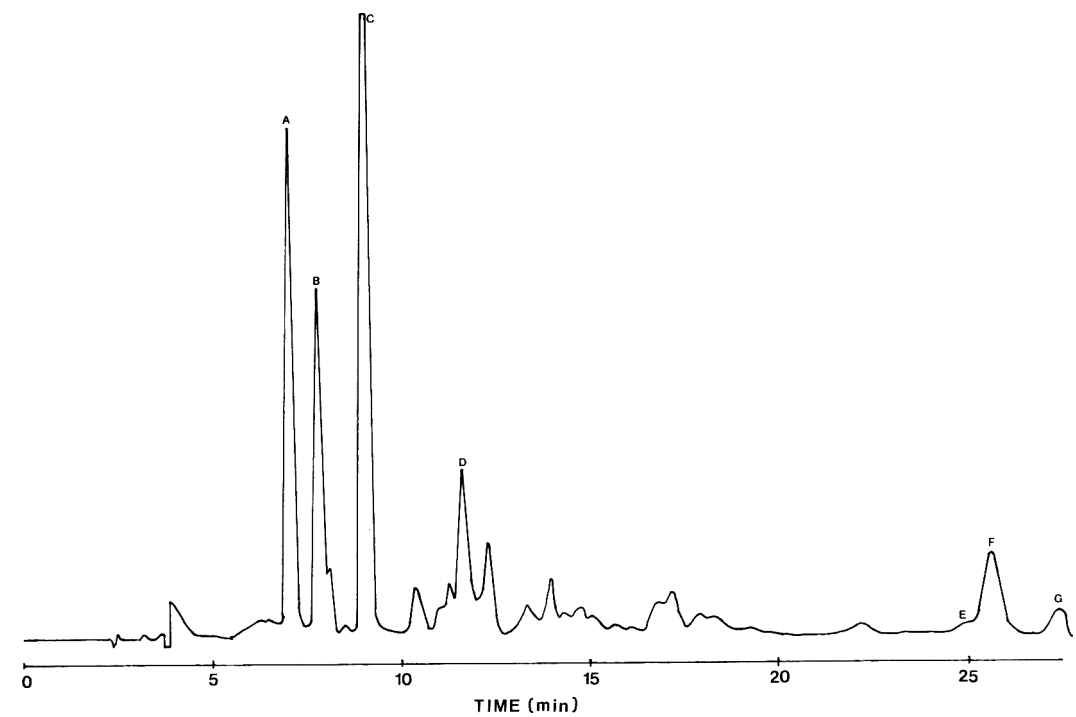

Figure 2. Liquid chromatogram of 5 μL commercial sample: A, rotenone; B, deguelin; C, aromatic solvent in formulation; D, pyrethrin II; E, unknown pyrethrin; F, pyrethrin I; G, BHT.

Table 1. Analysis of liquid formulations containing rotenone and pyrethrins[a]

	Rotenone, %			Pyrethrins, %		
Sample	Form.	Found	CV, %	Form.	Found	CV, %
1	1.00	1.29	1.99	0.80	0.75	2.49
2	1.00	1.28	1.43	0.80	0.77	2.38
3	1.00	1.26	1.49	0.80	0.80	2.58

[a]Means of 6 determinat ons for each sample.

Table 2. Analysis of formulations containing rotenone or pyrethrins[a]

	Rotenone, %			Pyrethrins %		
Sample	Form.	Found	CV, %	Form.	Found	CV, %
Powdered conc.	36.76	43.45	2.43	—	—	—
Powdered conc.	42.90	36.54	2.56	—	—	—
Liquid	12.00	9.67	3.24	—	—	—
Dust	5.00	4.61	2.58	—	—	—
Aerosol	—	—	—	0.20	0.22	4.09

[a]Means of 6 determinations for each sample.

Identity of the major pyrethrins (pyrethrins I and II) was confirmed by isolating pyrethrin I and pyrethrin II on the normal phase chromatographic system of Mourot et al. (2). These isolated pyrethrins were reinjected into the reverse phase system developed here. Our preliminary identification held to be true.

To demonstrate the accuracy and reproducibility of this LC method, 3 formulations were analyzed 6 times each (Table 1). The percent coefficients of variation for rotenone were all < 2, and for pyrethrins all < 3. The analyses were performed over a period of 6 days with one analysis per sample per day. Also, it can be seen from Table 1, that the rotenone concentration for these products was greater than the guarantee while the pyrethrins were all at or near the guarantee. These higher rotenone values are not unusual because many companies are still using older methods for analyzing the technical material.

The linearity of response for both rotenone and pyrethrin I was determined at 240 nm. The concentration of rotenone and pyrethrins could be determined by direct comparison of peak height for a sample and standard because both were linear over a wide concentration range. In fact if the sample concentration was higher or lower than the standard peak a direct comparison still could be made.

This method does not specify an internal standard, but BHT could be used as the internal standard if desired. For example, THF stabilized with BHT was used as the standard and sample solvent systems because both pesticides are highly soluble in THF, and BHT protects the pesticides from peroxide build-up. BHT elutes from the column immediately following but completely resolved from pyrethrin I. Therefore, one could use unstabilized THF and add a known amount of pure BHT.

Piperonyl butoxide (PBO) is frequently formulated with pyrethrins. It was chromatographed under these LC conditions, and eluted from the column between pyrethrins II and I. Although PBO does not interfere with the analysis, it cannot be quantitated in this system because one of the nonpesticide ingredients in these formulations elutes near the same time. It may be possible to analyze other formulations for PBO if they contain different nonpesticide constituents.

To demonstrate the applicability of this method to products that contain either rotenone or pyrethrins, a number of these were analyzed (Table 2). Four of the 5 were rotenone products; 3 were found to contain less rotenone than the guarantee. Again with some companies still using the older methods to analyze their formulations, any answer is possible. The reproducibility however was excellent.

To further test this method for rotenone analyses, we analyzed 2 additional complex formulations that were also analyzed by previously reported LC methods (1, 5). One sample was a 0.2% dust containing naphthalene and sulfur while the other was a 5% liquid formulated with numerous petroleum distillates. Although each of these 2 samples was analyzed only once by each method, there was good agreement among all methods.

Two formulations containing pyrethrins were also tested. The results of one, a low level pyrethrin, are given in Table 2; the amount found was only slightly greater than the guarantee, with good reproducibility. Another pyrethrin formulation (0.60%) was analyzed once. The percent rotenone found was 0.054%. Again, agreement was good.

In conclusion, this LC method for the simultaneous analysis of rotenone and pyrethrins is simple, rapid, and precise, considering the complexity of both pesticides and their formulations. Also this method is applicable to formulations containing either rotenone or pyrethrin and to very low concentrations.

Acknowledgments

The authors thank Penick Corp. for supplying the formulations and inert ingredients for this study.

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Determination of Polychlorinated Dibenzo-*p*-Dioxins in Technical Pentachlorophenol

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Thirty-four technical pentachlorophenol samples from 4 different manufacturers were analyzed for the presence of hexa-, hepta-, and octachlorodioxins. A double column procedure for extraction and cleanup was used for processing the samples, followed by gas chromatography-mass spectrometry for qualitative and quantitative determinations. All runs were performed isothermally on a packed glass column of 3% Dexsil 400. Octachloronaphthalene was used as an internal standard for quantitative estimations. The amounts of chlorodioxins found ranged from 1 to 38.5 ppm hexachlorodioxin, 119 to 562 ppm heptachlorodioxin, and 361 to 1723 ppm octachlorodioxin. Interfering polychlorinated diphenyl ethers and polychlorinated dibenzofurans were also observed in these samples.

Pentachlorophenol (PCP) is a general biocide used throughout the world, primarily as a wood preservative. In Canada, pentachlorophenol with its sodium salt has been registered for use since 1949, and approximately 1.8 million kilograms are marketed annually. Technical grade PCP has been shown to contain a number of phenolic and neutral polychlorinated reaction products (1–3) which arise during the manufacturing process (4). The phenols include the tri- and tetrachlorophenols, phenolic predioxins (5), and isopredioxins (2), while the neutral contaminants include the polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and diphenyl ethers, chlorinated cyclohexanones, and hexachlorobenzene. The data on the polychlorinated dibenzo-*p*-dioxin content of PCP have been reviewed and summarized by the Environmental Health Committee, U.S. Environmental Protection Agency, in 1978 (6). The highly toxic, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin has not been found in commercial PCP made in the United States (7).

Agriculture Canada, under its program on studies of microcontaminants in various pesticides, has carried out a survey on polychlorinated dioxin content of technical PCP being marketed in Canada by 4 different suppliers; the results obtained are reported in this paper.

Experimental

Apparatus

(a) *Gas chromatograph/mass spectrometer.*—Finnigan 4000 quadrupole mass spectrometer interfaced to an INCOS data system and coupled to a gas chromatograph. GC operating conditions: glass column 1.2 m × 2.0 mm id packed with 3% Dexsil 400 on Chrom W (AW); injector 270°C; column 275°C; helium carrier gas flow rate 40 mL/min. MS operating conditions: ionization 70 eV; ion source 260°C; interface 260°C; emission current 400 μA. Chlorinated dioxins were monitored using specific masses: m/z 390 and 392 for hexachloro-, 424 and 426 for heptachloro-, 458 and 460 for octachlorodioxins, and 402 and 404 for the internal standard octachloronaphthalene.

(b) *Gas chromatograph/high resolution mass spectrometer.*—VG ZAB-2F mass spectrometer interfaced to an INCOS data system coupled to a Varian 3700 gas chromatograph. GC operating conditions: glass column 1.2 m × 2.0 mm id packed with 3% Dexsil 400 on Chrom W (AW); injector 280°C; column 280°C; helium flow rate 30 mL/min. MS operating conditions: ionization 70 eV; ion source 260°C; interface 260°C; trap current 200 μA; accelerating voltage 8000 V; scan time 1.0 s. MID windows monitored: m/z 385–396 for hexachloro-, 419–430 for heptachloro-, 455–466 for octachlorodioxin, and 398–409 for octachloronaphthalene. Mass spectrometer was operated at a resolution of 2000. A 1.8 m × 2 mm id glass column with 3% Dexsil 400 gave better separation of hexachlorodioxin isomers (see Figures 1 and 2) but increased analysis time.

(c) *Glass columns.*—35 cm × 2.2 cm id and 25 cm × 1.4 cm id with 250 mL glass reservoir and a Teflon stopcock.

Reagents

(a) *Neutral alumina.*—(Fisher A-540). Activated at 110°C overnight.

(b) *Basic alumina.*—(Woelm). Activity 1.

(c) *Ottawa sand.*—20–30 mesh (Fisher Scientific).

(d) *Solvents.*—Dichloromethane, hexane, benzene, and toluene, all glass-distilled (Caledon Laboratories, Ontario, Canada).

Table 1. Levels of hexa-, hepta-, and octachlorodioxins in technical pentachlorophenol (34 batches; 4 manufacturers)

Sample	% Active Ingredient[a]	Chlorodioxin, ppm[b] Hexa-	Hepta-	Octa-
1	89.4	5.8	179.3	764.3
2	89.4	7.5	147.4	612.0
3	89.4	3.9	141.2	434.3
4	89.4	4.6	158.7	455.0
5	89.4	3.6	156.9	439.3
6	89.4	1.9	163.9	655.0
7	89.4	3.6	125.6	503.4
8	89.4	2.9	131.4	361.0
9	89.4	3.4	119.0	401.2
10	89.4	4.0	156.9	465.0
11	89.4	1.6	158.2	666.8
12	89.4	1.8	168.7	706.6
		Av. (3.7)	(150.6)	(537.1)
13	89.3	38.5	514.0	1335.0
14	89.3	36.8	526.0	1723.0
15	89.3	36.3	562.0	1644.0
16	89.3	37.5	483.0	1380.0
17	89.3	31.7	458.0	1274.0
18	89.3	29.2	438.0	1331.0
19	89.3	29.6	330.0	795.0
20	89.3	29.3	405.0	1188.0
		Av. (33.6)	(465.0)	(1334.0)
21	89.0	4.6	232.1	730.3
22	89.0	2.1	157.3	671.9
23	89.0	4.2	180.7	618.0
24	89.0	2.5	146.1	528.1
25	89.0	2.5	197.3	713.5
26	89.0	2.8	220.3	638.2
27	89.0	2.0	134.8	380.9
28	89.0	6.1	213.5	573.0
29	89.0	1.9	185.1	603.4
30	89.0	1.0	124.8	507.9
		Av. (2.9)	(179.2)	(596.4)
31	88.0	1.0	195.8	1058.5
32	88.0	2.0	293.4	1284.1
33	88.0	1.4	258.4	1590.9
34	88.0	1.0	153.4	1115.9
		Av. (1.3)	(225.2)	(1262.3)

[a]Average of % active ingredient found in 3 samples of each batch.
[b]Results have been adjusted for 100% active ingredient.

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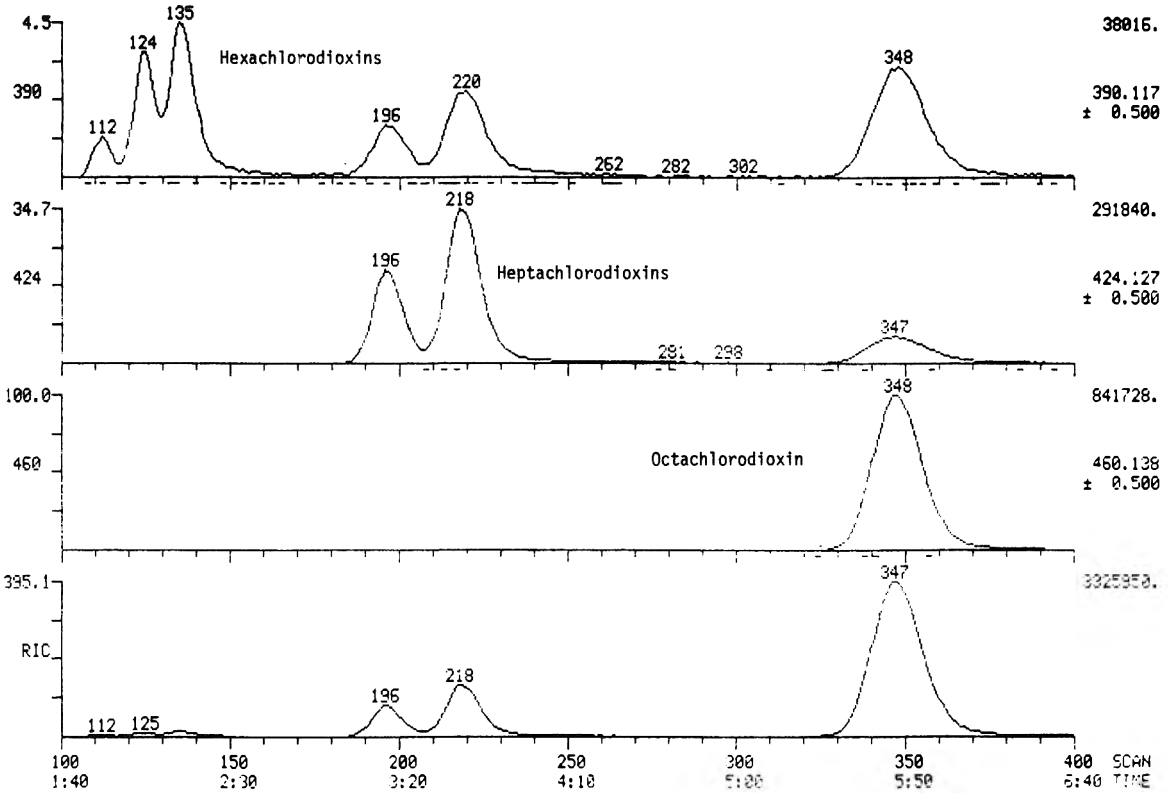

Figure 1. Typical MID-mass chromatogram indicating hexa-, hepta-, and octachlorodioxins found in technical PCP.

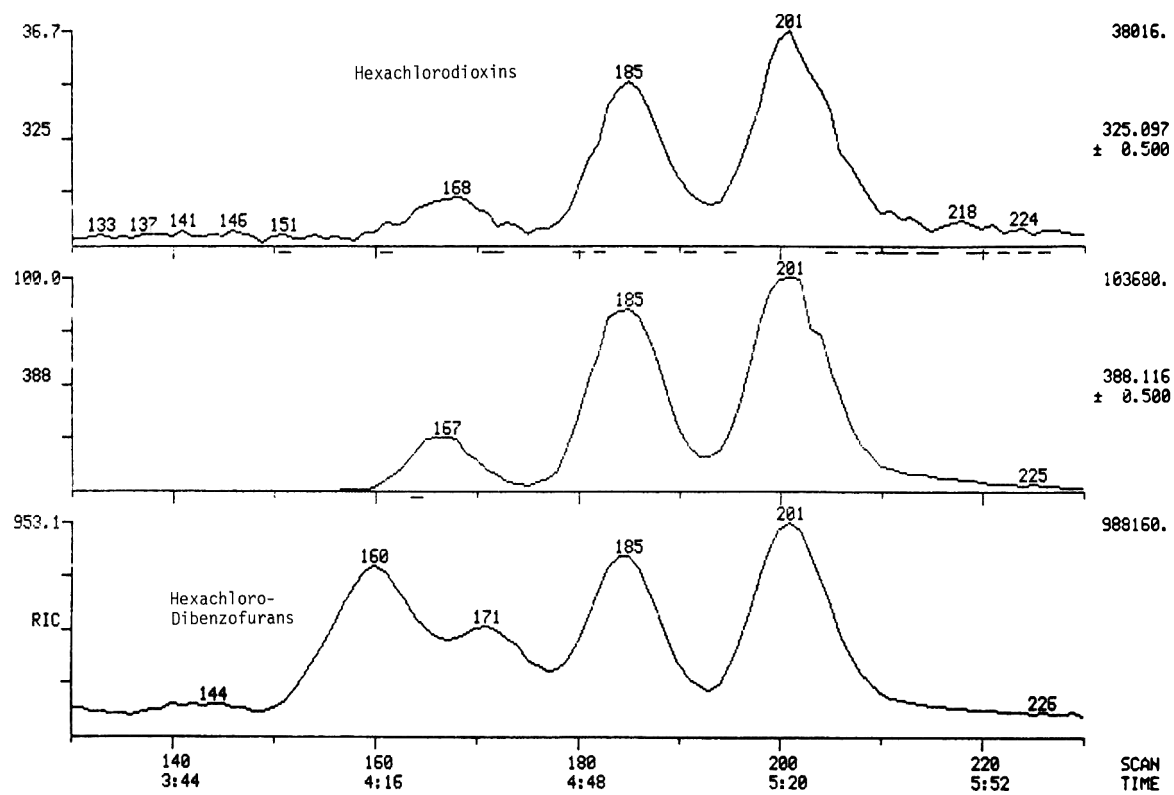

Figure 2. MID-mass chromatogram showing peaks of hexachlorodioxins at scan numbers 168, 185, and 201. Total ion bottom trace with peaks at 160 and 171 scan numbers are hexachlorodibenzofurans.

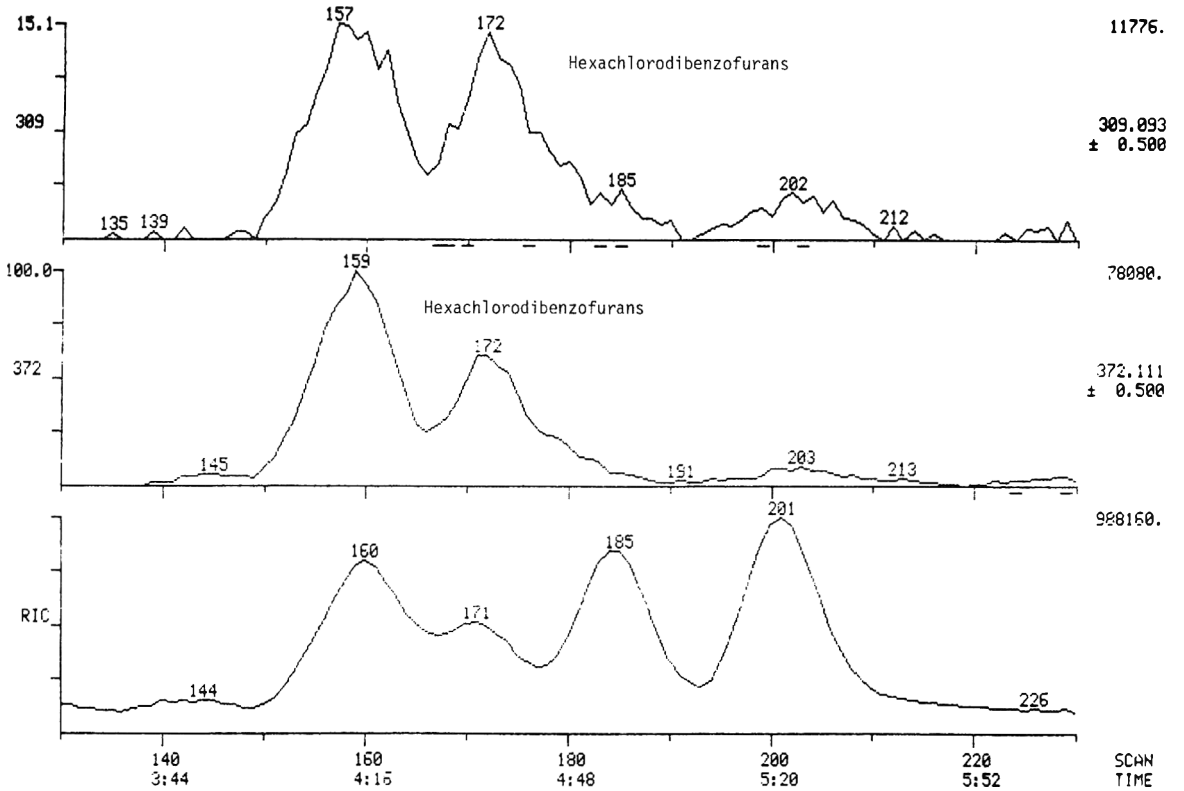

Figure 3. Mass chromatogram with response at m/z = 372 (M+) and 309 (M-COCl) confirming presence of hexachlorodibenzofurans.

Procedure

Prepare neutral alumina column, using 35 cm × 2.2 cm id glass column. Fill column with benzene up to junction of narrow stem and reservoir; using glass rod, pack small plug of glass wool and top it with Ottawa sand to ca 1 cm height. Pour 50 g neutral alumina into column while letting solvent slowly elute. Top alumina with anhydrous Na_2SO_4 to height of 1 cm. Accurately weigh 500 mg technical PCP in 25 mL Erlenmeyer flask and dissolve in 5 mL benzene (if needed, add small amount of methanol, drop-wise, until solution is achieved). Transfer sample solution onto alumina column and elute column with 150 mL benzene. Reduce eluate to 1–2 mL under reduced pressure on rotary evaporator.

Prepare basic alumina column using 25 cm × 1.4 cm id glass column, 2% dichloromethane in hexane as solvent, and 15 g basic alumina activity I (Woelm) as described above. Quantitatively transfer concentrate onto basic alumina column. Elute with 100 mL CH_2Cl_2–hexane (2 + 98) and discard. Collect next fraction of 100 mL CH_2Cl_2–hexane (30 + 70). Concentrate this fraction to 1–2 mL under reduced pressure on rotary evaporator; then remove solvent completely under gentle stream of nitrogen. Quantitatively transfer residue to 15 mL graduated centrifuge tube with toluene. Dilute to 10 mL and analyze by using GC/MS.

Results and Discussion

The levels of polychlorinated dibenzo-*p*-dioxins (PCDDs) found in the 34 technical PCP samples analyzed are shown in Table 1 and represent results on products from 4 different sources. These samples were analyzed for the presence of hexa-, hepta-, and octachlorodioxins only. The amount of these PCDDs in PCP originating with different manufacturers ranged from 1 to 38.5 ppm hexachlorodioxin, 119 to 562 ppm heptachlorodioxin, and 361 to 1723 ppm octachlorodioxin. The hexachlorodioxin results are a composite of 3 isomers, i.e., 1,2,3,6,7,8- and 1,2,3,6,7,9-, and possibly 1,2,3,6,8,9-hexachlorodioxin. Efforts are under way to develop an isomer-specific method of analysis. The heptachlorodioxin results are also a composite of the 2 possible isomers and the 1,2,3,4,6,7,8-chloro isomer was usually the most abundant (Figure 1).

Recoveries of hexa-, hepta-, and octachlorodioxin from spiked samples were greater than 90% when dioxins were added in the 1–2000 ppm range. All samples contained polychlorinated dibenzofurans and polychlorinated diphenyl ethers as well as dioxins. These compounds generally co-elute with the PCDDs and would interfere with a GC analysis using electron capture detection but can easily be distinguished by mass spectrometry. Figure 2 shows a mass chromatogram of a PCP extract with 3 hexachlorodioxin isomers eluting at scan numbers 168, 185, and 201. These are confirmed as dioxins by the responses at m/z = 388 (M+) and 325 (M-COCl). However, the bottom trace which is the total ion current shows peaks at scan numbers 160 and 171; Figure 3 is a mass chromatogram indicating these peaks to be hexachlorodibenzofurans by their response at m/z = 372 (M+) and 309 (M-COCl). The identity of different PCDDs was established by performing a full scan at a resolution of 2000. This was easily achieved due to the presence of high levels of these dioxins. All the analytical runs on GC/MS were performed under isothermal conditions. Quantitative determination was carried out with octachloronaphthalene as an internal standard.

It was also observed that amounts of different PCDDs found in a batch of samples originating with a particular supplier vary in a narrow range, whereas the amounts can vary substantially from those of another manufacturer. This points out the fact that fine tuning the experimental conditions in a manufacturing process could affect the final amount of dioxin contamination in the technical PCP.

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Liquid Chromatographic Determination of Triadimefon in Technical and Formulated Products: Collaborative Study

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A liquid chromatographic method for the determination of triadimefon (Bayleton™) in triadimefon technical and formulated products has been developed and subjected to a collaborative study with 7 participating collaborators. Formulations were extracted with mobile solvent and analyzed by normal phase chromatography, with 4-chlorophenyl sulfoxide as an internal standard. Collaborators were furnished with standards and samples of technical products, 50% wettable powders, and 25% wettable powders for analysis. Coefficients of variation of the values obtained on these samples were 1.42, 0.82, and 1.05%, respectively. The method has been adopted official first action.

Triadimefon, 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanone, is available in several formulations of wettable powder. Triadimefon is a widely used systemic fungicide which is highly effective against various rusts and mildews on fruits, vegetables, cereals, trees, and turf. Triadimefon, a relatively new fungicide, has been evaluated in a few published residue methods by gas chromatography (1–6). Liquid chromatography (LC) offers a quick and efficient separation of triadimefon for quantitation. Reverse phase LC systems were investigated and found to be nonspecific.

Collaborative Study

A normal phase LC method was developed and submitted to 7 collaborating laboratories. Collaborators were also furnished duplicate subsamples of technical materials, formulations, a reference standard, 4-chlorophenol, and the internal standard. The standard solution was spiked with 4-chlorophenol to ensure adequate separation from triadimefon and internal standard. The study was designed according to suggestions given by Youden and Steiner (7). The results of the collaborative study are reported in this paper.

Triadimefon Technical and Pesticide Formulations

Liquid Chromatographic Method

First Action

CIPAC–AOAC Method

(Method is suitable for tech. triadimefon and formulations with triadimefon as only active ingredient.)

This report of the Associate Referee was presented at the 98th Annual International Meeting of the AOAC, Oct. 28–Nov. 2, 1984, at Washington, DC.

Submitted for publication August 20, 1984.

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.* (1985) **68**, March issue.

6.A31 ***Principle***

Triadimefon is detd by liq. chromatgy, using 4-chlorophenyl sulfoxide as internal std.

6.A32 ***Apparatus***

(a) *Liquid chromatograph.*—Able to generate >7 MPa (>1000 psi) and measure *A* at 275 nm.

(b) *Chromatographic column.*—250 × 4.6 mm id packed with ≤10 μm silica gel capable of resolving 4-chlorophenol from triadimefon and internal std peaks (Du Pont Zorbax-Sil or equiv.). *LC operating conditions.*—Column temp. ambient; mobile phase flow rate 1.5 mL/min (ca 500 psi); chart speed 0.5 cm/min; injection vol.

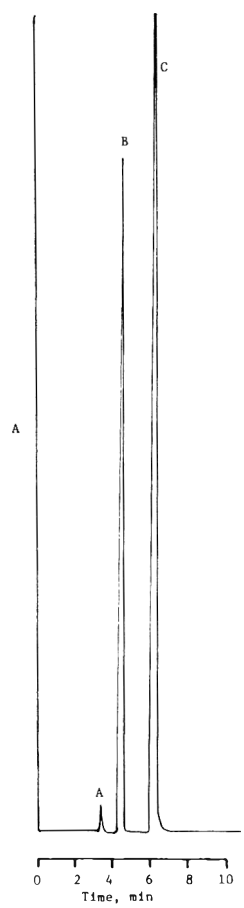

FIG. 6:A1—LC chromatogram of 4-chlorophenol (A), 4-chlorophenylsulfoxide (B), and triadimefon (C)

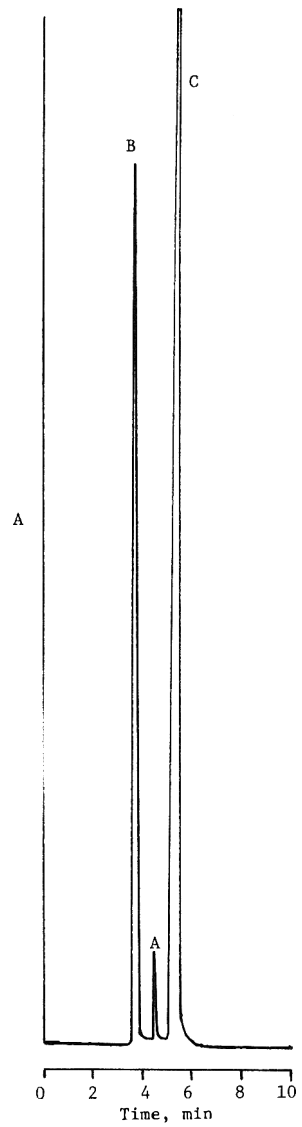

Figure 1. LC chromatogram of 4-chlorophenol (A), 4-chlorophenyl-sulfoxide (B), and triadimefon (C) on a Whatman Partisil 5 analytical column with butyl chloride–ethanol 100 + 1.

20 μL; *A* range 0.320 AUFS; retention times: 4-chlorophenyl sulfoxide ca 4.0 min, triadimefon ca 5.9 min. Pump LC mobile phase until system is equilibrated 15 min (flat baseline). Allow 6.5 min between injections.

(c) *Mechanical shaker.*

(d) *Filters.*—0.45 μm porosity (Gelman Acrodisc-CR or equiv.).

6.A33 ***Reagents***

(a) *Butyl chloride.*—LC grade or distd in glass (Burdick and Jackson Laboratories, Inc.).

(b) *Ethanol.*—Anhyd., 200 proof.

(c) *Mobile phase.*—Butyl chloride–EtOH (100 + 1). Pipet 10 mL anhyd. EtOH into 1 L butyl chloride, mix, and degas.

(d) *4-Chlorophenol stock soln.*—Weigh ca 20 mg 4-chlorophenol into 100 mL vol. flask and dil. to vol. with mobile phase.

(e) *4-Chlorophenyl sulfoxide internal std soln.*—About 275 mg/250 mL mobile phase; sonicate to dissolve.

(f) *Triadimefon reference std soln.*—Mobay Chemical Corp., Agricultural Chemicals Div., PO Box 4913, Kansas City, MO 64120. Accurately weigh ca 200 mg ref. std into 100 mL vol. flask. Pipet 10 mL chlorophenol stock soln into flask. Pipet 20 mL internal std soln into flask, dil. to vol. with mobile phase, and mix well. Filter portion of soln for LC analysis.

6.A34 ***Preparation of Sample***

Accurately weigh amt sample contg ca 200 mg triadimefon into 100 mL vol. flask. Pipet 20.0 mL internal std into flask. Add ca 50 mL mobile phase and shake 20 min. Dil. to vol. with mobile phase and mix. Filter portion of soln for LC analysis.

6.A35 ***Determination***

Inject triadimefon std soln and adjust operating parameters to cause triadimefon to elute in 5.5–6.0 min. Adjust injection size and attenuation to give largest possible on-scale peaks. Chlorophenol in std injection must be resolved from triadimefon and internal std peaks (Fig. **6:A1**). If not, change silica columns.

Using same injection vol. for all sample and std injections, make repetitive injections of std and calc. response ratios by dividing peak area (or ht) of triadimefon by that of internal std peak. Response ratios must agree within ± 1%. Average duplicate response ratios obtained with std solns.

Inject duplicate aliquots of each sample soln. Average duplicate response ratios for each sample soln. Response ratios must agree within ± 1%. If not, repeat detn, starting with std injections.

Reinject std soln twice. Average response ratios of stds immediately preceding and following sample injection. These must agree within ± 1%. If not, repeat detn.

6.A36 ***Calculation***

$$\text{Triadimefon, wt \%} = (R/R') \times (W'/W) \times P$$

where R and R' = av. response ratios for sample and std solns, resp.; W' and W = wt (mg) of triadimefon std and sample solns, resp.; and P = purity of triadimefon std (%).

Results and Discussion

A complete set of results was received from each of 7 collaborators (Table 1). A variety of equipment was used for

Table 1. Collaborative results of LC analysis of triadimefon technical and formulations[a]

	Technical		50% WP		25% WP	
Coll.	A	B	A	B	A	B
1	91.58	91.78	50.75	50.39	25.14	25.37
2	92.93	93.75	49.40	50.00	25.42	24.70
3	94.54	94.20	50.33	50.44	25.06	24.79
4	95.42	94.92	50.89	50.68	25.12	25.03
5	94.77	93.22	48.26[b]	47.83[b]	24.57	24.91
6	95.74	94.76	50.28	50.72	25.22	25.36
7	94.67	95.16	50.59	50.54	25.27	25.23
Mean	94.10		50.42		25.08	
S_x	1.33		0.41		0.26	
S_o	0.58		0.25		0.24	
S_L	1.20		0.33		0.11	
CV_x, %	1.42		0.82		1.05	
N	14		12		14	

[a]A and B are cuplicate subsamples.
[b]Values > 2 S_d from average are omitted from calculations (8).

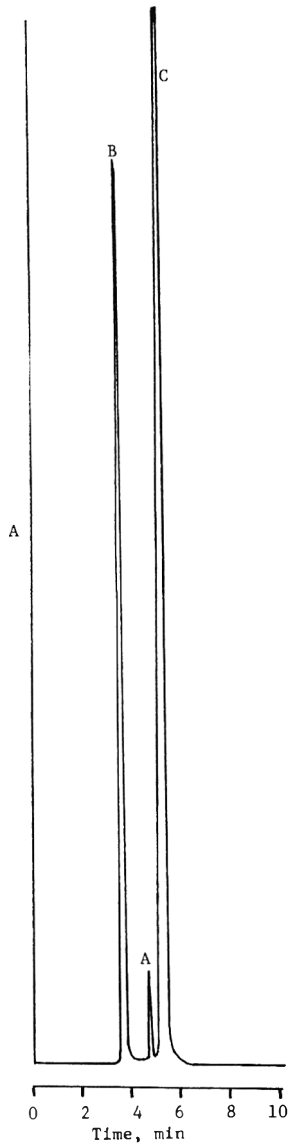

Figure 2. LC chromatogram of 4-chlorophenol (A), 4-chlorophenyl-sulfoxide (B), and triadimefon (C) on a Whatman Partisil 5 analytical column with butyl chloride–ethanol 100 + 0.5.

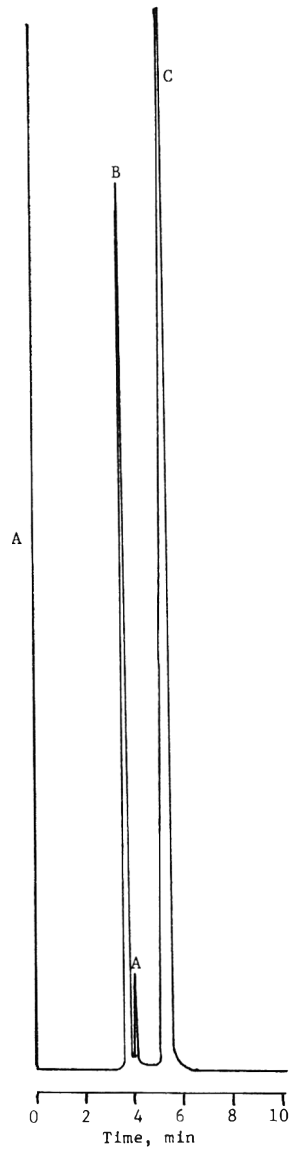

Figure 3. LC chromatogram of 4-chlorophenol (A), 4-chlorophenyl-sulfoxide (B), and triadimefon (C) on a Whatman Partisil 5 analytical column with butyl chloride–ethanol 100 + 1.5.

the analyses. Collaborators used 4 brands of pumps, 4 brands of detectors, 6 types of columns, and 6 brands of injectors. The sample volumes injected varied from 10 to 20 μL, pressures obtained were 3.9–9.8 MPa, and flow rates used were 1.5–1.6 mL/min. Five collaborators determined response ratios with data systems, while 2 collaborators used peak height measurements.

Previous personal experience with analysis of triadimefon by gas chromatography has shown it to give higher values and poorer reproducibility than does LC. This LC method resolves all known impurities in triadimefon from the active ingredient and internal standard.

The LC separation and quantitation of triadimefon was originally evaluated by reverse phase chromatography; however, after extensive work it appeared that a reverse phase LC system could not be devised which would separate several known impurities (e g., 1-(4-chlorophenoxy)-4-chloro-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanone) from triadimefon. Therefore, further work concentrated on the development of a normal phase LC method.

Initially, an acceptable normal phase method was developed using spectrophotometric grade chloroform containing about 0.75% ethanol as a stabilizer. The mobile solvent was chloroform–2,2,4-trimethylpentane (TMP) (70 + 30). Large volumes of analyses began to reflect separation problems resulting from the variability in ethanol stabilizer present in different batches of chloroform. This resulted in modifying the method to an isocratic mixture of chloroform (no alcohol stabilizers)–TMP–ethanol (70 + 30 + 0.75). Extensive use of this system gradually resulted in new problems. During long use, peak shapes began to skew and peak heights began to fluctuate, as did retention times. The solution to these problems was converting the mobile phase to butyl chloride–ethanol (100 + 1). Extensive use of this system did not show the problems encountered with chloroform mobile phases. Butyl chloride is readily available and fairly inexpensive. It offers several advantages over chloroform: It has a UV cutoff of only 220 nm, it does not need stabilizers, and it eliminates the need of a tertiary solvent system for triadimefon.

During the method development, it became apparent that the separation of 4-chlorophenol (a typical impurity) from the triadimefon and internal standard was a function of column packing and ethanol concentration. Figure 6:A1 shows a typical chromatogram using Du Pont Zorbax Sil as the column

packing and butyl chloride–ethanol (100 + 1) as the mobile phase. Figure 1 shows a similar separation substituting Whatman Partisil 5 packing. While the 4-chlorophenol retention time is perhaps less desirable in Figure 1, the resolution in each figure is excellent. Varying the ethanol concentration for the Partisil 5 ODS-3 column from butyl chloride–ethanol (100 + 0.5) to (100 + 1.5) produces chromatograms typical of Figures 2 and 3. Most data systems are capable of handling these interferences adequately. Likewise, these variations in ethanol concentration are within typical errors in solvent system preparation. The collaborators used the following columns: Whatman Partisil 10 and Partisil 5, E. Merck Lichrosorb SI 60 5 μm, Du Pont Zorbax Sil 7 μm and Zorbax Sil 5 μm, and Applied Science Adsorbosphere silica 5 μm. All of the collaborators achieved excellent resolution without varying the ethanol concentration in their mobile phases from (100 + 1).

Recommendation

It is recommended that the LC method be adopted official first action as an AOAC-CIPAC method.

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Oliver O. Bennett, Jr, Kansas State Board of Agriculture, Topeka, KS

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David F. Tomkins, Monsanto Agricultural Products Co., Muscatine, IA

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Gas Chromatographic Determination of Chlordimeform in Pesticide Formulations: Collaborative Study

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A gas chromatographic (GC) procedure for the determination of chlordimeform in emulsifiable concentrate formulations containing about 46% active ingredient was collaboratively studied using the matched pair scheme. Chlordimeform was extracted from the formulation with methylene chloride containing diethyl terephthalate as the internal standard, chromatographed on CBWX-20M, and detected by flame ionization. Determinations on the 4 samples by 20 government, university, and industrial collaborators using peak area measurements showed within-laboratory repeatability of better than 1%. Reproducibility was 1.2% for the formulation. The method has been adopted official first action.

Chlordimeform [*N'*-(4-chloro-*o*-tolyl)-*N,N*-dimethylformamidine] is an insecticide and ovicide. In formulations, it acts primarily as an ovicide for the control of bollworm and tobacco budworm on cotton. Chlordimeform is the active ingredient of the products Galecron® (registered trademark of Ciba-Geigy Corp., Agricultural Div.) and Fundal™ (registered trademark of Schering AG, FRG).

Methods of analysis based on gas and liquid chromatography were reviewed (1). This collaborative study evaluated a gas chromatographic method in which the internal standard was recently modified.

Collaborative Study

Two sets of matched pair samples of chlordimeform, Galecron 4EC formulation, together with standard, practice sample, internal standard, column packing, and detailed guidelines were sent to 22 collaborators in Europe and the United States. All collaborators were asked to pack and condition a column and to make at least one practice run for familiarization. The collaborators were asked to make a single GC determination for each sample from duplicate injections, and to report area integration and peak height measurements. The collaborators were also requested to submit the raw data and the chromatograms.

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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 *J. Assoc. Off. Anal. Chem.* (1985) **68**, March issue.

Table 1. Percent chlordimeform calculated by comparison with internal standard area (closely matched pairs)

Calculation	Laboratory																				Theory
	1	3	4	8	11	12	13	14	17	18	19	20	22	23	25	26	27	28	29	30	
Chlordimeform—1C	45.71	46.53	46.60	47.18	46.65	45.95	46.64	47.29	45.69	47.86	46.98	46.31	46.90	46.85	46.46	46.67	46.75[a]	46.16	45.88	47.14	46.50
Chlordimeform—2C	47.69	47.70	47.84	48.10	47.36	47.20	48.33	47.51	47.47	49.15	47.71	47.17	47.83	48.09	47.61	47.96	49.46	46.77	47.56	48.13	47.50
Differences, 2C – 1C	1.98	1.17	1.24	0.92	0.71	1.25	1.69	0.22	1.78	1.29	0.73	0.86	0.93	1.24	1.15	1.29	2.71	0.61	1.68	0.99	1.00
Totals, 1C + 2C	93.40	94.23	94.44	95.28	94.01	93.15	94.97	94.80	93.16	97.01	94.69	93.48	94.73	94.94	94.07	94.63	96.21	92.93	93.44	95.27	94.0
$(T_i - \bar{T})^2$	0.9025	0.3464	0.0081	0.8649	0.1156	1.4400	0.3844	0.2025	1.4161	7.0756	0.1156	0.7569	0.1444	0.3481	0.0784	0.0784	—	2.0164	0.8281	0.8464	—
Chlordimeform—3C	38.44	37.08	37.43	37.51	37.66	36.77	36.23	37.64	37.01	37.49	37.26	37.26	37.12	37.24	36.95	37.51	37.66	37.47[b]	36.71	37.10	37.40
Chlordimeform—4C	34.58	34.89	35.30	35.50	35.06	34.80	34.25	35.13	34.75	35.92	35.15	35.18	35.19	35.32	34.94	35.47	36.05	40.33	34.49	35.15	35.40
Differences, 3C – 4C	3.86	2.19	2.13	2.01	2.60	1.97	1.98	2.51	2.26	1.57	2.11	1.94	1.93	1.92	2.01	2.04	1.61	2.86	2.22	1.95	2.00
Totals, 3C + 4C	73.02	71.97	72.73	73.01	72.72	71.57	70.48	72.77	71.76	73.41	72.41	72.30	72.31	72.56	71.89	72.98	73.71	77.80	71.20	72.25	72.8
$(T_i - \bar{T})^2$	0.4225	0.1600	0.1296	0.4096	0.1225	0.6400	3.5721	0.1600	0.3721	1.0816	0.0016	0.0049	0.0036	0.0361	0.2304	0.3721	1.7956	—	1.3689	0.0144	—

[a]Lab. 27 eliminated as an outlier.
[b]Lab. 28 eliminated as an outlier.

Chlordimeform in Pesticide Formulations

Gas Chromatographic Method

First Action

AOAC–CIPAC Method

6.A09 ***Principle***

Chlordimeform is extd with CH_2Cl_2 and detd by flame ionization gas chromatgy, using diethyl terephthalate as internal std. Identity is verified simultaneously by comparing retention times with std.

6.A10 ***Apparatus***

(a) *Gas chromatograph.*—Capable of temp. program, preferably equipped with auto-injector, flame ionization detector, and integration capabilities.

(b) *Chromatographic column.*—2 mm id × 1.83 m (6 ft) glass column packed with 3% CBWX-20M on 80–100 mesh Gas-Chrom Q. Condition column ≥24 h at 225°, using carrier gas at ca 20 mL/min. Operating conditions: injector 250°; detector 250°; column 170° for 22 min, then to 225° at 20°/min, and hold 15 min; He carrier gas flow ca 25 mL/min. Retention times for internal std and chlordimeform are ca 11 and 14.8 min, resp.

6.A11 ***Reagents***

(a) *Internal std soln.*—4 mg/mL. Dissolve 4.0 g diethyl terephthalate in CH_2Cl_2 and dil. to 1 L with CH_2Cl_2. Check internal std soln for interfering components by injecting an aliquot into chromatograph.

(b) *Chlordimeform std soln.*—Accurately weigh 100 mg chlordimeform std of known purity (Ciba-Geigy Corp., Production Technical Dept, PO Box 18300, Greensboro, NC 27419) into 4 oz bottle. Pipet 50.0 mL internal std soln into bottle, cap, and shake 30 min.

6.A12 ***Preparation of Sample***

Accurately weigh amt of sample contg ca 100 mg chlordimeform into 4 oz bottle. Pipet 50.0 mL internal std soln into bottle, cap, shake 30 min to ext.

6.A13 ***Determination***

Set integration parameters, and stabilize instrument by injecting 1–3 μL aliquots of std soln until area ratios of chlordimeform to internal std vary less than 2% for successive injections. Using same established injection vol. as for std, inject sample. Sample area ratio should be ±10% of std area ratio. Inject 2 aliquots of std and 2 aliquots of sample followed by 2 aliquots of 2nd sample and 2 aliquots of std. Repeat sequence until all samples are analyzed. Calc. response factor, R, for each injection:

$$R = \text{peak area (or ht) chlordimeform/peak area (or ht) internal std}$$

$$\text{Chlordimeform, \%} = (R/R') \times (W'/W) \times P$$

where R and R' = av. response factor for sample and std solns, resp.; W and W' = mg sample and std, resp.; and P = purity (%) of std.

Results

Twenty collaborators analyzed 4 samples. These included a matched pair of a 4E emulsifiable concentrate differing by 1.0% and a matched pair of a modified 4E emulsifiable concentrate differing by 2.0%. All of the participants performed the desired number of analyses. Twenty of the participants reported peak area data, and only 14 reported peak height data.

Results were reviewed, tabulated, and statistically analyzed (2). Outliers were detected using 99% confidence limits. Statistical analysis was performed by Edwin Glocker, Committee A statistician. Peak area, peak height, and statistics are given in Tables 1, 2, and 3.

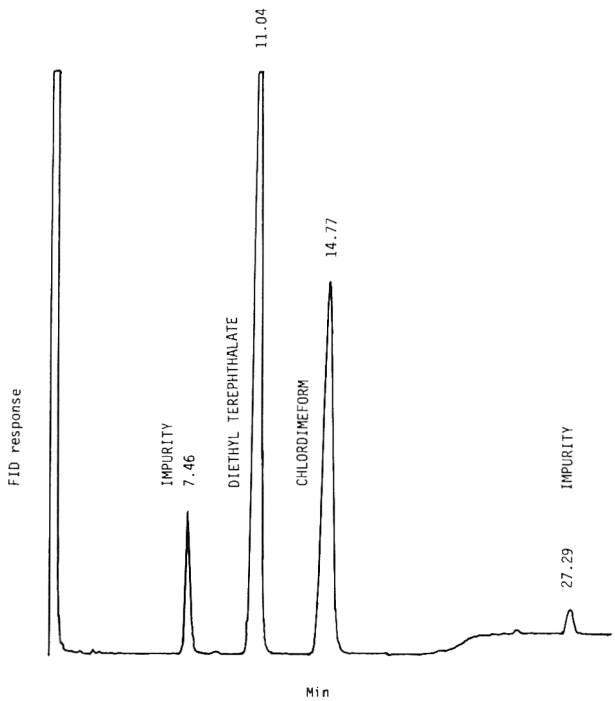

Figure 1. Typical chromatogram of chlordimeform formulation.

The differences in calculated means for area data between sample pairs are 1.15 and 2.15. This compares well with the theoretical differences of 1.00 and 2.00 and provides validation for accuracy.

A wide variety of gas chromatographs, integrators, and data systems as well as both automated and hand injection were used. Helium and nitrogen carrier gases and columns of 2, 3, and 4 mm id and 1.2 m (4 ft) to 2 m (6.6 ft) length were used. Most collaborators performed analyses on a single day, but a few used 2 days. Minor modifications and variation of available instrumentation were expected. A typical chromatogram of the sample is presented in Figure 1.

Collaborator comments on the method were favorable. Some would like to shorten the 30 min extraction time and reduce active ingredient and internal standard concentration.

Several collaborators commented on the long temperature program. Time and temperature are necessary to cover all potential sample compositions and age.

Since the pure crystalline analytical standard melts about 32°C, problems were encountered in sending the samples. Samples were shipped in dry ice but several were received melted. Once melted, it is difficult to maintain homogeneity so second shipments of the standard were sent. This problem is addressed internally by using a characterized 4EC as standard.

Conclusion

As the results reveal, the analytical method tested is rugged and accurate and yields reproducible results. No collaborator had difficulty with the method. This study shows essentially no difference between the values and statistics of 2 data forms: peak area and peak height. Peak height analysis is being replaced by modern integrators of peak area.

Recommendation

The Associate Referee recommends that this temperature-programmed gas chromatographic method for the determination of chlordimeform in formulations be adopted official first action. The lowest concentration tested was 35.4% and the highest concentration tested was 47.5%. The preferred method of quantitation is peak area measurement.

Acknowledgments

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D. Bradway, EPA-NEIC, Denver, CO

D. Carlson, McLaughlin Gormley King Co., Minneapolis, MN

L. Carpenter, Ciba-Geigy Corp., Agricultural Div., McIntosh, AL

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Table 2. Percent chlordimeform calculated by comparison with internal standard peak height (closely matched pairs)

	Laboratory														
Calculation	1	4	8	11	12	14	17	19	20	22	23	25	29	30	Theory
Chlordimeform—1C	46.21	59.93[a]	47.09	46.33	46.29	46.86	46.87	46.93	46.43	47.00	47.12	46.43	45.64	47.16	46.50
Chlordimeform—2C	47.24	53.32	48.08	47.35	47.32	47.14	47.37	47.57	47.57	47.45	48.24	47.60	47.26	47.83	47.50
Differences, 2C − 1C	1.03	—	0.99	1.02	1.03	0.28	0.50	0.64	1.14	0.45	1.12	1.17	1.62	0.67	1.00
Totals, 1C + 2C	93.45	109.25	95.17	93.68	93.61	94.00	94.24	94.50	94.00	94.45	95.36	94.03	92.90	94.99	94.0
$(T_i - \bar{T})^2$	0.5600	—	0.9409	0.2704	0.3481	0.0400	0.0016	0.0900	0.0400	0.0625	1.3456	0.0289	1.6900	0.6241	—
Chlordimeform—3C	38.26	36.07[a]	37.50	37.39	37.26	37.31	37.07	36.97	37.07	36.97	37.41	37.02	36.51	36.93	37.40
Chlordimeform—4C	35.36	45.55	35.43	34.92	34.76	35.28	34.86	35.05	35.03	35.15	35.43	34.93	34.25	35.03	35.40
Differences, 3C − 4C	2.90	—	2.07	2.47	2.50	2.03	2.21	1.92	2.04	1.82	1.98	2.09	2.26	1.90	2.00
Totals, 3C + 4C	73.62	81.62	72.93	72.31	72.02	72.59	71.93	72.02	72.10	72.12	72.84	81.95	70.76	71.96	72.8
$(T_i - \bar{T})^2$	0.3721	—	0.0064	0.4900	0.9801	0.1764	1.1664	0.9801	0.8281	0.7921	0.0289	79.924	5.0625	1.1025	—

[a]Lab. 4 eliminated as an outlier.

Table 3. Statistical summary for chlordimeform collaborative study

			Statistic				
Sample	*N*	Mean	$S_x(=S_d)$	$S_L(=S_b)$	$S_o(=S_r)$	CV_x	CV_o
Peak area:							
Pr 1, 2	38	47.2 (46.6,47.8)	0.541	0.444	0.310	1.15	0.66
Pr 3, 4	38	36.2 (37.3,35.1)	0.458	0.305	0.342	1.27	0.94
Peak height:							
Pr 1, 2	26	47.1 (46.6,47.5)	0.400	0.303	0.260	0.85	0.55
Pr 3, 4	26	36.1 (37.2,35.0)	0.369	0.301	0.213	1.02	0.59

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Capillary Gas Chromatographic Determination of Cypermethrin in Formulations: Collaborative Study

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A method is described for the determination of cypermethrin, 3-(2,2-dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylate cyano-(3-phenoxyphenyl)methyl ester, in technical and formulated material by capillary gas chromatography (CGC). Samples of technical or formulated material are dissolved in CH_2Cl_2 containing dicyclohexyl phthalate as internal standard. The solution is injected into a gas chromatograph fitted with a flame ionization detector and capillary column of 25 m × 0.32 mm fused silica with a thick film OV-1 phase at 240°C. Injection is made into a heated injection port fitted with an antidiscrimination device in a split mode. Peak areas obtained at retention times of the internal standard and active ingredient are measured with an integrator. The quantity of cypermethrin is determined by comparing the internal standard and active ingredient peak areas with those obtained from a calibration solution containing known amounts of internal standard and pure active ingredient. Five samples were chosen for collaborative study: technical cypermethrin, 70% liquid concentrate, 3 lb/US gal. emulsifiable, 3 lb/US gal. oil concentrate, and 40% wettable powder. Twelve collaborators carried out replicate determinations on each sample on separate days. Coefficients of variation between laboratories (CV_R) were 2.13 for the technical, 2.94 for the emulsifiable concentrate, 3.51 for the liquid concentrate, 2.66 for the wettable powder, and 2.29 for the oil concentrate. The method was adopted official first action.

Cypermethrin, 3-(2 2-dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylate cyano-(3-phenoxyphenyl)methyl ester is a new broad spectrum synthetic pyrethroid used principally in cotton and soybeans. It is highly active against worms and weevils in cotton and in pest control applications against German cockroaches (1). It is the active ingredient of the products Cymbush® (Registered Trademark of ICI PLC, London, UK), Ammo® (Registered Trademark of FMC Corp., Philadelphia, PA, USA), Barricade® and Ripcord® (Registered Trademarks of Shell International, London, UK). It is one of several chemicals discovered by M. Elliott and patented by the National Research and Development Corp. in England. Synthetic pyrethroids are synthetic relatives of the natural pyrethrin insecticides extracted from the pyrethrum flower. These chemicals all have much improved UV stability over the natural products, and can be used widely in agriculture.

Methods of analysis for cypermethrin based on gas chromatography (GC), liquid chromatography (LC), and capillary gas chromatography (CGC) are available in the literature (2, 3). The Collaborative International Pesticides Analytical Council (CIPAC) is currently collaborating on methods using packed column gas chromatography and liquid chromatography (2). Cypermethrin has 4 stereo isomers. GC methods of analysis normally merge the 4 chromatographic peaks of the active ingredient into one. This, potentially, can incorporate inactive impurities into the determination of active ingredient and would require separate determination of isomer ratios. This collaborative study on a CGC method for technical and formulated materials has the advantage of totally resolving each of the isomers from impurities.

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This report of the Associate Referee was presented at the 98th Annual International Meeting of the AOAC, Oct. 28–Nov. 2, 1984, 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.* (1985) **68**, March issue.

Cypermethrin in Pesticide Formulations

Capillary Gas Chromatographic Method

First Action

(Method is suitable for tech. and formulated cypermethrin.)

6.A01 *Principle*

Sample is dissolved in CH_2Cl_2 contg dicyclohexyl phthalate, and 1.0 μL is injected into capillary GC in split mode, with flame

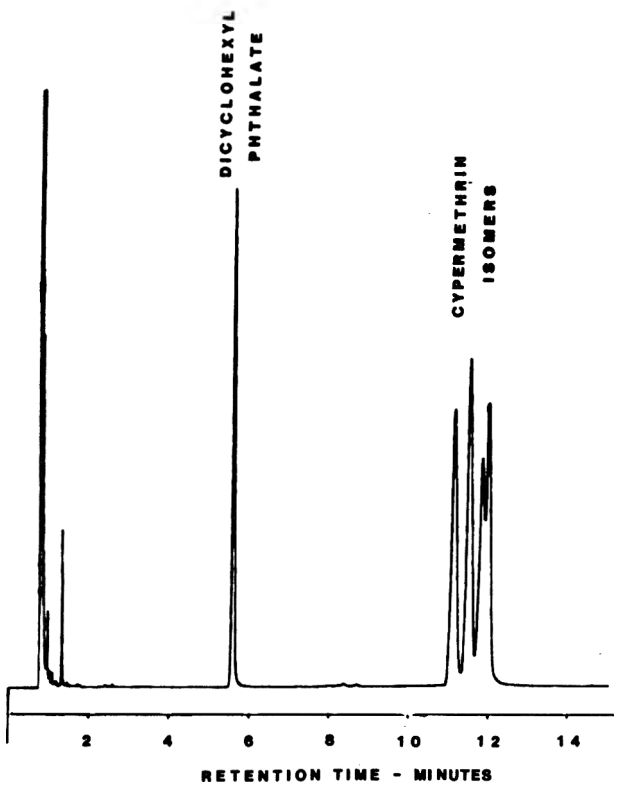

Figure 1. Typical capillary GC chromatogram of cypermethrin formulation; see text for chromatographic conditions.

ionization detection. Peak areas are measured for each cypermethrin isomer and dicyclohexyl phthalate and compared with those from std injection.

6.A02 *Apparatus*

(a) *Capillary gas chromatograph.*—With heated, glass-lined split mode injection port, flame ionization detector, and automatic sample injector. Temps (°)—column 240, injection port 250, detector 250; gas flows (mL/min)—He carrier gas 2.75, split vent 200 (split ratio 72.6:1), septum purge 0.5–1.0, He auxiliary gas to detector 30, H 60, air 240; column head pressure 15–20 psig; sample size 1.0 μL; retention times (min)—cypermethrin isomers: *cis* A, 11.18, *trans* C, 11.55, *cis* B, 11.85, *trans* D, 12.02, internal std, 5.58. Adjust parameters to assure sepn of 4 peaks and peak hts ca 60–80% full scale on chart at quoted retention times. Sepn of isomer peaks is critical to avoid inclusion of impurity peaks in active ingredient calcn.

(b) *Column.*—25 m × 0.32 mm (id) fused silica column with thick film OV-1 phase (Hewlett Packard, Avondale, PA 19311, Cat. No. 19091–62025). Precondition 1 h at 260° before use.

6.A03 *Reagents*

(a) *Dicyclohexyl phthalate internal std soln.*—Weigh 0.9 g dicyclohexyl phthalate (Eastman Kodak Co., Cat. No. P-2550), dissolve in CH_2Cl_2, and dil. to 500 mL. Check internal std soln for interfering components by injecting 1.0 μL into chromatograph. Store in tightly capped bottles to avoid evapn.

(b) *Cypermethrin std soln.*—Accurately weigh ca 100 mg cypermethrin std of known purity (ICI Americas, Inc., PO Box 208, Goldsboro, NC 27530) into scintillation vial. Pipet 10.0 mL internal

Table 1. Peak height data from collaborative study of cypermethrin

	Sample									
	1		2		3		4		5	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	91.70	96.90	37.50	36.80	70.40	70.50	46.20	43.80	37.10	38.10
	95.80	93.20	36.40	34.20	70.90	71.00	43.00	42.10	38.50	35.30
2[a]	92.48	88.57	37.42	35.31	71.50	72.33	44.98	42.25	37.86	38.35
	89.01	89.44	36.79	35.94	73.19	69.74	44.34	41.33	38.69	36.92
3	84.25	88.56	36.95	37.71	70.21	70.26	40.81	42.92	40.30	38.74
	88.17	89.29	36.83	35.07	69.83	68.31	41.10	41.43	36.09	37.98
4	89.22	89.95	36.21	34.97	71.58	70.04	43.30	40.38	37.93	38.24
	91.80	91.60	36.32	36.34	71.00	70.32	42.82	42.27	38.63	37.08
5[b]	—	—	—	—	—	—	—	—	—	—
6[c]	—	86.17	—	34.99	—	68.76	—	40.87	—	37.01
		88.27		37.19		69.85		40.79		36.80
7[d]	90.06	90.15	35.35	36.49	70.96	71.07	43.40	44.06	38.22	38.19
	89.69	89.68	37.82	37.44	70.90	70.06	42.16	41.44	37.95	36.97
8[e]	90.39	89.60	35.59	35.28	74.08	69.95	42.56	43.24	37.16	37.44
	(61.08*	92.05)	35.66	35.24	71.89	72.15	43.28	43.27	37.53	36.74
9[f]	91.31	95.44	34.55	37.16	68.80	73.63	43.46	40.68	37.35	39.29
	88.15	90.51	35.03	35.63	69.74	72.01	44.30	43.00	35.97	36.15
10	90.94	93.59	(41.35*	38.31)	(75.39‡	67.03)	42.35	43.97	(36.73	44.03*)
	89.24	86.30	32.22	37.28	(79.05*	74.62)	43.93	44.07	40.43	36.09
11	85.93	81.69	(39.20‡	32.81)	73.56	70.76	—	—	41.37	37.42
	91.53	92.82	(35.42	41.21*)	73.41	76.83			39.13	37.18
12[g]	92.07	84.95	36.22	34.07	71.59	70.77	42.73	39.23	39.18	36.44
	92.13	86.13	36.89	34.46	70.63	71.29	42.89	42.12	39.18	36.16
13[h]	88.92	92.33	(30.63*	34.81)	(65.32‡	73.37)	38.52	40.11	(28.67*	35.79)
	83.67	88.20	36.42	35.68	(64.28*	67.74)	38.67	39.82	34.89	37.25

[a]2 μL injections were made on Day 2 to try to improve reproducibility.
[b]Collaborator felt resolution was not good enough for peak height calculations.
[c]No Day 1 peak height data because of internal standard override.
[d]25 m OV-101 column used at 100:1 split ratio and a flow rate of 2 mL/min.
[e]25 m OV-101 column used at 100:1 split ratio and a flow rate of 2 mL/min.
[f]25 m CP5 SIL CB column used at 150:1 split ratio and 1 mL/min flow rate. Column temp. 220°C.
[g]No raw data available.
[h]Variable injection size 4-fold spread.
* = Outlier value at 99% control limits.
‡ = Outlier difference at 99% control limits.
( ) = Omitted at 99% control limits.

Table 2. Peak area data from collaborative study of cypermethrin

	Sample									
	1		2		3		4		5	
Coll.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1	89.00	88.70	35.60	35.50	70.10	69.40	42.60	41.80	37.50	36.90
	89.70	88.80	35.80	35.40	69.80	69.20	42.30	41.90	37.10	36.80
2[a]	92.17	89.47	37.17	35.55	71.85	73.11	44.63	43.16	38.27	38.82
	89.49	90.38	37.12	36.70	72.59	72.22	44.65	42.60	38.65	37.71
3	85.42	90.61	36.56	36.53	71.13	70.22	42.13	42.34	39.41	37.97
	88.64	88.42	36.75	35.02	71.16	68.06	42.31	42.38	37.06	38.31
4	89.74	89.78	36.10	35.76	70.36	70.01	42.38	41.95	37.34	37.72
	90.34	90.01	35.82	35.86	69.92	69.92	42.23	41.61	37.54	37.33
5[b]	91.04	91.52	35.40	36.04	70.37	71.80	44.00	43.98	37.53	37.77
	(—	91.22)	35.49	35.91	71.13	71.32	43.58	43.73	38.00	38.67
6	90.40	87.70	35.91	35.63	(61.91*	72.16)	(45.15	36.59*)	39.52	37.77
	(106.62*	88.77)	38.39	37.13	(97.90*	72.38)	44.29	40.57	39.74	37.22
7[c]	90.58	92.89	35.93	36.55	70.54	71.11	42.77	43.57	37.46	38.77
	89.86	92.47	35.22	36.06	69.79	71.97	42.49	43.51	37.15	38.71
8[d]	93.50	91.56	37.78	37.15	(78.09*	70.14)	44.39	43.53	39.74	37.79
	(59.36*	91.51)	38.87	36.73	73.87	70.61	43.89	43.29	39.38	37.68
9[e]	92.51	90.37	36.36	36.39	70.97	69.03	43.38	41.99	37.43	37.88
	93.32	87.90	35.63	35.69	71.04	70.94	43.64	42.99	37.57	38.30
10	91.46	93.32	39.13	36.56	76.62	65.49	(41.21	46.72*)	(35.47	44.40*)
	92.72	88.82	38.02	37.77	76.54	73.27	43.37	44.48	38.25	36.51
11	86.34	88.95	36.29	32.89	68.40	69.46			(44.12*	37.96)
	90.58	93.67	(37.16	43.74*)	(65.78	82.09*)	—	—	(32.37±	38.12)
12[f]	90.19	92.11	36.52	36.12	69.92	70.46	41.57	41.79	38.55	38.57
	91.67	92.73	36.84	36.68	69.58	71.07	41.28	42.01	38.01	38.60
13[g]	87.94	89.68	(31.58*	35.29)	65.04	74.51	40.27	42.26	(29.07*	36.31)
	(82.03*	91.13)	37.63	36.15	66.39	69.11	40.50	44.59	36.03	37.78

[a]2 μL injections were made on Day 2 to try to improve reproducibility.
[b]Used unpacked fritted insert as supplied by Varian; used a 25 m × 0.32 mm methyl silicone column.
[c]25 m OV-101 column used at 100:1 split ratio and a flow rate of 2 mL/min.
[d]25 m OV-101 column used at 100:1 split ratio and a flow rate of 2 mL/min.
[e]25 m CP5 SIL CB column used at 150:1 split ratio and 1 mL/min flow rate. Column temp. 220°C.
[f]No raw data available.
[g]Variable injection size 4-fold spread.
* = Outlier value at 99% control limits.
± = Outlier difference at 99% control limits.
( ) = Omitted at 99% control limits.

std soln into vial, cap, and shake to dissolve. Store in tightly capped bottles to avoid evapn.

6.A04 ***Determination***

(a) *Liquid and technical samples.*—Accurately weigh amt sample contg ca 100 mg cypermethrin into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake to dissolve.

(b) *Powder formulations.*—Accurately weigh amt sample contg ca 100 mg cypermethrin into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake on wrist-action shaker 10 min. Let insoluble inerts settle 10 min before analysis.

Inject 2 or more 1.0 μL aliquots of std soln to optimize instrument and integration parameters and to stabilize instrument. Monitor response factor until results agree ±2%. Inject 4 aliquots of std soln and 2 aliquots of sample in succession. Calc. response factor, R, for each injection; and take means for std and sample for calcn:

$$R = \frac{\text{total area of 4 cypermethrin isomer peaks}}{\text{area internal std peak}}$$

$$\text{Cypermethrin, \%} = (R/R') \times (W'/W) \times P$$

where R and R' = response factors for sample and std solns, resp.; W' and W = mg std and sample, resp.; and P = purity (%) of std.

Results and Discussion

Eleven collaborators analyzed 5 samples. Two of these collaborators supplied 2 sets of data, making a total of 13 sets. These included technical cypermethrin (Sample 1), 3 lb/US gal. emulsifiable concentrate formulation (Sample 2), 70% liquid concentrate (Sample 3), 40% wettable powder (Sample 4), and 3 lb/US gal. oil concentrate (Sample 5). Collaborators were supplied with an internal standard and cypermethrin analytical standard in addition to the samples. They were loaned columns because of the expense of purchasing columns specifically for this study. They were requested to stabilize their equipment and establish good resolution of active ingredient and internal standard (Figure 1). They were requested to analyze each sample, using duplicate injections, on the first day and to repeat the whole analysis on the second day from fresh solutions. They were asked to quantify results by using peak height and peak area measurements and to supply data, including chromatograms, to the Associate Referee.

Results were reviewed, tabulated, and sent for statistical analysis to Edwin Glocker, Committee A statistician, who used 99% confidence limits to detect outliers within laboratory pairs and between laboratories. These have been marked separately in the tables and were eliminated from the statistical summary. Tables 1, 2, and 3 show peak height and peak area data and statistics, respectively.

It was evident from telephone calls at the beginning of the study that many collaborators did not use capillary gas chromatography routinely for quantitative measurements. Several collaborators had difficulty in getting satisfactory reproducibility using the split injection technique. A separate letter was distributed to all collaborators pointing out the need to

Table 3. Statistical summary for cypermethrin collaborative study

Statistic	Sample 1	2	3	4	5
			Peak Height Data		
Mean	89.77	35.98	71.18	42.33	37.70
S_o	2.39	1.32	1.67	1.26	1.46
CV_o	2.66	3.67	2.35	2.98	3.86
S_x	3.15	1.32	2.41	1.70	1.46
CV_x	3.51	3.67	3.38	4.02	3.86
N	44	38	42	42	42
			Peak Area Data		
Mean	90.37	36.38	70.67	42.79	37.97
S_o	1.76	0.85	2.48	1.06	0.87
CV_o	1.95	2.35	3.51	2.47	2.29
S_x	1.93	1.07	2.48	1.14	0.87
CV_x	2.13	2.94	3.51	2.66	2.29
N	44	48	44	44	44

use an antidiscrimination device in the injection port of their instruments. Most instrument manufacturers supply these devices as accessories. They normally include a small amount of packing material held in a glass liner by silane treated glass wool to allow even heating of the injected sample and to ensure that the gas split contains an equivalent amount of internal standard and active ingredient. Two collaborators appeared to have problems even though they used these devices. The raw data supplied by one collaborator showed a 4-fold variation in internal standard counts across the range of sample and standard injections, clearly indicating an injection problem. Although, theoretically, the internal standard method of analysis should correct for sample volume, in practice, where there is a need to integrate a complex series of peaks like the 4 cypermethrin peaks which are not always baseline resolved, the actual size of the peaks can alter the way the integrator handles area determination on those peaks. It was evident from the raw data supplied by at least 2 of the collaborators that integrators did not handle peak analysis consistently throughout the study. Chromatographic separation in each of the data sets appears to be good, and there was no evidence of impurity interference.

One collaborator reported problems with the CH_2Cl_2 solvent bubbling in his injection syringe because of the altitude of his laboratory. This probably could have been overcome by using chloroform as an alternative solvent; however, no data are available on this alternative.

The coefficient of variation by peak height measurement was unacceptable for routine control laboratory work. The coefficient of variation from peak area measurement was larger than would be expected for a normal GC method of analysis. Surprisingly, the liquid formulations (Samples 2 and 3) had the highest coefficient of variation, while the technical material (Sample 1), which was an extremely viscous and difficult-to-handle material, had the lowest coefficient of variation. Reviewing the results, it appeared that several collaborators had extremely good within-laboratory reproducibility, while other laboratories had poor within-laboratory reproducibility. It was postulated, therefore, that the high coefficient of variation resulted from the inexperience of certain collaborators in using capillary GC for routine quantitative measurement. This should improve as more methods are published by AOAC using capillary GC. Sample 3 was a 1.28-fold dilution of Sample 1 by weight. The analytical results confirm the accuracy of this dilution.

Recommendations

It is recommended that the capillary gas chromatographic method for determination of cypermethrin in technical and formulated material be adopted official first action. The lowest concentration tested was 36.4%, and the highest concentration tested was 90.4%. Liquid and solid formulations can be handled by the method. The preferred method of quantitation is by peak area measurement.

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R. Stringham, Indiana State Chemists Laboratory, West Lafayette, IN

C. Weisskopf, State of California, Sacramento, CA

The Associate Referee also thanks Sally Sasser for assistance in the preparation and shipment of samples.

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DRUGS IN FEEDS

Liquid Chromatographic Determination of Nicarbazin in Feed

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A liquid chromatographic method was developed for the determination of nicarbazin (4,4'-dinitrocarbanilide.2-hydroxy-4,6-dimethylpyrimidine) in chicken feed. Ground feed was extracted with hot dimethylformamide, filtered, and then cleaned up on an alumina column. The nicarbazin was eluted from the column with ethanol and quantitated using a reverse phase C-18 column, with a methanol-water mobile phase and ultraviolet detection at 344 nm. Recoveries at a typical use level of 100 μg/g feed averaged 98% with a standard deviation of 3%. Samples fortified at levels as low as 0.1 μg/g were analyzed with 92% recovery. The detection limit is 1 ng, and the response is linear between 4 and 1000 ng. Feed additives in combination with nicarbazin do not interfere with recovery.

Nicarbazin is used to prevent intestinal coccidiosis in chickens. At levels between 0.01 and 0.02% in finished feed, hatchability, number, size, and appearance of eggs are affected; therefore, nicarbazin is not approved for use with laying hens. Nicarbazin must be withdrawn from feed 4–5 days before slaughter, depending on the drug combination used.

In the AOAC colorimetric method for the determination of nicarbazin in feed, the isolated 4,4'-dinitrocarbanilide moiety is mixed with alcoholic sodium hydroxide to yield a yellow solution (1,2). This method gives recoveries of 94–98% with standard deviations of about 13% with 0.01% nicarbazin in feeds (1). A modification of this procedure, which includes both liquid-liquid extraction and differential acid-base absorbance measurements, allows the determination of 1 μg/g levels (3). Some of the disadvantages of the AOAC method are: nonspecificity; interference from feed additives such as furazolidone, nitrofurazone, and nihydrazone; requirement of processed standards and blanks; and time dependence of the absorbance of the final derivative.

Polarographic (4) and gas chromatographic (5) methods have also been used to determine nicarbazin. A liquid chromatographic (LC) method for nicarbazin in tissue has been reported (6) as well as a method to detect just the 2-hydroxy-4,6-dimethylpyrimidine moiety (7).

By combining dimethylformamide (DMF) extraction, alumina column cleanup, reverse phase LC separation, and ultraviolet detection of the 4,4'-dinitrocarbanilide, we have developed a rapid, sensitive method to determine nicarbazin in feeds. There are no interferences with this method, which is designed to operate in the 0.1–200 μg/g range.

METHOD

Reagents

(a) *Solvents.*—Distilled-in-glass methanol and LC grade DMF (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442); 95% ethanol (USP, U.S. Industrial Chemicals Co., Tuscola, IL 61953).

(b) *Water.*—Double deionized.

(c) *Alumina.*—80–200 mesh (No. A540, Fisher Scientific, Fair Lawn, NJ 07410).

(d) *Nicarbazin.*—Reference standard (Merck Chemical Division, Rahway, NJ 07065).

(e) *Standard solutions.*—*Stock solution.*—100 μg/mL. Accurately weigh 0.01 g nicarbazin and transfer to 100 mL volumetric flask; dissolve in DMF at 50°C, cool, and dilute to volume with DMF. *Intermediate solution.*—10 μg/mL. Pipet 10 mL stock solution into 100 mL volumetric flask; dilute to volume with ethanol. *Working solutions.*—1.0 and 0.1 μg/mL. Pipet 10 and 1 mL intermediate solution into separate 100 mL volumetric flasks and dilute to volume with ethanol. Standard solutions are stable for several weeks stored in the dark.

(f) *Feed additives.*—Streptomycin and zinc bacitracin (U.S. Pharmacopeial Convention, Rockville, MD 20852); lincomycin (Control Reference Standard, Upjohn Co., Kalamazoo, MI 49001); procaine penicillin (Pfizer Inc., New York, NY 10017); roxarsone (Salsbury Laboratories, Charles City, IA 50616); furazolidone and nitrofurazone (U.S. Pharmacopeial Convention).

(g) *Mobile phase.*—Methanol-water (75 + 25). Filter through type FH 0.5 μm Millipore filter (Millipore Corp., Bedford, MA 01730).

Apparatus

(a) *Liquid chromatograph.*—Waters Associates (Milford, MA 01757) Model 680 controller, Model 590 solvent delivery module, Model U6K universal injector, Model 450 variable wavelength detector, and Servogor Model 120 recorder. Operating conditions—temperature ambient, flow rate 1 mL/min, wavelength 344 nm, absorbance 1.0–0.02 absorbance unit full scale.

(b) *Chromatographic tube.*—30 cm × 22 mm id, coarse filtering (Kontes Glass Co., Vineland, NJ 08368).

(c) *Chromatographic column.*—20 cm × 4.6 mm id LiChrosorb reverse phase, C-18, 10 μm (Hewlett Packard).

(d) *Feed grinder.*—Microjet 10-ZM 1, equipped with 0.5 mm screen (Micro Materials Corp., Westbury, NY 11590).

Column Preparation

Add 30 ± 1 g alumina to dry column and gently tap. Keep column dry until just before use. Add 40 mL DMF and let permeate alumina; stir alumina with long narrow rod until no air bubbles remain. Let DMF elute to 1 cm above column.

Extraction and Separation

Weigh 10.0 g feed into 125 mL Erlenmeyer flask. Add 50.0 mL DMF, heat to between 100 and 140°C for 15 min on hot

Table 1. Recovery of nicarbazin from feed spiked during initial DMF extraction

No. detns	Added, μg/g	Av. rec., ± SD, μg/g	CV, %	Av. rec., %
17	102	100 ± 3	3.3	98
11	27.4	26.8 ± 0.9	3.5	98
8	2.04	1.95 ± 0.10	5.1	96
3	1.02	0.92 ± 0.05	5.4	91
3	0.102	0.094 ± 0.016	17	92

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Table 2. Recovery[a] of 2.04 μg nicarbazin/g from feed spiked with other feed additives

Feed additive	Additive added, mg/g	Nicarbazin Rec., μg/g	Nicarbazin Rec., %
Furazolidone	0.33	2.12	104
Lincomycin.HCl	0.020	1.92	94
Nitrofurazone	0.55	1.94	95
Procaine penicillin G	0.12	1.98	97
Roxarsone	0.060	1.80	88
Streptomycin	0.090	1.92	94
Zinc bacitracin	0.11	2.04	100
All of the above at the indicated levels		1.90	93

[a]Duplicate analyses.

Table 3. Recovery of nicarbazin from dry spiked feed

No. detns	Added, μg/g	Av. rec., ± SD, μg[a]	CV, %	Av. rec., %
8	100	95 ± 5	5	95
8	200	187 ± 16	9	94

[a]Recovery for the 100 and 200 μg/g samples, using the official AOAC method (2), was 98 ± 3 and 201 ± 16 μg/g, respectively.

plate, filter through paper, and pipet 25.0 mL onto prepared alumina column. Drain solvent to 1 cm above bed, wash column with three 10 mL portions of DMF, then wash with three 5 mL portions of ethanol. Discard eluates. Elute nicarbazin with 30 mL ethanol and collect in 50 mL volumetric flask. Dilute to volume with ethanol.

At feed levels below 5 μg nicarbazin/g, collect eluate in 50 mL pear-shape flask and rotary evaporate to dryness at 65°C. Dissolve nicarbazin in 2.00 mL warm methanol.

Liquid Chromatography

Inject 5–25 μL sample, spiked sample, or blank into liquid chromatograph, bracket with standard injections, and calculate amount of nicarbazin by external standard method. Retention time of nicarbazin is ca 6 min.

Results and Discussion

Table 1 summarizes the recoveries of nicarbazin from chicken feed wet spiked during the initial DMF extraction. The recoveries ranged from 91 to 98% for nicarbazin concentrations ranging from 0.1 to 100 μg/g. All recoveries were based on external standards. The 0.1 μg/g detection level is 1000–2000 times less than the therapeutic concentration (8). The calibration curve was linear between 4 and 1000 ng.

Niarbazin is approved for use in chicken feed with other additives (8) such as those given in Table 2. Table 2 summarizes the recoveries of nicarbazin in the presence of typical amounts of these additives. Recoveries ranged from 88 to 104% with an average of 96%. When all of the additives were present in one sample, the recovery was 93%. None of these additives gave peaks on the LC chromatogram when they were carried through the extraction and cleanup procedure. Furazolidone and nitrofurazone did not interfere with this LC procedure, but interfered with the official AOAC method. Both methods use similar extraction and cleanup procedures.

Table 3 summarizes the results of dry spiking the feed before extraction. When the LC method was compared to the AOAC colorimetric method, the recoveries and precision were similar; however, the AOAC method is not as rugged since the absorbance must be measured within 5 min of adding the sodium hydroxide solution. Time is not a factor with the LC procedure.

The dry spiked feed utilized a premix, and a feed premix containing 1.00 mg nicarbazin/g was made by mixing 0.100 g powdered nicarbazin with 100 g ground feed for 24–48 h. The ground feed was ground to pass through a 0.5 mm screen. The 100 and 200 μg nicarbazin/g dry spikes were then prepared by mixing 1.00 or 2.00 g of the premix with 9.00 or 8.00 g feed, respectively.

The final 30 mL ethanol fraction containing nicarbazin was diluted to 50.0 mL with ethanol and used directly for feed concentrations >5 μg nicarbazin/g. For feed containing <5 μg nicarbazin/g, the final 30 mL ethanol fraction was evaporated to dryness, and methanol was used to dissolve the nicarbazin. These recoveries were low (76%) and erratic if the methanol was cold. Warming the methanol gave consistent recoveries as indicated in Table 1.

The amount of alumina used in the column was critical when 25 mL ethanol eluate was collected as specified in the AOAC method (2). When 40 or 20 g alumina was used in place of the specified 30 g, the recovery of 100 μg/g decreased to <50%. When 25 g alumina was used, the recovery was 98%; with 35 g, the recovery was 49%. Increasing the ethanol eluant from 25 to 30 mL gave recoveries of 98% with either 25 or 35 g alumina. Also, the recoveries decreased 20% if the

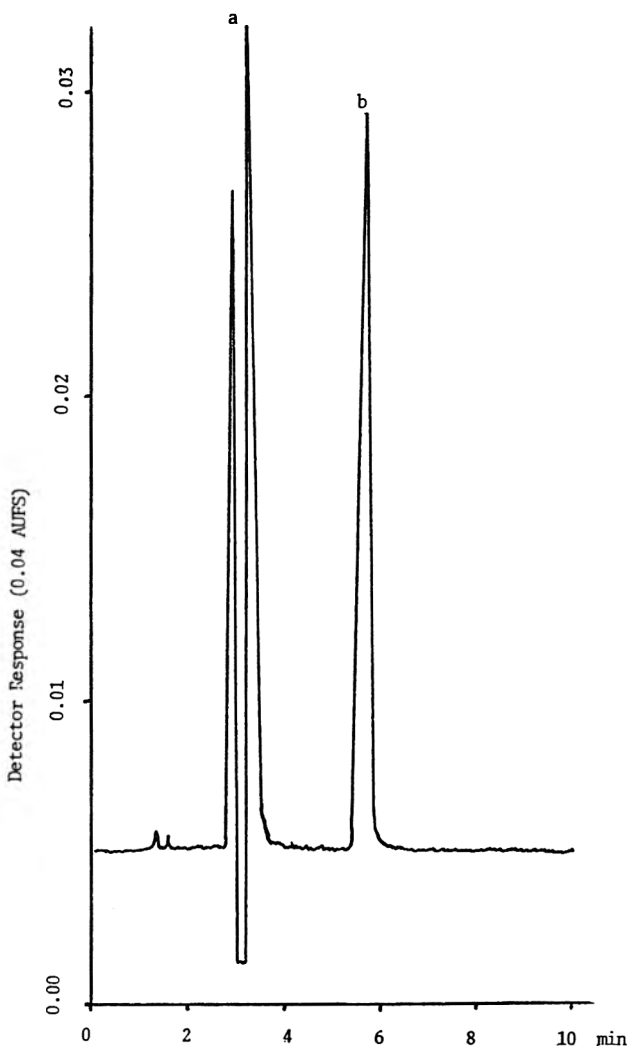

Figure 1. LC chromatogram of 10 g feed spiked with 100 μg nicarbazin/g (a is solvent and b is nicarbazin). Twenty μL final solution (10 μg nicarbazin/g) injected. AUFS = absorbance unit full scale.

alumina-DMF slurry set for 48 h before use. The DMF underwent cleavage, releasing ammonia.

Figure 1 is a typical chromatogram for spiked feed. A blank feed gave only the solvent peak.

In summary, the LC method can be used to quantitatively measure nicarbazin in chicken feed from typical use levels of 200 μg/g down to cross contamination levels of 0.1 μg/g. Normal feed additives do not interfere. The method is rapid, and involves only one column separation. Ten samples can be analyzed in 8 h.

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Liquid Chromatographic Determination of Morantel Tartrate in Cattle Feed

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A liquid chromatographic (LC) method is proposed for measuring 0.485–0.970% morantel tartrate in cattle feeds. The drug is leached from feed, diluted, separated from interfering substances on a silica column, and measured in the effluent stream by 313 nm spectrophotometric detection. Two potential degradation products, i.e., *cis*-isomer of morantel tartrate and *N*-(3-methylaminopropyl)-*trans*-3-(3-methyl-2-thienyl)acrylamide, and a related anthelmintic, i.e., pyrantel tartrate, do not interfere. Average recovery of drug from liquid spiked samples and laboratory blends was 98–100% with a maximum coefficient of variation (CV) of 2.3%. Results for pelleted and crumbled commercial scale feeds ranged from 94 to 102% of label claim, with a maximum CV of 1.5%.

Morantel tartrate (1,4,5,6-tetrahydro-1-methyl-2-(*trans*-2-(3-methyl-2-thienyl)vinyl)pyrimidine tartrate, Rumatel®) is a bovine anthelmintic agent (1) that has been approved for use in cattle feeds at levels of 0.485 and 0.970%. No methods for measuring this drug in feed have been reported in the literature and no collaborative studies have been initiated. The method in the New Animal Drug Application for this product involved ion-pair liquid-liquid partitioning followed by dual wavelength spectrophotometric measurement. The method proposed here involves the following sequence of steps: (a) Morantel tartrate is leached from feed with methanolic HCl solution, (b) the leachate is clarified by centrifugation and diluted with acetonitrile, (c) interfering substances are removed by liquid chromatography on a silica column, and (d) morantel tartrate is measured in the effluent stream by monitoring ultraviolet absorption at 313 nm.

The LC system separates morantel tartrate from 2 potential degradation products: the *cis*-isomer of morantel tartrate, which is a photodecomposition product, and *N*-(3-methylaminopropyl)-*trans*-3-(3-methyl-2-thienyl)acrylamide, which is an alkaline decomposition product. Pyrantel tartrate, a closely related anthelmintic, is also separated from morantel tartrate by the LC system. The performance of the method was satisfactory with respect to accuracy, precision, linearity, and applicability to pelleted rations.

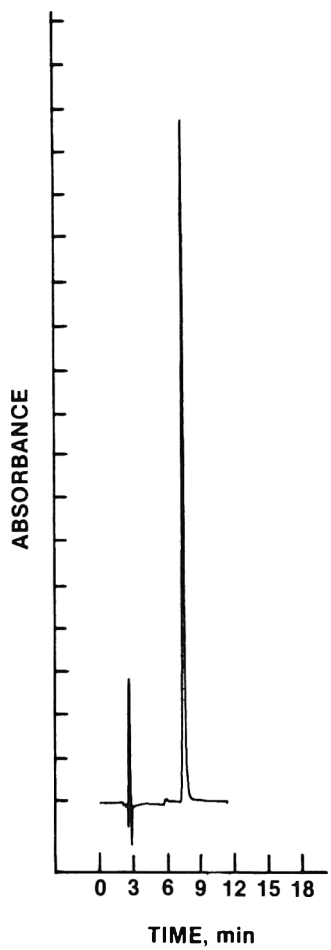

Figure 1. Liquid chromatogram for morantel tartrate reference solution B.

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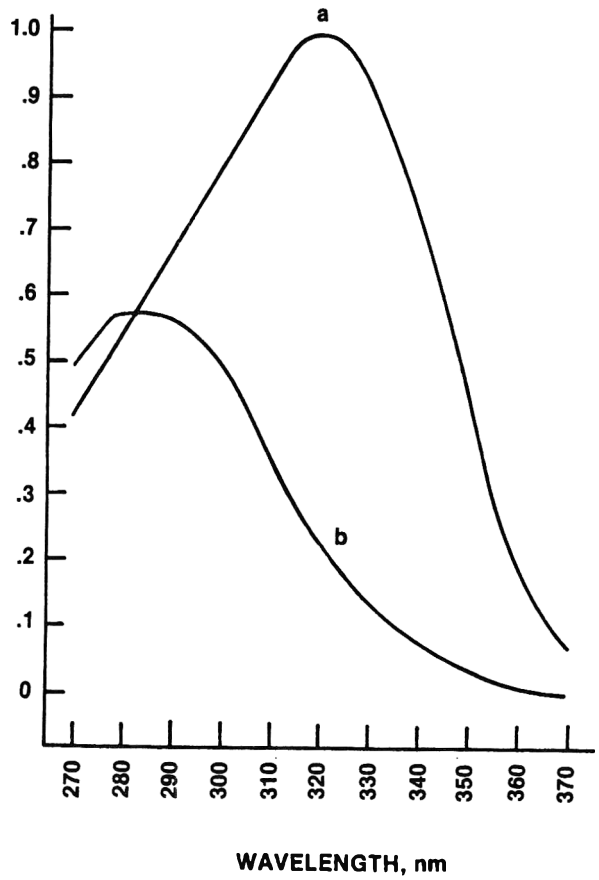

Figure 2. Ultraviolet absorption spectra for isolated 7.3 min LC fraction before irradiation (a) and after irradiation (b) with intense 366 nm light.

Earlier we reported on the use of a similar LC system in a method for 0.0106–0.106% pyrantel tartrate in swine feed (2). These relatively low pyrantel tartrate levels required sample cleanup prior to chromatography, and the use of standard additions to correct for the effect of the matrix on recovery. Such measures were not necessary for the analysis of morantel tartrate because of the higher drug levels involved.

METHOD

(Caution: Solutions of morantel tartrate are light-sensitive. Protect standard solutions and extracts from direct natural or artificial light.)

Reagents

(a) *Mobile phase.*—Acetonitrile–acetic acid–water–diethylamine (950 + 20 + 20 + 10). Filter under vacuum through 5 μm Mitex® filter, Cat. No. LSWP04700 (Millipore Corp., Bedford, MA 01730). Degas by stirring 10 min under vacuum.

(b) *Methanolic HCl solution.*—0.1N. Slowly add 8.5 mL HCl to 992 mL of mixture of water and methanol (1 + 1), and mix.

(c) *Morantel tartrate standard solutions.*—Prepare fresh daily from morantel tartrate reference standard (available from Pfizer Inc., Lee's Summit, MO 64063). *Standard solution A.*—Dissolve 25.0 mg reference standard in enough methanolic HCl solution to make 100.0 mL. *Standard solution B.*—Volumetrically dilute 5.00 mL standard solution A to 100.0 mL with acetonitrile.

Apparatus

(a) *Liquid chromatograph.*—Model 98750, 3000 psi pump, and Model 440 spectrophotometric detector (Waters Associates, Milford, MA 01757), or equivalent, operated at flow rate of 1.6 mL/min and detector wavelength at 313 nm, 0.2 AUFS. Model 385 recorder (Linear Instruments Corp., Irvine, CA 92714), or equivalent, operated at 5 mV and 12 cm/h chart speed. Model CV-6-UHPa-N60 injection valve with 25 μL injection loop (Valco Instruments Co., Inc., Houston, TX 77055), or equivalent.

(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), or equivalent at 180 cycles per min.

Preparation of Samples

Accurately weigh 5 g ground feed into 250 mL glass stopper flask. Pipet methanolic HCl solution into flask: 100 mL for 0.485% morantel tartrate or 200 mL for 0.97% morantel tartrate. Shake 30 min in reciprocating shaker. Transfer ca 40 mL extract to 50 mL glass-stopper centrifuge tube and centrifuge 10 min at 2000 rpm. Pipet 5 mL supernate into 100 mL volumetric flask, dilute to mark with acetonitrile, and mix. Centrifuge as before. Supernate is sample solution S.

Determination

Condition column with repetitive injections of standard B. Measure response as either height or area. When responses for 2 successive injections of B are within ± 1%, inject standard and sample solutions S in the order B, S_1, S_2, B.

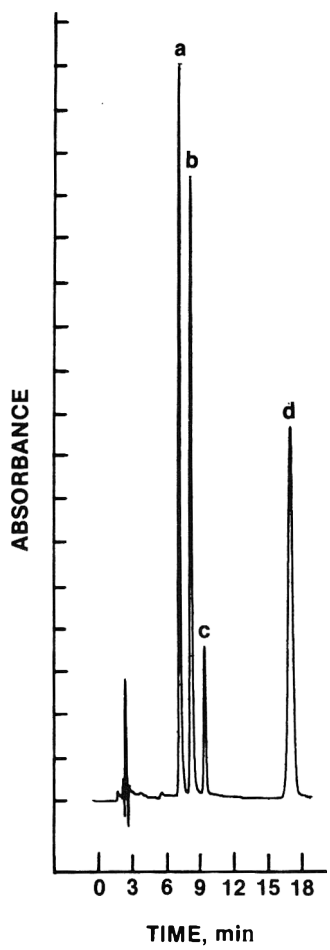

Figure 3. Liquid chromatogram for feed b containing 0.485% each of morantel tartrate (peak a), pyrantel tartrate (peak b), *cis*-isomer of morantel tartrate (peak c), and *N*-(3-methylaminopropyl)-*trans*-3-(3-methyl-2-thienyl)acrylamide (peak d).

Table 1. Assay of feeds spiked with morantel tartrate solution

Statistic	Morantel tartrate found, %		
	0.500% added[a]	0.500% added[b]	0.971% added[b]
	0.493	0.498	0.963
	0.496	0.499	0.962
	0.501	0.499	0.959
	0.497	0.500	0.957
	0.500	0.502	0.946
Av.	0.497	0.500	0.957
SD	0.003	0.002	0.007
CV, %	0.6	0.4	0.7
% of added	99	100	99

[a]Prepared with ration a.
[b]Prepared with ration b.

Table 2. Assay of laboratory blends

Statistic	Morantel tartrate found, %		
	0.483% added[a]	0.481% added[b]	0.969% added[b]
	0.457	0.481	1.006
	0.479	0.465	0.965
	0.478	0.482	0.955
	0.481	0.474	0.950
	0.482	0.476	0.968
Av.	0.475	0.476	0.969
SD	0.010	0.007	0.022
CV, %	2.1	1.5	2.3
% of added	98	99	100

[a]Prepared with ration a.
[b]Prepared with ration b.

Calculate response factor F for each standard injection as follows: F = [(% purity/100) × (g std/100 mL std A) × (5 mL std A/100 mL std B)]/response std B.

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

$$\% \text{ morantel tartrate} = [F \times \text{response of sample S} \times (\text{mL MeOH-HCl soln}/5 \text{ mL extract aliquot}) \times 100 \text{ mL sample S} \times 100]/\text{g feed}$$

Note: mL MeOH-HCl soln = 100 for 0.485% morantel tartrate and 200 for 0.97% morantel tartrate.

Results and Discussion

Morantel tartrate responds in the LC system as a single sharp peak with a retention time of 7.3 min and a capacity factor of 1.9 (Figure 1). Assay conditions provide 50% full scale deflection (5 mV recorder input) for approximately 0.28 μg morantel tartrate. The identity of the peak was confirmed by analysis of the pooled eluant fraction from several injections of a concentrated morantel tartrate reference solution. The ultraviolet absorption spectrum of the pooled fraction was identical to that of morantel tartrate (see curve a, Figure 2). Further, a photolytically induced spectral shift was observed following irradiation of the same sample with intense 366 nm light. The latter spectrum (see curve b, Figure 2) corresponded to that of the *cis*-isomer photolytic degradation product of morantel.

Two types of rations were used to evaluate the proposed method. The first, ration a, was a top dressing formulation consisting primarily of dehydrated alfalfa meal with mineral oil lubricant. The second, ration b, was a 12.8% protein cattle finisher ration consisting of ground yellow corn, alfalfa meal, soybean meal, cane molasses, soybean oil, and various vitamin and mineral supplements.

When nonmedicated portions of rations a and b were assayed as directed for 0.485% drug in feed, no feed-related components eluted at or near the retention time of morantel tartrate. Two potential degradation products of morantel tartrate, *cis*-isomer and *N*-(3-methylaminopropyl)-*trans*-3-(3-methyl-2-thienyl)acrylamide, and one related anthelmintic, pyrantel tartrate, responded in the assay procedure but were well resolved from morantel tartrate. A chromatogram for the analysis of feed b containing 0.485% each of morantel tartrate, the 2 potential degradation products, and pyrantel tartrate is shown in Figure 3. Separation factors, relative to morantel tartrate, were 1.4, 3.0, and 1.2 for *cis*-isomer, *N*-(3-methylaminopropyl)-*trans*-3-(3-methyl-2-thienyl)acrylamide, and pyrantel tartrate, respectively.

Linearity was evaluated over a range of 70 to 130% of label claim potency (0.485%). Known quantities of morantel tartrate in methanolic HCl solution were added to samples of nonmedicated feed a to simulate analytical samples containing 0.340, 0.485, and 0.631% drug. Methanolic HCl solution was then added to bring the total volume of solvent in each sample to 100 mL. A similar set of samples was set up without feed present to evaluate the effect of the feed matrix on linearity. All samples were assayed as described under *Method*, starting at "Shake 30 min . . . ." Regression analysis of the data demonstrated that the slope was linear and extrapolated to a near zero intercept. No significant differences in slope (s), intercept (i), or correlation coefficient (c) were observed between samples containing feed (i.e., s = 134, i = 0.002%, c = 0.9999) and samples without feed (i.e., s = 134, i = 0.001%, c = 0.9999).

Table 3. Assay of commercial scale crumbles[a]

Statistic	Morantel tartrate found, %		
	Lot 1	Lot 2	Lot 3
	0.488	0.463	0.475
	0.501	0.454	0.476
	0.478	0.449	0.479
	0.497	0.459	0.470
	0.503	0.451	0.467
	0.494	—	—
	0.502	—	—
	0.494	—	—
	0.497	—	—
	0.493	—	—
Av. ± SD	0.495 ± 0.007	0.455 ± 0.006	0.473 ± 0.005
CV, %	1.5	1.3	1.0
% of label claim	102	94	98

[a]Label claim = 0.485%. All lots made with ration a.

Three sets of liquid spiked samples were prepared as described above with a nominal drug content of 0.485% for 2 sets and 0.970% for the third. Assay of these samples showed good recovery and reproducibility (Table 1).

Three small laboratory blends, weighing approximately 250 g each, were carefully prepared by mechanically tumbling a closed vessel containing premix and feed. The nominal morantel tartrate content was 0.485% for 2 of the blends and 0.970% for the third. Results for 5 determinations on each blend showed good accuracy and precision (Table 2).

The proposed method was applied to refrigerated samples of 3 commercial scale lots that had been manufactured 3 to 4 years earlier from feed formulation a. These lots were prepared under controlled conditions to contain 0.485% morantel tartrate and were pelleted and crumbled. Sampling procedures conformed to AOAC recommended practices. The results (Table 3) were within 6% of label potency and exhibited good precision. These data indicate that the method is applicable to aged, pelleted feeds.

Acknowledgment

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TECHNICAL COMMUNICATIONS

Improved Cleanup for Gas Chromatographic Determination of Propiconazole Residues in Soil, Wheat Grain, Straw, and Leaves

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A simple and sensitive method is described for determination of propiconazole, a new type of broad-spectrum systemic fungicide, in soil, wheat grain, straw, and leaves. Pesticide residues in or on grain and green plant materials are extracted with methanol (or a mixture of methanol and water (4 + 1), for soil), partitioned into methylene chloride, and cleaned up on an alumina column for grain and soil or an activated charcoal column for green plant materials. The amount of residue is quantitatively measured by gas chromatography using an alkali flame ionization detector in the nitrogen-sensitive mode. Recoveries from soil, grain, and green plant materials fortified at 0.1–5 mg/kg are better than 80%. The practical detection limits of this method are 0.01 mg/kg in grain and soil and 0.02 mg/kg in green plant materials.

Propiconazole (1-((2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl)methyl)-1*H*-1,2,4-triazole) is the active ingredient of TILT®, a new, broad-spectrum, systemic fungicide with biological activity against powdery mildew, rust scab, and leaf spot diseases on many crops, especially wheat.

A supervised trial is being carried out to evaluate the residue dissipation of the fungicide in wheat and its environmental safety; therefore, we needed an analytical method for determination of the fungicide residue in or on soil, wheat grain, straw, and leaves.

Only one study has been reported for the determination of propiconazole residues in soil, plant materials, and water (1). The method involves extraction with methanol, partition into methylene chloride, cleanup on an alumina column, a further cleanup by gel permeation chromatography for green plant materials, and final detection by gas chromatography with a Carbowax 40M column and flame ionization detection.

Unfortunately, the method mentioned above could not be used in our laboratory, because we lacked the gel permeation chromatographic system and certain chemicals. The proposed analytical method described here was investigated as an alternative for determining the fungicide residue in soil, grain, and green plant materials. The method involves effective extraction, partitioning between aqueous methanol and methylene chloride, and cleanup on a basic alumina column or an activated charcoal chromatographic column. The final measurement is carried out by using gas chromatography with nitrogen-phosphorus detection.

Experimental

Apparatus

(a) *Gas chromatograph.*—Perkin-Elmer Sigma 2, equipped with alkali flame ionization detector operating in nitrogen-sensitive mode and 1 mV 056 Hitachi recorder. Operating conditions: temperatures (°C)—injection port 250, column 220, detector 250. High purity nitrogen carrier gas, 40 mL/min. Detector gases: air 100 mL/min, hydrogen 3 mL/min.

(b) *GC column.*—1 m × 2 mm id glass column packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q.

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(c) *High-speed homogenizer.*—Ultra-Turrax (Janke & Kunkel KG, Staufen i.Br., FRG).

(d) *Filtration apparatus.*—Modified, coarse (3–4 μm) sintered glass funnel, 165 × 80 mm id (Changchun Glass Ware Factory, Changchun, China).

(e) *Shaking machine.*—(Nanjing Soil Facility Factory, Nanjing, China).

(f) *Evaporating apparatus.*—Rotary evaporator (The First Glass Ware Factory, Tianjin, China).

(g) *Chromatographic column.*—300 × 15 mm id with stopcock (The First Glass Instrument Factory, Shanghai, China).

Reagents

(a) *Solvents.*—All analytical grade reagents including petroleum ether (60–90°C), acetone, methylene chloride, and methanol used in this experiment were distilled in glass before use.

(b) *Sodium sulfate.*—Anhydrous, reagent grade.

(c) *Saturated sodium chloride solution.*—Dissolve analytical grade NaCl in pure water until some solid particles remain undissolved.

(d) *Basic alumina.*—(Wushi State Firm, Shanghai, China). Heat 18 h at 110°C and store in air-tight glass container.

(e) *Activated charcoal.*—GR grade (E. Merck, Darmstadt, FRG). Mix one part activated charcoal, 2 parts pure grade magnesium oxide, and 4 parts Celite 545 and agitate 1 h on shaking machine; store in air-tight glass bottle.

(f) *Fungicide standard solutions.*—Propiconazole standard (> 99%) (obtained from Ciba-Geigy Ltd as a gift). Prepare stock solution in acetone, working standard solution in acetone–petroleum ether (1 + 1, v/v), and spiking solution in acetone.

Sample Preparation

Representative 500–1000 g samples of soil, grain, and green plant material (straw and leaves) were taken from supervised trial plots. Green plant samples were cut by knife into 0.5 cm pieces. Mature grain samples were ground into 20 mesh flour. All samples were stored in a freezer at −25°C until analysis.

Extraction

(a) *Soil.*—Weigh 20 g wet soil sample into round aluminum box (75 mm diameter, 75 mm height). Measure moisture content in soil sample by drying in 110°C oven to constant weight. According to percentage water in sample determined, weigh another 20 g soil sample (taking dry weight into account), pesticide-free or fortified at 0.1–2.0 mg/kg, into 250 mL flat-bottom extraction flask. Add appropriate water and 80 mL methanol to estimate 20% water content in extraction solution. Stopper flask with polyethylene cap and let stand overnight; then agitate 1 h on shaking machine.

(b) *Grain.*—Weigh 20 g ground wheat flour, pesticide-free or fortified at 0.1–0.5 mg/kg, into 250 mL flat-bottom extraction flask. Add 100 mL methanol and let set overnight; then agitate 1 h on shaking machine.

Table 1. Percent recovery of propiconazole fortified sample

Sample	Fortification level, mg/kg	Recovery, % mean ± SD (n)
Soil	0.1	99.3 ± 10.6(5)[a]
	0.5	91.5 ± 11.8(5)
	2.0	81.3 ± 3.2(4)
Straw and leaves	0.5	93.8 ± 8.1(7)
	2.0	90.7 ± 10.1(6)
	5.0	97.8 ± 13.0(4)
Grain	0.1	88.6 ± 6.1(4)
	0.5	96.8 ± 9.7(4)

[a]Number of replications.

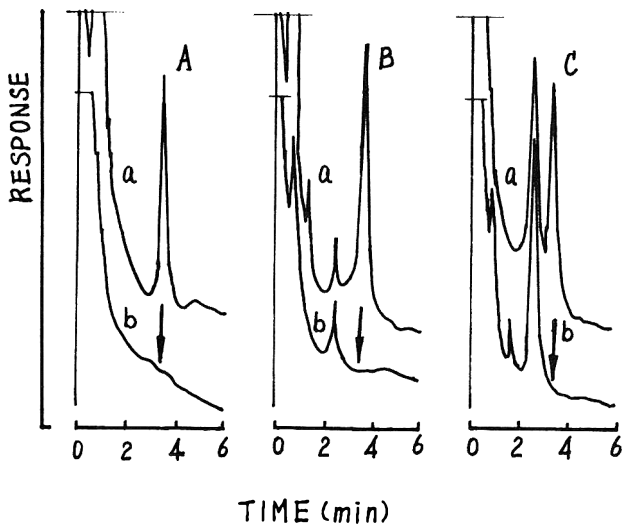

Figure 1. Gas chromatograms of typical samples of (A) soil: a, fortified at 0.1 mg/kg of propiconazole; b, untreated; (B) wheat grain: a, fortified at 0.1 mg/kg of propiconazole, b, untreated; (C) green plant material: a, fortified at 0.5 mg/kg of propiconazole; b, untreated.

Note: A or B represents 20 g sample, 4 mL final solution, 4 μL injection (equivalent to 20 mg sample); C is 10 g sample, 10 mL final solution, 4 μL injection (equivalent to 4 mg sample). Arrow indicates position of propiconazole peak.

(c) *Straw and leaves.*—Weigh 10 g chopped green plant sample, pesticide-free or fortified at 0.5–5.0 mg/kg, into 200 mL beaker. Add 100 mL methanol; then homogenize 3 min on high-speed homogenizer.

Filter slurry obtained from extraction step **a, b,** or **c** through coarse sintered glass funnel containing 1 cm Celite 545 by vacuum from water aspirator. Wash flask or beaker with two 15 mL portions of methanol and pour each wash onto funnel. Transfer entire filtrate to 500 mL pear-shape separatory funnel. Add 20 mL saturated NaCl solution and 100 mL water for soil and green plant materials or 200 mL water for grain. Extract residue 3 times with 50, 50, and 20 mL CH_2Cl_2. Combine organic phase and dry it by passing through a filter funnel (80 mm diameter) containing plug of cotton and 2 cm anhydrous Na_2SO_4. Evaporate organic phase to near dryness by rotary evaporator with 35°C water bath.

Cleanup

(a) *Column A.*—Prepare this column by adding enough basic alumina to glass column to yield 7 cm length; add 1 cm charcoal, MgO, and Celite 545 mixture, and add 1 cm anhydrous Na_2SO_4 to top of column.

(b) *Column B.*—Prepare this column by adding suitable amount of mixture of charcoal, MgO, and Celite 545 to yield column 7 cm length; then add 1 cm anhydrous Na_2SO_4 to top of column. Quantitatively transfer concentrated extract onto top of column A for soil and grain extracts, or column B for green plant extract with 4 mL acetone-petroleum ether (1 + 1, v/v). Elute column A with 80 mL mixture of CH_2Cl_2–petroleum ether–acetone (2 + 2 + 1, v/v/v). Discard first 10 mL fraction and collect remaining eluate. Elute column B with 80 mL CH_2Cl_2–petroleum ether (3 + 2, v/v) and collect entire eluate. Evaporate collected eluates to almost dryness by rotary evaporator with 35°C water bath.

GC Analysis

Dissolve residue with acetone–petroleum ether (1 + 1, v/v) and adjust to 4 mL. Inject 1–4 μL final solution into gas chromatograph. Dilute final solution with acetone–petroleum ether (1 + 1, v/v) as needed to yield peak height ca 10–60% full scale deflection. Compare retention time and height of peak in sample with those obtained by injecting several appropriate aliquots of propiconazole standard for qualitative and quantitative analyses, respectively.

Results and Discussion

Under GC conditions described earlier, the retention time of propiconazole is 3.5 min.

Before detection, the gas chromatographic system was checked for linearity by injecting several amounts of propiconazole standard. It was found that the detector response had excellent linearity for a range of propiconazole quantities at least from 0.4 to 2.4 ng.

The minimum detection limit of an analytical method mainly depends on the size of the background response of control samples (2). Gas chromatograms obtained from control samples of soil, grain, and green plant materials did not show any significant interfering peaks at the position of propiconazole. As low as 0.2 ng propiconazole could be easily observed on a chromatogram, so the minimum detection limits of this method were 0.01 mg/kg for soil and grain based on a 20 g sample, 4 mL final solution, and 4 μL injection, or 0.02 mg/kg for green plant materials based on a 10 g sample, 4 mL final solution, and 4 μL injection. It was possible to obtain a lower detection limit, but it seemed to be unnecessary. In fact, the minimum detection limits of 0.01 mg/kg for soil and grain and 0.02 mg/kg for green plant materials were sufficient to assess the safety of the fungicide on wheat crop.

The recoveries of propiconazole from soil, grain, and green plant materials fortified at 0.1 to 2.0 mg/kg concentration levels are shown in Table 1. Generally, the recoveries of propiconazole fortified at the various concentration levels were better than 80%.

Initially, 2 other gas chromatographic columns were investigated for separation of propiconazole during the development of this method. They were (a) 1 m × 2 mm id glass column packed with 3% Carbowax 40M on 80–100 mesh Gas-Chrom Q; operating conditions: injector 250°C, detector 250°C, column 225°C, flow rate of nitrogen carrier gas 40 mL/min; (b), 1.8 m × 2 mm id glass column packed with 2.5% OV-225 on 80–100 mesh Chromosorb W (AW-DMCS); operating conditions: injector 260°C, detector 260°C, column 220°C, flow rate of nitrogen carrier gas 50 mL/min. Attempts to separate propiconazole on either column were unsatisfactory. The OV-17 column was found to be the best for separating the fungicide.

We also noted that the ratio of volumes between aqueous phase and methylene chloride phase in the partitioning step should be taken into account. If the ratio was less than 5, an emulsion occurred easily in the partitioning step for grain. On the other hand, it may be difficult to extract the fungicide from aqueous phase if the ratio is high (based on the *p*-value

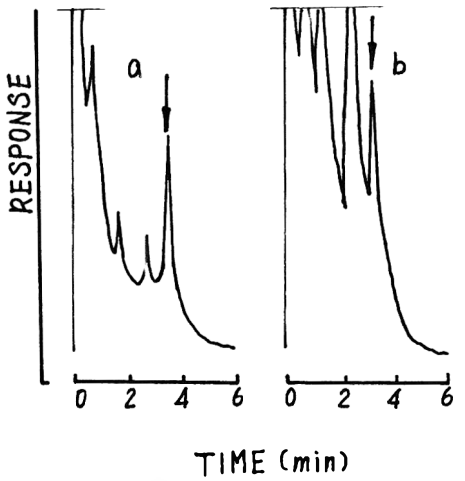

Figure 2. Gas chromatograms of straw and leaves taken at harvest time: a, after treatment with column A; b, before treatment with column A (for 10 g sample of residue-containing material, 4 mL final solution, 4 μL injection; propiconazole residue in sample equals 0.325 mg/kg).

theory). It is strongly recommended that the ratio of volumes between aqueous and methylene chloride phases be selected to range from 6 to 8.

Considering the instability and variability of many pesticides, it might not be necesary to dry soil samples before analysis. It seems more valid to directly analyze wet soil samples than to analyze air-dried ones.

The green plant material and soil samples were taken from the supervised trial plots at various intervals after the final application. Control samples were collected at the same time. Chromatograms of control soil samples taken at different times were almost identical. No interfering peaks were observed at the position of propiconazole on the chromatograms of control soil and grain samples (see Figure 1A and 1B). However, an unknown peak was found near the position of propiconazole on the chromatogram of control green plant material (see Figure 1C). In most cases, the peak was not large enough to affect the quantitation of propiconazole residue in green plant materials. However, the peak on the chromatogram of the sample collected at harvest time was often so large that it did affect the accuracy of quantitation of propiconazole residue (see Figure 2b). Fortunately, the interfering peak could be easily eliminated or sharply reduced by using a further cleanup step: passing the concentrated eluate through column A (see Figure 2a).

Activated charcoal has been used for determination of organophosphorus pesticide residues in agricultural products, especially in green plant materials (3, 4), because it allows effective separation of pigments from pesticide residues. In addition, activated charcoal has some advantages for cleanup of green plant extracts in contrast with gel permeation chromatography: less time consuming, less expensive, and more convenient. Using an activated charcoal column instead of a gel permeation chromatographic system for cleanup of green plant extract was successful in our laboratory.

Acknowledgments

The authors thank Qin Tai-qun and Tian Shu-xian for their skillful technical assistance.

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Calibration of Skim Milk-Test Bottles

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The method described uses a 250 μL syringe to calibrate skim milk-test bottles. Mercury is injected into the bore of the test bottle, and the amount is recorded. The bore of the bottle can contain 100 μL mercury, and those bottles shown to contain between 98 and 102 μL are acceptable for certification.

Babcock milk-test and cream-test bottles used in testing milk and cream for payment to producers usually are tested and certified by an official agency using methods described by AOAC (*Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. **16.065**). The test apparatus commonly used delivers mercury into the calibrated bore of the Babcock bottle, and the amount of mercury delivered is read at 3 points on the graduated scale of the test bottle. The apparatus is calibrated with a standard test bottle. Bottles are rejected if they do not fall within prescribed limits: ± 1 graduation (0.1%) on the milk-test bottle, and ± 1 graduation (0.5%) on the cream-test bottle.

Recently we were asked to test and certify skim milk-test bottles. The usual test equipment could not be used because the entire bore of the skim milk-test bottle, calibrated from 0 to 0.5% in 0.01% increments, can contain a total of only 0.1 mL (100 μL or 2 μL per calibrated increment). The usual test apparatus cannot be calibrated to the degree of accuracy needed, nor can the skim milk test-bottle be accommodated on the equipment. Furthermore, a standard skim milk-test bottle is not available to calibrate the equipment. We therefore developed the following method for calibration of skim milk-test bottles.

Experimental

Mercury is used and other testing parameters are followed according to AOAC method **16.065.**

Materials and Methods.—Use 250 μL syringe (Rheodyne 250A RLC, Scientific Glass & Instruments, Inc., Houston, TX 77001). Slip over end of needle on syringe ca 1 cm piece of Teflon tubing (id 0.028 mm, wall thickness 0.012 mm, No. AWG TW Natural, Penntube Plastics, Clifton Hgts, PA). Cut off and fit over Teflon tubing ½ cm end of single, service-type pipet tip (Type BR-35 yellow, disposable pipet tips, Bio-Rad Laboratories, Richmond, CA 94804). Tip may be glued onto tubing for security (Figure 1).

Calibration of skim milk-test bottle.—Clamp bottle in horizontal position for easy manipulation and reading. Fill syringe with mercury and insert tip of adapted needle into bore of test bottle. Inject mercury slowly until it reaches 0.5% calibration on bore of test bottle, and read μL on calibrated portion of syringe. Inject more mercury into bore of bottle until 0% point is reached, and read amount on syringe. Then withdraw mercury back into syringe. Difference between 2 readings is volume (μL) of mercury contained between 0 and 0.5% calibrated portion of skim milk-test bottle. Even though syringe is calibrated in 5 μL increments, it can be read to 2.5 μL with good accuracy.

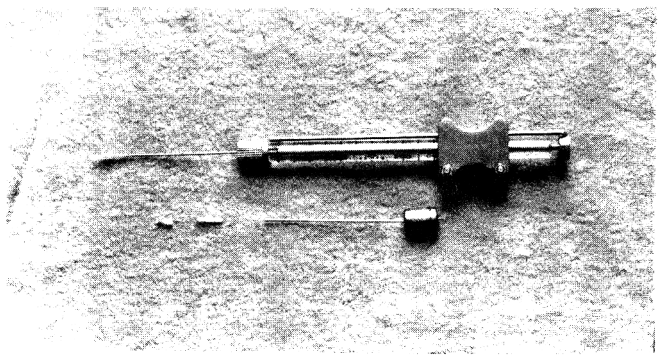

Figure 1. Calibration of skim milk-test bottles.

Results and Discussion

We have tested 72 skim milk-test bottles submitted for certification and found the mean amount of mercury contained between the 0 and 0.5% calibrations to be 100.4 μL, with a standard deviation about the mean of 0.78 μL, and a range from 98 to 102 μL. Because no standard skim milk-test bottle is available for comparison, we have assumed that the correct amount of mercury that should be contained between the 0 and 0.5% calibrations is 100 μL. Thus each subdivision on the calibrated scale can contain 2 μL of mercury.

Using the same criterian for rejection or acceptance of milk-and cream-test bottles, **16.065(a)***(3)*, the calibrated scale on any skim milk-test bottle that contained an amount of mercury within ± 1 subdivision (2 μL) would be acceptable for certification. All 72 bottles tested met this criterian and were certified.

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Improved Procedure for Determination of Acrylonitrile in Foods and Its Application to Meat

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The previously described headspace-gas chromatographic procedure for the determination of acrylonitrile (AN) in several foods, with N/P selective detection, has been modified to include packaged luncheon meats. The loss of AN during equilibration at 100°C in meat samples as well as the previously described loss in cold pack cheese and peanut butter has been studied. The loss of AN could be prevented by the addition of 10% phosphoric acid, which increases the acidity of the food–acid–salt slurry to pH 1.2–1.5. This acidification permits detection of AN at 2 ppb (5% FSD at 16 × 10^{-2} amp/mV) in all foods studied. AN was not detected in 10 samples of luncheon meat packaged in AN-based plastic which contained up to 2.6 ppm AN.

Previously, we reported a general procedure for the determination of acrylonitrile (AN) in margarine, honey butter, cold pack cheese, and peanut butter (1). In this technique, the entire contents of the container were blended with water and sodium chloride at < 5°C, aliquots were sealed in crimp-top headspace vials, and the vials were equilibrated 2 h at 100°C. The headspace was then sampled with a heated syringe and acrylonitrile was determined by gas chromatography with an N/P selective detector. Propionitrile (PN) was included as an internal standard and calibration curves for each food type were constructed.

At that time, it was noted that AN in fortified samples of peanut butter and cold pack cheese decreased after 1 h equilibration whereas PN, the internal standard, equilibrated to a steady level. It was speculated that this loss of AN during equilibration was due to a decomposition or reaction with proteinaceous material in the sample. Nevertheless, acceptable repeatability was attained using a 2 h equilibration, despite the continually decreasing concentration of AN, and a successful collaborative study resulted in the method being approved official first action (2).

Consistently low recoveries (60–67%) from peanut butter suggested that further study on the loss of AN during analysis was warranted. Furthermore, the determination of AN in luncheon meat by using the same procedure resulted in even lower recoveries with rapid losses during equilibration. Luncheon meat packages can be produced from plastic resins of high AN content. These high nitrile "barrier" resins have low oxygen permeability and give a longer shelf life for meat, but do not directly contact the food because a polyethylene layer is laminated to the food side of the resin. However, residual AN could conceivably migrate through the polyethylene layer into the meat, but, as mentioned above, methodology is not adequate to detect this migration.

This report describes our studies on the analytical deficiencies outlined above. The loss of AN during analysis was investigated and an improved procedure was developed which overcame this loss in the previously studied proteinaceous foods. This procedure was also shown to be applicable to luncheon meats. Ten luncheon meat samples as well as their laminated containers were then analyzed for residual AN. No migration was found.

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This paper was presented at the 96th Annual Meeting of the AOAC, Oct. 25–28, 1982, at Washington, DC.

METHOD

Apparatus

(a) *Gas chromatograph.*—As described previously (1).

(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 (3) or equivalent. Heat syringe as described previously (1). Use 2 in. 22 gauge side port needle. Mark metal plunger with annular groove to facilitate repeatable 1 mL sampling.

(c) *Water bath.*—As described previously (1).

(d) *Headspace sample vials.*—As described previously (1).

(e) *Pipet.*—Mohr or serological, 10 mL.

(f) *Blender.*—Sorvall, with different size containers.

Reagents

Caution: Acrylonitrile is a carcinogen. Observe all necessary safety precautions. Carry out all manipulations and dilutions in fume hood.

(a) *Margarine, honey butter, cold pack cheese, peanut butter, luncheon meat.*—As purchased in local retail stores. Packaged in glass or non AN-based plastic as available.

(b) *Acrylonitrile (AN).*—Available from Aldrich Chemical Co.

(c) *Propionitrile (PN).*—Available from Aldrich Chemical Co.

(d) *Phosphoric acid solution.*—Add H_3PO_4 to water and dilute to give 10% (v/v) aqueous H_3PO_4 solution.

(e) *Standard solutions.*—Refrigerate all solutions. Prepare all solutions using procedures and dilutions described previously (1), noting that MEHQ (methyl ether of hydroquinone, *p*-methoxyphenol) is not used in distilled water.

Preparation of Calibration Curves

Prepare calibration curves using dilutions and procedures described previously (1) except use 10% H_3PO_4 in place of water (with 1% MEHQ) whenever the latter is indicated. For meat samples, PN is obscured by interfering peaks; therefore, plot H_a (peak height of AN) vs ppb AN and draw best line through points.

Preparation of Sample

Prepare sample for analysis as described previously (1) except use 10% H_3PO_4 in place of water (with 1% MEHQ) whenever the latter is indicated. For luncheon meat, to detect possible AN migration, open refrigerated sample and remove only the slice contacting laminated plastic. For comparable blank samples, remove slice from middle of package.

Headspace Technique

Follow headspace procedure as described previously (1). With locking-type syringe, obtain reproducible headspace samples by aligning mark (1 mL) on metal syringe plunger with hexagonal plunger stop of syringe. After sampling, and before withdrawing needle from headspace sample vial, lock headspace sample in syringe by depressing button. Insert needle of syringe into gas chromatographic injection port, open locking button, and depress plunger to inject sample. For luncheon meat samples, 3 min after AN elutes, increase

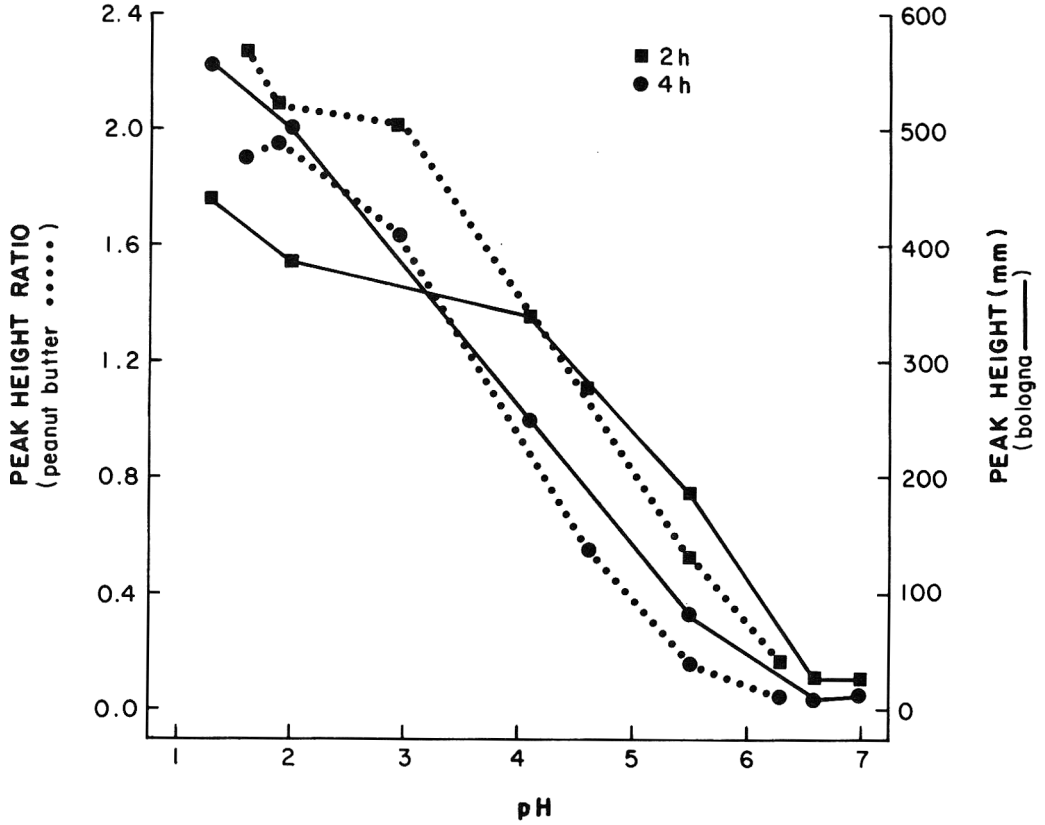

Figure 1. AN peak height for luncheon meat or AN–PN peak height ratios for peanut butter vs pH after 2 or 4 h equilibration when spiked at 100 ppb AN.

oven temperature to 200°C until baseline begins to decrease and then re-equilibrate oven at initial temperature.

Calculation

Calculate ppb AN as before (1). For luncheon meat samples determine average H_a from duplicate samples and determine ppb AN from calibration curve.

Results and Discussion

The loss of AN during analysis of peanut butter and cold pack cheese described previously (1) was attributed to reaction with proteinaceous material in the food. With luncheon meat samples, using the same food–water (1% MEHQ)–salt slurry, a similar loss occurred over time. These losses could be due to an enzymatic process or a simple chemical reaction. Acrylonitrile has been shown to bind irreversibly to proteins and react with reduced glutathione during in vitro experiments (4). Furthermore, chemical reactions, such as nucleophilic addition of thiols, have been widely studied (5). Possible enzymatic or simple chemical reaction of AN could be minimized by the addition of compounds with an analogous α,β-unsaturated structure. These compounds, if in high enough concentration, could favorably compete with AN for an enzyme or nucleophile. However, in our studies, a 1000-fold excess (weight basis) of acrylic acid, methacrylic acid, or methacrylonitrile added to peanut butter slurry with 100 ppb AN still resulted in significant loss of AN.

Acidic pH is known to inactivate enzymes; furthermore, the addition reaction of thiols to AN is known to be base-catalyzed and at lower pH this reaction would be slower. Therefore, food:buffer/acid mixtures were spiked at 100 ppb AN to investigate the effect of pH on AN stability. Figure 1 demonstrates the effect of pH on peak height or relative peak height after heating for 2 or 4 h for peanut butter and luncheon meat. Thus, a pH of 2 or less was necessary to prevent loss of AN during the headspace equilibration for peanut butter or luncheon meat.

For the luncheon meat and peanut butter data in Figure 1, salt was not included in the sample homogenate. When salt was added, peak heights for AN and PN increased by 1.4. Furthermore, addition of salt to the peanut butter–10% phosphoric acid slurry reduced the slurry viscosity to a runny consistency which facilitated pipet transfer. Phosphoric acid was chosen over citric or oxalic acid as the acidic slurry component, primarily because of its availability.

For the luncheon meat samples, peaks arising from a meat–acid interaction after 2 or 4 h heating interfered with the PN internal standard. This interference contributed 40–50% of the total PN peak height at pH 1.3, 10–25% at pH 2, and 10–15% at pH 4.1 or 5.5. Only at pH 6.6 or 7.0 did the interference disappear. Thus, for luncheon meat, PN was not used as an internal standard and AN as an external standard was used. For peanut butter, the interference with PN was only 1–4% and its occurrence was not related to pH. Other possible internal standards were investigated for luncheon meat, including butyronitrile, isobutyronitrile, and 3-butenitrile. They were not suitable because longer equilibration times were needed. In luncheon meat samples, other peaks with retention times and peak heights greater than those of PN were also formed, and it was necessary to elute these peaks by increasing the column temperature after each sample.

The loss of AN during the analysis of luncheon meat at pH > 2 was further investigated. Unacidified meat slurries were heated for 5 h at 100°C, cooled, spiked, and equilibrated for 2 h at 100°C. Acidified samples were left 5 h at room temperature, neutralized with sodium hydroxide to pH 5.5, spiked, and equilibrated for 2 h at 100°C. In both of these experiments, the loss of AN still occurred. Additional samples of unacidified slurries were spiked and left at room temperature for 1 and 2 h, acidified, and equilibrated as above. In this

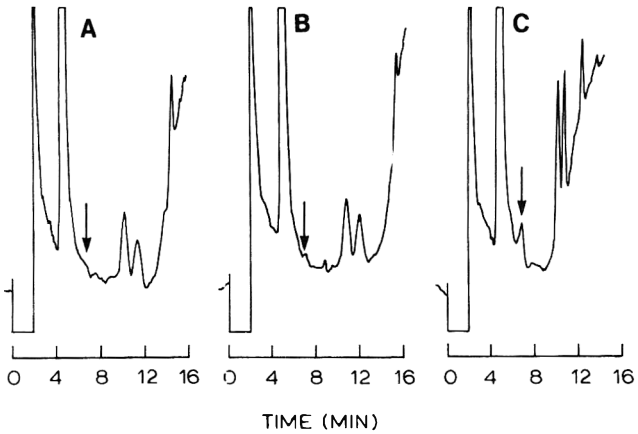

Figure 2. Chromatograms of headspace from same package of luncheon meat: A, slice from middle of package; B, slice contacting package; C, slice from middle of package spiked with 5 ppb AN. Arrow indicates retention time of AN. Column temperature increased to 200°C after 13, 13, and 9 min for A, B, and C, respectively.

case no loss of AN occurred. When non-acidified samples, spiked with AN and heated to 100°C for 2 h, were cooled, acidified, and re-equilibrated, AN was not recovered. Acidification, therefore, could not recover the reacted/degraded AN. These experiments indicate that the loss of AN occurs during the equilibration as the sample is heated to 100°C, that the agents responsible for the loss are not removed or inactivated by heat or by acid, and that a chemical rather than enzymatic process is involved.

Samples of honey butter, cold pack cheese, peanut butter, and margarine with 10% H_3PO_4 and salt as 1:1:0.5 slurries were then spiked with 100 ppb of both AN and PN. Heating for periods from 2 to 5 h showed little if any change in the AN/PN peak height ratio, with the peak heights themselves being relatively constant after equilibrium had been obtained. The peak heights for AN were similar to those obtained with the luncheon meat. The prevention of AN loss with an acidic slurry also increases the detection of AN at low levels compared to matrices in which AN degrades. Spiking luncheon meat and the above foods at 5 ppb suggest ca 2 ppb AN (5% FSD at 16×10^{-12} amp/mV) could be considered the detection limit for all foods This is a different situation than that reported previously (1) for non-acidified food slurries, in which the AN response for different foods varied by a factor of 3. The AN detection with the acidified samples was also increased in the developed procedure with the use of a locking gas-tight syringe. The pressurized 1 mL headspace is ca 1.8 times a non-pressurized sample. The amount of AN that partitions into the headspace at 100°C for the various foods examined ranged from 10 to 13%.

Repeatability studies for luncheon meat (bologna) were carried out at 5, 50, and 100 ppb. The coefficients of variation ($n = 6$) for the AN peak heights were 17.5, 9.1, and 6.6%, respectively. For comparison, the previously reported (1) coefficients of variation of the AN–PN peak height ratios for margarine, honey butter, cold pack cheese, and peanut butter spiked at 20 ppb ranged from 7.5 to 10.2%. At 100 ppb, they ranged from 1.9 to 7.1%. These results show a slight increase in precision when an internal standard is used. With the acidic food slurries, linear calibration curves were obtained using the AN–PN peak height ratios for the 4 foods described above. A linear calibration curve of AN peak height vs ppb AN was also obtained for luncheon meat.

With the stability of AN assured in a number of food matrices, a limited survey of luncheon meats was then undertaken. Seventeen samples packaged in "blister-packs" were purchased locally. Infrared examination of the constituent parts of the package showed 10 package fronts with absorptions at 2250 cm^{-1}, indicative of a nitrile-based polymer. The meat slice next to the nitrile-based polymer in these samples was then analyzed for AN according to the developed procedure. Slices of meat from the center of the package served as blanks or were spiked at 5 ppb. Figure 2 shows chromatograms of the center slice blank (A), the plastic-contacting slice (B), and the center slice spiked at 5 ppb (C). These chromatograms indicate that 2 ppb AN (ca 5% FSD at 16×10^{-12} amp/mV) in meat would be readily detectable and can, as mentioned above, be considered as the detection limit. The small peak in B indicated by the arrow is less than this detection limit. No AN was detected in any of the plastic-contacting slices from the 10 samples of luncheon meat examined. These 10 samples represented 5 food companies and a variety of luncheon meats such as mock chicken, ham, salami, pizza loaf, and several types of bologna. No interferences close to the AN peak were noted. Figure 2 also illustrates the interfering peaks arising after the AN elutes and as the temperature is increased.

The 10 blister-packs showing the nitrile absorption mentioned above were further analyzed. A polyethylene layer which contacted the food was physically separated from the AN-based layer. In 2 samples, the separated polyethylene was 23% by weight. Intact plastic samples, comprised of the 2 layers, were cut into small pieces, 1 g was weighed into a headspace vial containing propylene carbonate, and PN was added as an internal standard. After heating 1.5 h at 100°C to dissolve the AN-based polymer, the vial was swirled to ensure mixing and further equilibrated for 0.5 h. A 1 mL headspace sample was then taken and analyzed using N/P detection. A blank AN-based resin, containing low amounts of AN, was prepared by stripping AN from a plastic-propylene carbonate solution with nitrogen and then spiking for quantitative measurements. The results were corrected for the weight of polyethylene in the laminate. All 10 samples analyzed were found to contain AN with levels ranging from 1.59 to 2.58 ppm.

The proposed method, with an acidic sample matrix, presents a modification of an earlier procedure (1) and permits the analysis of AN in luncheon meat and other protein-containing foods without loss during analysis. With this acid medium, and a locking syringe, an increased and approximately equal method sensitivity is attained for all foods examined. Acidified luncheon meat, however, gives peaks which interfere with PN, and it cannot be used as an internal standard. With an external standard, slightly higher repeatability is found. AN was not detected in 10 samples of various luncheon meats packaged in AN-based plastic.

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Report on the Twenty-Eighth Annual Meeting of the Collaborative International Pesticides Analytical Council (CIPAC)

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Wageningen, The Netherlands

The 28th Annual Collaborative International Pesticides Analytical Council (CIPAC) Meeting was held in Baltimore, MD (USA), at the Baltimore Community College. The meeting was organized by Warren R. Bontoyan, U.S. member of CIPAC.

Mr. Bontoyan welcomed the participants and introduced Steve Schatzow, Director of the Pesticides Program of the U.S. Environmental Protection Agency (EPA). Mr. Schatzow welcomed CIPAC members on behalf of EPA, and said that the role CIPAC plays in terms of method development is important. He indicated that the development of standard methods to support regulation is important for health protection and for international trade, and that the participation of many countries in developing these standard methods of analysis is essential. Mr. Schatzow further said that EPA has an interest in the impurities and breakdown products of pesticides. In this context, Mr. Schatzow referred to the 2,3,7,8-TCDD study and CIPAC's part in developing a provisional method for 2,3,7,8-TCDD in 2,4,5-T acid and technical esters. In closing, Mr. Schatzow wished CIPAC a fruitful meeting.

David MacLean, Executive Director of AOAC, also addressed the CIPAC meeting, and said he was pleased that CIPAC would be present at the 100th Anniversary of AOAC. He referred to the close cooperation between the 2 organizations over the last 10 years, which has resulted in the adoption of 22 CIPAC/AOAC pesticide formulations methods of 59 adopted by AOAC during this period. He indicated that the agreement between AOAC and CIPAC is an excellent model for collaboration between AOAC and other organizations.

Jean Henriet, Chairman of CIPAC, thanked Mr. Schatzow and Dr. MacLean for their welcome addresses and expressions of support for CIPAC's work. He also thanked Mr. Schatzow for allowing Mr. Bontoyan time from his regular duties to organize the meeting. Dr. Henriet also pointed out that scientists from the People's Republic of China, Finland, South Korea, and Thailand were attending their first CIPAC meeting.

The annual CIPAC Symposium was part of an AOAC/CIPAC Symposium Program, organized by Alan Hanks, West Lafayette, IN and James Launer, Salem, OR. The symposium, *Analysis of Pesticide Products, Impurities, Degradation Methods, and Environmental Concerns*, included papers presented by industry and government scientists from the USA, West Germany, Japan, Canada, and the UK.

New members elected as representatives of the governments of the Philippines, West Germany, and Ireland were C. Gaston, W. Dobrat, and D. O'Sullivan, respectively. CIPAC also elected S. Bailey (UK), W. R. Bontoyan (USA), H. Bosshardt (Switzerland), H. Povlsen (Denmark), and S. Rasero (Spain) as the Committee of Management.

Summary of the CIPAC decisions made at the 28th Meeting

Code No.	*Name*	*Status of the Method*
1. +51. +85.	2,4-D + mecoprop + dicamba	The HPLC method for aqueous solutions, *JAOAC* **67,** 837 (1984), was accepted as *provisional* AOAC–CIPAC method.
52.	dalapon	The HPLC method, *JAOAC* **66,** 1390 (1983), was accepted as *provisional* AOAC–CIPAC method.
74.	dichlofluanid	The provisional HPLC method, CIPAC/3129, was accepted as *full* CIPAC method.
80.	propoxur	The HPLC method for propoxur technical and formulations, *JAOAC* **67,** 497 (1984), was accepted as *provisional* AOAC–CIPAC method.
146.	dazomet	The titrimetric-CS_2 evolution method for technical granules, CIPAC/3184/M, was accepted as *full* CIPAC method.
165.	methiocarb	The HPLC method for methiocarb technical and formulations, *JAOAC* **67,** 492 (1984), was accepted as *provisional* AOAC–CIPAC method.
206.	benomyl	The HPLC method for benomyl wettable powders, *JAOAC* **67,** 302 (1984), was accepted as *provisional* AOAC–CIPAC method.
232.	bendiocarb	The HPLC method for bendiocarb technical and wettable powders, CIPAC/3212/M, will become a *provisional* AOAC–CIPAC method after adoption as an official first action method by AOAC.
239.a	pirimiphos-methyl	The GLC method for the determination of pirimiphos-methyl technical and formulations, CIPAC/3174/M, was accepted as *full* CIPAC method.
261.	bupirimate	The GLC method for the determination of bupirimate technical and formulations, CIPAC/3176/M, was accepted as *full* CIPAC method.

Summary of the CIPAC decisions made at the 28th Meeting (continued)

277.	chlordimeform	The GLC method will become a *provisional* AOAC–CIPAC method after adoption as an official first action method by AOAC and after the data have become available.
283.	metribuzin	The GLC method, *JAOAC* **67,** 840 (1984), for metribuzin formulations was adopted as *provisional* AOAC–CIPAC method.
331.	permethrin	The GLC method for the determination of total permethrin and the *cis-trans* ratio in technical material and formulations, CIPAC/3169/M, was accepted as *full* CIPAC method.
332.	cypermethrin	The GLC method for the determination of total cypermethrin in technical material and formulations, CIPAC/3171/M, was accepted as *full* CIPAC method. The HPLC method for the determination of the diastereoisomer ratio of cypermethrin total, cypermethrin in technical material and formulations, CIPAC/3172/M, was accepted as *full* CIPAC method.
333.	deltamethrin	The HPLC method for the determination of deltamethrin in technical material and formulations, CIPAC/3218/m, was adopted as *tentative* CIPAC method.
350.	pyrazophos	The provisional HPLC method for determination of pyrazophos emulsifiable concentrates, CIPAC/3127/M, was adopted as *full* CIPAC method. The method for the technical material will remain provisional.
352.	triadimefon	The provisional HPLC method for triadimefon technical and formulations, CIPAC/3181/M, was adopted as *full* CIPAC method.
364.	phoxim	The HPLC method for the determination of phoxim in technical material and formulations, CIPAC/3188/(M), was accepted as *provisional* CIPAC method.
366.	bentazon	The HPLC method for the determination of bentazon in technical material and water soluble concentrates, CIPAC/3186/M, was accepted as *provisional* CIPAC method.
395.	fluazifop-butyl	The GLC method for the determination of fluazifop-butyl in formulations, *JAOAC* **67,** 499 (1984), was accepted as *provisional* AOAC–CIPAC method.
Imp. 2	ETU	The HPLC method for the formulation of ETU in ethylenebisdithiocarbamates, CIPAC/3167/M, was accepted as *full* CIPAC method (Referee method). The paper chromatographic method, CIPAC/3167/M2, was accepted as *full* CIPAC method.
Imp. 3	chlorophenols and -cresols	The HPLC method with UV detection for the determination of 2,4-dichlorophenol and 4-chloro-2-methylphenol in phenoxy alkanoic herbicides, CIPAC/3111 and 3112/(M), was adopted as *provisional* CIPAC method.
PP. 239.a	pirimiphos-methyl	The method for the preparation of pure pirimiphos-methyl, CIPAC/3192, was accepted.
PP. 261.	bupirimate	The method for the preparation of pure bupirimate, CIPAC/3193, was accepted.
PP. 242.	ethirimol	The method for the preparation of pure ethirimol, CIPAC/3194, was accepted.
RE 140	octacosane	Method CIPAC/3195 was accepted.
MT 114	correction for interfering peaks	The method for correction of interfering peaks, CIPAC/3196, was accepted.

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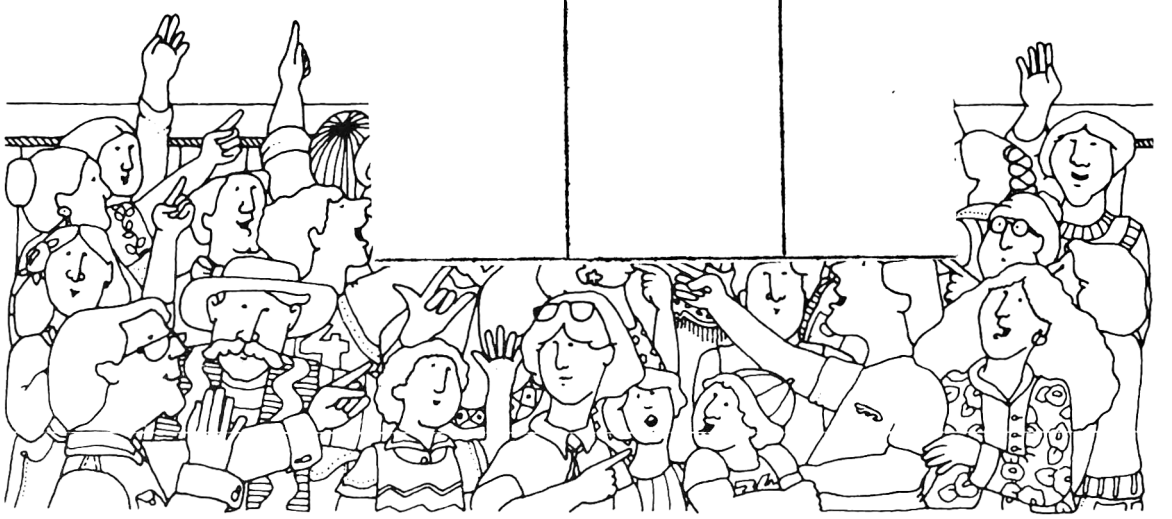

HIGHLIGHTS

SYMPOSIA

Safety Training of Laboratory Analysts and Managers—Chairman: Robert Bianchi

Quality Assurance—Chairman: Susan G. Anderson

Immunotoxicity of Pesticides and Other Toxic Compounds: Its Mechanisms and Up-to-date Methodology for Evaluation—Chairman: Thomas S. S. Mao

Chromatography of Amino Acids and Determination of Amino Acid Composition of Nutritionally Important Protein Sources—Chairman: Charles W. Gehrke

Nutritional Analyses—Chairman: Robert C. Benedict

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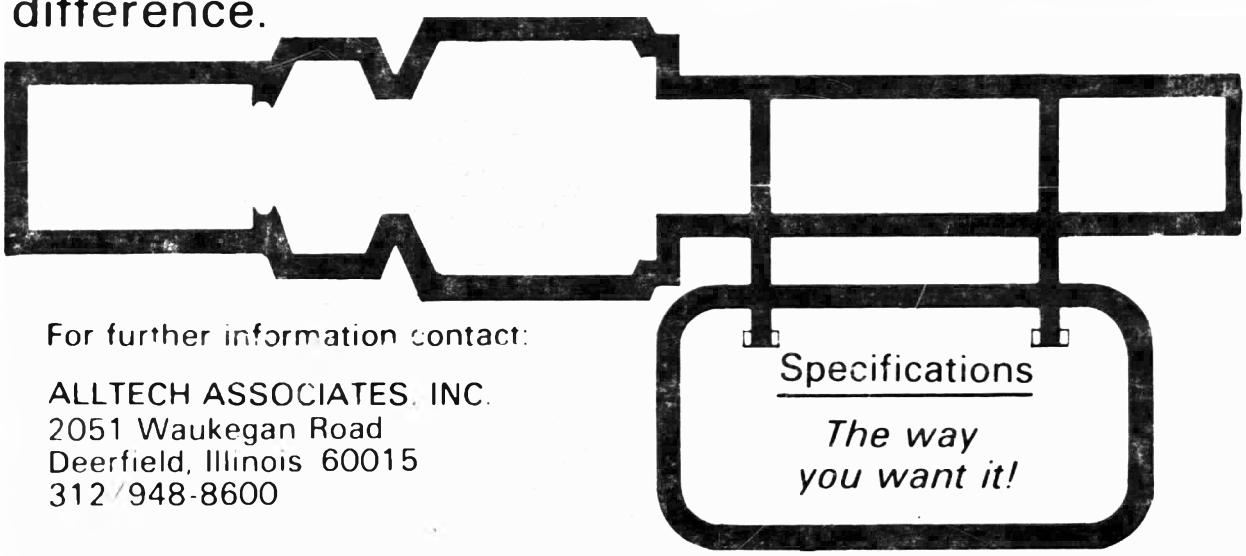

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