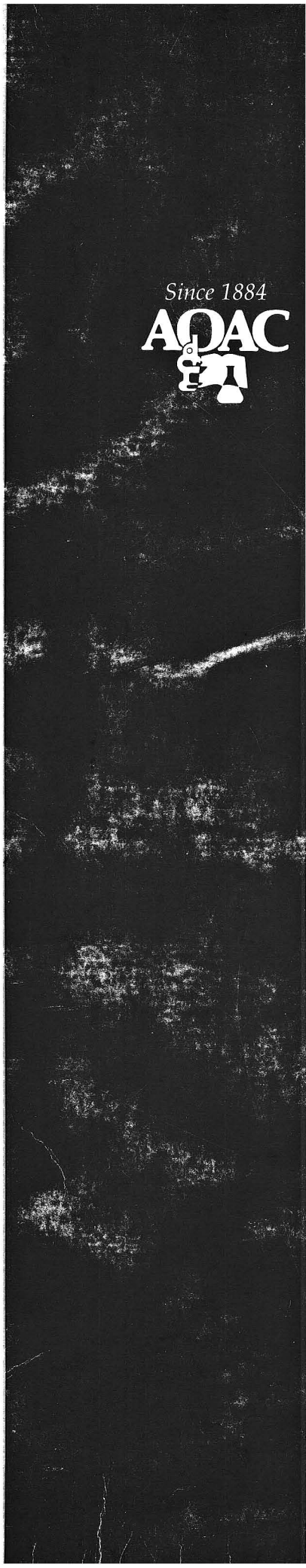
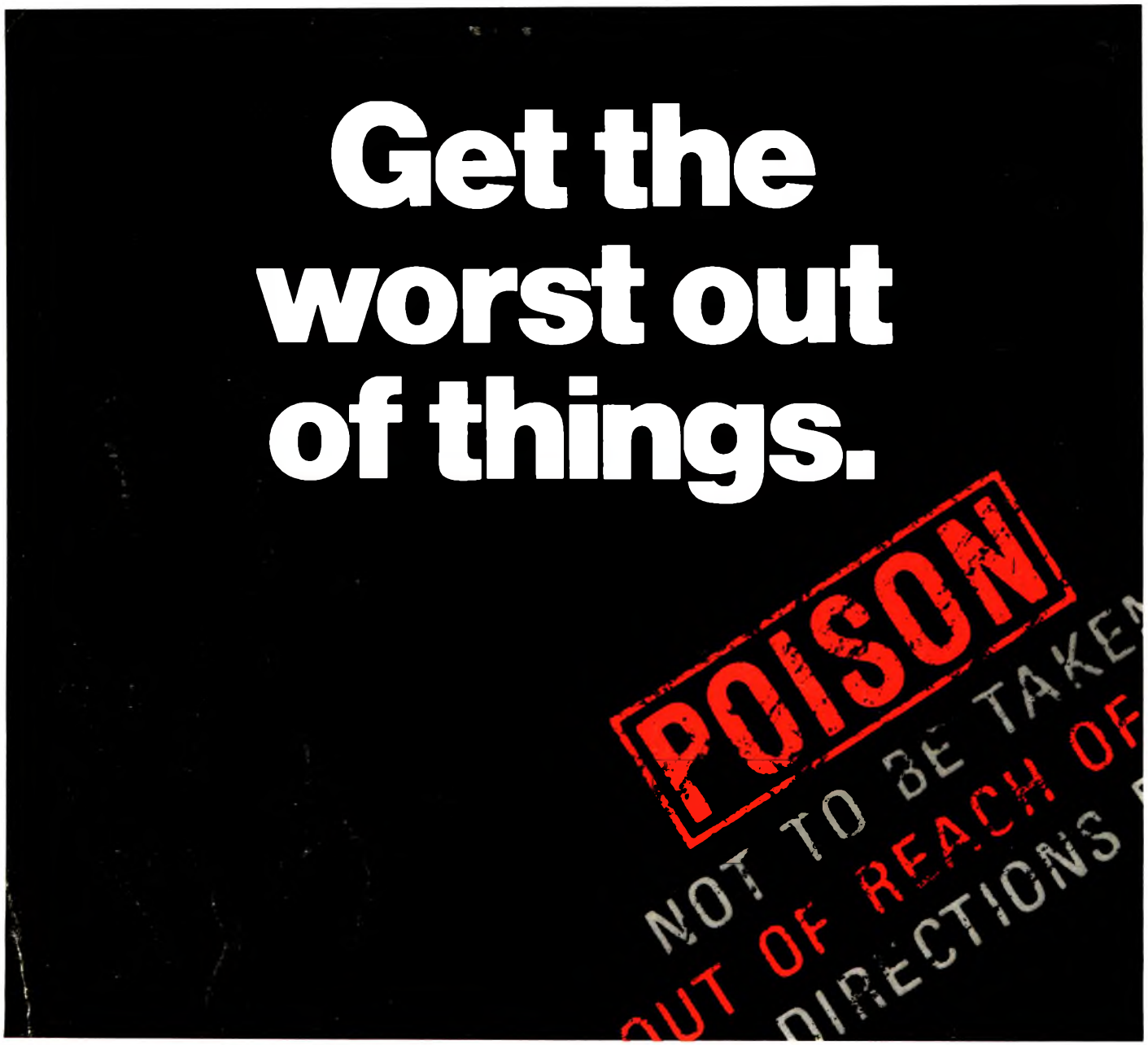


ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

NOVEMBER/DECEMBER 1987
VOL. 70, NO. 6

JANCA 70(6) 931–1112 (1987)
ISSN 0004-5756

Get the worst out of things.



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SGE's Universal Trace Residue Extractor – Unitrex – gets the worst out of your samples. Unitrex has been developed as an efficient and economical clean-up system for the recovery of pesticides and organic residues from meat fats, butter, cheese, vegetable oils and a wide range of samples.

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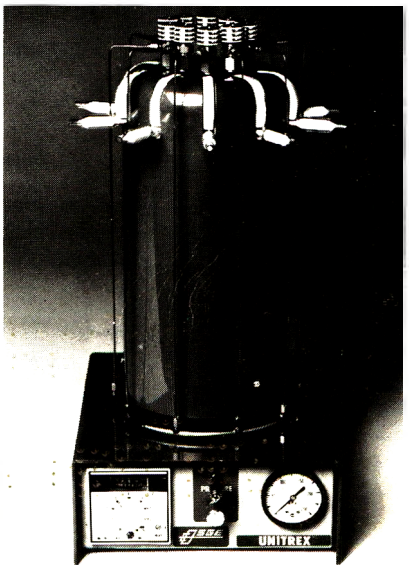

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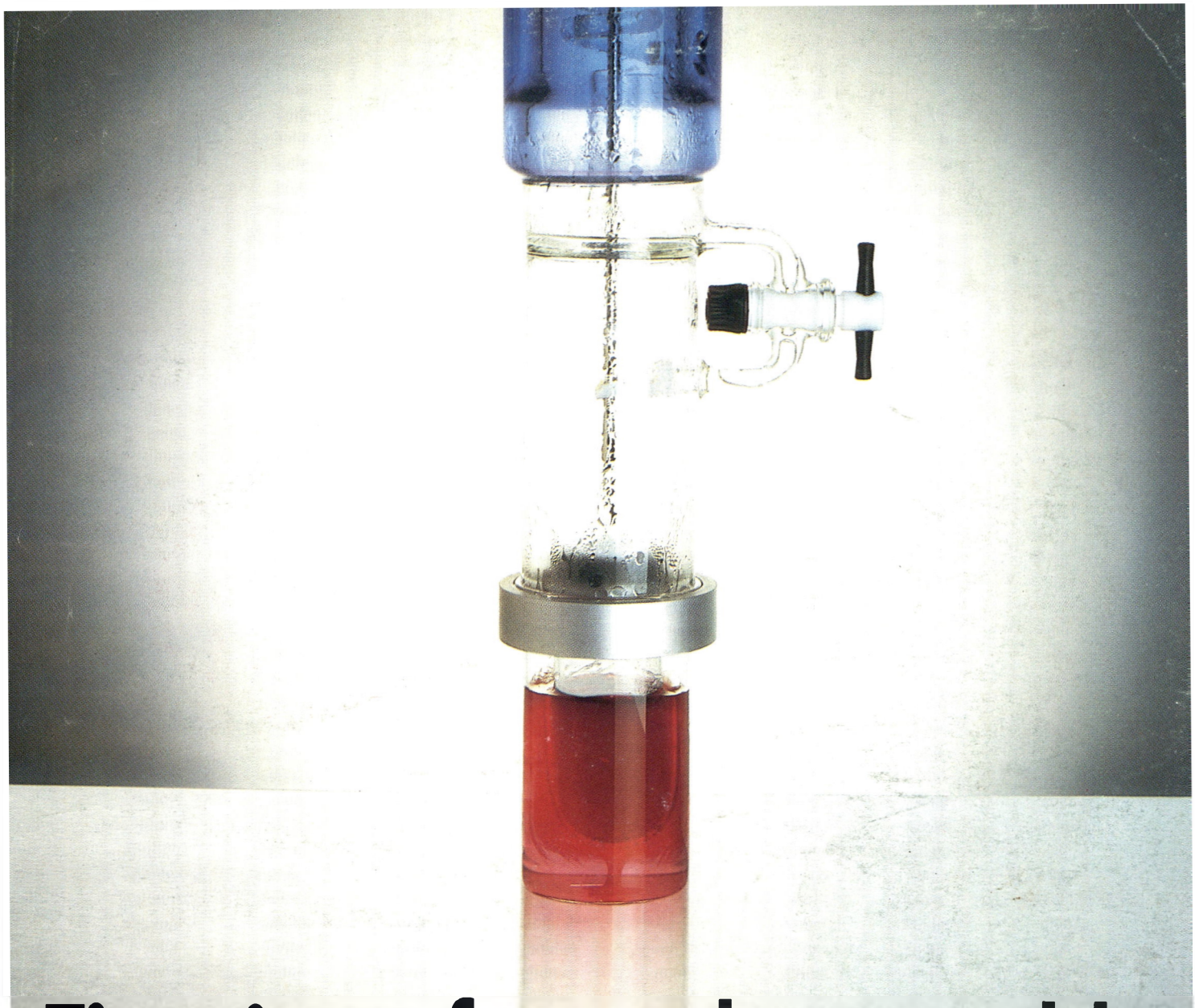

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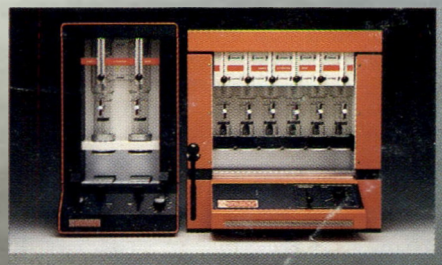

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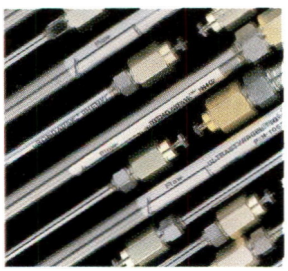

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ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

JOURNAL

NOVEMBER/DECEMBER 1
VOL. 70, NO
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 The *Journal of the Association of Official Analytical Chemists* (ISSN 0004-5756) is published bimonthly by the Association of Official Analytical Chemists, 1111 N 19th St, Suite 210, Arlington, VA 22209. **Subscriptions** for Vol. 70 (1987) are: Members, $84.57 in U.S., $94.57 outside U.S.; Nonmembers, $99.50 in U.S., $109.50 outside U.S. Second class postage paid at Arlington, VA, and additional mailing offices. Abstracted and/or indexed in Analytical Abstracts, Biological Abstracts, Chemical Abstracts, Current Advances in Ecological Sciences, and Current Contents. Printed in USA **POSTMASTER:** Send address changes to AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209.

Drugs

Microbiological Methods

Extraneous Materials

Pesticide and Industrial Chemical Residues

Technical Communications

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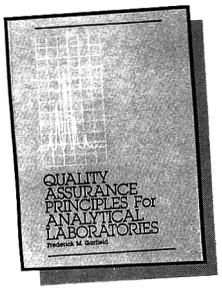
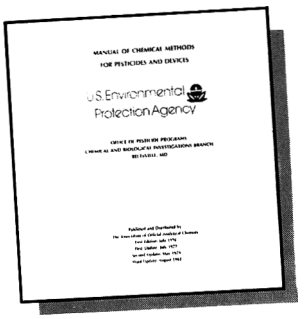


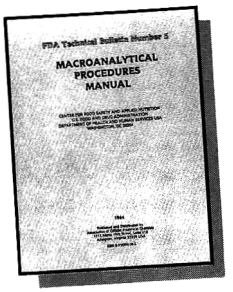
☐ **Quality Assurance Principles for Analytical Laboratories**

1984. 224 pp. Softbound, ISBN 0-935584-26-9.
Members: $41.25 in U.S., $44.25 outside U.S.
Nonmembers: $45.50 in U.S., $48.50 outside U.S.
A handbook for initiating or improving a laboratory quality assurance program.



☐ **EPA Manual of Chemical Methods for Pesticides and Devices**

1983. 1363 pp. With spectra. Includes three supplements and binder. ISBN 0-935584-23-4.
Members: $61.95 in U.S., $64.95 outside U.S.
Nonmembers: $68.50 in U.S., $71.50 outside U.S.
EPA and State laboratory compilation of over 300 currently used methods for analyzing pesticide formulations.



☐ **Macroanalytical Procedures Manual—FDA Technical Bulletin No. 5**

1984. 176 pp. Three hole drill with binder.
ISBN 0-935534-28-5.
Members: $26.25 in U.S., $27.75 outside U.S.
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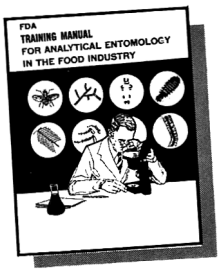
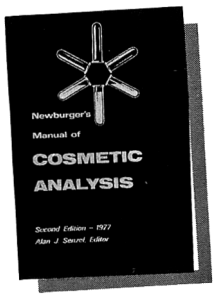


☐ **FDA Training Manual for Analytical Entomology in the Food Industry**

1978. 184 pp. Looseleaf. ISBN 0-935584-11-0.
Members: $26.75 in U.S., $27.75 outside U.S.
Nonmembers: $29.50 in U.S., $30.50 outside U.S.
With the aid of this text, organizations can set up their own in-house training.



☐ **Newburger's Manual of Cosmetic Analysis**

1977. 150 pp. Softbound. ISBN 0-935584-09-9.
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Bio-Rad's new Type 31 pipet tip is designed for reproducible pipetting with Eppendorf Ultra-Micro pipets, both fixed volume and digital. This tip offers the same advantages as other Bio-Rad pipet tips, and it also features the special shank design needed for proper function with very low sample volumes. Bio-Rad Laboratories.

Circle No. 128 on reader service card.

Liquid Chromatography Sample and Reagent Preparation Kit

To aid in the selection of the optimum sample preparation method, Bio-Rad offers a liquid chromatography sample and reagent preparation kit containing filters, disposable columns, 5 ion exchange media, and step-by-step instructions for each application. Bio-Rad Laboratories.

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Tabletop Cone Blender

The "Y" cone blender from Sitco combines a Multipex universal power module with a double cone vessel for tabletop laboratory use. The power module also accommodates other processors for applications such as blending and wet granulating. Sitco.

Circle No. 130 on reader service card.

Hybridoma/Enzyme Immunoassay Screening Kit

Beckman Instruments, Inc., has introduced a new hybridoma/enzyme immunoassay kit which provides all reagents necessary for testing 4000 supernates or forty 96 well plates. The kit includes affinity-purified antibody labeled with horseradish peroxidase, which has a turnover rate 10 times greater than commonly used enzymes. ABTS, the substrate included in the Beckman kit, will not cause premature color development often associated with other substrates. Beckman Instruments, Inc.

Circle No. 131 on reader service card.

Column Heater

The chief distinguishing feature of Analytichem International's new column heater is blocks that accommodate columns of several different sizes ranging up from ¼ in. Analytichem International, Inc.

Circle No. 132 on reader service card.

Variable Wavelength UV-Vis Detector

The SP8450, a variable wavelength UV-visible liquid chromatographic detector, is designed with simplified, long-life optics for sensitivity and stability. The detector uses fiber optics technology and reduces stray light by eliminating lenses and mirrors. Spectra-Physics Autolab Division.

Circle No. 133 on reader service card.

Ultra-Small Column

Liquid chromatography columns with 1.5 μm, nonporous Monospher sorbent particles enable chemists to perform quick, sensitive LC biopolymer separations. The ultra-small silica spheres facilitate both reverse-phase and normal-phase separations of large sample capacities without a loss of resolution. EM Science.

Circle No. 134 on reader service card.

Pump for Low Pressure Solvent Delivery

Bio-Rad has introduced Econo-Column, a 2 channel, variable speed peristaltic pump, which is efficient for applications such as chromatography, sample transfer, circulation systems, pH control, clinical chemistry, and flow cell colorimetry. Bio-Rad Laboratories.

Circle No. 135 on reader service card.

Cell for Preparative Electrophoresis

An alternative for protein purification is provided by the Rotofor cell, capable of achieving greater than 10-fold purification of a protein sample in a single 4 h run, from Bio-Rad Laboratories. The new cell incorporates a cylindrical focusing chamber, divided into 20 discrete compartments, that rotates around its axis. During electrofocusing, proteins move to the point in the pH gradient where they have a zero net charge. After they are focused, the solution in each compartment can be rapidly and easily collected. Bio-Rad Laboratories.

Circle No. 136 on reader service card.

Total Sulfur Analysis

Antek Instruments announces the availability of Pyrofluorescent sulfur systems. These instruments yield accurate, precise analyses of total sulfur content in solid, liquid, and gaseous samples. They cover an analytical range from ppb to 50% and produce final results in 30 s to 10 min. Antek Instruments, Inc.

Circle No. 137 on reader service card.

Cloning Pack and Labeling System

A Dual Promoter Cloning Pak, the SP6/T7 Pak, which includes 2 dual promoter vectors, pDP-1 and pDP-2, allows subsequent transcription by either T7 or SP6 RNA polymerase when producing RNA probes. The SP6/T7 polymerase labeling system allows efficient production of high-specific-activity RNA probes. Du Pont.

Circle No. 138 on reader service card.

Air and Nitrogen Generators

Chrompack, Inc., has announced 2 portable, self-contained gas generators for laboratory use. A new air generator can supply clear, dry, oil-free air for up to 6 gas chromatographs. A new nitrogen unit turns atmospheric air into clean, pure laboratory-grade nitrogen. Chrompack, Inc.

Circle No. 139 on reader service card.

Trace Sulfur and Chlorine Analyzer

Dohrmann announces a new automated microcoulometric sulfur analyzer, the MCTS 130/120, which is easy to use, is simply calibrated, and performs data analysis with statistics. Dohrmann.

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Method Development Liquid Chromatography System

A special configuration of Beckman's System Gold Personal chromatograph meets the particular needs of method development laboratories. Analytical method sets can be created and stored automatically and then recalled to evaluate and modify them at any time. The system uses an IBM PC-AT workstation with color graphics and customized Beckman chromatography software, a binary solvent delivery module, and a simultaneous dual wavelength UV-visible detector module. Beckman Instruments, Inc.

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Autosampler for Liquid Chromatography

Reliability and simplicity of opera-

tion and a free 5 yr warranty are available to purchasers of the SP8775, an autosampler for liquid chromatography, from Spectra-Physics. The SP8775 offers many of the same features and capabilities of the SP8780. Spectra-Physics.
Circle No. 142 on reader service card.

Single or Multiple Pump LC Gradient System

Micromeritics Instrument Corp. offers the 7600 Series for either single-pump, low-pressure gradients or multiple-pump, high-pressure runs. A control interface module and a small hand-held unit together control either one or two Micromeritics Model 760 liquid chromatography pumps. The system software allows multiple methods and steps with up to 6 external events per step. As many as 5 methods can be linked together and 10 different gradient methods can be stored. Micromeritics Instrument Corp.
Circle No. 143 on reader service card.

New Liquid Chromatography Cartridge Columns

Analytichem International, Inc., has introduced Quick Elut liquid chromatography columns, which enable the analyst to tailor the chemistry of the sample preparation sorbent to the analytical column packing. Analytichem International.
Circle No. 144 on reader service card.

Freeze-Drying System

With the E7480 Cyro freeze-drying system, materials researchers, biologists, and food technologists can obtain improved image quality when performing scanning electron microscopy. The E7480 removes water from specimens while preserving their essential structure. It can be modified for use in transmission electron microscopy. BioRad/Polaron Division.
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Redox Chemiluminescence Detector

The new redox chemiluminescence detector for gas chromatography from Sievers Research, Inc., used for the selective detection of oxygen-, sulfur-, nitrogen-, and phosphorus-containing compounds in the presence of saturated hydrocarbons, air, water, and other unreactive species, has demonstrated at least a 3-fold increase in sensitivity over the previous model. Sievers Research, Inc.
Circle No. 146 on reader service card.

Chemical Spill Treatment Kit

A choice of 3 Spill-X spill control agents is available in either individual spill treatment kits for acid, caustic, or solvent spills, or in a multipurpose kit with 2 of each type of agent. All are free-flowing, color-coded dry chemicals that will not themselves produce toxic by-products when used as the manufacturer directs in chemical spills.
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Chromatography Detector Linearizer

Varex Corp. has announced the avail-

ability of a chromatography detector linearizer, Model LD2.00. Developed primarily for evaporative type light-scattering detectors, the LD2.00 will also work with other detectors known to have a nonlinear output with increasing sample concentration. Varex Corp.
Circle No. 148 on reader service card.

Diode Array Detector for Real Time Multiwavelength Measurement

With LiChroGraph L-3000's diode array detector, researchers are able to perform real time LC analysis. Multiwavelength measurement features a 70 element diode array that covers the 200–360 nm portion of the ultraviolet range or the 360–520 nm portion of the visible light spectrum with 2.5 nm accuracy. Peak purity can be verified either by examining absorption ratios at 2 different wavelengths or by comparing spectra from different points in the chromatographic run. EM Science.
Circle No. 149 on reader service card.

Nitrogen Determinations

Antek Pyro-chemiluminescent nitrogen systems can detect and quantitate the total nitrogen content of solid, liquid, or gas samples. Two systems are available: Model 703C, designed for analyzing liquids, gases, and some solids, and Model 707C, with microprocessor-controlled temperature programming for analysis of gases, liquids, and solid samples. Antek Instruments, Inc.
Circle No. 150 on reader service card.

Laser Light Scattering Mass Detector

Varex Corp. announces the second generation of its laser light scattering mass detector for liquid chromatography/gel permeation chromatography, the LLSD MK II. Varex Corp.
Circle No. 151 on reader service card.

Gel Permeation Chromatography Software

GPC Plus is the name given software from Spectra-Physics Autolab Division that is designed to facilitate the calibration and molecular weight distribution calculations required by gel permeation chromatography (or size exclusion chromatography). The chip can be installed in the memory module of the Spectra-Physics SP4270 computing integrator. Spectra-Physics Autolab Division.
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FOR YOUR INFORMATION

Meetings

November 1987: Eastern Ontario/Quebec AOAC Regional Section Meeting. Contact: Milan Ihnat, Agriculture Canada, Land Resource Centre, Ottawa, Ontario K1A 0C6, telephone 613/995-5011.

January 17–20, 1988: Conference on in vitro Options to Animal Tests, sponsored by U. S. Pharmacopeial Convention, Inc., Radisson Suite Resort, Marco Island, FL. Contact: Alice E. Kimball, U. S. Pharmacopeial Convention, Inc., 12601 Twinbrook Parkway, Rockville, MD 20852, telephone 301/881-0666.

Spring, 1988: Northeast AOAC Regional Section Meeting. Contact: Gerald Roach, FDA, 599 Delaware Ave, Buffalo, NY 14202, telephone 716/846-4494.

March 24–25, 1988: National Committee for Clinical Laboratory Standards Annual Meeting, "Testing Today and Tomorrow," Sheraton Meadowlands, East Rutherford, NJ. Contact: John V. Bergen, NCCLS, 771 E Lancaster Ave, Villanova, PA 19805, telephone 215/525-2435.

April 26–28, 1988: AOAC Infant Formula Conference II, Radisson Francis Marion Hotel, Charleston, SC. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

May 12–13, 1988: "Controlled Release: Science and Technology 1988," Victorian College of Pharmacy, Melbourne, Australia. Organized by Royal Australian Chemical Institute, Polymer Division. Contact: R. C. Oppenheim, Victorian College of Pharmacy, Ltd, 381 Royal Parade, Parkville, Victoria 3052, Australia, telephone +61 3 387 7222.

June 1988: Pacific Northwest AOAC Regional Section Meeting. Contact: John Neilson, Neilson Research Corp., 446 Highland Dr, Medford, OR 97504, telephone 503/770-5678.

June 1988: Southeast AOAC Regional Section Meeting. Contact: Frank Allen, Environmental Protection Agency, Reg 4 ESD, Athens, GA 30613, telephone 404/546-3387.

June 7–10, 1988: "Distilled Beverage Flavour: Recent Developments," University of Stirling, Stirling, Scotland. Organized by Sensory Panel of the Society of Chemical Industry Food Group. Contact: J. R. Piggott, Food Science Division, Department of Bioscience and Biotechnology, University of Strathclyde, 131 Albion St, Glasgow G1 1SD, Scotland, telephone 041-552 4400, ext. 2150, telex 77472 UNSLIB G.

June 20–22, 1988: Midwest AOAC Regional Section Meeting, Columbia, MO. Contact: Howard Casper, North Dakota State University, Veterinary Diagnostic Laboratory, Fargo, ND 58102, telephone 701/237-7529.

August 21–24, 1988: "Bioavailability 88: Chemical and Biological Aspects of Nutrient Availability," University of East Anglia, Norwich, UK. Sponsored by Federation of European Nutrition Societies, Federation of European Chemical Societies—Working Party on Food Chemistry, Royal Society of Chemistry, and Royal Society of Chemistry—Food Chemistry Group. Contact: Bioavailability 88, ARFC Institute of Food Research, Norwich Laboratory, Colney Lane, Norwich, Norfolk NR4 7UA, UK, telephone (0603) 56122, telex 975453, telefax (0603) 58939.

August 29–September 1, 1988: 102nd AOAC Annual International Meeting and Exposition, spotlight on "Biotechnology," The Breakers, Palm Beach, FL. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

September 21–23, 1988: 6th International Symposium on Isotachophoresis and Capillary Zone Electrophoresis, Vienna, Austria. Organized by Institute for Analytical Chemistry of the University of Vienna. Contact: E. Kendler, Institute for Analytical Chemistry, University of Vienna, Wahringer Strasse 38, A-1090 Vienna, Austria, telephone (0222) 34 46 30-47 or 53.

January 31–February 3, 1989: 17th Australian Polymer Symposium, "Polymers in a Hostile Environment," Griffith University, Brisbane, Australia. Organized by Royal Australian Chemical Institute, Polymer Division. Contact: D. J. T. Hill, Chemistry Department, University of Queensland, Brisbane 4067, Australia.

March 30–31, 1989: National Committee for Clinical Laboratory Standards Annual Meeting, Sheraton Society Hill, Philadelphia, PA. Contact: John V. Bergen, NCCLS, 771 E Lancaster Ave, Villanova, PA 19085, telephone 215/525-2435.

August 2–7, 1989: 32nd International Congress of Pure and Applied Chemistry, IUPAC, Stockholm International Fairs, Stockholm, Sweden. Organized by Swedish National Committee for Chemistry, Royal Swedish Academy of Sciences. Contact: IUPAC, % Stockholm Convention Bureau, PO Box 6911, S-102 39 Stockholm, Sweden, telephone +46 8 23 09 90, telex +11556, telefax +46 8 34 84 41.

September 25–28, 1989: 103rd AOAC Annual International Meeting and Exposition, The Clarion Hotel, St. Louis, MO. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

September 9–13, 1990: 104th AOAC Annual International Meeting and Exposition, The Clarion Hotel, New Orleans, LA. Contact: Margaret Ridgell, 1111 N 19th St, Suite 210, Arlington, VA 22209, telephone 703/522-3032.

Courses of Study

The Foundation for Advanced Education in the Sciences (FAES) is completing cooperative agreements with several colleges and universities in the Washington, DC, area to accept direct transfer credit of equivalent FAES courses in recognized degree programs. FAES students, thus, may enroll for a degree at one of the cooperating institutions and may take a large number of the courses on the NIH campus in the evening program. The credit transfer will be direct and not subject to negotiation or reduction by the transferring school. Courses taken at FAES will count towards degree programs at the cooperating institutions. Courses will cost $40/credit hour. Contact: Sara Bahn, Foundation for Advanced Education in the Sciences/NIH, 4311 Lynbrook Dr, Bethesda, MD 20814, telephone 301/951-5180.

Short Course for Europe

An AOAC short course, Quality Assurance Short Course for Analytical Laboratories, will be held at the University of Florence in Florence, Italy, December 2–4, 1987. Contact: Margareet Lauwaars, PO Box 153, 6720 AD, Bennekom, The Netherlands, telephone +31-8389-18725.

Rund Is Incoming AOAC President

Robert C. Rund was named 1987–1988 President of the AOAC at the 101st AOAC Annual International Meeting and Exposition held in San Francisco, CA, Sept. 14–17, 1987.

Robert Rund has a long history of service to AOAC. He served as an Associate Referee from 1957 to 1971 and

About AOAC Regional Sections

Benefits

Low Cost Opportunities to:

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- Learn and Improve Leadership Skills
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- Gain Exposure for Your Work and the Work of Your Staff
- Make Valuable Contacts
- Share Common Problems and Solutions with Fellow Analysts
- Become Better Acquainted with the Work of Your Peers

History

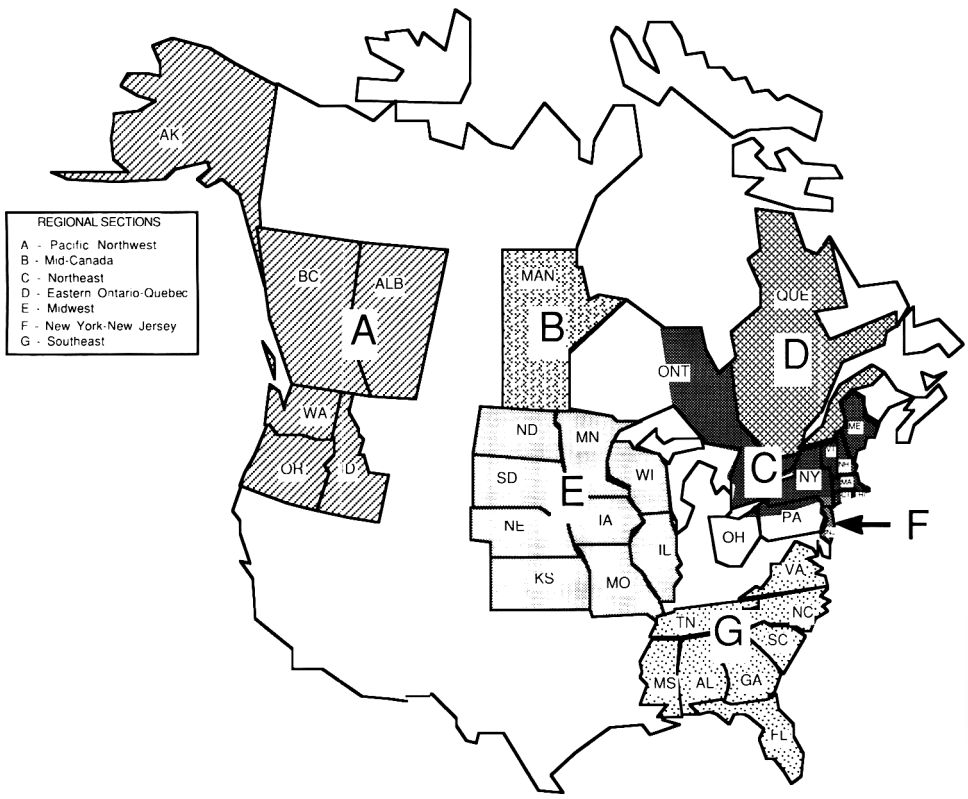
Organization of regional sections under the auspices of AOAC was begun in 1981. The purpose was to provide a mechanism whereby AOAC members and other laboratory analysts could get together regularly and at low cost to share common interests and problems, find ways to solve these problems by providing practical educational seminars and hands-on training workshops, and learn about and become involved in the AOAC methods validation process.

Who May Join

Membership in a Regional Section is open to any individual interested in the purpose of the Regional Section and resident or working within its boundaries. Officers of the section must be members of national AOAC.

Where They Are

There are currently seven chartered AOAC regional sections in the U.S. and Canada: the Pacific Northwest, Midwest, Northeast, Southeast, New-York-New Jersey, Eastern Ontario-Quebec and Mid-Canada – all outlined on the enclosed map. Each regional section is organized by a local volunteer committee with the assistance of the AOAC national office. Several additional sections are in various stages of becoming organized.



Since 1884 AOAC

Join, Help Organize, and Attend an AOAC Regional Section!

as a General Referee from 1969 to 1984. Over the years, he has been a member of the Committee on Safety, the Committee on Statistics, and the Committee on International Coordination. From 1976 to 1986, he was on the Editorial Board, serving as its chairman from 1981. He was elected to the Board of Directors in 1983 and became President-Elect in 1986. AOAC recognized his contributions to the organization by naming him a Fellow in 1975.

A 1948 graduate of Millikin University, Decatur, IL, Rund worked in progressively more responsible positions as a chemist for Swift & Co. before going to Purdue University in 1966. He is currently Senior Administrator in the Office of the Indiana State Chemist at Purdue. As such, he administers the Indiana Commercial Fertilizer Law and the Indiana Agricultural Ammonia Law and programs thereunder. Among other professionally related activities, he was editor of the official publication of the Association of American Plant Food Control Officials from 1970 to 1981; he participated in a quality control seminar at Centro de Estudos de Fertilizantes, São Paulo, Brazil, 1979; and he was chairman of the U.S. Technical Advisory Group of ISO Technical Committee 134, Fertilizers and Soil Conditioners, for AOAC. His professional affiliations include the American Institute of Chemists, of which he is a Fellow, American Society for Quality Control (Senior Member), the American Chemical Society, The Fertiliser Society of London, and the American Association for the Advancement of Science.

Outside his working hours, Rund is actively involved in his church, enjoys golfing and fishing, and recently obtained his private pilot certificate. He and his wife, Betty, have 2 daughters and a grandson.

1987–1988 AOAC Board of Directors

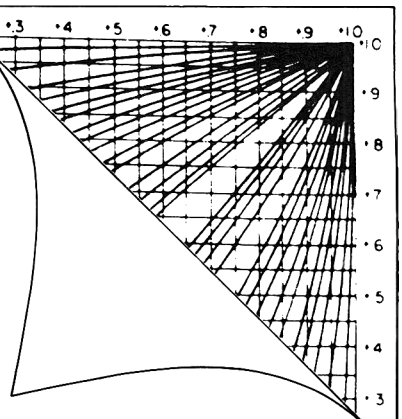
The following individuals will serve on the 1987–1988 Board of Directors with newly named AOAC President Robert C. Rund: President-Elect Odette L. Shotwell, Secretary/Treasurer Thomas G. Alexander, Directors Thomas P. Layloff, Albert W. Tiedemann, and H. Michael Wehr, and Past-President Frank J. Johnson.

General Referee Award to Ross

The 1987 winner of the General Referee Award, granted by the Official Methods Board in recognition of outstanding leadership and substantial contribution to method development, is P. Frank Ross, General Referee for Veterinary Analytical Toxicology. In that capacity, Ross oversees method development in 17 topic areas. Under his leadership, 4 official methods have been adopted.

Frank Ross is an analytical chemist employed at the National Veterinary Services Laboratories of the U.S. Department of Agriculture in Ames, IA. He has been actively involved with AOAC for several years, beginning with a presentation at the Annual Meeting in 1974.

AOAC Announces the Publication of an Indispensable Statistical Reference Book



USE OF STATISTICS TO DEVELOP AND EVALUATE ANALYTICAL METHODS

Grant T. Wernimont, Author
William Spendley, Editor

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

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

Other features include scores of specific statistical analyses of real-life data, many useful statistical tables, and very complete references.

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

Contents

1985. xvi + 183 pages. Softbound. ISBN 0-935584-31-5.
Price—Members: $47.55 in U.S., $50.55 outside U.S.; Nonmembers: $52.50 in U.S., $55.50 outside U.S.

To obtain book, send order with your name and address and check to:

AOAC, 1111 N. 19th Street, Suite 210-J, Arlington, Virginia 22209 USA

(USA funds only)

Collaborative Study of the Year Award—1987

The Official Methods Board named Elaine A. Bunch winner of the Collaborative Study of the Year Award for 1987 for "Analysis and Identification of Dexamethasone in Bulk Drugs and Elixirs." Bunch works for FDA in Seattle.

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

Alred and Williams Named to Posts in England

The 1987–1989 president of the Association of Public Analysts (APA) in England is John Barry Alred, Public Analyst, Greater Manchester Council. Alexander Williams is the new director of the Department of Trade and Industry's Laboratory of the Government Chemist, succeeding Ronald Coleman, who served as Government Chemist for 5 years. During his career with Monsanto Co., Southern Instrument, and National Physical Laboratory, Williams has engaged in research programs on electronic instrument development, radioactivity, neutron dosimetry, and the use of isotopes in analysis.

UK National Nutrient Databank

The Royal Society of Chemistry (RSC) and the Ministry of Agriculture, Fisheries and Food (MAFF) of the United Kingdom are collaborating to produce a new UK National Nutrient Databank developed from *The Composition of Foods* begun by R. A. McCance and E. M. Widdowson in the 1930s and last published as the 4th Edition in 1978. New data will be provided by MAFF from analyses specially commissioned for the databank; these data will be supplemented by data from other sources. Before inclusion in the published databank, all data will be reviewed by an expert committee, and the selected nutrient values will become the accepted UK figures.

The database will be available on tape and possibly as an online service, but the major use will probably be in combination with manipulative software on a microcomputer. The data will also be made available for use by other software vendors for inclusion in their packages.

For further information, contact Ian Unwin, Product Manager, The Royal Society of Chemistry, The University, Nottingham NG7 2RD, UK, telephone (0602) 507400, telex 37488.

NBS Updates

In a new joint program, the National Bureau of Standards (NBS) and the U.S. Department of Agriculture (USDA) will develop standard reference materials containing certified concentrations of nutrients—fat, starch, sugar, vitamins, and nutrient elements such as potassium, iron, zinc, and sodium. These materials will be used to evaluate studies of nutrient roles in health and disease, to establish dietary nutrient requirements, and to monitor foods for nutrients and contaminants. For more information, contact John Henkel, National Bureau of Standards, Gaithersburg, MD, 20899, telephone 301/975-2762.

A new publication by NBS, *The ABC's of Standards-Related Activities in the United States* (NBSIR 87-3576), provides information on the history of standardization, types of standards, private standards groups in the United States, standards development procedures, and the benefits and problems of standardization such as participation in the standards process by qualified consumer representatives. For copies, send a self-addressed mailing label to Maureen A. Breitenberg, A629 Administration Bldg, National Bureau of Standards, Gaithersburg, MD 20899, telephone 301/975-4031.

Mass Spectral Database for PCs Available from NBS

The National Bureau of Standards (NBS), the Environmental Protection Agency (EPA), and the Mass Spectrometry Data Centre (MSDC) in Nottingham, England, jointly maintain the NBS/EPA/MSDC Mass Spectral Database, heretofore available as a computer-magnetic tape format and as a 7000 page reference. NBS has recently announced a new PC version that is adapted for easy access by the bench scientist to large numbers of spectra. The search system is fast enough to allow the user to conduct multistep interactive searches. It is designed to be stored on a hard disk of any AT- or XT-class PC, where it occupies between 8 and 15 megabytes, depending on how many search options are needed by the user. The programs search the database either for spectra of specific chemicals according to chemical name, chemical formula, molecular weight, or Chemical Abstracts Registry Number or for spectra which have preselected characteristics such as peaks at certain masses.

For information on the database or to obtain a license agreement, contact Office of Standard Reference Data, A320 Physics Bldg, National Bureau of Standards, Gaithersburg, MD 20899, telephone 301/975-2208.

ISO Standards Published

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

ISO 1854-1987: Whey cheese—Determination of fat content—Gravimetric method (Reference method). $20.00.

ISO 6651 (2nd Ed.)-1987: Animal feeding stuffs—Determination of aflatoxin B_1 content. $20.00.

VDI Guidelines

Verein Deutscher Ingenieure (VDI)-Commission on Air Pollution Prevention has published the following guidelines. German-English editions are available, at the prices indicated, from Beuth Verlag GmbH, PO Box 1145, D-1000 Berlin 30, FRG.

VDI 2267. Part 4: Chemical Analysis of Particulates in Ambient Air. Determination of Lead, Cadmium and Their Inorganic Compounds as Part of the Dust Precipitation by Atomic Absorption Spectrometry. DM 36,70.

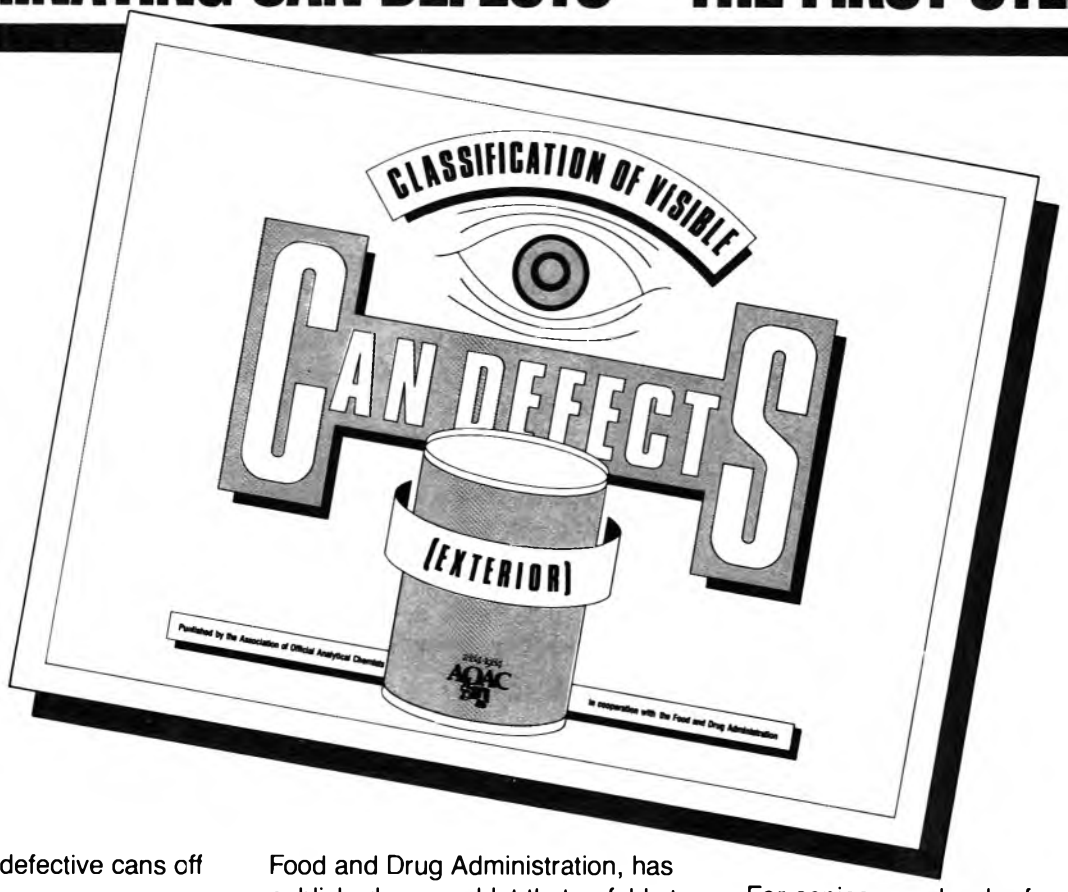
VDI 2267, Part 6: Chemical Analysis of Particulates in Ambient Air. Measuremen: of the Mass Concentration of Cadmium by Atomic Absorption Spectrometry. DM 36,70.

VDI 2268, Part 1: Chemical Analysis of Particulate Matter.Determination of Ba, Be, Cd, Co, Cr, Cu, Ni, Pb, Sr, V, Zn in Particulate Emissions by Atomic Spectrometric Methods. DM 41,50.

VDI 2452, Part 3: Gaseous Air Pollution Measurement. Measurement of Fluoride Ion Concentration. Sorption Method with Prepared Silver Balls and Heated Membrane Filter. DM 41,50.

VDI 2463, Part 9: Particulate Matter Measurement. Measurement of Mass Concentration in Ambient Air. Filter

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″ × 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, send order form and check to:

Association of Official Analytical Chemists
1111 North 19th Street, Suite 210
Arlington, VA 22209 USA
Telephone: (703) 522-3032

Minimum Order:
1 package of 10 charts
$40.00 plus $3.00 postage

Second package
$30.00 plus $3.00 postage

Each additional package
$25.00 plus $3.00 postage

I enclose $_____ for the following quantity of Can Defects charts:

Minimum order:

_____ First package of 10 charts @ $40.00 plus $3.00 postage $_____

_____ Second package of 10 charts @ $30.00 plus $3.00 postage _____

_____ Additional packages of 10 charts @ $25.00 plus $3.00 postage _____

Total _____

Name _____

Company _____

Address _____

City, State, Zip _____

Method. LIS/P Filter Device. DM 41,50.

VDI 3863, Part 1: Measurement of Gaseous Emission. Determination of Acrylonitrile. Gas Chromatographic Method. Grab Sampling. DM 36,70.

EPA Project Summaries

Some organic contaminants of water, the trihalomethanes (THMs), are primarily the by-products of the chlorination process used to disinfect water. Environmental Protection Agency (EPA) Method 510.1, "The Determination of the Maximum Total Trihalomethane Potential," was developed to determine the reasonable maximum total THMs currently present within a system and is applicable to the finished drinking water for those groundwater supplies that have failed the test for the presence of excess disinfectant. A collaborative study was conducted to determine the acceptability and understandability of the method before use in the regulated community. A major conclusion from the study was that the method is particularly complex and therefore susceptible to failure when applied by inexperienced analysts—although in the hands of a qualified analyst it does work. The full report, "USEPA Method Study, Method 510.1, the Determination of the Maximum Total Trihalomethane Potential" (Order No. PB 87-170 825/AS), can be obtained for $13.95 (subject to change) from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, telephone 703/487-4650. The EPA project officer, Harold Clements, can be contacted at: Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

A project was designed to determine statistically the length of time a sample can be stored for analysis of 10 National Pollutant Discharge Elimination System (NPDES) compliance parameters and 2 Safe Drinking Water Act (SDWA) parameters. The 10 NPDES parameters were phenols, cyanide, mercury, ammonia, nitrate plus nitrite, fluoride, total Kjeldahl nitrogen, total phosphorus, total organic carbon, and sulfide. The 2 SDWA parameters were nitrate and fluoride. The experimentally determined maximum holding times (MHTs) from this study were longer than proposed MHT values published in the *Federal Register* and longer than those recommended by EPA for all parameters except mercury and cyanide. The complete report, "Development of Preservation Techniques and Establishment of Maximum Holding Times: Inorganic Constituents of the National Pollutant Discharge Elimination System and Safe Drinking Water Act" (Order No. PB 87-132 833/AS), can be obtained for $18.95 (subject to change) from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, telephone 703/487-4650. The EPA project officer, Daniel F. Bender, can be contacted at: Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

Newly Adopted Methods

The following methods were approved interim official first action by the respective methods committees and by the chairman of the Official Methods Board and were adopted official first action at the AOAC 101st Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA: by the Methods Committee on Drugs and Related Topics—(*1*) Liquid Chromatographic Determination of Flucytosine in Capsules, submitted by D. Shostak and C. Klein (Food and Drug Administration, New York Regional Laboratory, Brooklyn, NY), (*2*) Reverse Phase Liquid Chromatographic Determination of Dexamethasone Acetate and Cortisone Acetate in Bulk Drug Substance and Dosage Forms, submitted by L. L. Ng (Merck Sharp and Dohme, West Point, PA), and (*3*) Resolution and Analysis of the Enantiomers of Amphetamine by Liquid Chromatography on a Chiral Stationary Phase, submitted by M. C. Alembik and I. W. Wainer (St. Jude's Children's Research Hospital, Memphis, TN); by the Methods Committee on Foods II—(*1*) Determination of Dextran in Raw Cane Sugar by the Roberts Copper Dextran Method, submitted by M. A. Clarke and M. A. Godshall (Sugar Processing Research, Inc., New Orleans, LA), (*2*) Determination of Sulfur Amino Acids and Tryptophan in Foods, submitted by M. C. Allred and J. L. MacDonald (Ralston Purina Co., St. Louis, MO), (*3*) Rapid Identification of Color Additives, Using the C_{18} Cartridge, submitted by M. L. Young (Food and Drug Administration, New York Regional Laboratory, Brooklyn, NY), and (*4*) Food Chemicals Codex Gas Chromatographic Method for Mixed Tocopherols Concentrate, submitted by A. J. Sheppard (Food and Drug Administration, Washington, DC); by the Methods Committee on Microbiology—(*1*) Temperature-Independent Pectin Gel Method for Aerobic Plate Count in Dairy and Nondairy Food Products, submitted by J. N. Roth (RCR Scientific, Inc., Goshen, IN), (*2*) Elevated Temperature Method for Recovery of *Vibrio cholerae* from Oysters, submitted by A. DePaola, M. L. Motes, and R. M. McPhearson (Food and Drug Administration, Fishery Research Branch, Dauphin Island, AL), and (*3*) Fluorogenic Assay for Rapid Detection of *Escherichia coli* in Chilled and Frozen Foods, submitted by L. J. Moberg, M. K. Wagner, and L. A. Kellen (General Mills, Inc., Minneapolis, MN); and by the Methods Committee on Feeds, Fertilizers, and Related Topics—Liquid Chromatographic Determination of Triamino-*s*-Triazine Used as Nitrogen Source in Urea Mixes, submitted by B. P. Arcement (MCI AgSystems, Inc., Donaldsonville, LA) and H. N. Levy III (A & E Testing, Inc., Baton Rouge, LA).

Copies of the methods are available from the AOAC office.

Interim Method

The following method has been approved interim official first action by the Methods Committee on Microbiology and the Chairman of the Official Methods Board: ImmunoBand Method for Detection of *Salmonella* in Foods, submitted by R. S. Flowers and M. J. Klatt (Silliker Laboratories, Chicago Heights, IL). The method will be submitted for adoption official first action at the 102nd AOAC Annual International Meeting, Aug. 29–Sept. 1, 1988, at Palm Beach, FL. Copies of the method are available from the AOAC office.

BOOKS IN BRIEF

Preparative Liquid Chromatography. Edited by B. A. Bidlingmeyer. *Journal of Chromatography Library 38.* Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1987. 330 pp. Price: United States $97.50; Dfl. 200.00. ISBN 0-444-42832-1.

This book is intended to assist the researcher in achieving rapid solutions to the most challenging purification requirements. Chapters 1 and 2 are mainly discussions of strategy using applications only to illustrate points. Chapters 3–8 are entirely application-focused.

Experimental Design: A Chemometric Approach. By S. N. Deming and S. L. Morgan. Published by Elsevier Science Publishers, PO Box 211, 1000 AE Amsterdam, The Netherlands, 1987. 294 pp. Price: United States $100.00; Dfl. 225.00. ISBN 0-444-42734-1.

Experimental design is approached from the point of view of the experimenter rather than that of the statistician. The book introduces the reader to the fundamentals of experimental design. Systems theory, response surface concepts, and basic statistics serve as a basis for the further development of matrix least squares and hypothesis testing.

USAN and the USP Dictionary of Drug Names, 1988 edition. Published by United States Pharmacopeial Convention, Inc., 12601 Twinbrook Parkway, Rockville, MD 20852, 1987. 708 pp. Price: $69.50, quantity discounts for 25 or more copies. ISBN 0-913595-23-3.

After 2 editions in which this dictionary provided 3 separate lists of names, this edition returns to the original format of a single main list that includes all names in one alphabetic sequence. All international nonproprietary names (INN) published by the World Health Organization from the start of the INN program in 1953 through 1986 are included. In all, the 1988 edition lists more than 24 000 entries.

Advances in Chromatography, Volumes 26 and 27. Edited by J. Calvin Giddings, Eli Grushka, and Phyllis R. Brown. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY, 1987. Vol. 26, 424 pp.; Vol. 27, 384 pp. Price: United States and Canada $79.75; elsewhere $95.50 (both volumes). ISBN Vol. 26 0-8427-7664-X, Vol. 27 0-8247-7770-0.

Volume 26 includes papers on topics ranging from liquid chromatography for therapeutic drug monitoring and determination of toxicity to application of fleuric devices to gas chromatographic instrumentation. Volume 27 includes derivatization in liquid chromatography, characterization of unsaturated aliphatic compounds by gas chromatography/mass spectroscopy, and others.

Applications of Mass Spectrometry in Food Science. Edited by John Gilbert. Published by Elsevier Applied Science Publishers, Crown House, Linton Rd, Barking, Essex LG11 8JU, UK, 1987. 442 pp. Price: £60.00; $99.00. ISBN 1-85166-081-X.

This book should be of interest to food scientists in studies involving small molecules that can be chromatographed, large molecules such as proteins and inorganic constituents, and those molecules involved with the classification of foods with common characteristics. The techniques discussed were chosen for inclusion to illustrate the range of compound types for which mass spectrometry can be applied. The book also includes chapters on fast atom bombardment.

Quality Assurance of Chemical Measurements. By John K. Taylor. Published by Lewis Publishers, Inc., PO Drawer 519, Chelsea, MI 48118, 1987. 335 pp. Price: $59.95. ISBN 0-87371-097-5.

Chapters in Taylor's book proceed from a statement of the concept of quality assurance to discussions of principles of good measurement, principles of quality assurance, and evaluation of measurement quality.

Biological Substances. International Standards and Reference Reagents 1986. Published by the World Health Organization, Geneva, Switzerland, 1987. Available in the United States from WHO Publications Center USA, 49 Sheridan Ave, Albany, NY 12210; buyers from other countries write World Health Organization, Distribution and Sales Service, 1211 Geneva 27, Switzerland, for information regarding how to order. 94 pp. Price: United States $9.00; Sw. fr. 15. ISBN 92-4-154213-6. Available in English; French and Spanish versions in preparation.

Information on the availability and specifications of international biological standards and reference reagents is offered in this volume, published to help ensure worldwide uniformity in the designation of the potency or specificity of biological preparations that are used in the prophylaxis, therapy, or diagnosis of disease.

Regulating Pesticides in Foods: The Delaney Paradox. By the Committee on Scientific and Regulatory Issues Underlying Pesticide Use Patterns and Agricultural Innovation, Board on Agriculture, National Research Council. Published by National Academy Press, 2101 Constitution Ave, NW, Washington, DC 20418, 1987. 288 pp. Paperbound. Price: $19.95. ISBN 0-309-03746-8.

The impact of the Delaney Clause on agricultural innovation and on the public's dietary exposure to potentially carcinogenic pesticide residues in foods is systematically evaluated. This report of the committee confronts hard questions: Does the zero-risk standard of the Delaney Clause protect the public from cancer-causing pesticide residues in food? Can a negligible-risk standard provide greater protection from potentially cancer-causing residues than the zero-risk standard? How can the EPA reduce dietary cancer risk while preserving economically valuable pesticides? Will adequate alternatives be available if the zero-risk standard of the Delaney Clause is invoked on pesticides registered before 1978?

The Chemistry of Acid Rain: Sources and Atmospheric Processes. Edited by Russell W. Johnson and Glen E. Gordon. *ACS Symposium Series 349.* Published by the American Chemical Society, 1155 Sixteenth St, NW, Washington, DC 20036, 1987. 337 pp. Price: United States and Canada $59.95; export $71.95. ISBN 0-8412-1414-X.

This 27 chapter volume describes the growing understanding of the sources and chemistry of acidic species in the environment. A historical perspective and a summary of past research are presented in the first chapter. Subsequent chapters are divided into sections covering receptor models, cloud chemistry and physics, kinetics, wet and dry deposition, experimental methods, and fundamental processes.

Agricultural Research for a Better Tomorrow. Commemorating the Hatch Act Centennial, 1887-1987. From "Research: Tomorrow's Challenges," a forum sponsored by the U.S. Department of Agriculture and the National Association of State Universities and Land-Grant Colleges, March 2–3, 1987. Published by the U. S. Department of Agriculture, Washington, DC, 1987. 194 pp.

The 20 papers published in this volume are based on a forum held in Washington, DC, as part of a celebration of the centennial of the signing of the Hatch Act of 1887, which created a national system of state agricultural experiment stations in conjunction with the land-grant universities. The papers are organized into 6 sections: Part I. Celebrating the Past, Looking to the Future; Part II. Global Agriculture: Will America Compete?; Part III. America's Changing Consumer Habits: Where Are They Headed?; Part IV. Natural Resources and Environmental Concerns: Who's Concerned? Why?; Part V. America's Rural Environment: Where Are We Going and Why Are We Concerned? Part VI. William Henry Hatch Centennial Year Lecture—"Plant Hormone Research: A Continuing Challenge."

AOAC ANNUAL INTERNATIONAL MEETINGS & EXPOSITIONS

April 26-28, 1988
INFANT FORMULA CONFERENCE II
The Radisson Francis Marion Hotel
Charleston, South Carolina

June 1988
SOUTHEAST
REGIONAL SECTION MEETING

June 20-22, 1988
NORTHEAST
REGIONAL SECTION MEETING
Lowell University, Lowell, MA

August 29-September 1, 1988
102nd ANNUAL INTERNATIONAL MEETING & EXPOSITION
The Breakers, Palm Beach, FL
Program: Analytical Methodology including spotlight on Biotechnology

June 20-22, 1988
MIDWEST
REGIONAL SECTION MEETING
Holiday Inn West, Columbia, MO

September 25-28, 1989
103rd ANNUAL INTERNATIONAL MEETING & EXPOSITION
The Clarion Hotel, St. Louis, MO

June 23-24, 1988
PACIFIC NORTHWEST
REGIONAL SECTION MEETING
(with banquet, speaker and exposition)
Evergreen College, Olympia, WA

September 10-13, 1990
104th ANNUAL INTERNATIONAL MEETING & EXPOSITION
The Clarion Hotel, New Orleans, LA

For further information, contact: The AOAC Meetings Department, AOAC, 1111 N. 19th St., Ste. 210, Arlington, VA 22209, or call in the U.S. (703) 522-3032 or outside the U.S. +1-703-522-3032.

INSTRUCTIONS TO AUTHORS

Scope of Articles and Review Process

The *Journal of the AOAC* publishes articles that present, within the fields of interest of the Association: unpublished original research; new methods; further studies of previously published methods; background work leading to development of methods; compilations of authentic data of composition; monitoring data on pesticide, metal, and industrial chemical contaminants in food, tissues, and the environment; technical communications, cautionary notes, and comments on techniques, apparatus, and reagents; invited reviews and features. Emphasis is on research and development of precise, accurate, sensitive methods for analysis of foods, food additives, supplements and contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment. The usual review process is as follows: (*1*) AOAC editorial office transmits each submitted paper to appropriate subject matter editor, who solicits peer reviews; (*2*) editor returns paper to author for revision in response to reviewers' comments; editor accepts or rejects revision and returns paper to AOAC editorial office; (*3*) AOAC editorial staff edits accepted papers, returns them to authors for approval, and transmits approved manuscripts to typesetter; (*4*) typesetter sends page proofs to author for final approval.

General Information

Follow these instructions closely; doing so will save time and revision. For all questions of format and style not addressed in these instructions, consult recent issue of *Journal* or current edition of *Council of Biology Editors Style Manual.*

1. Write in clear, grammatical English.
2. To Managing Editor, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209, submit typewritten original plus 3 photocopies (1 side only, white bond, 8½ × 11 in. [21½ × 28 cm]) of complete manuscript in order as follows—1. Title page; 2. Abstract; 3. Text (introduction, method or experimental, results and/or discussion, acknowledgments, references); 4. Figure captions; 5. Footnotes; 6. Tables with captions, one per page; 7. Figures.
3. **DOUBLE SPACE all typed material.** Manuscripts not double spaced will be returned for retyping. Do not right justify or use proportional spacing; avoid hyphenation.
4. Use letter quality printer for word-processed manuscripts; manuscripts prepared on dot matrix printers of less than letter quality may be refused.

Format and Style

1. **Title page** (separate sheet, **double spaced**): Title of article, authors' names (full first, middle initial if any, full last), authors' addresses including mail codes.
2. **Abstract** (separate sheet, **double spaced**): ≤200 words. Provide specific information, not generalized statements.
3. **Text** (consecutive sheets, **double spaced**):

   Introduction. Include information on why work was done, previous work done, use of compound or process being studied.

   Method or Experimental. Consult recent issue of *Journal* for proper format. Separate special reagents/apparatus from details of procedure and list in sections with appropriate headings; list in generic and performance terms, avoid use of brand names. (Common reagents/apparatus or those which require no special treatment need not be listed separately.) Place detailed operations in separate sections with appropriate headings (e.g., Preparation of Sample, Extraction and Cleanup). Include necessary calculations; number of significant figures must reflect accuracy of method. Use metric units for measurements of quantities wherever possible. Write *Method* (recommendation for use of specific method) in imperative voice ("Add 10 mL . . . Heat to boiling . . . Wash flasks"); write *Experimental* (description of laboratory experiment) in passive or active voice ("Ten mL was added . . . We heated to boiling . . . Flasks were washed"). Note hazardous and/or carcinogenic chemicals.

   Results/Discussion. Cite tables and figures consecutively in text with Arabic numerals. *Do not* intersperse tables and figures in text.

   Acknowledgments. Give brief thanks (no social or academic titles) or acknowledge financial aid in this section.

   References. Submitted papers or unpublished oral presentations may not be listed as references; cite them in text as unpublished data or personal communications. Cite all references to previously published papers or papers in press in numerical order in text with number in parentheses on line (*not* superscript). List references numerically in "References" in *exactly* (arrangement, punctuation, capitalization, use of ampersand, etc.) styles of examples shown below or see recent issue of *Journal* for less often used types of entries. Follow *Chemical Abstracts* for abbreviations of journal titles.

   JOURNAL ARTICLE REFERENCE

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

   BOOK CHAPTER REFERENCE

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

   BOOK REFERENCE

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

   OFFICIAL METHODS REFERENCE

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

4. **Figure captions** (separate sheet(s), **double spaced**): Designate all illustrations, including schemes, as figures and include caption for every one. Identify curves (See **Figures**) and include all supplementary information in caption rather than on face of figure. Spell out word Figure.
5. **Footnotes** (separate sheet, **double spaced**): Avoid use of footnotes to text. Include "Received . . . Accepted . . ." line; location/date of presentation, if appropriate; present address(es) of author(s); identification of corresponding author, if not senior author; proprietary disclaimers; institution journal series numbers.
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and

TRANSACTIONS OF THE
101ST AOAC ANNUAL INTERNATIONAL MEETING AND EXPOSITION

SPECIAL REPORT

Recommendations for Preparing Test Samples for AOAC Collaborative Studies of Microbiological Procedures for Foods

WALLACE H. ANDREWS
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Preparation of test samples for microbial collaborative studies poses problems not encountered in studies on chemical analytes. For Associate Referees who are considering a collaborative study of a microbiological procedure for food analysis, these problems have not been adequately addressed. Types of contamination (natural or artificial), number of test samples required, analyte selection, proper controls, and container selection are addressed herein. The discussion is a supplement to the guidelines contained in the *Handbook for AOAC Members*.

Anyone who has undertaken, or attempted to undertake, an AOAC collaborative study of a microbiological procedure has quickly discovered that specific guidelines for conducting these studies are lacking. The guidelines published in the *Handbook for AOAC Members* (1) apply primarily to collaborative studies of chemical procedures, although they have been adapted with some success for certain microbiological procedures. Before undertaking a collaborative study, however, the Associate Referee must be concerned with such factors as selection and optimization of the procedure, ruggedness testing, statistical design, and scientific clearance of the collaborative study protocol.

Perhaps the most difficult factor confronting the Associate Referee is the preparation of food samples to be used in the collaborative study, particularly in microbiology where the use of living microorganisms as analytes is unique. The presence of these organisms may be somewhat more difficult to predict than that of chemical substances because of their nonuniform distribution in foods and their varying ability to survive in foods over time. This paper addresses some of the specific concerns of the Associate Referees in food microbiology and furnishes preliminary guidelines for preparing food samples for use in the collaborative study of a procedure to identify or enumerate microbiological analytes.

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Food Types Required

Should the food types used in the collaborative study be the same as those used in the in-house validation study? The selection of foods for the collaborative study is determined by the applicability of the method. Before the collaborative study is conducted, an in-house validation study may be necessary to provide a basis for making a recommendation about the applicability of a particular procedure. The applicability should be determined for both the analyte being identified or enumerated and the food type being analyzed. For the analyte, a decision must be made regarding the procedure's usefulness for identifying or isolating more than one species, serotype, toxin-producing group, or other microbiological entity. For the food type, the Associate Referee must decide whether to recommend the procedure for identifying the analyte in one specific food, in a group or category of related foods, or in all foods.

Many microbiological procedures subjected to collaborative study have been approved for identifying an analyte in all foods. This approval is both scientifically valid and practical, provided that supporting data from the in-house validation study are given. The broader the range of recommended applicability, the more extensive the validation study must be. After completing the validation study, the Associate Referee must decide whether to use the same or different foods in the collaborative study. It would be inappropriate, if not impossible, to suggest a single response or solution to a situation having so many variations. Consider the following 3 situations:

(*1*) Suppose a procedure that is to be recommended for identifying *Salmonella* in all foods was subjected to an in-house validation study in which it was tested with 20–25 different food types. On the basis of results of that study, the procedure would now be ready to be studied collaboratively. In this situation, 5 or 6 food types should be included in the collaborative study with half of the food types being the same as those used in the validation study.

(*2*) Consider a procedure with a moderately restricted applicability, e.g., identification and enumeration of coliforms in eggs and egg products. It would be appropriate in this case to conduct an in-house validation study to demonstrate the efficiency of the procedure for enumerating a variety of coliforms (*Escherichia coli, Enterobacter aerogenes,* and *Klebsiella*) in frozen and unfrozen liquid whole egg, egg albumen, egg yolk, and dried egg powder. For the collaborative study, the enumeration of 2 coliform types in 3 or 4 kinds of eggs or egg products should be sufficient.

(*3*) Consider a procedure with a very restricted applicability, e.g., enumeration of *E. coli* in the Eastern oyster, *Crassostrea virginica.* Because of the limited applicability of this type of procedure, the scope of the in-house validation study may be proportionately reduced. Both the in-house validation study and the collaborative study could be restricted to the enumeration of one specific analyte, *E. coli,* in one specific matrix, *C. virginica.*

Thus, the in-house validation study is a valuable adjunct to the collaborative study for confirming the applicability of a procedure. Its use should remain flexible, however, to cover the various microbiological situations that may arise.

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For both the in-house validation study and the collaborative study, foods that are normally homogeneous or that can be inoculated to give homogeneous distribution of the analyte(s) should be used. Although liquids and powdered or dried, finely textured foods may present problems with respect to a homogeneous analyte distribution, these foods are easier to work with than most other food types. In some cases, however, the use of nonhomogeneous foods may be unavoidable, e.g., when the procedure specifically applies to the identification of the analyte in a nonhomogeneous food.

Types of Contamination

Naturally Contaminated Foods

At least some of the foods used in the in-house validation and collaborative studies should be naturally contaminated. Compared with foods that have been spiked or artificially contaminated, naturally contaminated foods more accurately reflect what exists in nature. Unfortunately, in nature, microorganisms are not uniformly dispersed throughout the food. Thus, the same precautions that must be taken to achieve uniform dispersal of the analyte in artificially contaminated foods must be taken with naturally contaminated foods. Multiple levels of contamination in these foods may be difficult to achieve, particularly if the level of contamination is low. If the concentration of the analyte is relatively high, and this is not often the case, then the naturally contaminated food may be diluted with the same type of food, not containing the analyte, to achieve one or more additional levels of contamination.

Artificially Contaminated Foods

Although the ideal would be to use naturally contaminated foods, these foods may not be available, and the Associate Referee may have to use artificially contaminated foods. An advantage of artificially contaminated foods, however, is that a range of contamination levels can be used to establish method sensitivity, or the lowest level of analyte identifiable by the procedure. In addition, with spiked foods, the inocula can be adjusted to the desired final levels of contamination; with naturally contaminated foods, contamination levels must be used as they exist. Another advantage of artificially contaminated foods is that the expanded applicability of a procedure can be readily documented. The Associate Referee interested in identifying an analyte in all foods will undoubtedly have to spike at least some of the food types to obtain the necessary range of coverage.

As with naturally contaminated foods, homogeneous dispersal of the analyte is equally important in spiked foods. Low-moisture, powdered foods should ordinarily be inoculated with a dry analyte. One approach is to lyophilize, or freeze-dry, the analyte in a substrate that will not cause segregation of analyte and food material. For example, if a batch of nonfat dry milk is to be contaminated with *E. coli,* reconstituted nonfat dry milk may be used as the menstruum for lyophilizing the *E. coli* cells. After freeze-drying, the dried inoculum is added to the uncontaminated nonfat dry milk and mixed. Reconstituted nonfat dry milk is by far the most favored substrate for freeze-drying microbial cells. If a food other than nonfat dry milk is to be contaminated with *E. coli,* the reconstituted nonfat dry milk may still be used as the freeze-drying menstruum. In this case, the amount of freeze-dried milk containing the analyte inoculum would be so negligible that the integrity of the product to be contaminated would not be altered.

Low-moisture, nonpowdered foods, e.g., seeds, grains, peppercorns, kernels, and nut meats, should not be inoculated with a lyophilized analyte because food material and analyte would segregate. Such foods should be submerged in an inoculum bath, drained, and dried. Air drying of foods contaminated with pathogenic organisms, however, requires special safety precautions.

Liquids and high-moisture comminuted foods such as ground beef or sausage patties may be directly inoculated with liquid inocula. Raw chicken and other poultry may be comminuted, inoculated, and kneaded. High-moisture foods such as liquid eggs and liquid egg products should be blended, inoculated, and reblended. Foods that tend to stratify, e.g., peanut butter, can be softened by warming gently and then can be inoculated and homogenized. Chocolate may be melted and inoculated, homogenized with an electric mixer, and then allowed to solidify before being distributed into test samples. Ice cream can be softened, inoculated, mixed, and refrozen. Although the physical nature of the refrozen product will be somewhat altered, this approach seems preferable to direct inoculation of unsoftened ice cream. Shellfish can be preblended with diluent and then pooled, inoculated, and mixed. The concentration of analytical media for preblended shellfish, however, requires adjustment because of the initial addition of diluent.

For some foods, e.g., refrigerated cheese and pasta, there appears to be no alternative to simple, direct inoculation of the individual foods. One could contaminate one or more miscible ingredient(s) and then process these foods to simulate the manufacture of the final product, but that approach is not within the realm of most microbiological laboratories.

One final approach for artificial contamination of foods, aerosolization, is mentioned only to emphasize that this procedure would pose a serious safety hazard. Aerosolization of inocula should not be used to contaminate foods except under

highly extenuating circumstances and with extensive safety precautions.

When it is necessary to use artificially contaminated foods, the Associate Referee must decide whether to use inocula containing stressed (injured) or nonstressed (uninjured) cells. Because many of the foods in today's diets have been subjected to some type of processing, injured cell inocula should be used in most situations (2–23). Foodborne analytes may be injured by freezing; freezing and prolonged storage; freezing, storage, and thawing; freeze-drying; heating; acid treatment; chlorination; ethylene oxide spraying; and gamma radiation. Thus, in preparing inocula for artificially contaminated foods, the type and degree of stress should simulate conditions in nature to the greatest extent possible.

Since almost all processed, nonperishable foods are stored to some extent before consumption, artificially contaminated foods of this type should be stored or aged for an appriopriate period. A storage time of at least 1–2 weeks has been used in past collaborative studies. This period is considered reasonable and practical for most situations. For perishable foods, however, a prolonged storage period may not be indicated. During storage of artificially contaminated food, the analyte will equilibrate or stabilize, which is helpful in determining its "die off" rate, or survival, in the food over an extended period. It is necessary to make a reasonably accurate prediction of the level(s) of analyte in the collaborative study test foods on the day of initiation of analyses. Inoculum levels that are too low can increase the possibility of a nonuniform distribution of the analyte or produce a high percentage of negative analytical results. Unrealistically high inoculum levels would not be appropriate because almost any procedure would be able to discern the analyte. Instead, the levels should be realistic, demonstrating the lowest level of analyte identifiable by the procedure. Moreover, the levels should be chosen within a range having regulatory and/or human health significance.

In a few instances, it is permissible, even advisable, to use nonstressed inocula to prepare artificially contaminated foods, e.g., when the analyte is to be isolated from foods expected to be contaminated after processing, as in the procedure for identification of *E. coli* in pasteurized crabmeat.

Number of Test Samples Required

Analytical Procedures Providing Quantitative Data

How many test samples are required for a collaborative study? Undoubtedly, this is the question asked most frequently by individuals contemplating a collaborative study. For a procedure providing quantitative data, the *Handbook* (1) states that, for minimum collaborative study, each of 5 participating laboratories should examine 3 sample pairs for a total of 30 data points for statistical treatment. Although this guideline was originally intended for collaborative studies of chemical procedures, these 3 sample pairs have been interpreted to mean 3 levels of contamination when the guideline is adapted for a microbiological collaborative study. It should be emphasized, however, that this design is the absolute minimum.

How many test samples are required for a collaborative study? Undoubtedly, this is the question asked most frequently . . .

Analytical Procedures Providing Qualitative Data

For a study of a procedure providing qualitative data, no written guidelines are available. Thus, it is rather difficult to give uniform guidelines because each collaborative study protocol in food microbiology is currently judged on an individual case basis. Recently, several procedures (24, 25) for the rapid identification of *Salmonella* in foods have received official action status. In each of those studies, at least 6 laboratories compared the proposed rapid procedure with the official method for the isolation of *Salmonella* in 5 aliquots at each of 2 levels of contamination for a minimum of 5 foods. This protocol will probably be used as a model for subsequent collaborative studies of procedures providing qualitative data. However, the number of different foods required may vary, depending on the recommended applicability of the procedure and the extent of the in-house validation study. A comparative collaborative study will be required when the objective is either to compare a proposed alternative procedure with an existing official method or to replace an existing official method with an alternative procedure.

Analyte Selection

Inclusion of Nonanalytes

To determine the specificity of the procedure and to increase both the rigor of the evaluation and the naturalness of artificially contaminated foods, it may be desirable to include nonanalyte substances, e.g., competing organisms, interfering substances, and mimics or simulative analytes (not to be confused with atypical analytes). It is advantageous—even necessary in some instances—to treat the food so that most or all of the indigenous microflora are killed before the food is artificially inoculated with the analyte. Suppose, for example, an Associate Referee is interested in officially validating a procedure for the identification of coliforms in liquid milk. Normally, this product may be expected to contain psychrophilic organisms, which, under conditions of prolonged storage, would proliferate and overgrow any coliforms present, rendering the latter undetectable. The Associate Referee may consider heating the liquid milk under specified conditions to reduce substantially or to eliminate the psychrophilic population. In that situtation the Associate Referee may want to consider including nonanalyte organisms in the treated milk in addition to the analyte to be identified. These nonanalytes should be nonpsychrophilic organisms which can be expected to be encountered in liquid milk.

Multiple Analytes

The microbiologist is often called upon to develop and validate a procedure to isolate various strains of *E. coli*, serotypes of *Salmonella*, types of toxin produced by *Staphylococcus aureus* or *Clostridium botulinum*, etc. In that instance, the Associate Referee must demonstrate the ability of a procedure to recover a broad range of multiple strains, species, serotypes, or toxin-producing types from foods that have been naturally and/or artificially contaminated. This determination may be made from the data accumulated from both the in-house validation study and the collaborative study.

Analyte Pools

Suppose the Associate Referee wants to validate a procedure for the isolation of *Salmonella* from foods. It would be both impractical and unreasonable to require data showing that the procedure can isolate the more than 1700 *Salmonella*

serotypes currently recognized. In this instance, the Associate Referee would conduct an extensive in-house validation study before performing the collaborative study. The in-house validation study should demonstrate the effectiveness of the proposed procedure for analyzing 20–25 food types naturally or artificially contaminated with *Salmonella*. One approach would be to use foods that are contaminated with a single *Salmonella* serotype each. Another approach, ensuring at least the same degree of confidence as the first in recommending broad-range applicability of a procedure, would be to use various food types, each contaminated with a pool (2–3 or more) of serotypes. Compared with using foods contaminated with a single serotype, the second approach would provide a wider data base with respect to the number of serotypes tested.

Atypical Analytes

The purpose of including atypical analytes in a collaborative study is not to "trick" the analyst but rigorously to establish the limitations of a procedure. For example, most *Salmonella* strains isolated from all foods do not utilize lactose. In some dairy foods, however, the percentage of lactose-positive *Salmonella* strains may be substantially higher. Thus, in designing the collaborative study protocol of a procedure for identifying *Salmonella* in foods, the Associate Referee should assess the ability of the proposed procedure to isolate atypical lactose-positive strains as well as typical lactose-negative strains, particularly if the procedure is applicable for dairy foods. The principle would, of course, apply in any analogous situation.

Randomization Versus Preselection of Analytes

The final choice of analytes to be used in the collaborative study is based on microbiological, statistical, and ethical considerations. If the decision were solely a microbiological one, the analyte selection would be based on such factors as relative species prevalence, pathogenicity, and typicality. This decision, however, would unavoidably bias the selection of isolates. If the decision were purely a statistical one, the bias would be minimized or eliminated; however, the choice of analytes would put equal emphasis on highly prevalent and rarely occurring strains, extremely pathogenic and mildly pathogenic types, and typical and highly unusual isolates. Finally, there is the dilemma of what to do when it is known, before the collaborative study is undertaken, that the procedure is not effective for isolating specific strains or is not applicable for the analysis of specific food types. It is incumbent upon the Associate Referee to make these shortcomings known with the appropriate precautionary statements in the descriptions of the official method.

The final choice of analytes to be used in the collaborative study is based on microbiological, statistical, and ethical considerations.

Amount of Food Material Inoculated

Test Samples Spiked by Originating Laboratory

Having decided on the numbers and types of foods and analytes to be used in the collaborative study, the Associate Referee must decide how much food material is to be inoculated. There are 2 approaches. Either a large amount of food may be inoculated, mixed, and subdivided into test samples, or the individual test samples may be inoculated directly.

Compared with the alternative approach, bulk inoculation is likely to result in a more uniform dispersal of the analyte. This approach has been used successfully in several recent collaborative studies of microbiological procedures and has been especially adaptable for the contamination of large amounts of dry, powdered foods. Suppose, for example, an Associate Referee needs to prepare a 2000 g bulk of soy flour containing *E. coli*. After a strain, or a pool of several strains, of *E. coli* is frozen in reconstituted nonfat dry milk, the lyophilized contents are pulverized to a fine powder and added to 50 g uninoculated nonfat dry milk in a sterile plastic bag. This 50 g seed inoculum is then mixed manually, sprinkled over the 2000 g bulk of powdered food, and thoroughly mixed manually for even distribution. The degree of homogeneity of analyte distribution may be determined by a most probable number test of the analyte in test samples taken from several different sites in the bulk amount of contaminated food.

Direct spiking of the individual test samples may be useful for foods that are difficult to manipulate in large bulk amounts (e.g., ground beef) or for inoculated foods that cannot be homogenized in any practical manner (e.g., perishable cheese and pasta). Each of the individually inoculated test samples should be mixed thoroughly to ensure homogeneous distribution of the analyte.

Test Samples Spiked by Collaborators

Inoculation by the collaborators should be used only as a last resort and only under highly unusual or extenuating circumstances. Analyst bias may be reduced in such cases if inocula are coded.

Reserves

Each collaborative study test sample should include a small amount of additional material since some may be spilled during weighing or may be lost in a laboratory accident. Weighing the actual test portion from a larger test sample could be considered part of the analytical procedure. Finally, the originating laboratory should have several extra complete sets of test samples on hand in the event of loss or damage during shipment.

Controls

Positive Controls

The use of proper controls in a microbiological collaborative study is frequently overlooked. Although some of these controls are the responsibility of the collaborating analyst, others are prepared by the originating laboratory.

Two types of positive controls should be considered in designing the collaborative study protocol. The first type, the media or culture control, ensures that the media are performing properly. It is prepared by inoculating the initial medium with the analyte and proceeding through the entire analytical protocol just as with the collaborative study test samples. Because the initial medium will be inoculated with a pure culture of the analyte, the number of cells in this control may be higher than that in some of the collaborative study test samples.

The second type of positive control, the sample control, serves the same purpose as the media or culture control. Food

material comprising the sample control is inoculated with analyte by the Associate Referee. The samples are then carried through the entire analytical procedure by both the collaborators and the originating laboratory, just as are the collaborative study test samples. This control may encompass the media or culture control and is actually preferable since the effect of food material on the performance of the media is considered. The level of analyte in this control does not have to approach the sensitivity level of the procedure, but should be high enough to ensure that viable analyte cells are present on the day analyses are initiated.

The use of proper controls in a microbiological collaborative study is frequently overlooked.

Negative Controls

Four types of negative controls should be considered in designing the collaborative study protocol. The first, the negative media control, ensures that the analytical media are not contaminated with the analyte. The initial uninoculated medium is carried through the entire analytical protocol just as are the collaborative study test samples.

The second type of negative control, the culture control, demonstrates either the appearance of the nonanalyte(s) (e.g., competing organisms) on various media, or it shows that the nonanalyte(s) will not grow on media used in the test procedure. This control is prepared by inoculating the initial medium with the nonanalyte and carrying the medium through the entire procedure in the same manner as are the collaborative study test samples.

The third type of negative control, the sample control, provides evidence that test samples are not being cross-contaminated with the analyte and ensures the absence of endogenous analyte in the food material. This control is prepared by removing a test sample from a food that has been found negative for the analyte, placing it in the initial medium, and carrying the medium through the protocol in the same manner as are the collaborative study test samples. The negative sample control may encompass the negative media control.

The fourth type of negative control, the environmental control, offers reasonable assurance that the environment is not a source of the analyte. With a few exceptions, this control is prepared by exposing the initial medium to the open air environment during all analytical operations on the first day of analyses. For example, this control may be an open flask of lactose broth if the procedure is for the identification of *Salmonella* in dried egg powder; an open flask of Trypticase soy broth for identifying *Salmonella* in dried active yeast; an uncapped tube of lauryl tryptose broth for the enumeration of coliforms; or a plate of exposed Baird-Parker medium for the enumeration of *S. aureus*. However, if the procedure is intended for the enumeration of the total aerobic microflora in a food, exposing a petri dish of plate count agar to the open atmosphere during the entire analysis would not be appropriate. An adequate environmental control in this instance would be to expose a petri dish of plate count agar to the open atmosphere for 15 min. Growth of no more than 15 colonies on the incubated plate would ensure that the laboratory environment is suitable for performing a total plate count determination. After the initial day of analysis, all negative environmental controls are treated in the same way as collaborative study test samples. The negative environmental control may encompass the negative media control.

Test Sample Containers

Composition of Container Material

Containers used for collaborative study test samples should be clean, dry, and sterile. They must be made of material that is not toxic, inhibitory, or bacteriostatic for the analyte and will not change the level or the nature of the analyte. They must be made of material that is resistant to punctures, tears, or rips that would lead to cross-contamination of test samples. Sterile containers such as leakproof metal cans or plastic jars, bags, or packets are always preferable to glass containers, which may break. If plastic bags or packets with twist wires for closure are used, special precautions should be taken to ensure that these wires do not puncture adjacent bags.

Labeling

Each collaborative study test sample should bear its own unique number. Analytical bias may be reduced by coding test samples from a series of randomized numbers rather than repeatedly using a "1 to 10" numbering sequence (26). Each coded, randomized number should be typed or written clearly on tape or other waterproof labeling material.

Packaging

Perishable foods should be packaged to maintain their original condition. Refrigerated test samples should be maintained at 0–4.4°C, whereas frozen test samples should be kept frozen at −20°C. Refrigerated test samples can be packed with frozen gel packs, and frozen test samples can be kept frozen by maintaining contact with dry ice. A temperature control should be included to establish the temperature of the test samples upon receipt by the collaborating laboratories. This control may be a thermometer that is included in the package and protected against damage during shipment. The temperature control may be an additional test sample exactly like the others that are to be analyzed. If this is not practical, water collected in a container that is like the other sample containers will suffice as a temperature control for refrigerated test samples. For frozen test samples, ethylene glycol may be used.

A complete set of test samples is placed in an insulated container and shipped to each collaborating laboratory. If test samples are frozen, the insulated container should be prechilled. Immediately upon receipt at the laboratory, the thermometer should be read and the temperature should be recorded. If an additional test sample is included as a temperature control, a prechilled thermometer should be used to read the temperature.

It is recommended that one set of test samples be packaged, shipped to the destination of one of the collaborating laboratories (preferably the most distant), and returned to the originating laboratory, where the temperature control is read.

Precautions for packaging nonperishable foods are not as extensive as those for perishable foods. Nonetheless, care should be taken to minimize damage during shipment by insulating the containers with foam chips or other suitable material. Sample containers for these foods may be placed within a noninsulated package for shipment. Ordinarily, a temperature control will not be needed.

The package containing the collaborative study samples

should be identified with hazard labels, if appropriate, and sent in accordance with federal regulations. Enclosed in the package should be complete instructions for storage of the test samples until analyses can be initiated. Refrigerated foods should be kept at 0–4.4°C, but should be analyzed as soon as possible, provided that all collaborators initiate analysis on the same day. Frozen foods should be kept frozen at −20°C until analyzed. Normally, nonperishable foods may be stored at room or ambient temperature.

Concluding Remarks

Considerable attention must be given to many factors in the preparation of test samples for an AOAC collaborative study of a microbiological procedure. This discussion reflects the author's own opinion and is intended as a supplement to, not a replacement for, the guidelines contained in the *Handbook* (1). It is hoped that this paper will stimulate others working in the area to convey their ideas on the various aspects of conducting microbiological collaborative studies, e.g., ruggedness testing, statistical design, and AOAC clearance procedures.

This discussion reflects the author's own opinion and . . . it is hoped that [it] will stimulate others working in the area to convey their ideas on the various aspects of conducting microbiological collaborative studies . . .

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FOCUS ON PESTICIDE REGULATORY ANALYSIS

Regulatory Perspective of Pesticide Analytical Enforcement Methodology in the United States

MARTIN F. KOVACS, JR, and CHARLES L. TRICHILO
Environmental Protection Agency, Office of Pesticide Programs, Hazard Evaluation Division, Residue Chemistry Branch, Crystal Mall Building No. 2, Arlington, VA 22202

A critical overview is presented of the current regulatory problems encountered by the U.S. Environmental Protection Agency in evaluating the adequacy of pesticide analytical enforcement methodologies submitted in support of proposed pesticide tolerances. One of these problems is the development and validation of appropriate, adequate enforcement analytical methods which account for all free or bound/conjugated residue components of the "total toxic residue" in the commodities of concern. Also included is a detailed discussion of suggested improvements in the development and validation of these enforcement methods, for example, integrating radiolabeled metabolism studies with the subsequent development and validation of proposed analytical enforcement methodologies. New procedures are proposed to facilitate the availability of analytical methods to enforcement agencies and other organizations during the method validation process. Future initiatives to use the collaborative study process in the development and validation of *Pesticide Analytical Manual,* Volume 2, enforcement methods for contemporary pesticides are also discussed.

Background and Regulatory Objectives

The U.S. Environmental Protection Agency (EPA), under authority of the Federal Food, Drug and Cosmetic Act (FFDCA) Section 408 of which applies to raw agricultural commodities and Section 409 of which applies to processed food or feed, is responsible for the registration of all pesticide products sold or distributed in the United States. Before a pesticide can be registered for use on a food or feed crop, a tolerance or exemption from a tolerance for residues of that pesticide must be established. Tolerances are normally established as a result of a petition that contains all the data necessary to establish the tolerance. A tolerance is the legal maximum residue concentration of pesticide chemical allowed in food or feed. If residues exceed the tolerance or if residues exist where no tolerance is established, the product may be considered adulterated and can be seized by the U.S. Food and Drug Administration (FDA), the U.S. Department of Agriculture (USDA), or a state enforcement agency.

The amended Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (40 CFR 158.125) (1) requires that the registration of any pesticide include analytical methods that will measure or quantitate the residue resulting from the proposed use. Pesticide residue analytical methods are developed on the basis of results of plant and animal metabolism studies conducted with radiolabeled compounds. Such studies characterize the *qualitative* nature of the total terminal residue in the raw agricultural food or feed commodities and animal commodities, and facilitate a chemical definition of the "total toxic residue" in these commodities. In a regulatory context, total toxic residue is defined as the sum of the parent pesticide and its degradation products, metabolites (free or bound) and impurities that are considered to be of toxicological significance, and therefore warrant regulation. Registrants must then develop pesticide residue analytical method(s) that are suitable, appropriate, and adequate to gather residue data and/or to enforce the proposed tolerance(s) of the total toxic residue in the commodities of concern.

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The analytical methodology developed by the registrant must determine all components of the total toxic residue, must be specific for the analyte, and must be capable of determining the residues of concern at the proposed tolerance level. In addition, the methodology should not involve reagents or equipment not available to enforcement agencies (namely, FDA, USDA, and state enforcement agencies) and should not require more than 24 h/sample for analysis.

To ascertain whether the analytical methods developed by the registrant are adequate to enforce the proposed tolerances, and to assure that FDA, USDA, or state enforcement agencies and other interested parties can use the methods, EPA conducts a method trial. Method trials are required if the pesticide is new, if the analytical methods are new or unfamiliar, or if the food or feed crop is known to be difficult to analyze because of high oil content, high or low moisture content, or high sugar content. Method trials are also required on samples of animal origin to support initial permanent tolerance requests on meat, milk, poultry, and eggs.

If the method performs satisfactorily in the trial and is acceptable for enforcement, and once a permanent tolerance is established, the method is made available to interested parties by publication or reference in the FDA *Pesticide Analytical Manual,* Volume 2 (PAM II) (2). EPA also has instituted procedures to make analytical methods readily available between the time of publication of a permanent tolerance and publication of the method in PAM II. The availability is announced in the *Federal Register* publication of permanent tolerances, and information on methods validated in EPA laboratories is available from EPA, Office of Pesticide Programs, Program Management and Support Division, Information Services Branch, 401 M St, SW, Washington, DC 20460. Methods are also available to enforcement agencies before a permanent tolerance is established if a FIFRA Section 18 emergency exemption or a temporary tolerance is in effect.

Current Regulatory Requirements

Current test requirements are stated in 40 CFR 158.125 (1): "A residue method for enforcement of tolerances is needed whenever a numeric tolerance is proposed. Exemptions

from the requirement of a tolerance will also usually require an analytical method. Analytical methods used to enforce residue limits for emergency exemptions, temporary tolerances, and permanent tolerances must be available for use by enforcement agencies and thus may not be claimed as confidential business information. For all food uses, data on whether the FDA/USDA multiresidue methodology would detect and identify the pesticide are required."

Pesticide Assessment Guidelines, Subdivision O, Residue Chemistry (3) is a nonregulatory companion to 40 CFR 158 and describes protocols in Sec. 171-4 (b) which may be used to perform food, feed, or tobacco residue testing to support the registration of pesticides under FFDCA and FIFRA. These guidelines provide information to aid the registrant in the development and validation of appropriate and adequate residue analytical methods.

The data requirements specified in Sec. 171-4 (b) address the general requirements for residue analytical methods and the factors to be considered by the registrant in performing an adequate method validation. Specific requirements are also delineated for the determination of extraction efficiencies for the procedures, the method or methods used to measure the total toxic residue as qualitatively identified in metabolism studies, and the requirements applicable to regulatory methods.

For the purposes of this discussion, we will first address the latter requirement in detail. Overall, regulatory method(s) proposed by the registrant should be as simple as possible to minimize the cost of monitoring for pesticide residues. A method that may be valid for gathering residue data is not necessarily suitable for enforcement purposes. In general, an appropriate enforcement method should not require the use of either a sample blank or an internal or procedural standard, neither of which may be available to enforcement analysts. The enforcement method also should not require the use of either exotic equipment or reagents, should be reasonably rapid in execution, should be sufficiently specific to measure and identify the residues of interest in the presence of other pesticides present in the subject commodities, and should be sufficiently sensitive in relation to the tolerance proposal. Finally, the enforcement method should be available for distribution to enforcement agencies and organizations, that is, it cannot be claimed as "confidential business information."

The method(s) proposed for enforcement by the registrant may be subjected to a method trial in EPA's laboratories, as discussed earlier.

Historical Examples and Regulatory Enforcement Problems

During the evaluation of methodology in the registration, re-registration (registration standards), and special pesticide review programs of EPA, examples were noted of old PAM II analytical enforcement methods that fell into 2 analytical problem categories: (*1*) The methods did not *qualitatively* determine all components of the total toxic residue as *redefined* by contemporary radiolabeled metabolism studies, or (*2*) the methods were unreliable because they only partially *quantitated* the total toxic residue in the various sample matrixes for which a permanent tolerance was established. Due to the confidential business information contained in EPA's registration files, identities of the subject pesticides used in the few examples cited in the following discussion could not be revealed; they have been referred to in a generic sense.

A specific example of the first category involved a PAM II method that failed to account for an entire class or group of metabolites arising from a herbicide registered for use on corn and soybeans. These metabolites, which originated from the parent pesticide and were identified by a contemporary radiolabeled plant metabolism study, were subsequently judged by EPA toxicologists as comprising a significant portion of the total toxic residue in corn and soybeans for which permanent tolerances were already established. The metabolites in question were converted by the usual acid hydrolysis procedure described in the method to a common chemical moiety which, in turn, was neither detected nor measured by the original PAM II enforcement methodology.

An example of the second category involved a PAM II method developed for the analysis of a herbicide, registered for use on wheat, barley, and soybeans, that was not thoroughly tested during its developmental stages by adequate validation/recovery studies. In a method trial, this inadequate testing subsequently resulted in questionable or variable recoveries for all components of the total toxic residue on sample matrixes (raw agricultural commodities and animal commodities) for which permanent tolerances were eventually proposed or established. Extraction efficiencies of additional PAM II methods, as identified in EPA's registration files, were also not adequately determined as evidenced by sample treatment and extraction procedures which did not facilitate the release and measurement of all bound or conjugated components of the total toxic residue.

EPA Initiatives to Improve Enforcement Method Development

Publication of Guidelines and Related Regulatory Documents

Current initiatives by EPA to obtain better and more consistent analytical methodology data involve publication of the following documents: *Pesticide Assessment Guidelines,* Subdivision O, Residue Chemistry (3), Data Reporting Guidelines (DRG) for "Analytical Method(s)" (4), and Standard Evaluation Procedure (SEP) for "Analytical Method(s)" (5).

Each DRG is designed to aid the registrant in formatting or organizing data and information submitted to EPA. This improved organization should clarify ambiguities in interpretation of specific portions or subsections of the existing guidelines and thereby facilitate the agency's review process.

Each SEP is designed to ensure comprehensive and consistent evaluation of major scientific topics in reviews of data submitted to EPA and to provide interpretive policy guidance where appropriate. Each SEP will be used in conjunction with the appropriate *Pesticide Assessment Guidelines* subsection and companion DRG.

Integration of Radiolabeled Metabolism Studies with Analytical Method Development

Adequate registrant responses to the following 3 critical questions should largely prevent proliferation of PAM II enforcement methods which are not adequate for the analysis or which produce unreliable results.

(*1*) Were all the necessary compounds (parent plus metabolites comprising the total toxic residue) and test matrixes tested in the analytical method(s) validation/recovery study? *Prior* to the development and validation of the proposed enforcement analytical methodologies, the registrant must conduct a detailed radiolabeled metabolism study that adequately identifies or characterizes *all* components of the total toxic residue, whether free or bound/conjugated, in the

raw agricultural or animal commodities for which a tolerance is sought. EPA's DRG "Nature of the Residue: Plants" (6) details reporting requirements relative to the conduct of such a radiolabeled plant metabolism study.

(*2*) Were the extraction efficiencies of the analytical method(s) determined? This determination is important for dry raw agricultural commodities, processed substrates, and aged residue samples; it is especially important if the presence of bound residue is suspected. The investigator also is directed to the above-named DRG on plant metabolism for general guidance on analytical method extraction efficiency data reporting requirements. For a more detailed discussion of the regulatory significance of bound pesticide residues and the need for development of analytical methodology to quantitatively measure these residues, the 1986 paper by Kovacs (7) should also be consulted.

(*3*) Were the method(s) tested on samples that had been derived from the radiolabeled plant and/or animal metabolism studies? This is necessary to determine extraction efficiency and percentage of total residue determined by the method(s). The following protocol selected from EPA's registration files is not to be considered a model procedure but merely one suggestion for integrating the conduct and interpretation of results obtained from radiolabeled plant metabolism studies with the subsequent development and validation of proposed enforcement methodologies.

Samples of green corn forage that had previously been treated with the phenyl-ring-labeled ^{14}C octanoate ester of the parent herbicide at twice the recommended label rate were analyzed along with untreated controls and controls fortified with the octanoate ester of the herbicide at phenol levels equivalent to about 0.5, 5, and 10 times the proposed tolerance level on the corn forage. The total radioactive residue (ppm), expressed as parent phenol equivalent, in these forage samples was determined by oxidative combustion and liquid scintillation counting (LSC) and was considered to be the theoretical ppm in the sample before extraction and analysis. This figure, in turn, was compared with the total corrected ppm found in the final quantitation to determine if the analytical method would accurately measure the entire residue present in the sample. The radiochemical extraction efficiency of the enforcement analytical method was monitored at each step during sample hydrolysis/extraction/cleanup to arrive at an overall ^{14}C extraction efficiency. These determinations were made by LSC analysis of aliquots of the samples (control, fortified controls, and ^{14}C-treated samples) after each step. The percent efficiency was determined by dividing the disintegrations per minute (dpm) left at the end of the procedural step by the dpm present at the start of the step. A gas chromatograph equipped with a ^{63}Ni electron-capture detector was used to quantitate the phenol equivalents of the parent herbicide in untreated control samples, fortified control samples, and ^{14}C-treated samples.

The results of this validation study indicated a good correlation of the gas chromatography results with the theoretical ppm values obtained by oxidative combustion and LSC, which, in turn, indicated that the enforcement method would accurately determine the parent herbicide and its phenol metabolite when they are present in residue samples. In addition, the results for the ^{14}C-treated samples, plus the recovery results found by fortifying control samples run through the procedure, showed that >93% of the ^{14}C residue in the treated samples was extracted.

The investigator should be cautioned, however, that before validation studies of this type are attempted, it is imperative that the total terminal residue (in the raw agricultural commodity or other sample matrix to be analyzed) be adequately characterized to ascertain what components of this residue need to be subjected to the validation procedure. In the example just cited, this requirement was satisfied in that the octanoate ester and the phenol metabolite of the parent compound together comprised about 93% of the total radioactive measurements in the treated corn forage samples.

EPA Initiatives to Improve Enforcement Method Validation

The remainder of this paper will address various initiatives by EPA to improve the quality of analytical methods being published in PAM II by making procedural changes in the method tryout (MTO) process. It is anticipated that these changes will increase EPA's monitoring capabilities, prevent unnecessary delays in the pesticide registration process, and improve EPA's overall effectiveness in this area. Also to be discussed are future initiatives under consideration by EPA for using non-EPA MTO procedures for certification of analytical methods, including the possible role of collaboratively studied methods.

Use of Multiresidue Protocols

An EPA notice in the *Federal Register* (51, No. 187, p. 34249, September 26, 1986) informed and reminded all pesticide registrants of their responsibility to provide additional analytical data on their pesticides for the standard multiresidue protocols in the *Pesticide Analytical Manual,* Volume I. These FDA protocols are available from the National Technical Information Service as an addendum to the residue chemistry guidelines (8). This action implemented the requirements of 40 CFR 158.125 (b) (15) that EPA published on October 21, 1984, namely, that "for all food uses, data on whether FDA/USDA multiresidue methodology would detect and identify the pesticide are required."

The availability of additional analytical residue data generated by these multiresidue protocols at federal, state, or local (municipal) levels will eventually reduce the overall cost of residue monitoring programs by increasing analytical capabilities and flexibility. Under current budgetary restrictions, accessibility of these additional analytical residue data will provide pesticide monitoring programs with the ability to screen inexpensively and efficiently for the presence of large groups or classes of pesticide residues. Only when multiresidue protocols do not permit adequate detection and quantitation of residues of concern will their re-analysis by specific PAM II enforcement methodology be necessary.

Use of Methods in Peer-Reviewed Journals

EPA currently accepts some methods for publication in PAM II without a method tryout. These methods have been subjected to a peer review by editors and reviewers of scientific journals (e.g., *Journal of Agriculture and Food Chemistry, Analytical Chemistry, Journal of the Association of Official Analytical Chemists,* and *The Analyst*). Methods accepted in this manner are assigned an alphabetical character, not a Roman numeral, for PAM II publication. In the introduction to PAM II, a caveat warns users of alphabetical character methods that although EPA regards those thus designated as workable, they have not been tested in federal laboratories; therefore, they may need additional validation before and during use. However, it should be pointed out that the vast majority of methods published in PAM II have undergone method validation prior to publication.

Timing of MTO Requests

Many of the newer pesticides, such as pheromones, insect growth regulators, or other biological pest control agents, are often chemically complex in nature or resemble natural products. These pesticides are applied to the target crop at extremely low rates; consequently, their residues must be analytically validated during the MTO at extremely low fortification levels in often complex sample matrixes by complex instrumentation operated at their limits of detection. Until recently, the MTO was conducted at or near the end of EPA's review process. However, because of the frequent analytical difficulties encountered with these new pesticide chemicals, more lead time was required in the registration review process. To alleviate this problem, EPA has initiated MTO requests earlier in the petition review process—at the beginning of the first permanent tolerance request, at a point during various petition amendment reviews, or possibly at the end of the first temporary tolerance/experimental use permit review.

Future Use of the Collaborative Study Process

The procedures we have just described to initiate method validation earlier in the registration review process allow the residue analyst more time to deal effectively with the analytical complexities of contemporary pesticides. Current initiatives by EPA, as discussed earlier, will also increase the availability of analytical methods to all other interested parties, including federal and state enforcement agencies, during method validation. Together, earlier initiation of the method validation process and more timely availability of analytical methods under investigation will enhance possibilities of using the collaborative study process in the development of validated and workable PAM II enforcement methods for contemporary pesticides.

As part of this collaborative study process, EPA would invite the participation of state regulatory or nonregistrant private laboratories, such as those of food processing companies and major food trade associations, as well as the Association of Official Analytical Chemists with its well-known and effective network of collaborators and General and Associate Referees.

The collaborative study approach contemplated will not extend or lengthen EPA's overall registration review process. Any time saved by starting early in the petition process will allow an equal amount of time for more collaborative inputs from the users of these enforcement methods. The extended time frame for method validation will also ensure that many complex analytical methodology problems will be dealt with in a more timely and effective manner.

If the collaborative study approach is adopted by EPA as part of the regulatory process, it may, in the short term, add a new dimension to the agency's current method validation procedure. More important, it may, in the long term, be the most effective approach to ensure the development of methods which are precise, accurate, and reliable enough to enforce future tolerances.

Although the problems scientists face today in the area of pesticide residue methodology are indeed complex and frustrating, and at times may appear insurmountable, in the final analysis, these same scientists working together in a collaborative spirit, that is, cooperating in a joint intellectual effort or common venture, can address these problems and resolve them to the benefit of all concerned.

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Importance of Quality Assurance in Canadian Pesticide Analysis

HENRY B. S. CONACHER
Health and Welfare Canada, Food Research Division, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada

Three important elements of the pesticide quality assurance program in the Health Protection Branch of Canada are described—the sampling protocol, the repository of pesticide standards, and the check sample program of the Federal Interdepartmental Committee on Pesticides. All play an important role in ensuring the production of sound, valid pesticide residue data. Efforts to improve each element will continue in the future.

The Health Protection Branch (HPB), Health and Welfare Canada, is accountable, among a variety of other responsibilities, for ensuring the safety of the Canadian food supply—one aspect of which is the control of pesticide residues in foods. HPB fulfills these responsibilities by establishing maximum (permitted) residue levels (MRLs) for pesticides in foods and by establishing programs to ensure compliance with these MRLs. Although several other federal and provincial agencies also play a major role in the control of pesticides, the present paper will mainly address activities within HPB relating to pesticide residues in food.

Most of the surveillance and monitoring programs for pesticide residues are conducted in Central Research Laboratories in Ottawa and in 5 Regional Laboratories located across Canada. It is, therefore, extremely important that the data produced by these laboratories be accurate, reliable, legally defensible, and adequate for the purpose.

To achieve these ends, an intensive quality assurance program is in place for pesticide residue analyses. The main elements of this program for pesticides differ little from those for any other class of compounds under investigation (1). These elements include sampling, continuity of sample integrity, identification and assurance of purity of standards, analytical methods used and their application in the laboratory, confirmation of results, proficiency testing, and sound documentation.

While many of these elements are common from country to country, some may have more relevance and may be unique to the Canadian pesticide situation. These would include the sampling protocol used in the monitoring and surveillance of foods for pesticide residues; the repository of pesticide standards; and the role of the Federal Interdepartmental Committee on Pesticides (FICP) Check Sample Program.

Sampling Protocols

The objective of any national pesticide sampling project is to determine the state of compliance of selected food commodities in the marketplace with regard to selected pesticides. To be assured of absolute compliance with established MRLs, a considerable number of samples of every commodity, both domestic and imported, would have to be analyzed for every possible pesticide residue. Since this is not practical, decisions must be made as to which pesticides should be analyzed, which pesticides should be measured in each commodity, and how many samples should be analyzed for each commodity and pesticide selected.

In connection with the last item, a choice can be made on the basis of statistical considerations. Assuming that the number of lots of any given commodity on the market is very large, the upper limit (95% confidence limit) of the possible percentage of unsatisfactory lots varies with the number of specimens analyzed as shown in Figure 1 (2). For example, if only 25 samples (lots) are analyzed and all are satisfactory, approximately 15% of the lots on the market could be unsatisfactory. If, however, 200 samples are analyzed for each commodity, and all are satisfactory, the percentage of unsatisfactory lots that could be on the market is approximately 2.5%.

In practice, within HPB, 100 samples for each commodity and pesticide combination are taken. If all are satisfactory, one can say with 95% confidence that no greater than 5% of the lots on the marketplace are unsatisfactory. If 2 of the samples are unsatisfactory, the possible percentage of unsatisfactory lots could be as high as 7.5%.

In addition to the question of how many total specimens have to be taken, the sampling within each lot must be considered, as must subsequent subsampling within the laboratory. The present procedure within HPB for minimum sample size within a lot is as follows for fruit and vegetables:

Small fruits and vegetables	3 lb
Medium fruits and vegetables	3 lb or 10 units (whichever is greater)
Large fruits and vegetables	3 lb or 3 units (whichever is greater)

It is important to note that, certainly in some cases with heterogeneous lots (e.g., aflatoxins in peanuts [3]), the variation due to the method is often the smallest component of the overall variation (13%, as opposed to 67% for sampling and 20% for subsampling [3]). Thus, expensive and time-consuming efforts in methodology may in fact result in only a small reduction in the overall variation. Efforts may be more appropriately directed, in some cases, to other areas such as subsampling.

The entire area of sampling is one in which considerable error can arise and one which continues to receive much attention and interest by regulatory agencies worldwide.

Repository of Standards

Since 1956, when the first analysts from the 5 HPB Regional Laboratories were trained in the analysis of foods for pesticide residues, the need for a supply of appropriate pesticide standards has been most apparent, and the responsibility for providing those standards has remained with the Central Research Laboratories.

The inventory of standards consists of numerous glass, screw-cap vials of pesticides and metabolites, which are stored under restricted access in a walk-in freezer. Each vial is la-

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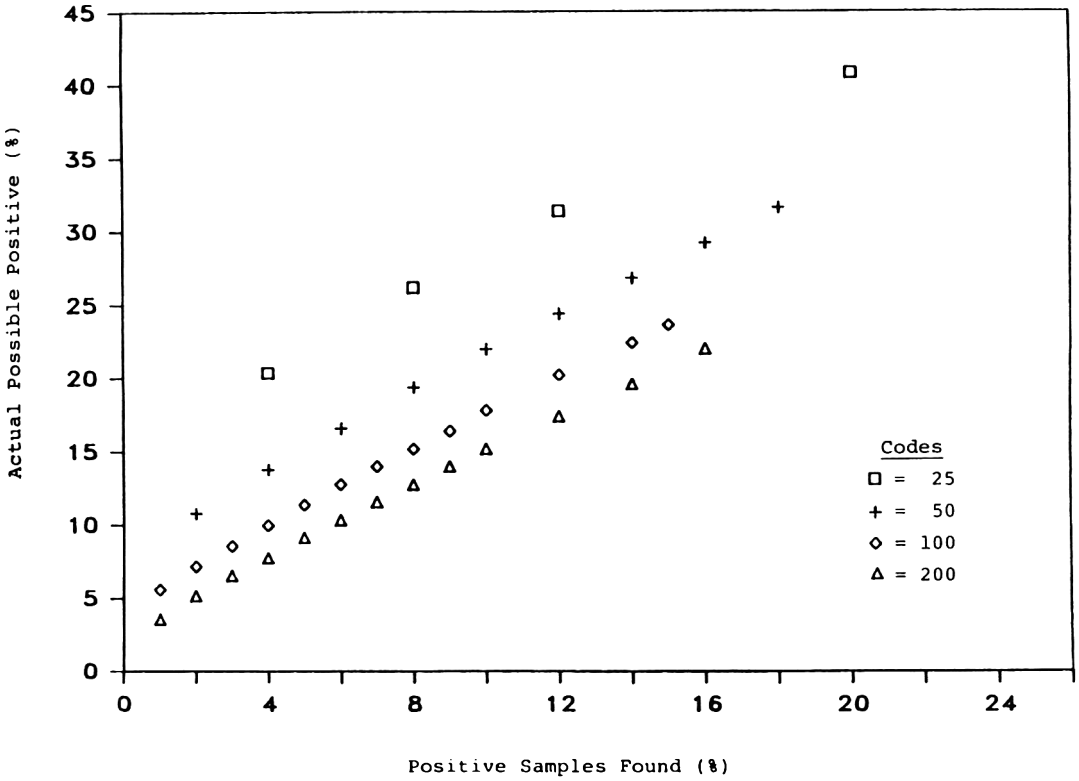

Figure 1. Calculation of upper confidence limits (95%) for varying numbers of samples.

beled with a unique number for identification purposes, and an alphabetical card index system provides information on alternative names, source, purity, lot number, date received, available documents, etc.

Since the early 1980s, a more effective in-house authentication system for standards has been in effect. Compounds are authenticated by mass spectrometry (MS) and comparison with published, recognized spectra. In some cases where MS authentication is not appropriate, nuclear magnetic resonance is used. A typical authentication certificate is shown in Figure 2. Discussions are continually underway concerning the extent of authentication necessary, particularly regarding purity, and also concerning the extent of documentation required to establish, without question, the continuity of possession of standards from Central Laboratories to Regional Laboratories.

AUTHENTICATION REPORT
RAPPORT DE CERTIFICATION

analytical standard : étalon analytique

STRUCTURE

Source and lot
Origine et lot

M.W.
P.M.

Purity
Pureté %

Reçu date received:

HPB lot no de la DGPS:

HPB TEST RESULTS
RESULTATS D'ESSAI DE LA DGPS

Mass spectrometry E.I. .....
Spectrométrie de masse C.I. .....

Signature and date ..... Satisfactory yes oui / no non Satisfaisant

Comments:
Commentaires:

Packed by ..... Empaqueté par

..... date

Figure 2. Authentication certificate for pesticide standards.

The list of standards is circulated among HPB laboratories semiannually; at present, some 500 compounds are in the inventory. A similar in-house inventory of pesticide standards is also maintained by Agriculture Canada.

Check Sample Program

The FICP check sample program, originally devised in 1967 and subsequently revamped in 1978 (4), was intended to be applicable to all pesticide residue analytical laboratories in Canada. It was designed to establish or verify the performance of a laboratory by the analysis of homogeneous samples of known residue levels. In addition to playing a most important role in performance assessment, it also leads to much improved communication among laboratories regarding pesticide analytical problems.

Currently, 72 laboratories participate in the program (which is administered by Agriculture Canada) with distribution among agencies as follows: federal, 34; provincial, 13; universities and hospitals, 7; and private 18. Some of the members do not participate in the analysis of check samples because they are involved only in specialized research projects and consider (rightly or wrongly) that participation is not appropriate to their work. Nevertheless, they remain members to keep in contact with general activity in the pesticide analytical community.

The present program outline is shown in Table 1. There are 8 subprograms, all of which have been relatively active in the last few years and have sampled a wide variety of substrate/pesticide combinations. The type of information

Table 1. FICP check sample program outline

Subprogram	Substrates	Distribution[a]	Pesticides
Soils	soil	3	2,4-D picloram atrazine
Foods	tallow, strawberries, potatoes	4	captan iprodione carbofuran chlorophenols
Water	standards, sediments, water	3	common organochlorines phenoxy acids
Fish	fish, eels, cod liver oil	6	DDE, mirex PCBs
Forest substrates, insecticides	fish, soil, balsam fir needles	3	pirimicarb aminocarb mexacarbate carbaryl
Forest substrates, herbicides	soil	1	hexazinone
Wildlife	herring gull lipids and homogenates	3	DDE, mirex, PCBs heptachlor epoxide chlordane oxychlordane dieldrin
Feeds	grains	2	triallate malathion carbathion permethrin lindane chlorpyrifos

[a] Number of check sample projects conducted in last 5 years.

that can be acquired can best be illustrated using the strawberry–captan/iprodione (foods) and the fish–PCBs (fish) check sample studies (taken from reports of the coordinators).

Strawberries—captan and iprodione.—This check sample study, using field-treated strawberries (at recommended treatment rates), was in fact conducted twice. In the first study, in 1983, excessively high interlaboratory variations in captan level were encountered that were subsequently traced to losses of captan occurring during transportation. The samples had been deliberately shipped unfrozen to simulate normal transportation from the field. Unfortunately, this instability and subsequent variation in level precluded assessment of the methodology used. However, as a result of this exercise, a considerable amount of research was done and much useful information was generated on captan breakdown under various storage conditions, on its instability on fruit, and on sampling problems.

Table 2. Levels of captan and iprodione found in strawberries

Lab.	Captan, ppm	Iprodione, ppm
1	2.3	1.3
2	1.7	0.9
3	1.6	—[a]
4	3.0	—
5	2.4	1.0
6	2.5	0.9
7	2.9	1.5
8	2.2	1.1
9	4.4	—
10	0.3[b]	—
Av. ± CV, %	2.5 ± 31	1.1 ± 19

[a] Not analyzed.
[b] Not included in statistics, outlier.

To check the methodology in use for captan and iprodione, a second study, in which the samples were shipped frozen, was conducted in 1984. The results are shown in Table 2. The coefficient of variation (CV) for captan results (±31%) was still higher than desirable, even allowing for all the different methods used. However, it was similar to that obtained within the coordinator's own laboratory, supporting a conclusion that the variation was probably due to sampling and captan instability and not to the methodology. The CV for iprodione results (iprodione is a much more stable compound than captan) was much more acceptable (±19%) for determinations at the 1 ppm level (5).

Fish—PCBs.—There has been continuing discussion at the national and international level on the best approach to quantitate PCBs in biological samples (6). The fish check sample program has shed considerable light on analytical problems in this area. In a recent check sample study using fish naturally contaminated with PCBs as the substrate, laboratories were asked to determine the PCB level by using a common quantitation technique (7) and by using their own quantitation technique on both a conventional packed column and a capillary column if one was available. The results, summarized in Table 3, indicate the considerable differences in level and in variation that can be obtained, depending on the method of quantitation used.

Table 3. Levels of PCBs found in fish homogenate

No. of labs	Quantitation technique	Level found, ppm ± CV, %
8	common	2.20 ± 14
6	own	2.78 ± 27
5	own + capillary	3.23 ± 30

There has been a definite trend among analysts in the past few years to move more and more into the measurement of individual isomers by capillary chromatography. Curiously enough, the capillary results (Table 3) would not indicate this as the approach to take. However, these poor results in the fish check sample were probably due to the fact that different isomers were chosen for measurement and that columns with different stationary phases were used. More consistent results would likely be obtained through the measurement of specified isomers on capillary columns with similar stationary phases.

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PRESERVATIVES

Spectrometric and Liquid Chromatographic Determination of Natamycin in Cheese and Cheese Rind

WILLEM G. DE RUIG and JACOBUS J. VAN OOSTROM
State Institute for Quality Control of Agricultural Products, PO Box 230, 2700 AE Wageningen, The Netherlands
KOOS LEENHEER
Netherlands Controlling Authority for Milk and Milk Products, Leusden, The Netherlands

Methods for determining natamycin content of cheese rind and cheese are presented. Cheese and rind samples are extracted with methanol and the fat precipitated by cooling the sample solution in methanol-water at −15 to −20°C for ca 1 h. Natamycin levels are measured by UV spectrometric detection at absorbance minimum 311 nm, maximum 317 nm, and at exactly 329 nm, or by LC separation over Lichrosorb RP-8 column with detection at 303 nm. For measuring low levels, a concentration step is provided. The method is applicable to natamycin in cheese rind and in the interior of the cheese. Detection limit is 0.5 mg/kg. The method is suitable for controlling a maximum tolerance of natamycin on the cheese rind, at a level of 1 mg/dm², and for detecting migration of natamycin into the cheese.

Natamycin or pimaricin is a white to creamy white, almost tasteless, and almost odorless, crystalline powder. It is a fungicidal antibiotic and antimycotic of the polyene macrolide group and is produced by the actinomycete *Streptomyces natalensis*. The chemical formula is $C_{33}H_{47}NO_{13}$ and the molecular weight is 665.74. The structural formula is shown in Figure 1.

Natamycin is widely used on cheese and sausages. In the dairy industry, it is applied in cheese coatings and is superior to alternative products in preventing mold formation without affecting the taste and appearance of the cheese.

Official clearances for the use of natamycin as a preservative for cheese have been granted by 26 countries. Natamycin is also of interest to international bodies such as Codex Alimentarius, the International Dairy Federation, and the European Economic Community (1–7).

Methods for determining the natamycin content of cheese, based on microbiological assay, spectrometry, thin-layer chromatography, and liquid chromatography (LC) with UV detection, have been published (8–13). The behavior of natamycin and its determination have been thoroughly studied by the Netherlands State Institute for Quality Control of Agricultural Products (RIKILT) in cooperation with the Netherlands Institute for Dairy Research (NIZO) and the Netherlands Controlling Authority for Milk and Milk Products (COZ) (14, 15). Two methods of analysis, based on spectrometric determination (16) and LC detection (17), have been developed, and a series of national collaborative studies has been conducted to enhance the methods. These studies made clear that the microbiological assay method is not appropriate for quantitative measurements. Spectrometric and LC methods are presented here which have been tested in an international interlaboratory study, the results of which are also published in this journal (18).

METHOD

Apparatus

(a) *Slicer.*—One apparatus for cutting portions of cheese rind 5 mm thick × 3 cm wide (see Figure 2 for example), and one for cutting thin slices of cheese, ≤1 mm thick (see Figure 3).

(b) *Grinder or blender.*

(c) *Knife.*—Sharp, for cutting slices of cheese into small pieces.

(d) *Magnetic stirrer or shaking machine.*

(e) *Conical flasks.*—200 and 100 mL, colored glass, with ground-glass stoppers.

(f) *Syringes.*—10 mL, disposable.

(g) *Membrane microfilters.*—0.45 and 0.20 μm pore size, resistant to attack by alcohol solutions.

(h) *Paper filters.*—Folded, 15 cm diameter (S&S, No. 595 1/2).

(i) *Funnels.*—About 7 cm diameter.

(j) *Freezer.*—Operating at −15 to −20°C.

(k) *Disposable cartridges.*—Sep-Pak C_{18} (Waters, Cat. No. 51910) or equivalent. Activate with 3–5 mL methanol, then wash with 10 mL water. Use if concentration of filtered extracts is necessary.

(l) *Spectrometer.*—Pye Unicam, Model SP1700, or equivalent, suitable for registering UV spectrum between 300 and 340 nm, equipped with cells having 1 cm optical pathlength and recorder.

(m) *LC apparatus.*—Waters, Model 6000, or equivalent, equipped with UV detector, recorder, and/or integrator. *Analytical column:* 150 mm × 4.6 mm id, packed with 5 μm Lichrosorb RP-8, or equivalent. *Guard column:* 100 mm × 2.1 mm id, packed with 30–40 μm Perisorb RP-8, or equivalent. The following operating conditions are recommended: Mobile phase, methanol–water–acetic acid (60 + 40 + 5) (v/v/v); flow rate, 1 mL/min; detector, 303 nm, 0.005 AUFS; recorder, 10 mV; theoretical plate count, minimum 1500. *Note:* When an analytical column other than that specified above is used, the methanol–water ratio may have to be changed. The relative amount of acetic acid, however, is essential to keep the absorption maximum at 303 nm.

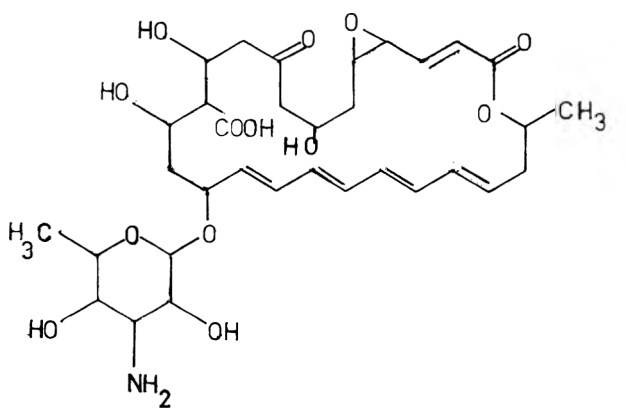

Figure 1. Structural formula of natamycin.

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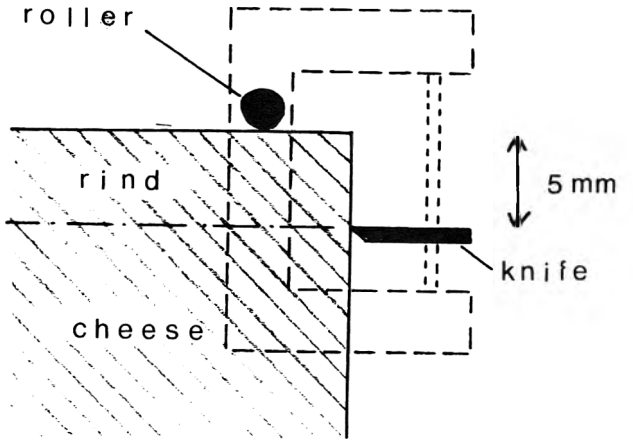

Figure 2. Slicer for cutting portions of cheese rind 5 mm thick.

Reagents

(a) *Aqueous methanol.*—Mix analytical grade methanol and water (2 + 1) (v/v).

(b) *Natamycin standard.*—With known natamycin content (Gist Brocades, Delft, The Netherlands). *Note:* Natamycin is unstable in aqueous methanol, and so measurements must be made as rapidly as possible.

(c) *Standard solution.*—Immediately before use, dissolve natamycin preparation containing 50 mg of 100% natamycin in 100 mL methanol. Dilute 5 mL of solution to 50 mL with aqueous methanol. Dilute 5 mL of diluted solution to 50 mL with aqueous methanol to make a final solution of 5 μg/mL. Concentration of final standard solution must be close to that of sample solution as described below in *Sample Preparation.* Adapt final dilution if necessary.

Sample Preparation

Use whole cheese or representative segment of cheese. Cheese rind includes outer 5 mm layer of cheese and coating layer if present.

Cheese rind.—Cut cheese into sectors so that width of cheese rind is not more than ca 3 cm. Using slicer, remove whole rind to a thickness of 5 mm from all portions. From the rind, cut a rectangular piece 20–40 cm^2 and determine its area in square centimeters and its mass in grams. Carefully grind whole rind, including weighed and measured piece, and mix thoroughly. Immediately weigh, to the nearest 10 mg, ca 10 g prepared sample and transfer to 200 mL conical flask. After preparing each sample, clean all tools that have been in contact with the cheese or cheese rind, first with hot water and then with methanol, and dry thoroughly, e.g., with a stream of compressed air.

Interior of cheese.—After removing rind as described above, remove a slice ≤1 mm thick encompassing whole outer section of the cheese, using the fine slicer. Cut slices of cheese into 0.5 cm^2 pieces and mix thoroughly. Immediately weigh, to the nearest 10 mg, ca 5 g prepared sample and transfer to 100 mL conical flask. After preparing each sample, clean tools as described above.

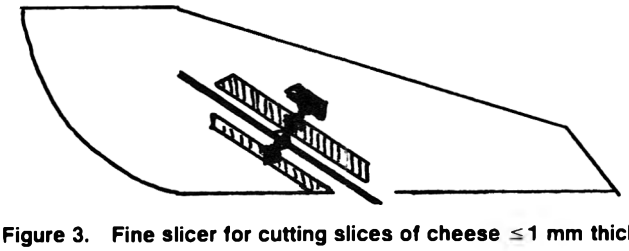

Figure 3. Fine slicer for cutting slices of cheese ≤1 mm thick.

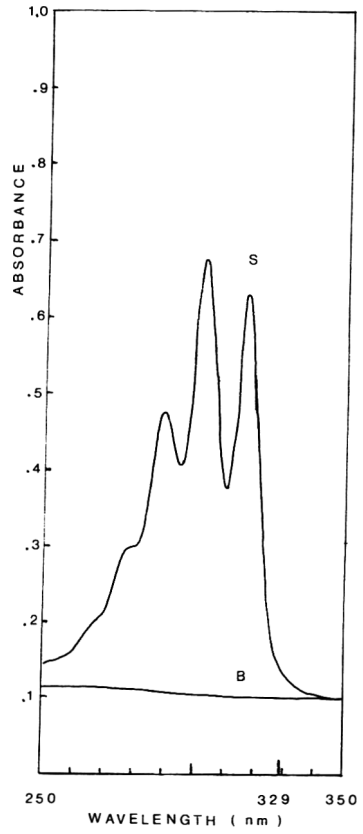

Figure 4. Spectrometric detection of (S) standard solution and (B) blank.

Extraction of Sample Solutions

To cheese rind add 100 mL methanol; to cheese add 50 mL methanol. Stir contents of conical flask 90 min on magnetic stirrer or shake 90 min in a shaking machine. To cheese rind solution add 50 mL water; to cheese solution add 25 mL water. To precipitate the fat, immediately place conical flask into freezer at −15 to −20°C and let stand ca 1 h. Filter cold extract through folded paper filter, discarding first 5 mL of filtrate. (*Note:* Filtration must be conducted while suspension is still cold to avoid the risk of turbid filtrates.) Bring filtrate to room temperature. Withdraw portion of filtrate with syringe and pass through 0.45 μm membrane filter and then through 0.20 μm membrane filter.

Concentration of Filtered Extract for Determination of Low Natamycin Content

Decide whether concentration of ca 5 or 10 times is desired on basis of required detection limit and pipet 25 or 50 mL, respectively, of sample solution into beaker. Add 50 or 100 mL water, respectively, and mix. With a syringe, inject diluted sample solution through activated Sep-Pak C_{18} cartridge at ca 25 mL/min. Rinse cartridge with 10 mL water and elute natamycin with 3 mL methanol.

Spectrometric Determination

Calibration.—Record spectrum of standard solution in the range 300–340 nm. Measure absorbance at maximum ca 317 nm, at minimum ca 311, and at exactly 329 nm. Use aqueous methanol as a blank. See Figure 4 for example of spectrum of standard solution. Exact positions of maximum at 317 and minimum at 311 nm may vary slightly because of ap-

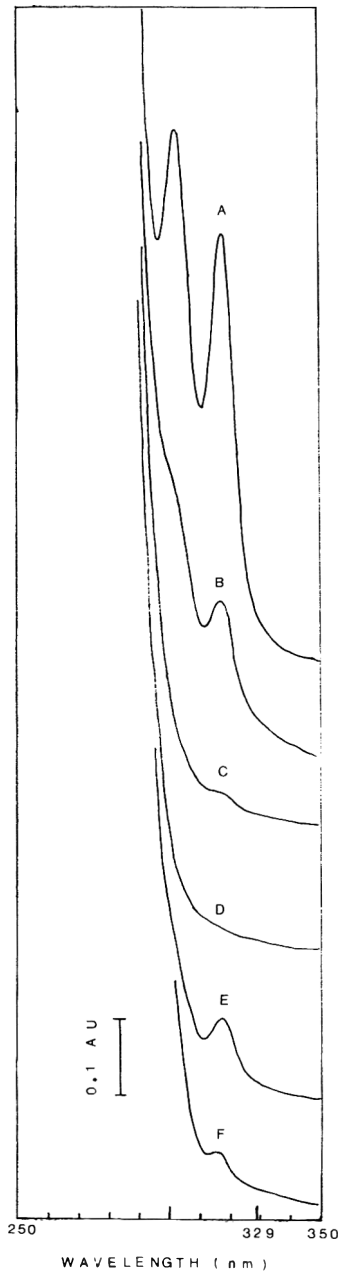

Figure 5. Spectrometric detection of cheese and cheese rind samples. A, cheese rind, 61 mg/kg; B, cheese rind, 15 mg/kg; C, cheese, 1.7 mg/kg; D, cheese, 0.3 mg/kg; E, cheese, 1.7 mg/kg, after concentration 5×; F, cheese, 0.3 mg/kg, after concentration 10×.

paratus calibration. Always use actual maximum and minimum values.

Measurement of sample solution.—For direct spectrometric measurement, use at least 3 mL filtrate.

Record spectrum of sample solution in the range 300–340 nm. Measure absorbance at maximum of ca 317 nm, at minimum of ca 311 nm, and at exactly 329 nm. Use aqueous methanol as a blank. See examples of spectra of sample solutions in Figure 5. (*Note:* The presence of spices, particularly pepper, in the cheese can interfere with the results. It will be obvious by distortion of the absorbance curve, as shown in Figure 5.)

If natamycin concentration of sample is so low that detection is very difficult or impossible (signal-to-noise ratio <3) but determination is nevertheless required, proceed according to *Concentration of Filtered Extract.* Then, add 1.5 mL water to eluate and mix. Withdraw solution with a syringe and filter through 0.45-μm membrane filter and then through a 0.20-μm membrane filter into a cell, and record sample as mentioned above. For examples of UV spectra of sample solutions, see Figure 6.

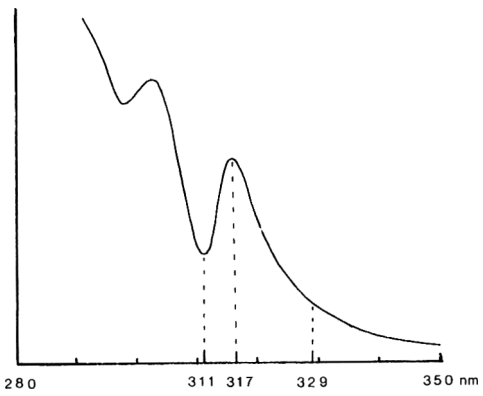

Figure 6. UV spectrum of sample solution containing natamycin.

Calculation.—Calculate natamycin concentration, C_s, of sample (mg/kg) as follows:

$$C_s = (V/m_1) \times (E_s/E_N) \times C_N \qquad (1)$$

where C_N is the natamycin concentration of the natamycin standard, μg/mL; E_N is the net absorbance of the natamycin standard solution at ca 317 nm; E_s is the net absorbance of the sample solution at ca 317 nm; m_1 is the mass of the sample portion, g; and V is total volume of sample solution, mL. E_N can be taken from the UV spectrum of the standard solution, using the straight line between the absorbance at ca 311 and 329 nm as a base line, or can be calculated from

$$E_N = (E_1)_N - \tfrac{2}{3}(E_2)_N - \tfrac{1}{3}(E_{329})_N \qquad (2)$$

where $(E_1)_N$ is the maximum absorbance at ca 317 nm; $(E_2)_N$ is the minimum absorbance at ca 311 nm; and $(E_{329})_N$ is the absorbance at 329 nm. E_s can be taken from the UV spectrum of the sample solution, using the straight line between the absorbance at ca 311 and 329 as a base line (Figure 6), or can be calculated from

$$E_s = (E_1)_s - \tfrac{2}{3}(E_2)_s - \tfrac{1}{3}(E_{329})_s \qquad (3)$$

where $(E_1)_s$ is the maximum absorbance at ca 317 nm; $(E_2)_s$ is the minimum absorbance at ca 311 nm; and $(E_{329})_s$ is the absorbance at 329 nm. In the case of the sample solution of cheese taken from below the rind, C_s is the natamycin content resulting from migration of the natamycin into the cheese. In the case of the sample solution of cheese rind, calculate

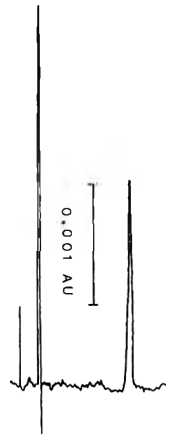

Figure 7. LC detection of standard solution, 0.5 μg/mL.

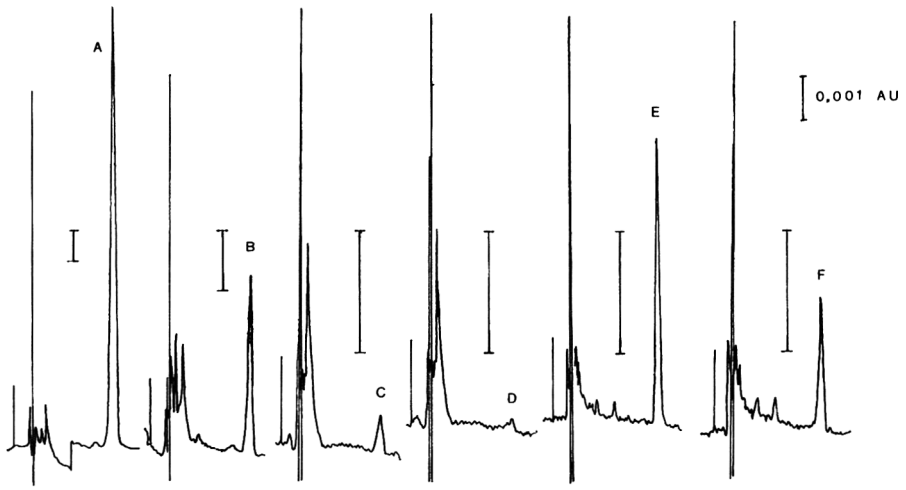

Figure 8. LC detection of sample solutions. A, cheese rind, 61 mg/kg; B, cheese rind, 15 mg/kg; C, cheese, 1.7 mg/kg; D, cheese, 0.3 mg/kg; E, cheese, 1.7 mg/kg, after concentration 5×; F, cheese, 0.3 mg/kg, after concentration 10×.

amount of natamycin on surface of cheese rind, C_s' (mg/dm²), out of C_s as follows:

$$C_s' = 0.1\ C_s \times (m_t/a) \qquad (4)$$

where m_t is the mass of the weighed piece of cheese rind, g, and a is the area of the weighed piece of cheese rind, cm². The constant 0.1 is used to convert kg to g and cm² to dm². If the filtered extract is concentrated, correct calculated values of C_s and C_s' by dividing by 5.6 for ca 5 times concentration and 11.1 for ca 10 times concentration.

LC Determination

Calibration.—Pipet 1, 2, 4, 6, and 8 mL standard natamycin solution, concentration 5 μg/mL, into a series of 50 mL volumetric flasks and dilute to volume with aqueous methanol. Solutions contain 0.1, 0.2, 0.4, 0.6, and 0.8 μg/mL, respectively. Inject 20 μL of each solution into chromatograph. Measure peak area or height and plot values found on y-axis against injected quantities, in μg/mL, on the x-axis. See Figure 7 for an example of a chromatogram of a standard solution.

Measurement of sample solution.—Inject 20 μL sample solution into chromatograph. As rapidly as possible, measure peak area or height having same retention time as standard natamycin solutions. (*Note:* The presence of spices, particularly pepper, in the cheese can cause an interfering peak at the same retention time as natamycin. Separation can be achieved by a gradient elution program.) If peak area or height for sample solution is so low that interpolation on the calibration graph is impossible or almost impossible but determination is nevertheless required, proceed according to *Concentration of Filtered Extract.* Then, dilute eluate to 5 mL with methanol and inject 20 μL sample solution into chromatograph and continue as mentioned above. For examples of LC chromatograms of sample solutions, see Figure 8.

Figure 9. Classification of UV spectrometric peaks: 1, a distinct peak in the spectrum, quantitative calculation without problem; 2, a peak is present, but not quantitatively calculable; 3, a very weak slope is present; 4, no deviation from a blank is observed.

Calculation.—The concentration of natamycin in the injected aliquot of the sample solution can be found by interpolation of the calibration graph. Calculate natamycin concentration, C_s, of sample (mg/kg) from

$$C_s = (V/m_t) \times C_m \qquad (5)$$

where C_m is the measured concentration of natamycin in the sample solution, μg/mL; m_t is the mass of the sample portion, g; and V is the total volume of the solution, mL. In the case of the sample solution of cheese taken from below the rind, C_s is the natamycin content resulting from migration of natamycin into the cheese, as noted above. Calculate natamycin in the sample solution of cheese rind in mg/dm² rind surface according to equation 4, above.

If filtered extract has been concentrated, correct calculated values of C_s and C_s' by dividing by 5 for 5 times concentration and by 10 for 10 times concentration.

Results and Discussion

Repeatability and Reproducibility

Repeatability and reproducibility of both methods according to an interlaboratory study carried out in 1984 by 36 laboratories on 8 samples (18) are shown in Table 1.

Limit of Detection

The limit of detection can be defined as the amount of analyte giving a response that is 3 times the noise. We have

Table 1. Repeatability and reproducibility of spectrometric and LC methods for determining natamycin in cheese and cheese rind

	Level[a]		Spectrometry		Liquid chromatography	
	mg/kg	mg/dm²	CV_r, %[b]	CV_R, %	CV_r, %	CV_R, %
Direct determination	60	4	5.9	12.2	9.3	20.6
	15	1	6.2	11.9	7.1	25.7
10× concn	1.3	0.08	16.5	35	23.4	37
	0.3	0.02	42.5	60	29	39

[a] mg/dm² calculated from mg/kg for cheese rind with thickness of 5 mm and density of 1.3.

[b] Relative repeatability, $r_{rel} = 2.8 \times CV_r$; relative reproducibility, $R_{rel} = 2.8 \times CV_R$.

Table 2. Signal-to-noise ratios for 1.7 mg/kg and 0.3 mg/kg levels of natamycin in cheese samples as determined by liquid chromatography

	Level C (1.7 mg/kg)				Level D (0.3 mg/kg)			
	Sample 3		Sample 6		Sample 5		Sample 7	
Lab.	Direct	After concn	Direct	After concn	Direct	After concn	Direct	After concn
1	—[a]	—	—	—	—	—	—	—
2	—	—	4.5	47	—	—	—	—
3	4	26	11	32	—	3.7	—	7.5
4	—	4	—	7	—	5	—	2.5
5	10	—	—	—	—	5.8	—	—
6	25	—	13	—	10	—	1.5	—
7	1.6	—	2.2	—	—	2.1	—	4.1
8	0[b]	—	0	6.3	—	2.7	—	3.0
9	0	—	0	—	0	—	0	—
10	4.6	30	4.6	—	1.5	11	1.5	11
11	0	7.6	0	—	0	—	0	—
12	3.6	22	5.3	10.6	0	4.3	0	1.5
13	1.8	3	2	2.1	0	1.3	0	0
14	—	8.3	—	6.7	—	1.6	—	0
15	—	4.3	—	4.4	—	1.6	—	1.4
16	6.5	47	7	50	2.7	22	3.3	20
17	17	50	17	51	4.5	24	4	24
18	4	4.6	3.3	18.8	2.7	11	1.5	14
19	0	39	0	53	0	8	0	18
20	—	80	—	12.3	—	11	—	—
21	0	4.2	0	15	0	4.8	0	0

[a] — = Missing.
[b] 0 = No signal observed.

calculated signal-to-noise (S/N) ratios from the LC chromatograms of 21 participants of the interlaboratory study (18). The S/N ratios for level C (1.7 mg/kg) and level D (0.3 mg/kg) are presented in Table 2. It can be concluded that even at the lowest concentration (level D), most participants found S/N ratios >3. However, 30% found S/N <3. The wide variation in S/N ratios indicates that the LC method may be less rugged than the spectrometric method.

With spectrometric detection, the UV spectrum is practically without noise. Therefore, UV spectra of interlaboratory study participants have been divided into 4 classes (Figure 9): class 1, a distinct peak in the spectrum, quantitative calculation without problem; class 2, a peak is present, but not quantitatively calculable; class 3, a very weak slope is present; class 4, no deviation from a blank is observed. The results relative to these classifications are presented in Table 3. When classes 1 and 2 were "detectable" and classes 3 and 4 were "not detectable," then, after concentration, all results were detectable at level C (1.7 mg/kg) and 68% were detectable at level D (0.3 mg/kg). That is the same percentage as found for the LC method at level D. On the basis of these results, the limit of detection is fixed arbitrarily at 0.5 mg/kg for both methods.

Table 3. UV spectra classifications for 1.7 mg/kg and 0.3 mg/kg levels of natamycin in cheese samples

	UV spectrum classification number[a]							
	Level C (1.7 mg/kg)				Level D (0.3 mg/kg)			
	Sample 3		Sample 6		Sample 5		Sample 7	
Lab.	Direct	After concn	Direct	After concn	Direct	After concn	Direct	After concn
1	3	1	3	1	4	2	4	2
2	—[b]	1	—	1	—	2	—	1
3	3	1	3	1	4	2	4	2
4	4	2	4	2	4	3	4	3
5	3	1	2	1	4	2	4	2
6	3	1	3	1	4	3	4	2
7	4	2	4	2	4	3	4	3
8	3	1	3	1	4	1	4	1
9	3	1	3	1	4	1	4	1
10	2	1	3	1	4	2	4	2
11	—	—	3	2	—	—	—	—
12	3	1	3	2	4	3	4	3
Summary								
Class 1		9		8		2		3
Class 2		2		4		5		5
Class 3		0		0		4		3
Class 4		0		0		0		0

[a] See Figure 9 for depictions of classes 1, 2, 3, and 4 UV spectra.
[b] Missing.

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Determination of Natamycin in Cheese and Cheese Rind: Interlaboratory Collaborative Study

WILLEM G. DE RUIG
State Institute for Quality Control of Agricultural Products, PO Box 230, 2700 AE Wageningen, The Netherlands

Collaborators: M. Barnett; E. C. Conchie; J. Crow; Z. Cvak; J. Daenen; A. Dupont; V. Eckelmans; G. Engel; R. Fawcett; W. Frede; Führling; J. P. Geerts; Ch. Gertz; C. N. Grange; M. Guldborg; A. J. Harrison; B. Hoffmann; V. Husbands; I. Ibanez Rico; M. Iwaida; T. E. Johnson; H. Köbler; J. Leenheer; T. Linazo Iglesias; S. Littman; J. M. Nuijens; H. Overström; J. A. Palgrave; S. v. d. Reek; C. Repelius; W. G. de Ruig; Schneider; M. J. Scotter; D. Scuffam; J. H. Shelton; M. T. Stijve; R. Tiebach; E. Tsigaridas; Ch. R. Winné

A collaborative test on the determination of natamycin in cheese and cheese rind was conducted. Participants were from 37 laboratories in 13 countries. Eight samples, consisting of 4 duplicates, were investigated by a spectrometric method and a liquid chromatographic (LC) method. The spectrometric method gave good results (coefficient of variation [CV] = 12%) and the LC method with ultraviolet detection gave reasonable results (CV = 25%) for levels down to 15 mg/kg (0.9 mg/dm²). For very low levels, a preconcentration step is necessary, but even then quantitation is poor (CV = 35-37%) for both methods at 1.7 mg/kg, although the presence of natamycin can be detected qualitatively. For a level of 0.3 mg/kg, quantitation is poor (CV = 39%) for the LC method and impossible (CV = 60%) for the spectrometric method.

Methods for determining natamycin in cheese rind and in cheese are described by de Ruig et al. (1). The methods consist of extraction of cheese and cheese rind in methanol, precipitation of fat by cooling sample solution 1 h at −15 to −20°C, and measurement by spectrometry or liquid chromatography with UV detection. For detection of low levels of natamycin, a concentration step is included.

Internationally, methods of determination have been discussed by the European Economic Community (EEC) Working Group on Additives and by the Joint IDF/ISO/AOAC Group of Experts on Additives (E 43). Both groups felt a collaborative study was desirable. In the United Kingdom, the Food Science Division of the Ministry of Agriculture, Fisheries and Food also was planning a collaborative study on a national level. It was concluded that one collaborative study would be preferable, to be organized by the Netherlands State Institute for Quality Control of Agricultural Products (RIKILT). In 1983, a pilot collaborative study was conducted, with 9 laboratories in 4 countries (2). That pilot study resulted in minor alterations of the methods.

In the collaborative study, conducted in 1984 and reported here, 37 collaborators in 13 countries participated.

This study concerns the determination and detection steps only. Although the sampling procedure and the preparation of the laboratory sample can substantially contribute to the inaccuracy of the method, they are beyond the scope of this study.

Collaborative Study

Two methods of measuring the amount of natamycin were tested: a spectrometric method and a liquid chromatographic method with UV detection. The aims of the study were to determine the applicability of both methods, for various concentration levels; the reliability of both methods, for various concentration levels, in terms of statistical parameters; whether both methods give corresponding results; whether false results may be obtained by interference of degradation products of natamycin; and the recovery of the methods.

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Samples

The main problem in preparing samples was that neither the cheese as such nor natamycin on the rind is stable. In the Netherlands intercomparisons, no comparable results were obtained when there were differences in duration and conditions of storage before analysis. For a worldwide intercomparison, samples must be stable under normal conditions. In earlier investigations, it had been found that natamycin in cheese rind that is homogenized, lyophilized, and then packed in brown glass bottles under nitrogen is stable for a longer period. Such samples were used successfully in the pilot international study.

For the present study, each participant received 8 samples, prepared in May 1984, consisting of lyophilized cheese rind or cheese packed in brown bottles under nitrogen. Each sample was ca 15 g, which is sufficient for one analysis. Participants were advised to store samples in a refrigerator.

Samples were blind duplicates containing 4 natamycin levels and were dispatched under code numbers. Participants were not informed whether they received duplicates or split-level samples.

Samples are grouped below according to their characteristics.

Group A.—Samples 1 and 4; cheese rind containing high level (above EEC limit) of natamycin; cheese production date, Apr. 10, 1984; treated 4 times with cheese coating containing 0.005% natamycin during the period May 9–16, 1984.

Group B.—Samples 2 and 8; cheese rind containing low level (ca EEC limit) of natamycin; cheese production date, July 15, 1982; treated 3 times with cheese coating containing 0.0125% natamycin during the period July 15–Aug. 31, 1982, and 2 times with 0.005% natamycin during 1983.

Group C.—Samples 3 and 6; cheese, inner part, ground, treated with natamycin and homogenized; containing natamycin at level above detection limit.

Group D.—Samples 5 and 7; cheese, inner part, ground, treated with natamycin and homogenized; containing natamycin at very low level.

From earlier investigations, it could be expected that levels of groups A and B could be determined directly without concentration of samples, that the level of group C had to be concentrated, and that the level of group D was at or below the detection limit. Although no blank samples were dispatched, the results for group D could have been construed as false positives.

Homogeneity

The natamycin contents (mg/kg) of 5 lots of each sample, as dispatched to the participants, were as follows: Group A—61.7, 65.3, 60.0, 61.2, 59.3; mean, 61.4; standard deviation (SD), 2.22; coefficient of variation (CV), 3.6%. Group B—14.5, 15.2, 14.3, 14.5, 14.5; mean, 14.6; SD, 0.35; CV, 2.4%. Group C (5× concn)—1.5, 1.8, 1.3, 1.8, 1.5; mean, 1.52; SD,

Table 1. Interlaboratory results of spectrometric determinations of natamycin in unconcentrated cheese and cheese rind samples

	Natamycin, mg/kg							
	Group A		Group B		Group C		Group D	
Lab.	Sample 1	Sample 4	Sample 2	Sample 8	Sample 3	Sample 6	Sample 5	Sample 7
1	63.20	59.40	16.00	13.20	0.80	0.40	n.d.[a]	n.d.
3	61.99	55.88	14.75	12.85	n.d.	n.d.	n.d.	n.d.
4	61.37	56.79	14.01	13.83	1.01	1.19	n.d.	n.d.
5	62.10	60.90	16.50	15.90	1.50	1.35	n.d.	n.d.
6	59.40	60.20	15.90	16.00	1.17	1.17	n.d.	n.d.
7	64.36	65.42	15.29	14.02	n.d.	0.85	n.d.	n.d.
8	66.79	64.21	17.30	16.93	n.d.	n.d.	n.d.	n.d.
9	63.03	69.10	17.10	19.20	n.d.	n.d.	n.d.	n.d.
10	59.45	44.47	14.52	14.12	2.37	n.d.	n.d.	n.d.
11	57.08	47.46	12.82	13.83	n.d.	n.d.	n.d.	n.d.
12	70.60	65.20	15.90	16.80	n.d.	n.d.	n.d.	n.d.
13	63.90	56.47	18.80	17.75	1.60	1.71	1.30	0.40
14	75.90	67.10	18.70	15.50	2.90	2.60	n.d.	n.d.
15	59.53	57.13	14.63	14.61	n.d.	n.d.	n.d.	n.d.
16	62.30	61.20	13.60	13.20	0.76	n.d.	n.d.	n.d.
17	74.70	78.20	16.30	16.30	1.60	1.90	0.50	0.50
18	67.14	66.59	13.92	15.85	1.29	1.00	n.d.	n.d.
19	63.80	63.80	12.80	15.00	1.10	0.90	n.d.	n.d.
20	73.50	70.80	14.80	15.70	0.40	0.90	n.d.	n.d.
21	61.90	57.40	14.90	15.30	2.90	3.00	1.70	n.d.
22	60.66	56.56	15.40	14.53	0.84	1.25	n.d.	n.d.
23	54.06	51.34	10.68	12.15	2.10	2.30	8.38	0.42
24	47.50	40.50	18.10[b]	10.10[b]	n.d.	n.d.	n.d.	n.d.
25	73.50	63.90	15.60	15.80	1.30	0.80	n.d.	n.d.
26	68.25	63.42	16.85	18.26	0.63	0.63	0.39	0.38
27	75.90	70.70	17.60	16.40	n.d.	1.20	n.d.	n.d.
28	64.83	62.50	14.50	14.66	1.66	1.66	n.d.	n.d.
29	62.82	63.47	15.63	15.27	n.d.	n.d.	n.d.	n.d.
30	65.81	67.67	15.68	15.06	1.23	n.d.	n.d.	n.d.
31	65.50	61.10	17.00	16.20	0.70	n.d.	n.d.	n.d.
32	68.93	66.37	14.21	15.37	n.d.	n.d.	n.d.	n.d.
33	65.95	59.55	14.65	16.50	2.80	2.70	n.d.	n.d.
34	66.58	60.72	14.45	16.12	0.62	1.47	n.d.	n.d.
35	44.00	43.17	10.67	9.50	0.22	n.d.	n.d.	n.d.
36	65.49	67.44	15.81	13.60	n.d.	n.d.	n.d.	n.d.
37	67.12	65.12	15.67	15.30	n.d.	n.d.	n.d.	n.d.

[a] Not detectable.
[b] Outlier.

Table 2. Interlaboratory results of LC determinations of natamycin in unconcentrated cheese and cheese rind samples

	Natamycin, mg/kg							
	Group A		Group B		Group C		Group D	
Lab.	Sample 1	Sample 4	Sample 2	Sample 8	Sample 3	Sample 6	Sample 5	Sample 7
1	58.74	53.43	14.69	12.15	1.85	n.d.[a]	n.d.	n.d.
2	63.00	60.20	15.10	15.60	n.d.	n.d.	n.d.	n.d.
3	52.58	49.83	15.66	12.63	n.d.	n.d.	n.d.	n.d.
4	58.62	54.04	15.57	14.56	1.65	2.47	n.d.	n.d.
5	62.43	61.66	16.57	13.88	1.93	2.31	0.77	0.58
7	63.76	68.04	17.10	17.66	1.77	1.93	0.48	0.46
10	54.37	31.04	13.54	13.14	n.d.	n.d.	n.d.	n.d.
11	54.96	35.04	7.56[b]	14.29[b]	n.d.	n.d.	n.d.	n.d.
12	69.40	67.00	17.80	16.40	2.60	1.90	0.60	0.50
13	85.10[b]	17.09[b]	25.90[b]	12.55[b]	3.13	2.43	1.90	0.14
14	77.60	85.00	18.00	19.80	1.80	2.50	n.d.	n.d.
16	39.80	34.20	n.d.[b]	n.d.[b]	n.d.	n.d.	n.d.	n.d.
17	63.80	63.60	14.10	14.70	1.80	1.80	n.d.	n.d.
18	62.56	67.42	14.38	14.38	n.d.	n.d.	n.d.	n.d.
19	56.50	57.80	10.50	12.20	n.d.	n.d.	n.d.	n.d.
20	64.58	50.70	13.95	13.95	2.06	2.79	n.d.	n.d.
24	54.30	49.20	15.70	13.80	n.d.	n.d.	n.d.	n.d.
25	81.70	69.60	17.20	18.50	n.d.	n.d.	n.d.	n.d.
27	68.60	61.90	9.90	9.64	n.d.	1.00	n.d.	n.d.
28	66.33	60.33	12.17	12.50	1.33	1.33	0.33	0.33
29	53.15	53.52	13.50	13.38	1.50	1.04	0.15	0.15
30	64.84	65.87	15.77	14.87	2.63	2.32	1.18	1.20
31	87.00	92.00	23.00	25.00	8.00	n.d.	n.d.	n.d.
32	68.70	66.64	8.11	9.25	n.d.	n.d.	n.d.	n.d.
35	42.67	42.33	13.50[b]	4.73[b]	n.d.	2.00	n.d.	n.d.
36	65.10	69.00	23.00	23.60	n.d.	n.d.	n.d.	n.d.
37	71.00	66.20	21.10	24.40	n.d.	n.d.	n.d.	n.d.

[a] Not detectable.
[b] Outlier.

0.28; CV, 17.8%. Group D (10× concn)—0.35, 0.43, 0.47, 0.42, 0.41; mean, 0.42; SD, 0.04; CV, 9.5%.

Degradation Products

Degradation products (3) may interfere with natamycin and give rise to incorrect results. With LC determination, these degradation products have a shorter retention time, so that 2 separate peaks are obtained. The spectrometric method is less specific, and degradation products may seemingly enhance the results for natamycin. One aim of this study was to test whether this occurs in practice when the described method is applied. If so, the spectrometric results will be higher than LC results. This will be the case particularly for older cheeses where more degradation products can be expected. Therefore, group B (samples 2 and 8) was prepared from an extremely old cheese that had been treated with natamycin throughout 2 years. Especially for this group, remarkable differences between the 2 methods can thus be expected.

Recovery Tests

For determination of recovery, participants were asked to analyze 2 other samples, which they prepared, as follows: Cut a piece of rind to a thickness of ca 5 mm from a hard type of domestic cheese. Grind the rind and homogenize. Weigh 10 g ground rind into 200 mL conical flask. Dissolve 100 mg natamycin reference sample (91.6% natamycin) in 50 mL methanol. Dilute 1:10 with methanol. Add 1 mL of this solution to the contents of the conical flask. The concentration in the sample is thus $0.916 \times 20 = 18.32$ mg/kg. Proceed as described by De Ruig et al. (1) starting from *Extraction of Sample Solutions*.

Results

The results reported by the participants are shown in Tables 1–4. Recoveries of the spectrometric and LC detection are reported in Tables 5 and 6. In these tables, the natamycin levels are given only in mg/kg. The amount in mg/dm^2 can be calculated, taking into account the surface (Y) and the mass (X) of the laboratory sample; in the collaborative study the values were given in the protocol, namely, $X = 15$ g, $Y = 25$ cm^2, so that

$$C'(\text{mg/dm}^2) = 0.1(15/25)C = 0.06C(\text{mg/kg}).$$

Results were sent in a provisional form to all participants to check the data. In some cases, where something obviously seemed to be wrong, the institute in question was contacted. It was found that some institutes had not corrected for the standard natamycin being 91.6%. After a questionnaire was sent, some participants corrected their results, and the data in this report have been corrected where necessary. Because the method was not conducted as prescribed, results from 7 laboratories were excluded in the evaluation of this collaborative study. Deviations included use of other LC column, mobile phase, or flow rate, variations in pretreatment, and results of a second experiment with less sample.

Table 3. Interlaboratory results of spectrometric determinations of natamycin in concentrated cheese and cheese rind samples

	Group B				Group C				Group D			
Lab.	Concn level	Sample 2, mg/kg	Concn level	Sample 8, mg/kg	Concn level	Sample 3, mg/kg	Concn level	Sample 6, mg/kg	Concn level	Sample 5, mg/kg	Concn level	Sample 7, mg/kg
1	—[a]	—	—	—	10	1.26	10	1.18	10	0.14	10	0.18
3	—	—	—	—	10	0.84	5	1.37	10	n.d.[b]	10	0.26
4	—	—	—	—	10	0.92	10	1.10	10	0.18	10	0.27
5	—	—	—	—	5	1.32	5	1.56	10	0.31	10	0.30
7	—	—	—	—	10	0.87	10	0.94	10	0.21	10	0.25
10	—	—	—	—	5	1.09	10	0.85	10	0.24	10	0.37
11	5	10.60	—	—	5	1.07	10	1.21	10	0.42	10	0.05
12	—	—	—	—	10	1.50	10	1.10	10	n.d.[c]	10	n.d.[c]
13	—	—	—	—	—	—	5	1.82	5	0.52	5	0.38
14	—	—	—	—	—	—	—	—	10	n.d.[c]	10	n.d.[c]
15	5	11.88	5	5.31	10	0.61	10	n.d.	10	n.d.[c]	10	n.d.[c]
17	—	—	—	—	10	1.20	10	1.50	10	0.30	5	0.30
18	—	—	—	—	5	1.58	10	1.25	5	0.16	5	0.28
19	—	—	—	—	10	0.60	10	0.30	10	n.d.[c]	10	n.d.[c]
20	—	—	—	—	10	1.17	10	0.66	10	0.09	10	n.d.
22	—	—	—	—	10	1.04	10	1.15	5	0.16	10	0.15
23	—	—	—	—	10	4.35[c]	—	—	—	—	10	0.64
24	—	—	—	—	10	1.50	10	1.42	10	0.28	10	n.d.
25	—	—	—	—	5	1.60	5	1.60	5	0.70	5	0.50
27	—	—	—	—	10	1.26	10	1.64	10	0.38	10	0.41
28	—	—	—	—	5	1.67	5	1.83	10	0.33	10	0.33
29	—	—	5	13.39	10	1.01	10	0.88	10	n.d.[c]	10	n.d.[c]
30	—	—	—	—	5	1.82	5	1.78	10	0.30	10	0.30
31	5	35.20	5	54.10	10	13.70[c]	10	6.70[c]	10	2.40[c]	10	n.d.[c]
32	—	—	—	—	10	1.43	10	1.23	10	0.16	10	0.50
33	—	—	—	—	—	—	10	2.00	—	—	—	—
34	5	12.44	5	5.73	10	0.55	10	0.73	10	n.d.[c]	10	n.d.[c]
35	—	—	10	6.50	—	—	10	0.75	10	0.17	10	0.10
36	—	—	—	—	5.6	1.19	5.6	1.29	5.6	n.d.[c]	5.6	n.d.[c]
37	—	—	—	—	5.5	1.34	5.5	1.18	5.5	n.d.[c]	5.5	n.d.[c]

[a] Missing.
[b] Not detectable.
[c] Outlier.

Table 4. Interlaboratory results of LC determinations of natamycin in concentrated cheese and cheese rind samples

	Group B				Group C				Group D			
Lab.	Concn level	Sample 2, mg/kg	Concn level	Sample 8, mg/kg	Concn level	Sample 3, mg/kg	Concn level	Sample 6, mg/kg	Concn level	Sample 5, mg/kg	Concn level	Sample 7, mg/kg
1	—[a]	—	—	—	10	1.26	10	1.15	10	0.35	10	0.26
2	—	—	—	—	10	1.10	10	1.30	10	n.d.[b,c]	10	n.d.[c]
3	—	—	—	—	10	0.92	5	1.46	10	0.24	10	0.32
4	—	—	—	—	10	0.92	10	1.37	10	0.22	10	0.23
5	—	—	—	—	5	1.00	5	1.08	10	0.31	10	0.31
7	—	—	—	—	5	1.31	5	1.48	10	0.32	10	0.23
10	—	—	—	—	5	0.79	10	0.82	10	0.27	10	0.24
11	5	7.42	—	—	5	1.13	10	2.12	10	0.36	10	0.17
12	—	—	—	—	—	—	5	1.50	5	0.50	5	0.40
14	—	—	—	—	—	—	—	—	10	0.30	10	0.50
16	10	11.60	10	11.70	—	—	—	—	—	—	—	—
17	—	—	—	—	10	1.10	10	1.30	10	0.40	5	0.40
18	—	—	—	—	5	1.46	5	1.77	10	0.31	10	0.38
19	—	—	—	—	10	1.10	10	0.22	10	n.d.[c]	10	n.d.[c]
20	—	—	—	—	10	1.26	10	0.73	10	0.29	10	0.18
24	—	—	—	—	10	2.90	10	2.30	10	1.00[c]	10	0.90[c]
25	—	—	—	—	5	1.60	5	1.70	5	0.70	5	0.40
27	—	—	—	—	10	1.21	10	1.45	10	0.36	10	0.38
28	—	—	—	—	5	1.67	5	1.83	10	0.33	10	0.33
29	—	—	—	—	10	1.35	10	0.90	10	0.15	10	n.d.[c]
30	—	—	—	—	5	2.15	5	2.12	10	0.42	10	0.43
32	—	—	—	—	10	0.94	10	1.28	10	0.18	10	0.43
35	—	—	10	8.27	10	0.88	10	2.02	10	1.45[c]	10	n.d.
36	—	—	—	—	5.5	2.10	5.5	2.00	5.5	0.70	5.5	0.40
37	—	—	—	—	5.5	2.00	5.5	2.30	5.5	0.40	5.5	0.50

[a] Missing.
[b] Not detectable.
[c] Outlier.

Table 5. Recovery of natamycin by spectrometric method from cheese rind samples spiked by individual laboratories

		Sample 1		Sample 2		Recovery	
Lab.	Added, mg/kg	Found, mg/kg	Rec., %	Found, mg/kg	Rec., %	Mean, %	Diff., %
1	40.00	42.20	105.50	38.20	95.50	100.50	10.00
2	—[a]	—	—	—	—	—	—
3	20.00	18.40	92.00	—	—	92.00	—
4	20.00	15.50	77.50	16.20	81.00	79.25	3.50
5	20.00	19.50	97.50	20.30	101.50	99.50	4.00
6	18.30	13.00	71.04	—	—	71.04	—
7	20.00	17.20	86.00	17.42	87.10	86.55	1.10
8	20.00	19.51	97.55	19.36	96.80	97.18	0.75
9	20.00	18.90	94.50	17.80	89.00	91.75	5.50
10	—	—	—	—	—	—	—
11	20.00	21.00	105.00	21.80	109.00	107.00	4.00
12	20.00	19.20	96.00	19.40	97.00	96.50	1.00
13	20.00	17.50	87.50	17.00	85.00	86.25	2.50
14	20.60	19.50	94.66	—	—	94.66	—
15	18.32	17.67	96.45	17.83	97.33	96.89	0.87
16	18.00	12.84	71.33	13.20	73.33	72.33	2.00
17	19.45	19.80	101.80	18.70	96.14	98.97	5.66
18	19.83	17.90	90.28	18.70	94.31	92.29	4.03
19	20.00	18.70	93.50	18.00	90.00	91.75	3.50
20	19.57	19.00	97.09	19.60	100.15	98.62	3.07
21	20.00	17.10	85.50	—	—	85.50	—
22	19.02	19.42	102.10	—	—	102.10	—
23	20.00	19.28	96.40	17.60	88.00	92.20	8.40
24	18.30	13.00	71.04	—	—	71.04	—
25	18.40	15.60	84.78	—	—	84.78	—
26	20.00	12.47	62.35	13.24	66.20	64.28	3.85
27	20.00	16.40	82.00	17.60	88.00	85.00	6.00
28	21.35	20.27	94.94	19.87	93.07	94.00	1.87
29	20.07	18.52	92.28	—	—	92.28	—
30	18.32	17.54	95.74	17.74	96.83	96.29	1.09
31	20.00	20.50	102.50	19.10	95.50	99.00	7.00
32	20.00	27.46	137.30	25.68	128.40	132.85	8.90
33	19.70	21.00	106.60	—	—	106.60	—
35	20.12	15.66	77.85	26.00	129.26	103.56	51.40
36	18.47	18.55	100.43	—	—	100.43	—
37	20.00	19.83	99.15	—	—	99.15	—

[a] Missing.

Table 6. Recovery of natamycin by LC method from cheese rind samples spiked by individual laboratories

		Sample 1		Sample 2		Recovery	
Lab.	Added, mg/kg	Found, mg/kg	Rec., %	Found, mg/kg	Rec., %	Mean, %	Diff., %
1	40.00	39.41	98.53	30.42	76.05	87.29	22.48
2	50.00	51.00	102.00	45.70	91.40	96.70	10.60
3	20.00	18.30	91.50	—[a]	—	91.50	—
4	20.00	19.10	95.50	19.10	95.50	95.50	0.00
5	20.00	20.80	104.00	20.40	102.00	103.00	2.00
7	20.00	18.55	92.75	18.76	93.80	93.28	1.05
8	—	—	—	—	—	—	—
10	20.00	19.09	95.45	19.31	96.55	96.00	1.10
11	20.00	22.00	110.00	17.55	87.75	98.88	22.25
12	20.00	20.50	102.50	20.70	103.50	103.00	1.00
13	20.00	20.87	104.35	19.31	96.55	100.45	7.80
14	20.60	23.00	111.65	—	—	111.65	—
16	18.00	15.20	84.40	14.40	80.00	82.22	4.44
17	—	—	—	—	—	—	—
18	19.83	15.90	80.19	20.10	101.37	90.78	21.18
19	20.00	20.30	101.50	9.40	47.00	74.25	54.50
20	19.57	14.97	76.49	16.88	86.25	81.37	9.76
21	—	—	—	—	—	—	—
22	—	—	—	—	—	—	—
23	—	—	—	—	—	—	—
24	18.30	17.40	95.08	—	—	95.08	—
25	18.40	16.00	86.96	—	—	86.96	—
26	—	—	—	—	—	—	—
27	20.00	14.60	73.00	12.40	62.00	67.50	11.00
28	—	—	—	—	—	—	—
29	20.07	17.84	88.89	—	—	88.89	—
30	18.32	19.05	103.99	19.02	103.82	103.90	0.16
31	20.00	24.00	120.00	20.00	100.00	110.00	20.00
32	20.00	16.30	81.50	15.80	79.00	80.25	2.50
35	20.12	14.00	69.60	26.50	131.74	100.67	62.14
36	18.47	16.40	88.79	—	—	88.79	—
37	20.00	18.90	94.50	—	—	94.50	—

[a] Missing.

Evaluation of Results

The results of laboratories that applied the method correctly were statistically evaluated according to ISO 5725. According to this standard, Cochran's maximum variance test is used to test the repeatability within laboratories, and Dixon's outlier test is used to test the precision between laboratories. Outliers were rejected, stragglers were kept in.

Values reported as "not detectable" were considered to be outliers and were not included in the calculations of results. As was expected, levels in group A and B samples could be determined without concentration, group C levels could barely be determined, and group D levels could not be determined at all without preconcentration. Therefore, only the direct determinations for groups A and B and the determinations after concentration for groups C and D were evaluated and are reported in Table 7. The results are summarized in Table 8.

Applying the t test for random samples on the spectrometric and the LC results shows that there is no significant difference in the results of both methods at all 4 levels investigated; $t < 1.96$ in all cases. In the spectrometric method, there could be a risk that degradation products contributed to absorbance, thus giving rise to significantly higher values. Especially for group B samples (made from very old cheese and thus probably containing a high level of degradation products) this should be the case. However, such interference did not occur here as shown by the values.

The repeatability and reproducibility of the spectrometric method are better than those of the LC method for groups A and B. For group C they are comparable, and for group D, liquid chromatography is the better method.

Conclusions

The "true values," i.e., the arithmetic means of the mean values of natamycin detected by the spectrometric and the LC methods, of the samples can be estimated to be: group A, 61.7 mg/kg, 3.70 mg/dm^2; group B, 15.4 mg/kg, 0.92 mg/dm^2; group C, 1.31 mg/kg, 0.08 mg/dm^2; and group D, 0.30 mg/kg, 0.018 mg/dm^2.

More collaborators were able to conduct the spectrometric determination than the liquid chromatographic. Moreover, more difficulties were reported for the LC determination. Apparently, the spectrometric determination is more rigid and straightforward and easier to perform than the LC determination.

Group A levels of natamycin can be determined directly by both methods. However, for the spectrometric method, the coefficients of variation within laboratories, CV_r, and those among laboratories, CV_R, are less than half of those for the LC method. There is no need to apply the concentration step for this level.

Group B levels can be measured directly by both methods, too. The CV_r is about the same, but the CV_R of the LC method is twice that of the spectrometric method. For this level, too, concentration is unnecessary.

About one-third of the laboratories were not able to determine group C levels in unconcentrated samples: spectrometric, 11 out of 37; liquid chromatographic, 13 out of 32.

Table 7. Mean values of natamycin in groups A and B unconcentrated samples and in groups C and D concentrated samples determined by spectrometric and LC methods

	Direct				After concentration			
	Group A		Group B		Group C		Group D	
Lab.	Spectrom.	LC	Spectrom.	LC	Spectrom.	LC	Spectrom.	LC
1	61.30	56.09	14.60	13.42	1.22	1.21	0.16	0.31
2	—[a]	61.60	—	15.35	—	1.20	—	—
3	58.94	51.21	13.80	14.15	1.11	1.19	0.13[b]	0.28
4	59.08	56.33	13.92	15.07	1.01	1.15	0.23	0.23
5	61.50	62.05	16.20	15.23	1.44	1.04	0.31	0.31
6	59.80	—	15.95	—	—	—	—	—
7	64.89	65.90	14.66	17.38	0.91	1.40	0.23	0.28
8	65.50	—	11.12	—	—	—	—	—
9	66.07	—	18.15	—	—	—	—	—
10	51.96	42.71	14.32	13.34	0.97	0.81	0.31	0.26
11	52.27	45.00	13.32	—	1.14	1.63	0.24	0.27
12	67.90	68.20	16.35	17.10	1.30	1.50[c]	—	0.45
13	60.19	—	18.28	—	1.82[c]	—	0.45	—
14	71.50	81.30	17.10	18.90	—	—	—	0.40
15	58.33	—	14.62	—	0.31[b]	—	—	—
16	61.75	37.00	13.40	—	—	—	—	—
17	76.45	63.70	16.30	14.40	1.35	1.20	0.30	0.40
18	66.87	64.99	14.89	14.38	1.42	1.62	0.22	0.35
19	63.80	57.15	13.90	11.35	0.45	0.66	—	—
20	72.15	57.64	15.25	13.95	0.92	1.00	0.05[b]	0.24
21	59.65	—	15.10	—	—	—	—	—
22	58.61	—	14.93	—	1.10	—	0.16	—
23	52.70	—	11.42	—	—	—	0.64[c]	—
24	44.00	51.75	—	14.75	1.46	2.60	0.14[b]	—
25	68.70	75.65	15.70	17.85	1.60	1.65	0.60	0.55
26	65.84	—	17.56	—	—	—	—	—
27	73.30	65.25	17.00	9.77	1.45	1.33	0.40	0.37
28	63.67	63.33	14.58	12.34	1.75	1.75	0.33	0.33
29	63.15	53.34	15.45	13.44	0.95	1.13	—	0.08[b]
30	66.74	65.36	15.37	15.32	1.80	2.14	0.30	—
31	63.30	89.50	16.60	24.00	—	—	—	0.43
32	61.65	67.67	14.79	8.68	1.33	1.11	0.33	0.31
33	62.75	—	15.58	—	2.00[c]	—	—	—
34	63.65	—	15.29	—	0.64	—	—	—
35	43.59	42.50	10.09	—	0.75[c]	1.45	0.14	—
36	66.47	67.05	14.71	23.30	1.24	2.05	—	0.55
37	66.12	68.60	15.49	22.75	1.26	2.15	—	0.45
n[d]	36	26	35	23	27	23	20	20
$\bar{x}$	62.50	60.80	15.19	15.49	1.21	1.43	0.28	0.34
s_x	7.14	11.90	1.68	3.91	0.41	0.47	0.15	0.11
$s_{\bar{x}}$	1.19	2.33	0.28	0.82	0.079	0.098	0.034	0.025
Δ	1.70		−0.293		−0.222		−0.059	
s_Δ	2.62		0.86		0.125		0.042	
t	0.65		−0.34		−1.77		−1.40	

[a] Missing.
[b] Duplicate not detectable.
[c] Duplicate omitted.
[d] n = number of evaluated laboratories;
$\bar{x}$ = total mean value = mean of the mean values;
s_x = standard deviation of the mean values;
$s_{\bar{x}}$ = standard deviation of the total mean value $= s_x/\sqrt{n}$;
Δ = difference between spectrometric and LC methods; $= \bar{x}_{Sp} - \bar{x}_L$;
s_Δ = standard deviation of the difference $= s(\bar{x}_{Sp} - \bar{x}_L) = \sqrt{(s_x)^2_{Sp} + (s_x)^2_L}$;
t = t test = Δ/s_Δ.

After the samples were concentrated, all participants could obtain measurable results. CV_r is slightly better for the spectrometric detection, and CV_R is comparable.

Most participants were unable to detect group D levels in unconcentrated samples (spectrometric, 28 out of 37; liquid chromatographic, 17 out of 29). After the samples were concentrated, however, 20 out of 27 laboratories detected natamycin spectrometrically, and 23 of 24 obtained results by using liquid chromatography. The CVs for liquid chromatography were better than those for spectrometry.

To judge the applicability of the methods, we can apply, arbitrarily, the following classifications for CV_R: 1–15%, good; 16–30%, reasonable; 31–45%, poor; and >45%, not detectable. The breakdown of these classifications by group and method is shown in Table 9.

Recovery at a level of 20 mg/kg is approximately 100%.

Results by the spectrometric and LC methods are not significantly different. No contribution of natamycin degradation products by the spectrometric method was observed.

The difficulties encountered due to interference from paprika and pepper in the analysis of fresh cheeses was referred to the Joint IDF/ISO/AOAC Group of Experts. The occurrence of this phenomenon will be obvious from the complete spectrum, which would be altered, but it can be misinterpreted when only the 3 relevant wavelengths are measured. Therefore, in case of spectrometric determination, measurement of the complete spectrum is obligatory. No further essential alterations in the method were necessary.

The method has been adopted by the Joint IDF/ISO/AOAC Group of Experts on Selected Food Additives eventually to become an IDF/ISO Standard Method. It fulfills the requirements of the EEC, and the ad hoc EEC working group has adopted an unambiguous method for analysis of cheese rind and cheese based on this method.

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M. Barnett, Dr. Augustus Voelcker and Sons Ltd, London, UK

E. C. Conchie, Ruddock and Sherratt Public Analyst's Laboratory, Chester, UK

J. Crow, Dairy Technical Services, Melbourne, Australia

Z. Cvak, Vyzkumny ustav mlékarensky, Prague, Czechoslovakia

J. Daenen, Food Inspection Service, Maastricht, The Netherlands

W. G. de Ruig, State Institute for Quality Control of Agricultural Products, Wageningen, The Netherlands

A. Dupont, Laboratoire Central de Recherche et d'Analyse, Massy, Essonne, France

V. Eckelmans and Ch. R. Winné, Centraal Laboratorium Ministerie van Economische Zaken, Brussels, Belgium

G. Engel, Institut für Mikrobiologie Bundesanstalt für Milchforschung, Kiel, FRG

R. Fawcett. Kent County Analytical Laboratory, Kent, UK

W. Frede, Chemische und Lebensmitteluntersuchungsanstalt im Hygienischen Institut, Hamburg, FRG

J. P. Geerts, Cheese Control Station "Friesland" Leeuwarden, The Netherlands

Ch. Gertz, Chemische Untersuchungsanstalt, Hagen, FRG

Führling, Landesanstalt für Lebensmittel, Arzneimittel und gerichtliche Chemie, Berlin, FRG

C. N. Grange, Bostock Hill and Rigby, Birmingham, UK

M. Guldborg, National Food Institute, The Central Lab-

Table 8. Overall interlaboratory results expressed as repeatability and reproducibility

	Direct						After concentration					
	Group A			Group B			Group C			Group D		
Measurement	Spectrom.	LC	Sign. diff.	Spectrom.	LC	Sign. diff.[a]	Spectrom.	LC	Sign. diff.	Spectrom.	LC	Sign. diff.
Number of participants	36	27	NA	36	27	NA	29	23	NA	29	24	NA
Number of outliers	0	1	NA	1	4	NA	2	0	NA	9	4	NA
Outliers, %	0	4	NA	3	15	NA	7	0	NA	31	17	NA
Natamycin, mg/dm²												
x[b]	3.75	3.65	no	0.91	0.93	no	0.071	0.085	no	0.016	0.020	no
r[c]	0.61	0.96	no	0.16	0.19	no	0.034	0.057	yes	0.019	0.017	no
2.8 s_L[d]	1.13	1.90	yes	0.26	0.65	yes	0.062	0.070	no	0.019	0.016	no
R[e]	1.29	2.13		0.31	0.68		0.070	0.089		0.028	0.023	
Natamycin, mg/kg												
x[b]	62.5	60.8	no	15.2	15.5	no	1.19	1.43	no	0.27	0.34	no
r[c]	10.4	16.0	no	2.7	3.1	no	0.56	0.95	yes	0.32	0.28	no
2.8 s_L[d]	18.8	31.7	yes	4.4	10.8	yes	1.03	1.16	no	0.32	0.25	no
R[e]	21.5	35.5		5.1	11.3		1.17	1.49		0.46	0.38	
Coefficients of variation												
CV_r, %[f]	5.9	9.3	NA	6.2	7.1	NA	16.5	23.4	NA	42.5	29	NA
CV_L, %[g]	10.6	18.4	NA	10.2	24.7	NA	31	29	NA	42.6	26	NA
CV_R, %[h]	12.2	20.6	NA	11.9	25.7	NA	35	37	NA	60	39	NA

[a] no = no significant difference ($\alpha < 0.01$) between spectrometric and LC methods.
yes = significant difference ($\alpha < 0.01$) between spectrometric and LC methods.
[b] Content, weighted mean value.
[c] Repeatability value = 2.8 S_r, where S_r = repeatability (within-laboratory) standard deviation.
[d] S_L = between-laboratory standard deviation.
[e] Reproducibility value = 2.8 S_R, where S_R = reproducibility (overall among laboratories). Standard deviation, $S_R = \sqrt{S_r^2 + S_L^2}$.
[f] Repeatability relative standard deviation as a percent.
[g] Between laboratories relative standard deviation as a percent.
[h] Reproducibility relative standard deviation as a percent, $CV_R = \sqrt{CV_r^2 + CV_L^2}$.

Table 9. Applicability of spectrometric and LC methods according to level of natamycin in cheese and cheese rind samples

	Group, mg/kg (mg/dm²)			
Method	A, 60 (3.8)	B, 15 (0.9)	C, 1.7 (0.1)	D, 0.3 (0.02)
Spectrometric, direct	good[a]	good	not detectable	not detectable
Spectrometric, after concentration	—	—	poor	not detectable
LC, direct	reasonable	reasonable	not detectable	not detectable
LC, after concentration	—	—	poor	poor

[a] Good = 1–15%; reasonable = 16–30%; poor = 31–45%; not detectable = >45%.

oratory, Div.A: Nutrients and Food Additives, Søborg, Denmark

A. J. Harrison, County of Avon Scientific Services, Bristol, UK

B. Hoffmann, Chemisches Landesuntersuchungsamt Nordrhein-Westfalen, Munster, FRG

V. Husbands, Department of Regional Chemist, Strathclyde Regional Council, Glasgow, UK

I. Ibanez Rico, Laboratorio Arbitral Central de Fraudes, Madrid, Spain

Masahiro Iwaida, Nestlé K. K., Kobe Port, Japan

T. E. Johnson, Herbert J. Evans and Partners, Camarthen, UK

J. Leenheer, Netherlands Controlling Authority for Milk and Milk Products, Leusden, The Netherlands

H. Köbler, Chemische Landesuntersuchungsanstalt Stuttgart, Stuttgart, FRG

T. Linazo Iglesias, Laboratorio Agrario del Estado en Madrid, Madrid, Spain

S. Littman, % Chem. Untersuchungsamt, Hamm, FRG

J. M. Nuijens, Food Inspection Service, Haarlem, The Netherlands

H. Overström, SMR Central Laboratorium, Malmö, Sweden

J. A. Palgrave, Moir and Palgrave, London, UK

S. v. d. Reek, DMV-Campina B. V., Veghel, The Netherlands

C. Repelius, Gist-Brocades NV, Delft, The Netherlands

Schneider, Milchwirtschaftliche Untersuchungsanstalt München der Landesvereinigung der Bayerischen Milchwirtschaft e.V., München, FRG

M. J. Scotter, Ministry of Agriculture, Fisheries and Food, London, UK

D. Scuffam, Laboratory of the Government Chemist, London, UK

J. H. Shelton, Leo Taylor and Lucke, Bedford House, London, UK

M. T. Stijve, Société d'Assistance Technique pour Produits Nestlé S. A., La Tour de Peilz, Switzerland

R. Tiebach, Max von Pettenkofer Institut des Bundesgesundheitsamtes, Berlin, FRG

E. Tsigaridas, General Chemical State Laboratory, Athens, Greece

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DECOMPOSITION

Evaluation of Precision Estimates for Fiber-Dimensional and Electrical Hygrometers for Water Activity Determinations

WILLIAM H. STROUP and JAMES T. PEELER[1]
Food and Drug Administration, Division of Food Chemistry and Technology, Cincinnati, OH 45226
KENT SMITH
Food and Drug Administration, Division of Field Science, Rockville, MD 20857

The precision of instruments used in 3 collaborative studies conducted within the Food and Drug Administration over a 4-year period (1981, 1982, 1984) for water activity (a_w) determinations according to the official AOAC method is evaluated. Calibration responses of the instruments were tested for linearity over the a_w range from 0.75 to 0.97. Average absolute percent difference between predicted and assigned a_w values for the linear model ranged from 0.3 to 0.7% for a fiber-dimensional hygrometer (Abbeon) and 3 electrical hygrometers (Beckman, Rotronics, and Weather Measure). The calibration responses for another electrical hygrometer (Hygrodynamics) were nonlinear. The fiber-dimensional hygrometer yielded mean a_w values and precision estimates that did not differ significantly from those obtained with the electrical hygrometers for $(NH_4)_2SO_4$ slush, KNO_3 slush, sweetened condensed milk, pancake syrup, and cheese spread. However, the mean a_w value for a soy sauce was 0.838 for the electrical hygrometers compared with 0.911 for the fiber-dimensional hygrometer. The fiber-dimensional hygrometer was affected by a volatile component(s) in the soy sauce that caused an erroneously high a_w value. Pooled estimates of reproducibility (S_X) in the 3 studies were 0.008 for the fiber-dimensional hygrometer and 0.010 for the electrical hygrometers; these values were not significantly different from those reported in the study that verified the current official AOAC method.

The Food and Drug Administration (FDA) laboratories use the official AOAC method (1, 2) tc measure the water activity (a_w) values of foods. Accurate and repeatable a_w measurements are needed by FDA to support the exemption of low-acid canned foods with a finished product a_w of 0.85 or less from the requirements of the *Code of Federal Regulations* (3). These measurements are also needed to determine the appropriate level of humectants for canned foods that receive a thermal processing less than that required for commercial sterility.

Within the framework of this AOAC method, several instruments with different operating principles may be used for a_w measurement. These operating principles include change in electrical conductivity of immobilized salt solutions, change in electrical capacitance of polymer thin films, dew point by chilled-mirror technique, longitudinal change in dimensions of water-sorbing fiber, partial water vapor pressure by manometric system, and relative weight of moisture sorbed by anhydrous hydrophilic solid. A multiinstrument study (2) determined that a_w measurements by the described method can be made with an accuracy and precision within ± 0.01 with instruments using immobilized salt sensors (Beckman, Rotronics, and Hygrodynamics). However, a valid statistical analysis could not be made for other instruments with different operating principles listed in the method because too few laboratories were using them at the time (2). The objectives of this study were to determine the precision estimates for a fiber-dimensional hygrometer which was used by an insufficient number of laboratories in the original study (2) and to compare the precision estimates for this fiber-dimensional hygrometer with those for selected electrical hygrometers from the original study.

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[1] Division of Microbiology.

Experimental

Five instruments were used to measure a_w: the Abbeon hygrometer, which is based on the longitudinal change in the dimensions of a water-sorbing fiber; the Beckman, Hygrodynamics, and Rotronic hygrometers, which are based on a change in electrical conductivity of immobilized salt solutions; and the Weather Measure hygrometer, which is based on a change in the electrical capacitance of polymer thin films. The Hygrodynamics instruments were operated with narrow-range sensors, so 2 sensors were used (color-coded as violet and gray by the vendor) to obtain the necessary coverage over the a_w range from 0.75 to 0.97. All other hygrometers evaluated in this study used a single sensor over this a_w range.

Sixteen to 18 laboratories participated in each of the 3 intra-FDA collaborative studies. All samples were analyzed in duplicate for a_w values by using the AOAC method (1). In the first collaborative study, 5 salt slushes used to calibrate the Abbeon hygrometers were chosen and prepared by each participating laboratory from 9 salts ($a_w > 0.742$) listed in the official AOAC method. In the last 2 studies, the same 5 salt slushes (NaCl, KBr, KCl, $BaCl_2$, and K_2SO_4) were prepared by each participating laboratory to calibrate all instruments. Each laboratory submitted the instrument readings for the calibration responses and the test samples as well as the calculated a_w values.

For test samples, 2 salt slushes (KNO_3 and $[NH_4]_2SO_4$) and 2 foods (a fermented soy sauce and a pancake syrup) were portioned from pooled lots of reagent grade salts and products, respectively. In addition, 2 foods (14 oz cans of a sweetened condensed milk and 8 oz jars of a cheese spread) were selected at random from a bulk purchase of foods with the same container code.

Results and Discussion

Instrument Calibration Responses

In AOAC method **32.004-32.009**, a best-fitting line or curve is drawn through a plot of the meter readings for 5 salt slushes vs their assigned a_w values to determine the calibration response. The a_w values are then determined from the meter readings for the test samples by using the calibration response. However, most investigators, including FDA analysts, prefer to use a mathematical model to characterize the calibration response for all a_w instruments whenever possible. This approach tends to minimize between-analyst bias associated with freehand drawing of a best-fitting line. The calibration responses for the a_w instruments used in this study were analyzed for linearity by using the method of least-squares regression (4). The average absolute percent differences between the observed meter readings and the values predicted from the linear model were calculated by the meth-

Table 1. Precision estimates for hygrometers (Abbeon) used in 1981 collaborative study[a]

Statistic		KNO_3 slush	Sweetened condensed milk
Mean a_w[b]	$\bar{x}$	0.928	0.853
Repeatability[c]	S_0	0.004	0.004
Among laboratories[c]	S_L	0.007	0.008
Reproducibility[c]	S_x	0.008	0.009
$(S_0/\bar{x}) \times 100$	CV_0	0.4	0.4
$(S_L/\bar{x}) \times 100$	CV_L	0.8	1.0
$(S_x/\bar{x}) \times 100$	CV_x	0.9	1.1

[a] No electrical hygrometers were used in this study.
[b] Data from 14 laboratories submitted in duplicate.
[c] Calculated as a standard deviation.

od of Davis (5). These average absolute percent differences ranged from 0.3 to 0.7% for all hygrometers except the Hygrodynamics instruments.

When a mathematical model is used to characterize the calibration points, 2 types of errors can occur: experimental error associated with the generation of the meter reading, and failure of the mathematical model to fit the calibration response. Since the calibration responses for each instrument consisted of a single meter reading for each salt slush, no measure of replicate error was available for the calibration. Consequently, the residual sum of squares calculated for each response could not be used to obtain estimates of lack of fit for the model and the experimental error. We then assumed that the repeatability variances (Tables 1–3) for the test samples are a reasonable estimate for the replicate error associated with the calibration responses. Thus, the residual error for the calibration responses can be compared to the estimated value for replicate error, and a statistical test can be performed to indicate when a mathematical model fails to adequately "fit" the calibration response. The pooled estimate for replicate standard deviation (SD) calculated from Tables 1–3 was 0.004 with 259 degrees of freedom (df). The F-ratio was computed for the calibration curves (4). As an example of this calculation, consider that a linear regression from an Abbeon instrument yielded a correlation coefficient of $r = 0.9995$. The residual mean square was 0.000005505

Table 2. Precision estimates for hygrometers used in 1982 collaborative study

Statistic		$(NH_4)_2SO_4$ slush	Soy sauce
Abbeon hygrometers			
Mean a_w[a]	$\bar{x}$	0.803	0.911
Repeatability[b]	S_0	0.004	0.005
Among laboratories[b]	S_L	0.006	0.009
Reproducibility[b]	S_x	0.007	0.010
$(S_0/\bar{x}) \times 100$	CV_0	0.5	0.6
$(S_L/\bar{x}) \times 100$	CV_L	0.8	1.0
$(S_x/\bar{x}) \times 100$	CV_x	0.9	1.1
Electrical hygrometers			
Mean a_w[c]	$\bar{x}$	0.810	0.838
Repeatability[b]	S_0	0.004	0.002
Among laboratories[b]	S_L	0.007	0.011
Reproducibility[b]	S_x	0.008	0.011
$(S_0/\bar{x}) \times 100$	CV_0	0.5	0.3
$(S_L/\bar{x}) \times 100$	CV_L	0.9	1.3
$(S_x/\bar{x}) \times 100$	CV_x	1.0	1.3

[a] Data from 13 laboratories submitted in duplicate.
[b] Calculated as a standard deviation.
[c] Data from 5 laboratories submitted in duplicate. Electrical hygrometers used included 2 from Hygrodynamics and 1 each from Rotronics, Beckman, and Weather Measure.

Table 3. Precision estimates for hygrometers used in 1984 collaborative study

Statistic		KNO_3 slush	Syrup	Cheese spread
Abbeon hygrometers				
Mean a_w[a]	$\bar{x}$	0.930	0.849	0.946
Repeatability[b]	S_0	0.003	0.004	0.005
Among laboratories[b]	S_L	0.004	0.005	0.006
Reproducibility[b]	S_x	0.005	0.006	0.008
$(S_0/\bar{x}) \times 100$	CV_0	0.4	0.4	0.5
$(S_L/\bar{x}) \times 100$	CV_L	0.4	0.6	0.7
$(S_x/\bar{x}) \times 100$	CV_x	0.6	0.7	0.8
Electrical hygrometers				
Mean a_w[c]	$\bar{x}$	0.931	0.852	0.946
Repeatability[b]	S_0	0.002	0.002	0.003
Among laboratories[b]	S_L	0.008	0.013	0.010
Reproducibility[b]	S_x	0.009	0.013	0.011
$(S_0/\bar{x}) \times 100$	CV_0	0.2	0.2	0.4
$(S_L/\bar{x}) \times 100$	CV_L	0.9	1.5	1.1
$(S_x/\bar{x}) \times 100$	CV_x	0.9	1.5	1.1

[a] Data from 16 laboratories submitted in duplicate.
[b] Calculated as a standard deviation.
[c] Data from 8 laboratories submitted in duplicate. Electrical hygrometers used included 4 from Hygrodynamics, 2 Rotronics, and 2 Beckman.

with 3 df. If we assume as a worst case that the variance is all due to lack of fit, then $F_{3,259} = (0.000005505/0.00001477) = 0.37$. This is not significant at the $\alpha = 0.05$ level. Consequently, a mathematical model for a calibration response that yields a significant F-ratio indicated an unsatisfactory (lack-of-fit) model to characterize that response. These tests show that the linear model adequately fits the calibration responses for all hygrometers used in this study except the Hygrodynamics instruments.

The vendor for the Hygrodynamics instruments provides a calibration curve that is nonlinear for each narrow-range sensor. We attempted to characterize these curves with several common mathematical models; however, we were unable to find a simple model that yielded a nonsignificant lack of fit between the observed and predicted meter readings. Consequently, when the Hygrodynamics instruments are used, the a_w of test samples still should be determined graphically from a "best-fitting" curve drawn through the calibration points.

Effect of Potassium Nitrate Slush on Calibration Response

A disagreement exists among investigators on the appropriate assigned a_w value for KNO_3 slush at 25°C. The 2 most commonly assigned values are 0.925 and 0.936 (6). In the official AOAC method, the a_w value of 0.936 was assigned for KNO_3 slush on the basis of the recommendations of Greenspan (7). In the 1981 collaborative study, 9 of the participating laboratories used KNO_3 slush in generating their calibration responses. The participating laboratories submitted the meter readings for their calibration responses as well as for test samples; consequently, we were able to simulate the effect of this disagreement on the means and precision estimates in calculating the a_w values for test samples.

Table 4 shows the simulated mean and precision estimates for KNO_3 slush and sweetened condensed milk when it is assumed that the KNO_3 slush had an a_w value of 0.936, the KNO_3 slush had an a_w value of 0.925, and the KNO_3 slush was omitted and a 4-point calibration response was used. The means and precision estimates from the a_w values for these 2 tests were not significantly different ($\alpha = 0.05$ level) when calculated from the calibration responses under the 3

Table 4. Simulated means and precision estimates of fiber-dimensional hygrometers used in 1981 collaborative study, obtained by using different assigned a_w values for KNO_3 slush[a]

Statistic		KNO_3 slush as test samples			Sweetened condensed milk		
		I[c]	II[d]	III[e]	I	II	III
Mean a_w	$\bar{x}$	0.928	0.924	0.925	0.850	0.849	0.850
Repeatability[b]	S_0	0.003	0.003	0.003	0.003	0.003	0.003
Among laboratories[b]	S_L	0.007	0.006	0.007	0.010	0.009	0.010
Reproducibility[b]	S_r	0.008	0.007	0.008	0.010	0.010	0.010
$(S_0/\bar{x}) \times 100$	CV_0	0.3	0.3	0.3	0.4	0.4	0.4
$(S_L/\bar{x}) \times 100$	CV_L	0.8	0.6	0.8	1.2	1.1	1.2
$(S_r/\bar{x}) \times 100$	CV_r	0.9	0.8	0.8	1.2	1.2	1.2

[a] Based on results from 9 of the 16 laboratories in the 1981 collaborative study that used KNO_3 slush in determining their calibration responses.
[b] Calculated as a standard deviation.
[c] KNO_3 slush assigned an a_w value of 0.936.
[d] KNO_3 slush assigned an a_w value of 0.925.
[e] Four-point calibration response obtained by deleting KNO_3 slush value.

assumed conditions. No effect was shown on the mean a_w value for the sweetened condensed milk, whereas the mean a_w value for the KNO_3 slush as a test sample was lowered by 0.004 units. Consequently, an error associated with the assigned a_w value for KNO_3 had limited effects on the calculated a_w for test samples because a 5-point calibration response was used which diluted an error associated with any one calibrating slush. The results in Table 4 also indicate that an a_w value of 0.925 is a better estimate of the a_w for KNO_3 slush than 0.936 when the Abbeon hygrometer is used.

Precision Estimates for Three Collaborative Studies

Tables 1, 2, and 3 show the precision estimates for the 1981, 1982, and 1984 collaborative studies, respectively. The components of variance were estimated independently by the method of Youden and Steiner (8) for each test sample after approximately 4% of the data were deleted as outliers. In all 3 tests, the reproducibility was better for the salt slush than for the food product(s).

For the Abbeon hygrometer, the pooled reproducibilities were 0.009, 0.009, and 0.006 for 1981, 1982, and 1984, respectively. For the electrical hygrometers, the pooled reproducibilities were 0.010 and 0.011 for 1982 and 1984, respectively (Table 5). Although Stoloff (2) calculated the reproducibility for each electrical instrument separately, we pooled his a_w data for the NaCl slush, the KCl slush, and the fudge sauce on the Beckman, Hygrodynamics, Rotronic, and Weather Measure instruments. This pooled reproducibility was calculated to be 0.008. A Bartlett's test (4) comparing the 5 pooled reproducibilities (as variances) from these studies with the variance adapted from Stoloff (2) showed no significant difference between any of the variances. Consequently, the FDA laboratories demonstrated a reproducibility for a_w determinations that does not differ significantly from that reported in the study used to develop the official AOAC method (2).

Of the 3 food products tested on more than one instrument, only the soy sauce exhibited a significant product-instrument interaction (the a_w value depended on the instrument used to measure it). The mean a_w value for the soy sauce was 0.911 when the Abbeon hygrometer was used compared with 0.838 when the electrical hygrometers were used (Table 2). The salt content for this soy sauce is approximately 17% (F. A. Phillips, FDA, private communication); consequently, the a_w value should be reduced to approximately 0.87 on the basis of its salt content alone (10). A volatile component(s) in this product may have affected the a_w measurement for the Abbeon hygrometer and caused an erroneously high reading.

Table 5. Summary of reproducibility variance by instrument and year

Instrument	Year	Variance ($\hat{\sigma}_r^2$)	Degrees of freedom[a]	Standard deviation ($\hat{\sigma}_r$)
Abbeon	1981	0.0000752	36	0.009
Abbeon	1982	0.0000795	31	0.009
Abbeon	1984	0.0000415	20	0.006
Electricals	1982	0.0000972	9	0.010
Electricals	1984	0.0001168	12	0.011
Stoloff[b]	1978	0.0000681	51	0.008

[a] Based on a linear combination of χ^2 variates (9).
[b] Electrical hygrometer data pooled for Beckman, Hygrodynamics, Rotronic, and Weather Measure instruments for NaCl slush, KCl slush, and fudge sauce.

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COSMETICS

Determination of Cinnamyl Anthranilate in Perfume, Cologne, and Toilet Water by Liquid Chromatography with Fluorescence Detection

FRANCOIS X. DEMERS, RONALD L. YATES, and HENRY M. DAVIS
Food and Drug Administration, Division of Colors and Cosmetics, Washington, DC 20204

A liquid chromatographic method with fluorescence detection was developed for the determination of cinnamyl anthranilate in perfumes and other fragrance compositions. The method was evaluated by conducting recovery studies of 10 different commercial fragrance compositions to which cinnamyl anthranilate had been added at levels of 0.1, 0.5, and 1.0 mg/mL. Recoveries ranged from 91 to 103% with a mean of 97% and a standard deviation of ±3.3%.

Cinnamyl anthranilate is a synthetic flavor and fragrance ingredient which is manufactured by condensing isatoic anhydride with cinnamyl alcohol (1). Its use in food was prohibited in 1985 after it was found to cause cancer in laboratory animals (2). To assess the hazard to human health from its use as a fragrance ingredient, the Food and Drug Administration needs to know to what extent cinnamyl anthranilate is present in cosmetics.

In the absence of mandatory registration of cosmetic product compositions, the use of cinnamyl anthranilate must be determined by chemical analysis. The purpose of this study was to develop a method suitable for determining cinnamyl anthranilate in alcoholic and hydroalcoholic cosmetic products. A paper chromatographic method has been reported for the identification of anthranilates, including cinnamyl anthranilate (3). The anthranilates were visualized by their fluorescence under UV light and identified by R_f values.

The analytical method described here uses a combination of liquid chromatography (LC) and fluorometry for the respective separation and determination of cinnamyl anthranilate in fragrance compositions.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Waters Model 204 equipped with Model 6000A solvent pump and Model U6K universal injector (Waters Associates, Milford, MA 01757). Operating conditions: isocratic, flow rate of 1.5 mL/min.

(b) *LC detector.*—Waters Model 420-E fluorescence detector equipped with F4T5/BL fluorescence source, 340 nm excitation filter, and 395 nm emission filter (Waters Associates).

(c) *LC column.*—μBondapak C_{18}, 10 μm irregular particles, 30 cm long × 4.2 mm id (Waters Associates).

Reagents

(a) *Methanol.*—LC or spectrophotometric grade.

(b) *Water.*—LC or spectrophotometric grade.

(c) *Acetic acid.*—ACS grade.

(d) *Ammonium acetate.*—ACS grade.

(e) *LC solvent.*—Methanol–water–acetic acid (350 + 150 + 1) containing 0.8 g ammonium acetate/100 mL.

(f) *Cinnamyl anthranilate.*—98% purity (Pfaltz & Bauer, Inc., Stamford, CT 06902). *Caution:* animal carcinogen.

(g) *Standard solutions.*—Prepare stock solution of cinnamyl anthranilate in methanol at concentration of 2.5 mg/mL. Prepare working solutions with concentrations of 0.1, 0.2, 0.5, and 1.0 mg/mL by dilution of stock solution with methanol.

Determination

Pump LC solvent through column for ca 15 min, using isocratic conditions and flow rate of 1.5 mL/min. Inject ca 10 μL working solution (0.1 mg/mL), adjusting gain to keep emission intensity of cinnamyl anthranilate peak at 50–90% full scale. Repeat until retention volumes are reproducible. If cinnamyl anthranilate does not elute in 9–12 min, adjust water–methanol ratio to achieve elution in the required time. Increasing the methanol content reduces elution time. Cinnamyl anthranilate should have an asymmetry factor of ca 1.2 at 10% peak height. If the factor exceeds 1.3, change LC columns. After system has been equilibrated, inject known volume (ca 10 μL) of each working solution into liquid chromatograph. Adjust attenuation to keep peaks at 50–90% full scale. Next, inject known volume (ca 10 μL) of cosmetic fragrance. If height of cinnamyl anthranilate peak for cosmetic fragrance exceeds range of peak heights for standard solutions, estimate cinnamyl anthranilate concentration by comparing peak heights and make appropriate dilution of cosmetic fragrance.

Calculation

Measure peak height of each standard solution injected and adjust values to a common attenuation. Construct standard curve by plotting micrograms cinnamyl anthranilate vs peak height. Measure cinnamyl anthranilate peak height for cosmetic fragrance injected and obtain weight of cinnamyl anthranilate from standard curve. Calculate concentration of cinnamyl anthranilate in cosmetic fragrance as follows:

$$\text{Cinnamyl anthranilate, } \mu\text{g/mL} = 1000 W_x / V_i$$

where W_x = μg cinnamyl anthranilate from standard curve, and V_i = μL cosmetic fragrance injected into liquid chromatograph. Adjust attenuation to keep peaks at 50–90% full to prepare a more dilute solution, multiply above equation by the dilution factor. Prepare a standard curve daily.

Results and Discussion

Recovery studies were conducted by using 10 different commercial fragrance oils. The fragrances were prepared as 10% solutions of the fragrance oils in 95% ethanol. Each was analyzed by the described method to make certain that none of them contained cinnamyl anthranilate. Cinnamyl anthranilate was then added to 4 of the fragrances at the 0.1 mg/mL level, to 3 at the 0.5 mg/mL level, and to 3 at the 1.0

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mg/mL level. A chromatogram of a typical spiked fragrance is shown in Figure 1. Each fragrance was analyzed in triplicate; peak heights were averaged, and the amount of cinnamyl anthranilate was obtained from the standard curve. Recoveries are shown in Table 1.

Recovery studies were conducted with 10 different matrixes to maximize the standard deviation, i.e., to obtain a realistic value in terms of what would be expected when a series of different fragrances is analyzed.

Because of the complexity of fragrance formulations, it is almost certain that some compounds will coelute with cinnamyl anthranilate and, therefore, such compounds are sources of potential interference. The mean recovery (97%) indicates that there are, at least for some fragrances in this group, systematic negative errors. The negative interferences encountered in fluorescence analysis are due to compounds that cause quenching and filtration effects. Quenching can be identified by reanalysis of a fragrance after dilution. A relative increase in fluorescence emission indicates that quenching is responsible for at least some of the negative interference. Dilution does not significantly increase relative emission when filtration effects are the sources of negative interference. One fragrance from each concentration level was diluted 4-fold with 95% ethanol and reanalyzed to determine the nature of the negative interferences. Recoveries improved significantly for one fragrance, indicating that quenching was the primary interference. The other 2 fragrances showed slight improvements in recoveries, indicating that interferences were caused by filtration effects.

If a given fragrance contains a compound that causes negative interference, the magnitude of the interference will be constant at a specified dilution. A negative interference will have an adverse effect on recovery that is proportionally greater for the lower levels of cinnamyl anthranilate. When a series of recovery studies is done with a fragrance of this type, the regression line of a plot of cinnamyl anthranilate found vs cinnamyl anthranilate added will have a slope that is greater than 1.0. A regression line with a slope of 0.942 was obtained by plotting the recovery data from this investigation. This result indicates that the fragrances, as a group, used for the low-level recovery studies had negative interferences of a lower magnitude than those fragrances used for recovery studies at the higher levels.

One fragrance (No. 4) contained an apparent positive interference, which could not, however, be verified by analysis of the unspiked sample.

Positive interferences are caused by compounds that coelute with cinnamyl anthranilate and have fluorescence emission under the conditions used in this method. Positive interferences can be identified after the cinnamyl anthranilate is extracted into dilute HCl from an ethyl ether solution of the fragrance. Analysis of the ether solution shows an emission peak near the retention time of cinnamyl anthranilate when a positive interference is present.

For this method, the smallest measurable quantity of cinnamyl anthranilate was experimentally determined to be 20 ng when the signal-to-noise ratio was 3. This quantity is equivalent to a cinnamyl anthranilate concentration of 2 μg/mL in a fragrance. Statistical analysis of the recovery data, however, shows that quantitation is not reliable below 22 μg/mL. Because cinnamyl anthranilate is highly fluorescent, it was expected that smaller quantities could be determined. The reason for the higher limit of reliable measurement is the use of a fluorescence detector with fixed excitation and emission filters. The excitation and emission wavelengths for cinnamyl anthranilate are 351 and 409 nm, respectively. The best combination of available filters was 340 nm excitation and 395 nm emission. The bandpass characteristics of these filters indicate a 27% transmittance of excitation energy and a 22% transmittance of emission energy. To determine cinnamyl anthranilate at concentrations lower than 22 μg/mL, it is necessary to use a fluorescence detector equipped with single or dual monochrometers.

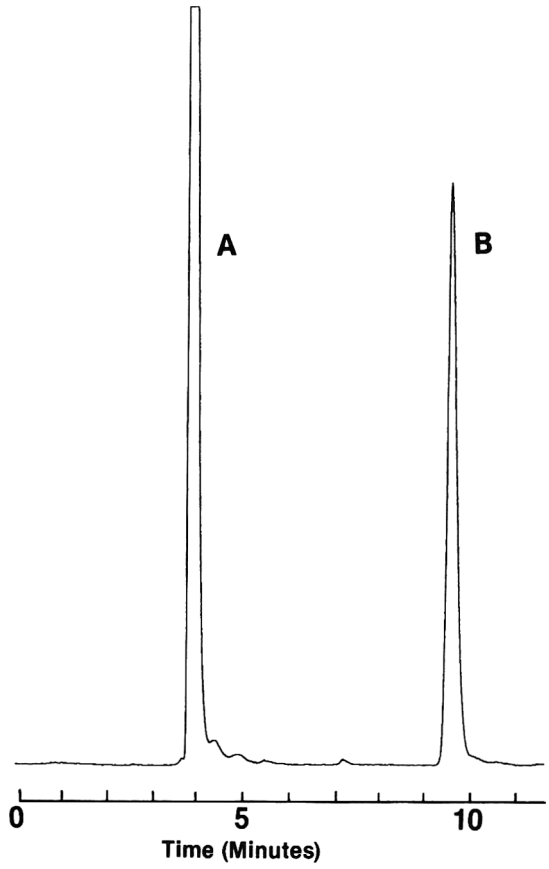

Figure 1. LC chromatogram of typical fragrance spiked with cinnamyl anthranilate. A, methyl anthranilate; B, cinnamyl anthranilate.

In conclusion, the proposed method is rapid, and the lower limit of determination is adequate for the products analyzed. Despite the inherent selectivity of the LC-fluorescence system, however, it must be assumed that interferences will

Table 1. Recovery of cinnamyl anthranilate from commercial fragrances

Fragrance	Cinnamyl anthranilate, μg/mL Added	Found	Rec., %
1	100	99	99
2	100	94	94
3	100	99	99
4	100	103	103
5	500	480	96
6	500	480	96
7	500	480	96
8	1000	950	95
9	1000	910	91
10	1000	980	98
Mean (n = 10)			97
SD			±3.3
Slope			0.942 ± 0.0143
Intercept			0.0058 ± 0.0088
Standard error of regression			±0.0023
Correlation coefficient			0.9989
Lower limit of reliable measurement, μg/mL			22

occasionally occur. Because of the great complexity of fragrances, it is impossible to screen for all potential interferences. The possibility of interferences, however, can be minimized by extracting the cinnamyl anthranilate into dilute HCl from an ethyl ether solution of the fragrance. The acid extract is then made basic and the cinnamyl anthranilate is extracted into ethyl ether. The ether is evaporated, the residue is dissolved in methanol, and the solution is analyzed by LC-fluorometry. We did not choose to make this extraction step part of the method because, in our experience, the contributions of positive and negative interferences are relatively small.

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Screening Cosmetic Products for *N*-Nitroso Compounds by Chemiluminescent Determination of Nitric Oxide

HARDY J. CHOU, RONALD L. YATES, and JOHN A. WENNINGER
Food and Drug Administration, Division of Colors and Cosmetics, Washington, DC 20204

Cosmetic products were screened for total *N*-nitroso compounds by chemiluminescent measurement of nitric oxide liberated by the reductive cleavage of the *N*-nitroso group. The cosmetic was first partitioned between methylene chloride and water to separate polar and nonpolar *N*-nitroso compounds. Each extract was then examined for the presence of *N*-nitroso compounds by adding the cleavage reagent and sweeping the nitric oxide formed into a chemiluminescent analyzer. Although the method is not intended to be quantitative, recovery studies were conducted to determine measurable levels. Recovery studies of polar *N*-nitroso compounds were conducted by adding *N*-nitrosodiethanolamine (NDELA) to a cream, a shampoo, and a lotion at 3 levels, i.e., 80, 320, and 960 ppb, and then determining NDELA by the method. Recoveries ranged from 48 to 83% (mean 68%; SD = 11.9). For recoveries of nonpolar *N*-nitroso compounds, 100, 200, and 500 ppb of *N*-nitrosomethyltetradecylamine were added to the 3 cosmetic products. Recoveries ranged from 58 to 70% (mean 63%; SD = 5.3).

Carcinogenic *N*-nitrosamines are pervasive in the environment at parts-per-billion levels (1–3). *N*-Nitrosamines have also been reported to occur in food (4) and some manufactured products such as cutting fluids (5) and baby bottle rubber nipples (6). Fan et al. (7) reported the presence of *N*-nitrosodiethanolamine (NDELA) in cosmetic products. A survey of several hundred cosmetic products by our laboratory demonstrated that approximately 40% of those products containing the NDELA precursors diethanolamine and/or triethanolamine also contained detectable levels of NDELA (8). Recently, cosmetic products were found to contain *N*-nitrosomethyldodecylamine (9), *N*-nitrosomethyltetradecylamine (NMTDA) (9), *N*-nitrosomethyloctadecylamine (10), *N*-nitrosomorpholine (11; Thermedics, Inc., Woburn, MA, 1985, private communication), *N*-nitrosodimethylamine (11), and *N*-nitrosodiisopropanolamine (11).

Cosmetics are formulated with a large number of different amines, amides, and their derivatives; thus, it is likely that other *N*-nitroso derivatives of amines and amides could occur as contaminants in these products. Because of the possibility that cosmetics may contain a number of different *N*-nitroso compounds, it would be useful to have a screening procedure that indicates the presence of one or more of these compounds before attempting to determine specific *N*-nitrosamines.

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Several approaches have been used as screening procedures to estimate total *N*-nitroso compounds in different matrixes (12–17). The approach most frequently used involves the pyrolytic or chemical cleavage of the *N*-nitroso group to form nitric oxide, which is then measured by its chemiluminescent reaction with ozone. This general approach has been used to determine extractable *N*-nitroso compounds in food (13), biological matrixes (12), and environmental samples (14). Brennan and Frank (17) estimated the total water-soluble *N*-nitroso content of cosmetic products by acid-catalyzed denitrosation of the *N*-nitroso compounds followed by chemiluminescent determination of the nitric oxide formed. Recently, Waters et al. (18) discussed the pitfalls in determining *N*-nitroso compounds as a group.

This paper describes a procedure for screening cosmetics for total *N*-nitroso compounds. The cosmetic product is partitioned between methylene chloride and water to separate it into polar and nonpolar *N*-nitroso compounds. The respective cleavage reagents are then added to each extract and the nitric oxide formed is swept into the chemiluminescent analyzer. Because of the possibility of positive interferences, results that indicate the presence of *N*-nitroso compounds should be verified by liquid chromatography- or gas chromatography-thermal energy analysis (LC-TEA or GC-TEA, respectively) to determine which, if any, *N*-nitrosamines are present.

METHOD

Note: Nitrosamines are potential carcinogens. Therefore, care should be exercised in handling these materials.

Apparatus

(a) *Nitric oxide analyzer.*—Thermal Energy Analyzer (Model 502, Thermedics, Inc., Woburn, MA 01888-1799). Operating conditions: catalytic furnace temperature, 550°C; cold trap, consisting of 12 cm id Dewar flask filled with slush of powdered dry ice-ethanol; carrier gas, helium, with flow rate adjusted to obtain pressure of 1.5 torr; oxygen flow rate adjusted to obtain increase of pressure from 1.5 to 2.5 torr.

(b) *Degassing apparatus.*—Similar to that used by Drescher and Frank (14). Glass impinger with 10 mm medium frit and 24/40 male fitting is connected to 25 mm × 17 cm test tube (80 mL).

(c) *Cold trap.*—Glass mini-impinger (20 cm × 13 mm od)

filled with 6N NaOH to level of gas entrance tube. Cold trap is placed in Dewar flask containing mixture of ice and water.

(d) *Absorption trap.*—10 cm × 6 mm stainless steel tubing packed with anhydrous sodium carbonate. Glass wool plugs are placed in each end, and unit is fitted with Swagelok connectors. Trap is heated at 150°C overnight before use.

(e) *Electronic integrator.*—Model 3390A, Hewlett-Packard Co., Avondale, PA 19311.

(f) *Chromatographic tube.*—25 cm × 1 cm id glass with coarse porosity fritted glass disc and Teflon stopcock.

Reagents

(a) *Methylene chloride.*—Distilled-in-Glass (Burdick & Jackson Laboratories, Inc., Muskegon, MI 69442).

(b) *N-Nitrosodiethanolamine.*—Gift from Battelle Columbus Laboratories, Columbus, OH. Purity and identity were verified by LC and GC/mass spectrometry. Prepare stock solution in water (0.1 mg/mL). Prepare working solution by diluting stock solution (1 μg/mL).

(c) *N-Nitrosomethyltetradecylamine.*—Gift from American Health Foundation, Valhalla, NY. Stock (0.1 mg/mL) and working (1 μg/mL) solutions were prepared in chloroform.

(d) *Ion exchange resin.*—Dowex 1-X8, 50–100 mesh (Bio-Rad Laboratories, Richmond, CA 94804).

(e) *Reductive cleavage reagent.*—Dilute 1.7 mL 48% hydrobromic acid (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) to 25 mL with glacial acetic acid. Add 2 mL acetic anhydride.

(f) *Sodium iodide solution.*—Dissolve 2.5 g sodium iodide (ACS reagent grade, J. T. Baker Chemical Co.) in water and dilute to 25 mL. Prepare daily and store at 4°C. When added to system containing acetic and sulfuric acids during analysis of aqueous extract, this solution constitutes cleavage reagent for polar compounds.

(g) *Ammonium sulfamate solution.*—Prepare 0.25M solution of ammonium sulfamate (Mallinckrodt Inc., St. Louis, MO 63134) in 1.5N HCl.

(h) *Acetic acid, acetic anhydride, sulfuric acid, and hydrochloric acid.*—ACS reagent grade (J. T. Baker Chemical Co.).

Preparation of Sample

Disperse 1 g cosmetic product in 10 mL warm water. Add 0.2 mL ammonium sulfamate solution and extract in separatory funnel with three 20 mL portions of methylene chloride. Separate aqueous (upper) layer from methylene chloride (lower) layer. Dry methylene chloride extract over 25 g anhydrous sodium sulfate for minimum of 2 h and then filter through short bed (10 g) of anhydrous sodium sulfate. Wash sodium sulfate with three 15 mL portions of methylene chloride and add washings to filtrate.

If quantitative estimate of *N*-nitroso compounds is desired, measure extract volume or adjust to known volume by dilution or partial evaporation. Store methylene chloride extract in stoppered flask over sodium sulfate.

Adjust acidity of aqueous extract to pH 3.5 or less by dropwise addition of 6N HCl. Measure final volume of aqueous extract. If analysis cannot begin immediately, store in dark, preferably under refrigeration.

Analysis of Aqueous Extract

Connect, in series, carrier gas, degassing apparatus, cold trap, and absorption trap to liquid chromatograph-thermal energy analyzer inlet, using ¼ in. od Teflon tubing. (A diagram of this arrangement appears in ref. 16.) Place thermal energy analyzer in operation, using conditions given for nitric oxide analyzer, *Apparatus* (a). Turn stopcocks to bypass position, remove test tube, and add 5 mL NDELA working solution, 13 mL glacial acetic acid, and 3.5 mL H_2SO_4. Mix, add 1 mL of 10% sodium iodide solution and immediately place tube on degassing apparatus. Turn stopcocks to allow carrier gas to pass through reaction mixture. Continue purge until no further evolution of nitric oxide is observed on integrator readout. Return stopcocks to bypass position. Analyze 5 mL cosmetic aqueous extract by same procedure. Make quantitative estimate of nitrosamines in cosmetic product by relating integration values of test sample and standard.

If broad, low peak is observed on the recorder, the cosmetic product may contain nitrate; remove as follows. Slurry 5 mL ion exchange resin with 2N HCl and transfer to chromatographic tube. Wash with two 25 mL portions of 2N HCl and two 25 mL portions of water. Add aqueous extract to column and elute with 25 mL water at rate of 2 mL/min. Discard first 3 mL eluate; collect next 20 mL and reserve for analysis.

Analysis of Methylene Chloride Extract

Remove cold trap and absorption trap from system. Place 20 mL methylene chloride extract and 0.2 mL reductive cleavage reagent (e) in test tube. Proceed as above. For quantitation use 20 mL NMTDA working solution.

Results and Discussion

Original investigations involved the development of a single solvent method to screen cosmetics for total *N*-nitroso compounds. Several polar and semipolar solvents such as isobutyl alcohol were examined. In that study, the cleavage reagent for the analysis of aqueous extracts described in this method (10% sodium iodide solution) was used for denitrosation and NMTDA was used for recovery studies. The NMTDA was not sufficiently soluble in 40% propyl alcohol, and recoveries were low and nonreproducible. Isobutyl alcohol and *N,N*-dimethylformamide resulted in a high detector background, possibly due to impurities in the solvents. These results led to development of the 2-solvent approach.

The reductive cleavage of *N*-nitroso compounds and nitrite in aqueous solutions proceeds rapidly and gives rise to well-defined peaks on the integrator printout. Nitrite, therefore, is a direct interference. The reduction of nitrate proceeds relatively slowly, however, and peaks are broad and poorly defined (19). Because of their slow reduction rate to nitrites, nitrates do not cause serious interference until they are present at levels of 10 ppm or more. Nitrite, however, interferes at all but the lowest levels and must be removed from the sample before determination of *N*-nitroso content. Although the ion exchange column used to remove nitrate also removes nitrite fairly efficiently, low levels of both nitrate and nitrite still remain in the eluate. Cox (19) reported levels of 10–50 ppb nitrite and nitrate in the eluate. Nitrate does not interfere at these levels, but 50 ppb nitrite could be an interference in determining low levels of nitrosamines. Also, the ion exchange resin may be ineffective for nitrite removal if the cosmetic product contains relatively large amounts of strong anions that would compete effectively for cationic sites.

Removal of nitrite by chemical reduction is a simpler and more effective approach than ion exchange. The effectiveness of ammonium sulfamate was evaluated by adding it to a cosmetic product along with nitrite at levels ranging from 0.01 to 10 μg/g and then proceeding with analysis. The absence of response when the denitrosating reagent (10% sodium iodide solution) was added to the acidified aqueous

Table 1. Recovery of *N*-nitrosodiethanolamine (NDELA) from a lotion, a shampoo, and a cream

Cosmetic product	NDELA Added, ng/g (ppb)	NDELA Found, ng/g (ppb)	Av. rec., %
Lotion	80	56, 58, 54	70
	320	250, 243, 237	76
	960	720, 691, 700	73
Shampoo	80	66, 64, 65	81
	320	218, 205, 202	65
	960	768, 816, 797	83
Cream	80	37, 39, 40	48
	320	170, 166, 176	53
	960	624, 595, 643	65
Mean (n = 9)			68
SD (n = 27)			11.5

extracts indicated that nitrite had been removed. Cosmetic products containing the preservatives 2-bromo-2-nitro-1,3-propanediol or 2-hydroxymethyl-2-nitro-1,3-propanediol may contain relatively high levels of nitrite because of the stepwise degradation of these compounds in solution (20, 21). If nitrite is not removed from these samples, gross analytical errors may result.

Although the method is designed as a screening procedure to detect the presence of *N*-nitroso compounds in cosmetic products, it is useful to have a quantitative estimate of the total *N*-nitroso content. For this reason, a series of recovery studies was conducted. In studies of polar *N*-nitroso compounds, levels of NDELA corresponding to 80, 320, and 960 ppb were added to a lotion, a shampoo, and a cream in solution. The products were analyzed before recovery studies to determine if they contained *N*-nitroso compounds, nitrites, or any other compounds which might give a positive response. The products used were free of positive interferences. The spiked products were taken through the procedure and the aqueous extracts were examined for NDELA. Results are given in Table 1. The method has a limit of determination of 50 ppb (based on NDELA) in the test samples.

In recovery studies of nonpolar *N*-nitroso compounds, NMTDA was added to a lotion and a cream at levels of 100, 200, and 500 ppb. These test samples were then analyzed by the method described for methylene chloride extract. Results are shown in Table 2. Initial studies resulted in very low or variable recoveries. An investigation of the problem indicated that water dissolved in the methylene chloride extract was largely responsible for the low efficiency of the cleavage reagent. After prolonged drying of the methylene chloride extract over anhydrous sodium sulfate, recoveries improved to better than 60% and were more reproducible. The method has a limit of determination of approximately 50 ppb (based on NMTDA) in the test samples.

Table 2. Recovery of *N*-nitrosomethyltetradecylamine (NMTDA) from a cream and a lotion

Cosmetic product	NMTDA Added ng/g (ppb)	NMTDA Found, ng/g (ppb)	Av. rec., %
Lotion	100	58, 57, 59	58
	200	120, 122, 125	61
	500	346, 350, 340	69
Cream	100	58, 58, 59	58
	200	124, 122, 120	61
	500	350, 340, 360	70
Mean (n = 6)			63
SD (n = 18)			5.1

Table 3. Analysis of commercial cosmetic products for water-soluble *N*-nitroso content

Cosmetic product	NDELA, ppb, by LC-TEA[a]	Total nitric oxide, ppb[b]
Lotion	ND[c]	110
Lotion	1240	1700
Cream	350	410
Cream	ND	ND
Makeup	ND	370
Shampoo	ND	ND

[a] Ref. 22.
[b] Calculated as NDELA for comparison. NDELA to nitric oxide factor = 0.224.
[c] ND = Not detected.

An LC-TEA method (22) was compared with the denitrosation method described here by examining 6 commercial cosmetic products. For purposes of comparison, the LC-TEA method was used to determine NDELA, and the denitrosation procedure for total *N*-nitroso compounds was applied only to the aqueous extract. Results, shown in Table 3, were roughly comparable for 4 cosmetic products. In 2 products, however, the denitrosation procedure indicated the presence of polar nitrosamines, whereas the LC-TEA method showed no NDELA to be present. It is possible, however, that the signal obtained from these test samples was due to false positives instead of *N*-nitroso compounds. Some commonly occurring organic nitro compounds were studied to determine their potential for interference. Aqueous solutions containing 100 ppm of the cosmetic preservatives 2-bromo-2-nitro-1,3-propanediol and 5-bromo-5-nitro-1,3-dioxane were prepared and analyzed. No measurable levels of nitric oxide were found. Nonpolar interferences were also studied. Methylene chloride solutions containing 100 ppm of the common nitro fragrance compounds musk ambrette, musk ketone, and musk xylol were prepared and examined by the denitrosation method. No measurable levels of nitric oxide were found. These results demonstrate that false-positive responses may be observed in analyses of some cosmetic products. The method, therefore, is intended only for the preliminary screening of these products. A positive response from the screening procedure should be followed by standard LC-TEA or GC-TEA methods for verification of specific *N*-nitrosamines.

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DRUGS

Determination of Aspirin and Salicylic Acid by Reverse-Phase Liquid Chromatography

DOROTHY R. HEIDEMANN, EDWARD S. SCHULENBERG, and WILLIAM H. SMITH
Sandoz Consumer Health Care Group (Sandoz Pharmaceuticals Corp.), Analytical Research Department, Lincoln, NE 68501-3288

Buffered solid dosage forms containing aspirin, magnesium hydroxide, and aluminum hydroxide are blended with acidic ethanol to extract the aspirin and salicylic acid rapidly. The resulting preparation is then immediately injected onto a 4.6 mm × 3 cm 5 μm reverse-phase column. Aspirin and free salicylic acid are determined simultaneously. The run time is <2 min. The total time from the initiation of sample extraction to completion of the separation is <5 min.

The analysis of aspirin (I) (Figure 1) and free salicylic acid (II), the major hydrolysis product of aspirin, as well as the other minor salicylates, salicylsalicylic acid (III), and acetylsalicylsalicylic acid (IV) has been performed in many different ways. Classical wet methods (1–3) have been largely replaced by more rapid and specific chromatographic separations. However, the rapid hydrolysis of the aspirin molecule to salicylic acid has been a limiting factor for typical reverse-phase separations. As a result, aspirin and salicylic acid are often determined by normal phase chromatography since the hydrolysis of the aspirin is significantly reduced in a normal phase separation in which the sample preparation, as well as the mobile phase, is free from water (4, 5). However, for practical reasons, reverse-phase separations are frequently preferred by analysts. Separations by reverse phase that minimize or compensate for hydrolysis during the analysis of the product have been reported (6–9).

For satisfactory analysis of aspirin by reverse-phase chromatography, 2 prerequisites must be met: (*1*) the extraction from the tablet matrix must be rapid, complete, and performed with a nonaqueous solvent, and (*2*) the run time of the separation must also be rapid. Normal grinding of the tablet prior to extraction can, in itself, contribute to the formation of salicylic acid and result in falsely high results for this hydrolysis product. Furthermore, the presence of buffering agents such as aluminum and magnesium hydroxide can make the aspirin and salicylic acid more difficult to retrieve. Consequently, without a rigorous method of extraction, the recovery of aspirin and salicylic acid can be incomplete. Compensation of aspirin hydrolysis during the chromatographic separation is possible if standard and sample preparations hydrolyze at the same rate. Unfortunately, excipients in the formulation can alter the hydrolysis rate of the aspirin. Thus, it is preferable to keep hydrolysis to a minimum or, if possible, a negligible level.

The method described here for analysis of buffered aspirin products is rapid and reliable. A household food blender is used for extraction. The liquid chromatographic (LC) system uses an inexpensive guard column.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Equipped to operate as follows: flow rate, 3.0 mL/min; UV detection, 205 nm; temperature, ambient; absorbance path length, 10 mm with rise time of 0.1 s (Lambda Max 481, Waters Associates, Milford, MA 01757).

(b) *Column.*—RP-8 Spheri-5 stainless steel, 3 cm × 4.6 mm, packed with spherical particles, RP-8 packing, 5 μm size (Brownlee Labs, Santa Clara, CA 95050).

(c) *Solvents.*—LC grade methanol, 85% phosphoric acid, hydrochloric acid, anhydrous denatured ethanol.

(d) *Extraction solvent.*—Anhydrous alcohol containing 1% hydrochloric acid by volume.

(e) *Mobile phase.*—Methanol–water–85% phosphoric acid, 70 + 30 + 3, v/v/v.

(f) *Blender.*—Osterizer with mini-blend jars.

(g) *Automatic dispenser.*—100 mL (Repipet, Labindustries, Inc., Berkeley, CA 94710).

(h) *Standards.*—Aspirin (Dow Chemical), salicylic acid (Fisher Scientific), salicylsalicylic acid (Pfaltz & Bauer, Waterbury, CT 06708), and acetylsalicylsalicylic acid (Mobay Chemical).

(i) *Samples.*—Commercial tablets, label claim: aspirin, USP 500 mg; magnesium oxide, USP 150 mg; dried aluminum hydroxide gel, USP 150 mg. The studies were conducted on tablets exposed for various periods of time to stress of 25, 30, 40, and 50°C at ambient humidity as well as at 35°C with 90% relative humidity. This method has also been used to assay other laboratory formulations of buffered aspirin products containing either 325 mg aspirin or calcium carbaspirin (a calcium salt of aspirin) equivalent to 325 mg aspirin. The standard preparations were adjusted accordingly.

Standard Preparation

(a) *Aspirin.*—Weigh 500 mg standard into 100 mL volumetric flask. Dilute to volume with extraction solvent. Dilute 5 mL to 100 mL with anhydrous alcohol (0.25 mg/mL). Use within 60 min.

(b) *Salicylic acid.*—Weigh 500 mg standard into 100 mL volumetric flask. Dilute to volume with anhydrous alcohol. Dilute 1 mL to 100 mL with extraction solvent. Dilute 5 mL to 100 mL with anhydrous alcohol for a 1% salicylic acid standard (0.0025 mg/mL).

Sample Preparation

Transfer 5 tablets to 8 oz blender jar. Add 100 mL extraction solvent and blend at high speed for 90 s. Filter a portion of blended sample through glass fiber paper (Whatman, GF/A) and dilute 1 mL to 100 mL with anhydrous alcohol. Immediately inject 10 μL into chromatograph and compare to chromatograms for standard preparations of aspirin and salicylic acid.

Results and Discussion

Because of the rapid analysis time (<2 min), hydrolysis of the aspirin is nominal during chromatographic separation. Consequently, the difference in the hydrolysis rate of the standard and sample due to other excipients present in the tablet is reduced to a negligible amount. Since the analysis

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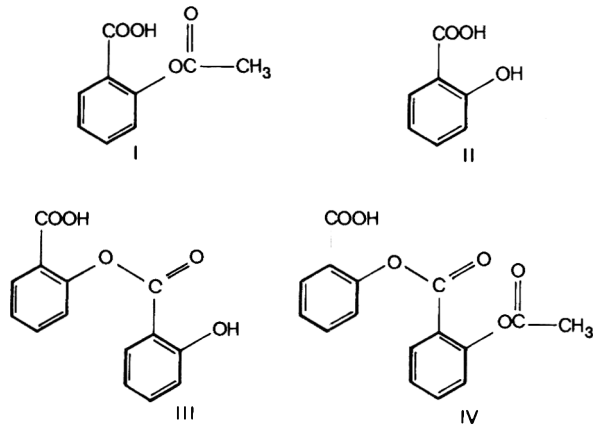

Figure 1. Structures of aspirin and degradation products. (I) aspirin, (II) salicylic acid, (III) salicylsalicylic acid, (IV) acetylsalicylsalicylic acid.

time is similar to the extraction time, the analyst can extract the sample, inject it immediately, and prepare the next sample during the 2 min needed to perform the chromatographic separation.

The salicylic acid peak demonstrated excellent linearity in a range that represented a 1 to 20% hydrolysis of aspirin to salicylic acid. The coefficient of correlation was 1.000 with a y-intercept of -0.002. Because of the high degree of linearity, it was not necessary to match the salicylic acid standard with the sample. A recovery of 1.95% salicylic acid (mean of 2 injections) was obtained for a 2% salicylic acid standard addition to the sample blank. The standard deviation (SD) for salicylic acid in 10 replicate determinations of a fresh sample having a trace level of 0.4% salicylic acid was 0.012 (Table 1). The relative standard deviation improves as the salicylic acid level increases.

The injection is slightly overloaded with aspirin to obtain a peak of measurable size for trace levels of salicylic acid. Consequently, the linearity for aspirin requires that the standard be matched within 5% of the sample for accurate results. On 10 replicate determinations performed by 3 chemists on a fresh sample of the commercial tablet, the recovery averaged 100.1% of theoretical aspirin with an SD of 1.3% (Table 1). A recovery of 99.4% of theoretical (mean of 2 injections) was obtained for the standard addition of aspirin to the sample blank. The analysis of highly stressed tablets showed a good mass balance for aspirin and salicylic acid (Table 2). No blank interference was observed. The hydrolysis rate was 0.014%/min for the standard preparation. System suitability limits selected for the analysis of both aspirin and salicylic acid are: coefficient of variation $\leq 1.8\%$, tailing factor ≤ 2.0, and resolution ≥ 2.0. For the normalized aspirin data in Table 2, the coefficient of variation is 0.44%.

The 4.6 mm × 3 cm column is sold as a guard column. However, the efficiency of the column is sufficient to perform the separation (Figure 2). The cost of this column is nominal compared to the cost of a typical analytical column. The short column length minimizes total elution time.

The separation was not only stability-indicating for aspirin in the presence of salicylic acid but also for the additional minor salicylates, (III) and (IV) (Figure 3). Trace amounts of those compounds may be present in aspirin products, particularly if magnesium or calcium stearates have been used in the formulation as lubricants (6). However, because levels of these salicylates were not more than 0.1% of theoretical even in abnormally high stressed samples, the down-

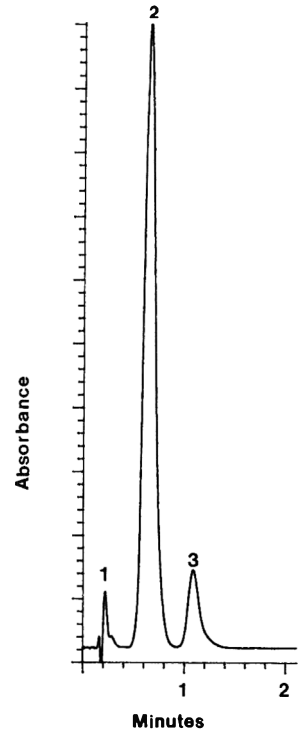

Figure 2. Chromatogram of aspirin and salicylic acid in a buffered product. (1) excipients, (2) aspirin, (3) salicylic acid.

field elution did not cause significant interferences with later injections.

In addition to being suitable for buffered aspirin and buffered carbaspirin, the extraction and separation are also suitable for aspirin products containing antihistamines and decongestants. Phenylpropanolamine, chlorpheniramine, pheniramine, and pyrilamine do not interfere with the chromatographic separation of aspirin or salicylic acid.

The unique design of the blender jar used in this method allows for the rapid sample handling required in the analysis. The single opening with the blender jar reduces the time required to seal the container. The blades remove easily for cleaning. By having a number of blender jars and blades, it is practical to prepare a large number of samples serially. The blender blades become dull over time and as a result, recovery can be reduced. Therefore, it is important to replace or sharpen the blades when they become dull or worn.

The analysis is semiautomated by the use of an automatic dispenser for delivery of extracting solvent, an automatic injector, and computerized data handling.

Table 1. Replicate data obtained by chemists I, II, and III

Replicate	Chemist	Aspirin, % theoretical	Free salicylic acid, %/theoretical aspirin content
1	I	99.8	0.40
2	I	102.1	0.37
3	I	99.9	0.40
4	I	100.4	0.40
5	I	100.6	0.39
6	I	100.4	0.41
7	II	101.9	0.41
8	II	99.6	0.39
9	III	97.9	0.39
10	III	98.6	0.39
Mean		100.1	0.40
SD		1.3	0.012

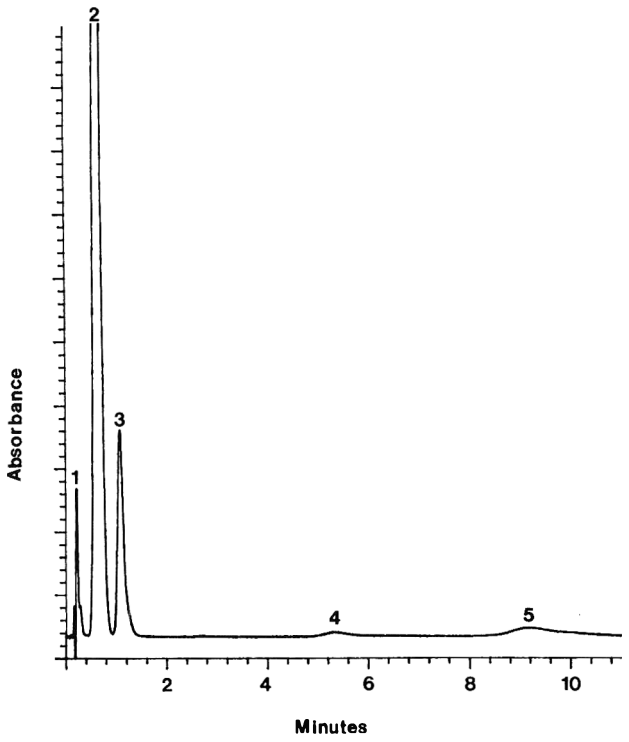

Figure 3. Chromatogram of buffered aspirin product spiked with minor salicylates. (1) excipients, (2) aspirin, (3) salicylic acid, (4) acetylsalicylsalicylic acid, (5) salicylsalicylic acid.

Conclusions

This analysis provides a rapid, accurate, and economical method for determining aspirin and salicylic acid in buffered tablets. The design of the blending equipment provides a unit which is completely sealed and can easily be cleaned for serially prepared samples. The extraction solvent can be safely handled by the analyst without special precautions. The sample preparation can be synchronized with the chromatographic separation for convenient serial analysis. The analysis can be semiautomated by the use of a dispenser for delivery of extracting solvent, an automatic injector, and computerized data handling.

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Table 2. Mass balance of aspirin and hydrolyzed aspirin in various laboratory formulations—stressed buffered tablets

Storage conditions[a]	Aspirin, % theoretical: Duplicate preparations, 1 injection each	Aspirin, % theoretical: Mean	Hydrolyzed aspirin, % theoretical: Duplicate preparations, 1 injection each	Hydrolyzed aspirin, % theoretical: Mean	Mass balance
30 mo., 25°C	98.8, 97.1	97.9	1.3, 1.7	1.5	99.4
30 mo., 30°C	97.7, 97.5	97.6	2.0, 1.9	2.0	99.6
30 mo., 25°C	98.2, 98.9	98.6	1.9, 1.7	1.8	100.4
30 mo., 30°C	97.9, 98.5	98.2	2.2, 1.8	2.0	100.2
24 mo., 25°C	97.8, 97.9	97.8	1.3, 1.4	1.4	99.2
24 mo., 30°C	97.0, 97.5	97.3	1.7, 1.7	1.7	99.0
24 mo., 25°C	96.7, 97.7	97.2	1.5, 1.3	1.4	98.6
24 mo., 30°C	96.4, 97.3	96.8	1.6, 1.7	1.7	98.5
1 mo., 35°C	97.2, 96.9	97.0	4.1, 4.1	4.1	101.1
1 mo., 40°C	96.5, 96.1	96.3	4.5, 4.8	4.7	101.0
1 mo., 50°C	93.2, 91.9	92.6	7.9, 7.9	7.9	100.5

[a] Ambient relative humidity for 25°C, 30°C, 40°C, and 50°C samples; 90% relative humidity for 35°C samples.

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Liquid Chromatographic Determination and Identification Tests for Dexamethasone in Bulk Drugs and Elixirs: Collaborative Study

ELAINE A. BUNCH
Food and Drug Administration, 909 First Ave, Seattle, WA 98174

Collaborators: J. F. Brower; D. D. Hughes; R. H. Johnson; M. Perales-Uribe; W. T. Smith; S. Yuen; R. H. Albert (*Statistical Consultant*)

A normal phase liquid chromatographic method for the determination of dexamethasone in bulk drugs and elixirs was collaboratively studied by 6 laboratories. The method uses a silica column, water-modified acetic acid-methanol-methylene chloride mobile phase, cortisone internal standard, and photometric detection at 254 nm. Collaborators were supplied blind duplicate samples of 3 bulk drugs, 2 commercial elixirs, and 1 authentic elixir. Dexamethasone elixir dosage level is 0.5 mg/5 mL. Mean recovery of dexamethasone from the authentic elixir formulated to contain 0.471 mg/5 mL was 94.5%. (Authentic elixirs were found to stabilize about 6% below the theoretical concentration.) Mean recovery for the bulk drugs was between 97.1 and 100.1%. Mean coefficients of variation for bulk drug and elixir samples were less than 0.8% and 3.6%, respectively. Identification tests for dexamethasone by thin-layer chromatography, infrared spectroscopy, and relative LC retention times, as well as the gas chromatographic determination of alcohol in the elixirs were also collaboratively studied. Mean recovery of alcohol from the synthetic elixir was 98.6%. The mean coefficient of variation for alcohol for all samples analyzed was less than 1.4%. The LC method for dexamethasone in drug substance and elixirs, the identification tests, and the GC method for alcohol in dexamethasone elixirs have been adopted official first action.

Dexamethasone is an adrenocorticosteroid which is commercially available in tablets, elixirs, aerosols, and ophthalmic suspensions. The United States Pharmacopeia compendial assay of the drug substance is a reverse phase liquid chromatographic (LC) method (1–3) which has undergone frequent revision. For example, both the mobile phase composition and the length of the column used for this assay were changed in the 20th revision and its supplements. The revised USP XX method for the drug substance, using a column packed with octylsilane (C_8), is retained in the 21st revision. The liquid-liquid extraction procedure followed by the blue tetrazolium colorimetric reaction for the elixir in USP XX was also replaced by LC analysis in the 21st revision. This method specifies a column packed with octadecylsilane (C_{18}) and a methanol–water mobile phase rather than the acetonitrile–water mobile phase that is used for the drug substance. It has been observed in this laboratory that the compendial liquid chromatographic methods do not resolve dexamethasone from its configurational isomer, betamethasone, and do not exhibit the selectivity for closely related steroids which is found in normal phase LC analysis (4, 5) as depicted in Figure 1. In addition, as the percentage of acetonitrile is increased, the spectral profile and absorptivity of dexamethasone undergo changes around the 254 nm wavelength, which is used for quantitation.

The USP identification tests for the drug substance are (a) UV absorptivity, (b) an IR spectral profile (KBr disc), and (c) thin-layer chromatography (TLC). However, these tests have some deficiencies. The UV absorptivity test is not specific because it depends on the Ring A chromophore common to corticosteroids; the IR test does not specify the same recrystallization solvent for sample and standard, which will present a problem if polymorphism is present; and the TLC system will not resolve the alpha and beta configurational isomers. The elixir TLC identification test is subject to interference from excipients added as preservatives. In addition, in the USP assay for the alcohol content of the elixir, the internal standard, acetonitrile, is usually incompletely resolved from the ethanol peak (6).

Degradation of dexamethasone occurs most frequently in Ring A and/or the Ring D side chain (Figure 2). Degradation of Ring A is light-catalyzed; the decomposition routes for the Ring D side chain are more varied and common. Basic-, acidic-, and metal-catalyzed degradation products of the C_{17} side chain have been identified. The predominant Ring D decomposition products are the C_{20}-etio acid, C_{17}-ketone, and the C_{21}-glyoxal (7). The normal phase LC method resolves dexamethasone from its decomposition products as do the TLC identification tests.

Prior work in this laboratory (8) was incorporated by Walters and Dunbar (5, 9) in the development of a normal phase LC method. Walter's method for hydrocortisone drug substance and tablets was collaboratively studied and adopted by AOAC (10). The method developed by this author is similar to that of Walters. The main differences are the use of cortisone as the internal standard and the change from ethylene chloride to methylene chloride as the base solvent for the mobile phase. This modified LC method (5), a modified alcohol assay (3), a modified infrared (3), and 2 TLC identification tests, as well as comparison of relative LC retention times, have been subjected to intra- and interlaboratory collaborative studies. The results of these studies are the subject of this report.

Collaborative Study

Six collaborators participated in the study. The author's results are listed as collaborator 7 in the tables. Each of the 6 collaborators received 12 samples, labeled 1–12, as 6 blind duplicate pairs. Samples 1–6 were bulk drug substances, and 7–12 were elixirs. All samples were to be analyzed for dexamethasone. Infrared, LC, and TLC identification tests were required for 3 drug substance samples. All elixir samples were to be analyzed for alcohol, and a TLC identification test was required for 3 elixir samples.

Sample pair 7/11 represented an authentic elixir formulated at 94% of the commercial level with benzoic acid as the preservative. Sample pairs 8/12 and 9/10 were commercial elixirs. Methyl and propyl parabens were used as preservatives by the formulator of sample pair 9/10. Drug substance sample pairs were 1/5, 2/6, and 3/4. TLC, infrared, and relative LC retention times were required as identifica-

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The recommendation of the Associate Referee was approved interim official first action by the General Referee, the Committee on Drugs and Related Topics, and the Chairman of the Official Methods Board. The method was adopted official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) **71**, January/February issue.

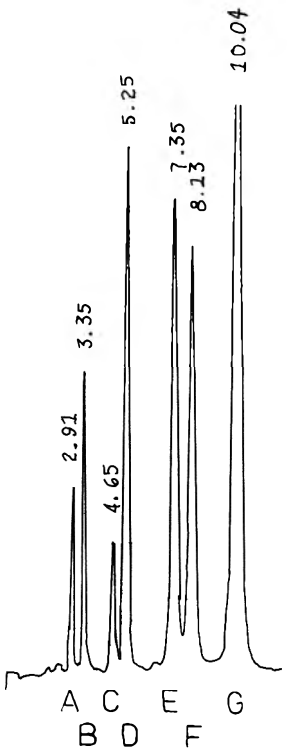

Figure 1. Liquid chromatogram of 7 related corticosteroids. Retention times in min. Flow rate = 1.6 mL/min. Key: (A) Cortisone acetate; (B) hydrocortisone acetate; (C) cortisone; (D) prednisone; (E) dexamethasone; (F) betamethasone; (G) prednisolone.

tion tests for samples 1, 2, and 4. Samples 8, 9, and 11 required a TLC identification test.

Each collaborator also received an instruction sheet, copy of the method, report form, vial of internal standard, vial of betamethasone, vials containing drug substance samples, and small bottles of elixir. For the analysis of the elixir, each collaborator was instructed to consider the samples to be formulated as 0.5 mg dexamethasone/5 mL.

Dexamethasone in Drug Substance and Elixirs

Quantitative and Identification Methods

First Action

Principle

Dexamethasone content in drug substance and elixir is detd by normal phase LC using quaternary mobile phase with controlled H_2O content, UV detection at 254 nm, and cortisone as internal std. Identity is confirmed in bulk drug substance and elixir by TLC and in drug substance by IR spectroscopy and relative LC retention time ratios. Alcohol content in elixir is detd by GC on porous polymer column using internal std and flame ionization detector.

Liquid Chromatographic Method

Apparatus

(a) *Liquid chromatograph.*—Model 8100 (Spectra-Physics, 3333 N First St, San Jose, CA 95134-1995) equipped with Model 100-10 photometric detector (Hitachi/NSA, 460 E Middlefield Rd, Mountain View, CA 94043), 15–30 μL injection valve (Valco Instruments Co., Inc., P.O. Box 55603, Houston, TX 77255), and Model CR1A integrator (Shimadzu Scientific Instruments, Inc., 7102 Riverwood Rd, Columbia, MD 21046). Equiv. LC system, UV detector, autosampler, and strip chart recorder may be used. LC pumping system in which bubbles develop in mobile phase is unsuitable. 1 μg dexamethasone should produce 50% full scale response with appropriate detector and recorder or integration settings at 254 nm. Mobile phase flow rate 1.2 mL/min at ambient temp.

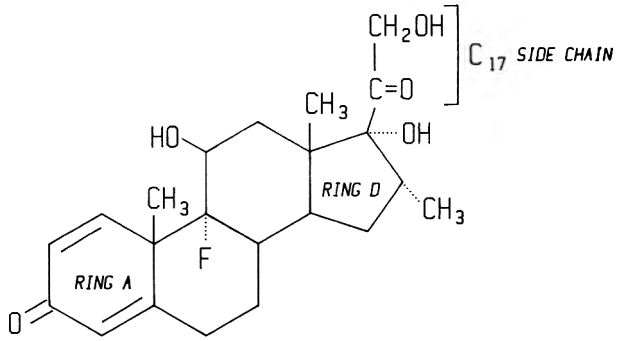

Figure 2. Dexamethasone.

(b) *Chromatographic column.*—Stainless steel, 25 cm × 4.6 mm id, packed with 5 μm Zorbax-Sil (E.I. du Pont de Nemours and Co.), or equiv. meeting appropriate LC system suitability requirements. 3 cm × 4.6 mm id stainless steel guard column packed with 10 μm silica particles may be used. If necessary, dry silica column by eluting with 20 mL. CH_2Cl_2–HOAc–2,2-dimethoxypropane (90 + 2 + 2 v/v/v).

Reagents

(a) *Solvents.*—Glacial HOAc (J. T. Baker or equiv.), UV grade MeOH and CH_2Cl_2 (Burdick and Jackson Laboratories, Inc., or equiv.), and distd-in-glass H_2O.

(b) *Methanol soln.*—Pipet 5.0 mL H_2O into 100 mL vol. flask and dil. to vol. with MeOH.

(c) *Mobile phase.*—Pipet 1.0 mL glacial HOAc and 45.0 mL MeOH soln into 1 L vol. flask, and dil. to vol. with CH_2Cl_2. Degas mixt. Adjust MeOH content to obtain retention times of approx. 6 and 9 min for cortisone and dexamethasone, resp. Cortisone retention time should be used for mobile phase composition adjustments; increased MeOH content decreases retention time.

(d) *Sodium bicarbonate soln.*—1M. Dissolve 8.4 g $NaHCO_3$ in 100 mL H_2O.

(e) *Internal std soln.*—Dissolve 30 mg cortisone (Sigma Chemical Co., or equiv.) in 4.0 mL MeOH and dil. to 100.0 mL with CH_2Cl_2.

(f) *Dexamethasone std soln.*—4.0 mg/100 mL. Transfer ca 25 mg accurately weighed USP Ref. Std Dexamethasone (previously dried 30 min at 105°) to 25 mL vol. flask, and dissolve in and dil. to vol. with MeOH. Transfer 2.0 mL aliquot of this soln to 50 mL vol. flask contg 6.0 mL internal std soln, and dil. to vol. with CH_2Cl_2. Do not filter thru membrane filter.

Sample Preparation

(a) *Drug substance.*—Prep. as directed for *Dexamethasone std soln,* using 25 mg dexamethasone. Do not filter thru membrane filter.

(b) *Elixir.*—Transfer accurately measured 10 mL portion of Dexamethasone Elixir, contg 1 mg dexamethasone, to 125 mL separatory funnel, add 5 mL 1M $NaHCO_3$ soln, and ext with four 20 mL portions of CH_2Cl_2. Collect exts in 250 mL separatory funnel contg 5 mL H_2O. Back-wash combined exts and filter thru cotton wet with CH_2Cl_2 into suitable beaker. Rinse H_2O back-wash and 125 mL separatory funnel consecutively with 10 mL CH_2Cl_2. Filter this rinse into beaker. Evap. filtrate on steam bath under jet of air to approx. 10 mL and quant. transfer with CH_2Cl_2 to 25 mL vol. flask contg 1.0 mL MeOH and 3.0 mL internal std soln. Dil. to vol. with CH_2Cl_2. Do not filter thru membrane filter.

Determination

Equilibrate column with mobile phase at 1.2 mL/min. Monitor response at 254 nm. Make 3 replicate injections of dexamethasone std soln. Using either peak area or peak ht measurements for each injection, calc. coefficient of variation (CV) of peak response ratios of dexamethasone to internal std. In suitable system, CV should be ≤2.5% and resolution factor, R_s, for dexamethasone peak and internal std peak should be ≥3. Make duplicate injections of std and sample solns and det. response ratio for each. Relative retention ratios of dexamethasone to internal std should agree within ±2.0%.

If relative retention ratios differ by >2.0%, then dry silica column as described in *Apparatus* (**b**).

Calculations

Calc. content of dexamethasone as follows:

Drug substance:

$$\text{Dexamethasone, mg} = 625 \times C \times (RR/RR')$$

Elixirs:

$$\text{Dexamethasone, mg/5 mL} = 12.5 \times C \times (RR/RR')$$

where C = final concn of std soln (mg/mL), and RR and RR' = av. response ratio for peak ht or area of analyte to that of internal std for sample and std solns, resp.

Thin Layer Chromatographic Identification

Apparatus, Reagents, and Test Solutions

(**a**) *Thin layer plates.*—Glass, 20 × 20 cm, coated with 250 μm layer of silica gel G with fluorescence indicator (Analtech Cat. No. 02011 [Analtech Inc., 75 Blue Hen Dr, PO Box 7557, Newark, DE 19711], or equiv.).

(**b**) *Developing solns.*—(*1*)*Drug substance.*—$CHCl_3$–diethylamine (2 + 1). (*2*) *Elixir.*—$CHCl_3$–acetone–glacial HOAc (80 + 40 + 1).

(**c**) *TLC std test solns.*—(*1*) *Drug substance.*—Prep. 1 mg/mL soln of USP Ref. Std Dexamethasone in CH_2Cl_2–MeOH (1 + 1). (*2*) *Elixir.*—Evap. 10 mL of *Dexamethasone std soln* (**f**) just to dryness on steam bath. Dissolve residue in 1 mL CH_2Cl_2–MeOH (1 + 1). Prep. individual 400 μg solns of dexamethasone (Sigma Chemical Co., or equiv.) and cortisone in CH_2Cl_2–MeOH (1 + 1) to serve as chromatgc identification stds.

(**d**) *TLC sample test solns.*—(*1*) *Drug substance.*—Prep. as directed for *TLC std test soln.* (*2*) *Elixir.*—Evap. 10 mL of elixir sample prepn (**b**) just to dryness on steam bath. Dissolve residue in 1 mL CH_2Cl_2–MeOH (1 + 1).

Chromatography

Equilibrate suitable chromatgc tank with appropriate developing solv. Spot 5 μL of each test soln ca. 2.5 cm from bottom of coated plate. Let spots dry and develop chromatogram until solv. front has moved 10 cm from origin. Remove plate, mark solv. front, air-dry plate, and locate spots under shortwave UV light. For drug substance, R_f of major spot in sample test soln corresponds to that for std test soln. For elixir, relative R_f of dexamethasone to cortisone for TLC sample test soln corresponds to that for TLC std test soln.

Infrared Spectroscopic Identification

Drug substance.—Prep. KBr dispersions from previously dried sample and std material. Scan spectra between 2.5 and 15.0 μm. Compare sample and std spectra. If difference appears, dissolve portions of both sample and std in CH_3CN, evap. solns to dryness, and repeat test on residues. Sample and std prepns exhibit maxima at same wavelength.

Identification by Relative Retention Times

Drug substance.—Compare retention ratios of main peak to internal std peak obtained for dexamethasone std soln and for assay sample prepn as directed in LC assay. Ratios that do not differ by >2.0% confirm identity.

Alcohol in Elixir

Gas Chromatographic Method

Apparatus and Reagents

(**a**) *Gas chromatograph.*—Model 5830A, with flame ionization detector and electronic integrator (Hewlett-Packard), or equiv. Operating conditions: column temp. 165° and N gas flow adjusted so that 2-propanol elutes in 3–5 min.

(**b**) *Chromatographic column.*—Glass, 6 ft × 4 mm id, packed with 80–100 mesh copolymer of ethylvinylbenzene and divinylbenzene that has nominal surface area of 500–600 sq m/g and av. pore diam. of 0.0075 μm. This material has been washed with org. solvs and acids and then preconditioned in bulk in O-free atm. Super-Q (Alltech Associates Cat. No. 2735 [Applied Science Labs, 2051 Waukegan Rd, Deerfield, IL 60015]) has been found to be suitable.

(**c**) *Internal std soln.*—Dil. 5.0 mL 2-propanol with H_2O to 250 mL.

(**d**) *Alcohol std soln.*—Dil. 5.0 mL absolute alcohol with H_2O to 250 mL. Pipet 10 mL of this soln and 10 mL internal std soln into 100 mL vol. flask and dil. to vol. with H_2O.

Preparation of GC Column

With small plug of silanized glass wool in end of column, apply vac. to exit of column and add packing in small amts to inlet end. With aid of gentle vibration, pack column firmly. Condition column overnight at 225° with slow N flow. Check column for voids after conditioning. Gently vibrate column to remove voids. Check column performance by injecting alcohol std soln and calcg following: resolution ≥3; RSD <1.5% for alcohol peak area relative to 2-propanol peak area with 6 replicate injections; and tailing factor ≤2.0 for alcohol.

Sample Preparation

Pipet 4 mL elixir and 10 mL internal std soln into 100 mL vol. flask and dil. to vol. with H_2O.

Determination and Calculation

Inject ca 5 μL each of sample and std solns in duplicate. Calc. % alcohol in elixir as follows:

$$\%\ \text{alcohol (v/v)} = (RR/RR') \times C \times D$$

where RR and RR' = av. response ratio for peak area of analyte to that of internal std for sample and std, resp.; C = % alcohol in std soln; and D = sample diln factor.

CAS-50-02-2 (dexamethasone)

Results and Discussion

For the drug substance and elixir determinations, the collaborators used 5 different brands of liquid chromatographs, both manual injection and autosamplers (3 brands), and either electronic integrators (3 brands) or strip chart recorders. Calculations were based on manual measurement of height (3 collaborators), integrator areas (2 collaborators), and integrator heights (2 collaborators). No differences attributable to mode of calculation were noted. For the elixir alcohol analysis, the collaborators used 5 brands of gas chromatographs and either electronic integrators (4 brands) or strip chart recorders. Calculations were based on manual area calculation (1 collaborator), integrator areas (4 collaborators), and manual height measurement (1 collaborator). The alcohol method requires that area, not peak height, measurements be used for calculation. One collaborator did not complete the alcohol analyses because the column packed for the study did not meet the system suitability requirements. Because of documented problems with alcohol analyses with Poropak Q columns (6), collaborators were instructed to terminate the alcohol analysis if the system suitability test requirements were not met. Two collaborators chose to complete the alcohol analyses even though their reported replicate standard injections and tailing requirements, respectively, did not meet the study protocol. These data were included in the statistical treatment because the reported values were close to the study limits.

The study data, Tables 1–3, were statistically evaluated for within- and between-laboratory variance. No data points were discarded for the elixir dexamethasone analyses. An individual result from collaborator 4 for sample 1 was eliminated as a Dixon (11) outlier for the dexamethasone bulk drug substance analyses. Individual results from collaborator 2 for

Table 1. Collaborative results of dexamethasone bulk drug substance assay (% by weight on dried basis)

	Sample blind duplicate pairs					
Coll.	1/5[a]		2/6		3/4	
1	97.3	97.0	100.8	100.4	99.6	100.0
2	97.0	96.3	100.1	100.2	98.9	99.0
3	97.3	96.2	101.2	99.9	99.1	99.7
4	94.2[b]	96.4	98.8	99.1	98.6	98.6
5	97.4	98.0	99.4	101.3	100.4	100.3
6	97.7	96.9	99.7	99.6	99.3	98.8
7	97.5	97.8	100.1	100.7	99.4	99.4
Av.	97.1		100.1		99.4	
s	0.5752		0.7502		0.5839	
CV, %	0.59		0.75		0.59	

[a] Standard purity as received from manufacturer.
[b] Outlier, eliminated by Dixon's test criteria (11).

samples 10 and 12 and from collaborator 4 for sample 10 were eliminated as Dixon outliers for the elixir alcohol analyses.

After the data set was normalized by dividing each data point by its respective sample group average, pooled results for each analysis were statistically evaluated by the ranking and analysis of variance criteria presented by Steiner (11). For the alcohol analyses, the average calculation for sample group 9/10 did not include the outlier data point, 6.99%. Inclusion of this data point would cause a positively biased average and skew the statistical analysis. The author's results are reported as collaborator 7.

For the 3 bulk drug substance analyses, the coefficients of variation, CVs, were between 0.59 and 0.75% (Table 1). For 42 analyses, the pooled mean analysis, the CV for repeatability (CV_o, within-laboratory error), and the CV for reproducibility (CV_x, within- and between-laboratory error) were 99.8%, 0.81%, and 0.75%, respectively (Table 4). Laboratory 4 was identified as an outlier by the Thompson and Willke ranking test (11), and those results were eliminated. For the remaining 36 analyses, the pooled mean, CV_o, and CV_x were 100.2%, 0.36%, and 0.40%, respectively (Table 4). For the elixir dexamethasone analyses, the CVs were between 1.74 and 3.55% (Table 2). The pooled mean, CV_o, and CV_x were 99.9%, 2.11%, and 1.19%, respectively (Table 4). For the elixir alcohol analyses, the CVs were between 0.87 and 1.34% (Table 3). For 36 analyses, the pooled mean, CV_o, and CV_x were 100.0%, 4.61%, and 4.13%, respectively (Table 4). Laboratory 2 was identified as an outlier by the Thompson and Willke ranking test (11), and those results were eliminated. For the 30 remaining analyses, the pooled mean, CV_o, and CV_x were 99.5%, 0.82%, and 0.89%, respectively (Table 4).

The values for the system suitability tests for the dexamethasone drug substance and elixir assays are reported in Table 5. All of the collaborators successfully met the study system suitability test requirements by a substantial margin. Participants were not required to repeat these tests if the same chromatographic system was used for both analyses. The collaborators were told that prior to sample analyses their silica columns could be dried quickly with the methylene chloride–acetic acid–2,2-dimethoxypropane solvent system described by Bredeweg et al. (12). Careful control of the water content on the silica column is necessary for reproducible results in normal phase liquid chromatography. The drug substance analysis reflects the precision and accuracy of the normal phase chromatographic system. One collaborator had difficulty with bubble formation in the pumping system with the methylene chloride-based solvent

Table 2. Collaborative results of dexamethasone elixir assay (mg/5 mL)

	Sample blind duplicate pair formulations					
	Authentic		Commercial			
Coll.	7/11[a]		8/12[b]		9/10[b]	
1	0.456	0.451	0.465	0.469	0.491	0.504
2	0.448	0.444	0.490	0.491	0.522	0.522
3	0.444	0.437	0.519	0.512	0.509	0.517
4	0.452	0.442	0.505	0.484	0.539	0.539
5	0.442	0.442	0.486	0.485	0.510	0.513
6	0.424	0.450	0.475	0.480	0.483	0.499
7	0.448	0.445	0.458	0.482	0.508	0.512
Av.	0.445		0.486		0.513	
Av., %	89.0		97.2		102.6	
Rec., %	94.5					
s	0.00775		0.01723		0.01594	
CV, %	1.74		3.55		3.11	

[a] Formulated to contain 0.471 mg/5 mL.
[b] Label declaration 0.5 mg/5 mL.

Table 3. Collaborative results of elixir alcohol assay (% alcohol)

	Sample blind duplicate pair formulations					
	Authentic		Commercial			
Coll.	7/11[a]		8/12[b]		9/10[b]	
1	ND[c]	ND	ND	ND	ND	ND
2	5.01	5.01	4.98	5.40[d]	5.22	6.99[d]
3	4.95	4.99	4.95	4.97	5.22	5.12
4	4.79	4.96	4.81	4.93	5.12	4.96[d]
5	4.85	4.93	4.90	4.82	5.10	5.15
6	4.96	4.94	4.89	4.92	5.16	5.14
7	4.88	4.90	4.96	4.99	5.13	5.09
Av.	4.93		4.92		5.14	
Av., %	98.6		98.4		102.8	
Rec., %	98.6					
s	0.06612		0.06083		0.04478	
CV, %	1.34		1.24		0.87	

[a] Formulated to contain 5% by volume.
[b] Label declaration 5% by volume.
[c] ND = not determined. Column packed for this analysis did not meet system suitability requirements.
[d] Outlier; eliminated by Dixon's test criteria (11).

Table 4. Statistical analysis of pooled interlaboratory results

Statistic	Drug substance, % by weight		Elixir, 0.5 mg/5 mL decld	Alcohol, 5% by volume decld	
No. of analyses	42	36[a]	42	36	30[a]
Pooled mean[b]	0.998	1.002	0.999	1.000	0.995
Pooled mean, as decld	99.8	100.2	0.500	5.00	4.98
s_o[c]	0.0081	0.0036	0.0211	0.0461	0.0081
s_x[d]	0.0075	0.0040	0.0119	0.0413	0.0089
CV_o,[c] %	0.81	0.36	2.11	4.61	0.82
CV_x,[d] %	0.75	0.40	1.19	4.13	0.89

[a] Data from laboratories 4 and 2 were eliminated for the drug substance and alcohol analyses, respectively. These laboratories were found to be outliers based on Thompson and Willke ranking criteria (11).
[b] Pooled results were normalized through the division of each sample point by its sample group average. Outliers were not eliminated from any data sets for the drug substance or elixir for these calculations. One outlier (132.3% from the average) was eliminated from the alcohol data set when the average was calculated for the 30-point data pool.
[c] SD_o and CV_o are the repeatability (within-laboratory error) standard deviation and coefficient of variation, respectively.
[d] SD_x and CV_x are the reproducibility (within- and between-laboratory error) standard deviation and coefficient of variation, respectively.

Table 5. LC conditions for collaborative study of dexamethasone

			Drug substance			Elixir	
Coll.	Column	Calculation mode	R[a]	CV,[b] %	RRT[c]	R	CV, %
1	Spherisorb S5W	manual height	4.6	0.47	1.35	6.4	1.33
2	Zorbax-Sil	integrator area	7.0	0.18	1.44	NC[d]	—
3	Zorbax-Sil	integrator height	6.1	0.48	1.41	NC	—
4	Zorbax-Sil	manual height	NC	—	1.42	7.5	0.55
5	Zorbax-Sil	manual height	11.2	0.013	1.55	9.0	0.43
6	Zorbax-Sil	integrator area	8.8	0.17	1.45	9.8[e]	1.2
7	Zorbax-Sil	integrator height	5.0	0.21	1.48	NC	—
Study[f]	Silica	height or area	≥3	≤2.5	≤2.0%[g]		

[a] Resolution for dexamethasone and internal standard cortisone for system suitability test.
[b] Coefficient of variation for 3 replicate standard injections for system suitability test.
[c] RRT = relative retention time of dexamethasone to cortisone.
[d] NC = not calculated.
[e] Second Zorbax-Sil column was used.
[f] Collaborative study requirements.
[g] Maximum difference allowed between standard and sample relative retention times.

system. It appears that this particular manufacturer's pumps tend to form air bubbles with more volatile solvents. However, another collaborator used the same manufacturer's instrument and did not report any apparent difficulties. Methylene chloride also has a tendency to form bubbles in this manufacturer's autosampler. This solvent system has been successfully used by the author with 4 different designs of pumping systems but not including one from this particular manufacturer. Normal phase systems have greater selectivity for related corticosteroids (4, 5) than do previously reported reverse phase systems (13–16).

Brower (17) and Walters (9) have reported collaborative study results for prednisolone and hydrocortisone with a similar normal phase solvent system and samples prepared in methylene chloride. The major difference in the LC mobile phase is that ethylene chloride was used in place of methylene chloride. Because of the toxicity, flammability, lower capacity for water, slightly poorer selectivity for corticosteroids, and solvent contamination problems associated with ethylene chloride, the author chose to use methylene chloride. However, the previously cited studies indicate that satisfactory results could be obtained by substituting ethylene chloride if air bubbles in the system become a major problem. The methanol content might have to be adjusted to obtain the required separations.

The recovery for the authentic elixir was 94.5% of the theoretical 0.471 mg dexamethasone/5 mL. One month after preparation, the author recovered 94.8%. The author had prepared and analyzed 2 other authentic elixirs prior to the collaborative study. The USP XX (1) blue tetrazolium method (BT) and the proposed LC method were both used. For Authentic No. 1, the average of 7 analyses was 98.4% (CV 1.00%) by the BT method and 98.9% (CV 0.88%) by LC analysis. All analyses of Authentic No. 1 were within 1 week of preparation. For Authentic No. 2, the average of 5 analyses by the BT method was 99.0% (CV 1.76%); the average of 6 LC analyses was 96.5% (CV 0.77%). The USP assay was within a few days of preparation; the LC analyses were 1 month later. The author's original LC analyses of the commercial elixirs used for this study were 7.2% higher than the analyses prior to issuing the study. Thirty-one months had elapsed between these analyses. Equivalent results are obtained when the blue tetrazolium and LC analyses are done at the same time. Decomposition of the steroid is detected by LC analysis a month after formulation. That decomposition continues slowly is evidenced by the change in the assay of the 2 commercial products. According to Das Gupta (18), hydrocortisone, a related corticosteroid, appears to be unstable in both water and polyethylene glycol ointment base. He also noted that, when mixed with water, either alcohol or glycerin had a stabilizing effect on the steroid. He further noted that decomposition occurs both in Ring A and in the C_{17} dihydroxyacetone side chain and commented that the blue tetrazolium method is not a stability-indicating assay. Excipients for Authentic No. 1 included alcohol, benzoic acid, methyl paraben, and propyl paraben. Authentic No. 2 did not include the preservatives, but alcohol, glycerin, and propylene glycol were present. The collaborative study authentic sample incorporated alcohol, benzoic acid, and glycerin. Both commercial elixirs contained alcohol and glycerin, but one used benzoic acid, and the other used methyl and propyl paraben as preservatives.

The elixir preservatives do not interfere in the LC analysis. Benzoic acid is removed during the extraction; methyl and propyl parabens elute just prior to the internal standard, cortisone. No interference was noted in any of the chromatograms submitted by the collaborators. See Figure 3 for a typical chromatogram when methyl and propyl parabens are present. For the pooled elixir results, the within-laboratory error is larger than the between-laboratory error. This may be attributed to the loss of small amounts of the steroid in the extraction step of the procedure or to incomplete pipet delivery of the slightly viscous product since the within- and between-laboratory error is quite close for the drug substance analyses. The average of all the analyses for each sample set agrees quite well with the author's results, indicating that this difference is not significant.

For the alcohol analysis, the collaborative study specified a column packed with a copolymer of ethylvinylbenzene and divinylbenzene marketed as Poropak Q, and which preferably had been Soxhlet-extracted with chloroform prior to conditioning the column. Chloroform extraction has been reported as a method to reduce tailing of alcohols (7). Subsequent to the issuance of the study, a commercially available, purified form of Poropak Q, Super Q, was identified. On the basis of an evaluation in this laboratory, it appears that this material does not require special treatment to meet the system suitability tests. For this reason, Super Q or its commercial equivalent is specified in this report rather than Poropak Q.

The alcohol method collaborated in this study was a modification of the USP XX (2) and XXI (3) Method II. The USP method has a close correlation to a method for ethanol, isopropanol, or acetone in drugs by Falcone (19), which was

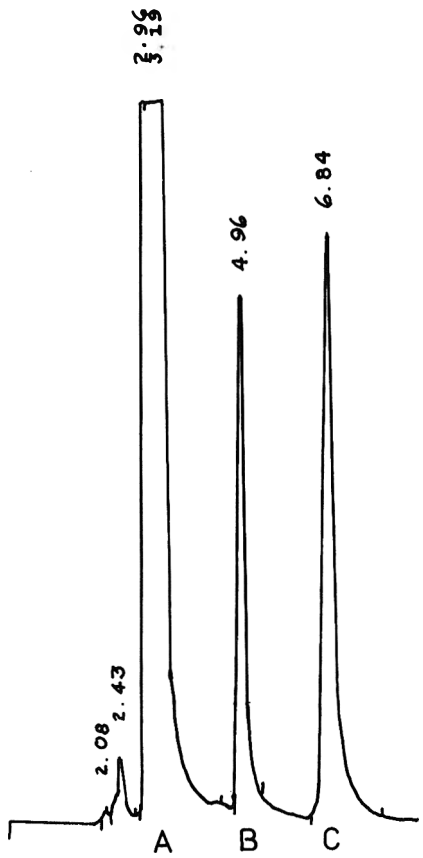

Figure 3. Collaborative study chromatogram with the least resolution achieved for an elixir with methyl and propyl paraben preservatives. Retention times in min. Flow rate = 1.2 mL/min. Key: (A) Propyl and methyl paraben; (B) cortisone; (C) dexamethasone.

collaboratively studied in 1973 and adopted by AOAC (20). The USP method specifies a smaller mesh size and lower column temperature. Both methods use acetonitrile as the internal standard. It has been documented (7) that ethanol and acetonitrile frequently are not resolved on the polymer packing that is commercially available now. For that reason, 2-propanol was chosen as the internal standard for this study.

In accordance with the study instructions, one collaborator

Table 6. GC conditions for collaborative study of alcohol in elixirs

Coll.	R[a]	CV,[b] %	T[c]	Chloroform extn[d]	Date packed	Calculation mode
1	ND[e]			yes	6/82	
2	3.6	0.17	1.4	yes	5/83	integrator area
3	4.0	1.8	1.2	no	11/82	integrator area
4	3.8	1.18	1.2	no	1/83	manual area
5	3.6	0.70	1.2	no	12/82	manual height
6	3.3	0.23	2.2	no	10/82	integrator area
7	4.0	0.60	1.2	yes	88/82	integrator area
Study[f]	≥3	≤1.5	≤2.0	yes		area

[a] Resolution for alcohol and internal standard 2-propanol for system suitability test.
[b] Coefficient of variation for 6 replicate standard injections for system suitability test.
[c] Tailing factor for alcohol for system suitability test.
[d] Method recommends chloroform extraction of Poropak Q packing to improve peak shape for polar compounds such as alcohol.
[e] ND = not determined. Column packed for this study did not meet system suitability requirements.
[f] Collaborative study requirements.

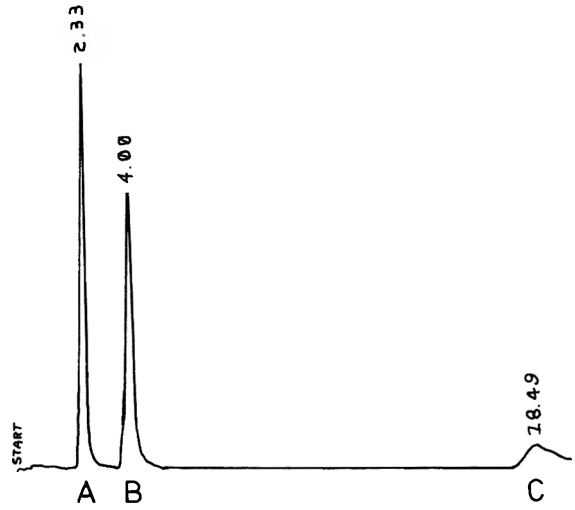

Figure 4. Gas chromatogram for alcohol analysis. Retention times in min. Key: (A) Alcohol; (B) 2-propanol; (C) propylene glycol.

did not complete the analyses because of poor column performance and the inability to meet any of the system suitability tests. Two other analysts submitted data even though the requirements of at least one of the 3 system suitability tests, including the generous tailing factor of 2 for alcohol (Table 6), were not met. The USP method requires a tailing factor of not more than 1.5. Inspection of the chromatograms revealed that random changes in peak height due to peak broadening continue to be a problem that requires the use of peak area to obtain accurate results. One collaborator commented on a late eluting peak, polyethylene glycol, as a possible source of interference (Figure 4). All the chromatograms were examined for possible interference from this peak and none was noted.

Identification of dexamethasone was confirmed by thin-

Table 7. Conditions and results for thin layer identification tests

Drug Substance					
Coll.	R_f[a]	R_f[b]	RR_f[c]	Solv. front, mm	Plate source
1	0.27	0.34	0.79	100	Analtech Silica Gel GF
2	0.44–0.45	0.57	0.79	103	E. Merck F254
3	0.28	0.38	0.74	109	Supelco Redi-coat-H
4	0.32	0.40	0.80	100	Analtech Silica Gel GF
5	0.30	0.39	0.77	108	Analtech Silica Gel GF
6	0.26–0.27	0.31	0.84[c]	145	Analtech Silica Gel GF
7	0.27	0.34	0.79	100	Whatman LK5F Silica Gel
Elixir					
Coll.	R_f[a]	R_f[d]	RR_f[e]	Solv. front, mm	Plate source
1	0.32	0.38	0.84	137	Analtech Silica Gel GF
2	0.45–0.46	0.56	0.81	100	E. Merck F254
3	0.27–0.28	NR[f]		145	Supelco Redi-coat-H
4	0.43	0.49	0.87	100	Analtech Silica Gel GF
5	0.30	0.37	0.81	150	Analtech Silica Gel GF
6	0.32	NR		119	Analtech Silica Gel GF
7	0.28	0.33	0.85	100	Baker Si250F Silica Gel

[a] Dexamethasone R_f.
[b] Betamethasone R_f.
[c] Relative R_f of dexamethasone to betamethasone. Coefficient of variation is 2.8%. Relative R_f for collaborator 6 is an outlier by Dixon's test (11).
[d] Cortisone R_f.
[e] Relative R_f of dexamethasone to cortisone. Coefficient of variation is 3.1%.
[f] NR = not reported.

layer chromatography, relative LC retention time, and IR spectroscopy for the drug substance and by a second TLC system for the elixir. For the drug substance, each collaborator was also asked to spot betamethasone, the configurational isomer of dexamethasone. The TLC system easily resolved this pair. Although the collaborators used a variety of commercial TLC plates and reported a wide range of R_f values for both TLC identification tests, the CVs for relative R_f values for each test are around 3% (Table 7). All collaborators successfully identified dexamethasone by the TLC test. The range of relative LC retention times was 1.35 to 1.55. The largest reported difference in relative retention time, 0.7%, was well within the required ±2.0% (Table 5). One sample specified for the IR spectroscopy identification test had a different crystalline structure and exhibited previously unreported polymorphism (21). Three of 6 collaborators not only successfully identified the polymorphic form of dexamethasone from its IR spectrum but also found that the method resolved the spectral differences. Of the remaining 3 collaborators, one missed the spectral difference, one submitted such poor quality spectra that the polymorphism was only suggested, and the third clearly did not exhibit polymorphism. It has been found in this laboratory that prolonged grinding with KBr powder changes the crystalline form so that the polymorphic effect is reversed. Cohen (7) reports that 2 crystalline forms of dexamethasone have been observed so that polymorphism should be expected in its IR spectrum.

Recommendation

It is recommended that the LC method for dexamethasone in bulk drug substance and elixirs, with identification by TLC, IR, and relative LC retention times for bulk drug substance and TLC for elixirs be adopted official first action. It is further recommended that the GC method for alcohol in elixirs be adopted official first action.

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Determination of Terbutaline Sulfate in Dosage Forms by Liquid Chromatography with Electrochemical Detection

WILLIAM L. CHILDRESS[1]
Food and Drug Administration, 585 Commercial St, Boston, MA 02109

A liquid chromatographic method with electrochemical detection has been developed for the determination of terbutaline sulfate in dosage forms. A cyanopropyl bonded-phase column is used with a mobile phase consisting of methanol–0.1M monobasic potassium phosphate containing 0.1M sodium heptanesulfonate and 1mM disodium ethylenediaminetetraacetate (15 + 85). The compound of interest is detected at a glassy carbon electrode held at a potential of +0.9 V vs silver–silver chloride. The response is linear from 0 to 10 μg/mL terbutaline sulfate. The method is applicable to tablet composites, individual tablets, dissolution determinations, and injections. Results and supporting data are reported for the above analyses.

The bronchodilator terbutaline sulfate is formulated at levels of 2.5 or 5 mg in tablets or at 1 mg/mL in injections. The compendial method for the determination of terbutaline sulfate in both products is a color development method in which the analyte is oxidized to a colored complex with potassium ferricyanide in alkaline solution (1). This method has been successfully used for automated analyses (2), but it requires precise timing in the addition of the reagents, and therefore, the accuracy and precision obtainable for a manual analysis could suffer. In addition, the method is neither specific nor stability indicating. An improved colorimetric method has been recently developed (3), but the same limitations apply. Finally, the sensitivity of the method is not as good as desirable in the case of dissolution determinations, where more dilute analyte solutions are encountered.

One obvious alternative is liquid chromatography (LC). Williams and coworkers (4) have used LC with fluorescence detection for the determination of terbutaline sulfate and related compounds. They used a reverse-phase C_{18} column and an ion-pairing reagent in the mobile phase and stated that the method is applicable to formulations containing terbutaline sulfate, although they present no results for actual formulations.

The phenolic structure of terbutaline sulfate suggests that it might be amenable to electrochemical detection, which, if applicable, would provide sensitivity comparable to that obtained with fluorescence detection. Lin and coworkers used LC with electrochemical detection to determine terbutaline and related betamimetics in plasma (5). Their method also involves a reverse-phase, ion-pairing system, with detection by oxidation at a glassy carbon electrode. They report that sensitivity of the method is in the low nanogram range for on-column injections—adequate for the most dilute analyte solutions likely to be encountered in dosage form analyses.

This paper reports the application of LC with electrochemical detection to the determination of terbutaline sulfate in dosage forms. Tablet formulations were analyzed for assay value, content uniformity, and dissolution. Injections were also assayed, and linearity and recovery studies were performed.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Model M45 constant-flow pump (Waters Associates, Milford, MA 01757); Model 7125 fixed-loop injection valve (Rheodyne, Inc., Cotati, CA 94928); cyanopropyl bonded-phase column, μ-Bondapak CN, 30 cm × 3.9 mm (Waters); Model LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN 47906) with glassy carbon working electrode, silver–silver chloride reference electrode, and stainless steel auxiliary electrode; and Model 4270 recording integrator (Spectra-Physics, San Jose, CA 95134). Operate at ambient temperature.

(b) *Dissolution apparatus.*—(Hanson Research Corp., Northridge, CA 91324). Equipped with rotating baskets and held at 37.0 ± 0.5°C by circulating water bath (GCA/Precision Scientific, Chicago, IL 60647).

(c) *Membrane filters.*—Nylon-66, porosity 0.45 μm (Rainin Instrument Corp., Woburn, MA 01801).

(d) *Ultrasonic bath.*—Model SC-150TH (Sonicor, Copiague, NY 11726).

(e) *Filter paper.*—Whatman No. 1 (Whatman, Inc., Clifton, NJ 07014).

Reagents

(a) *Terbutaline sulfate standard.*—U.S. Pharmacopeial Convention, Inc., Rockville, MD 20852, Cat. No. 64350, or secondary standard assayed according to *U.S. Pharmacopeia* (1). Dry at 105°C for 3 h.

(b) *Terbutaline sulfate standard solution.*—2.5 to 5.0 μg/mL in water, depending on the analysis being performed. Accurately weigh reference standard, dissolve in water, and serially dilute to desired concentration. Filter through membrane filter prior to injection into liquid chromatograph.

(c) *Mobile phase.*—Dissolve 13.6 g monobasic potassium phosphate and 2.2 g sodium heptanesulfonate in ca 800 mL water. Add 10 mL 0.1M disodium ethylenediaminetetraacetate (EDTA) and dilute to 1 L with water. Mix 850 mL of this solution with 150 mL LC grade methanol, filter through membrane filter, and degas by stirring 10 min under vacuum.

Sample Preparation

(a) *Tablets.*—Accurately weigh not less than 20 tablets and grind to pass No. 60 sieve. Transfer accurately weighed portion of powder equivalent to 5 mg terbutaline sulfate to 100 mL volumetric flask and add ca 80 mL water. Sonicate 15 min, dilute to volume with water, and filter through paper, discarding first 20 mL filtrate. Dilute 5.0 mL filtrate to 100.0 mL with water and filter through membrane filter.

For content uniformity determination, place individual tablet in 100 mL volumetric flask with 80 mL water. Let tablet completely disintegrate prior to sonication (10 to 15 min with occasional shaking). Filter as described above. Dilution scheme may be altered depending on tablet strength.

(b) *Injections.*—Pipet aliquot of injection equivalent to 5 mg terbutaline sulfate into 100 mL volumetric flask and dilute to volume with water. Dilute 5.0 mL of this solution to 100.0 mL with water and filter through membrane filter.

(c) *Dissolution.*—Proceed according to *U.S. Pharmacopeia* (1). Rotate baskets at 100 rpm. Use 900 mL water as dissolution medium; time is 45 min. (Data reported herein are based on a 60 min dissolution time.) At the end of specified time period, withdraw aliquot from each vessel and filter through membrane filter.

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[1] Present address: Food and Drug Administration, 109 Holton St, Winchester, MA 01890.

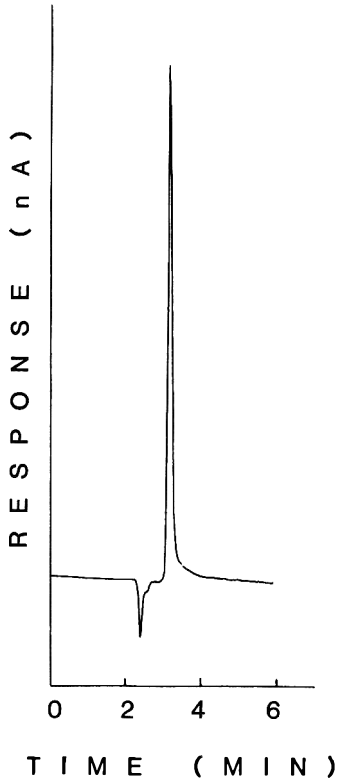

Figure 1. Typical chromatogram of standard solution of terbutaline sulfate. Detector potential +0.9 V vs Ag–AgCl, 20 nA full scale; other conditions are described in text. Chromatograms of sample solutions were identical.

Procedure

Set instrument flow rate to 1.0 mL/min and set working electrode potential to +0.9 V vs Ag–AgCl. Adjust detector sensitivity and/or recorder attenuation so that 20 μL standard solution gives ca half-scale deflection. Inject five 20 μL portions of standard solution. System is suitable if coefficient of variation (CV) of peak responses of 5 standard injections is not more than 2.0%.

Inject replicate 20 μL aliquots of standard and sample solutions into liquid chromatograph and calculate average peak response for each. Calculate amount of terbutaline sulfate in sample solution by the following formula:

$$\text{Terbutaline sulfate in sample, mg} = [(Ru/Rs)CD]/1000$$

where C = concentration of terbutaline sulfate in standard solution, μg/mL; D = dilution factor; Ru = average peak response of sample solution; Rs = average peak response of standard solution. For tablet assay only, multiply above result by (A/W), where A = average tablet weight and W = weight of sample.

At the end of each working day, flush system with ca 100 mL each of water and methanol, in that order. Flush system with ca 100 mL water prior to introduction of mobile phase.

Condition electrode weekly by operating at +1.5 V for 5 min, −1.5 V for 10 min, and at working potential, all with mobile phase flowing through system. This procedure has been shown to maintain detector sensitivity while greatly reducing the need for electrode polishing (6).

Results and Discussion

The previously published methods for the analysis of terbutaline sulfate by LC use reverse-phase, ion-pair chromatography (4, 5). Initially, an attempt was made to follow the procedure described by Lin and coworkers (5), which uses an octadecylsilane bonded-phase column and a mobile phase consisting of 15% methanol in a 0.1M phosphate buffer containing sodium octanesulfonate as the ion-pairing agent. A small amount of disodium EDTA was also included, a common practice when electrochemical detection is to be used. That system was duplicated, except that sodium heptanesulfonate was used as the ion-pairing agent rather than sodium octanesulfonate which was unavailable at the time. When terbutaline sulfate was injected, the retention time was excessively long. Before resorting to a different mobile phase, we tried a more polar cyanopropyl bonded-phase column. Terbutaline sulfate eluted at a retention time of approximately 4 min, with a flow rate of 1.0 mL/min. This system was used for all analyses described here. A typical chromatogram is shown in Figure 1.

Table 1. Results of LC and electrochemical detection of terbutaline sulfate in tablets

Sample[a]	Label, mg	% of label: Assay	% of label: Content uniformity[b]	% of label: Dissolution[c]	Rec., %
A	2.5	99.2	91.7–96.2	95.1–101.4	97.1
B	5	97.2	92.3–101.6	98.8–107.4	99.6
C	5	100.0	92.0–99.8	99.6–108.6	98.4

[a] A, Geigy lot 14210; B, Merrell-Dow lot 403126; C, Geigy lot 27600.
[b] Range for 10 tablets.
[c] Range for 6 tablets.

To determine if the system was linear in the range of interest, a series of solutions of terbutaline sulfate at 2 to 10 μg/mL was prepared, and each solution was injected in triplicate. A linear curve having a correlation coefficient of 0.9998 was obtained using a least-squares regression. The plot passed through the origin, within experimental error.

Three commercial samples of terbutaline sulfate tablets and 2 samples of terbutaline sulfate injection were analyzed using the proposed procedure. The tablets were analyzed for assay value, content uniformity, and dissolution. The injections were assayed only. A recovery study was performed for each tablet assay by the method of standard addition. For the injections, a synthetic mixture was prepared from terbutaline sulfate and sodium chloride in the concentrations stated on the product label, and the resulting solution was adjusted to pH 3.0–5.0. Because both commercial samples had the same formulation, only one synthetic mixture was prepared. Results of tablet assays ranged from 97.2 to 100.0% of label. Individual tablet determinations ranged from 91.7 to 101.6%, and dissolution results ranged from 95.1 to 108.6%. Recoveries of added standard ranged from 97.1 to 99.6%. For the injections, assay values were 100.9 and 101.0% of label, while the synthetic formulation showed a recovery of 98.5%. Results are summarized in Tables 1 and 2.

No interferences were noted in any of the chromatograms. Although it is possible to perform the analysis using UV detection, more baseline noise was encountered in this mode (7). Because the more sensitive electrochemical detector needed to be used for the dissolution determination, it was

Table 2. Results of LC and electrochemical detection of terbutaline sulfate in injections

Sample[a]	Concn, mg/mL	Assay, %
D	1	100.9
E	1	101.0
Synthetic	1.024	98.5

[a] D, Merrell-Dow lot 404067; E, Geigy lot 75079020.

deemed preferable to use it for the entire analysis. Sample preparation consists simply of extraction and dilution with water. Solutions of terbutaline sulfate in the mobile phase showed a fairly rapid decrease in peak response with time, whereas straight aqueous solutions showed constant peak responses for several days. For solutions in mobile phase, no other peaks were observed, indicating that the degradation product(s) is not electroactive at the chosen potential. Therefore, the straight aqueous extraction and dilution scheme was chosen.

Reproducibility was checked by injecting 5 consecutive aliquots of the standard solution prior to each sample analysis. Coefficients of variation of the peak responses were always less than 2.0% and were routinely in the range of 1.2–1.5%.

Use of LC with electrochemical detection has thus been shown to be an alternative to previously described methods for the determination of terbutaline sulfate in dosage forms. It is reasonably rapid, potentially stability indicating, reproducible, relatively uncomplicated, and linear over the concentration range of interest. The use of electrochemical detection for analyses such as dissolution, which require greater sensitivity, has been demonstrated to be applicable in this case and may prove to be a valuable technique for other drugs having electroactive functional groups.

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Titrimetric Determination of Nonesterified Fatty Acids in Intravenous Fat Emulsions

TERRY D. CYR, ROBERT C. LAWRENCE, and EDWARD G. LOVERING

Health and Welfare Canada, Health Protection Branch, Bureau of Drug Research, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada

A titrimetric method, suitable for use at a limit of 5 mEq/L, has been developed for the determination of total nonesterified fatty acids in intravenous fat emulsion preparations. The method differentiates titrant consumed by the nonesterified fatty acids from that consumed by egg yolk phospholipids, usually present as an emulsifying agent. The total nonesterified fatty acids in 8 products from 4 manufacturers were in the range from 0.4 to 3.8 mEq/L. The mean standard deviation of the method is 0.09 mEq/L.

A method is needed to serve as a limit test for total nonesterified fatty acids (which include free acids and their sodium salts), usually present at levels below 5 mEq/L, in intravenous fat emulsions. These emulsions typically contain 10 or 20% soybean or safflower oil, 1.2% egg yolk phospholipids as an emulsifying agent, 2.2% glycerol for isotonicity, and sodium hydroxide for pH adjustment, in water for injection (1). Phosphatidic acids—weakly acidic components of egg yolk phospholipids—are a potential interference in any titrimetric method. The procedure for total nonesterified fatty acids described in this paper distinguishes titrant consumed by the nonesterified fatty acids from that consumed by the phosphatidic acids. Typically, the latter titrant exceeds that required by the fatty acids.

Intravenous fat emulsions are not official in the *U.S. Pharmacopeia* (2) or the *British Pharmacopoeia* (3), but soybean oil is official in both (as Soya Oil in BP). The USP requirement for neutralization of free fatty acids in 10 g soybean oil is not more than 2.5 mL of 0.020M sodium hydroxide, which is equivalent to 0.50 mEq/L in 10% fat emulsion. The BP limit for acid value (mg potassium hydroxide to neutralize 1 g oil) is not more than 0.6, which corresponds to 1.07 mEq/L in 10% fat emulsion. A proposed USP monograph for egg yolk phospholipid (4) specifies that the free fatty acids in 1.0 g sample require for neutralization not more than 5.0 mL of 0.10M sodium hydroxide, equivalent to 6 mEq/L in 10% fat emulsion. There is also an AOAC method for free fatty acids in crude and refined oils, based on titration (5). None of these methods provide for the presence of phosphatidic or other weak organic acids.

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METHOD

Apparatus and Reagents

(a) *Automatic recording titrator.*—Mettler Memotitrator, equipped with Mettler DG 112 glass calomel electrode with ground-glass junction for nonaqueous titrations.

(b) *Egg yolk phospholipid fractions.*—Sigma Chemical Co. (St. Louis, MO 63178).

(c) *Stearic acid.*—Gold Label (Aldrich Chemical Co., Inc., Milwaukee, WI 53201).

(d) *Sodium stearate.*—BDH Chemicals (Poole, England).

(e) *Primary standard.*—Potassium hydrogen phthalate (Fisher Scientific).

Solutions

(f) *Potassium hydrogen phthalate standard solution.*—Transfer 39 mg, accurately weighed and previously dried at 120°C for 2 h, to Erlenmeyer flask; dissolve in 10 mL water, add 40 mL ethanol, and mix.

(g) *Sodium hydroxide solution.*—Standardize 0.1M sodium hydroxide by titration to potentiometric end point against potassium hydrogen phthalate solution **(f)**.

(h) *Sulfuric acid solution.*—Standardize 0.025M sulfuric acid to potentiometric end point against solution **(g)**.

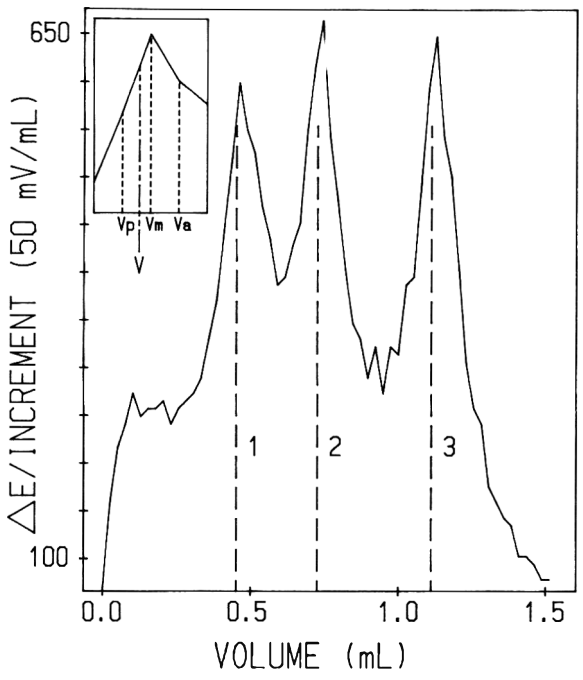

Figure 1. First derivative of titration curve for 10% intravenous fat emulsion, Lot A1. Peaks 1, 2, and 3 correspond to sulfuric acid, free fatty acid, and phosphatidic acid end points, respectively. Insert represents peak at end point 1, where Vp, Vm, and Va are the volumes of titrant that correspond to Ep, Em, and Ea, respectively, and V is the volume calculated at the equivalence point.

(i) *Spiking solution.*—7.26 mg/mL stearic acid in isopropyl alcohol.

Procedure

Set automatic recording titrator equipped with glass calomel electrode to record voltage change after increments of 0.025 mL titrant. Three titrations are required for each sample to be analyzed. Make all intravenous fat emulsion transfers with a "to contain" pipet and rinse pipet with a portion of the ethanol addition required for each sample. Prepare and titrate samples consecutively, as follows:

(I) Test preparation: Transfer 10.0 mL fat emulsion to titration vessel and add a total of 40.0 mL ethanol, including pipet rinsings.

(II) Spiked preparation: Transfer 10.0 mL fat emulsion, 10.0 mL spiking solution, and a total of 30.0 mL ethanol, including pipet rinsings, to titration vessel.

(III) Spike blank: Pipet 10.0 mL spiking solution and 40.0 mL ethanol into titration vessel.

To each of the above, add 1.0 mL 0.025M sulfuric acid solution and 3 drops of 1.0% phenolphthalein in ethanol. Place vessel in titrator bracket, insert electrode that has been rinsed for at least 3 min in ethanol, continuously purge vessel with flow of nitrogen, and stir rapidly for 1 min. Titrate with standardized sodium hydroxide (g) and record voltage changes to well past the end points, as shown by pink color of indicator.

Calculation

The end points, in order of total titrant added, are due to excess sulfuric acid, total nonesterified fatty acids, and the phosphatidic acid component of the egg yolk phospholipids (Figure 1). Equivalence points were determined by interpolation according to the procedure specified by the manufacturer of the automatic titrator (6). An example of the interpolation procedure is given below. Milliequivalents of nonesterified fatty acid per liter are given by $1000 \times M(V_2 - V_1)/A$ where M is the concentration of the standard sodium hydroxide solution (mol/L), V_1 and V_2 are the amounts of titrant (mL) required to reach the first and second equivalence points, respectively, and A is the volume (mL) of sample taken. The concentration of nonesterified fatty acid in the test preparation is equal to that in the spiked preparation less the spike blank.

Table 1. Abridged data from a typical titration of nonesterified fatty acids

Cumulative NaOH, mL	Voltage change, mV
0.25	3.8
0.50	5.2
0.75	5.8
0.425	12.6
0.450	14.6[a]
0.475	13.4
0.700	15.0
0.725	16.2[b]
0.750	13.2
.075	14.4
.100	15.8[c]
.125	13.2

[a] First end point: excess sulfuric acid.
[b] Second end point: total nonesterified fatty acids.
[c] Third end point: phosphatidic acids.

Abridged data from a typical run are presented in Table 1. For a given end point, Ep, Em, and Ea are the potential changes per increment of titrant just prior to, at, and just after the maximum (Em) voltage change, respectively. Vp and Vm are the total volumes of titrant added to achieve incremental potential changes Ep and Em, respectively (Figure 1).

For $Ep \geq Ea$,

$$V = Vp + Qp \times Vi, \quad (1)$$

where V is the volume of titrant added to the equivalence point, Vi is the increment volume (0.025 mL in this work), and

$$Qp = (18Rp - 5Rp^2 - 10RpSp^2 - 3)/20(1 - RpSp^2) \quad (2)$$

where $Rp = Ea/Ep$, and $Sp = Ep/Em$.

If Ep is less than Ea, then Eq. 1 becomes

$$V = Vm - Qa \times Vi \quad (3)$$

where

$$Qa = (18\ Ra - 5\ Ra^2 - 10\ Ra/Sa^2 - 3)/20(1 - Ra/Sa^2) \quad (4)$$

and where $Ra = Ep/Ea$ and $Sa = Ea/Em$. Thus, from the data in Table 1 and Eqs. 1–4, the cumulative total volume of titrant added to reach equivalence points 1, 2, and 3 are calculated to be 0.440, 0.707, and 1.084 mL, respectively, and the total nonesterified fatty acid is 2.67 mEq/L.

Results and Discussion

Total nonesterified fatty acids and phosphatides were determined in 3 samples of intravenous fat emulsions from 4 manufacturers. Results are presented in Table 2.

Excess sulfuric acid is added to the intravenous fat emulsion samples prior to titration to convert any sodium salts of the fatty acids to the free acid forms. The first equivalence point is due to excess sulfuric acid. When the amount of added sulfuric acid was increased (compare recovery exper-

Table 2. Nonesterified fatty acid, phosphatide, and sodium salt concentrations in intravenous fat emulsion products[a]

Product	Lot No.	Lipid, wt%	Fatty acid, mEq/L (RSD%)	Phosphatides, mEq/L (RSD%)	Na salt, mEq/L	Expir. date
Prod. A	A1	10	2.7 (0.7)	3.8 (1.1)	0.6	5/86
Spiked[b]			5.5 (2.5)	3.7 (3.9)		
Spike subtracted[c]			2.6 (5.1)			
Prod. A	A2	20	3.8 (5.1)	3.3 (4.6)	1.7	12/86
Spiked			6.5 (2.1)	3.5 (3.9)		
Spike subtracted			3.4 (4.1)			
Prod. B	B1	10	0.4 (8.9)	3.9 (2.2)	ND[d]	1/87
Spiked			3.5 (0.7)	3.8 (0.5)		
Spike subtracted			0.6 (30)			
Prod. B[e]	B2	20	0.4 (29)	3.7 (7.8)	ND	1/87
Spiked			3.3 (2.2)	3.6 (3.9)		
Spike subtracted			0.4 (18)			
Prod. C	C1	10	1.3 (1.0)	3.7 (0.9)	0.2	6/86
Spiked			4.3 (4.4)	4.8 (13)		
Spike subtracted			1.4 (14)			
Prod. C	C2	20	1.1 (4.6)	3.6 (8.6)	0.2	12/86
Spiked			3.9 (0.9)	3.7 (2.9)		
Spike subtracted			1.0 (2.9)			
Prod. D[e]	D1	10	0.4 (21)	3.9 (3.3)	0.1	9/87
Spiked			3.5 (5.4)	3.4 (5.6)		
Spike subtracted			0.6 (30)			
Prod. D[e]	D2	20	0.5 (35)	3.5 (6.7)	ND	4/87
Spiked			3.3 (4.5)	3.6 (2.3)		
Spike subtracted			0.4 (40)			

[a] Each result is the average of 4 determinations.
[b] Spiked as described in the method.
[c] After subtraction of spike blank.
[d] None detected.
[e] In samples containing low fatty acid levels, the first and second end points were not always separated. This leads to unusually high standard deviations. This occurred in one of 4 determinations for products B2 and D2—both 20% lipid by weight.

iments A and B, Table 3), the amount of titrant required to reach the first equivalence point also increased.

The second equivalence point is due to the free fatty acids in the sample. In recovery experiment C, Table 3, the intravenous fat emulsion was spiked with stearic acid; only the amount of titrant consumed at the second equivalence point increased. Experiment D recovered a spike of sodium stearate. The amount of titrant to reach the sulfuric acid equivalence point was reduced because a portion of the acid was consumed in converting sodium stearate to stearic acid, and the free fatty acid equivalence point was correspondingly increased. The levels of fatty acids present as sodium salts are given in Table 2.

The third equivalence point is due to the phosphatidic acid component of the phospholipids in the intravenous fat emulsion. In recovery experiments E, F, and G (Table 3) the emulsions were spiked with egg yolk phospholipid fractions III-E, IX-E, and X-E, respectively (7). These are 99, 60, and 60% phosphatidyl choline, respectively. The latter 2 lead to an increase in the titrant consumed at the third equivalence point, but type III-E, being 99% phosphatidyl choline, does not. The egg yolk phospholipids used in intravenous fat emulsions are believed to be unfractionated (8).

Table 3. Effect of various solutions on titrimetric end points[a]

Expt	Solution	NaOH volume between equivalence points, mL 1–0	2–1	3–2
A	1 mL 0.05M H_2SO_4	0.473	0.346	0.34
B	2 mL 0.05M H_2SO_4	0.996	0.341	0.353
C	1 mL 0.05M H_2SO_4 Stearic acid	0.458	0.678	0.315
D	1 mL 0.05M H_2SO_4 Sodium stearate	0.348	0.702	0.310
E	Phosphatidyl choline type III-E (120 mg)	0.477	0.333	0.344
F	Phosphatidyl choline type IX-E (120 mg)	0.464	0.356	0.663
G	Phosphatidyl choline type X-E	0.482	0.379	0.790

[a] Solutions were added to 10 mL of product C2.

Relative standard deviations, (RSD) are given in Table 2. The relative error is high at low free fatty acid levels, but at levels over 1.0 mEq/L, the RSD is generally <5%. Phosphatide levels are between 3.3 and 3.9 mEq/L throughout, with one result outside this range at 4.8 mEq/L. The RSDs vary considerably; all but one are less than 10%, and most are less than 5%. Recoveries of free fatty acids from the spiking experiments are indicative of the accuracy of the method. The concentrations of nonesterified fatty acids determined directly and those determined after subtraction of the spike blanks are presented in Table 2. The results agree within 0.2 mEq/L for all samples except A2 for which the difference is 0.4 mEq/L. The average standard deviation of the spiked samples is 0.01 mEq/L compared to 0.09 mEq/L for the unspiked samples. The difference between the amount of sulfuric acid added (1.0 mL, 0.025M) and that found, as measured by the volume of titrant consumed to the first equivalence point, is indicative of fatty acids in the sodium salt form. The highest level found was 0.17 mEq/100 mL in lot No. A (Table 2).

Conclusion

The method described in this paper is suitable for the determination of total nonesterified fatty acids in intravenous fat emulsions, with an RSD of <5% at a limit of 5 mEq/L.

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Colorimetric Determination of a Polymeric Quaternary Ammonium Antimicrobial Preservative in an Ophthalmic Solution

RALPH M. GOOD, JR, JOHN C. LIAO, M. JOAN HOOK, and CATHY L. PUNKO
Alcon Laboratories, Inc., PO Box 6600, Fort Worth, TX 76115

A simple colorimetric method is presented for the determination of Polyquad®, a polymeric quaternary ammonium antimicrobial preservative, in an ophthalmic solution. Polyquad and magnesium are coprecipitated by ion pairing with Ponceau S, a highly colored sulfonate dye, at an acidic pH. The sample is centrifuged and the supernate analyzed spectrophotometrically. The concentration of Polyquad is determined by the decrease in dye concentration. Formulation excipients such as disodium edetate, dextran, and hydroxypropylmethylcellulose did not interfere with the quantitation. The method is specific, accurate, precise, and stability-indicating for the quantitation of 10 ppm Polyquad.

Polyquad® or Onamer M®, a polymeric quaternary ammonium compound chemically known as α-4-[1-tris(2-hydroxyethyl)ammonium chloride-2-butenyl]poly-[1-dimethyl ammonium chloride–2-butenyl]-ω-tris(2-hydroxyethyl)-ammonium chloride, is a mixture of high-molecular-weight polymers (5000–10 000 amu) (Figure 1). Water-soluble polymers such as Polyquad show great potential for use as preservatives in the pharmaceutical industry. Polyquad has been shown to be as effective as the most active commercially available quaternary ammonium compounds when evaluated as a hard-surface disinfectant and is low in toxicity (1).

The ion pairs formed between acid dyes and quaternary ammonium compounds can be used in the quantitation of both moieties (2–9). In the proposed method, Polyquad is specifically quantitated in an aqueous pharmaceutical formulation containing disodium edetate, dextran, and hydroxypropylmethylcellulose. Polyquad and magnesium are coprecipitated by ion-pairing with Ponceau S (3-hydroxy-4-[-2-sulfo-4-(4-sulfophenylazo)phenylazo]-2,7-naphthalene disulfonic acid, tetrasodium salt), at an acidic pH. Ponceau S is a highly colored sulfonate dye which has been used as a protein stain (10, 11). Its structure is shown in Figure 2. The sample is centrifuged and the supernate analyzed spectrophotometrically. The Polyquad concentration is determined by the loss in dye concentration.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.*—Perkin-Elmer Model 559A double beam (Perkin-Elmer Corp., Norwalk, CT 06856) with 1 cm disposable plastic cuvettes (Evergreen Scientific, Los Angeles, CA 90058).

(b) *Test tubes.*—20 mm × 125 mm glass, with Teflon-lined caps.

(c) *Centrifuge.*—Beckman Model TJ-6 horizontal swinging rotor centrifuge capable of 2500 rpm (Beckman Instruments, Inc., Palo Alto, CA 94304).

(d) *Syringe.*—For standard curve, Hamilton gas-tight 100 μL syringe (Hamilton Co., Reno, NV 89510).

(e) *Ponceau S solution.*—0.2 mg/mL aqueous (Aldrich Chemical Co., Milwaukee, WI 53201).

(f) *Magnesium sulfate solution.*—5% magnesium sulfate in 1% (v/v) sulfuric acid (J. T. Baker Chemical Co.).

(g) *Saline solution.*—0.9% NaCl aqueous.

(h) *Polyethylene glycol 4000 solution.*—Average mol wt ≈ 4000 amu, 1.2% aqueous (Union Carbide Corp., Atlanta, GA 30329).

(i) *Freon–Tween solution.*—0.05% Tween 21 (polysorbate 21) (ICI Americas, Wilmington, DE 19897) in freon-113 (J. T. Baker). *Note:* Freon-113 should be used with adequate ventilation, and skin contact should be avoided.

(j) *Standard Polyquad solution.*—0.36% w/v aqueous (Stepan Co., Northfield, IL 60093).

Preparation of Standard Curve

Dilute a weighed aliquot of Polyquad standard with water to yield a 1250 ppm solution. Add 30, 40, and 50 μL aliquots of this solution to separate 5.0 mL aliquots of saline in test tubes. Treat each of the these working standards as described under *Assay.* Corrected absorbances are plotted against concentration to obtain a straight line which demonstrates a significant positive *y*-intercept.

Preparation of Sample

Dilute sample solutions containing >10 ppm Polyquad with saline to 10 ppm. This method was validated for Polyquad concentrations from 7.5 ppm to 12.5 ppm. Place a 5.0 mL aliquot of sample solution into a test tube and treat as described under *Assay.*

Preparation of Blanks

Place a 5.0 mL aliquot of saline into a test tube and treat as described under *Assay.*

Assay

Pipet 5.0 mL polyethylene glycol solution into each of the working standards, blanks, and products. Immediately cap test tubes and mix by shaking thoroughly 15 s. Add 2.0 mL Ponceau S solution to each tube and vortex immediately 15 s. Dispense 2.0 mL of magnesium sulfate solution into each test tube and immediately vortex 15 s to mix thoroughly. Add 5.0 mL freon–Tween solution into each tube, cap tubes, and immediately shake 30 s. Let samples stand at room temperature 40 min to achieve complete reaction. Centrifuge test tubes 10 min at 2500 rpm. Measure absorbance of dye at 510 nm against water. Correct standard and sample absorbances by subtracting from blank absorbance. Determine sample concentrations by comparison with the standard curve.

Results and Discussion

It is likely that Polyquad molecules are anchored and stretched on the linear polyethylene glycol chains, thus open-

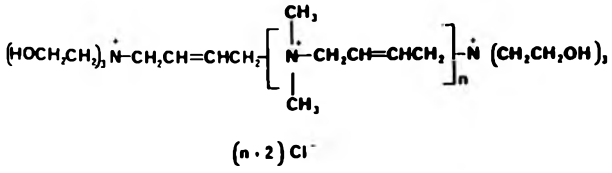

Figure 1. Structure of all *trans* Polyquad.

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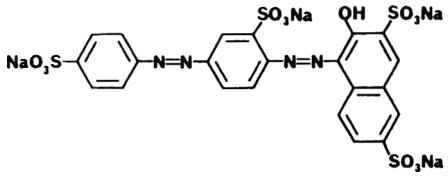

Figure 2. Structure of Ponceau S.

ing them for ion pairing with Ponceau S and coprecipitation with magnesium ions. Both polyethylene glycol 4000 and polyethylene glycol 8000 were evaluated and found to be satisfactory. The volume of the polyethylene glycol solution also serves to reduce the viscosity of the formulation matrix by dilution. Freon is used to remove the turbidity from the aqueous phase before spectrophotometric measurement. Chloroform is unsuitable for this purpose because it will not clarify the aqueous phase. The addition of Tween to the freon causes the precipitate to remain at the aqueous layer–freon interface, thereby reducing the tendency of the particles to float up into the aqueous layer. However, the absence of Tween in the freon produces similar results. Saline is used in the standard and reagent blanks to produce an environment similar to that in the products.

The drug formulation vehicle (without Polyquad) was spiked at 3 concentrations on 3 different days to demonstrate the reliability and recovery of the proposed method. Recoveries (Table 1) were calculated using a standard curve. The precision of the method was verified by the analysis of 10 product samples (Table 2) which exhibited a 1.3% relative standard deviation.

Table 1. Recoveries and standard curves from spiked drug formulations

Concn, ppm	Recoveries, %		
	Curve 1	Curve 2	Curve 3
7.59	97, 98	100, 97	100, 99
10.1	101, 101	99, 99	102, 102
12.7	98, 99	98, 98	99, 98
Mean recovery, % 99			
RSD, % 1.6			
Slope AU/ppm	0.0582	0.0623	0.0607
y-Intercept, AU	0.108	0.0887	0.0845
R^2	0.995	0.999	0.996
RSD, %	3.61	2.97	2.84

Table 2. Polyquad product precision data

Sample No.	Blank corrected response, AU	Concn found,[a] ppm
1	0.722	10.4
2	0.742	10.8
3	0.729	10.6
4	0.729	10.6
5	0.725	10.5
6	0.728	10.5
7	0.738	10.7
8	0.742	10.8
9	0.740	10.8
10	0.735	10.7

Mean concn, ppm 10.6
RSD, % 1.34
[a] Theoretical product concentration is 11.0 ppm.

Method specificity was demonstrated by the analysis of forcefully degraded drug formulation vehicles and vehicle standards. No significant interferences were observed. Benzalkonium chloride and chlorhexidine digluconate at comparable product concentrations also exhibited no significant interference.

The data indicate that the method is precise, accurate, sensitive, specific, and stability indicating for the analysis of Polyquad in an ophthalmic solution.

Acknowledgments

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Synthesis, Identification, and Acute Toxicity of Some *N*-Alkyl Derivatives of 3,4-Methylenedioxyamphetamine

F. TAYLOR NOGGLE, JR
Alabama Department of Forensic Sciences, Wire Rd, Auburn, AL 36849
JACK DERUITER, SAMUEL T. COKER, and C. RANDALL CLARK[1]
Auburn University, School of Pharmacy, Department of Pharmacal Sciences, Auburn, AL 36849

A series of *N*-alkyl derivatives of 3,4-methylenedioxyamphetamine (MDA) was prepared in an effort to characterize these potential drugs of abuse. These secondary amines were synthesized via reductive amination of the corresponding ketone with alkylamines. The ultraviolet absorption spectra for these compounds produced almost equally intense absorbance at 234 and 285 nm. The compounds were separated by liquid chromatography using reverse phase (C_{18}) procedures with a ternary mobile phase mixture. Toxicity studies showed all of the compounds to have LD_{50} values similar to *N*-methyl MDA (MDMA).

The pharmacological actions of 3,4-methylenedioxyamphetamine (MDA) allow for its classification as a hallucinogen (1). It has other atypical effects such as a low potential to produce severe sensory disruption; however, MDA became a popular drug of abuse primarily because of its enhancing effect on empathy (2).

Methylation to yield the secondary amine, MDMA, produces significant changes in the pharmacological properties: a shorter duration of effect, a general decrease in potency, and elimination of the hallucinogenic properties. However, the empathy-enhancing properties are retained and appear to be more pronounced in MDMA (3). MDMA is claimed to have unique properties in psychotherapy, reducing the anxiety that normally accompanies the discussion of emotionally unpleasant events (4). The recent appearance of this drug on the street market as "Ecstasy" indicates the popularity and potential for abuse of this drug. Its popularity is probably due to its mild effects and its ability to facilitate interpersonal communication (5).

The work of Hardman et al. (6) compared the toxicity and behavioral aspects of MDA and MDMA to mescaline in 5 animal species. The LD_{50} for MDA was significantly lower than that for mescaline, with the lethality of MDMA intermediate between the two. The toxicity for MDA ranged from 2.68 times that of mescaline in the mouse to 17.65 in the monkey. Similar values for MDMA were 2.04 in the mouse and 5.89 in the monkey.

The recent appearance of the *N*-ethyl derivative of MDA in clandestine drug samples has prompted this investigation. In this study a series of *N*-alkyl derivatives of MDA has been prepared and characterized. The ultraviolet (UV) and nuclear magnetic resonance spectral properties, liquid chromatographic analysis, and acute toxicity data are reported.

Experimental

General

Melting points were determined in open glass capillaries using a Thomas-Hoover melting point apparatus and are uncorrected. All ^1H NMR spectra were measured in DMSO on a Varian T-60A spectrometer with an internal standard of tetramethylsilane. Infrared (IR) spectra were recorded on a Perkin-Elmer Model 1500 Fourier transform infrared spectrophotometer. The UV absorption spectra were determined using a Shimadzu 160 spectrophotometer. Solutions for UV studies were prepared in 0.1N H_2SO_4 at a concentration of 1×10^{-4}M.

Synthesis of N-alkyl-MDA Analogs

Method A.—A solution of the ketone (1.78 g, 10 mmol) in 50 mL 70% aqueous ethylamine was stirred at reflux for 30 min. The mixture was then cooled to room temperature, and 2.0 g $NaBH_4$ was added portionwise over a period of 10 min. The mixture was then stirred at reflux for 30 min, cooled in an ice bath, and acidified to pH 1 with concentrated HCl. The resultant aqueous suspension was extracted 3 times with 75 mL each of $CHCl_3$ and then was made basic (pH 14) with 1N NaOH. The basic aqueous solution was extracted 3 times with 100 mL each of $CHCl_3$, and the combined $CHCl_3$ extracts were dried over $MgSO_4$. Filtration, followed by evaporation of the filtrate solvent, yielded the product base as a brown oil. Treatment of the base with ethereal HCl gave a white solid which was recrystallized from ethanol–ether to give *N*-ethyl methylenedioxyamphetamine HCl as a fine white granular solid (Table 1).

Method B.—Sodium cyanoborohydride (1.9 g, 30 mmol) was added portionwise to a solution of the ketone (1.78 g, 10 mmol) and 5.0 mL amine in 50 mL acetonitrile. This mixture was stirred at room temperature and 1.0 mL glacial acetic acid added. After stirring for 2 h, more glacial acetic acid was added (1.0 mL) and the mixture was stirred an additional 30 min. The reaction mixture was then extracted twice with 100 mL each of ether, and the combined ether extracts were washed successively with 100 mL 1N NaOH, 100 mL saturated $NaHCO_3$ solution, and 100 mL water. The ether solution was then extracted twice with 100 mL each of 3N HCl, and the combined HCl extracts were washed with 100 mL $CHCl_3$. The aqueous acid solution was then made basic (pH 12) with 2N NaOH, and this suspension was extracted 3 times with 150 mL each of $CHCl_3$. The combined $CHCl_3$ extracts were washed with 200 mL water and dried over Na_2SO_4. Filtration, followed by evaporation of the filtrate, gave the product base as an oil. The base was converted to the corresponding HCl or HBr salt after treatment with ethereal HCl or HBr, respectively, and recrystallized from mixtures of ethanol and ether (Table 1).

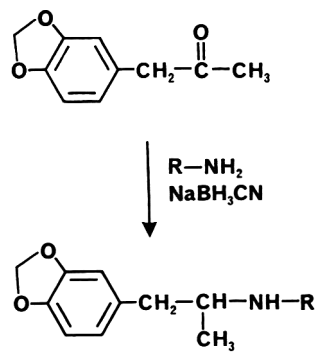

Figure 1. Synthesis procedure for preparation of *N*-alkyl analogs of 3,4-methylenedioxyamphetamine.

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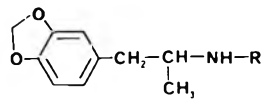
[1] Address correspondence to this author.

Table 1. *N*-Alkyl methylenedioxyamphetamine derivatives and their acute toxicities in mice



Compound No.	R	Synthetic method	Yield, %	mp, °C	Formula	LD_{50}, mg/kg (95% confidence limits)
1	$-CH_2CH_3$	A	31	197–198	$C_{12}H_{17}NO_2 \cdot HCl$	102 (96–108)
2	$-CH_2CH_2CH_3$	B	42	183–186	$C_{13}H_{19}NO_2 \cdot HCl$	102 (96–108)
3	$-CH(CH_3)_2$	B	63	174–179	$C_{13}H_{19}NO_2 \cdot HCl$	116 (109–123)
4	$-CH_2CH_2CH_2CH_3$	B	10	180–183	$C_{14}H_{21}NO_2 \cdot HBr$	85 (77–93)
5	$-CH_2CH(CH_3)_2$	B	9	175–178	$C_{14}H_{21}NO_2 \cdot HCl$	132 (124–140)
6	$-CH(CH_3)CH_2CH_3$	B	46	100–110	$C_{14}H_{21}NO_2 \cdot HCl$	104 (98–110)
MDA	-H					68 (50–92)[a]
MDMA	$-CH_3$					97 (89–106)[a]

[a] Data from Ref. 6.

Chromatographic Procedures

The liquid chromatograph consisted of a Waters Associates (Milford, MA 01757) Model 6000A pump, U6K injector, 440 UV detector with dual-wavelength accessory operated at 254 and 280 nm, and Houston Instrument (Austin, TX 78753) Omniscribe dual-pen recorder. The column was 30 cm × 3.9 mm id packed with μBondapak C_{18} (Waters Associates), and the mobile phase consisted of pH 3.0 phosphate buffer–methanol–acetonitrile–triethylamine (500 + 100 + 25 + 1). The mobile phase flow rate was 1.5 mL/min and the UV absorbance detector was operated at 0.2 AUFS. Sample solutions for analysis were prepared in methanol and all separations were done at ambient temperature.

Acute Toxicological Evaluation

Male ICR Swiss mice were used from Southern Animal Farms, Prattville, AL. Food and water were available to the animals ad libitum, and animals were housed in temperature- and light-controlled quarters. The compounds were administered intraperitoneally as the salts in distilled water. The animals weighed between 30 and 40 g. The concentration of the solution injected was 1–2% so that the total volume never exceeded 0.5 mL. Three dosing levels were used with 6 animals per dose. Mortality was determined after 24 h for the LD_{50}. The Lithchfield and Wilcoxon (7) method was used to calculate the LD_{50} values with upper and lower confidence limits at the 0.05 level of significance.

Results and Discussion

The *N*-alkyl analogs of 3,4-methylenedioxyamphetamine (MDA) were prepared according to the synthesis procedure shown in Figure 1. The synthesis of these amines involves condensation of the carbonyl moiety of 3,4-methylenedioxyphenyl-2-propanone with the appropriate amine to yield the imine which is reduced in situ to the corresponding secondary amine. The imine is reduced by using sodium cyanoboro-

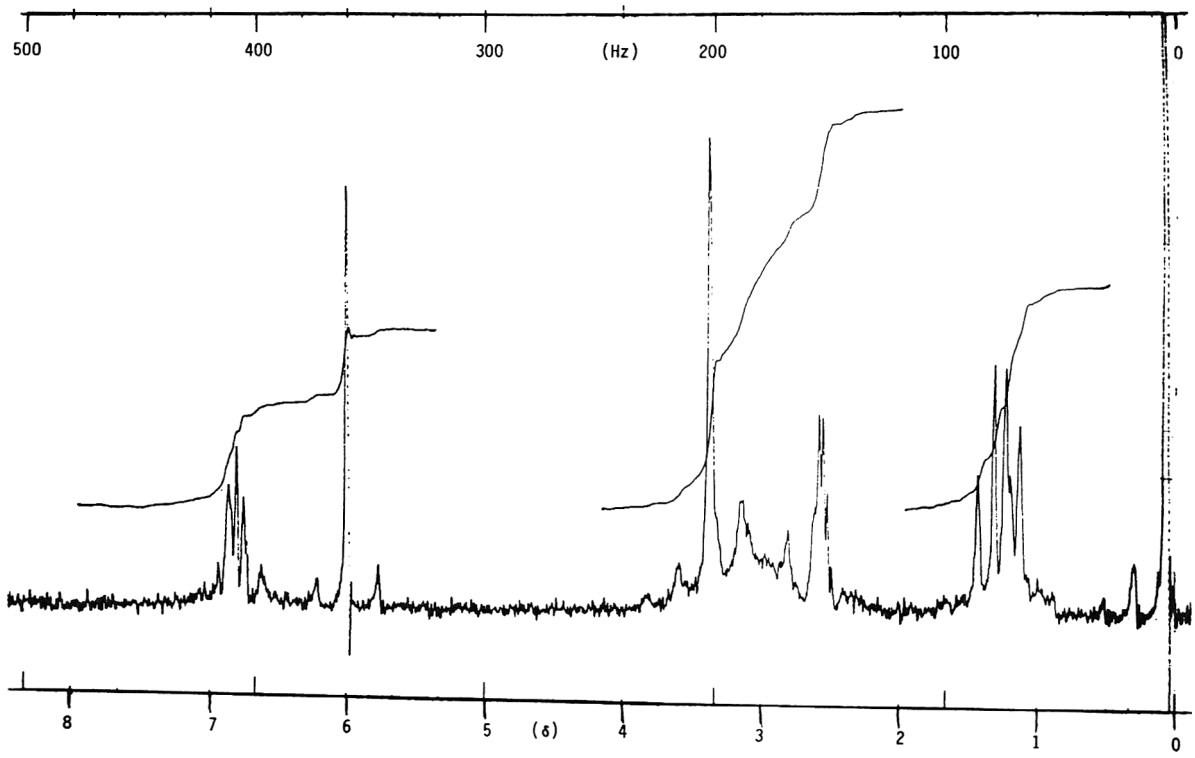


Figure 2A. Proton NMR spectrum for *N*-alkyl 3,4-methylenedioxyamphetamine compound 1. See Table 1 for structure of compound.

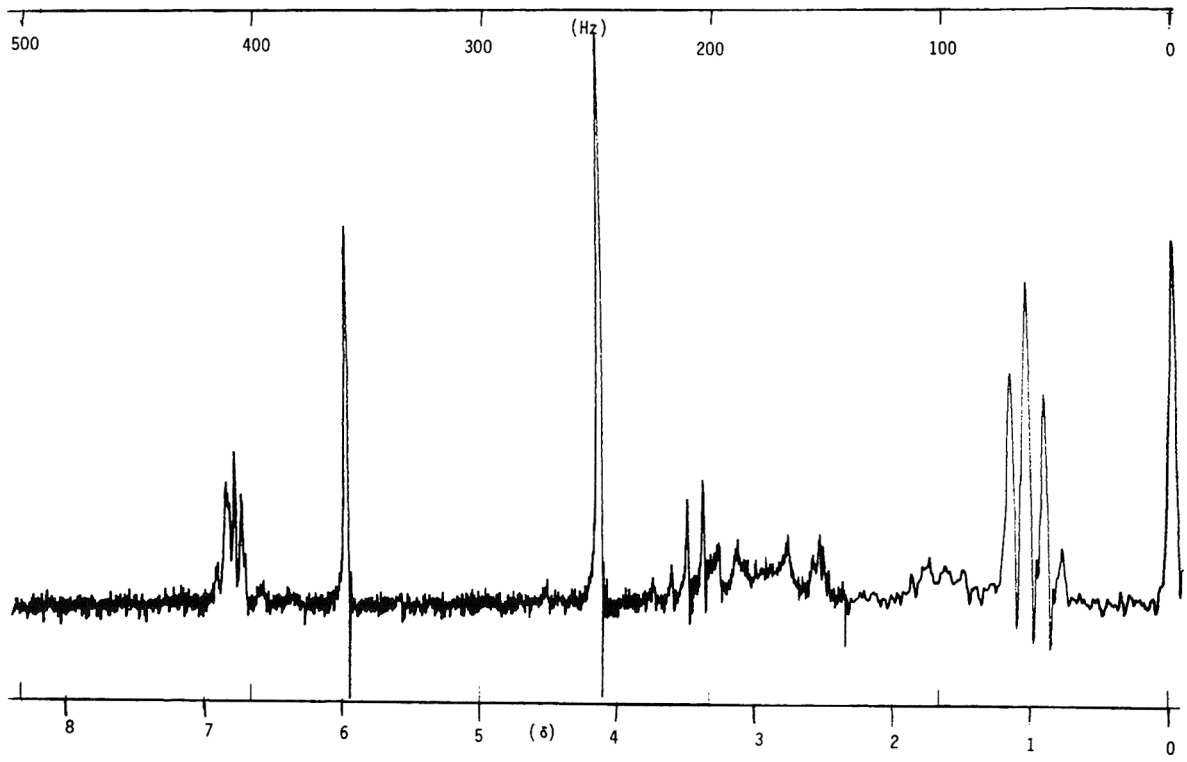


Figure 2B. Proton NMR spectrum for *N*-alkyl 3,4-methylenedioxyamphetamine compound 2. See Table 1 for structure of compound.

hydride, a hydride source selective for imines. Thus, the imines are reduced selectively as formed, and carbonyl reduction is not a competing reaction (8). The amines were converted to the hydrochloride salt using HCl in diethyl ether, except for compound 4 which was prepared as the HBr salt. The compounds prepared in this study included the C_1–C_4 *N*-alkyl derivatives of MDA; Table 1 summarizes the synthetic data obtained for these compounds. The proton NMR spectra are shown in Figures 2A–2F and are consistent with the assigned structures.

The UV absorption characteristics for all these compounds were quite similar, as expected. The 3,4-methylenedioxyphenyl group is the major chromophoric unit common to all these compounds. Figure 3 shows an example UV spectrum

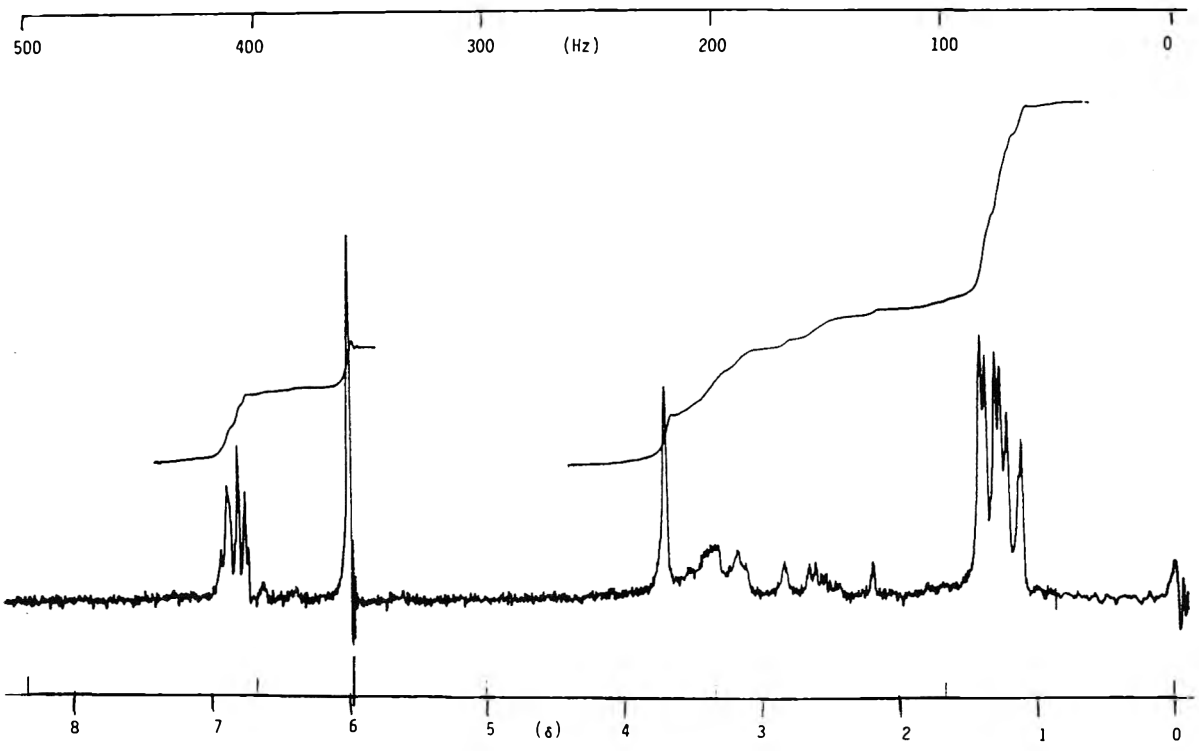


Figure 2C. Proton NMR spectrum for *N*-alkyl 3,4-methylenedioxyamphetamine compound 3. See Table 1 for structure of compound.

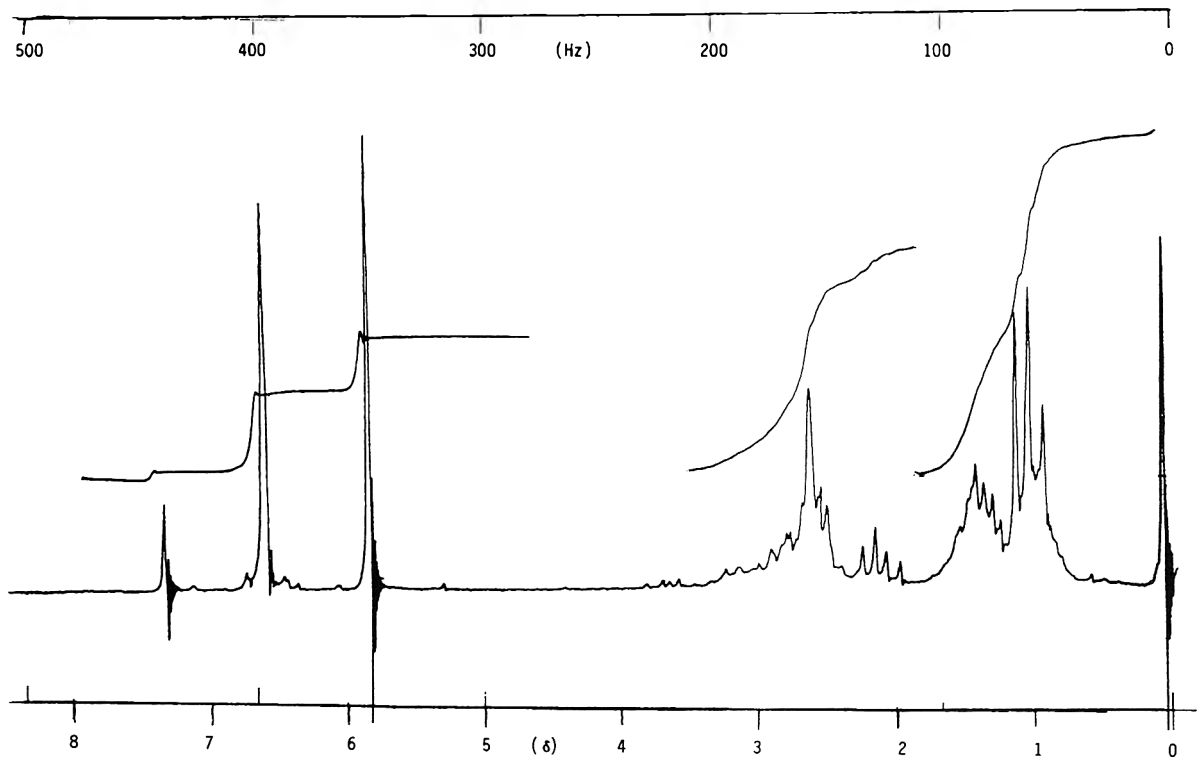


Figure 2D. Proton NMR spectrum for *N*-alkyl 3,4-methylenedioxyamphetamine compound 4. See Table 1 for structure of compound.

for the *N*-*n*-propyl-MDA analog. All the *N*-alkyl derivatives of MDA showed UV spectral properties similar to those of MDA. The major absorption bands occurred at 285 and 234 nm with the absorptivity slightly higher at the lower wavelength. The observed molar absorptivities were 3.3–3.7 × 10^3 L/mole-cm at 285 nm and 3.6–3.9 × 10^3 L/mole-cm at 234 nm. These absorptivities are considerably higher than the values for the amphetamines, which fall in the range of 2.0 × 10^2 L/mole-cm.

The liquid chromatographic separation of these MDA derivatives was accomplished using reverse phase techniques which consisted of a C_{18} stationary phase and a ternary mo-

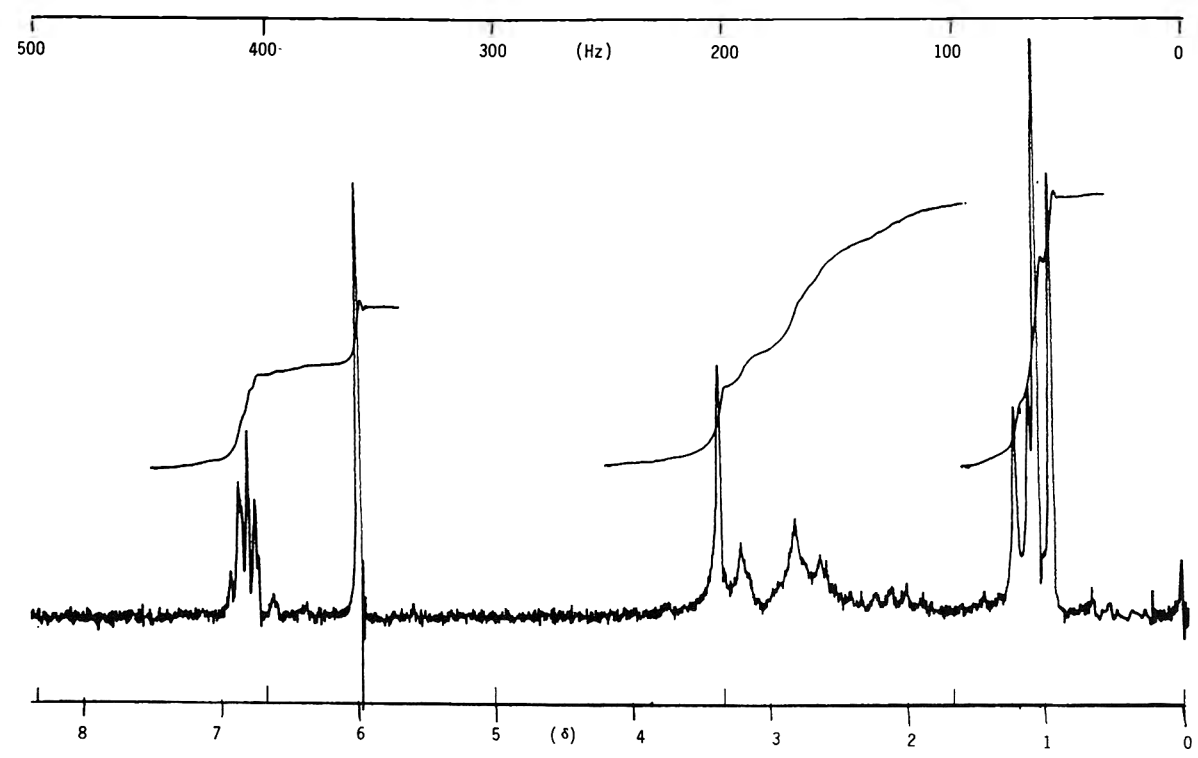


Figure 2E. Proton NMR spectrum for *N*-alkyl 3,4-methylenedioxyamphetamine compound 5. See Table 1 for structure of compound.

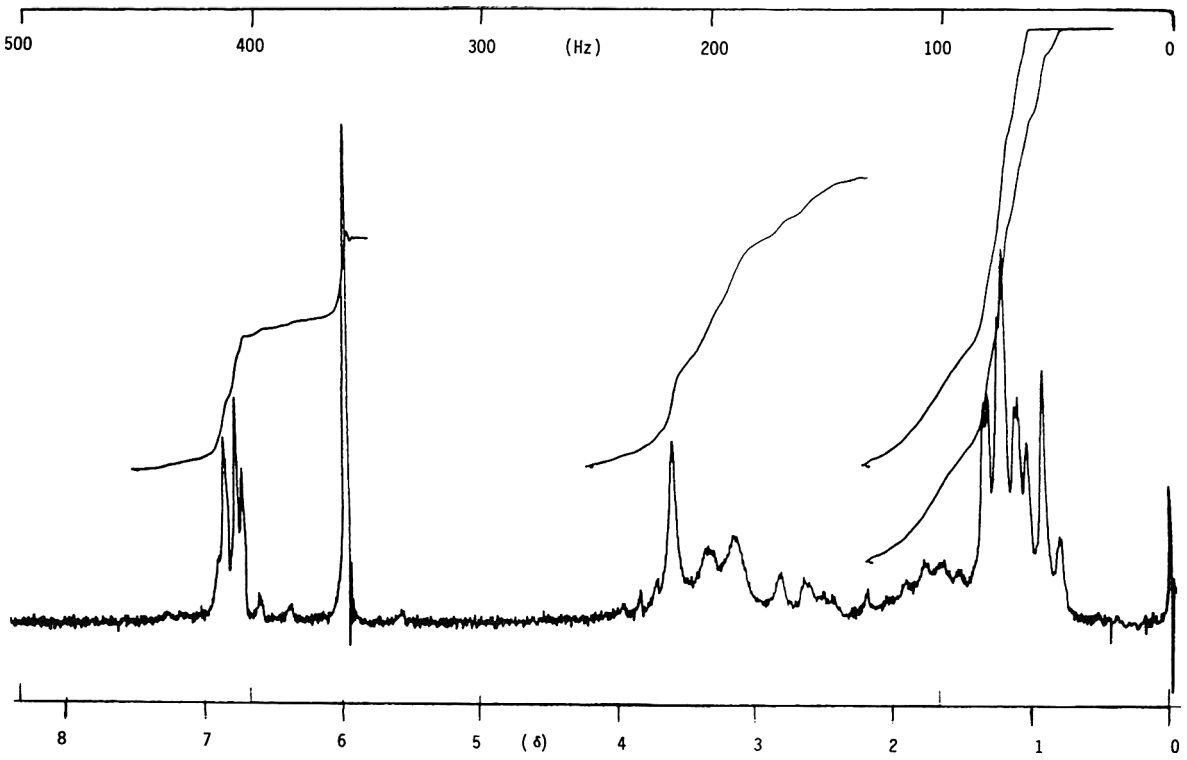


Figure 2F. Proton NMR spectrum for *N*-alkyl 3,4-methylenedioxyamphetamine compound 6. See Table 1 for structure of compound.

bile phase. The ternary mobile phase, which produced maximum resolution for these amines, consisted of pH 3 phosphate buffer, acetonitrile, and methanol containing triethylamine. The triethylamine was necessary to improve peak shape for the basic MDA compounds.

The chromatogram in Figure 4 shows the separation of all of the compounds described in Table 1 with the exception of the sec-butyl-MDA derivative. The elution order of the compounds is as expected, showing higher k' values for the more lipophilic compounds. The elution order essentially parallels the size of the alkyl group attached to nitrogen. The synthesis of the *N*-sec-butyl-MDA derivative via reductive amination of the appropriate ketone produces 2 diastereomeric products from racemic sec-butylamine. Figure 5 shows the presence of 2 diastereomeric forms of the sec-butyl-MDA. This separation was obtained using the mobile phase that produced the chromatogram in Figure 4. No attempts were made to maximize the resolution of these 2 diastereomers. These compounds, when added to the mixture separated in Figure 4, produce significant peak overlap with the isobutyl-MDA derivative. The chromatograms in Figures 4 and 5 were obtained using dual-wavelength UV detection at 254 and 280 nm. These amines are much stronger chromophores than the amphetamines and do not require chromophoric derivatization for detectability. The use of 254 and 280 nm for dual-wavelength detection produces large peak area ratios (absorbance ratios) since these wavelengths are very close to the absorbance minimum and maximum, respectively.

The acute toxicity (LD_{50}) data for the *N*-alkyl MDA derivatives were determined in male mice following intraperitoneal administration. The results of these studies are given in Table 1. These data indicate that *N*-alkylation of MDA produces a general decrease in toxicity. Hardman et al. (6) observed that *N*-methylation of both MDA and mescaline produced an increase in LD_{50} values when compared to the parent compounds. The LD_{50} for mescaline (6), 3,4,5-trimethoxyphenethylamine, is 212 mg/kg in mice for intraperitoneal administration. Thus, MDA and all its *N*-alkyl derivatives are considerably more toxic than mescaline. The increased toxicity of the MDA derivatives may be a result of their enhanced metabolic stability. Mescaline, a primary amine, is rapidly inactivated in central and peripheral tissues by oxidative deamination. The presence of the α-methyl moiety in MDA or both an α-methyl and *N*-alkyl moiety in MDMA and analogs 1–6 would retard oxidative destruction of these compounds by a steric mechanism and thereby increase their toxicity. The effect of *N*-methylation of amphetamine to yield methamphetamine (9), however, produces an increase in toxicity. The LD_{50} for amphetamine is 91 (86–96) mg/kg in mice (intraperitoneal) while the value for methamphetamine is 57 (52–62) mg/kg. The observed differences in the relative toxicities of amphetamine and methamphetamine compared to MDA and MDMA are consistent with earlier results (5) and provide additional support for the hypothesis that the amphetamines and MDA derivatives alter neurotransmission in the central nervous system by somewhat different mechanisms. The MDA derivatives

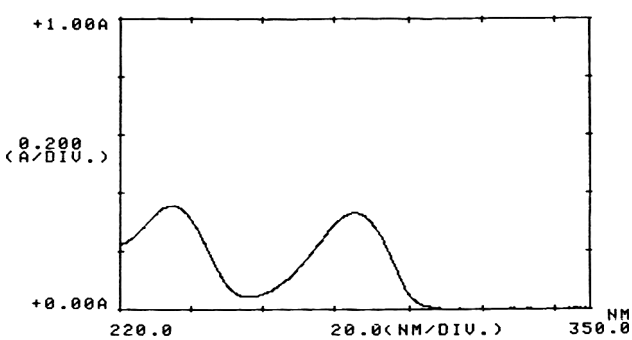


Figure 3. Ultraviolet absorption spectrum for *N*-*n*-propyl MDA (1×10^{-4}M in 0.1N H_2SO_4).

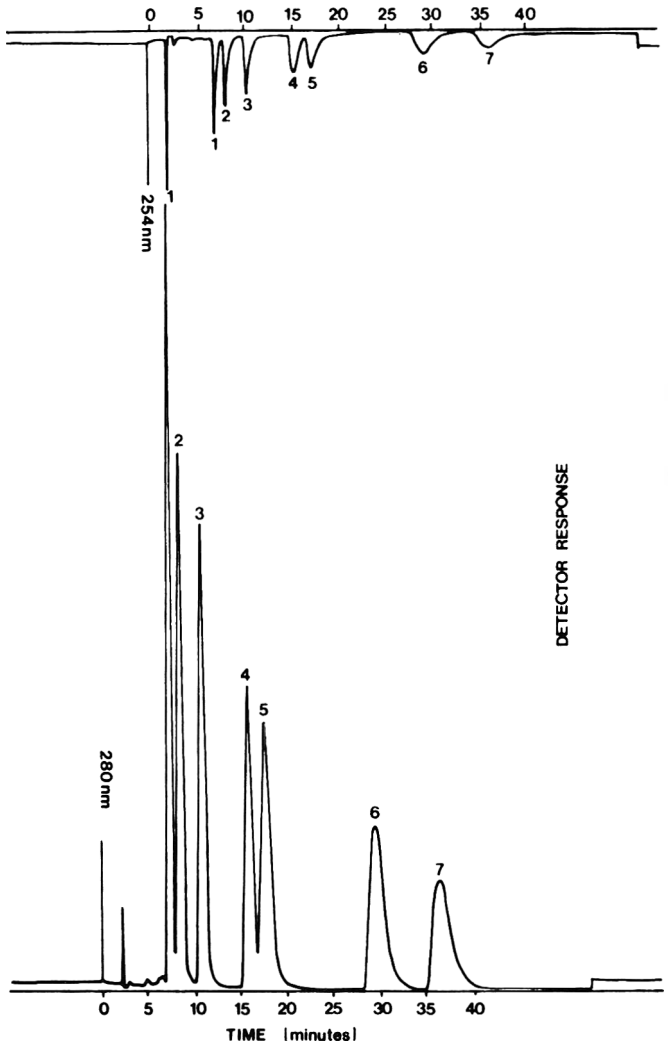


Figure 4. Reverse-phase liquid chromatographic separation of MDA and *N*-alkyl MDA derivatives at 254 and 280 nm. Peaks: 1, MDA; 2, *N*-methyl MDA; 3, *N*-ethyl MDA; 4, *N*-isopropyl MDA; 5, *N*-*n*-propyl MDA; 6, *N*-isobutyl MDA; 7, *N*-*n*-butyl MDA.

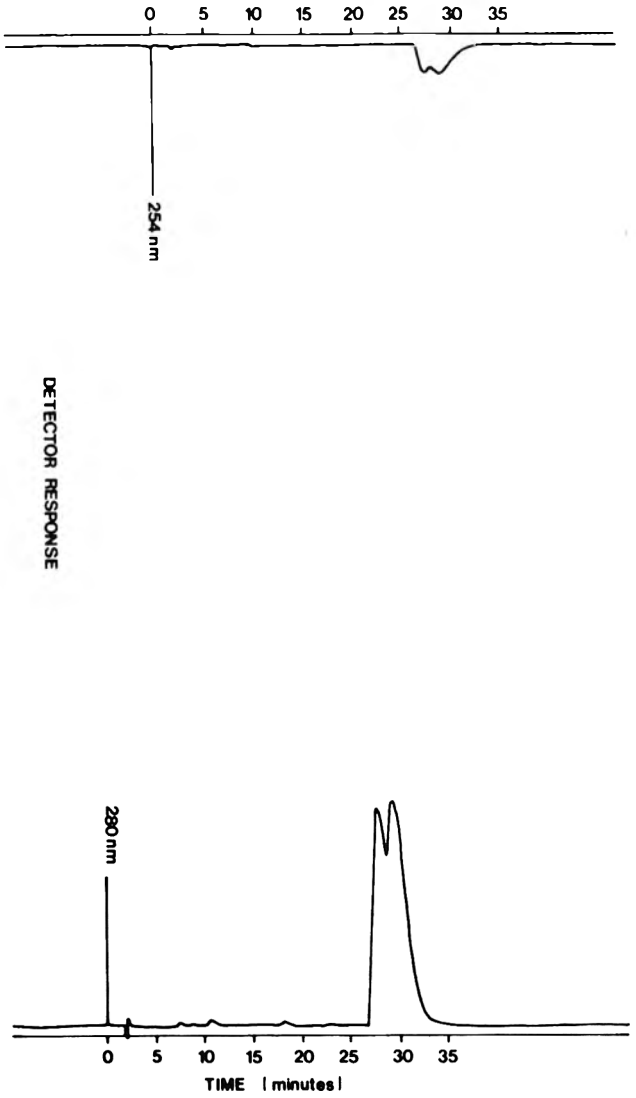


Figure 5. Reverse-phase liquid chromatographic analysis of *N*-sec-butyl MDA diastereomers.

evaluated in this study seemed to retain the peripheral sympathomimetic effects of the amphetamines such as salivation and the dopaminergic stereotyped effects. All compounds produced increased central nervous system activity, with death by clonic and tonic convulsions.

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Liquid Chromatographic Determination of Levodopa and Levodopa-Carbidopa in Solid Dosage Forms: Collaborative Study

SUSAN TING
Food and Drug Administration, New York Regional Laboratory, 850 Third Avenue, Brooklyn, NY 11232

Collaborators: L. Cohn; R. E. Draper; C. Easter; J. C. Illuminati; M. E. Richelieu; L. A. Rihbany; D. Shostak; R. H. Albert (*Statistical Consultant*); M. Friedberg (*Statistical Consultant*)

A liquid chromatographic method for the determination of levodopa in tablets and capsules and levodopa-carbidopa in tablets was collaboratively studied by 6 laboratories. Collaborators were supplied with duplicate powdered composites of levodopa (1 synthetic formulation, 1 commercial tablet, and 1 commercial capsule) and levodopa-carbidopa (1 synthetic formulation and 2 commercial tablets), along with individual levodopa-carbidopa tablets for content uniformity determinations. The repeatability coefficient of variation (CV_o) and reproducibility coefficient of variation (CV_x) for levodopa single component were 0.48 and 0.87%; for levodopa in combination, 0.50 and 0.90%; and for carbidopa, 0.77 and 1.20%, respectively. Overall, the recovery values for levodopa and carbidopa from synthetic formulations simulating tablets were 100.4 and 99.5%, respectively. The pooled CVD_o and CVD_x values for the individual tablet assays were 2.07 and 2.30% for levodopa, and 1.80 and 2.24% for carbidopa, respectively. The method has been adopted official first action for determination of the active ingredients in levodopa tablets and capsules and in levodopa-carbidopa tablets and for content uniformity testing in the combination dosage form.

In a previous paper (1), we reported the development of a rapid and specific liquid chromatographic (LC) method for the determination of levodopa and levodopa-carbidopa in solid dosage forms as well as of the corresponding impurities that are specified in the *U.S. Pharmacopeia* (2). In this regard, the USP methods (2) for the detection of these impurities are thin layer chromatographic and cation-exchange liquid chromatographic procedures, which are either time-consuming or insensitive. Assay results for commercial dosage forms obtained with the proposed method were in good agreement with those obtained with the compendial method (1). This paper presents the results of a collaborative study on the proposed LC method.

Collaborative Study

Blind duplicate samples of 3 powdered composites of levodopa (1 synthetic formulation, 1 commercial tablet, 1 commercial capsule) and 3 levodopa-carbidopa powdered composites (1 synthetic formulation and 2 commercial tablets) were sent to each of 6 participating laboratories along with 10 tablets of levodopa-carbidopa for content uniformity testing, a copy of the method, report forms for each set of samples, and vials containing standards. Collaborators were asked to perform one analysis per sample, and to forward their results along with the worksheets and chromatograms to the Associate Referee.

Submitted for publication May 29, 1987.
This report of the Associate Referee was presented at the 99th AOAC Annual International Meeting, Oct. 27–31, 1985, at Washington, DC.
The recommendation of the Associate Referee was approved interim official first action by the General Referee, the Committee on Drugs and Related Topics, and the Chairman of the Official Methods Board. The method was adopted official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) **71**, January/February issue.

Levodopa and Levodopa-Carbidopa in Solid Dosage Forms

Liquid Chromatographic Method

First Action

Principle

Levodopa in tablets or capsules and levodopa-carbidopa in tablets are detd by reverse phase liq. chromatgy on C_{18} column with 3% HOAc as mobile phase, and UV detection at 280 nm. Methyldopa is internal std for levodopa tablets or capsules; acetaminophen is internal std for levodopa-carbidopa tablets.

Apparatus

(a) *Liquid chromatograph.*—Isocratic system equipped with detector capable of monitoring *A* at 280 nm, suitable strip chart recorder, and injection valve with 20 μL sample loop.

(b) *Chromatographic column.*—300 × 3.9 mm id, μBondapak C_{18}, 10 μm particle size (Waters Associates, Inc), or equiv. column that meets suitability requirements.

(c) *Membrane filters.*—0.45 μm porosity (Millipore, or equiv.).

Reagents

(a) *Mobile phase.*—3% aq. HOAc.

(b) *Methyldopa internal std soln.*—2 mg/mL. Accurately weigh ca 200 mg USP Methyldopa Ref. Std into 100 mL vol. flask, add 50 mL 0.1N HCl, and sonicate to dissolve std. Dil. to vol. with mobile phase, and mix.

(c) *Acetaminophen internal std soln.*—0.5 mg/mL. Accurately weigh ca 125 mg USP Acetaminophen Ref. Std into 250 mL vol. flask, add 75 mL MeOH, and sonicate to dissolve std. Dil. to vol. with mobile phase, and mix.

(d) *Levodopa std soln.*—Just prior to use, dry USP Levodopa Ref. Std 4 h at 105°. Store in tightly covered, light-resistant container. Accurately weigh ca 100 mg dried std into 50 mL vol. flask. Add 30 mL 0.1N HCl, and sonicate to dissolve. Dil. to vol. with 0.1N HCl, and mix. Filter soln thru 0.45 μm membrane filter, discarding first 5 mL filtrate. Pipet 5 mL filtrate and 10 mL methyldopa internal std soln into 100 mL vol. flask, dil. to vol. with mobile phase, and mix.

(e) *Levodopa-carbidopa std soln.*—Dry USP Carbidopa Ref. Std to const wt at 100° under reduced pressure not exceeding 5 mm Hg. Store in tightly covered, light-resistant container. Accurately weigh ca 100 mg dried USP Levodopa Ref. Std **(d)** into 50 mL vol. flask. Add accurately weighed amt of dried carbidopa std so that carbidopa-to-levodopa ratio corresponds to that found in com. levodopa-carbidopa tablet. Add 30 mL 0.1N HCl, sonicate to dissolve, dil. to vol. with 0.1N HCl, and mix. Filter soln thru 0.45 μm membrane filter, discarding first 5 mL filtrate. Pipet 10 mL filtrate into 100 mL vol. flask, and add vol. of acetaminophen internal std soln so that acetaminophen concn is 1.25 times carbidopa concn. Dil. to vol. with mobile phase, and mix.

Sample Preparation

(a) *Levodopa tablets or capsules.*—Weigh and finely powder ≥20 tablets or composite contents of 20 capsules. Weigh portion of powder equiv. to ca 100 mg levodopa into 50 mL vol. flask, and proceed as directed under levodopa std soln **(d)**, beginning "Add 30 mL 0.1N HCl . . ."

(b) *Levodopa-carbidopa tablets.*—Weigh and finely powder ≥20 tablets. Weigh portion of powder equiv. to ca 100 mg levodopa into

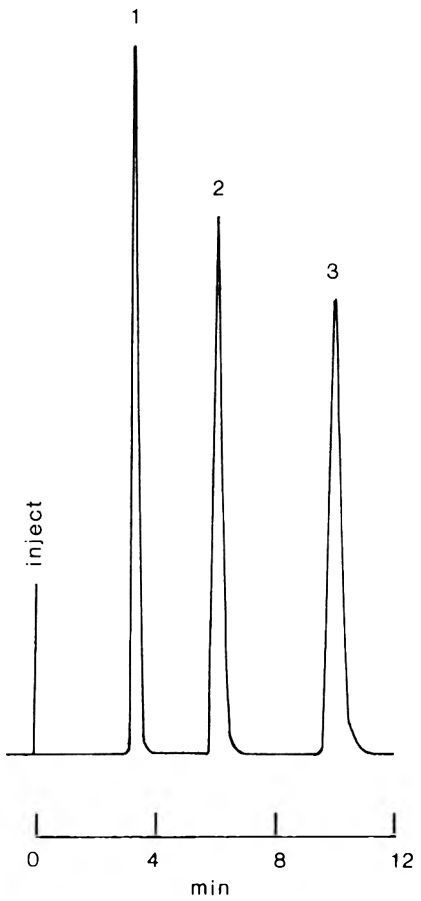

Figure 1. Chromatogram of (1) levodopa (0.64 AUFS); (2) carbidopa (0.08 AUFS); (3) acetaminophen (0.08 AUFS), internal standard.

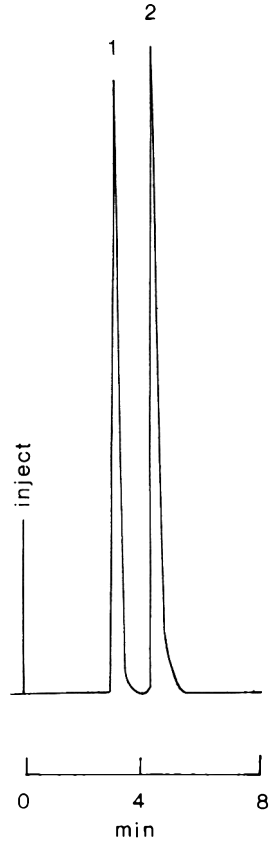

Figure 2. Chromatogram of (1) levodopa; (2) methyldopa, internal standard.

50 mL vol. flask, and proceed as directed under levodopa-carbidopa std soln (e), beginning "Add 30 mL 0.1N HCl . . ."

(c) *Levodopa-carbidopa tablets for content uniformity determination.*—Dissolve 1 tablet in sufficient 0.1N HCl to prep. soln contg 2 mg levodopa/mL. Filter soln thru 0.45 μm membrane filter, discarding first 5 mL filtrate. Pipet 10.0 mL filtrate into 100 mL vol. flask, add acetaminophen internal std soln (15 mL for levodopa-carbidopa 100/25 tablets, 5 mL for all other dosage levels), dil. to vol. with 0.1N HCl, and mix.

Table 1. Collaborative results for levodopa in commercial and synthetic solid dosage forms[a]

Coll.	Found, % of declared: 250 mg tablet[b]	Found, % of declared: 500 mg capsule[c]	Synthetic formulation,[d] rec., %
1	100.8	97.5	101.3
	101.0	96.2	101.4
2	100.3	97.5	99.6
	100.5	97.5	101.4
3	99.2	95.9	100.9
	99.2	96.0	101.4
4	99.3	95.6	99.9
	98.9	95.1	100.1
5	99.8	97.3	97.7
	99.9	97.0	100.5
6	100.6	97.6	101.0
	101.9	97.2	101.1
Av.	100.2	96.7	100.7
SD	0.92	0.88	0.70
CV, %	0.92	0.91	0.70
Pooled CVD_o, 0.48%			
Pooled CVD_x, 0.87%			

[a] Samples furnished as blind duplicates.
[b] Dosage level 250 mg/tablet; average tablet weight 349.7 mg.
[c] Dosage level 500 mg/capsule; average capsule weight 566.0 mg.
[d] Prepared to simulate tablets containing 100 mg levodopa.

Table 2. Collaborative results for levodopa (LD) and carbidopa (CD) combined in commercial tablets and synthetic formulation[a]

	Found, % of declared: Tablet 1[b]		Found, % of declared: Tablet 2[c]		Synthetic formulation,[d] rec., %	
Coll.	LD	CD	LD	CD	LD	CD
1	104.1	105.7	101.3	101.0	100.9	95.9
	104.1	105.0	101.8	102.0	101.0	97.7
2	102.5	105.2	100.2	102.2	98.9	100.7
	102.5	102.7	100.6	101.4	99.1	99.8
3	104.0	103.1	102.2	102.8	101.1	99.1
	103.8	103.2	102.1	101.3	101.4	100.0
4	102.5	103.7	101.0	102.1	100.0	99.9
	101.7	103.4	100.9	102.3	98.9	100.6
5	103.1	103.6	101.0	101.6	99.3	100.4
	101.6	102.3	100.7	101.1	101.1	99.2
6	102.4	104.9	101.3	102.1	99.1	101.1
	101.7	103.5	101.7	102.4	99.7	100.2
Av.	102.8	103.9	101.2	101.9	100.0	99.5
SD	0.96	1.08	0.62	0.57	0.99	1.46
CV, %	0.94	1.04	0.61	0.56	0.99	1.46
Levodopa: pooled CVD_o, 0.50%; pooled CVD_x, 0.90%						
Carbidopa: pooled CVD_o, 0.77%; pooled CVD_x, 1.20%						

[a] Samples furnished as blind duplicates.
[b] Dosage level 100 mg LD and 10 mg CD; average tablet weight 225.8 mg.
[c] Dosage level 100 mg LD and 25 mg CD; average tablet weight 232.4 mg.
[d] Prepared to simulate tablets containing 100 mg LD and 10 mg CD.

Table 3. Collaborative results (% of declared found) for content uniformity assays of levodopa (LD, 100 mg)–carbidopa (CD, 25 mg) individual tablets

	Coll. 1		Coll. 2		Coll. 3		Coll. 4		Coll. 5		Coll. 6	
Tablet	LD	CD	LD	CD	LD	CD	LD	CD	LD	CD	LD	CD
1	103.3	104.6	101.1	101.5	102.6	102.0	101.3	103.8	105.8	103.3	103.3	100.1
2	100.5	100.1	101.7	102.9	99.8	99.2	100.3	103.9	99.8	99.7	99.7	101.0
3	100.5	102.7	99.3	100.6	101.4	102.0	98.5	101.5	97.8	98.1	103.0	103.8
4	104.7	106.0	99.8	100.9	104.9	104.4	101.7	105.3	101.5	100.7	102.4	103.5
5	103.7	103.2	98.8	101.1	100.4	99.6	100.5	103.8	102.3	101.5	101.2	103.3
6	102.4	101.2	98.5	100.0	101.8	102.0	100.6	107.0	100.2	101.1	95.3	98.6
7	104.5	105.8	101.8	102.4	104.7	104.0	101.5	106.0	104.1	103.1	103.5	102.8
8	105.7	104.2	102.3	100.1	104.0	103.6	98.0	102.3	101.0	99.8	105.9	103.4
9	107.4	109.0	102.3	100.1	102.1	102.4	99.4	104.5	104.1	102.7	102.7	102.3
10	103.7	103.6	102.0	102.4	102.3	102.4	101.7	106.3	103.3	100.4	99.3	100.5
Av.	103.6	104.0	100.8	101.2	102.4	102.2	100.4	104.4	102.0	101.0	101.3	101.9

LD, CVD_o, 2.07%
LD, CVD_x, 2.30%
CD, CVD_o, 1.80%
CD, CVD_x, 2.24%

System Suitability Test and Assay

Equilibrate LC system with mobile phase at 1.5 mL/min. Inject 20 μL std soln. Approx. retention times are levodopa, 3 min; methyldopa, 4.5 min; carbidopa, 5 min; and acetaminophen, 9 min. Calc. resolution factor, R, as follows:

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

where t_2 and t_1 = retention times of the 2 components, and W_2 and W_1 = corresponding widths of bases of peaks obtained by extrapolating relatively straight sides of peaks to baseline. R between levodopa and carbidopa and between carbidopa and acetaminophen should be >3.5. R between levodopa and methyldopa should be >2.

Change flow rate to improve resolution. For levodopa-carbidopa tablets, change detector sensitivity between levodopa peak (approx. 0.64 AUFS) and carbidopa peak (approx. 0.08 or 0.04 AUFS). Set detector sensitivity to 35–95% AUFS. If necessary, adjust vol. of internal std soln added to sample soln and std soln to obtain satisfactory detector response for std soln. Inject std soln 5 times and compare peak hts. Calc. CV as follows:

$$\text{CV, \%} = \frac{100}{\bar{X}}\left[\frac{\sum_{i=1}^{N}(X_i - \bar{X})^2}{N-1}\right]^{1/2}$$

where $\bar{X}$ = mean of set of n measurements, and X_i = an individual detn of ratio of peak ht of analyte to peak ht of internal std. In suitable system, CV = ≤2.0%.

Proceed with sample analysis by injecting 20 μL each of sample soln and corresponding std soln.

Calculation

Using peak ht ratios R and R' relative to internal std, calc. mg drug/tablet or capsule as follows:

$$\text{mg/tablet or capsule} = (R/R') \times C \times (D/W) \times T$$

where R and R' = peak ht ratios for sample and std solns, resp.; C = concn of std soln, mg/mL; W = wt of sample taken, mg; D = sample diln; and T = av. tablet or capsule wt, mg.

CAS-59-92-7 (levodopa)

CAS-38821-49-7 (carbidopa)

CAS-28860-95-9 (carbidopa anhydrous)

Results and Discussion

Figures 1 and 2 represent typical chromatograms from the LC analysis of levodopa and levodopa-carbidopa tablets, respectively. Tables 1 and 2 summarize the collaborative results for the assay of levodopa and levodopa-carbidopa solid dosage forms. Statistical evaluation of these data showed the overall repeatability (D_o) and reproducibility (D_x) coefficients of variation (CV) to be 0.48 and 0.87% for levodopa single component, 0.50 and 0.90% for levodopa in combination, and 0.77 and 1.20% for carbidopa in levodopa-carbidopa combination, respectively. The synthetic tablet formulation given to the collaborators was prepared by mixing the tablet excipients with a quantity of each drug that was equivalent to that found in a commercial tablet. Overall, the recovery values for levodopa and carbidopa from the synthetic formulation were 100.4 and 99.5%, respectively.

Collaborative results for the content uniformity testing of levodopa-carbidopa commercial tablets are presented in Table 3. The pooled CVD_o and CVD_x were 2.07 and 2.30% for levodopa, and 1.80 and 2.24% for carbidopa, respectively.

None of the collaborators reported problems with the proposed method. The formula for the suitability tests was included in the method following a suggestion by Collaborator 1. Collaborator 2 indicated that using a detector sensitivity setting that will provide greater sensitivity than that recommended in the proposed method may result in a noisy baseline with tablet samples that contain low levels of carbidopa (10 mg/tablet). Although the Associate Referee did not detect this problem in the chromatograms submitted by Collaborator 2, the prospective analyst is advised of the possibility of achieving satisfactory detector responses while maintaining the same experimental settings for all tablet strengths if the volumes of filtrate used for sample preparation and/or standard preparation are doubled. Under these conditions, detector responses will still be linearly related to concentrations of drug injected ranging from 0.05 to 0.40 mg/mL of levodopa, and from 0.9 to 12.8 mg/mL of carbidopa.

Collaborator 5 also questioned the necessity of manually switching detector settings when going from the levodopa peak to the carbidopa peak, and commented on the hindrance that such a step may offer to a laboratory attempting to use a totally automated LC system. This instrumental adjustment, which is required because of the large differences in concentration between 2 drugs, can be both facilitated and rendered amenable to automation by using an electronic integrator. Collaborator 5 commented that the addition of an internal standard solution to the sample preparation will increase the chances for error because the sample preparation will become diluted. The Associate Referee decided to retain

the use of an internal standard solution because its use at the recommended volume will have no significant effect on the concentration of the sample, and, more important, it will improve the precision of the method.

Recommendation

The Associate Referee recommends that the LC method for the assay of levodopa tablets and capsules and levodopa-carbidopa tablets, and for content uniformity testing of levodopa-carbidopa tablets be adopted official first action.

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Ronald E. Draper, Food and Drug Administration (FDA), San Francisco, CA

James C. Illuminati, FDA, Philadelphia, PA

Mary Ellen Richelieu, FDA, Kansas City, MO

Linda Anne Rihbany, FDA, Winchester Engineering and Analytical Center, Winchester, MA

Donald Shostak, FDA, Brooklyn, NY

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MICROBIOLOGICAL METHODS

Rapid Fluorogenic Enumeration of *Escherichia coli* in Selected, Naturally Contaminated High Moisture Foods

PAUL L. POELMA, CLYDE R. WILSON, and WALLACE H. ANDREWS
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

An assay for the enzyme glucuronidase was used to determine the presence of *Escherichia coli* in selected, naturally contaminated high moisture foods. Raw pork sausage, ground turkey, and ground beef were inoculated into tubes containing the substrate 4-methylumbelliferyl beta-D-glucuronide (MUG) in lauryl tryptose (LT) medium. After incubation at 35°C for 24 h, the inoculated LT-MUG tubes were examined under longwave ultraviolet light for the presence of a fluorogenic glucuronidase end product. A fluorescing tube indicated the presumptive presence of *E. coli*. The 10 day most probable number method of the AOAC and the LT-MUG procedure gave comparable recoveries of *E. coli*.

An assay to determine the presence of *Escherichia coli* can be used to assess the sanitary quality of food. The most probable number (MPN) technique, which is recommended by AOAC for the enumeration of *E. coli* and other coliforms (1), takes up to 4 days; confirmation of the organisms as *E. coli* requires an additional 6 days.

Enumeration of *E. coli* by fluorogenic assay for the enzyme glucuronidase offers a substantial reduction in analytical time. Kilian and Bulow (2) reported that this enzyme was produced by 97% of *E. coli* strains but by few other enteric genera. To test for glucuronidase, an appropriate concentration of 4-methylumbelliferyl beta-D-glucuronide (MUG) is added to the medium during preparation. After incubation of the inoculated tubes, a fluorescent end product of the MUG substrate is determined by using a longwave (366 nm) ultraviolet (UV) light. In a previous investigation of sunflower seeds, crabmeat, and walnuts artificially contaminated with *E. coli*, Andrews et al. (3) established that MUG should be incorporated into lauryl tryptose (LT) broth rather than EC medium for those particular foods and that 24 h was adequate for incubation of the LT–MUG broth. The objectives of this study were to establish the optimal concentration of MUG in the LT broth and to evaluate the performance of the LT–MUG medium for the examination of foods naturally contaminated with *E. coli*.

Experimental

Food Products

Raw pork sausage, ground turkey, and ground beef were purchased at grocery stores in the Washington, DC, area and stored 2–8°C for a maximum of 5 days before analysis.

Microbiological Analysis

The protocol used to compare the relative efficiency of LT–MUG broth with that of the AOAC method is shown in Figure 1. For each experiment, 3 replicate 50 g portions were withdrawn from each food and blended in a Waring commercial blender for 2 min at 14 000 rpm. Serial 10-fold dilutions were made in Butterfield's phosphate buffer and inoculated into LT and LT–MUG broths. The lauryl sulfate broth was prepared from the dehydrated product obtained from Difco Laboratories, Detroit, MI. Both the AOAC and the LT–MUG methods were then evaluated.

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For the AOAC method, all LT broth tubes producing gas were subcultured at 35°C to EC medium after 24 and 48 h incubation. Inoculated EC tubes were incubated in a water bath at 45.5°C; at 24 and 48 h intervals, tubes producing gas were streaked to Levine's eosin methylene blue (EMB) agar. Colonies typical for *E. coli* were confirmed by indole, methyl red, Voges-Proskauer, and citrate utilization (IMViC) reactions, lysine decarboxylase, hydrogen sulfide production, malonate, oxidase, urease, cellobiose, gram stain, and ability to produce gas upon reinoculation of the pure culture into LT broth.

For the LT–MUG method, serial dilutions of the foods were inoculated into LT–MUG broth (containing either 50

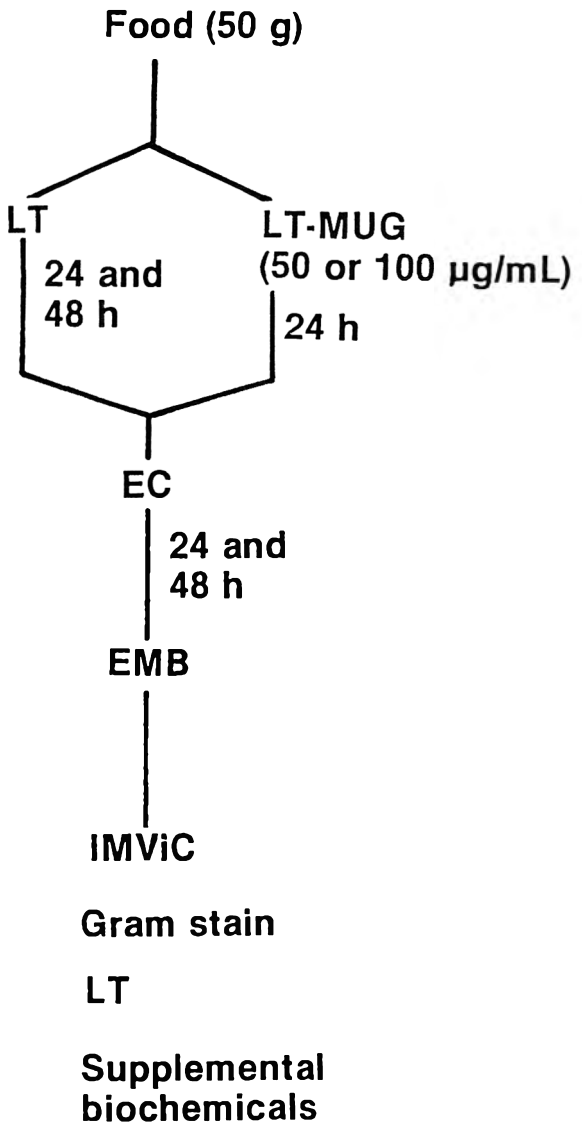

Figure 1. Protocol for comparing AOAC and fluorogenic MPN methods for *E. coli* in selected, naturally contaminated high moisture foods.

Table 1. Comparison of AOAC and fluorogenic MPN methods for the enumeration of *E. coli* in selected, naturally contaminated high moisture foods

	Mean $\log_{10}$ *E. coli* MPN values/g[a]		
		LT–MUG, μg MUG/mL	
Trial	AOAC	100	50
Pork sausage			
1	1.099	1.238	1.452
2	0.862	0.564	1.100
Ground turkey			
1	0.255	0.543	0.543
2	1.038	0.967	0.774
Ground beef			
1	1.745	1.655	1.655
2	1.593	1.231	1.300

[a] MPN values were determined on 3 replicates for each method.

Table 2. Correlation of fluorescence and the presence of *E. coli* in selected, naturally contaminated high moisture foods

MUG concentration, μg/mL	Reactions of media containing MUG			No. of tubes containing *E. coli*	
	Growth	Gas	Fluorescence	Rec. (+)	Not rec. (−)
Pork sausage					
100	+	+	+	29	3[a]
	+	+	−	0	23
	+	−	+	0	2[a]
	+	−	−	0	24
	−	−	+	0	0
	−	−	−	0	36
50	+	+	+	34	1[a]
	+	+	−	1[b]	19
	+	−	+	0	1[a]
	+	−	−	0	21
	−	−	+	0	0
	−	−	−	0	38
Ground turkey					
100	+	−	+	26	9[a]
	+	−	−	0	41
	+	−	+	0	0
	+	−	−	0	13
	−	−	+	0	0
	−	−	−	0	19
50	+	−	+	26	10[a]
	+	−	−	0	37
	+	−	+	0	0
	+	−	−	0	19
	−	−	+	0	0
	−	−	−	0	16
Ground beef					
100	+	+	+	38	4[a]
	+	+	−	0	17
	+	−	+	0	0
	+	−	−	0	28
	−	−	+	0	0
	−	−	−	0	21
50	+	+	+	38	2[a]
	+	+	−	1[b]	18
	+	−	+	0	1[a]
	+	−	−	0	26
	−	−	+	0	0
	−	−	−	0	21

[a] False-positive reaction (*E. coli* not recovered from fluorescent-positive tubes).

[b] False-negative reaction (*E. coli* recovered from fluorescent-negative tubes).

Table 3. Summary of discrepant reactions for *E. coli* obtained with fluorogenic MPN methods

	LT–MUG concentration, μg/mL			
	100		50	
Food	False-positive	False-negative	False-positive	False-negative
Pork sausage	5	0	2	1
Ground turkey	9	0	10	0
Ground beef	4	0	3	1

or 100 μg MUG/mL) and incubated for 24 h at 35°C. Tubes were examined for fluorescence at a wavelength of 366 nm. All LT–MUG tubes were subcultured to EC medium, incubated in a water bath at 45.5°C, and streaked to Levine's EMB agar at 24 and 48 h intervals. Suspect *E. coli* colonies were confirmed as described for the AOAC method. In preparing the LT–MUG broth, appropriate amounts of MUG were dissolved in warm water, and compensation was made for the amount of water used to dissolve the MUG. After preparation, the LT and the LT–MUG broth media were dispensed into tubes containing inverted fermentation vials and were sterilized by autoclaving.

Caution: For safety, analysts should wear glasses with UV-treated lenses when observing fluorescence in LT–MUG broth.

Statistical Analysis

MPN values were converted to the log base 10, and the mean log value was calculated for the replicates analyzed by each method. For each food, an analysis of variance was made of the log-transformed data values. Duncan's multiple comparison procedure (4) was used to assess differences for each pair between the method mean log values at $\alpha = 0.05$.

Results and Discussion

Several investigators (5–7) have been successful in using MUG in LT broth for the rapid enumeration of *E. coli*. Koburger and Miller (8), however, reported that incorporation of MUG into LT broth for determining the presence of *E. coli* in oysters was not successful because of the interference of an endogenous glucuronidase in the oysters themselves. Their assay, therefore, was modified by incorporating the MUG into EC broth rather than LT broth, even though the LT broth is preferable for completing the analysis in 24 h.

A comparison was made of 2 concentrations of MUG (50 and 100 μg/mL) in LT broth to determine which concentration gave the more decisive reactions. The LT–MUG medium containing the 2 levels of MUG was then compared with the AOAC method for the enumeration of *E. coli* in naturally contaminated foods (Table 1). For both trials with each food type, the mean log MPN values obtained with either concentration of LT–MUG did not differ significantly from those obtained with the AOAC method. Growth, gas production, and fluorescence in LT–MUG media with recovery (+) and without recovery (−) of *E. coli* from naturally contaminated foods are shown in Table 2. The different combinations of positive and negative reactions in the LT–MUG media were correlated with the number of tubes demonstrating the presence (recovered) or absence (not recovered) of *E. coli*. A false-positive fluorogenic method reaction was one in which the LT–MUG medium fluoresced but *E. coli* was not present or was not recovered by the confirmatory procedure. A false-negative fluorogenic method reaction was one in which the LT–MUG medium did not fluoresce but *E. coli* was recovered.

For pork sausage, the LT–MUG test (100 μg MUG/mL) reactions for growth, gas production, and fluorescence, respectively, were +, +, +, but *E. coli* was not recovered (false-positive reactions) from 3 tubes. This method also gave 2 false-positive reactions for the +, −, + combination. At 50 μg MUG/mL, the MUG test gave a single false-positive reaction for the +, +, + and the +, −, + combinations, and a false-negative reaction with the +, +, − combination.

Ground turkey had the highest number of discrepant reactions (+,+, +), giving 9 false-positive reactions at 100 μg MUG/mL and 10 at 50 μg MUG/mL. For this product, no false-negative reactions were obtained with either concentration of MUG in LT broth.

With ground beef, the LT–MUG test (100 μg MUG/mL) gave false-positive reactions with the +, +, + combination (4 tubes), but no false-negative reactions. The analysis of this product with 50 μg MUG/mL gave false-positive reactions with the +, +, + combination (2 tubes) and the +, −, + combination (1 tube). The only other discrepant reaction at this MUG concentration was a single false-negative reaction occurring with the +, +, − combination. A summary is given of total discrepant reactions obtained with 2 levels of LT–MUG (Table 3). The discrepant reaction values were combined for trials 1 and 2. These data demonstrate no significant differences in false-positive and false-negative reactions when MUG was used at either of 2 concentrations in LT broth. Because of the high cost of the MUG substrate, a concentration of 50 μg MUG/mL is recommended for analysis of the 3 specific food types used in this study.

Several problems are associated with the MUG test. One of these is autofluorescence of the glass test tube containing the MUG broth medium (3). To eliminate this potential problem, we screened all glass test tubes used in this study. Autofluorescence can also be caused by the presence of glucuronidase, which is endogenous in certain foods, such as oysters and other mollusks; however, this enzyme did not cause a problem in our study because fluorescence was observed in only 2 tubes from which *E. coli* was not recovered. Another problem is that certain enteropathogenic strains of *E. coli* do not possess the enzyme glucuronidase and thus give a negative MUG reaction (Michael Brodsky, Ministry of Health, Toronto, Ontario, Canada, and Khalil Rayman, Health and Welfare Canada, Ottawa, Ontario, Canada, 1986, personal communication). Finally, because reading of the fluorogenic reaction is often subjective, appropriate positive and negative culture controls must be included for each experiment or sample analysis.

A major advantage of the LT–MUG test is the reduction of analytical time. The LT–MUG procedure also offers a significant reduction in media and labor costs. Thus, the fluorogenic LT–MUG test is a reliable alternative to the current AOAC method for the enumeration of *E. coli* in selected, naturally contaminated high moisture foods.

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Enumeration of *Clostridium perfringens* Spores in Human Feces: Comparison of Four Culture Media

STANLEY M. HARMON and DONALD A. KAUTTER
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Enumeration of *Clostridium perfringens* spores was compared using 4 culture media. Duplicate 1 g portions of 35 stools (25 from *C. perfringens* food poisoning outbreaks and 10 from normal stools) were heat treated 20 min at 75°C and tested on tryptose-sulfite-cycloserine (TSC) agar, trypticase-soy-blood (TSB) agar, lactose-sulfite (LS) medium, and iron milk (IM) medium. Dilutions were plated directly onto TSB and TSC, and a 3-tube most probable number determination was made with each specimen in LS and IM incubated at 45°C. TSB was easiest to use and nonhemolytic food poisoning strains were readily differentiated from the normal hemolytic biotype on this medium. Confirmed counts on TSC and TSB were similar for all specimens, but counts of 8 of 25 outbreak specimens were 2–4 log units lower in LS and IM than on plating media; spores in specimens associated with 2 of 5 outbreaks were intolerant of the elevated temperatures. Results showed that elevated temperature MPN methods in LS and IM are inappropriate for the examination of outbreak stools.

A recent study (1) has confirmed the value of enumerating *Clostridium perfringens* spores in feces of food poisoning patients as a means of confirming outbreaks. Although they are effective, traditional plating methods are laborious and require specialized anaerobic apparatus; therefore, a study was made to determine the efficacy of 2 elevated-temperature, most probable number (MPN) methods described in a series of papers published in both the United States and in France (2–7) for enumeration of *C. perfringens*. Results of the iron milk (IM) medium method of St. John et al. (7) and the lactose-sulfite (LS) method of Beerens et al. (4), both elevated-temperature, MPN methods, were compared with traditional plating methods using tryptose-sulfite-cycloserine (TSC) and trypticase-soy-blood (TSB) agars. The outbreak specimens examined were stools obtained from persons affected in 1 of 5 different food poisoning episodes; control specimens were from normal, healthy persons.

To minimize sampling error and to determine the replicability of results, duplicate determinations were made with each specimen. Enumerations were made by each method, and the advantages and disadvantages of each method were evaluated, particularly the ability to differentiate food poisoning strains from normal intestinal flora.

Experimental

Fecal Samples

Outbreak stools were collected by personnel from various state health departments and shipped frozen to the microbiology laboratory, Food and Drug Administration (FDA), where they were stored frozen until examined. Normal stools were handled and examined in the same manner as outbreak stools.

Culture Media

IM medium was prepared as described by St. John et al. (7) and used on the day of preparation. LS medium was prepared from the ingredients specified by Beerens et al. (4, 5). LS base was routinely stored at 4°C for as long as 7 days before use. Filter-sterilized components were added to the deaerated medium just before use. The plating media and procedures used were those previously described (8). TSB agar plates were purchased from Baltimore Biological Laboratories, Cockeysville, MD 21030.

Test Procedures

Stool specimens weighing 1 g were suspended in 9 mL sterile 0.1% peptone and heated 20 min at 75°C to kill vegetative cells and to encourage germination of *C. perfringens* spores. Suspensions were cooled in cold tap water, diluted in 0.1% peptone, and tested in the 4 different culture media. Triplicate tests were made of 1 mL volumes in IM and LS broths; 1 mL volumes were also pour-plated in TSC agar. A 0.1 mL volume of the 10-fold lower corresponding dilution was plated in duplicate on TSB agar, using a sterile glass rod spreader. The inoculated plates were incubated for 20–24 h at 37°C, and presumptive *C. perfringens* colonies were counted. The presence or absence of complete hemolysis on TSB agar was noted. (TSB plates were sometimes stored at 5°C for several hours to allow development of the partial hemolysis zones characteristic of *C. perfringens*.) Each 3-tube series in IM and LS media was incubated in a water bath at 45 ± 0.5°C. The IM tubes were examined at 18 and 24 h for evidence of stormy fermentation, which is characterized by coagulation and fracturing of the curd into a spongy mass. LS broth was checked after 24 h of incubation for the formation of a black precipitate (sulfite reduction) and fermentation of lactose, as indicated by the presence of gas trapped in a Durham vial.

Presumptive positive tubes were streaked on TSB agar plates, and colonies were selected for confirmation tests as described in the official AOAC method (8). Plate counts were performed for plates containing between 15 and 150 colonies. The corresponding counts in IM and LS were calculated by using the MPN tables in the FDA *Bacteriological Analytical Manual* (9). Enterotoxigenicity of isolates from outbreak stools was determined by culturing modified AEA medium and then testing culture supernates for *C. perfringens* enterotoxin by the ELISA method as previously described (1).

Results and Discussion

Spore counts obtained with outbreak stools are given in Table 1; counts with normal stools are given in Table 2. These data show that TSC and TSB agars were similarly effective for enumerating *C. perfringens* spores in stools of food poisoning patients and in normal stools as reported by Harmon et al. (1), but, in some instances, the spores were grossly underestimated in outbreak stools on IM and LS media, as shown in results for specimen 2 of outbreak No. 3, all specimens of outbreak No. 4, and specimens 2 and 5 of outbreak No. 5 (Table 1). Spore counts of specimens enumerated by the other 3 methods were compared to confirmed counts with TSC agar, which is currently the most widely accepted method (10). Counts for these 8 specimens in IM and LS media were 2–4 log units lower than the corresponding plate counts on TSC, and enterotoxigenic colonies usually could not be isolated from the positive culture tubes. Five of the 8 specimens that gave false negative results or lower counts in IM and LS media were associated with the same

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Table 1. *Clostridium perfringens* spores in stools of food poisoning patients counted on 4 culture media[a]

Outbreak No.	Stool No.	Spores (log_{10})/g of stools[b] TSC[c] trial 1	TSC[c] trial 2	TSB[d] trial 1	TSB[d] trial 2	IM[e] trial 1	IM[e] trial 2	LS[f] trial 1	LS[f] trial 2
	1	7.75	7.69	8.04	7.2[-]	7.66	8.04	7.97	7.38
	2	8.28	8.43	7.96	7.8[-]	8.38	7.38	7.66	7.97
	3	8.15	8.20	7.81	7.06	8.38	7.60	7.36	7.30
	4	6.85	6.98	6.49	6.76	6.66	7.38	6.36	7.04
1	5	8.98	—[g]	8.06	8.18	8.38	9.38	7.97	7.63
	1	7.36	7.50	7.54	7.36	7.66	8.04	8.04	7.60
	2	8.20	8.04	8.14	7.68	8.04	9.04	7.38	7.66
	3	8.02	7.88	8.04	7.75	8.38	7.97	7.66	8.17
	4	6.75	7.36	7.21	8.28	7.38	6.87	6.38	6.66
2	5	6.97	6.48	7.38	6.34	6.63	7.04	6.63	7.38
	1	7.00	6.34	6.91	6.28	7.04	7.66	6.38	5.17
	2	6.26	7.10	6.20	6.88	3.59	4.04	4.66	3.87
	3	6.99	6.34	7.04	6.99	3.38	4.66	4.04	5.96
	4	5.99	5.28	—[g]	5.10	4.38	5.36	4.38	<3.00
3	5	5.91	5.75	4.77	5.26	6.38	5.38	4.18	6.63
	1	5.90	6.15	6.20	5.38	3.32	2.38	1.18	2.38
	2	5.48	5.60	4.99	5.63	1.38	2.63	1.38	3.04
	3	6.51	7.38	6.50	7.04	3.97	<2.00	4.04	3.66
	4	5.85	5.65	6.18	5.32	3.17	4.38	3.66	3.04
4	5	7.54	7.00	6.68	7.03	3.63	3.18	4.36	3.18
	1	5.79	5.79	6.00	6.07	5.32	6.38	5.66	5.32
	2	5.72	—[g]	5.75	5.54	3.38	3.32	3.38	3.66
	3	3.88	4.60	3.99	4.04	3.66	3.38	<2.00	2.38
	4	4.95	4.20	4.78	4.97	5.38	5.04	5.66	4.63
5	5	4.75	5.48	4.07	—[g]	3.96	3.38	2.63	2.32

[a] Five specimens from each of 5 outbreaks.
[b] As determined in 2 separate trials.
[c] TSC agar (8) without egg yolk (pour plated).
[d] Trypticase-soy-sheep blood agar (surface plated).
[e] Iron milk medium (7).
[f] Lactose sulfite medium (4).
[g] No data available.

Table 2. *Clostridium perfringens* spores in stools of healthy adults counted on 4 culture media

Stool No.	Spores (log_{10})/g of stools[a] TSC[b] trial 1	TSC[b] trial 2	TSB[c] trial 1	TSB[c] trial 2	IM[d] trial 1	IM[d] trial 2	LS[e] trial 1	LS[e] trial 2
1	3.62	2.85	3.40	3.50	3.66	3.97	2.97	3.78
2	3.55	3.32	3.48	2.70	3.38	3.97	3.38	3.87
3	5.64	6.63	5.52	5.78	5.87	7.04	5.04	6.38
4	2.47	3.32	<3.00[f]	<3.00[f]	2.63	3.17	2.97	2.66
5	2.84	3.00	<3.00[f]	<3.00[f]	2.97	3.32	2.38	2.66
6	3.32	3.44	3.00	3.52	3.66	4.38	3.38	3.66
7	3.78	2.95	3.08	<3.00[f]	3.38	2.97	3.04	3.17
8	3.30	3.78	3.20	3.65	3.66	4.38	4.32	3.66
9	3.91	3.56	3.77	3.78	4.04	2.95	3.38	3.97
10	2.65	2.70	3.00	<3.00[f]	3.97	3.38	3.17	2.87

[a] As determined in 2 separate trials.
[b] TSC agar without egg yolk (pour plated).
[c] Trypticase-soy-sheep blood agar (surface plated).
[d] Iron milk medium (7).
[e] Lactose sulfite medium (4).
[f] None detected in 0.02 g of sample.

food poisoning outbreak, and the 3 remaining specimens were from separate outbreaks. In contrast, typical growth and similar counts were obtained with all 25 outbreak specimens on TSC and TSB agar.

Results from replicate trials with liquid media varied more than those on plating media; however, this was not of practical importance because precise counts are not needed to indicate *C. perfringens* as the probable cause of food poisoning. Although IM and LS gave rapid presumptive results, we found that food poisoning strains sometimes could not be reliably enumerated in these media, apparently because of intolerance of some *C. perfringens* strains for elevated temperature. Both IM and LS media were incapable of indicating the presence of enterotoxigenic *C. perfringens* in stools of all 5 patients tested from outbreak No. 4, and a highly variable response was noted with the specimens from outbreak No. 5, which contained a heterogeneous *C. perfringens* population. Two specimens, 2 and 5, associated with outbreak No. 5 were always negative for enterotoxigenic *C. perfringens* in IM and LS media, and the remaining 3 specimens yielded lower counts (Table 1). Counts with normal stools were similar on all of the media (Table 2) except for a few specimens that had spore populations too low to be detectable in the 0.02 g portion tested on TSB.

The MPN methods gave satisfactory results with some (77%) stools examined and thus might prove useful for screening stools in laboratories that lack the equipment and trained personnel to perform the standard tests; however, they are not reliable enough for regulatory work. A presumptive result with the elevated-temperature MPN methods is more reliable for stools than for foods because apparently few sporeformers that might give false positive results are present in stools. As our results show, false negative results are much more likely. Other disadvantages of the liquid media MPN methods are the need to prepare culture media or components just before use and the need to monitor incubation over a protracted period of time. The potential for spilling contaminated culture contents in the water bath with IM medium is also high. In general, the MPN methods were found to be more laborious and less appealing to the analyst. We are reluctant to accept elevated-temperature methods for general culturing of *C. perfringens*, and our experience in this regard has received support (A. H. W. Hauschild, Bureau of Microbial Hazards, Health Protection Branch, Ottawa, Canada, personal communication, 1986).

The TSB agar method, on the other hand, was very simple and yielded the most useful information of any of the 4 methods studied. Prepared TSB plates can be purchased for a modest price and can be stored up to 30 days without a demonstrable loss of effectiveness. Nonhemolytic colonies of enterotoxigenic strains were easily recognized on TSB and differentiated from the indigenous normal hemolytic biotype when it was present, and colonies of the characteristic type were easily selected for enterotoxin testing. Even though some enterotoxigenic isolates are hemolytic as noted elsewhere (1) and 1 of the 5 strains in this study was hemolytic, the TSB agar method is preferable to the other methods currently available because it provides the most information and can be performed with a minimum of effort and expense.

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EXTRANEOUS MATERIALS

Extraction of Light Filth from Whole Leaves of Alfalfa, Lemon Balm, Papaya, and Spearmint: Collaborative Study

MARVIN J. NAKASHIMA and LARRY E. GLAZE
Food and Drug Administration, Division of Microbiology, Washington, DC 20204

Collaborators: A. Bright; C. H. Crewe; Z. Curling; M.-A. Gardiner; A. Gladstein; A. Howlett; F. J. Leahy; E. Levesque; S. F. Schnittger; M. S. Von Stetina

Results are reported for a collaborative study to extend AOAC method 44.A06–44.A08 to extraction of light filth from whole leaves of alfalfa, lemon balm, papaya, and spearmint. A 5 g (spearmint) or 10 g (alfalfa, lemon balm, papaya) test portion is defatted with isopropanol in a simple reflux apparatus. Rat hairs, insect fragments, and whole insects are isolated by wet sieving on a No. 230 sieve, a deaerating boil in 40% isopropanol, and flotation with mineral oil–heptane (85 + 15) from Tween 80–Na_4EDTA (1 + 1) and 40% isopropanol in a Wildman trap flask. Each product was spiked at a different level. For rat hairs, recoveries averaged 82.2% from alfalfa, 88.9% from lemon balm, 80.6% from papaya, and 79.6% from spearmint. Recoveries of whole or equivalent insects from these products averaged 66.1, 218.8, 69.4, and 85.4%, respectively; recoveries of insect fragments from these products averaged 89.6, 94.4, 94.1, and 88.1%, respectively. The method has been adopted official first action for extraction of light filth from whole leaves of alfalfa, papaya, and spearmint. The extension of the method to lemon balm was not recommended because of interferences by intrinsic whole insects, which were the same species as the spike material.

This collaborative study was performed to extend the application of a method for the extraction of light filth from whole peppermint leaves (1, 2) to the leaves of alfalfa, lemon balm, spearmint, and papaya.

The method involves defatting a 5 g (spearmint) or 10 g (alfalfa, lemon balm, papaya) test portion with isopropanol in a simple reflux apparatus (3) for 10 min. Rat hairs, insect fragments, and whole insects are isolated by wet sieving on a No. 230 sieve, a deaerating boil in 40% isopropanol, flotation with mineral oil–heptane (85 + 15) from Tween 80–Na_4EDTA (1 + 1) and 40% isopropanol, and trappings in a Wildman trap flask.

Collaborative Study

Each product was spiked at a different level. The lemon balm spike consisted of 5 rat hairs (2.5–3.5 mm), 20 insect fragments (elytral squares of *Tribolium confusum*, ca 0.5 sq. mm), and 5 whole insects (adult *Lasioderma serricorne*, cigarette beetle). For alfalfa, the spike consisted of 10 rat hairs, 30 insect fragments, and 10 whole insects. The spearmint spike consisted of 15 rat hairs, 50 insect fragments, and 15 whole insects, and the papaya spike consisted of 20 rat hairs, 20 insect fragments, and 10 whole insects. Two preweighed test portions of each product together with 2 vials of each spike level were sent to each of 9 collaborators. The test portions were randomly numbered from 1 to 8; the spike vials were numbered to correspond to the appropriate test portions. The collaborators were instructed to report their results and analysis times and to return the extraction papers so that their results could be checked by the Associate Referee.

METHOD

See secs **44.A06–44.A08** (2): *Light Filth in Whole Peppermint Leaves, Flotation Method.*

Results and Discussion

This discussion emphasizes the Associate Referee's counts, because collaborators sometimes failed to recognize spike material or to distinguish it from intrinsic filth. Two analysts performed portions of the analysis in the same laboratory and were considered together as Collaborator 2.

Background striated hairs that were within the size range of the spike rat hairs resulted in overcounts for lemon balm in Table 1, as reflected in the mean recovery of 161.1%. The Associate Referee was able to differentiate the spike from background hairs by length and color; the actual mean recovery was 88.9%. Student's *t*-tests comparing the mean percentage recoveries for products or analyte levels (Table 1) showed significant differences ($P < 0.05$) for lemon balm (88.9%) and papaya (80.6%).

Mean recoveries of whole or equivalent insects (Table 2) were quite variable, as in the previous collaborative study of the method (1). Background *L. serricorne* occurred in lemon balm (251.2% mean recovery) and was confirmed by the Associate Referee (218.8% mean recovery). For alfalfa, papaya, and spearmint, the counts of Collaborators 8 and 9 included nonspike insects in their tallies, and Collaborator 3 counted any major body part, such as a thorax-abdomen or an abdomen, as an equivalent insect. As defined in the collaborative study instructions, an equivalent insect must have at least the head. Student's *t*-tests comparing the mean percentage recoveries for products or analyte levels (Table 2) showed significant difference ($P < 0.05$) for all comparisons with lemon balm because of the high level of intrinsic whole or equivalent insects. Mean recoveries from alfalfa (66.1%) and spearmint (85.4%) were significantly different at $P < 0.05$.

As shown in Table 3, recoveries of insect fragments were high. The results for Collaborators 8 and 9 were excluded as outliers because the counts reflected all the insect fragments in the samples rather than the added elytral squares. Collaborator 2 also attempted to count all insect fragments, but greatly undercounted them. The counts of Collaborator 3 were low because insect fragments that were not exactly square were not counted. Four counts of Collaborator 3 were judged outliers. Student's *t*-tests comparing the mean percentage recoveries in Table 3 showed significant differences

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The recommendation of the Associate Referee was approved interim official first action by the General Referee, the Committee on Microbiology, and the Chairman of the Official Methods Board. The method was adopted official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) **71**, January/February issue.

Table 1. Collaborative results for recovery of rat hairs (blind duplicates)

	Product (spike level)							
Coll.	Lemon balm (5)		Alfalfa (10)		Spearmint (15)		Papaya (20)	
1	14 (5)[a]	13 (6)	10 (9)	11 (9)	15 (12)	15 (13)	12	18 (17)
2	7 (5)	3	2 (7)	1 (5)	11	6 (9)	15 (14)	6 (16)
3	11 (4)	10 (5)	6 (9)	5 (9)	12	9 (12)	13 (14)	19 (18)
4	5	7 (3)	8	9	9 (8)	8 (9)	17 (16)	17
5	6 (4)	5 (4)	10 (9)	9	12	10 (9)	20 (16)	18 (17)
6	8 (5)	8 (5)	9 (8)	9 (8)	17 (15)	21 (17)	20 (19)	20 (17)
7	8 (3)	9 (5)	10 (7)	8	12 (11)	12 (13)	18 (17)	20 (15)
8	10 (5)	10 (3)	9 (8)	10 (8)	13 (12)	22 (14)	21 (17)	13 (16)
9	8 (5)	3 (5)	7 (9)	9	15	11	12 (15)	17
$\bar{x}$	8.1 (4.4)		7.9 (8.2)		12.8 (11.9)		16.4 (16.1)	
$\bar{x}$,[b] %	161.1 (88.9)A		78.9 (82.2)		85.2 (79.6)		82.2 (80.6)A	
SD								
Repeatability	1.6 (1.0)		0.9 (0.6)		2.9 (1.5)		3.7 (1.8)	
Reproducibility	3.1 (1.0)		2.8 (1.1)		4.3 (2.4)		3.9 (1.8)	
CV, %								
Repeatability	19.8 (22.7)		11.4 (7.3)		22.6 (12.6)		22.6 (11.2)	
Reproducibility	38.3 (22.7)		35.4 (13.4)		33.6 (20.2)		23.8 (11.2)	

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

[b] Same capital letters in this row represent Associate Referee values which are significantly different from one another by Student's t-test at $P < 0.05$.

($P < 0.05$) for lemon balm (94.4%) and spearmint (88.1%) and for papaya (94.1%) and spearmint (88.1%).

Table 4 compares the results of this collaborative study (the 4-botanicals study), the results of the previous collaborative study (1), and precollaborative experimental data. No comparisons with the previous study were made for papaya because there was no comparable spike level in the previous study. Student's t-tests comparing the mean percentage recoveries for the same spike levels from the 4-botanicals study and the previous study (Table 4) showed no significant differences for rat hairs ($P > 0.05$). For whole or equivalent insects, only the mean recoveries from lemon balm were significantly different ($P < 0.05$) because of the inflated counts reported in this study. For insect fragments, mean recoveries from lemon balm in this study were significantly different from mean recoveries from peppermint at the low spike level in the previous study.

In both series of t-test comparisons, no pattern was found that would suggest that these significant differences in recoveries were due to the product or to the spike levels used.

For the alfalfa samples, heavy plant material was found on the extraction papers returned by Collaborators 1 and 2, whereas the other collaborators returned extraction papers with noticeable but acceptable amounts of debris. Discussions with individuals in both laboratories revealed no reason for the excessive amounts of plant matter on the extraction papers from the alfalfa samples.

Stems from lemon balm samples were trapped in mineral oil; they probably floated to the surface because of incomplete deaeration. The stems could have been eliminated by discarding them after a thorough examination of the extraction papers for filth elements.

With the exception of the extraction papers returned by Collaborators 1 and 2 for the alfalfa samples, for which more than 1 filter paper should have been used, only 1 filter paper per sample would have been required if separate trappings had not been requested.

In this collaborative study, analytical time was slightly less than in the previous study: Extraction time averaged 2.4 h (range, 1.5–3.8 h) compared to 2.8 h for the previous study; extraction paper examination time averaged 0.4 h (range, 0.2–0.7 h) compared to 0.5 h for the previous study.

Table 2. Collaborative results for recovery of whole or equivalent insects (blind duplicates)

	Product (spike level)							
Coll.	Lemon balm (5)		Alfalfa (10)		Spearmint (15)		Papaya (10)	
1	14 (12)[a]	7	4	4	11 (10)	10	3[b] (2)	1[b] (1)
2	19 (12)	11 (10)	6 (5)	8 (7)	23 (13)	13 (10)	9	8 (9)
3	10 (8)	14 (12)	10 (8)	8 (6)	18 (15)	28 (16)	5	13 (8)
4	15 (14)	7	10	11	14	15 (16)	10	10
5	9 (8)	10	6	9	12	9	10	9
6	10	11	10	5	17 (16)	13	9	7
7	18	8 (7)	9	8	16	14	9	10
8	34[b] (5)[b]	36[b] (2)[b]	12 (2)	9 (2)	42 (5)[b]	21 (4)[b]	11 (2)	13 (2)
9	27 (19)	11 (10)	13 (10)	8 (3)	20 (14)	10 (7)	6 (9)	8 (4)
$\bar{x}$	12.6 (10.9)		8.3 (6.6)		17.0 (12.8)		9.2 (6.9)	
$\bar{x}$,[c] %	251.2 (218.8)A,B,C		83.3 (66.1)A,D		113.3 (85.4)B,D		91.9 (69.4)C	
SD								
Repeatability	5.9 (4.3)		2.1 (2.3)		6.6 (2.3)		2.2 (1.5)	
Reproducibility	5.9 (4.3)		2.6 (3.0)		8.1 (2.9)		2.2 (3.4)	
CV, %								
Repeatability	46.8 (39.4)		25.3 (34.8)		38.8 (18.0)		23.9 (21.7)	
Reproducibility	46.8 (39.4)		31.3 (45.4)		47.6 (22.6)		23.9 (49.3)	

[a] Associate Referee counts are in parentheses if different from those of collaborator or if either duplicate value is an outlier.

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

[c] Same capital letters in this row represent Associate Referee values which are significantly different from one another by Student's t-test at $P < 0.05$.

Table 3. Collaborative results for recovery of insect fragments (blind duplicates)

Coll.	Product (spike level)							
	Lemon balm (20)		Alfalfa (30)		Spearmint (50)		Papaya (20)	
1	38[a] (37)[a,b]	20 (19)	27 (25)	13 (12)	21 (21)[c]	26 (26)[c]	22 (20)[c]	8 (7)[c]
2	20[c] (18)	32[c] (19)	19 (20)	24 (25)	31 (35)	82 (39)	45 (18)	25 (18)
3	1[c] (19)	7[c] (19)	11[c] (29)	0[c] (28)	18 (44)	23 (50)	3 (19)	4 (19)
4	16 (20)	16 (19)	29	26 (28)	40 (42)	35	18 (19)	16
5	19	18	28	30	36 (38)	50 (49)	20 (19)	19
6	16 (18)	20	28 (30)	27	49	40	20	19
7	18	19	30	29 (30)	46 (47)	48 (50)	19	20
8[d]	461 (20)	343 (18)	251 (28)	330 (28)	412 (43)	358 (70)[e]	386 (19)	123 (20)
9[d]	374 (17)[c]	318 (18)[c]	127 (27)	105 (30)	357 (47)	206 (49)	239 (19)	160 (18)
$\bar{x}$	18.1 (18.9)		25.8 (26.9)		38.9 (44.0)		18.4 (18.8)	
$\bar{x}$,[f] %	90.5 (94.4)A		86.4 (89.6)		77.8 (88.1)A,B		92.1 (94.1)B	
SD								
Repeatability	1.4 (0.9)		4.4 (3.5)		14.5 (4.6)		6.6 (0.9)	
Reproducibility	1.6 (0.9)		5.1 (4.5)		16.5 (5.3)		10.4 (1.0)	
CV, %								
Repeatability	7.7 (4.8)		17.0 (13.0)		37.3 (10.4)		35.9 (4.8)	
Reproducibility	8.8 (4.8)		19.8 (16.7)		42.4 (12.0)		56.5 (5.3)	

[a] In the Dixon test for outliers, the values 38 and (37), which resulted from a spike of 40 insect fragments, were each given a proportionate value of 19 of 20.
[b] Associate Referee counts are in parentheses if different from those of collaborator or if either duplicate value is an outlier.
[c] Outlier by Dixon test; not included in calculations.
[d] All results for this collaborator were outliers by Dixon test; not included in calculations.
[e] In the Dixon test for outliers, the value (70), which resulted from a spike of 75 insect fragments, was given a proportionate value of 47 of 50.
[f] Same capital letters in this row represent Associate Referee values which are significantly different from one another by Student's *t*-test at $P < 0.05$.

Table 4. Comparison of mean percentage recoveries[a,b] of rat hairs (RH), whole or equivalent insects (W/E), and insect fragments (IF) from 4-botanicals study, previous study, and precollaborative experimental data

Source of recoveries	Product	RH	W/E[c]	IF[c]
4-Botanicals study	lemon balm	88.9 (5)	218.8A (5)	94.4B (20)
Previous study	peppermint	83.3 (5)	85.0A (5)	79.6B (20)
Experimental data	lemon balm	92.5 (20)	90.0 (10)	87.5 (20)
4-Botanicals study	alfalfa	82.2 (10)	66.1 (10)	89.6 (30)
Previous study	peppermint	87.5 (10)	80.0 (10)	88.3 (30)
Experimental data	alfalfa	86.7 (20)	86.6 (10)	87.5 (20)
4-Botanicals study	spearmint	79.6 (15)	85.4 (15)	88.1 (50)
Previous study	peppermint	82.2 (15)	77.2 (15)	84.8 (50)
Experimental data	spearmint	87.5 (20)	95.0 (10)	80.8 (20)
4-Botanicals study	papaya	80.6 (20)	69.4 (10)	94.1 (20)
Experimental data	papaya	80.0 (20)	66.7 (10)	83.3 (20)

[a] Obtained by Associate Referee.
[b] Spike levels in parentheses.
[c] Same capital letters in this column represent values which are significantly different from one another by Student's *t*-test at $P < 0.05$.

Recommendations

It is recommended that the application of the flotation method for the extraction of light filth be extended to the leafy botanicals alfalfa, spearmint, and papaya and that these applications of the method be adopted official first action. Recoveries were generally satisfactory, and the filter papers obtained were usually free of interfering debris. It is also recommended that the portion of the method title naming whole peppermint leaves be changed to leafy botanicals, with the individual botanicals listed.

Because a method to detect light filth in lemon balm is not greatly needed, and because interference of intrinsic filth (whole insects) made it difficult to evaluate filth recoveries of the method for this analyte, it is not recommended that the method be adopted for lemon balm.

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Comparison of Diethyl Ether and Ethyl Acetate as Extracting Agents for Recovery of *Ascaris* spp. and *Trichuris* spp. Eggs

RICHARD A. RUDE
Food and Drug Administration, Center for Microbiological Investigations, Minneapolis, MN 55401
JAMES T. PEELER
Food and Drug Administration, Division of Microbiology, Cincinnati, OH 45226
NORRIS G. RISTY
Food and Drug Administration, Center for Microbiological Investigations, Minneapolis, MN 55401

Ethyl acetate and diethyl ether were compared for their ability to recover *Ascaris* spp. and *Trichuris* spp. eggs from seeded milorganite, liquid sludge, and cabbage. Concentrations of 10, 100, and 1000 eggs/10 g test sample were prepared for 20 replicates of each product. The use of diethyl ether yielded fewer eggs/10 g than did ethyl acetate in 5 of 6 sets of data. For *Ascaris* spp., recovery from cabbage was 10 times higher with ethyl acetate at the higher concentration than with diethyl ether. For *Trichuris* spp., recovery from liquid sludge was slightly higher with diethyl ether for all egg concentrations. The other results ranged from 0 to 23% difference in recovery for the 2 agents. Depending on the parasites in question and the products to be screened, the substitution of ethyl acetate for diethyl ether may be significant.

Eggs of the pathogenic nematode parasites *Ascaris* spp. and *Trichuris* spp. can survive common methods of sewage treatment and contaminate food crops that are fertilized with sewage sludge or irrigated with sewage effluent. The Nacconol-ether centrifugation method is used to determine qualitatively the presence of *Ascaris* spp. and *Trichuris* spp. in crop irrigants, fertilizers, and on foods (1). The method uses diethyl ether, which is flammable, explosive, and toxic. Some laboratories are not equipped to handle diethyl ether safely and are reluctant to take the risk involved in this method.

Previous studies compared 4 methods: Nacconol-ether centrifugation (1), zinc sulfate flotation (2), saline centrifugation (2), and the water trough method (3). Nacconol-ether centrifugation gave the best recoveries and was selected for these parasites. However, a substitute was needed for the diethyl ether extraction step.

The Centers for Disease Control (CDC) screened several solvents for their ability to concentrate parasites. The flammability, density, cost, and carcinogenicity of the solvents were also evaluated. Ethyl acetate was found to be the most satisfactory in the CDC study (4). Our experiments were designed to compare the diethyl ether and ethyl acetate solvents.

Experimental

Apparatus

(a) *Gridded tissue culture dishes.*—Falcon Integrid 3030 (Becton, Dickinson and Co., Oxnard, CA).

(b) *Bolting cloth.*—100 μm pores. Nitex HC3-100 (Nitex Corp., Bern, Switzerland).

(c) *Pasteur pipets.*—229 mm, with cotton plug and rubber bulb.

(d) *Bottle shaker.*—Size 2, midpoint setting. International shaker.

(e) *Centrifuge.*—IEC HN-SII, DAMON/IEC Division.

Reagents

(a) *Formalin solution (10%).*—Add 730 mL water to 270 mL 37% stock formaldehyde solution.

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(b) *Nacconol 35 SL.*—Sodium linear alkylate sulfonate, 35% active (Stepan Chemical Co., Northfield, IL).

(c) *Nacconol solution (0.008%).*—3.7 mL Nacconol 35 SL in 16 L physiological saline.

(d) *Ether (ethyl) anhydrous.*—Reagent grade (Mallinckrodt Inc., Paris, KY).

(e) *Ethyl acetate.*—Analytical reagent (Mallinckrodt).

Determination

Ninety mL 0.008% Nacconol solution was added to seeded test portion in milk dilution bottle and was shaken vigorously 10 min on automatic bottle shaker. Piece of bolting cloth, 6 in. × 6 in., was soaked in 0.008% Nacconol solution immediately before use. Cloth was secured to top of 600 mL beaker with rubber band, and bottle's contents were poured through soaked bolting cloth into 450 mL beaker. Bottle was rinsed 3 times with 20 mL portions of 0.008% Nacconol solution, and rinses were poured through bolting cloth into beaker. Contents of beaker were stirred with glass stirring rod and then poured into 100 mL conical centrifuge tubes. Tubes were centrifuged 4 min at 900 × *g*, and supernate was decanted. One mL 0.008% Nacconol solution was added to tubes, and pellicle was resuspended. With Pasteur pipet, sediment was combined into 100 mL conical centrifuge tube. Emptied tubes were rinsed with 2 mL of 0.008% Nacconol solution, and rinse was added to 100 mL conical centrifuge tube containing sediment. Bulb was removed from Pasteur pipet, and pipet was rinsed through top into 100 mL conical centrifuge tube. Tube was centrifuged 4 min at 900 × *g*, and supernate was decanted and discarded. Eight mL of 10% formaldehyde solution was added, and pellicle was resuspended using vortex mixer. Pellicle was loosened with 4 mm glass rod when needed. Under chemical hood, 3 mL diethyl ether or ethyl acetate were added to tube. Tube was stoppered and shaken vigorously 30 s. Tube was centrifuged 4 min at 900 × *g*. Tube now contained supernate, organic plug, and pellicle. Supernate and organic plug were poured into safety can. One mL 0.008% Nacconol solution was added to tube and pellicle was resuspended. With Pasteur pipet, suspended contents were transferred into tissue culture dish. Tube was rinsed with 1 mL of 0.008% Nacconol solution and then rinse was poured into tissue culture dish. If background material was too heavy, contents were diluted into more tissue culture dishes. To count recovered parasites, entire tissue culture dish was scanned with inverted microscope, and results were recorded.

Statistical Methods

The distribution of eggs was recorded for each series of 20 replicates. A transformation of $\log_{10}$ (count + 1) was used to normalize the data in subsequent analyses. An analysis of variance (5) was performed on each food product with concentration and extraction solvent as factors. A univariate analysis was performed on *Ascaris* spp. and *Trichuris* spp. as was a multivariate analysis using both variables (5). Dun-

Table 1. Summary of analysis of variance results for *Ascaris* and *Trichuris* using 2 extraction agents

Factor		F-ratio *Ascaris*	F-ratio *Trichuris*	Degrees of freedom for F-ratio
Cabbage				
Concn	A	198.03[a]	2090.08[a]	2, 114
Extraction solvent	B	180.13[a]	2.90	1, 114
Interaction	A × B	27.95[a]	0.47	2, 114
Milorganite				
Concn	A	1319.53[a]	6633.60[a]	2, 114
Extraction solvent	B	0.02	7.23[a]	1, 114
Interaction	A × B	1.06	3.40[a]	2, 114
Sludge				
Concn	A	2136.50[a]	7691.21[a]	2, 114
Extraction solvent	B	0.03	7.42[a]	1, 114
Interaction	A × B	0.70	0.85	2, 114

[a] Significant at the $\alpha = 0.05$ level.

can's test (6) was performed to determine if the means differed significantly. The $\alpha = 0.05$ level was used for all tests.

Results

Results of the 6 statistical analyses are shown in Table 1. In each case, the null hypothesis was that means for concentrations and the 2 extraction solvents were equal. The null hypothesis that the interaction between the 2 factors (concentration and extraction agent) equals zero was also tested. All 6 concentration effects were significant and 3 of 6 solvent comparisons differed. Only 2 of the interactions were significant at the $\alpha = 0.05$ level.

Interaction occurs when results are inconsistent at different levels of factors. Significant interaction was observed in 2 of the analyses of variance (Table 1). The mean for *Ascaris* in cabbage illustrates the reason for the significant interaction ($F_{2, 114} = 27.95$). Means at the lowest concentration are the same order of magnitude, whereas the geometric means for the highest concentration differ more than tenfold. A multivariate analysis of variance was also computed when the variables *Ascaris* and *Trichuris* were considered together.

Table 2. Geometric means of parasite eggs/10 g for *Ascaris* in cabbage, milorganite, and sludge using diethyl ether and ethyl acetate

Extn solv.	Concn, egg/10 g 1000	100	10	Geometric mean for extn agent
Cabbage				
Diethyl ether	16[a]	4.4	2.2	5.4[b]
Ethyl acetate	285	28	3.7	31
Geometric mean for concentration	67[b]	11	2.9	
Milorganite				
Diethyl ether	262	26	3.4	28
Ethyl acetate	300	26	3.0	29
Geometric mean for concentration	280[b]	26	3.2	
Sludge				
Diethyl ether	389	42	2.2	42
Ethyl acetate	420	41	3.7	42
Geometric mean for concentration	404[b]	42	4.4	

[a] There were 20 replicate determinations for each mean.
[b] Means differ significantly at the $\alpha = 0.05$ level.

Table 3. Geometric means of parasite eggs/10 g for *Trichuris* in cabbage, milorganite, and sludge using diethyl ether and ethyl acetate

Extn solv.	Concn, egg/10 g 1000	100	10	Geometric mean for extn agent
Cabbage				
Diethyl ether	601[a]	51	5.7	56
Ethyl acetate	665	52	6.7	62
Geometric mean for concentration	632[b]	51	6.2	
Milorganite				
Diethyl ether	585	53	5.8	57[b]
Ethyl acetate	717	53	6.2	62
Geometric mean for concentration	648[b]	53	6.0	
Sludge				
Diethyl ether	715	67	5.7	67[b]
Ethyl acetate	649	59	6.7	62
Geometric mean for concentration	681[b]	63	6.3	

[a] There were 20 replicate determinations for each mean.
[b] Means differ significantly at the $\alpha = 0.05$ level.

The linear correlation between the 2 variables was 0.20, 0.08, and 0.16 for cabbage, milorganite, and sludge. Results from multivariate analysis did not modify the univariate results.

The experiments were designed so that the concentrations of parasites increase tenfold. Thus, the geometric means of the recovered parasites differed significantly ($\alpha = 0.05$ level) for concentrations. Tables 2 and 3 present the geometric means for concentrations and extraction solvents. The significant overall means for each concentration and extraction solvent are marked. Ethyl acetate extraction solvent yielded mean results equal to or greater than those treated with diethyl ether in 5 of 6 cases. The overall recovery by ethyl acetate was significantly higher for the recovery of *Ascaris* in cabbage and *Trichuris* in milorganite. In 1 of 6 tests (recovery of *Trichuris* in sludge) the diethyl ether mean was significantly higher than the mean observed from ethyl acetate results. However, the largest differences were observed for *Ascaris* in cabbage. Ethyl acetate recovered more than 10 times as many eggs at the high concentration as did diethyl ether. The other *Ascaris* results were 8 and 15 in recovery for the 2 solvents. No distortion of parasite morphology was observed with either extraction solvent.

Discussion

Ethyl acetate is a satisfactory and efficient extraction solvent for use in the Nacconol-ether centrifugation method. The organic material plug present after the final centrifugation was thicker and looser with ethyl acetate, but that had no effect on the recovery of parasites. Overall, the recovery rate using ethyl acetate was equal to or greater than that with diethyl ether.

Ethyl acetate is also less hazardous than diethyl ether. Both solvents are flammable; however, ethyl acetate is less flammable with a flashpoint of −4°C and a boiling point of 77°C as compared with −45°C and 34.5°C for diethyl ether. Ethyl acetate has narrower flammability limits: 1.4 to 7.6% per unit as compared with 1.9 to 48% per unit volume for diethyl ether. Both solvents are noncarcinogenic.

Based on the parasites and products tested in this study, it is concluded that ethyl acetate may be substituted for diethyl ether for the recovery of parasites.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Analysis of Phenols by Chemical Derivatization. V. Determination of Pentachlorophenol and 19 Other Chlorinated Phenols in Sediments

HING-BIU LEE, YVONNE D. STOKKER, and ALFRED S. Y. CHAU
Environment Canada, National Water Research Institute, Canada Centre for Inland Waters, 867 Lakeshore Rd, Burlington, Ontario L7R 4A6, Canada

A method for quantitative and isomer-specific analysis for pentachlorophenol and 19 other chlorophenols in sediment was developed. After acidification to pH <1, sediment samples were Soxhlet-extracted with acetone–hexane (59 + 41, v/v) for 20 h. Phenols in the organic extract were back-extracted into 2% $KHCO_3$ and were then acetylated with acetic anhydride and extracted with petroleum ether. After evaporation to a small volume, the acetates were cleaned up on a 5% deactivated silica gel column. Extracts were then analyzed on a 12 m OV-1 column interfaced to an electron-capture detector and on a 30 m SPB-5 column interfaced to a mass selective detector. The procedure has been validated with sediment samples fortified at 100, 10, and 1 ng/g levels of chlorophenols. Recoveries of dichloro- and the higher chlorophenols were generally between 80 and 95% at all 3 levels of fortification, whereas recoveries of monochlorophenols were between 65 and 85%. The 2 chloromethylphenols were less than 50% recovered, and recovery of phenol itself was erratic by this procedure. Using a 50 g sample size, the estimated detection limit was ca 0.2 ng/g.

Pentachlorophenol (PCP) has long been used as a wood preservative, and other chlorophenols are often used as precursors in the production of many phenoxyalkanoic herbicides and biocides. According to one report, PCP comprises over 60% of the 3200 tonnes of chlorophenols used annually in Canada (1). Residues of these phenols are reported in the environment and especially in industrial wastewaters and sludges. Because of the acute toxicity of PCP and other chlorophenols, routine methods for the monitoring of these chemicals in water and sediments are required. Analysis for chlorophenols in sediment samples is particularly important because phenols are retained in large quantities by municipal solid wastes, landfill leachates, and sediments (2–4). Several papers have been published on the extraction and analysis for PCP and a few other phenols in sediments (5–9). In previous publications, we have reported methods for isomer-specific analysis for chlorophenols in water by the formation of acetate (10), chloroacetate (11), and pentafluorobenzyl (PFB) ether (12) derivatives. Presumably because of its simplicity and ruggedness, the acetate procedure is a popular approach; it has been used by many workers (13–17). However, application of the acetate procedure to environmental samples is limited to phenols with 2 or more chlorine atoms if an electron-capture detector (ECD) is used for analysis, because the ECD sensitivity to monochloro- and nonchlorinated phenol acetates is poor. Recently, analysis for chlorophenol acetates by gas chromatography/mass spectrometry (GC/MS) has been reported (17–20). Since strong characteristic ions were observed for these acetates under electron impact (EI) conditions, GC/MS, operating in selected ion monitoring (SIM) mode, is potentially a highly specific and sensitive technique for phenol determination. The recent advent of the mass selective detector (MSD) interfaced with a capillary column GC has also provided fully automated acquisition of GC/MS data, but at lower cost. In this paper, we describe a method for the routine analysis for PCP and 19 chlorophenols in sediment samples by formation of acetate derivatives followed by quantitation with GC-ECD and GC-MSD.

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Experimental

Apparatus and Reagents

(a) *Gas chromatograph.*—Hewlett-Packard Model 5880A equipped with ^{63}Ni electron-capture detector, Model 7671A autosampler, level 4 terminal, and split-splitless capillary column injection port. GC column: 12 m × 0.2 mm id fused silica capillary column coated with cross-linked dimethyl silicone gum and surface-deactivated by siloxane (Hewlett-Packard part No. 19091-60312). Operating conditions: injection port, 200°C; detector, 300°C; column initial temperature, 70°C, hold 0.5 min, increase 10°/min to 120°, hold 5 min at 120°, increase 2°/min to 160°; splitless valve on for 0.5 min; carrier gas, helium at 10 psi; detector makeup gas, argon–methane (95 + 5) at 25 mL/min.

(b) *Gas chromatograph.*—Hewlett-Packard Model 5880A equipped with 5970B mass selective detector, Series 200 computer, 9133XV disc drive, a split/splitless injection port, level 2 terminal, and Model 7671A autosampler. Capillary column: 30 m × 0.25 mm id SPB-5 fused silica (Supelco, Ltd) directly interfaced to the electron-impact ion source for maximum sensitivity. Operating conditions: electron energy, 70 eV; injection port, 250°C; interface temperature, 280°C; column initial temperature, 70°C, hold 0.5 min, increase 30°/min to 120°, then increase 2.5°/min to 180°, hold 10 min at 180°C; splitless valve on for 0.5 min; carrier gas, helium at 4 psi; split vent flow, 50 mL/min.

(c) *Chlorophenol standards.*—Available from Aldrich Chemical Co. or Supelco, Inc. (Phenol Kit 27). 2,3,4,6-Tetrachlorophenol (Eastman Organic Chemicals). Prepare all stock solutions in toluene at 5 mg/mL. Prepare mixture of phenols in acetone at 50 μg/mL for spiking purposes. Store all solutions in the dark at 4°C.

(d) *Acetic anhydride.*—Distill AnalaR grade (BDH Chemicals) reagent 3 times and collect 138–140°C fraction for acetylation reactions.

Fortification of Sediment Samples

Spike 50 g sediment with 100 μL phenol mixture in acetone at appropriate concentrations. Mix well with spatula and equilibrate 30 min before extraction.

Extraction

Place sediment sample on top of 5 cm Celite 545 in 45 mm id glass thimble with coarse frit. Acidify samples to pH <1 with H_2SO_4 (1 + 1, v/v). Put thimble in Soxhlet extractor and extract sample 20 h with 350 mL acetone–hexane (59 + 41, v/v) at rate of 6–8 cycles/h. After extraction, add 50 mL 2% aqueous $KHCO_3$ to organic extract and evaporate solvent to ca 100 mL. Add 50 mL hexane to mixture to facilitate phase separation and drain aqueous layer into 250 mL volumetric flask. Extract organic layer 2 min with 40 mL of 2% $KHCO_3$ and drain aqueous fraction to above-mentioned 250

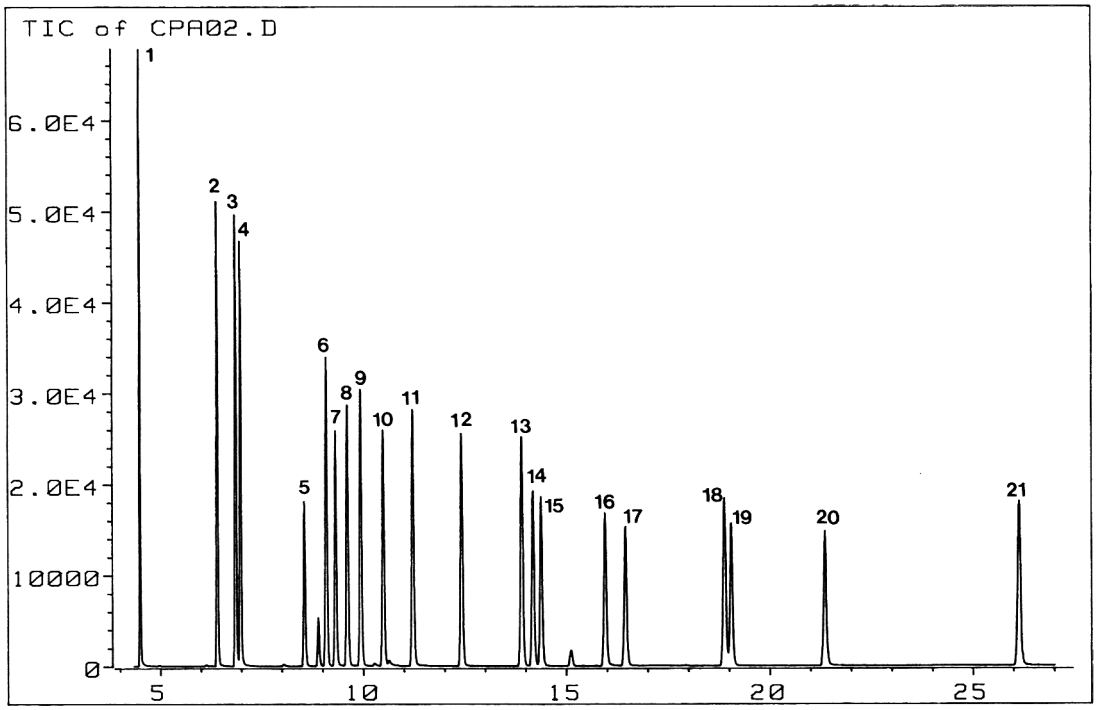

Figure 1. EI-GC-MSD total ion current trace of 21 phenol acetates as recorded on 30 m SPB-5 column. Refer to numbers in Table 2 for peak identification.

mL volumetric flask. Repeat extraction twice with 30 mL base each time. After final extraction, discard organic layer. To prepare calibration standard, spike known amounts of phenols directly to 150 mL of 2% $KHCO_3$ and proceed to derivatization and cleanup procedure described below.

Derivatization and Cleanup

To the 150 mL 2% $KHCO_3$ solution containing phenols, add 3 mL acetic anhydride and 25 mL petroleum ether (30–60°C). Stir sample *slowly* until evolution of CO_2 subsides and then stir vigorously for 30 min. Separate layers in separatory funnel and drain water sample back into original container. Collect organic layer in 250 mL round-bottom flask. Repeat extractive acetylation twice with 3 mL acetic anhydride and 25 mL petroleum ether each time. Dry organic extract with anhydrous sodium sulfate. Add 2 mL isooctane and evaporate solvent to ca 3 mL using a 3-stage Snyder column.

Prepare mini cleanup column by plugging long Pasteur pipet (23 × 0.5 cm id) with piece of silanized glass wool. Fill column with 5 cm of 5% deactivated silica gel. Tap column gently and add 5 mm anhydrous Na_2SO_4 at top. Rinse column with 5 mL hexane and discard washings. With Pasteur pipet, quantitatively transfer acetylated products to silica gel column. Elute column with 5 mL hexane and discard. Continue elution with 10 mL toluene. Collect this fraction and dilute to final volume of 10.0 mL. Inject 2 μL extract, in splitless mode, and analyze by GC-ECD (10) and by GC-MSD.

Table 1. Retention time windows and characteristic ions used for selected ion monitoring of chlorophenol acetates by GC-MSD

Ion group	Corresponding phenols	Retention time window, min	Characteristic ions m/z
1	Phenol	4.20–6.00	43, 94, 136
2	Chloro-	6.00–8.00	43, 128, 170
3	Dichloro- and chloromethyl-	8.00–12.00	43, 142, 162, 184, 204
4	Trichloro-	12.00–18.00	43, 198, 240
5	Tetrachloro-	18.00–23.00	43, 232, 274
6	PCP	23.00–27.00	43, 266, 308

GC-MSD Analysis of Phenol Acetates

(a) *Total ion scanning.*—Obtain abundance data of the major fragments for chlorophenol acetates by scanning from m/z 40 to 320 at a rate of 1.5 scans/s and a scan threshold of 10.

(b) *Selected ion monitoring.*—For quantitative purposes, monitor 3 characteristic ions for each group of phenols as shown in Table 1 and set dwell time to 100 ms for each ion. To maximize sensitivity, divide ions into 6 groups or reten-

Table 2. Characteristic ions, relative abundances, and relative response factors[a] of 21 chlorophenol acetates

No.	Parent phenol	Characteristic ions, m/z (rel. abundance) CH_3CO^+	(M-42)$^+$	M^+	Rel. response factors MSD[b]	ECD
1	Phenol	43 (28)	94 (100)	136 (10)	4.0	<0.01
2	2-Chloro	43 (59)	128 (100)	170 (12)	6.5	0.03
3	3-Chloro	43 (80)	128 (100)	170 (16)	7.0	0.03
4	4-Chloro	43 (43)	128 (100)	170 (11)	5.4	0.02
5	2-Chloro-5-methyl	43 (56)	142 (100)	184 (15)	2.2	0.01
6	2,6-Dichloro	43 (100)	162 (66)	204 (14)	8.3	0.74
7	4-Chloro-3-methyl	43 (49)	142 (100)	184 (13)	3.0	0.03
8	2,4-Dichloro	43 (100)	162 (99)	204 (11)	6.0	0.83
9	3,5-Dichloro	43 (100)	162 (51)	204 (14)	8.2	0.98
10	2,3-Dichloro	43 (100)	162 (52)	204 (15)	7.0	0.87
11	3,4-Dichloro	43 (93)	162 (100)	204 (14)	6.4	0.76
12	2,4,6-Trichloro	43 (100)	198 (50)	240 (7)	8.4	3.2
13	2,3,6-Trichloro	43 (100)	198 (36)	240 (8)	9.9	3.0
14	2,3,5-Trichloro	43 (100)	198 (40)	240 (10)	7.3	3.2
15	2,4,5-Trichloro	43 (100)	198 (59)	240 (7)	5.7	2.7
16	2,3,4-Trichloro	43 (100)	198 (58)	240 (7)	5.7	4.1
17	3,4,5-Trichloro	43 (100)	198 (61)	240 (12)	5.0	3.2
18	2,3,5,6-Tetrachloro	43 (100)	232 (39)	274 (12)	10.0	4.5
19	2,3,4,6-Tetrachloro	43 (100)	232 (54)	274 (8)	7.0	5.0
20	2,3,4,5-Tetrachloro	43 (100)	232 (59)	274 (9)	6.2	7.0
21	PCP	43 (100)	266 (51)	308 (10)	10.0	10.0

[a] PCP acetate = 10.0.
[b] Based on ion m/z = 43.

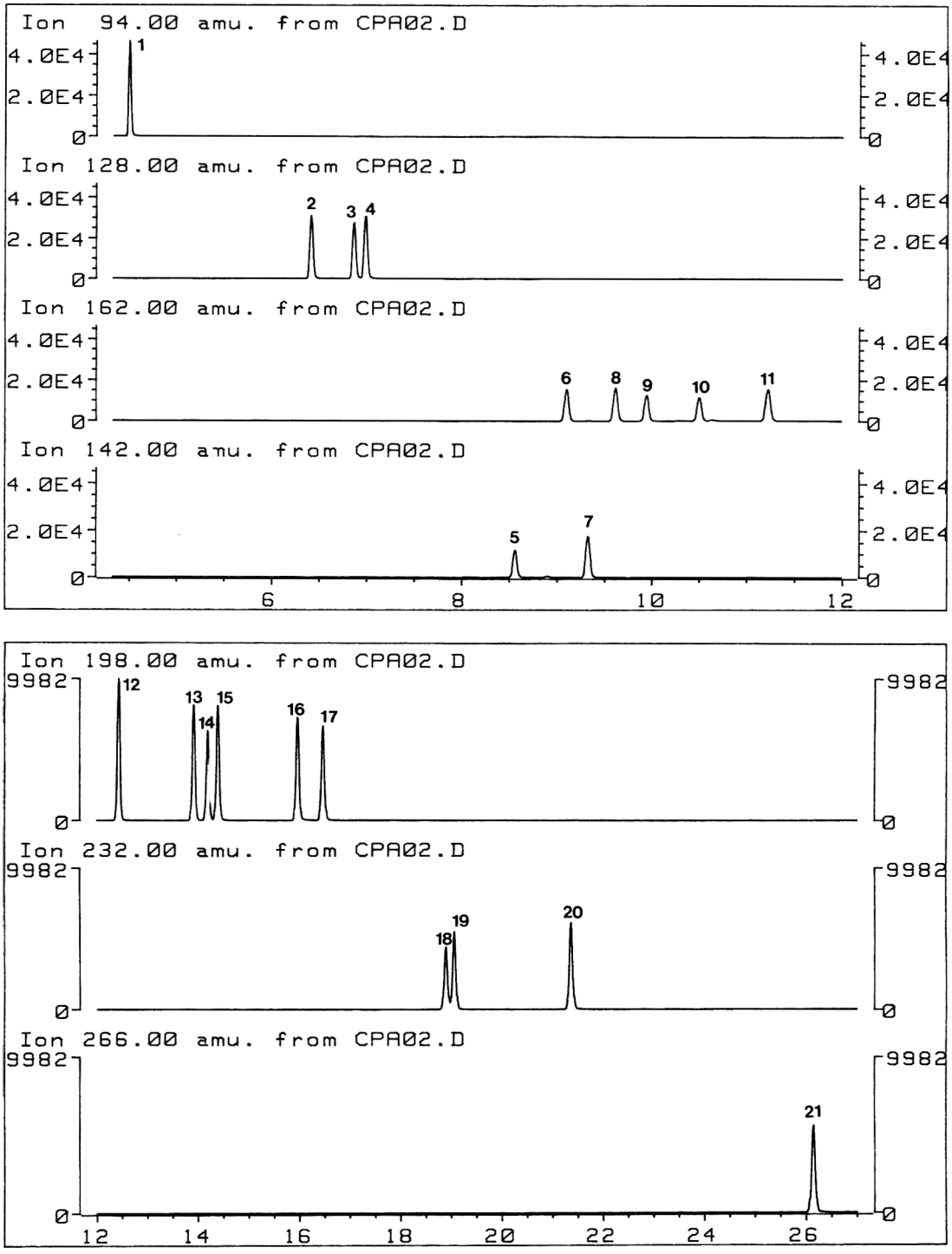

Figure 2. EI-GC-MSD selected ion monitoring of acetate derivatives. Top, phenol, monochlorophenols, dichlorophenols, and chloromethylphenols; bottom, trichlorophenols, tetrachlorophenols, and PCP.

tion time windows. Integrate the $(M-42)^+$ ion. If required, use phenanthrene-d_{10} as an internal standard to calibrate MSD response factors.

Results and Discussion

In a previous paper, we successfully demonstrated that 15 di-, tri-, tetra-, and pentachlorophenols in water samples can be conveniently analyzed in their acetate forms after an in situ acetylation reaction (10). With a combination of a high resolution capillary column and an electron-capture detector, isomer-specific analysis of the above 15 chlorophenols was feasible, and quantitative recoveries were obtained from surface water containing as low as 0.01 μg of phenols/L. Phenol, monochlorophenols, and chloromethylphenols are also acetylated by the same or similar procedures. However, because the electron-capture sensitivities of these acetates are a few hundred to over 1000 times lower than that of PCP acetate (Table 2), these derivatives are normally undetectable by an electron-capture detector at levels commonly found in environmental samples. A mass selective detector or other mass spectrometric detectors do not have a discriminating sensitivity effect against the nonchlorinated and monochlorinated phenols. For example, the relative response factors of the 21 phenols determined by the mass selective detector on the CH_3CO^+ ion (m/z = 43) are all within a factor of 5 and within a factor of 2 with the exception of 3 phenols (Table 2). Therefore, use of mass selective detection will allow the analysis of nonchlorinated and monochlorinated phenols at levels

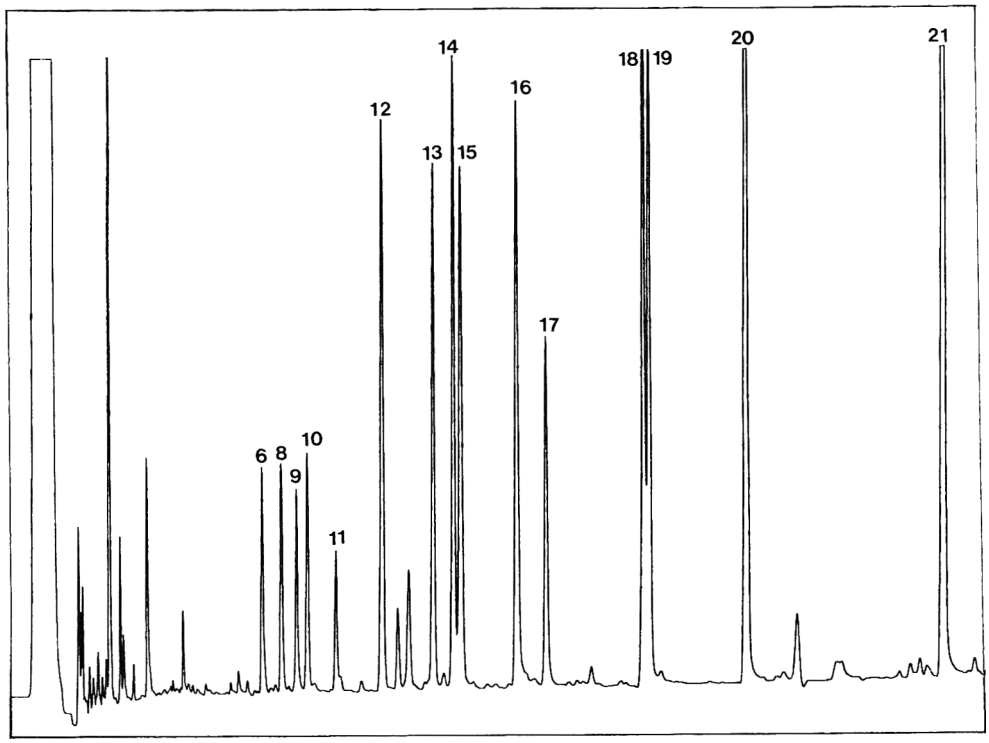

Figure 3. GC-ECD chromatogram of acetylated extract from a sediment sample fortified to 10 ng/g for each phenol.

similar to phenols of higher chlorination. A reconstructed total ion current chromatogram of the 21 phenol acetates is shown in Figure 1.

The mass spectrum of each chlorophenol acetate obtained under EI conditions included the following 3 characteristic masses: (*1*) the molecular ion (M^+), (*2*) the parent phenol moiety (M-42), and (*3*) the CH_3CO^+ fragment. The m/z values of characteristic masses for the 21 phenol acetate derivatives and their relative abundances are listed in Table 2. For acetates of chlorophenols, the molecular ions were low in abundance, and in all cases, they were less than 20% of their respective base peaks. In cases of phenol, the 3 monochlorophenols and 2 chloromethylphenols, the M-42 ions were the most abundant ions. For other phenols of higher chlorination, the most abundant ion was the CH_3CO^+ fragment.

For quantitative GC-MSD analysis for these acetate derivatives, selected ion monitoring (SIM) of the above-mentioned characteristic ions was used. Representative single ion chromatograms of phenol acetates at each level of chlorination are depicted in Figure 2, top and bottom. The presence of a phenol in question was confirmed if all 3 characteristic ions were present at the expected retention time and at a ratio of not more than ±20% deviation from the expected relative abundance. Once the presence of a phenol had been confirmed, the $(M-42)^+$ ion was used for quantitation to optimize sensitivity. Since the retention time windows of chlorophenol acetate do not overlap, the entire chromatogram can be subdivided into 6 ion groups, one for each level of chlorination corresponding to phenol through to PCP. In this case, further enhancement in sensitivity can be achieved by monitoring only 3 ions for those phenols expected in this window. Retention times for the acetates of the 2 chloromethylphenols fell into the retention time window of dichlorophenol acetates. Therefore, characteristic masses of these 2 groups of phenols were both monitored within this window.

Chlorophenols in sediments are generally extracted by the following 3 approaches: (a) with an aqueous buffer solution or a base at high pH (5, 21); (b) solvent extraction after the sediment is acidified to a low pH (6–9); or (c) steam distillation of sediments acidified to pH <1 (22, 23). All approaches provide satisfactory recoveries of PCP and a few other chlorophenols. In this work, sediment samples were acidified to pH <1 with H_2SO_4 (1 + 1) and were then Soxhlet-extracted with a mixture of acetone–hexane (59 + 41). This technique was used because the same method produced quantitative recoveries of various classes of compounds, such as PCBs (24), chlorobenzenes (25), and chlorinated insecticides, herbicides, and polyaromatic hydrocarbons (unpublished data). By using the same extraction technique, a multiclass, multiresidue method can be developed in the future.

Back extraction of chlorophenols into a base was a critical step in this method. Preliminary experiments indicated that over 90% recovery of all chlorophenols in a hexane solution could be achieved by 3 successive back extractions with 40 + 30 + 30 mL of 2% $KHCO_3$. Before an efficient back extraction of phenols could be performed on sediment extracts, the acetone and acids in the organic layer had to be removed. Acetone was evaporated in the presence of 50 mL 2% $KHCO_3$ using a 3-stage Snyder column with a heating mantle. The base was added as a keeper for the phenols during solvent evaporation and was also used to neutralize the free acids present in the sample extract.

To enhance phase separation during back extraction, the organic extract was evaporated to about 100 mL, then 50 mL of hexane was added to the concentrated extract.

Column cleanup was performed with a 5% deactivated silica gel column. Polar sediment coextractives that were not removed in the $KHCO_3$ partitioning step were removed by silica gel since they tended to stay on the column. Acetates of all chlorophenols were eluted in one fraction by 10 mL toluene. If the analyses of monochlorophenols are not required, a less-polar toluene–hexane eluant (1 + 1) can be used (10). If further evaporation of solvent is required, the

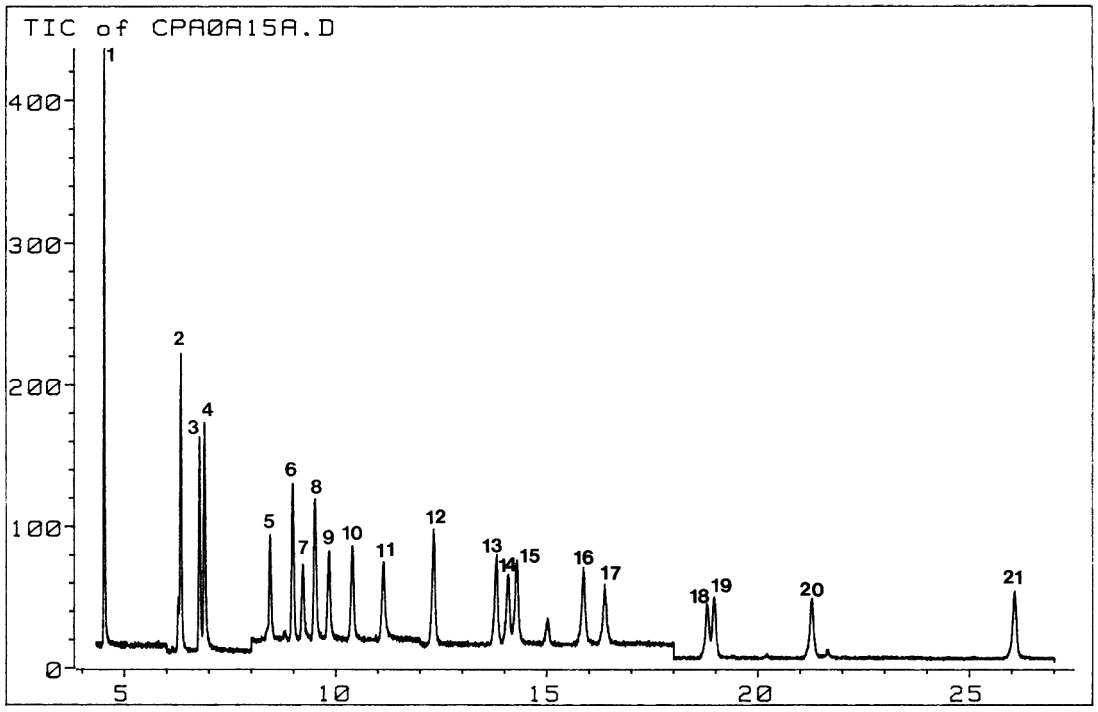

Figure 4. GC-MSD chromatogram of the same sample shown in Figure 3. Note that acetates of phenol, monochlorophenols, and chloromethylphenols are easily identified by this detector.

toluene should be replaced by acetone–hexane (5 + 95) in the column cleanup step.

In the present work, recovery data for chlorophenols were obtained at 100, 10, and 1 ng/g levels. Sediment samples used in the fortification experiments were prepared from a bulk composite sediment sample by mixing sandy, loamy, and clay-based sediments in a 2:1:2 ratio. After fortification, the sediment samples were equilibrated 30 min before acidification and Soxhlet extraction. Sediment extracts were then acetylated, cleaned up, and analyzed by GC-ECD and GC-MSD. At 100 and 10 ng/g levels, the extracts were sufficiently clean for reliable GC-ECD analysis of the di-, tri-, tetra-, and pentachlorophenols. At the 1 ng/g fortification level, this cleanup procedure did not produce extracts clean enough for ECD quantitation of the dichlorophenol derivatives, although useful results could still be obtained for the higher chlorophenols. In such cases, as well as for the analysis of monochlorophenols, a mass selective detector operating in the SIM mode was used to provide quantitative results.

Table 3. Mean percent recovery of chlorophenols and standard deviations for fortified sediment samples

Phenol	Fortification level, ng/g 100 (n = 5)	10 (n = 6)	1[a] (n = 6)
2-Chloro-	73 ± 6[a]	74 ± 7[a]	74 ± 7
3-Chloro-	76 ± 4[a]	79 ± 4[a]	86 ± 3
4-Chloro-	68 ± 6[a]	77 ± 5[a]	65 ± 8
2-Chloro-5-methyl-	47 ± 3[a]	41 ± 4[a]	51 ± 6
2,6-Dichloro-	84 ± 5	92 ± 9	76 ± 10
4-Chloro-3-methyl-	42 ± 3[a]	38 ± 4[a]	50 ± 7
2,4-Dichloro-	80 ± 5	82 ± 6	88 ± 6
3,5-Dichloro-	78 ± 4	77 ± 11	83 ± 3
2,3-Dichloro-	89 ± 5	94 ± 8	87 ± 4
3,4-Dichloro-	76 ± 4	77 ± 12	85 ± 5
2,4,6-Trichloro-	87 ± 4	87 ± 3	88 ± 5
2,3,6-Trichloro-	90 ± 2	94 ± 3	83 ± 5
2,3,5-Trichloro-	96 ± 2	93 ± 4	91 ± 3
2,4,5-Trichloro-	91 ± 2	95 ± 4	87 ± 2
2,3,4-Trichloro-	95 ± 2	94 ± 5	93 ± 2
3,4,5-Trichloro-	83 ± 4	86 ± 6	91 ± 7
2,3,5,6-Tetrachloro-	93 ± 5	90 ± 2	90 ± 4
2,3,4,6-Tetrachloro-	91 ± 3	93 ± 3	94 ± 8
2,3,4,5-Tetrachloro-	94 ± 2	95 ± 5	95 ± 5
PCP	85 ± 5	92 ± 5	96 ± 4

[a] Results obtained by MSD; other results obtained by ECD.

As shown in Table 3, recoveries of tri-, tetra-, and pentachlorophenols at all levels of validation were between 85 and 95%, whereas dichlorophenols were between 75 and 90% recovered. Recoveries of monochlorophenols were slightly lower at 65 to 85%. On the other hand, the 2 chloromethylphenols were only 40–50% recovered, and the recovery of phenol itself was erratic by this procedure. The method detection limit (26) for the 20 chlorophenols by MSD in this study was estimated as 0.2 ng/g based on a 50 g sample and a final volume of 1 mL. Acetylated extracts from sediment samples fortified to 10 ng/g for each phenol as analyzed by ECD and MSD are shown in Figures 3 and 4, respectively.

The effect of storage time on the recovery of chlorophenols was briefly studied. A set of sediment samples was fortified to 10 ng/g per phenol and stored at 4°C in the dark for 4 and 8 days before extraction and analysis. No significant change was observed in the recoveries of any of the chlorophenols after 4 or 8 days of cold storage as compared to the control samples which were spiked and extracted immediately.

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Reverse-Phase Liquid Chromatographic Determination of Paraquat and Diquat in Agricultural Products

TOSHIHIRO NAGAYAMA, TOSHIO MAKI, KIMIKO KAN, MAMI IIDA, and TAICHIRO NISHIMA
The Tokyo Metropolitan Research Laboratory of Public Health, Division of Food Hygiene, 24-1 Hyakunin-cho 3-chome, Shinjuku-ku, Tokyo, 160 Japan

A simple, rapid, highly sensitive liquid chromatographic method is described for the quantitative determination of paraquat and diquat residues in agricultural products. Paraquat and diquat are extracted with hot dilute hydrochloric acid and are cleaned up on an Amberlite CG-50 column, followed by reverse-phase liquid chromatography on an NH_2 column, with ultraviolet detection at 257 nm (paraquat) and 310 nm (diquat). The minimum detectable concentration of both paraquat and diquat was 0.5 ng per injection, which corresponds to a lower detection limit of approximately 0.02 μg/g in the original samples. Recoveries of paraquat and diquat added to various samples were greater than 79%, and averaged 91 and 90%, respectively, at the 0.1 and 1.0 μg/g spiking levels.

Paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride) and diquat (1,1'-ethylene-2,2'-bipyridylium dibromide) are effective contact desiccants and are widely used in preharvest desiccation of various crops, for postemergent nonselective weed control, and for aquatic weed control. Determinations of paraquat or diquat in water (1), blood (2–4), urine (4–6), biological fluids (7), and soft drinks (8) have been published, but it is difficult to detect small amounts of paraquat in agricultural products by these methods. Analysis of paraquat and diquat in agricultural products (9–16) has been reported. These methods include spectrophotometry (8–11), thin-layer chromatography (12), gas chromatography (4, 13), and liquid chromatography (LC) (2, 5, 6, 14–16). Other methods include polarography (17) and gas chromatography mass spectrometry (18, 19). Almost all of these methods require extensive sample treatment, and are time-consuming.

The purpose of the present investigation was to develop a simple analytical method that allows rapid analysis of low levels of paraquat and diquat residues in agricultural products.

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METHOD

Apparatus and Reagents

(a) *Blender.*—(Torio Science Co., Ltd, Tokyo, Japan).

(b) *Ultra-high speed homogenizer.*—Nihonseiki Model biomixer (Nihonseiki, Ltd, Tokyo, Japan).

(c) *Liquid chromatograph.*—Shimadzu Model LC-5A (Shimadzu, Ltd, Kyoto, Japan) with Rheodyne sample injector Model 7125 equipped with Model UVIDEC-100-III variable-wavelength ultraviolet (UV) detector (Japan Spectroscopic Co., Ltd, Tokyo, Japan). Chromatographic conditions: temperature, 20°C; flow rate, 1.0 mL/min; wavelengths, 257 and 310 nm; injection volume, 50 μL with 50 μL loop.

(d) *Data processor.*—Shimadzu Model C-R2A(X) with 2-channel module, Model INP-R2A, for integration and calculation.

(e) *Chromatographic column.*—Glass, 30 cm × 1.0 cm id.

(f) *LC column.*—Hibar LiChrosorb NH_2 (Cica-MERCK; Cat. No. 50376, Kanto Chemical Co., Inc., Tokyo, Japan), stainless steel, 25 cm × 4.0 mm id, particle size, 5 μm.

Reagents

(a) *Glass fiber filter paper.*—Toyo filter paper GA100 (Toyo Roshi Co., Ltd, Tokyo, Japan).

(b) *Resin for column chromatography.*—Amberlite CG-50 type 1 (Rohm and Haas Co., Philadelphia, PA). Condition resin as follows: Wash with water and 1N HCl, and then wash with water until pH is raised to 6.0. Discard water, and wash resin with 0.1M acetate buffer (pH 5.6). Store resin in 0.1M acetate buffer.

(c) *Acetate buffer.*—Dissolve 3.28 g reagent grade sodium acetate (anhydrous) in 400 mL water (0.1M sodium acetate

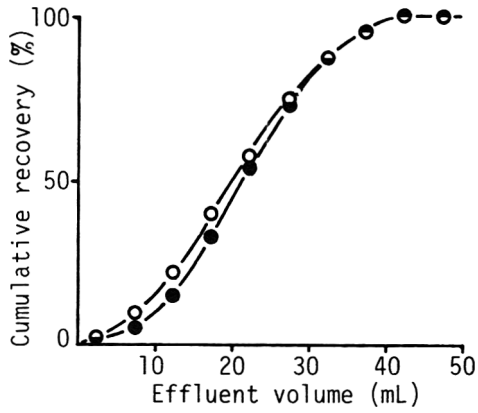

Figure 1. Elution profiles of paraquat and diquat from Amberlite CG-50 column. Eluant, 0.1N HCl–methanol. ●—●, paraquat; ○—○, diquat.

solution). Add 50 mL 0.1M acetic acid and adjust to pH 5.6 with 0.1M sodium acetate solution and 0.1M acetic acid.

(d) *Paraquat and diquat reference standard solutions.*—*Paraquat dichloride.*—Anhydrous, analytical grade, 99.2% (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Dehydrate 24 h in sulfuric acid desiccator under reduced pressure (below 30 mm Hg). Accurately weigh 50 mg dried paraquat dichloride, dissolve in methanol in 50 mL volumetric flask, and dilute to volume with methanol. *Diquat dibromide.*—Analytical standard, 1000 μg/mL methanol (NANOGENS, Inc., Watsonville, CA). Accurately transfer 1.0 mL of each standard solution to 10 mL volumetric flask and dilute to volume with methanol. Store all solutions in stoppered containers in a refrigerator, but use them at room temperature. Dissolve 1.07 g ammonium chloride in acetonitrile–methanol–water (2 + 1 + 1) in 100 mL volumetric flask. Accurately transfer 1.0 mL mixed standard solution to 100 mL volumetric flask and dilute to volume with ammonium chloride/acetonitrile–methanol–water solution. This mixed solution contains 1 μg/mL of each compound.

(e) *LC mobile phase.*—Dissolve 2.92 g sodium chloride in 150 mL water, add 100 mL methanol and 750 mL acetonitrile, and adjust pH of resulting solution to 3.0 with 8N phosphoric acid solution.

Extraction

Weigh 10 g sample, finely ground (mesh size less than ca 2 mm), into 100 mL volumetric flask, add ca 30 mL water, and homogenize using bio-mixer (a). Rinse shaft with additional small portions of water, add 25 mL 6N HCl, and dilute to volume with water. Heat for 30 min on steam bath and filter through glass fiber paper. Transfer 10 mL filtrate to 100 mL beaker, adjust pH to 5–6 with ammonia water, and add 50 mL 0.1M acetate buffer.

Column Chromatography

Prepare Amberlite CG-50 column as follows: Insert glass wool in bottom of chromatographic column (e) and add slurry of Amberlite CG-50 resin in 0.1M acetate buffer so that height of settled column is ca 5 cm. Add 50 mL water and then add sample solution to column. Rinse beaker with additional small portions of water and transfer rinses to column. Wash column with 10 mL water and 10 mL methanol at 3–4 mL/min. Drain and discard water and methanol from column. Elute paraquat and diquat with 50 mL 0.1N HCl–methanol at ca 3 mL/min, collecting eluate in 50 mL pear-shape flask. Evaporate eluate to dryness under reduced pressure on warm bath. Dissolve residue in 2 mL acetonitrile–methanol–water (2 + 1 + 1). Inject 50 μL portions of sample extract solution directly into liquid chromatograph and quantitate by data processor.

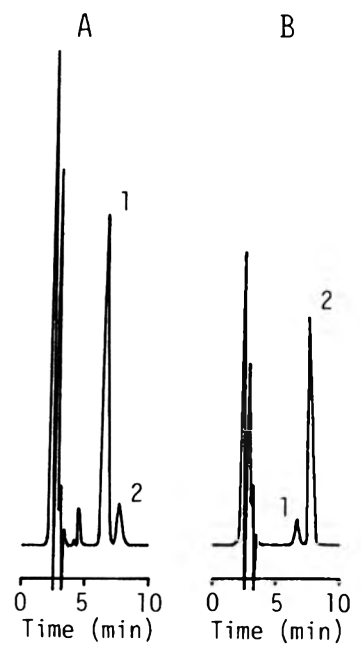

Figure 2. LC chromatograms of mixture of 1.0 μg/mL of paraquat dichloride (1) and diquat dibromide (2). A, detection at 257 nm; B, detection at 310 nm.

Recovery Experiments

Prepare 10 g samples of ground rice, wheat, and corn by adding 1 or 10 μg paraquat and diquat in 0.5 mL methanol. Seal containers with glass stoppers, mix contents by shaking for 1 min, and let stand for 1 day at approximately 20°C unstoppered to let solvent evaporate. For samples of cut potato, peach, and cabbage, transfer appropriate amounts of paraquat and diquat in methanol into containers, let solvent evaporate almost to dryness, and add samples. Seal containers with glass stoppers, mix contents by shaking for 1 min, and let stand in refrigerator for 1 day. Determine paraquat and diquat by LC analysis as described above.

Results and Discussion

Paraquat and diquat have a strong affinity for components in plant tissues, and even though they are water-soluble, they cannot be easily extracted once they have been sprayed onto and incorporated into the plant. Paraquat and diquat can be extracted almost completely by homogenizing the sample with the bio-mixer (a) and then heating the homogenized sample in acidic solution on a steam bath. However, a further cleanup step is necessary to detect low levels of paraquat and diquat by liquid chromatography. A chromatographic procedure using an Amberlite CG-50 column was used to purify and concentrate residues extracted from agricultural products. The cation-exchange resin in this column removes most coextracted materials from agricultural products.

Elution profiles from the Amberlite CG-50 column are presented in Figure 1. Both paraquat and diquat are eluted with 40 mL 0.1N HCl–methanol.

The LC analytical column is conditioned by passing the mobile phase through it for about 2 h before use. LC separation of paraquat and diquat standards is shown in Figure 2. Retention times are typically 6.5 and 7.8 min for paraquat and diquat, respectively.

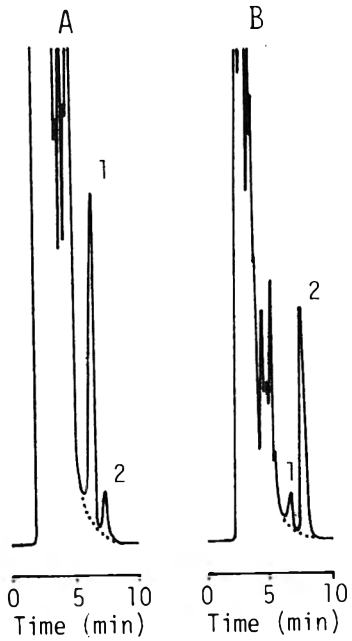

Figure 3. LC chromatograms of paraquat (1) and diquat (2) in rice. A, detection at 257 nm; B, detection at 310 nm. Dotted line, none added; solid line, 1.0 μg/g added.

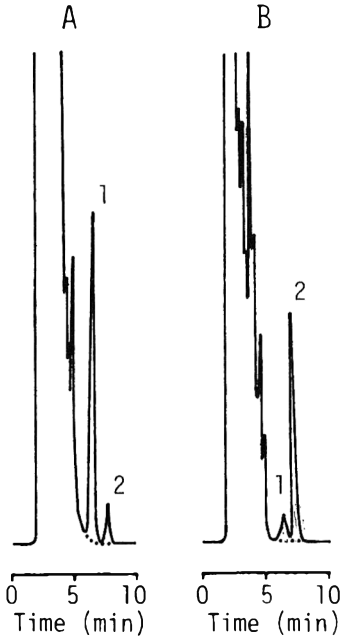

Figure 4. LC chromatograms of paraquat (1) and diquat (2) in peach. A, detection at 257 nm; B, detection at 310 nm. Dotted line, none added; solid line, 1.0 μg/g added.

Paraquat and diquat were added to rice and peach, and the samples were cleaned up on an Amberlite CG-50 column. Chromatograms of the purified samples (Figures 3 and 4) show that the naturally occurring background materials in the sample are well separated from paraquat and diquat, so that they cause minimal interference in the analyses. Other cleaned up samples gave similar chromatograms.

Linearity of the UV detector response for paraquat and diquat was verified by injecting 50 μL of eluant solutions in which the amount of paraquat dichloride and diquat dibromide varied from 1 to 50 ng per injection. Results were plotted as peak area vs amount of paraquat or diquat injected. The relationship was linear over the concentration range examined ($r = 0.9998$ and 0.9999). The lower limit of detection was approximately 0.5 ng (signal-to-noise ratio = 2) for both paraquat and diquat at 257 and 310 nm, at a range setting of 0.04 absorbance unit full scale. This corresponds to a lower detection limit of approximately 0.02 μg/g in the original samples. This limit of detection is very low, about 1/100–1/5 the lower detection limit of previous published methods (2, 5, 6, 14–16).

Results for recovery of paraquat and diquat added to rice, wheat, corn, potato, peach, and cabbage at concentrations of 0.1 or 1.0 μg/g are presented in Table 1. Recovery of paraquat from spiked samples ranged from 80 to 103% and averaged 91%; recovery of diquat ranged from 79 to 98% and averaged 90%. Peaks are sharp at an injection volume of 1 or 50 μL, which shows high sensitivity for paraquat and diquat. The proposed method was applied to 25 samples of commercial agricultural products obtained from retail stores; paraquat and diquat were not detected.

Table 1. Recovery by the proposed procedure of paraquat dichloride and diquat dibromide added to agricultural products[a]

	Paraquat dichloride added, μg/g		Diquat dibromide added, μg/g	
Sample	0.1	1.0	0.1	1.0
Rice	98 ± 3.5	103 ± 6.1	98 ± 5.0	92 ± 3.4
Wheat	84 ± 5.7	95 ± 3.0	90 ± 6.8	87 ± 4.1
Corn	90 ± 10.6	95 ± 6.4	90 ± 5.0	96 ± 7.5
Potato	89 ± 3.9	89 ± 8.0	93 ± 3.3	93 ± 7.8
Peach	93 ± 9.9	91 ± 7.6	90 ± 6.7	87 ± 5.5
Cabbage	84 ± 5.1	80 ± 3.4	81 ± 11.9	79 ± 4.1
Av. rec., %	90 ± 6.5	92 ± 9.5	90 ± 6.6	89 ± 7.6

[a] Average ± standard deviation of 3 determinations.

This method is a simple, accurate, rapid, quantitative procedure for determining paraquat and diquat in agricultural products. In particular, this proposed method gives a very low limit of detection, approximately 0.5 ng per injection, which corresponds to approximately 0.02 μg/g of paraquat or diquat in agricultural products. The method has a potential for application to the analysis of bipyridylium herbicide residues in various foodstuffs.

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2-Chloroethyl Fatty Acid Esters as Indicators of 2-Chloroethanol in Black Walnuts, Seasoning Mixes, and Spices

MARTIN P. YURAWECZ
Food and Drug Administration, Division of Food Chemistry and Technology, Washington, DC 20204

Residues of 2-chloroethyl fatty acid esters (CEEs) and 2-chloroethanol (ECH), by-products of ethylene oxide fumigation, were determined in black walnuts, seasoning mixes, and spices. Extracts containing ECH and CEE were cleaned up by previously described procedures, and residue levels were quantitatively determined using a gas chromatograph equipped with a halogen-selective electrolytic conductivity detector. All food products that contained CEE residues also contained ECH. ECH residues ranged from <0.2 to 880 ppm and were <0.2-7 times the CEE levels found.

Since 2-chloroethyl fatty acid esters (CEEs) were first reported in foods (1, 2), they have been among the highest level organic residues found in the U.S. Food and Drug Administration's Total Diet Studies. In the most recently published Total Diet Study reports (3, 4), for 1978–1982, estimates of the total dietary intake (μg/kg body weight/day) of 2-chloroethyl linoleate ($CEE_{18:2}$) ranged from 0.79 to 0.228 for adults and from 0.145 to 0.448 for toddlers.

CEEs and 2-chloroethanol (ethylene chlorohydrin or ECH) are by-products of ethylene oxide fumigation (2, 5). Furthermore, CEEs are converted in model digestive systems to ECH, and ECH is the focus of toxicological concern associated with CEE residues in foods (6). Levels of ECH found in spices (7–10) are similar to those of CEEs. The above facts emphasize the need to assess human exposure to these by-products in foods. Estimates of dietary intake are available for CEEs, which are recovered from a wide variety of foods by methods normally employed for determining organochlorine and organophosphate pesticides (11); however, dietary intake data are not available for ECH because no methods have been reported for determining it in composite foods, e.g., hot dogs, chili, etc.

This study is the first to investigate the relationship of residues of ECH and CEEs in spices, nuts, and seasoning mixes to determine the extent to which CEE residues are indicative of the presence of ECH. The ultimate goal of this research is to evaluate whether a relationship exists between ECH and CEE levels which could be used to estimate the human dietary exposure to both ECH and ECH bound to fatty acids (CEEs). ECH residues were determined in 46 samples of black walnuts, seasonings, and spices, and 21 of the samples were analyzed for CEEs. All of the food products used in this study were purchased from local grocery stores.

Experimental

Reagents

(a) *General reagents.*—See sec. **29.002** (11). Solvents and reagents were tested for interferences using the gas chromatographic (GC) parameters described below.

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(b) *2-Chloroethanol (ECH).*—Aldrich Chemical Co., Inc., Milwaukee, WI 53233, No. 18,574-4.

(c) *1-Chloro-2-propanol.*—Aldrich, No. 17,552-8.

(d) *2-Chloroethyl esters.*—Synthesized in manner similar to that previously described (2): Add 0.1–1.0 g fatty acid to 5–10 mL ECH. Add 1 drop of HCl and place solution on steam bath for 15 min. Transfer solution to 1 L separatory funnel with 100 mL pentane and 200 mL water. Shake 1 min, then discard aqueous layer. Wash pentane solution with three 200 mL portions of water, dry by passing through 50 g Na_2SO_4, then chromatograph on Florisil with 200 mL 15% ethyl ether (EE) in pentane. Evaporate solution on steam bath to remove pentane. Purities of CEEs were >95% in all cases as determined by gas chromatography using flame ionization detector.

Apparatus

(a) *General apparatus.*—See sec. **29.005** (11).

(b) *Gas chromatograph.*—Varian 3700 equipped with Tracor 560/700A Hall electrolytic conductivity detector operated in halogen-selective mode and following columns: (*1*) 6 ft (1.8 m) × 2 mm id 20% Carbowax 20M on 80–100 mesh Supelcoport; (*2*) 6 ft (1.8 m) × 2 mm id 5% SE-30 on 80–100 mesh Supelcoport. Operating conditions: solvent flow rate (mL/min)—*n*-propanol, 0.35, hydrogen reactant gas, 70, nitrogen carrier gas, 30; temperatures (°C)—injection port, 250, reactor base, 250, nickel reactor tube, 1000, column 1, 130, column 2, 150 raised 5°/min to 250; solvent vent, 1.75 min. Signal was recorded with Hewlett-Packard 3388A integrating terminal with attenuator set to give ½ full scale deflection (FSD) for ca 5 ng ECH, which elutes in 5 min using column 1. Attenuator was set to give ½ FSD for ca 15 ng 2-chloroethyloleate ($CEE_{18:1}$), which eluted in 17 min using column 2.

(c) *Gas chromatograph.*—Varian 3700 equipped with flame ionization detector and column 2 as described above. Operating conditions: as described above with detector temperature, 300°C; flame gases—hydrogen, 30 mL/min, and air, 300 mL/min. Attenuator was set to give FSD for ca 1 μg CEE.

(d) *Gas chromatograph.*—Hewlett-Packard 5880 series equipped with Finnigan MAT 700 ion trap detector and 25 m × 0.20 mm id 0.25 μm film thickness, 5% phenyl–95% methyl silicone-wall-coated open tubular capillary column. Operating conditions: temperatures (°C)—column, 60 for 1 min, raised to 200 at 30°/min, then raised 5°/min to 250, injector, 250, manifold, 230, transfer line, 250; helium flow set to 28 cm/s linear velocity; multiplier 1950 V. Full scan acquisition from 50 to 500 m/z was acquired at 1 scan/s, using IBM PC XT with software supplied with ion trap detector.

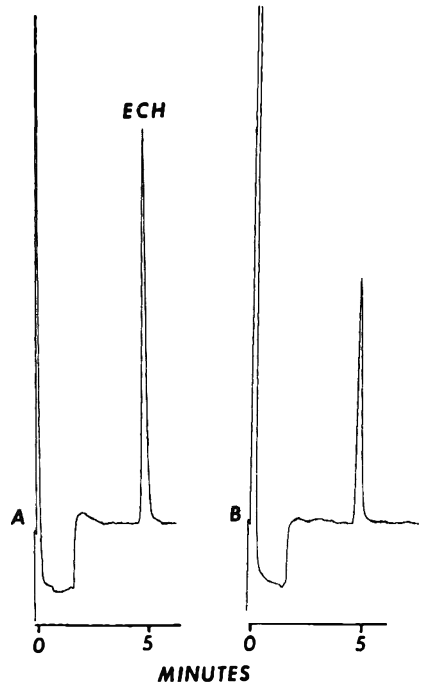

Figure 1. Chromatograms of ECH, using GC parameters in Apparatus (b), column 1: A, 5.58 ng ECH; B, 0.00965 mg equivalent injection of paprika extract containing 361 ppm ECH.

(e) *Gas chromatograph/mass spectrometer/data system.* — Finnigan MAT 4023 quadrupole gas chromatograph/mass spectrometer interfaced to INCOS 2300 data system equipped with 50 m × 0.25 mm Carbowax 20M silica-wall-coated open tubular capillary column. Operating conditions: temperatures (°C)—injection port, 150, separator transfer line, 180, column, 40 for 1 min, then increased to 200 and held constant, source, 220; helium carrier gas to 40 cm/s linear velocity. Spectrometer was operated in chemical ionization mode using methane reagent gas at 0.1 torr. Full positive ion scans from m/z 60 to 560 were acquired at rate of 1 s/scan.

Extraction and Cleanup

For ECH determination, test portions weighing from 2 to 10 g were extracted 4 h with 30 mL acetone–water (5 + 1) (7). The extract was filtered through sharkskin and, where appropriate, diluted with acetone before GC analysis. Spices and seasoning mixes were cleaned up for CEE determination using the AOAC method for organochlorine and organophosphate pesticides in dry products, sec. **29.011(d)** (11). Test portions were extracted with acetonitrile–water, partitioned into petroleum ether (PE), and chromatographed on Florisil. CEEs eluted in both the 6 and 15% EE/PE eluates, sec. **29.015** (11).

Black walnuts were extracted and cleaned up for CEE determination as follows: Ten g black walnuts was added to 100 mL glass-stopper graduated cylinder, 100 mL EE was added, and cylinder was shaken 10 s. Shaking was repeated for 10 s 12 h later, solution was filtered through 50 g Na_2SO_4 into 400 mL beaker, and EE was evaporated on steam bath. Walnut oil was dissolved in 100 mL PE; 15 mL aliquot of PE (1.5 g equivalent test portion) was cleaned up using acetonitrile–PE partitioning, sec. **29.014** (11), and was chromatographed on Florisil, sec. **29.015** (11).

Gas Chromatography

For CEE determination, the volumes of the Florisil eluates were adjusted to obtain 20–80% FSD in a 3–8 μL injection

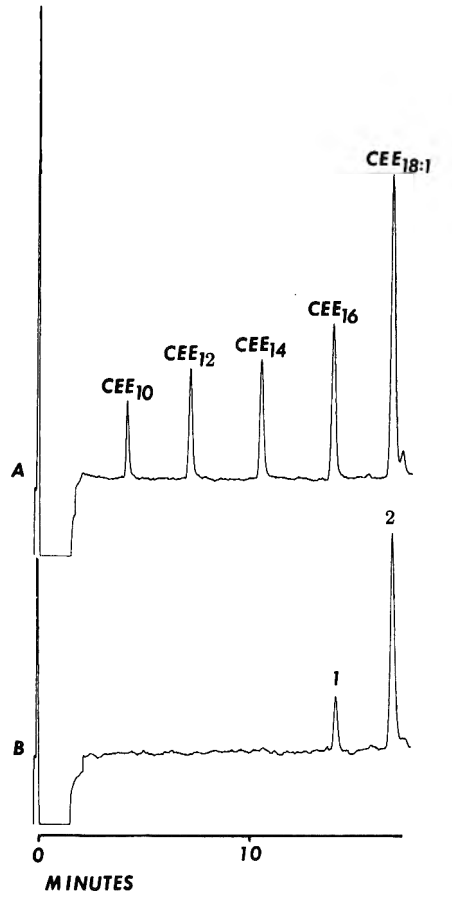

Figure 2. Chromatograms of CEEs, using GC parameters in Apparatus (b), column 2: A, 1.5 ng CEE_{10}; 3.0 ng CEE_{12}; 3.8 ng CEE_{14}; 7.6 ng CEE_{16}; 15.2 ng $CEE_{18:1}$; B, 0.069 mg equivalent injection of 6% EE/PE Florisil eluate of ground pepper showing presence of 1, 48 ppm CEE_{16} and 2, 187 ppm $CEE_{18:2}$.

for the major CEE residue present. The response was compared with that obtained for a comparable amount of reference material (external standard procedure). Using column 2 [Apparatus **(b)**], a 20 mg test portion equivalent injection could typically be used to determine ca 0.2 ppm 2-chloroethyl palmitate (CEE_{16}) or $CEE_{18:1}$ at a signal-to-noise ratio of 10:1.

ECH residues were determined using column 1 [Apparatus **(b)**]. Method sensitivity for a 1 mg equivalent injection, 3 μL/30 mL for a 10 g test portion, was typically 0.4 ppm with a 10:1 signal-to-noise ratio.

Results and Discussion

The selectivity of the Hall detector for halogen relative to hydrocarbon- and oxygen-containing compounds allows for determination of ECH without the need to isolate the diacetone alcohol (4-hydroxy-4-methyl-2-pentanone) which is present in the acetone–water (5 + 1) extraction solvent. Figure 1 shows chromatograms of 5.58 ng ECH and 0.00965 mg equivalent injection of paprika extract that contained 361 ppm ECH. These chromatograms are typical of the quality obtained for residues at levels >3 ppm. No extraneous responses were recorded at retention times >10 min. The column operating temperature was held constant at 130°C throughout this study, and typical total analysis time was <20 min/sample. Although extensive recovery studies on the extraction procedure have been previously reported (7), the commodities used here were not examined. In the present study, ECH was recovered from paprika (spike 3.6 ppm) at

Table 1. ECH and CEEs[a] found (ppm) in commercial products[b]

Product	ECH	CEE	Ratio ECH/CEE
Allspice	810	280	2.9
Allspice	830[c]	250[c]	3.5
Black pepper, ground	0.2	—[d]	—
Black pepper, ground	0.5	ND[e]	—
Black pepper, ground	45	—	—
Black pepper, whole	43	—	—
Black walnuts	1.9	6.7	0.29
Black walnuts	2.2	11	0.20
Black walnuts	2.3	10	0.23
Black walnuts	4.8	22	0.22
Chili powder	18	—	—
Chill powder	25	—	—
Chili powder, hot Mex. style	1.4	—	—
Chili powder, inst. chill mix	0.8	—	—
Chili powder, inst. chili mix	1.5	—	—
Chili seasoning mix	45	—	—
Cocoa	ND	—	—
Cocoa	ND	—	—
Mint flakes, dehydrated	0.6	—	—
Mushroom slices, freeze dried	ND	—	—
Mushroom slices, freeze dried	0.4	—	—
Nacho cheese mix	ND	—	—
Nacho cheese mix	14	—	—
Nutmeg, ground	18	7.7	2.3
Nutmeg, ground	16	—	—
Nutmeg, ground	30	—	—
Nutmeg, ground	37	—	—
Onion, powder	ND	—	—
Onion, powder	ND	—	—
Paprika	ND	ND	—
Paprika	0.1	ND	—
Paprika	0.8	—	—
Paprika	360	660	0.55
Paprika	513[c]	1034[c]	0.50
Parsley flakes	0.4	—	—
Red pepper, crushed	475	67	7.1
Red pepper, crushed	490[c]	97[c]	5.1
Red pepper, ground	3.6	2	1.8
Red pepper, ground	192	140	1.4
Red pepper, ground	356	268	1.3
Seafood seasoning	33	140	1.4
Taco seasoning mix	115[c]	48[c]	2.4
Taco seasoning mix	117	28	4.2
Vegetable flakes	0.35	—	—
Vegetable flakes	0.6	—	—
White pepper	0.78	ND	—

[a] Sum of C_{14}–C_{18} CEEs.

[b] Raw values, not uniformly rounded off to indicate proper number of significant figures.

[c] Qualitatively confirmed by GC/MS: ECH using Apparatus **(e)**, CEEs using Apparatus **(d)**.

[d] No analysis.

[e] ND = not detected for ECH (<0.1–<0.6 ppm), for CEEs (<0.2–<2 ppm).

93 and 97% and from walnuts (spike 1.8 ppm) at 80 and 84%. It was considered that these commodities were representative of those studied here and that comprehensive recovery experiments were unnecessary.

The chromatograms in Figure 2 show 1.5 ng 2-chloroethyl caprate (CEE_{10}), 3.0 ng 2-chloroethyl laurate (CEE_{12}), 3.8 ng 2-chloroethyl myristate (CEE_{14}), 7.6 ng CEE_{16}, 15.2 ng $CEE_{18:1}$, and 0.069 mg test portion equivalent injection of a 6% EE/PE Florisil eluate of ground pepper. In nutmeg, CEE_{14} was the major residue. In all other products, $CEE_{18:2}$ was the major residue. In all products, $CEE_{18:1}$, which has the same response as $CEE_{18:2}$ on the Hall detector, was used to quantitate residues eluting after CEE_{16}. CEE recoveries ranged from 73 to 112% in black walnuts and from 71 to 122% in paprika.

Food product selection was based primarily on previously reported findings (2, 7–10). Residue findings for the 46 products are presented in Table 1. The wide range of ECH levels found, 0.14–880 ppm, is similar to previously published values (7–10) as are the CEE levels, 2–1034 ppm (2). As indicated in Table 1, some results were qualitatively confirmed by mass spectrometry (MS). In addition, 1-chloro-2-propanol (average 15 ppm) was confirmed by GC/MS in 3 of the 4 black walnuts. No relationships were observed with residue levels vs various products or brands.

Ratios of ECH/CEE (total of C_{14}–C_{18}) ranged from 0.2 to 7.1 for those products that contained measurable levels of both residues. The ratios are probably determined by the fatty acid content of the products examined (2). ECH/CEE ratios were within a narrow range for particular products, e.g., ground red pepper 1.3–1.8 and black walnuts 0.20–0.29.

Conclusions

In all 17 test portions that contained CEE residues, ECH was also found. This demonstrates that CEE residues are indicators of the presence of ECH in the products examined. Furthermore, the relatively narrow range of ratios of ECH/CEEs for particular products suggests that by studying the food ingredients fumigated with ethylene oxide not examined in this work it may be possible to obtain an estimate of the total dietary human exposure to free and bound ECH (CEEs) using the existing Total Diet intake values for CEE residues (3, 4).

Acknowledgment

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Behavior of 78 Pesticides and Pesticide Metabolites on Four Different Ultra-Bond Gas Chromatographic Columns

JOHN F. SUPROCK and J. HOWARD VINOPAL
U.S. Army Environmental Hygiene Agency, Aberdeen Proving Ground, MD 21010

The gas chromatographic (GC) elution order and relative retention time data (compared to aldrin) are presented for 78 pesticides and pesticide metabolites on 4 different types of commercially available 2 mm id Ultra-Bond columns including Ultra-Bond 20M (20M), Ultra-Bond 20SE (20SE), Ultra-Bond 20M coated with 1% OV-210 (OV-210), and Ultra-Bond 20M coated with 0.5% OV-210 + 0.65% OV-17 (mixed phase). Relative retention time data (compared to parathion) are also represented for 19 organophosphorus insecticides on the 4 Ultra-Bond columns evaluated. Corresponding 4 mm id Ultra-Bond columns were evaluated at the same time as the 2 mm id columns, and results and comparisons for these larger-diameter columns are discussed. These data indicate that, with aldrin as a reference peak, a complement of the mixed-phase column and either the 20M, the 20SE, or the OV-210 column represents a useful chromatographic tool for dual-column analysis of pesticide residues. The 2 mm id columns were more useful in chromatographing later-eluting pesticides whereas the corresponding 4 mm id columns were more useful in chromatographing earlier-eluting pesticides.

Ultra-Bond® 20M column packing, introduced commercially in 1976 by RFR (now ULTRA Scientific), was the first in a family of gas chromatographic (GC) packings based on a method developed by Aue et al. (1). The product of this method was a packing having an ultrathin film of surface-bonded Carbowax 20M on highly deactivated Chromosorb W. Carbowax 20M can be used by itself or lightly coated (e.g., 1–5%) with common liquid phases.

Lorah and Hemphill (2) used this surface-modified support with an alkali flame detector to chromatograph intact several carbamate pesticides. Later, Hall and Harris (3), using electrolytic conductivity detection, determined relative retention indices for 24 carbamate pesticides on 6 different commercially available Ultra-Bond 20M and coated Ultra-Bond 20M surface-modified supports.

Our laboratory has been using the Ultra-Bond 20M column packings since 1978 for specific types of pesticide residue analyses, for confirmatory column determinations, and for certain difficult separations. The success achieved with the use of these packings has led us to further investigations on the GC behavior of numerous pesticides of different classes.

The chromatographic elution order and relative retention times of 78 pesticides and pesticide metabolites from 9 pesticide classes on 4 different Ultra-Bond GC columns using electron-capture detection are presented. The chromatographic data generated in the present study of Ultra-Bond columns should prove useful to laboratories involved in pesticide residue analyses. To our knowledge, information on the chromatographic behavior of many of the pesticides studied in this paper on Ultra-Bond columns has not been previously published.

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Ultra-Bond® is a registered trade name of ULTRA Scientific, Hope, RI 02831. Use of trade names does not imply endorsement by the U.S. Army but is intended only to assist in identification of a specific product.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

Experimental

Apparatus and Reagents

(a) *Gas chromatograph.*—Tracor Model 560 equipped with dual linearized ^{63}Ni electron-capture detectors. Operating conditions: argon–methane (95 + 5 v/v) carrier gas flowing at 25–30 ml/min (2 mm id column) or 55–60 mL/min (4 mm id column); temperatures, °C—detector, 325°, column, 190–205 (depending on column type used), inlet, 160 (an early Tracor 560 model with recessed septum retainer was used, necessitating a reduced inlet temperature to prevent rapid septum deterioration); detector saturation current, 6.0×10^{-9} Å.

(b) *Analytical balance.*—Electronic, Mettler Model AE163.

(c) *Ultra-Bond GC columns.*—Ultra-Bond 20M (20M), Ultra-Bond 20SE (20SE), Ultra-Bond 20M coated with 1% OV-210 (OV-210), and Ultra-Bond 20M coated with 0.5% OV-210 + 0.65% OV-17 (mixed phase) (ULTRA Scientific, Hope, RI 02831). Empty silanized glass columns—6 ft × 2 mm id or 6 ft × 4 mm id (Supelco, Inc., Bellefonte, PA 16823-0048)—were rinsed with acetone and petroleum ether, dried under nitrogen, and packed with appropriate Ultra-Bond packing, using house vacuum and hand-held vibrator. Silanized glass wool (Supelco) was used to plug column ends.

All Ultra-Bond columns were heat-conditioned 1 week at 230°C, using ultrahigh purity nitrogen as carrier with flow rates of 20–30 mL/mm for 2 mm id columns and 50–60 mL/min for 4 mm id columns. Columns were not connected to the detector during conditioning.

(d) *Solvents.*—Isooctane, toluene, methanol, acetone, petroleum ether, and ethyl acetate, distilled in glass (EM Science, Cherry Hill, NJ 08034, and Anachemia, Montreal, Quebec, Canada).

(e) *Analytical standards.*—Methyl esters of 2,4-D, 2,4,5-T, 2,4-DB, and silvex were obtained from PolyScience Corp. (Niles, IL 60648); all other standards were obtained from EPA Pesticides and Industrial Chemicals Repository (Research Triangle Park, NC 27711). Using appropriate solvent, primary standard solutions of 100–200 μg/mL concentration were prepared for each compound. Primary standard solutions were diluted with isooctane (and in some instances toluene) to achieve working range level (generally 25–75% full-scale deflection under GC operating conditions used). All analytical standards were prepared fresh before chromatography. All primary and secondary dilutions of analytical standards were stored in freezer at −15°C.

Chromatographic Procedures

In this study, solutions of individual compounds were chromatographed on each of the Ultra-Bond columns. Injection volumes of 6–8 μL were used. Standards of aldrin (or parathion) were not present in each injected solution, but were chromatographed at the beginning of each day and then rechromatographed after every fourth or fifth injection throughout the day. Relative retention time data were derived in most part from single chromatographic determinations at the working concentration level.

Table 1. Pesticides and pesticide metabolites (by pesticide class) subjected to GC on Ultra-Bond columns

Benzenhexachloride (BHC) insecticides:
- α-BHC
- β-BHC
- δ-BHC
- Lindane

DDT insecticides:
- *o,p'*-DDE
- *p,p'*-DDE
- *o,p'*-DDD
- *p,p'*-DDD
- *o,p'*-DDT
- *p,p'*-DDT

Cyclodiene insecticides:
- Aldrin
- *trans*-Chlordane
- *cis*-Chlordane
- α-Chlordene
- γ-Chlordene
- Chlordene
- Compound C
- Dieldrin
- Endosulfan I
- Endosulfan II
- Endosulfan sulfate
- Endrin
- Endrin aldehyde
- Heptachlor
- Heptachlor epoxide
- Oxychordane
- *trans*-Nonachlor

Organophoshorus insecticides:
- Carbophenothion
- Chlorfenvinphos
- Chlorpyrifos
- Cyanophos
- Diazinon
- Diazinon oxygen analog
- Dichlofenthion
- Dimethoate
- Ethion
- Fenitrothion
- Isofenphos
- Malathion
- Malathion oxygen analog
- Methyl parathion
- Monocrotophos
- Parathion
- Phencapton
- Ronnel
- Zytron

Miscellaneous insecticides:
- Methoxychlor
- Mirex

Chlorophenoxy and benzoic acid herbicides:
- 2,4-D, methyl ester
- 2,4-DB, methyl ester
- Dimethyl-2,3,5,6-tetrachloroterephthalate (DCPA)
- Dicamba, methyl ester
- Dichlorprop, methyl ester
- Fenac, methyl ester
- Methylchlorophenoxyacetic acid (MCPA), methyl ester
- Silvex, methyl ester
- 2,4,5-T, methyl ester

Triazine herbicides:
- Atrazine
- Propazine
- Simazine

Miscellaneous herbicides:
- Alachlor
- Benefin
- Bromacil
- Chloroallyl diethyldithiocarbamate (CDEC)
- Dichlobenil
- Nitrofen
- Oxadiazon
- Picloram, methyl ester
- Pronamide
- Propanil
- Trifluralin

Miscellaneous fungicides:
- Captan
- Chloroneb
- Chlorothalonil
- Ethazol
- Hexachlorobenzene
- Pentachloronitrobenzene (PCNB)
- Triadimefon

Results and Discussion

The pesticides and pesticide metabolites chromatographed in this study are listed (by pesticide class) in Table 1. Table 2 presents the chromatographic behavior and relative retention times (compared to aldrin) for the 78 pesticides and pesticide metabolites on the 4 different Ultra-Bond 2 mm id column types. Table 3 presents the chromatographic behavior and relative retention times (compared to parathion) for 19 organophosphorus insecticides on the same 4 Ultra-Bond 2 mm id columns.

The 78 pesticides and pesticide metabolites listed in Table 1 represent 9 diverse classes of pesticides. Standards for these compounds were obtained and prepared over a period of several years. As a result, not all of the compounds listed in Table 1 were available at the time a particular Ultra-Bond column type was being evaluated. The Ultra-Bond columns were evaluated in the following order: 20M, 20SE, OV-210, and mixed-phase. Of the compounds listed in Table 1, 61 were chromatographed on all 4 columns. The remaining 17 compounds were chromatographed only on the OV-210 and mixed-phase columns except for CDEC and PCNB which were chromatographed only on the mixed-phase column.

The absolute retention times for aldrin (measured from the beginning of the solvent peak) under the chromatographic operating conditions described above were 1.10 min for both the 20M and 20SE columns, 1.46 min for the OV-210 column, and 2.28 min for the mixed-phase column. The relative retention time (RRT) data (compared to aldrin) for the pesticides and pesticide metabolites listed in Table 2 were generated at slightly differing oven temperatures for the 4 columns. The oven temperature used for each of the 2 mm id columns represented the highest temperature at which the earliest eluting compound evaluated was separated from the solvent front on the corresponding 4 mm id columns. The 2 different sized columns were evaluated at the same time, and the results of these evaluations are discussed later in this section. In spite of slightly differing oven temperatures, several general statements regarding the RRT data of Table 2 can be made. The 20M and 20SE columns showed very similar RRTs for most of the compounds evaluated. The RRTs for most of the compounds evaluated on the OV-210 column were similar to the RRTs on the 20M and 20SE columns. However, overall, the RRTs for the 20M and 20SE columns were closer to each other than they were to the RRTs for the OV-210 column. The RRTs for most of the compounds on the mixed-phase column were dissimilar to the RRTs obtained using the other 3 columns. In general, the RRT values for the mixed-phase column were numerically lower than those for the 3 other Ultra-Bond columns.

Table 2. Chromatographic behavior and relative retention times (compared to aldrin) for 78 pesticides and pesticide metabolites on 4 different Ultra-Bond columns, using electron-capture detection

Pesticide[a]	Type of Ultra-Bond column[b] 20M (205°C)	20SE (190°C)	1% OV-210 (195°C)	0.5% OV-210–0.65% OV-17 (200°C)
Ethazol	—[c]	—	0.24	0.20
Dichlobenil	coelutes with solvent	coelutes with solvent	0.30	0.23
Benefin	—	—	0.38	0.28
Trifluralin	—	—	0.38	0.29
Chloroneb	—	—	0.38	0.30
Dicamba, methyl ester	coelutes with solvent	coelutes with solvent	0.41	0.32
MCPA, methyl ester	—	—	0.54	0.40
Dichlorprop, methyl ester	—	—	0.54	0.40
Hexachlorobenzene	0.41	0.41	0.41	0.44
Fenac, methyl ester	—	—	0.57	0.47
CDEC	—	—	—	0.55
2,4-D, methyl ester	0.81	0.80	0.84	0.55
α-BHC	0.79	0.76	0.76	0.59
Diazinon	0.81	0.96	0.73	0.59
Chlordene	0.70	0.68	0.68	0.59
Silvex, methyl ester	0.81	0.84	0.81	0.62
PCNB	—	—	—	0.63
Diazinon oxygen analog	1.00	1.15	0.92	0.66
Compound C	0.74	0.76	0.76	0.71
Heptachlor	0.93	0.93	0.92	0.85
2,4,5-T, methyl ester	1.30	1.32	1.35	0.87
Lindane	1.15	1.08	1.16	0.88
α-Chlordene	0.96	1.00	0.97	0.90
Dichlofenthion	1.15	1.23	1.14	0.90
Propazine	1.56	1.65	1.62	0.92
Aldrin[d]	1.00	1.00	1.00	1.00
2,4-DB, methyl ester	—	—	1.46	1.00
Alachlor	1.37	1.42	1.43	1.07
Pronamide	—	—	2.08	1.07
Atrazine	1.96	2.04	2.05	1.08
γ-Chlordene	1.30	1.32	1.38	1.19
Ronnel	1.48	1.50	1.59	1.20
Simazine	2.48	2.50	2.68	1.30
Chlorothalonil	—	—	1.73	1.32
β-BHC	2.24	2.00	2.35	1.32
Cyanophos	2.19	2.12	2.46	1.35
Chlorpyrifos	1.63	1.81	1.70	1.39
Oxychlordane	1.59	1.62	1.65	1.44
Zytron	2.15	2.35	2.32	1.52
Malathion oxygen analog	2.48	2.77	2.73	1.58
DCPA	2.04	2.12	2.30	1.61
Heptachlor epoxide	1.96	1.92	2.16	1.68
δ-BHC	—	2.93	3.22	1.73
Dimethoate	3.11	3.08	3.49	1.75
Monocrotophos	3.52	3.81	4.05	1.76
Malathion	2.56	2.81	2.73	1.78
trans-Nonachlor	1.93	2.08	2.05	1.83
trans-Chlordane	2.28	2.23	2.35	1.88
Triadimefon	—	—	3.22	1.93
cis-Chlordane	2.24	2.17	2.41	1.97
Methyl parathion	3.00	2.96	3.46	1.97
Isofenphos	—	—	2.86	2.02
Fenitrothion	3.11	3.19	3.59	2.07
Parathion	3.00	3.15	3.49	2.08
Endosulfan I	2.26	2.23	2.37	2.10
o,p'-DDE	2.48	2.50	2.73	2.10
Chlorfenvinphos	3.15	3.62	3.49	2.32
p,p'-DDE	3.15	3.15	3.54	2.62
Dieldrin	3.11	3.04	3.38	2.66
Oxadiazon	—	—	3.76	2.67
Propanil	5.93	6.31	7.11	3.00
Endrin	3.26	3.12	3.55	3.14
o,p'-DDD	4.41	4.46	5.11	3.34
Captan	4.70	4.73	5.38	3.47
o,p'-DDT	4.34	4.23	4.78	3.63
Picloram, methyl ester	6.89	6.96	8.24	3.77
Ethion	—	—	6.62	4.51
Endosulfan II	6.11	6.45	7.00	4.73
p,p'-DDD	7.07	7.27	8.49	4.90
Nitrofen	7.30	7.64	8.95	5.24
p,p'-DDT	6.81	6.96	7.97	5.31
Carbophenothion	7.26	8.12	8.43	5.62
Bromacil	12.89	14.20	15.76	6.10
Endrin aldehyde	9.26	8.85	10.53	6.30
Mirex	7.04	7.04	8.08	7.02
Endosulfan sulfate	11.59	12.40	14.26	8.20
Phencapton	11.89	13.70	14.27	9.23
Methoxychlor	14.93	16.00	18.24	10.87

[a] Listed in order of relative retention time on mixed-phase column.
[b] All columns were 2 mm id × 6 ft long; column oven temperatures are given in parentheses.
[c] Analytical standard for this pesticide was not available at the time this column was evaluated.
[d] Absolute retention times for aldrin (measured from the beginning of the solvent peak) on the 20M, 20SE, OV-210, and mixed-phase columns were 1.10, 1.10, 1.46, and 2.28 min, respectively.

The absolute retention times for parathion under the chromatographic operating conditions used were 3.30, 3.47, 5.10, and 4.74 min for the 20M, 20SE, OV-210, and mixed-phase columns, respectively. Regarding the RRTs (compared to parathion) for the organophosphorus insecticides listed in Table 3, it is apparent that the 20M and 20SE columns again have very similar RRTs for this group of pesticides. Also, the RRTs for the OV-210 column were similar to those on the 20M and 20SE columns for most of the organophosphorus compounds. In contrast to the RRT data of Table 2 (relative to aldrin), the RRT data for the organophosphorus compounds listed in Table 3 (relative to parathion) on the mixed-phase column were similar to those of the other 3 columns.

It appears that the data of Table 2, using aldrin as the reference compound, shows greater overall diversity in RRT values for the 4 columns evaluated than do the data of Table 3, using parathion as the reference compound. From a practical chromatography standpoint, with aldrin as a reference peak, we can conclude that a complement of the mixed-phase column and either the 20M, the 20SE, or the OV-210 column represents a useful and powerful tool for the dual-column identification, resolution, and confirmation of pesticide residues. The data presented in Table 2 represent perhaps the most significant contribution of this paper.

As mentioned earlier in this section, in addition to the evaluation of the four 2 mm id Ultra-Bond columns shown in Tables 2 and 3, a comparable evaluation was made with 4 mm id columns of Ultra-Bond 20M, 20SE, OV-210, and mixed phase. The chromatographic behavior and RRT data for the 4 mm id columns are not presented in tabular form in this paper since these data are essentially identical to the corresponding 2 mm id column data. Identical elution order and RRTs (compared to aldrin and parathion) were obtained with all the compounds studied on corresponding 4 mm id and 2 mm id Ultra-Bond columns. Some differences, however, were noted between the 2 mm and the 4 mm id columns. The absolute retention times for chromatographed com-

Table 3. Chromatographic behavior and relative retention times (compared to parathion) for 19 organophosphorus insecticides on 4 different Ultra-Bond columns, using electron-capture detection

	Type of Ultra-Bond column[b]			
Pesticide[a]	20M (205°C)	20SE (190°C)	1% OV-210 (195°C)	0.5% OV-210–0.65% OV-17 (200°C)
Diazinon	0.27	0.30	0.21	0.28
Diazinon oxygen analog	0.33	0.37	0.26	0.31
Dichlofenthion	0.38	0.39	0.33	0.43
Ronnel	0.49	0.48	0.46	0.57
Cyanophos	0.73	0.67	0.71	0.65
Chlorpyrifos	0.54	0.57	0.49	0.67
Zytron	0.72	0.74	0.67	0.73
Malathion oxygen analog	0.83	0.88	0.79	0.74
Dimethoate	1.04	0.98	1.00	0.82
Monocrotophos	1.16	1.21	1.17	0.83
Malathion	0.84	0.89	0.78	0.86
Isofenphos	—[c]	—	0.82	0.92
Methyl parathion	1.00	0.94	0.99	0.93
Fenithrothion	1.04	1.01	1.03	1.00
Parathion[d]	1.00	1.00	1.00	1.00
Chlorfenvinphos	1.05	1.15	1.00	1.10
Ethion	B	B	1.91	2.13
Carbonphenothion	2.39	2.57	2.44	2.57
Phencapton	3.96	4.35	4.13	4.46

[a] Listed in order of relative retention time on mixed-phase column.

[b] All columns were 2 mm id × 6 ft long; column oven temperatures are given in parentheses.

[c] Analytical standard for this pesticide was not available at the time this column was evaluated.

[d] Absolute retention times for parathion (measured from the beginning of the solvent peak) on the 20M, 20SE, OV-210, and mixed-phase columns were 3.30, 3.47, 5.10, and 4.74 min, respectively.

pounds on the 4 mm id columns were approximately twice as long as those obtained on the 2 mm id columns. This can be an advantage when early-eluting compounds are being chromatographed, but it is a disadvantage for later-eluting compounds. The electron-capture detector response (in terms of peak height) to the compounds listed in Table 1 was greater when these compounds were chromatographed on the 2 mm id columns than when these same compounds were chromatographed on the 4 mm id columns. For most of the compounds, at least a 2-fold detector response difference (in terms of peak height) was noted between the 2 mm id and 4 mm id columns. Overall, for the compounds listed in Table 1, the amount of material to give approximately 50% full-scale deflection under the GC operating conditions used ranged as follows: 0.015 ng (for hexachlorobenzene) to 200 ng (for monocrotophos) on the 2 mm id 20M column (the most sensitive 2 mm id column type evaluated); and 0.050 ng (for hexachlorobenzene) to 1400 ng (for monocrotophos) on the 4 mm id 20M column (the most sensitive 4 mm id column type evaluated). For some reason, captan could only be chromatographed on one of the 4 mm id columns—the mixed phase—whereas this compound was satisfactorily chromatographed on all four 2 mm id columns. With the 4 mm id 20SE column, and to a lesser extent with the corresponding 2 mm id column, some undesirable peak broadening was observed for compounds eluting later than aldrin. In general, most of the differences observed in this paper between corresponding 2 mm id and 4 mm id Ultra-Bond columns reflect known, basic GC principles.

Our laboratory plans to continue evaluating and obtaining RRT data for Ultra-Bond columns. Relative retention data for additional pesticides and pesticide metabolites using electron-capture detection will be completed for the 4 types of Ultra-Bond columns. We plan to generate Ultra-Bond RRT data for the organophosphorus insecticides listed in Table 1, as well as for additional organophosphorus compounds, using flame photometric detection. Also planned is the collection of Ultra-Bond RRT data for a series of triazine type pesticides using nitrogen-phosphorus detection.

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Extraction of Polycyclic Aromatic Hydrocarbons from Spiked Soil

MERVIN P. COOVER, RONALD C. SIMS,[1] and WILLIAM DOUCETTE
Utah State University, Utah Water Research Laboratory, Logan, UT 84322

A homogenization method was evaluated for extracting polycyclic aromatic hydrocarbons (PAHs) from soils. Fifteen PAHs were spiked and recovered from 2 soils at concentrations ranging from 1 to 1000 μg/g, using the homogenization method and a Soxhlet extraction method. Each extraction method performed well in removing the 15 PAHs from both soils over a broad range of concentrations. In general, Soxhlet extraction yielded slightly but significantly ($P < 0.05$) higher recoveries than did the homogenization method. The homogenization method, however, was easy to use, and the extraction step turnaround time was less than 15 min/sample. The method should be suitable for other applications requiring the extraction of hydrophobic organic compounds from soils.

Several techniques have been developed for the extraction of hydrophobic organic compounds from soils and sediments. The most widely used techniques include Soxhlet extraction (1–6), shake flask methods (7, 8), sonication (9), and homogenization methods (6, 10–13). Three important criteria in the selection of an extraction technique for quantitative analysis of environmental samples are recovery efficiency, processing time, and ease of use.

Factors affecting recovery efficiency include the physical and chemical nature of the sample matrix and the structure and concentration of the analyte. Correlations between soil organic matter content and sorption of nonpolar organic compounds from aqueous solution have been observed (14), which suggests that the organic matter fraction of a soil or sediment may influence extraction efficiency. Sorption is also influenced by the physical and chemical characteristics of the sorbate, indicating that recovery efficiencies must be determined for each analyte (14, 15). Extraction efficiencies have been observed to vary with analyte concentration (6) suggesting the need to investigate recovery efficiencies over the entire range of concentrations expected. Finally, processing time per sample must be considered, especially when the number of samples to be analyzed is large.

As part of a comprehensive laboratory research effort to investigate the biological degradation of polycyclic aromatic hydrocarbons (PAHs) present in vadose zone soils at hazardous waste sites (16), a study was undertaken to determine the efficiency of a homogenization technique for extraction of these compounds from soil. The 15 PAH compounds examined in this study were selected because of their presence in organic wastes associated with vadose zone soils (16) and because they are regulated as hazardous constituents by the U.S. Environmental Protection Agency (EPA). The degradation experiments involved the use of small soil microcosms to monitor the loss of PAHs over time in 2 soils. Each microcosm represented a discrete sample, and PAH concentrations in the incubating soil were periodically determined by solvent extraction of the entire contents of individual microcosms and quantitation of the PAHs recovered. The homogenization method was selected for evaluation because it permitted extractions to be performed directly in the microcosm glassware, thus minimizing sample handling. In addition, significant savings on the extraction solvent were expected over costs with a conventional Soxhlet extraction method.

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[1] Address correspondence to this author.

The extraction study consisted of spiking both experimental soils with a mixture of 15 PAHs at concentrations ranging from 1 to 1000 μg/g and extracting the compounds to determine recovery efficiency. A Soxhlet extraction procedure approved by EPA for the chemical characterization of solid wastes (17) was also evaluated for comparison.

Experimental

Experimental Design

Spiked soil extraction recoveries of 15 PAHs were determined in 2 soils by using a homogenization method and a Soxhlet extraction method. Extraction efficiencies at PAH concentrations of 100, 10, and 1 μg/g were evaluated for the following compounds: acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, benzo[*g,h,i*]perylene, and indeno[1,2,3-*cd*]pyrene. In addition, extraction efficiencies for the first 9 compounds were evaluated at a concentration of 1000 μg/g. The concentration ranges were selected to bracket levels typically found in the soils of controlled and uncontrolled hazardous waste sites containing PAHs. Triplicate extractions were performed at each experimental matrix position.

Apparatus and Reagents

(a) *Standard solutions.*—Solutions containing all 15 PAHs were prepared in LC grade dichloromethane for spiking soil samples. Compounds were ≥98% purity except for acenaphthylene (95%) and were obtained from Aldrich Chemical Co. (Milwaukee, WI 53201) and Foxboro Analabs (North Haven, CT 06473). PAHs of 99% purity (Foxboro Analabs) were used to prepare analytical standards in LC grade acetonitrile for liquid chromatographic (LC) analysis.

(b) *Tissumizer homogenization system.*—Tekmar (Cincinnati, OH 45222), consisting of Model SDT-1810 motor, Model SDT-182EN shaft and generator assembly, and Model TR-10 speed controller.

(c) *Liquid chromatograph.*—Shimadzu Model LC-6A, equipped with Model SCL-6A system controller, Model SIL-6A autosampler, Model C-R3A computing integrator, and Model SPD-6A variable wavelength UV detector.

(d) *LC column.*—LC-PAH, 4 mm × 15 cm ODS with 5 μm packing (Supelco, Bellefonte, PA 16823).

Spiked Soil Sample Preparation

Two soils, which differed in texture and organic matter content, were used as sample matrixes in the extraction study. The Durant clay loam contained 3% organic carbon by weight and was collected from a plot at an EPA Field Test Facility in Ada, OK. The Kidman sandy loam contained 0.5% organic carbon by weight and was obtained from a Utah State University agricultural experiment station in Kaysville, UT. Prior to use in the extraction study, both soils were air dried and screened to <2 mm.

Spiked soil samples were prepared in 125 mL Erlenmeyer flasks by pipetting 3 mL standard solution containing all 15 PAHs onto 10 g soil. After mixing the solution with soil, the solvent was allowed to evaporate at 20°C for 2 days prior to sample extraction.

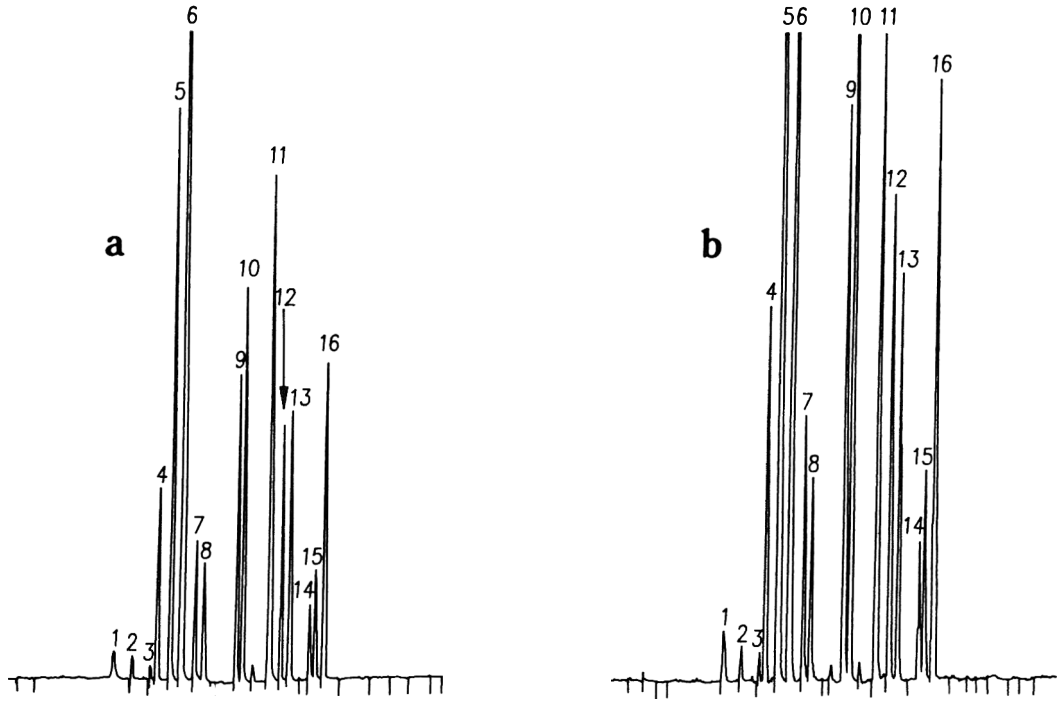

Figure 1. Chromatograms of (a) PAH standard and (b) PAH-spiked soil extract. Peak identification: 1, naphthalene; 2, acenaphthylene; 3, acenaphthene; 4, fluorene; 5, phenanthrene; 6, anthracene; 7, fluoranthene; 8, pyrene; 9, benz[*a*]anthracene; 10, chrysene; 11, benzo[*b*]flouranthene; 12, benzo[*k*]fluoranthene; 13, benzo[a]pyrene; 14, dibenz[*a,h*]anthracene; 15, benzo[*g,h,i*]perylene; 16, indeno[1,2,3-*cd*]pyrene.

Extraction and Analysis

All glassware was washed with soap and water, rinsed with deionized water, and heated in a muffle furnace at 550°C prior to use.

Soxhlet extraction of the soil samples was conducted following EPA Method 3540 (16). A 10 g soil sample was combined with 10 g anhydrous Na_2SO_4 in a solvent-cleaned cellulose thimble and was Soxhlet-extracted with 300 mL dichloromethane for 16 h which corresponded to ca 60 solvent cycles. The extract was then dried with anhydrous Na_2SO_4 and concentrated to between 0.5 and 1 mL in a Kuderna-Danish apparatus. The concentration extract was diluted to the desired volume with acetonitrile for LC analysis.

A 10 g soil sample was homogenized in the Tissumizer with 70 mL dichloromethane for 45 s. The solids were allowed to settle in the flask, and the supernate was transferred to a drying column containing anhydrous Na_2SO_4. The solids were reslurried with 40 mL solvent and transferred with rinsing to the drying column. Dried extracts were concentrated as described above for the Soxhlet extracts.

Extracts were analyzed by reverse phase LC using a gradient mobile phase program consisting of 2 min isocratic elution with 40% acetonitrile in water followed by 15 min linear gradient to 100% acetonitrile at a flow rate of 2 mL/min. Analytes were detected at a wavelength of 254 nm.

Results and Discussion

Method blanks, spiked blanks, and unspiked soil samples were processed through each analytical system before spiked soil samples were extracted. Both systems were free of interferences, and all 15 PAHs were quantitatively recovered when spiked blanks were processed. Chromatographic analysis of the unspiked soil extracts demonstrated that interferences contributed by natural soil components were minimal and that background soil PAH concentrations were below the detectable level of approximately 0.1 μg/g. Representative chromatograms of a PAH standard and a PAH-spiked soil extract, presented in Figure 1, demonstrate the degree of analyte resolution and freedom from interferences encountered during the study.

Percent recoveries of the 15 PAHs, using both the Soxhlet and Tissumizer extraction techniques, are summarized in Table 1. Efficiencies in this table represent averages of results from 9 or 12 extractions, depending on the concentration range investigated and reflect the influence of the 2 soils on extraction recovery independent of the effect of concentration. One-way analysis of variance was conducted on the factor concentration for each soil/method combination to

Table 1. Average PAH percent recoveries from sandy loam and clay loam soils by Soxhlet and Tissumizer extraction methods[a]

Compound	Tissumizer method		Soxhlet method	
	Sandy loam	Clay loam	Sandy loam	Clay loam
Acenaphthylene[b]	71 ± 12	85 ± 5	77 ± 4	82 ± 8
Acenaphthene[b]	77 ± 2	84 ± 6	93 ± 1	92 ± 1
Fluorene[c]	99 ± 1	96 ± 2	107 ± 2	105 ± 1
Phenanthrene[c]	102 ± 1	103 ± 3	109 ± 2	109 ± 1
Anthracene[c]	82 ± 1	85 ± 3	67 ± 3	71 ± 7
Fluoranthene[c]	94 ± 1	96 ± 6	109 ± 1	111 ± 1
Pyrene[c]	98 ± 2	100 ± 8	102 ± 2	101 ± 2
Benz[*a*]anthracene[c]	97 ± 2	99 ± 1	101 ± 1	103 ± 2
Chrysene[c]	96 ± 1	95 ± 1	104 ± 1	105 ± 1
Benzo[*b*]fluoranthene[d]	71 ± 1	70 ± 1	83 ± 0	83 ± 1
Benzo[*k*]fluoranthene[d]	104 ± 2	103 ± 2	113 ± 1	113 ± 1
Benzo[*a*]pyrene[d]	68 ± 3	70 ± 5	46 ± 1	49 ± 4
Dibenz[*a,h*]anthracene[d]	94 ± 4	97 ± 3	115 ± 3	118 ± 1
Benzo[*g,h,i*]perylene[d]	95 ± 1	98 ± 3	94 ± 1	95 ± 2
Indeno[1,2,3-*cd*]pyrene[d]	98 ± 1	102 ± 1	107 ± 0	108 ± 1

[a] Average ± 95% confidence interval, rounded to nearest percent.
[b] Concentrations represented: 10, 100, and 1000 μg/g.
[c] Concentrations represented: 1, 10, 100, and 1000 μg/g.
[d] Concentrations represented: 1, 10, and 100 μg/g.

Table 2. Average PAH percent recoveries at each concentration by Soxhlet and Tissumizer extraction methods[a]

	Tissumizer method				Soxhlet method			
	Concn, μg/g				Concn, μg/g			
Compound	1	10	100	1000	1	10	100	1000
Acenaphthylene	ND[b]	61 ± 21	86 ± 7	86 ± 7	ND	53 ± 2	124 ± 6	61 ± 14
Acenaphthene	ND	73 ± 4	83 ± 9	85 ± 4	ND	89 ± 2	96 ± 1	93 ± 2
Fluorene	99 ± 5	95 ± 4	97 ± 1	98 ± 1	105 ± 5	106 ± 2	109 ± 1	104 ± 1
Phenanthrene	113 ± 6	99 ± 3	99 ± 1	99 ± 1	112 ± 6	110 ± 3	110 ± 1	103 ± 1
Anthracene	61 ± 5	84 ± 4	91 ± 2	97 ± 6	44 ± 10	67 ± 5	92 ± 0	74 ± 15
Fluoranthene	87 ± 13	98 ± 1	100 ± 2	96 ± 2	107 ± 2	115 ± 4	114 ± 1	106 ± 1
Pyrene	77 ± 20	104 ± 4	108 ± 2	107 ± 3	100 ± 3	104 ± 5	103 ± 0	98 ± 1
Benz[*a*]anthracene	98 ± 4	98 ± 2	98 ± 1	97 ± 2	100 ± 3	101 ± 2	108 ± 0	100 ± 2
Chrysene	96 ± 3	97 ± 2	92 ± 1	96 ± 1	103 ± 1	106 ± 1	107 ± 0	100 ± 1
Benzo[*b*]fluoranthene	87 ± 2	64 ± 1	61 ± 0	ND	90 ± 1	79 ± 1	81 ± 0	ND
Benzo[*k*]fluoranthene	102 ± 3	104 ± 2	104 ± 1	ND	111 ± 1	112 ± 1	116 ± 1	ND
Benzo[*a*]pyrene	65 ± 8	64 ± 4	77 ± 2	ND	49 ± 6	29 ± 2	65 ± 1	ND
Dibenz[a,*h*]anthracene	82 ± 5	102 ± 6	103 ± 3	ND	127 ± 6	111 ± 1	112 ± 1	ND
Benzo[*g*,*h*,*i*]perylene	107 ± 4	91 ± 2	92 ± 1	ND	95 ± 3	88 ± 2	101 ± 1	ND
Indeno[1,2,3-*cd*]pyrene	104 ± 2	98 ± 2	98 ± 1	ND	110 ± 1	104 ± 1	109 ± 1	ND

[a] Average ± 95% confidence interval, rounded to the nearest percent (average of 6 recoveries, 3 from each soil).
[b] ND = not determined.

establish confidence intervals on the reported means. In general, high extraction efficiencies with good precision were obtained regardless of soil type or extraction method. Recoveries from both soils were essentially the same for a given extraction method despite the fact that the organic matter content of the clay loam was about 6 times the value for the sandy loam. Furthermore, recoveries were high for PAHs of both low and high molecular weight.

Extraction recoveries remained high over the range of concentrations tested, as demonstrated in Table 2. Efficiencies in this table represent averages of results from 6 extractions, 3 from each soil, and reflect the influence of concentration level on extraction recovery independent of the effect of soil type. Confidence intervals were again established using analysis of variance mean square errors.

Each extraction method performed well in removing the 15 PAHs from both soils over a broad range of concentrations. In general, Soxhlet extraction yielded slightly but significantly ($P < 0.05$) higher recoveries than did the homogenization method. In degradation experiments conducted in this laboratory, the homogenization method presented here minimized sample handling by permitting extractions to be performed directly in soil microcosm glassware. In addition, the method was easy to use and the extraction step turnaround time was less than 15 min/sample. The method should be suitable for other applications requiring the extraction of hydrophobic organic compounds from soils.

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Determination of Naptalam and Its Metabolite in Foods, as 1-Naphthylamine, Using Liquid Chromatography with Oxidative Electrochemical Detection

BRIAN L. WOROBEY and J BRIAN SHIELDS
Health and Welfare Canada, Health Protection Branch, Food Research Division, Ottawa, Ontario K1A 0L2, Canada

A new method is described for the determination of the herbicide naptalam and its metabolite 1-naphthylamine in several foods. The method is sensitive, selective, and extremely rapid compared with previously reported methods. Liquid chromatography with electrochemical detection (LC/ECD) is used to determine 1-naphthylamine produced from the metabolism or base hydrolysis of naptalam in asparagus, peaches, and cranberries. These foods were spiked with naptalam at 0.05 and 0.11 ppm and hydrolyzed with 30% NaOH with concomitant distillation of 1-naphthylamine. Aliquots of the distillate were injected onto a reverse-phase PRP-1 LC column for separation of 1-naphthylamine from coextractives near the solvent front and detection at an applied potential of +0.83 V using an amperometric electrochemical detector in the oxidation mode. Recoveries ranged from 89% ± 2% to 97% ± 8% for all foods at both spiking levels. Accuracy of these recoveries was confirmed by use of ^{14}C-radiolabeled naptalam and radioassay by liquid scintillation spectrometry of the ^{14}C-1-naphthylamine released.

The herbicide 2-[(1-naphthalenylamino) carbonyl] benzoic acid (*N*-1-naphthylphthalamic acid) (naptalam; Alanap®) or its sodium salt is registered for use on several fruits and vegetables in Canada and other countries. Naptalam breaks down rapidly into 1-aminonaphthalene (1-naphthylamine) and phthalic acid via metabolic and other degradative processes (1, 2). 1-Naphthylamine has been reported to be cytotoxic and genotoxic in mammals (3, 4), and its *N*-hydroxy metabolite is a potent, direct-acting carcinogen and mutagen (5). Naptalam residues in foods (plants, crops) are typically analyzed by hydrolyzing the naptalam to 1-naphthylamine, distilling off the 1-naphthylamine, partitioning against hexane, extracting with aqueous acetic acid, and forming a colored derivative by coupling 1-naphthylamine with diazotized sulfanilic acid (λ_{max} 535 nm) (6–8).

A more rapid method was sought for routine analysis of naptalam and 1-naphthylamine using the electrochemical detector. Electrochemical detection (ECD) monitors the changes in current associated with the reduction or oxidation of a sample component. When combined with liquid chromatography, it has proven to be a highly sensitive and selective method of instrumental analysis (9, 10). The LC/ECD analysis of 1-naphthylamine as a standard or in laboratory wastes has been previously reported (11–13). The present study was implemented to develop a more rapid, selective, and sensitive analysis for naptalam and 1-naphthylamine in several foods by using LC/ECD.

METHOD

Reagents

(a) *Standards.*—Naptalam, obtained as the sodium salt from U.S. Environmental Protection Agency (Washington, DC) at 94% stated purity; 1-naphthylamine, >99% purity (Aldrich Chemical Co., Milwaukee, WI 53201). *Safety precautions:* 1-Naphthylamine is genotoxic and is a suspected carcinogen and should be handled with due caution. Determine mass spectra of both compounds by direct inlet probe–mass spectrometry.

(b) *^{14}C-naptalam.*—Ring-labeled, sodium salt, 54.81 μCi, 11.86 mCi/mmol, radiochemical purity > 99% (UniRoyal Chemical Co., Naugatuk, CT 06770).

(c) *Standard solutions.*—Prepare in 0.1N aqueous $NaHCO_3$. Prepare 1-naphthylamine stock solution by dissolving weighed standard in minimum volume of acetonitrile followed by dilution with LC mobile phase. Prepare serial dilutions in mobile phase for working standards.

(d) *Mobile phase.*—Acetonitrile–0.15M aqueous *o*-phosphoric acid (40 + 60). Filter through 0.45 μm millipore filter and degas 5 min on Branson ultrasonic bath.

(e) *Zinc granules.*—80 mesh; use to maintain reducing conditions during hydrolysis (Fisher Scientific Co., Ottawa, Ontario, Canada).

(f) *Antifoam A.*—(Dow Corning Co., Toronto, Ontario, Canada).

(g) *Paraffin.*—(Fisher Scientific).

(h) *Solvents.*—Distilled-in-glass, pesticide grade (Caledon Chemical Co., Georgetown, Ontario, Canada).

(i) *o-Phosphoric acid.*—88% H_3PO_4 (sp gr 1.75), Analar grade (BDH, Toronto, Ontario, Canada).

Apparatus

(a) *Blender.*—Waring Blendor.

(b) *Hydrolysis and distillation apparatus.*—All-glass, equipped with splash head and condenser with receiving flask, as shown in Figure 1.

(c) *Heating plate.*—Corning PC-351 (Fisher Scientific Co., Ottawa, Ontario, Canada).

(d) *Magnetic stirrer.*—(Fisher).

(e) *LC apparatus.*—100 μL loop attached to Model 7125 Rheodyne injector (Cotati, CA 94928); injector connected to Model P/N 080040 Guard-Pak C_{18} guard column (Waters Associates, Milford, MA 01757), which is connected to PRP-1, 10 μm, 305 mm × 7 mm analytical column (Hamilton Co., Reno, NV 89502); Varian (Ottawa, Ontario, Canada) Model 4270 printer/plotter to record analog output from ECD. Operating conditions: column temperature, 20°C; chart speed, 0.5 cm/min; LC pump flow rate, 2 mL/min. Equilibrate 30 min before applying voltage to electrode. At end of day, purge 1 min with water followed by 2 min with methanol.

(f) *Electrochemical detector.*—Model LC-4B (Bioanalytical Systems, West Lafayette, IN 47906) amperometric detector with a glassy carbon working electrode. Set to oxidation mode (+) and apply optimum voltage of +0.83 V at 2 or 5 nA full-scale output current.

(g) *Liquid scintillation spectrometer.*—Beckman Model LS2800 (Beckman Instrument Co., Toronto, Ontario, Canada).

Spiking

Mascerate 300 g of a food (fresh peaches, asparagus, or cranberries) at medium speed for 30 s in a blender. Weigh 50 g of mascerated food into a beaker and spike with naptalam by dripping 0.5–1.0 mL aqueous spiking solution evenly onto surface of mascerate from a volumetric pipet and stirring 30 s with a glass rod. Spiking levels are 0.05 and 0.11 ppm. Spike foods with ^{14}C-naptalam at the rate of 0.5–1.0 ×

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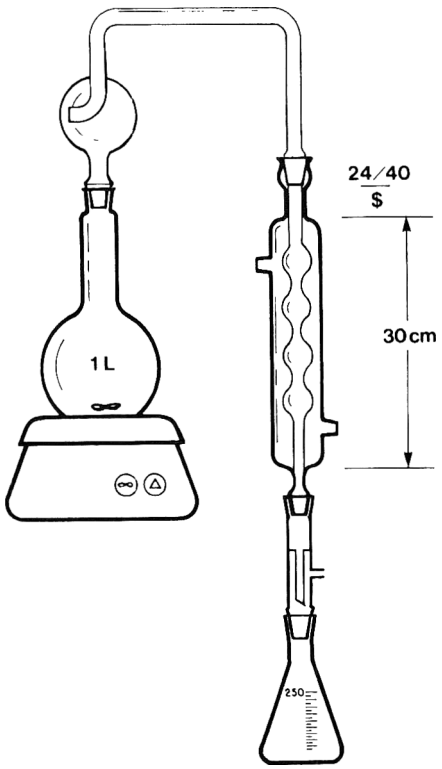

Figure 1. Diagram of all-glass apparatus used for the hydrolysis and distillation procedure.

10^6 dpm per 50 g of mascerate. Let spiked foods equilibrate 30 min before extraction.

Extraction

Transfer spiked food to 1 L boiling flask with 300 mL of 30% aqueous NaOH solution. To this, add 1 g zinc granules, 1 g paraffin, and 0.5 mL antifoam A. Attach flask to splash head and condenser with a receiving flask as shown in Figure 1. Heat with a heating plate, starting with a high rheostat setting for distillation and then reducing the heat to maintain distillation at a rate of ca 3 mL/min. Leave magnetic stirrer on high throughout distillation. If foaming occurs (especially for asparagus), apply a cold cloth to the splash head for ca 1 min. Cover transfer line between splash head and condenser inlet with aluminum foil and maintain distillation until 250 mL of distillate is collected.

LC/ECD Analysis

Inject distillates into 100 μL loop attached to Rheodyne injector. Completely fill loop for all injections. Inject samples in duplicate followed by a standard of similar peak height.

Perform quantitation for LC/ECD analysis by using external standard method and peak height comparisons. Calculate recoveries on the basis of 1-naphthylamine released from sodium salt (i.e., 0.457mol 1-naphthylamine/mol naptalam sodium salt) and correct for purity (94%) of naptalam.

Radioassay

Pipet two 1.0 mL aliquots of each distillate into glass counting vials. Add 15 mL Aquasol and acidify mixture to pH 3 with concentrated HCl. Determine ^{14}C-1-naphthylamine by counting 10 min in a liquid scintillation spectrometer. Determine quench correction by external standard counts related to an "H" number and determine percentage efficiency from a quench curve (% efficiency vs H number). Monitor chemiluminescence by using a random coincidence monitor accessory. Subtract background values from sample counts and then convert from cpm to dpm. Count all samples and standards as soon as possible after dispensing into counting vials.

Results and Discussion

During the development of the method just described, it was necessary to optimize several variables. Figure 2 illustrates the percentage recovery of naptalam from spiked cranberries (0.05 and 0.11 ppm) as a function of distillate volume.

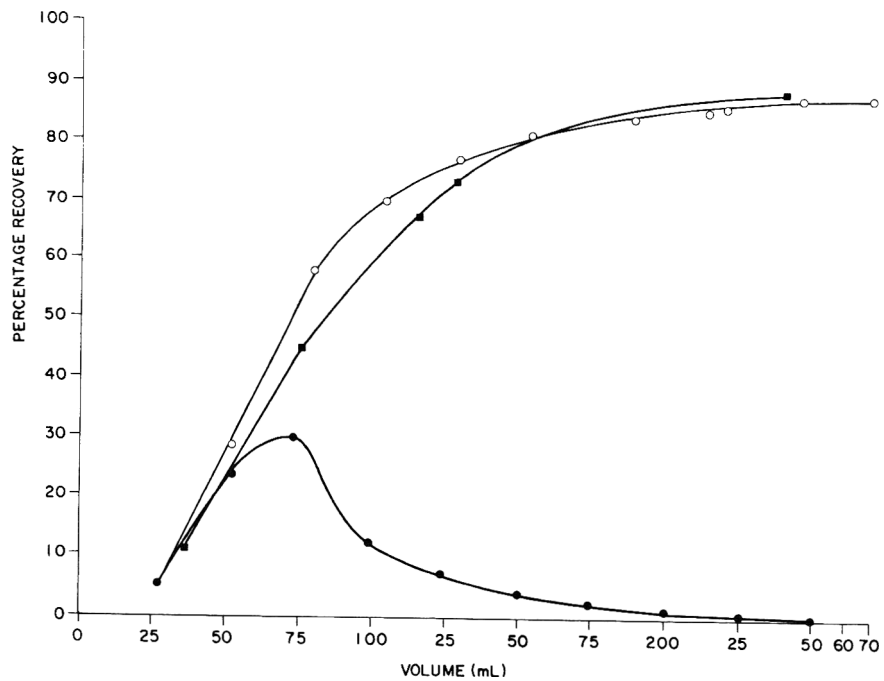

Figure 2. Percentage recovery of 1-naphthylamine from naptalam as a function of distillate volume collected (from spiked cranberries); (■) cumulative percentage recovery at 0.05 ppm, (○) cumulative percentage recovery at 0.11 ppm, (●) percentage recovery for separate 25 mL fractions (at 0.05 ppm level).

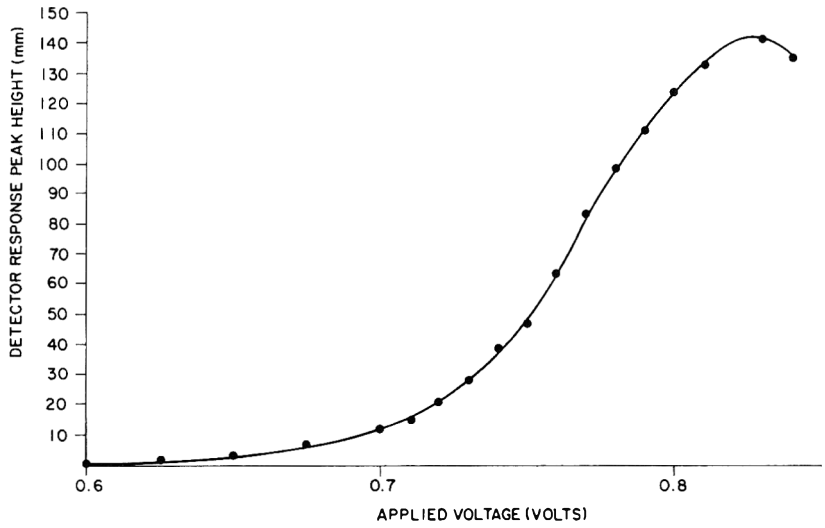

Figure 3. Voltammogram for 1-naphthylamine; ECD response (peak height, mm) as a function of applied potential (E°, V); 5 nA full scale and a 0.382 μg/mL standard.

From this graph, we were able to select 250 mL distillate as a suitable volume containing >80% 1-naphthylamine. The figure also illustrates that over half (approximately 58%) of the naptalam is hydrolyzed and distilled off in the first 75 mL (3 × 25 mL), but the remainder distills off slowly over the next 175 mL.

Figure 3 illustrates a voltammogram for 1-naphthylamine and is a graph of detector response (peak height) as a function of applied potential (voltage). From this curve it was possible to select the optimum applied voltage for the highest sensitivity towards 1-naphthylamine (+0.83 V). Linearity of the LC/ECD system was checked (Figure 4) and found to exhibit a wide linear range of 0.0035–3.95 μg/mL 1-naphthylamine with a lower detection limit of 0.30 ng per injection (S/N = 3/1). Typical LC/ECD chromatograms of extracts from blank and spiked foods are shown in Figure 5. The high degree of selectivity is evidenced by the absence of any peaks near 1-naphthylamine. The retention time was 11.1 min under

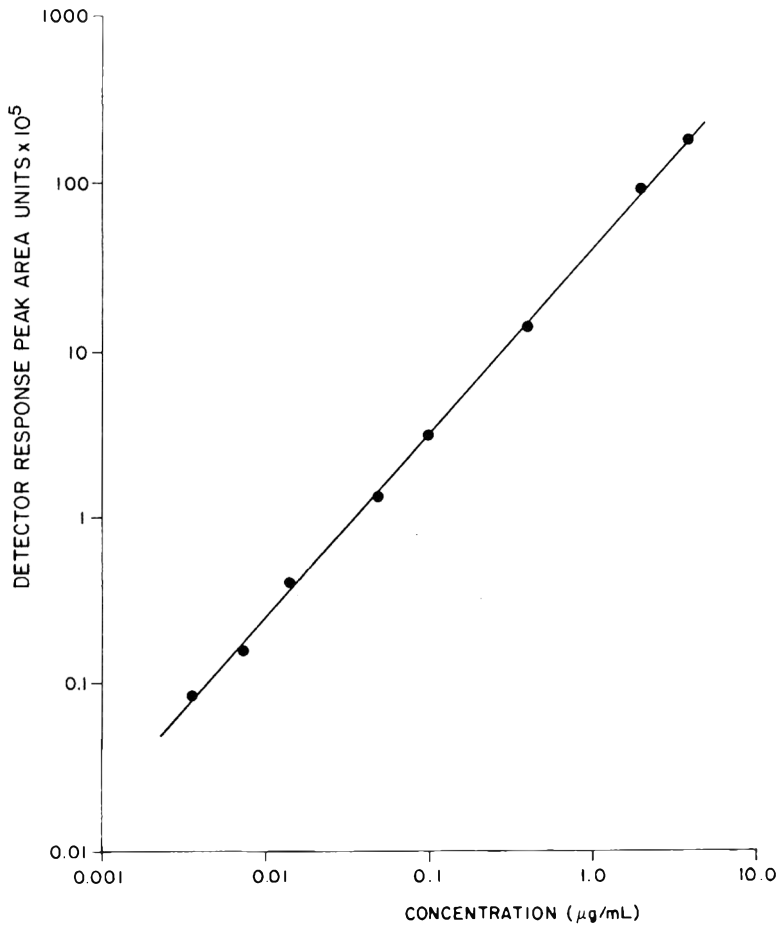

Figure 4. Linearity curve for 1-naphthylamine by LC/ECD. Log peak area units (×10⁵) vs 25 μL injected from standards 0.0035–3.85 μg/mL at 5 nA full scale, SD = ±1.5–10% for triplicate injections per data point.

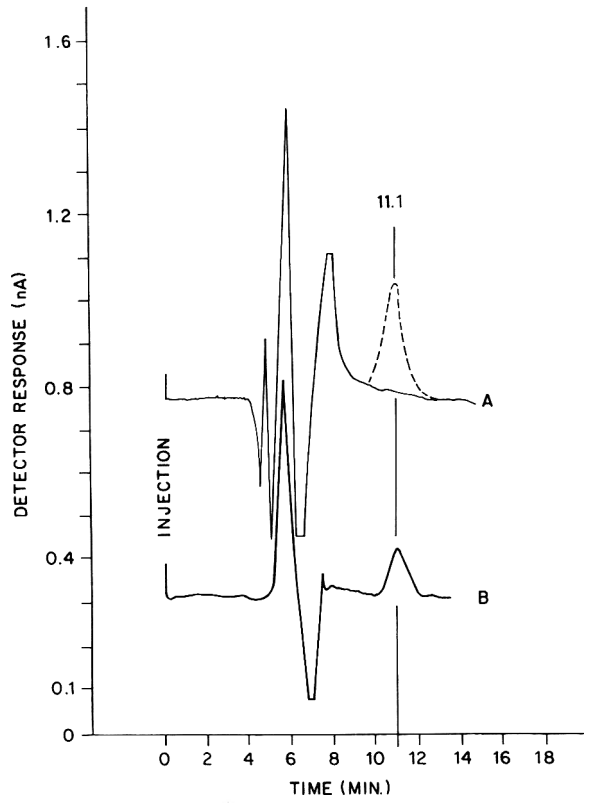

Figure 5. LC/ECD chromatograms. A, 1-naphthylamine standard (- - -) and control food extracts; B, spiked food extract (0.05 ppm).

the conditions described. Use of a preparative column facilitated repetitive injections of 100 μL of extracts without overloading the column.

Distillates were injected by LC/ECD and counted by liquid scintillation spectrometry (LSS) for comparison. Table 1 lists recoveries of naptalam from 3 foods at 2 spiking levels pertinent to negligible residue levels of ≤0.1 ppm (Food and Drug Act, Health and Welfare Canada). Recoveries were >88% as determined by LC/ECD analysis and >81% as determined by LSS analysis. Standard deviations (SD) were < ± 10% for all analyses and as low as ± 1%. LSS results were very similar to LC/ECD data, thus confirming the accuracy of the data by the latter method.

Several other observations are worth reporting. 1-Naphthylamine stock solutions are best prepared in the acidic mobile phase to reduce sorptive or degradation losses (1-naphthylamine was stable for 2–3 months in mobile phase). Large-volume pipetting (≥1.0 mL) is preferred because it reduces sorptive losses and increases accuracy. For example, a working standard at 0.14 μg/mL prepared by pipetting 10 μL stock solution and then diluting it for analysis was only one-fifth the peak area of a 0.14 μg/mL solution prepared by pipetting and then diluting 1.0 mL.

A standard curve of 1-naphthylamine at +0.75 V applied potential resulted in curvilinear responses at the lowest (0.02 μg/mL) and highest (0.8 μg/mL) concentrations. Attempts to distill off the 1-naphthylamine during the hydrolysis of naptalam without using antifoam A or paraffin were fruitless for

Table 1. Recoveries of naptalam from spiked foods

Sample	Spiking level, ppm	Recovery ± SD, % LC/ECD	Recovery ± SD, % LSS
Peaches	0.00	0 (3)[a]	0 (2)
	0.05	94 ± 2 (4)	95 ± 1 (2)
	0.11	96 ± 3 (4)	90 ± 3 (5)
Asparagus	0.00	0 (3)	0 (2)
	0.05	96 ± 7 (7)	91 ± 7 (5)
	0.11	96 ± 5 (5)	87 ± 4 (3)
Cranberries	0.00	0 (3)	0 (2)
	0.05	88 ± 8 (4)	81 ± 1 (2)
	0.11	94 ± 8 (4)	93 ± 9 (3)

[a] Number of replicates in parentheses.

all foods except cranberries. Chromatograms for canned peaches, asparagus, and cranberries were identical to those shown in Figure 5 for extracts of fresh foods. Three of the most common pesticide chloroaniline metabolite/degradation products—4-chloroaniline, 3-chloroaniline, and 3,4-dichloroaniline—did not interfere or coelute with 1-naphthylamine (retention times were 9.4, 13.8, and 49.6 min, respectively). Moving the auxilliary electrode connection from the cell to the waste stainless steel tube on the reference electrode reduced the baseline noise at 2 nA output from 2 mm to approximately 0.5 mm. This would permit an extrapolated lower detection limit of <0.030 ng/injection.

The electrochemical detector provides sensitivity (pg/injection) and selectivity (solvent front peaks only) and, in tandem with LC analysis, provides a rapid method for the determination of naptalam and its metabolite in foods, without derivatization and without preconcentration or cleanup of the distillate from base hydrolysis.

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Enzyme-Linked Immunosorbent Assay of Benomyl and Thiabendazole in Some Foods

W. HARVEY NEWSOME and PETER G. COLLINS
Health and Welfare Canada, Food Research Division, Bureau of Chemical Safety, Foods Directorate, Ottawa, Ontario K1A 0L2, Canada

Enzyme-linked immunosorbent assays were developed for benomyl as its decomposition product, methyl 2-benzimidazole carbamate, and thiabendazole in foods. Immunogens consisting of human serum albumin coupled to 2-succinamidobenzimidazole or 2-(2'-succinamido-4'-thiazolyl)benzimidazole were used in rabbits to raise antisera that were specific for the respective fungicides. Lower limits of quantitation of 0.35 ppm for benomyl and 0.03 ppm for thiabendazole were established without cleanup of the ethyl acetate extract. Recoveries of benomyl from 3 crops spiked at 0.5 to 10 ppm averaged 89% (range 73–109%) and of thiabendazole from 5 crops spiked at 0.1 to 2.0 ppm were 93% (range 81–105%).

The benzimidazole fungicides benomyl and thiabendazole are widely used on food crops to prevent storage rot, with maximum residue limits in Canada ranging from 0.4 to 10 ppm for thiabendazole and 0.5 to 10 ppm for benomyl or its decomposition product methyl 2-benzimidazole carbamate (MBC) in different foods.

Several methods are available in the literature for the determination of thiabendazole residues, including liquid chromatography (LC) with fluorometric (1, 2) or ultraviolet (UV) detection (3, 4) and gas chromatography (5). Similar procedures are available for benomyl, which is determined as its degradation product MBC, because benomyl is unstable in solution and decomposes rapidly to MBC by loss of its butylcarbamoyl side chain (6, 7). These methods have been reviewed by Baker and Hoodless (8).

In a previous report (9), a radioimmunoassay (RIA) method for benomyl was described which compared well in accuracy with liquid chromatography while affording a 5-fold increase in the rate of data generation. A disadvantage of the procedure was the requirement for a radiolabeled ligand and associated counting equipment and the problem of waste disposal. In the present study, enzyme-linked immunosorbent assay (ELISA) was investigated as a simpler means of screening large numbers of samples for benomyl and was extended to the determination of thiabendazole residues in the same extract.

METHOD

Reagents

(a) *2-Aminobenzimidazole, benzimidazole, 2-benzimidazoleurea, succinamide, succinic anhydride, o-phenylenediamine, thiourea, 3-bromopyruvic acid.*—(Aldrich Chemical Co., Inc., Milwaukee, WI).

(b) *Human serum albumin, bovine serum albumin, ovalbumin, goat anti-rabbit IgG-peroxidase conjugate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), Tween 20 (polyoxyethylene sorbitan monolaurate).*—(Sigma Chemical Co., St. Louis, MO).

(c) *Analytical standards.*— Thiabendazole (Merck Sharp and Dohme Canada, Ltd., Montreal, Quebec); benomyl, methyl 2-benzimidazole carbamate (DuPont of Canada Ltd, Montreal, Quebec).

(d) *Tri-n-butyl amine, isobutyl chloroformate.*—(Eastman Kodak Co., Rochester, NY).

(e) *2-Succinamidobenzimidazole (I).*—Prepare from 2-amino benzimidazole and succinic anhydride as previously described (10). Structure is shown is Figure 1.

(f) *2-Amino-4-carboxythiazole hydrobromide.*—Prepare by reacting 10.1 g of 0.13 mol thiourea with 16.7 g of 0.1 mol 3-bromopyruvic acid in 50 mL water as described by Sprague et al. (10). Filter white crystals which form on cooling, wash with acetone, and air-dry (yield 19 g, mp 280°C dec.).

(g) *2-(2'-Amino-4'-thiazolyl)benzimidazole.*—Heat 3.22 g of 14.3 mmol 2-amino-4-carboxythiazole hydrobromide with 2 g of 18.5 mmol *o*-phenylenediamine in 60 g polyphosphoric acid 3 h at 240°C. Pour cooled solution onto 200 g ice, stir, and filter insoluble material. Adjust to pH 6 with NaOH, remove resulting precipitate by filtration, wash with acetone, and dry (yield 4.9 g, mp 247–250°C). Electron impact mass spectrometry (EIMS) by direct probe gives the molecular ion of aminothiazolyl benzimidazole as the base peak at m/z 216.

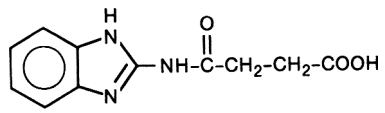
(h) *2-(2'-Succinamido-4'-thiazolyl)benzimidazole (II).*—Reflux 1.0 g of 20 mmol aminothiazolyl benzimidazole with 2.0 g of 20 mmol succinic anhydride 24 h in 10 mL pyridine. Add sufficient 1N HCl to bring pH to 1 and filter resulting precipitate. Wash the solid with water, then with methanol, and dry in vacuum desiccator to give 1.3 g product. Electron impact mass spectrometry gives a molecular ion at m/z 316 and base peak at m/z 298 for $M-H_2O$. Structure is shown in Figure 1.

(i) *Immunogens and coating antigens.*—Prepare immunogens for raising antisera to MBC or thiabendazole by coupling 2-succinamidobenzimidazole (I) or 2-(2'-succinamido-4'-thiazolyl)benzimidazole (II) to human serum albumin with EDC as previously described (9). Prepare antigen used to coat microtiter plates for MBC assay by coupling (I) to ovalbumin using the mixed anhydride procedure described for metalaxyl (11). For thiabendazole assay, use this procedure to couple (II) to gamma globulin.

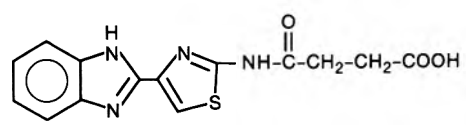
(j) *Buffers.*—Prepare phosphate-buffered saline, antiserum diluent, pH 9.6 coating buffer, citrate buffer, and peroxidase substrate as described previously (11). PBS–Tween washing solution contains 0.1% Tween 20 in PBS.

Microtiter Plate Preparation

Wash polystyrene microtiter plates having U-shape wells with 10% diethyl ether in ethanol and coat by incubation at



I



II

Figure 1. Structures of 2-succinamidobenzimidazole (I) and 2-(2'-succinamido-4'-thiazolyl)benzimidazole (II).

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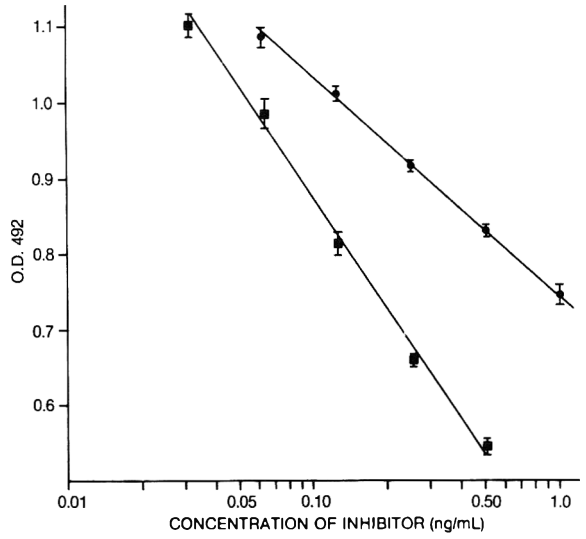

Figure 2. Inhibition curves for assay of thiabendazole (■——■) and MBC (●——●); values are the means of quadruplicate readings.

pH 9.6 as described (11), using 8 μg/mL of antigen for MBC or 2 μg/mL for thiabendazole.

Antisera

At monthly intervals, inject New Zealand rabbits with 0.5 mL each of a 1 mg/mL emulsion of immunogen in a 1:1 mixture of 0.14N NaCl and Freund's complete adjuvant (1 + 1). Collect blood and prepare serum 1 week after 6th and subsequent injections.

Sample Preparation

Cut crop material into small pieces and homogenize in Waring Blendor. Add 0.1–0.5 mL freshly prepared methanol solution of benomyl or thiabendazole to 10 g subsamples of crop homogenate in 125 mL boiling flask to give spiking concentrations of 0.5–10 ppm benomyl or 0.1–2.0 ppm thiabendazole. Add 40 mL ethyl acetate and 2 mL saturated Na_2CO_3 and reflux 15 min. Filter through Whatman No. 1 paper on Buchner funnel and bring filtrate to 50.0 mL in volumetric flask.

For thiabendazole assay, place 10 μL aliquot of extract in 12 × 75 mm glass tube and permit solvent to evaporate overnight in fume hood. For benomyl, remove solvent from 25 μL aliquot of 1:5 dilution of extract in the same fashion as for thiabendazole assay. Dissolve residue in 25 μL methanol.

Immunoassay of MBC

Add 1.0 mL of a 1:150 000 dilution of antiserum in diluent to tubes containing extract residue and to tubes containing 0, 0.06, 0.13, 0.25, 0.50, and 1.0 ng MBC in 25 μL methanol. After mixing and equilibrating for 15 min at room temperature, add 200 μL aliquots of sample and standard in quadruplicate to wells of sensitized plate and maintain 1 h at room temperature. Empty plate, wash 4 times with PBS–Tween, and determine amount of adsorbed antibody using second antibody consisting of goat anti-rabbit IgG coupled to horseradish peroxidase. Dilute commercial second antibody 1:1000 with antiserum diluent and add 225 μL to each well. After 30 min at room temperature, empty wells, wash 4 times with PBS–Tween, add 200 μL substrate, and permit reaction to proceed for 20 min in the dark. Terminate reaction with 50 μL of 2.5M sulfuric acid and read optical density (OD) at 490 nm on dual beam microtiter plate reader. Calculate concentration of analyte in samples by reference to least-squares plot of the log of the concentration against OD of standards run concurrently.

Table 1. Recovery of MBC from various commodities fortified with benomyl

Benomyl added, ppm	MBC recovered, %		
	Lemon	Grape	Apple
0.50	73	82	109
2.5	82	91	82
5.0	79	97	97
10	76	100	100

Table 2. Comparison of concentrations of various compounds required to inhibit binding by 50%

Compound	Concn, ng/mL	
	MBC assay	Thiabendazole assay
MBC	1.4	145
Thiabendazole	380	0.208
2-Benzimidazoleurea	7.0	225
2-Aminobenzimidazole	>500	>500
Benzimidazole	>500	>500
Succinamide	>500	—

Immunoassay of Thiabendazole

Prepare standards by diluting solutions of thiabendazole in 0.1N HCl 1:10 with 1:15 000 dilution of antiserum in diluent to give concentrations of 0, 0.03, 0.06, 0.13, 0.25, and 0.50 ng/mL. Add 1.0 mL of diluted antiserum to sample tubes, mix, and, after 30 min of equilibration at room temperature, transfer quadruplicate aliquots of standards and samples to sensitized microtiter plate. Permit antibody adsorption to occur for 1 h at 4°C, wash, and determine amount of adsorbed antibody as for MBC.

Results and Discussion

An ELISA procedure was developed which is capable of determining benomyl, as its degradation product MBC, in an ethyl acetate extract of fruit without cleanup. Compared to the RIA procedure (9), the ELISA method is less sensitive, producing a more shallow inhibition curve (cf., Figure 2). However, ELISA has a lower limit of detection, with 0.12 ng/mL producing a 20% inhibition in binding compared to 2 ng/mL for RIA. As with the RIA method, the initial extract did not produce interferences and satisfactory recoveries were obtained from 3 different commodities at concentrations ranging from 0.5 to 10 ppm (Table 1). Extracts of samples spiked at 2.5 ppm and above were diluted to bring the concentration of MBC within the linear portion of the standard curve. The lower limit of quantitation, defined as 10 standard deviations above background, was 0.35 ppm benomyl. A mean recovery of 91% and a within-run coefficient of variation (CV) of 12.7% was obtained from 6 samples of apple spiked at 0.35 ppm. A between-run CV of 15.8% was found by repeating the recovery study on 3 different occasions.

A comparison of the ability of related compounds to inhibit the binding of MBC antiserum is shown in Table 2. There was little cross-reactivity with thiabendazole and none with 2-aminobenzimidazole or benzimidazole. 2-Benzimidazoleurea was recognized to a moderate degree but does not occur as a metabolite and thus would not be expected to interfere with the determination of benomyl.

Table 3. Recovery of thiabendazole from various fortified commodities

Thiabendazole added, ppm	Thiabendazole recovered, %				
	Pear	Potato	Lemon	Grapefruit	Apple
0.10	81	83	98	95	88
0.20	95	91	105	93	85
0.50	92	96	94	90	96
1.0	87	92	96	86	91
2.0	94	92	98	98	105

The ELISA for thiabendazole was more sensitive than that for benomyl, as shown by the data in Figure 2. The lower limit of quantitation was below that of benomyl, being 0.03 ppm or less for a variety of extracts. Recoveries from 5 commodities spiked with 0.1–2.0 ppm thiabendazole exceeded 80% as indicated in Table 3. Replicate determinations (n = 6) on grapefruit spiked with 0.2 ppm of thiabendazole yielded a within-run CV of 7.3% and between-run CV of 11.7%.

The specificity of antiserum directed toward thiabendazole is indicated by the data in Table 2. There was a slight cross-reactivity with MBC, and in contrast to the MBC antiserum, little cross-reactivity with 2-benzimidazoleurea. Thus, thiabendazole, benomyl, and MBC may be determined in a single extract using the appropriate antisera and plate coating antigens.

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FEEDS

Comparison of LECO FP-228 "Nitrogen Determinator" with AOAC Copper Catalyst Kjeldahl Method for Crude Protein

ROSE A. SWEENEY and PAUL R. REXROAD
University of Missouri, Experiment Station Chemical Laboratories, Agriculture Building, Columbia, MO 65211

The LECO FP-228 "Nitrogen Determinator" was compared with the AOAC copper catalyst Kjeldahl method, 7.033-7.037, for the determination of crude protein in feed materials. The completely microprocessor-controlled instrument determines nitrogen by measuring the nitrogen gas following combustion of the sample; it was easy to operate and broadly applicable. A wide variety of feed materials of various nitrogen levels were analyzed in one mixed sequence. Results were precise, accurate, and rapid. Analysis time for one sample was approximately 3 min. Fourteen samples containing 2.5-15.5% N were selected for study and consisted of meals, grains, forages, and standard organic materials. The overall mean for the 14 samples by the LECO combustion method was 8.61% N compared with an overall mean of 8.58% N for the AOAC Kjeldahl method. Within-sample standard deviations for the LECO combustion method ranged from 0.013 to 0.052% N with a pooled standard deviation (SD) of 0.033% N for the 14 samples. Standard deviations for the AOAC Kjeldahl method ranged from 0.006 to 0.035% N with a pooled SD of 0.022% N. Combined average recovery of nitrogen from tryptophan, lysine·HCl, and EDTA determined by the LECO combustion method was 99.94% compared to 99.88% determined by the AOAC Kjeldahl method.

The search continues for "better" methods for crude protein determination. A new instrument for the determination of crude protein or "total nitrogen," the LECO Corp. FP-228 Nitrogen Determinator, employs combustion chemistry to determine nitrogen in a wide variety of materials. The instrument is completely controlled by an automated microprocessor. Once a sample is weighed (150 mg recommended) into a tin capsule, it proceeds automatically through the analysis. Different sample types covering a wide range of nitrogen can be analyzed in mixed sequence without recalibrating. Analysis time for one sample is about 3 min, and the result is printed directly as percent nitrogen or percent protein as selected. No postanalysis treatment is needed to arrive at the final result. The instrument has built-in routines to perform automatic calibration and blank determinations and will also compensate for normal changes in atmospheric pressure. Many other routines assist the operator in analyzing samples and in performing instrument diagnostics. A built-in alarm/message system will alert the operator when a reagent needs changing or when an improper condition is sensed. An autosampler is also available which can hold up to 50 samples for unattended analysis. The instrument is housed in a single cabinet, 31 in. high, 26 in. wide, and 27 in. deep, which is easily located on most any laboratory bench space. No special installation is required other than 230 V power and access to 3 gases: oxygen for combustion, helium used as a carrier, and an inert gas or compressed air to drive the valve system.

The operational advantages of the automated combustion method over manual Kjeldahl and other nitrogen methods such as the block digester are obvious. The key, of course, is how well the results of the combustion method compare with AOAC official methods for the determination of crude protein. This paper presents a study comparing the LECO combustion method with an AOAC Kjeldahl method with this objective in mind.

In our laboratory, major research efforts have been directed toward the development of nitrogen methodologies for both feeds and fertilizers. We have studied and published extensively on manual Kjeldahl methods, semiautomated and automated methods, and methods without digestion. In 1975, Wall and Gehrke (1) reported on an automated method for total protein nitrogen which used a block digester and Technicon AutoAnalyzer. This method is now an official AOAC method (2) for determining protein in feeds. Rexroad and Cathey (3) developed a modified macro-Kjeldahl method for crude protein which replaced mercury catalyst with copper. The copper catalyst method is official in AOAC (4) and is the method we chose to use in this study for comparison with the LECO combustion method.

It is appropriate that we point out that in 1967 the Dumas combustion method (5) for the determination of protein/nitrogen was successfully collaborated and in 1968 became an AOAC official method (6). It is debatable whether the combustion method implemented in the LECO instrument could become an official method by virtue of the successful 1967 collaboration of the Dumas method. A basic difference between the official Dumas and the current LECO method lies in the final detection technique. In general, both methods use combustion with oxygen at high temperature followed by reduction to liberate N_2, and similar techniques are used to remove excess oxygen. The Dumas method, however, measures evolved N_2 by volume over CO_2-absorbing KOH. This technique is inherent to the Dumas method and hence gives it its name. In the LECO instrument, the isolated N_2 is measured by thermal conductivity. This difference may be enough to justify a full or perhaps a mini-collaborative study of the LECO combustion method.

Experimental

Fourteen samples ranging in nitrogen content from 2.5 to 15.5% were selected for study. The sample set consisted of meals, grains, forages, 2 amino acid standards, and standard EDTA. All samples were analyzed in duplicate on 3 different days by the LECO combustion and AOAC Kjeldahl methods. The analyses by both methods were done during the same 2-week period to minimize any effects caused by changes in the moisture content of the samples. The meals, grains, and forages were ground to pass a U.S. No. 35 (0.5 mm) sieve and stored in tightly sealed polyethylene bottles. The amino acids and EDTA were dried for 2 h at 105°C and stored in a desiccator. The same dried standard EDTA used in the study was also used to calibrate the LECO instrument. The analyses done by the combustion method could have been done easily on 3 consecutive days, which could have had an influence on the results. To avoid this possibility, the com-

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bustion method runs were made several days apart and the instrument was completely powered down between runs.

LECO Combustion Method

Principle

A sample is combusted at 950°C in pure oxygen. The products of combustion, mainly CO_2, H_2O, NO_x, and N_2 plus excess O_2, are collected in a glass ballast volume and allowed to equilibrate. A portion of the combustion gases is then forced into a 10 mL aliquot loop, and the remainder is discarded.

The aliquot is swept via helium carrier gas through a train of chemicals which leaves only N_2 and helium carrier. Excess O_2 is converted to CuO and removed as the stream passes through hot copper turnings. NO_x products are converted to N_2 when heated in the presence of N-catalyst reagent. CO_2 is absorbed by Ascarite II reagent (silica impregnated with NaOH) and H_2O is absorbed by $Mg(ClO_4)_2$. The isolated N_2, carried by helium gas, is measured as it flows through a thermal conductivity cell containing 2 pairs of matched filaments—a reference and measure filaments. Cell output is multiplied by a calibration factor and a "K factor" which corrects for barometric pressure and ballast volume pressure. The final result is adjusted for blank and sample weight and displayed as percent nitrogen or as percent protein if a protein conversion factor had also been supplied.

Apparatus and Reagents

(a) *Nitrogen combustion analyzer and accessories.*—Model FP-228 with the following reagents and accessories is available from LECO Corp., 3000 Lakeview Ave, St. Joseph, MI 49085-2396: combustion crucibles No. 529-026; Ascarite II No. 183-001; iron chips No. 501-077; anhydrone ($Mg(ClO_4)_2$) No. 501-171; copper turnings No. 501-621; tin weighing capsules No. 502-040; N-catalyst 502-049; quartz wool No. 501-608.

(b) *Gases.*—Oxygen, 99.99% pure; helium, 99.99% pure; compressed air.

(c) *Balance.*—Accurate to 0.1 mg.

(d) *Barometer.*—Hg type, readable to 1 mm.

Determination

Operate instrument according to manufacturer's instructions. Use manufacturer's recommended settings for oxygen profile, furnace temperature (950°C), and other system constants. Let furnace and catalyst heater reach operating temperature and stabilize. Warm-up time takes ca 30 min from a cold start. Enter current barometric pressure. Establish system blank by running at least 3 blank determinations. Calibrate instrument at beginning of each day by selecting Calibrate System procedure to perform auto-calibration from at least 3 analyses (5 are recommended) of standard EDTA (9.59% N) or other suitable organic material of high purity and known nitrogen content. Weigh samples, or standards if calibrating instrument, into tin weighing capsules. Weigh 130–160 mg for samples containing 2–16% N. Larger weights having a volume of 0.5 mL or less may be taken for samples low in nitrogen; however, do not exceed 150 mg for samples containing 50% N. Sample size depends on a combination of factors including oxidation rate, volume of gas produced, and volume, 0.5 mL, of weighing capsule. Enter sample weights into instrument weight stack. The stack has a capacity to retain 50 weights.

Samples may be analyzed in one of 2 modes, manual or auto mode which requires the automatic sampler. In manual mode, press analyze key and drop first sample into open loading port. Press analyze key again to close port and start analysis. When analysis is complete, in ca 3 min, press analyze key again and drop next sample into loading port. Continue in this fashion for all samples. Samples are analyzed in the same order as their weights appear in the weight stack.

If automatic sampler is used, switch to auto mode and place samples in sampler tray in weight stack order. Press analyze key to begin. Samples will be automatically loaded and analyzed in sequence until weight stack is empty.

AOAC Kjeldahl Method

Follow AOAC copper catalyst Kjeldahl method (4).

Results and Discussion

Results obtained by the LECO combustion and AOAC Kjeldahl methods are shown in Table 1. Agreement between the 2 methods was good. The overall average of the 14 samples analyzed by the AOAC Kjeldahl method was 8.58% N compared to 8.61% N for the LECO combustion method. The accuracy of the LECO combustion method was measured by the recovery of nitrogen from standard organic materials (samples 12, 13, and 14). Average values for EDTA, tryptophan, and lysine·HCl were 9.58, 13.70, and 15.35% N, respectively, by the LECO combustion method. These compare with expected theory of 9.59% N for EDTA, 13.72% N for tryptophan, and 15.34% N for lysine·HCl. The average values for Kjeldahl were 9.57% N for EDTA, 13.68% N for tryptophan, and 15.36% N for lysine·HCl.

Overall, the Kjeldahl method gave slightly lower results than the LECO combustion method as can be seen from the tabulated differences, AOAC − LECO. Except for sample 11, all negative differences are less than or equal to −0.05. These small consistent differences result in a *t*-test which shows a significant difference between methods at the 95% confidence level. It would be interesting to see if this pattern would hold in a collaborative study. We feel the difference between methods indicated by these data is of no practical concern.

The largest difference between methods, for average results per sample was −0.19% N for sample 11. This difference was found in the high nitrate grass which contains about 0.5% N from nitrate. It is known that the Kjeldahl method used in this study does not completely recover nitrate-nitrogen, so it was judged appropriate to include this sample in the study because we were comparing dissimilar methods. The average value by the combustion method is higher, which indicates that the combustion method does recover more nitrate. Sample 11 was also analyzed by the sulfuric-salicylic manual Kjeldahl method (7), which is quantitative for nitrate. The average of 3 determinations by this method was 2.50% N, which falls between the combustion (2.62% N) and Kjeldahl (2.43% N) values obtained in the study. These data show that the difference is not easily resolved. Further study of this point is indicated.

Within-sample standard deviations for the LECO method ranged from 0.013 to 0.052% N and those for the Kjeldahl method ranged from 0.006 to 0.035% N. these precision data compare well with expected within-laboratory standard deviations (S_o) of 0.031 to 0.11 for samples containing 1.6 to 14.4% N, respectively. These S_o data are the performance criteria specified in AOAC *Official Methods of Analysis* for the Kjeldahl method (4) used in this study.

The Kjeldahl method was generally more precise than the LECO combustion method. Six of the 14 within-sample standard deviations by the LECO combustion method were ap-

Table 1. Comparison of AOAC Kjeldahl (7.037) and LECO combustion methods[a]

Sample	Description	AOAC Kjeldahl method			LECO combustion method			Diff., AOAC − LECO
		Av. % N	SD	RSD, %	Av. % N	SD	RSD, %	
1	cattle concentrate	6.64	0.007	0.11	6.69	0.020	0.31	−0.05
2	alfalfa pellets	2.76	0.015	0.53	2.77	0.031	1.11	−0.01
3	broiler finisher	3.42	0.008	0.24	3.42	0.013	0.39	0.00
4	soy protein conc.	13.98	0.026	0.19	14.02	0.052	0.37	−0.04
5	soybean meal	7.98	0.021	0.26	8.00	0.043	0.54	−0.02
6	blood meal	13.04	0.030	0.23	13.06	0.033	0.25	−0.02
7	dry milk	5.54	0.016	0.29	5.58	0.022	0.39	−0.04
8	feather meal	13.56	0.034	0.25	13.61	0.048	0.35	−0.05
9	meat meal	8.73	0.014	0.16	8.75	0.032	0.37	−0.02
10	hog feed	3.38	0.006	0.19	3.39	0.024	0.71	−0.01
11	high nitrate grass	2.43	0.024	1.00	2.62	0.048	1.85	−0.19
12	EDTA[b]	9.57	0.025	0.26	9.58	0.016	0.17	−0.01
13	tryptophan[c]	13.68	0.021	0.16	13.70	0.024	0.18	−0.02
14	lysine·HCl[d]	15.36	0.035	0.23	15.35	0.033	0.21	0.01
Av.		8.58	0.022[e]		8.61	0.033[e]		

[a] Each result is the average of 6 analyses.
[b] LECO calibration standard, theory = 9.59% N.
[c] Theory = 13.72% N.
[d] Theory = 15.34% N.
[e] Pooled SD = $\sqrt{\frac{\sum SD^2}{n}}$.

proximately twice those obtained by Kjeldahl. This difference in precision is most likely due to sampling variation in the matrix samples because the uniform materials, EDTA and the amino acids, did not follow this pattern. A smaller sample size ranging from 70 to 160 mg was taken for the combustion assays; most weighings were in the 130–160 mg range compared to a 500 or 1000 mg sample taken for the Kjeldahl assays. The amino acids and EDTA are very uniform materials, and sample size would not contribute much to variability, hence the standard deviations were nearly the same. Matrix samples 6 and 7 also had standard deviations of the same magnitude by both methods. Sample 7 was much more finely ground and more uniform in appearance than the other matrix samples. Sample 6 was blood meal. Added digestion difficulties for Kjeldahl may be more of a contributing factor for this sample.

The extremely precise Kjeldahl data obtained in this study allow us to see variations due to sample uniformity. A larger sample could have been taken for the combustion assays for many of the study samples without exceeding the range of the instrument, 0.01 to 50.0% N. The sample size taken was that recommended by the manufacturer (150 mg) or nearly so for the level of nitrogen being measured. From a practical standpoint, sample size also depends on the density of the material because the tin weighing capsule has a limited capacity of 0.5 mL. Weights less than 100 mg are common for light fluffy grasses (sample 11 in this study) which are harder to pack into the capsule. A larger capsule would be helpful overall and especially for the lighter materials.

Conclusions

The LECO combustion method has been shown in this study to agree with the AOAC copper catalyst Kjeldahl method for the determination of crude protein. The LECO combustion method provides a very attractive alternative to Kjeldahl and various other methods for the determination of crude protein in agricultural feeds, foods, and biological materials. The instrument is easy to operate, and results are obtained quickly and accurately over a wide mix of samples without repeated calibration. The 150 mg sample size recommended for the combustion method is reasonable. Using a small sample, however, does emphasize the importance of sample preparation. In our experience with the instrument we have found it to be well designed and reliable. We recommend that this method be considered for AOAC collaborative study to evaluate its official method potential.

Acknowledgments

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DRUG RESIDUES IN ANIMAL TISSUES

Liquid Chromatographic Determination of Sulfamoyldapsone in Swine Tissues

YUUKO S. ENDOH, RYOZO YAMAOKA, and NOBUO SASAKI
Ministry of Agriculture, Forestry and Fisheries, National Veterinary Assay Laboratory, 1-15-1 Tokura, Kokubunji, Tokyo 185, Japan

A liquid chromatographic (LC) method is described for the quantitative determination of sulfamoyldapsone (2-sulfamoyl-4,4'-diaminodiphenyl sulfone) in swine muscle, liver, kidney, and fat. Sulfamoyldapsone was extracted from tissues with acetonitrile saturated with *n*-hexane. The extract was washed with *n*-hexane saturated with acetonitrile, concentrated, and cleaned up by alumina column chromatography. Sulfamoyldapsone was separated on an ODS column by using acetonitrile–methanol–water (6 + 18 + 76) and was detected at 292 nm. Overall average recovery of sulfamoyldapsone added to tissues at levels of 0.1 and 0.5 μg/g was 93.3% ± 6.0. Detection limit was 0.02 μg/g in these tissues.

Sulfamoyldapsone (2-sulfamoyl-4,4'-diaminodiphenyl sulfone), an antitoxoplasmic agent developed in Japan, has been widely used as a prophylactic or therapeutic drug for pigs. The prophylactic (1, 2) and therapeutic (3) effects of this compound were studied in pigs, and investigations with tritium-labeled drug in mice and rats showed that residues remained for a longer time in the liver and kidney than in other tissues (4, 5). According to Japanese regulations, this drug must not be given in feed for 5 days before slaughter and must not be injected within 30 days before slaughter. Thus, a simple, reliable, and sensitive method for determination of this compound in swine tissues is necessary. At present, no method is available for determination of this compound in animal tissues, except in guinea pig serum (6). In contrast, many determination methods for other widely used antitoxoplasmic agents, pyrimethamine, and sulfadrugs in tissues of animals used for food have been reported (7–10). In the present paper, we describe a simple and sensitive method for determination of sulfamoyldapsone in swine muscle, liver, kidney, and fat tissues by reverse-phase liquid chromatography (LC).

METHODS

Reagents

All chemicals are analytical reagent grade.

(a) *Solvents.*—Acetonitrile, methanol, and *n*-hexane (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

(b) *Anhydrous sodium sulfate.*—(Wako Pure Chemical Industries Ltd.)

(c) *Alumina.*—Alumina Woelm B Akt. 1 (Woelm Pharma GmbH & Co., D-3440 Eschwege, FRG).

(d) *Sulfamoyldapsone.*—Purity 100% (Tanabe Seiyaku Co., Ltd, Osaka, Japan). *Standard solution.*—Dissolve 10.0 mg sulfamoyldapsone in 100 mL methanol. Dilute 5 mL aliquot of this solution to 100 mL with methanol to prepare working solution that contains 5 μg sulfamoyldapsone/mL.

(e) *Internal standard solution.*—Dissolve 100 mg acetanilide (standard for elementary analysis, E. Merck, Darmstadt, FRG) in 100 mL methanol. Dilute 30 mL of this solution to 100 mL with methanol to prepare solution that contains 300 μg acetanilide/mL.

(f) *Quartz wool.*—Fine (Nihon Chromato Works, Ltd, Tokyo, Japan).

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Apparatus

(a) *High-speed homogenizer.*—Bio-mixer BM-2 (Nihon Seiki Kaisha Ltd, Tokyo, Japan).

(b) *Meat mincer.*—Bonny Mincer (Bonny Co., Japan).

(c) *Evaporator.*—Rotary evaporator MINI Model RE-21 (Yamato Scientific Co., Tokyo, Japan).

(d) *Centrifuge.*—Model 90-3 (Sakuma Seisakusho, Tokyo, Japan).

(e) *Cleanup column.*—Place a small quartz wool plug at the bottom of a 24 cm × 10 mm id column, pack 6 g alumina into column with 40% methanol in acetonitrile, and wash with 50 mL of 40% methanol in acetonitrile before use.

(f) *Liquid chromatograph.*—Japan Spectroscopic Co. (Tokyo, Japan) Model Trirotar-III equipped with Model Uvidec-100-III detector set at 292 nm, and Shimadzu Seisakusho Ltd (Kyoto, Japan) Model C-R3A integrator for recording retention times and responses (measured as peak height or peak area). Column: 25 cm × 4.6 mm id stainless steel packed with Nucleosil 5 C_{18} (Machery-Nagel, D-5160 Dueren, FRG). Operating conditions: Mobile phase, acetonitrile–methanol–water (6 + 18 + 76); flow rate, 0.8 mL/min; column temperature, 40°C; chart speed, 5 mm/min; injection volume, 10 μL.

(g) *Ultrasonic generator.*—Ultrasoniccleaner IUC-2811 (San-Ei Seisakusho Ltd, Tokyo, Japan).

Tissue Samples

Five nonmedicated pigs were sacrificed, and the muscles, liver, kidneys, and fat were removed. Muscle tissues were minced and other tissues were chopped. Samples were stored frozen at −20°C wrapped in aluminum foil.

Extraction and Cleanup

Accurately weigh 10 g minced muscle or chopped liver, kidney, or fat, homogenize twice for 2 min with 50 mL acetonitrile saturated with *n*-hexane, and centrifuge 10 min at 3000 rpm. Add 10 g anhydrous sodium sulfate to supernate and let stand 30 min. Filter mixture through G-4 glass filter, wash filtrate twice with 40 mL *n*-hexane saturated with acetonitrile, and concentrate to ca 3 mL under vacuum on rotary evaporator at 40°C (complete evaporation results in some decomposition of sulfamoyldapsone and increased background interference from tissue components). Add 2 mL methanol and sonicate for 1–2 min to dissolve remaining residue and then apply to alumina column. Elute sulfamoyldapsone with 20 mL of 40% methanol in acetonitrile. Evaporate eluate to dryness under vacuum on rotary evaporator at 30°C and dissolve residue in 1 mL internal standard solution (e).

Analysis and Calculation

Inject 10 μL each of working standard and sample solution into chromatograph and measure peak area ratios with respect to area of internal standard peak. Calculate concentration in tissue by using the following formula:

Table 1. Recovery of sulfamoyldapsone from fortified swine tissues

Sample	Added, μg/g	Rec., %[a]	CV, %
Muscle	0.1	91.2	1.9
	0.5	95.0	2.4
Liver	0.1	94.8	3.5
	0.5	95.5	3.4
Kidney	0.1	98.2	2.9
	0.5	99.3	1.2
Fat	0.1	81.7	7.0
	0.5	89.8	2.8

[a] Each value is the average for 5 samples.

$$\text{Sulfamoyldapsone, } \mu g/g \text{ tissue} = R_s/R_w \times C_w \times 0.1$$

where R_s and R_w = peak area ratios of sample and working standard solutions injected, respectively, and C_w = concentration of sulfamoyldapsone in working standard solution (μg/mL).

Results and Discussion

Reverse-phase liquid chromatography was used for the determination of sulfamoyldapsone, on the basis of a previous study on LC analysis of sulfamoyldapsone (6). The ODS (Nucleosil 5 C_{18}) column gave better separation of sulfamoyldapsone from tissue components than did the octyl silane (Nucleosil 5 C_8) column. Maximum absorptions of sulfamoyldapsone were observed at 221 and 292 nm; 292 nm was selected for the detection of the compound in LC analysis because many interfering peaks derived from tissue components showed absorption at 221 nm.

We used acetonitrile saturated with *n*-hexane for extraction, and *n*-hexane saturated with acetonitrile for subsequent washing because sulfamoyldapsone is soluble in acetonitrile saturated with *n*-hexane. Methanol was also used as an extracting solvent instead of acetonitrile saturated with *n*-hexane. However, because methanol dissolved more interfering tissue components than did acetonitrile saturated with *n*-hexane, it was unfavorable for subsequent analysis. Furthermore, recovery from methanol extract was about 10% lower than that from acetonitrile saturated with *n*-hexane extract.

Because the concentrated extracts still contained many kinds of interfering materials derived from tissues, further cleanup was examined by using several column materials (silica, alumina, and Florisil). Of them, alumina was the most effective. Almost all interfering peaks could be removed by eluting with 20 mL 40% methanol in acetonitrile.

The optimal LC operating conditions given in *Apparatus* (f) were chosen after several trials. Figure 1 shows typical chromatograms from a swine kidney blank and a swine kidney fortified with 0.02 μg/g of sulfamoyldapsone. Under the conditions used, sulfamoyldapsone was well separated from the solvent front and from interfering components from tissues. Chromatograms of extracts from muscle, liver, and fat were similar (data not shown).

A calibration curve was prepared over the range of 2–100 ng sulfamoyldapsone. A plot of the ratios of the peak areas of sulfamoyldapsone to the area of the internal standard against the concentration of sulfamoyldapsone gave a straight line. Acetanilide was selected as the internal standard because its retention time was sufficiently longer than that of sulfamoyldapsone and its peak was well separated from other peaks. Recovery studies were conducted by adding 0.1 and 0.5 μg/g sulfamoyldapsone to 10 g tissue samples. These samples were analyzed by the present method. Table 1 shows the recovery data of sulfamoyldapsone. These recoveries are satisfactory for residue analysis. The detection limit of this method is as low as 0.02 μg/g, the concentration with the signal-to-noise ratio of 3.

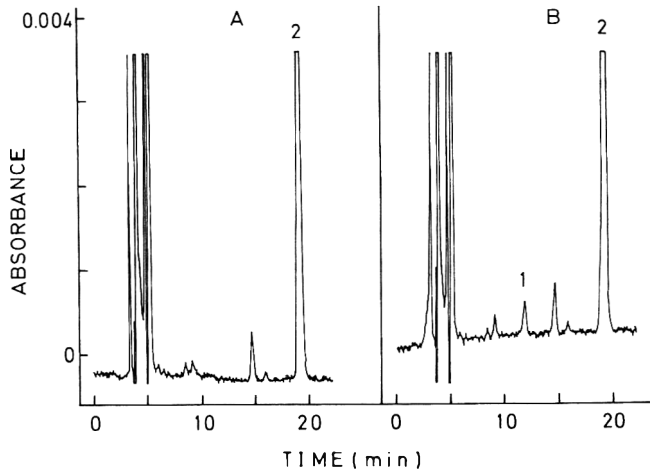

Figure 1. Typical chromatograms obtained from swine kidney tissues. A, blank; B, fortified with 0.02 μg/g sulfamoyldapsone. Peak 1, sulfamoyldapsone; peak 2, acetanilide internal standard.

Finally, we examined the interferences from other antimicrobial agents used in pigs: 5 antibiotics (chlortetracycline, oxytetracycline, tetracycline, chloramphenicol, and tylosin); 12 sulfonamides (sulfisoxazole, sulfadiazine, sulfisomidine, sulfathiazole, sulfamerazine, sulfamonomethoxine, sulfamethoxypyridazine, sulfamethazine, sulfadoxine, sulfaphenazole, sulfaquinoxaline, and sulfadimethoxine); 3 nitrofurans (nitrofurazone, nitrovin, and furazolidone); and 7 other drugs (trimethoprim, ormetoprim, diaveridine, pyrimethamine, nalidixic acid, carbadox, and olaquindox). Under our LC conditions, tetracyclines, tylosin, nitrovin, trimethoprim, ormetoprim, diaveridine, nalidixic acid, and pyrimethamine were not eluted until 50 min, and drugs other than nitrofurazone were well separated from sulfamoyldapsone and acetanilide (internal standard). Nitrofurazone could not be removed by treatment on the alumina column. However, because resolution of sulfamoyldapsone and nitrofurazone peaks was 0.98 at 1 μg/mL sulfamoyldapsone and 20 μg/mL nitrofurazone, and because sulfamoyldapsone was detected at 0.1 μg/mL in the presence of 10 μg/mL nitrofurazone, the interference of nitrofurazone could be marginal. Therefore, this method should be applicable to the routine determination of sulfamoyldapsone in commercial swine tissues.

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FOOD COMPOSITION

Determination by Liquid Chromatography with Electrochemical Detection of Cysteamine and Cysteine, Possible Precursors of *N*-Nitrosothiazolidine

JOHN W. PENSABENE, ROBERT C. DOERR, and WALTER FIDDLER
U.S. Department of Agriculture, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, PA 19118

A method is described that is selective, sensitive, rapid, and accurate for the quantitative measurement in meat products of both cysteamine and cysteine, potential precursors for *N*-nitrosothiazolidine (NTHZ) and *N*-nitrosothiazolidine-4-carboxylic acid (NTHZC), respectively. In general, a ground meat sample is homogenized with acetonitrile-formate buffer in the presence of dithiothreitol, and then is centrifuged, filtered, and recentrifuged in a disposable microfilter. The thiols are quantitated by liquid chromatography using an amperometric detector equipped with a gold/mercury electrode operated in the oxidative mode. Cysteamine was found in 6 of 20 samples of raw pork belly in concentrations ranging from 150 to 450 ppb, and cysteine was found in all samples in concentrations ranging from 2.4 to 36.5 ppm. Analysis for the thiols and their corresponding nitrosamines—NTHZ and NTHZC—of bacon before and after processing showed no correlation between cysteamine and cysteine levels before processing nor with nitrosamine levels after processing. Liquid chromatography with electrochemical detection was found to be an extremely selective technique to measure the 2 free sulfhydryl compounds in a complex food substrate.

The occurrence of *N*-nitrosothiazolidine (NTHZ) in smoked, cured meat products, particularly bacon, prior to home cooking has led to an investigation for the precursors of and mechanism for NTHZ formation. Unlike *N*-nitrosopyrrolidine, whose precursors are present in the adipose tissue of bacon (1), the precursors for NTHZ were found in the lean tissue (2). Several possible pathways have been proposed to account for the formation of NTHZ in bacon. In a model system study (3), it was found that cysteamine in meat can react with formaldehyde from smoke to form thiazolidine, which in turn reacts with nitrite to form NTHZ. It has also been hypothesized (4, 5) that cysteine reacts similarly to form *N*-nitrosothiazolidine-4-carboxylic acid (NTHZC), which can then thermally decarboxylate to form NTHZ. Although no correlation has been found between NTHZ and NTHZC in raw bacon (4), Sen et al. (6) reported a good correlation between NTHZC before and NTHZ after frying bacon. In elucidating the mechanism of NTHZ formation, it is important to ascertain whether cysteamine and cysteine are present in sufficient quantities to favor a particular pathway. Given this information, it will be easier to develop potential inhibitors or treatments to reduce the content of these nitrosamines in foods. This work takes on added importance since NTHZC has been found in human urine, and it also is claimed to be an indicator of in vivo nitrosamine formation (7).

At present, the only information available on the cysteamine content of edible meats comes from the analysis of kidney, liver, heart, and brain tissue from pork and beef (8, 9); for cysteine, the values are usually reported as the disulfide, cystine. This is due, in part, to the lack of an accurate quantitative method for these compounds in food products because cysteamine and cyteine are highly reactive and readily oxidized. However, Lunte (10) recently reported an effective method for the detection of cysteine and cystine in urine by liquid chromatography with electrochemical detection (LCEC). Using a gold/mercury electrode, we have developed a selective, rapid, and accurate method for determining low concentrations of cysteamine and cysteine in meat products. A description of the method and the results from the analysis of pork bellies for the 2 thiols in question are reported herein.

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METHOD

Reagents

(a) *Acetonitrile.*—Distilled-in-glass solvent (American Burdick and Jackson Labs, Muskegon, MI 49442).

(b) *Dithiothreitol.*—(Aldrich Chemical Co., Milwaukee, WI 53201.) Prepare fresh weekly at a concentration of 5 mg/mL in acetonitrile–formate buffer.

(c) *Cysteamine and cysteine.*—(Aldrich Chemical Co.) 1.0 ng/μL each in acetonitrile–formate buffer solution as standard in liquic chromatography with electrochemical detection.

(d) *Other reagents.*—Purchased from local suppliers and used without further purification.

(e) *Formate buffer.*—0.2M formic acid, 0.1 M potassium hydroxide, 0.0002M EDTA in 2 L water (pH 3.5).

(f) *Mobile phase.*—Acetonitrile–formate buffer solution (50 + 50 v/v).

(g) *Meat products.*—Fresh pork bellies were obtained from a local supplier within 2 h of slaughter and frozen at −18°C until analyzed. Thawed bellies were ground and thoroughly mixed prior to analysis. Bacon was prepared as described previously (3).

Apparatus

Usual laboratory equipment and the following items:

(a) *Homogenizer.*—VirTis Model 45 (VirTis Co., Inc., Gardiner, NY 12525) with 100 mL flask and U-shaped "Turbo-Shear" blades (No. 16-107).

(b) *Refrigerated centrifuge.*—Sorvall Model RC-5B (DuPont Co., Wilmington, DE 19898).

(c) *Microfilter.*—Centrex disposable microfilter unit (No. DF 101-1), 0.2 μm nylon (purchased from Schleicher and Schuell, Inc., Keene, NH 03431).

(d) *Liquid chromatograph-electrochemical detector.*—Altex Model 100A pumping system (Altex, Berkeley, CA 94710) with Rheodyne Model 7125 injector (Rheodyne, Cotati, CA 94928) and a 25 cm × 4.6 mm id 10 μm Partisil PXS 10/25 SCX ion exchange column (purchased from Whatman Ltd., Clifton, NJ 07014) interfaced to a Bioanalytical Systems LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN 47906) equipped with a Au/Hg electrode operated in the oxidative mode (+0.15 V) vs Ag/AgCl (10). Mobile phase flow rate, 1.1 mL/min. Under these operating

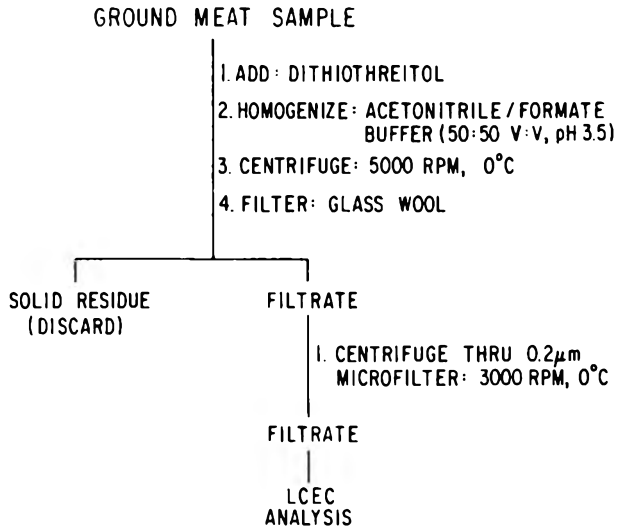

Figure 1. Flow diagram of cysteamine-cysteine LCEC Method.

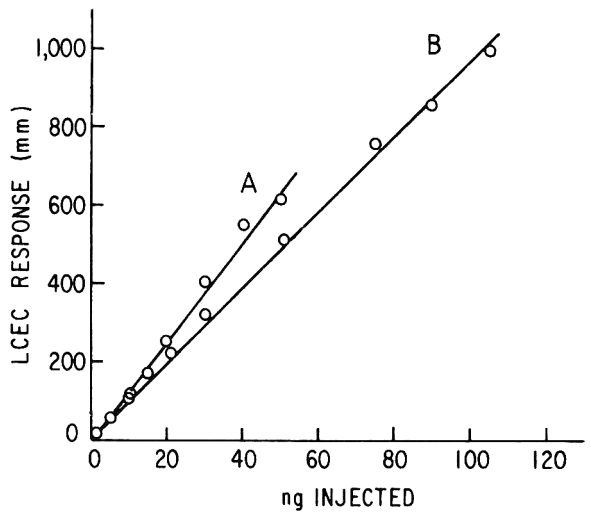

Figure 3. LCEC response for A, cysteamine, and B, cysteine.

conditions, the Au/Hg electrode is usually operational with good sensitivity and repeatability for ca 2 weeks before it is necessary to recoat the electrode.

Procedure

Note: N-Nitrosamines are potential carcinogens. Exercise care in handling these compounds.

(a) *Cysteamine–cysteine analysis.*—A flow diagram of this method is shown in Figure 1. Accurately weigh 10.0 ± 0.1 g ground pork belly into 100 mL VirTis flask. Add exactly 40 mL acetonitrile–formate buffer solution and 0.5 mL dithiothreitol solution. Homogenize sample 2 min at medium setting. Quantitatively transfer sample, using 9.5 mL-acetonitrile–formate buffer (total volume of solution is 50 mL), to 150 mL glass centrifuge bottle and centrifuge 30 min at 5000 rpm at 0–5°C. Filter sample through glass wool into 125 mL Erlenmeyer flask and transfer a 3 mL aliquot of filtered solution to the 2-stage microfilter unit. Centrifuge unit 20 min at 3000 rpm at 0–5°C. Remove lower section of microfilter, which contains the sample, for quantitation by LCEC.

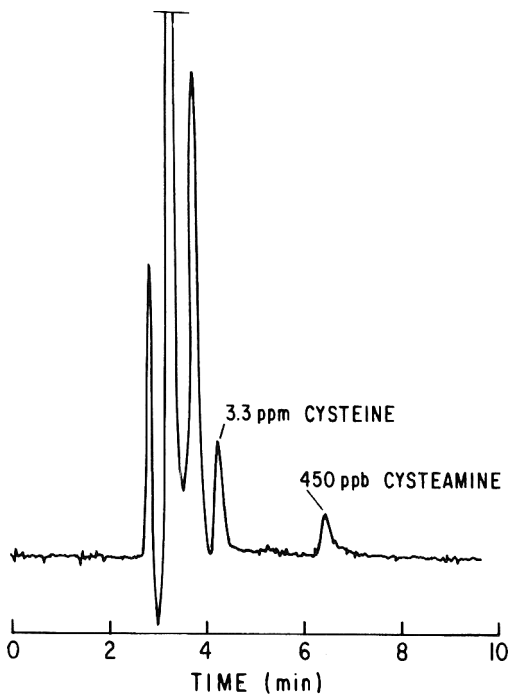

Figure 2. LCEC chromatogram of unprocessed pork belly.

(b) *Cysteamine–cysteine determination.*—A sample chromatogram of unprocessed pork belly is shown in Figure 2. Inject 15.0 μL cysteamine–cysteine standard (1 ng/μL) into LCEC at lowest attenuation that yields a peak suitable for quantitation. Repeat standard injection to ensure reproducibility of retention time and response. Inject 15.0 μL sample solution and measure peak heights. Calculate cysteamine–cysteine based on 10.0 g sample in 50 mL solution. The minimum detectable level for cysteamine was 100 ppb and for cysteine, 200 ppb.

(c) *N-Nitrosothiazolidine (NTHZ) and N-nitrosothiazolidine-4-carboxylic acid (NTHZC) analysis and determination.*—Perform procedure and requisite calculations for determining NTHZ and NTHZC in cured bacon as described previously (4, 11).

(d) *Statistical analysis.*—Perform according to the methods of Snedecor and Cochran (12).

Results and Discussion

The isolation and quantitation of cysteamine and cysteine from fresh or processed meat samples was performed using an extraction procedure designed to prevent the further oxidation of the free thiols. This was accomplished by adding the antioxidant dithiothreitol (DTT). Cleland (13) reported that in a model system at pH 7 or above, DTT is capable both of maintaining monothiols in the reduced state and of reducing disulfides quantitatively. We found, however, that although DTT at neutral or alkaline pHs can reduce cystamine (the disulfide of cysteamine), the rate of oxidation of cysteamine back to cystamine or another disulfide was so rapid under conditions present in a meat sample that cysteamine could not be accurately quantitated. Dupré and Aureli (14) also found that the oxidation of thiols to disulfides was faster in the alkaline range, especially in the presence of catalytic amounts of copper or other cations under mild oxidizing conditions. We found that by extracting cysteamine and cysteine in an acidic medium in the presence of DTT, both thiols remained intact and the symmetric disulfides—cystamine and cystine—if present, were quantitatively reduced to their corresponding thiols. Caldwell (15) also successfully reduced disulfides to thiols at pH 3.2 in the presence of DTT by heating the reaction to 100°C for 5 min. We found, however, that the reaction is so facile that the heating step

Table 1. Cysteamine and cysteine in raw pork belly

Sample No.	Cysteamine, ppb	Cysteine, ppm
1	198	17.9
2–5	ND[a]	10.5, 13.6, 7.8, 20.0
6	150	14.5
7	450	3.3
8	292	26.1
9	218	21.6
10–12	tr[b]	23.4, 16.1, 12.1
13, 14	ND	6.1, 7.6
15	170	36.5
16–18	ND	10.3, 10.3, 8.3
19, 20 (ham)	ND	14.5, 2.4

[a] ND = none detected.
[b] tr = trace (i.e., <100 ppb).

was unnecessary. In addition, extraction of the meat samples with the LCEC mobile phase solvent system both increased the recovery of the thiols and eliminated erratic detector response. Recovery of cysteamine and cysteine during the procedure was verified by adding known amounts of these compounds to the meat sample prior to analysis. Recovery of cysteamine fortified at the 200 ppb level was 82 ± 6.0%; recovery of the more prevalent cysteine spiked at the 2.5 ppm level was 89 ± 2.1%. The LCEC response for both cysteamine and cysteine was linear (Figure 3) over the range of 1 to 50 ng injected for cysteamine (equivalent to 100 ppb to 16 ppm) and from 1 to 110 ng injected for cysteine (equivalent to 200 ppb to 32 ppm).

Some typical concentrations of cysteamine and cysteine in unprocessed pork belly are shown in Table 1. Cysteamine concentrations ranged from none detected to 450 ppb; cysteine, from 2.4 to 36.5 ppm. To determine if there was a correlation between these 2 compounds and their corresponding nitrosamines, cysteamine and cysteine were measured before and after processing; NTHZ and NTHZC were measured after processing. Results are shown in Table 2. No correlation was found between cysteamine levels before and NTHZ levels after processing, nor between cysteine and NTHZC in the 9 pork belly samples. It is interesting to note that NTHZ was present in a few samples that contain no detectable cysteamine in the preprocessed pork belly. Because the NTHZ amine precursors were previously found in the lean tissue (2), and to eliminate the dilution effect of the adipose tissue, lean tissue was physically separated from the pork belly before processing. Again, no apparent correlation was found between the thiols and their corresponding nitrosamines.

It now appears that the mechanism of NTHZ formation is more complex than previously thought. In model system studies, it was shown that NTHZ forms from thiazolidine 3 times faster than from cysteamine; hence, the formation of thiazolidine from cysteamine and formaldehyde may be the limiting factor (3). Formaldehyde might also be limited in either concentration or exposure to the amine precursor, which may be present in the interior of the meat rather than just on the surface. The high concentrations of cysteine present in the samples analyzed suggest that it may contribute to NTHZ formation indirectly, even though no correlation was found between cysteine and NTHZ. Although the thermal conditions during processing are not sufficiently high for NTHZC or cysteine decarboxylation, as indicated in model systems (4), other components in meat may favor this pathway. Certainly, enzymatic decarboxylation would not be a consideration because meat enzymes are deactivated by the heat treatment The low levels of cysteamine compared to those of cysteine also suggest that cysteamine may be much more reactive than cysteine, thereby forming reaction products that might be much less efficient in forming NTHZ than cysteamine itself, but might be present in sufficiently high concentrations to contribute to the total NTHZ content of bacon.

Table 2. Cysteamine, cysteine, and nitrosamines in smokehouse processed bacon

	Before processing		After processing			
Sample No.	Cysteamine, ppb	Cysteine, ppm	Cysteamine, ppb	Cysteine, ppm	NTHZ,[a] ppb	NTHZC,[a] ppb
1	tr[b]	12.1	ND[c]	3.9	ND	350
2	ND	7.6	ND	5.3	10.8	510
3	ND	23.4	ND	17.4	ND	ND
4	ND	16.1	ND	13.9	ND	ND
5	292	26.1	ND	11.1	ND	ND
6	218	21.6	ND	14.4	ND	ND
7	ND	10.3	ND	2.1	9.8	453
8	ND	8.3	ND	2.3	12.7	368
9	ND	10.3	ND	8.0	4.3	576
10[d]	189	30.3	ND	4.7	1.5	498
11	114	8.0	ND	0.8	7.1	586
12	ND	12.6	ND	2.1	7.7	547
13	337	16.3	ND	19.2	5.9	1055
14	137	8.6	ND	1.2	7.3	486
15	159	9.9	ND	1.4	7.5	542

[a] NTHZ = *N*-nitrosothiazolidine; NTHZC = *N*-nitrosothiazolidine-4-carboxylic acid.
[b] tr = trace (i.e., <100 ppb).
[c] ND = none detected.
[d] Sample 10–15 are lean tissue.

In conclusion, we have shown that liquid chromatography with eletrochemical detection is a sensitive technique for measuring cysteamine and cysteine in extracts from a complex food substrate. The low redox potential of the gold/mercury electrode resulted in a high degree of selectivity. This method should be applicable to these and other thiol compounds in a wide variety of food products and biological samples.

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Anthocyanin Pigment, Nonvolatile Acid, and Sugar Composition of Red Raspberry Juice

GEORGE A. SPANOS and RONALD E. WROLSTAD
Oregon State University, Department of Food Science and Technology, Corvallis, OR 97331

Liquid chromatographic methodology for determination of red raspberry anthocyanin pigments, nonvolatile acids, and sugars was developed and applied to authentic juices pressed in our pilot plant as well as to samples of European origin. Sugar analysis utilized an Aminex HPX-87C column; the mean contents of sucrose, glucose, and fructose were 0.17, 2.59, and 2.97 g/100 mL, respectively. Citric, malic, and isocitric acids were best quantitated on two C-18 columns in series with refractive index detection; the mean content of citric, malic, and isocitric acids was 21.0 g/L, 872 mg/L, and 158 mg/L, respectively. Anthocyanins were resolved with high selectivity on a C-18 column using 15% acetic acid as organic modifier. The mean values for individual anthocyanins were: cyanidin-3-sophoroside, 74.2%; cyanidin-3-glucoside, 12.2%; cyanidin-3-glucorutinoside, 8.6%; cyanidin-3-rutinoside, 1.7%; and pelargonidin-3-sophoroside, 2.9%. Anthocyanin pigment content ranged from 23.8 to 101.0 mg/100 mL. Carbon stable isotope ratios ranged from −23.2 to −24.7‰ with a mean of −24.1‰. Additional data include color density, polymeric color, browning index, UV-visible spectra, pH, °Brix, titratable acidity, and Hunter color parameters.

The attractive color and flavor of red raspberries (*Rubus ideaus* L.) have led to increased demand for red raspberry juice and juice concentrates. Red raspberry juice is increasingly used in blended fruit drinks and in other formulated food products. The relatively high cost of red raspberry fruit makes red raspberry juice concentrates products of high economic value; prices in 1985 ranged from $70 to $75 per gallon for 68° Brix (% soluble solids) concentrate. The increased demand along with its high value makes red raspberry juice a likely target for adulteration. Much attention has been given to establishing methodology and criteria for detecting adulteration in more widely consumed fruit juices such as apple (1–3) and orange juices (3, 4) and, recently, in the popular cranberry juice cocktails (5–8). However, both methodology and the compositional data base for red raspberry juice are limited.

The purpose of this work was to develop methodology for analyzing red raspberry juice for sugar, nonvolatile acid, and pigment composition and to create a reference data base for authentic red raspberry juice.

METHOD

Samples

Red raspberry fruit samples (10–15 lb per sample) were obtained from Oregon North Willamette Station (ORNWS), Aurora, OR; Agriculture Canada Research Station (ACRS), Vancouver, British Columbia; and Western Washington Research and Extension Center (WWREC), Puyallup, WA. These samples had the following varietal makeup: Meeker (ORNWS, ACRS, and WWREC), Willamette (ORNWS and ACRS), and Skeena (ACRS). Fruit samples were washed, individually quick-frozen (IQF), and stored frozen at −12°C until needed. A New Zealand sample, variety Marcy, supplied in freeze-dried form by the Department of Scientific and Industrial Research (DSIRNZ), Auckland, New Zealand, was reconstituted to initial moisture with water before pressing.

Three juice samples of European origin, certified to be authentic by the supplier, were obtained from Bayernwald Fruchteverwertung GmbH, Hengersberg, West Germany. These had been pressed from Bavarian wild raspberries (picked from Buchelstein), Romanian wild raspberries (picked from central Cluj.), and Hungarian cultivated raspberries (northern Hungary, no name variety). The European samples had been pasteurized but not depectinized. After arrival, samples were stored frozen at −12°C. Before analysis, they were thawed and centrifuged.

Apparatus

(a) *Liquid chromatograph.*—Perkin-Elmer Series 400 (Perkin-Elmer Corp., Analytical Instruments, Norwalk, CT); Varian Model 5000 equipped with a column heater (Varian Instrument Group, Walnut Creek, CA).

(b) *Detectors.*—Varian UV 50 variable wavelength detector; Varian refractive index detector.

(c) *Integrators.*—Perkin-Elmer LCI-100; Hewlett-Packard HP 3380A (Hewlett-Packard Corp., Avondale, PA).

(d) *LC columns.*—Bio-Rad Aminex HPX-87C column, 300 × 7.8 mm id, fitted with Bio-Rad Carbo C 4 cm × 4.6 mm Micro-Guard column (Bio-Rad Laboratories, Richmond, CA); Supelcosil LC-18 (5 μm), 250 × 4.6 mm id column (Supelco, Inc., Bellefonte, PA); Nucleosil C-18 (10 μm), 250 × 4.6 mm id column (Alltech Associates, Inc., Deerfield, IL); ODS-10, 4 cm × 4.6 mm id, Micro-Guard column (Bio-Rad Laboratories).

(e) *Bath circulator.*—Lauda Model RMT-6 refrigerated circulator.

(f) *UV-visible spectrophotometer.*—Varian DMS 100 interfaced with Varian DS-15 data station.

(g) *Color difference meter.*—Hunter DP-25P-2 (Hunter Instruments, Reston, VA).

(h) *C_{18} minicolumn.*—Sep-Pak C_{18} (Waters Associates, Milford, MA). To activate, pass 5 mL methanol through cartridge, followed by 5 mL water.

(i) *Disposable Poly-Prep chromatography columns.*—Graduated, 0.8 × 4 cm, holding up to 2 mL chromatographic media and including an integral 10 mL reservoir (Bio-Rad Laboratories).

Reagents

(a) *LC mobile phase for nonvolatile acids.*—Phosphate buffer pH 2.4. Dissolve 27.2 g KH_2PO_4 in 1000 mL glass-distilled, deionized water and adjust pH to 2.4 with concentrated phosphoric acid. Filter through 0.45 μm Millipore filter, type HA (Millipore Corp., Bedford, MA), and degas.

(b) *LC mobile phase for sugars.*—200 mg $Ca(NO_3)_2$/1000 mL water.—Dissolve 200 mg $Ca(NO_3)_2$ in 1000 mL glass-distilled, deionized water. Filter through 0.45 μm Millipore filter, type HA, and degas.

(c) *LC mobile phase for anthocyanins.*—Solvent A: 15% acetic acid. Add 150 mL LC grade glacial acetic acid to 850 mL glass-distilled, deionized water, mix, filter through 0.45 μm Millipore filter, type HA, and degas. Solvent B: 15% acetic acid in methanol. Add 150 mL LC grade glacial acetic acid to 850 mL LC grade methanol, mix, filter through 0.45 μm Millipore filter, type HV, and degas. (Prepare solvent B fresh daily.)

(d) *LC mobile phase for anthocyanidins.*—Solvent A: 10%

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acetic acid. Add 100 mL LC grade glacial acetic acid to 900 mL glass-distilled, deionized water, mix, filter through Millipore 0.45 μm filter, type HA, and degas. Solvent B: acetonitrile–methanol (2 + 1). Add 600 mL LC grade acetonitrile to 300 mL LC grade methanol, mix, filter through 0.45 μm Millipore filter, type HV, and degas.

(e) *Organic acid standards 1.*—Add 1000 mg reagent grade D,L-malic acid and 200 mg reagent grade D,L-isocitric acid to 1000 mL volumetric flask and dilute to volume with water.

(f) *Organic acid standards 2.*—Add 1.500 g reagent grade citric acid to 100 mL volumetric flask and dilute to volume with organic acid standard solution 1.

(g) *Sugar standard solution 1 (internal standard).*—Add 6.500 g reagent grade mannitol to 250 mL volumetric flask and dilute to volume with sugar mobile phase, (b).

(h) *Sugar standard solution 2.*—Add 0.250 g reagent grade sucrose, 2.000 g reagent grade glucose, and 2.400 g reagent grade fructose to 100 mL volumetric flask and dilute to volume with sugar standard solution 1.

(i) *Enzymatic analysis kits.*—L-Malic acid, D-isocitric acid, and citric acid kits (Boehringer Mannheim Biochemicals, Indianapolis, IN).

(j) *Anion exchange resin.*—Use intermediate base anion exchange resin, Bio-Rex 5 (Bio-Rad Laboratories), chloride form, 50–200 mesh size, and 8.8 meq./g resin (dry basis) or 2.8 meq./mL of resin bed ion-exchange capacity. Slurry resin in water. Make 1.2 mL and 1.4 mL resin bed volume to prepare samples for sugar analysis and for acid analysis, respectively. Rinse packed bed with 5 mL water. Do not let bed become dry.

Production of Red Raspberry Juice

Let frozen fruit thaw at ambient temperature. Grind berries through hammer mill (Model D Comminuting Machine, W. J. Fitzpatrick Co., Chicago, IL) equipped with ½ in. diameter circular pore mesh at speed that produces about ⅛ berries. Heat crushed fruit to 45°C and depectinize by incubating with 0.1 mL Rohapect D5L liquid pectic enzyme (Rohm & Haas, Co., Philadelphia, PA 19105) per lb fruit. Add 1% cellulose as press aid and press in Willmes bag press (60 type, Moffet Co., San Jose, CA) with final pressure of 4.0 bar. Take 25–30 mL sample, add 0.05% diatomaceous earth, filter through Whatman No. 1 paper, and conduct alcohol test. If precipitate forms, incubate juice at 42°C with 0.1 mL Rohapect/1000 mL juice to complete depectinization. Pasteurize juice at 98–100°C for 11–13 s. Add 0.05% diatomaceous earth and filter through Whatman No. 1 paper. Store juice frozen.

Concentration of Samples

Concentrate ca 250 mL single strength juice on rotary evaporator (water bath, 35°C) to 50° Brix. For analysis, redilute with water to 10° Brix.

Titratable Acidity

Determine titratable acidity by using AOAC glass electrode method, sec. **22.059** (9). Express results in terms of anhydrous citric acid per 100 mL juice.

Spectral Analyses

For visible spectra, dilute single strength juice 1/6–1/14 (depending on pigment concentration) with water and scan from 700 to 400 nm. For UV spectra, dilute juice 1/270 with water and scan from 400 to 200 nm. Scanning conditions: 1.0 cm quartz cell, water as blank, 1 nm slit width, 50 nm/min scan rate. Recompute first and second derivative spectra from original zero order.

Measure anthocyanin concentration, color density, polymeric color, and browning index as described by Wrolstad et al. (10). Express anthocyanin concentration as mg cyanidin-3-glucoside per 100 mL juice, using the extinction coefficient for cyanidin-3-glucoside, $\epsilon = 29\,600$, reported by Blundstone and Crean (11).

Hunter Parameters

Set up instrument to read transmitted color, spectral component included (arrangement III). Calibrate instrument according to manufacturer's instructions. Read L, a, b and X, Y, Z parameters of single strength juice, using 0.5 cm pathlength cell.

$^{13}C/^{12}C$ Stable Isotope Ratios

Concentrated samples (ca 50° Brix) were sent to Coastal Science Laboratory (5321 Industrial Oaks Blvd, Suite 103, Austin, TX 78735) for analysis.

Nonvolatile Acids—LC Determination

Sample preparation.—Adjust pH of sample to 6–7 with concentrated ammonium hydroxide (NH_4OH). Prepare 1.4 mL Bio-Rex 5 resin bed in Poly-Prep column and rinse bed with 5 mL water. Carefully apply 3 mL sample to resin bed and wash bed with 3 mL water. Place test tube under column and elute acids with 3.5 mL 10% sulfuric acid followed by 3.5 mL water. Mix eluate well and pass it through activated Sep-Pak C_{18} cartridge. Discard first 3 mL, collect remaining eluate, filter through 0.45 μm Millipore filter (type HA), and inject onto LC system.

Chromatography.—Operate liquid chromatograph (Varian 5000) under following conditions: columns, Supelcosil LC-18 and Nucleosil C-18 fitted with ODS-10 Micro-Guard column; mobile phase, 0.2M KH_2PO_4 pH 2.4; flow rate, 0.7 mL/min; elution temperature, 25°C; detection, refractive index, 1 × attenuation; detection temperature, 20 ± 0.1°C (controlled with bath circulator); integrator, Perkin-Elmer LCI-100; injection volume, 50 μL.

Calculate organic acid composition by using external standard method and graphical interpretation of results. Take 1:0, 2:3, 1:1, and 1:3 dilution of organic acid standard solution 2, plot peak area (peak height for isocitric acid) vs concentration, and fit a curve with the linear regression model for each individual acid:

$$C = \alpha + \beta \times A \qquad (1)$$

where C is concentration, mg/mL; A is peak area (peak height for isocitric acid); α is curve intercept; and β is curve slope. Calculate concentration, C'_s, of individual acids in prepared sample from peak area (peak height for isocitric acid) and formula (*1*). Acid concentrations, C_s, in original sample can be calculated by the formula:

$$C_s = C'_s \times DF/R \qquad (2)$$

where R is recovery and DF is dilution factor. To determine recovery, R, of each individual acid, subject standard acid solution to anion exchange and Sep-Pak cleanup procedure, calculate concentrations, C'_{std}, of individual acids in treated mixture from peak area (peak height for isocitric acid) and equation (*1*), and compare to concentrations, C_{std}, in original mixture. Percentage recovery is given by the formula:

$$\%R = C'_{std} \times DF \times 100/C_{std} \qquad (3)$$

Table 1. General properties of red raspberry juice

Sample	Yield, %	Yield,[a] %	°Brix	pH	Titrat.[b] acidity	Titrat.[a,b] acidity	$\delta^{13}C$, ‰ (PDB)
North American and New Zealand samples[c]							
Meeker (ORNWS)							
(Repl. 1)	(68.7)	(62.5)	(9.1)	(3.19)	(1.77)	(1.95)	
(Repl. 2)	(81.9)	(61.4)	(7.5)	(3.19)	(1.45)	(1.93)	
Av. of repl. 1, 2	75.3	62.5	8.3	3.19	1.61	1.94	−24.7
Meeker (ACRS)	90.1	73.0	8.1	3.07	1.49	1.84	−23.3
Meeker (WWREC)	74.7	79.9	10.7	3.10	1.41	1.32	−24.4
Willamette (ORNWS)							
(Repl. 1)	(77.8)	(56.8)	(7.3)	(3.15)	(1.85)	(2.53)	
(Repl. 2)	(72.6)	(53.7)	(7.4)	(3.14)	(1.86)	(2.51)	
Av. of repl. 1, 2	75.2	55.6	7.4	3.15	1.86	2.51	−24.7
Willamette (ACRS)	91.5	59.5	6.5	3.02	1.80	2.78	−23.8
Skeena (ACRS)	86.4	69.1	8.0	2.94	1.68	2.10	−24.4
Marcy (DSIRNZ)	90.1	50.5	5.6	3.23	1.40	2.50	−23.2
Min.	74.7	50.5	5.6	2.94	1.40	1.32	−24.7
Max.	91.5	79.9	10.7	3.23	1.86	2.78	−23.2
Mean	83.3	64.3	7.8	3.10	1.61	2.14	−24.1
Std dev.	7.3	9.5	1.5	0.09	0.17	0.46	0.6
CV, %	8.8	14.8	19.1	3.0	10.6	21.5	−2.4
European samples							
Bavarian			7.7	3.17	1.59	2.06	
Romanian			8.4	3.23	1.23	1.46	
Hungarian			8.9	3.22	1.58	1.77	

[a] Normalized to 10° Brix.
[b] Expressed as g citric acid/100 mL.
[c] Samples prepared at Oregon State University pilot plant.

Nonvolatile Acids—Enzymatic Determination

Determine citric, D-isocitric, and L-malic acid content by following the procedure provided with Boehringer Mannheim test kits. Dilute samples with water, 1:50 for citric acid and 1:0 for isocitric acid analysis. For malic analysis, add 1 mL sample to 10 mL volumetric flask, adjust pH to 7–8 with 0.1N NaOH (estimate required volume of 0.1N NaOH from titratable acidity data), and dilute to volume with water. Monitor absorbance at 340 nm.

Sugars

Sample preparation.—Mix 5 mL juice with 3 mL sugar standard solution 1 (internal standard) and pass mixture through activated Sep-Pak C_{18} cartridge. Discard first 3 mL and collect remaining eluate. Apply 4 mL eluate to 1.2 mL Bio-Rex 5 resin bed. Discard first 2 mL, collect remaining eluate, mix, filter through 0.45 μm Millipore filter (type HA), and inject onto LC system.

LC determination.—Operate liquid chromatograph (Varian 5000) under following conditions: column, Bio-Rad Aminex HPX-87C; mobile phase, 200 mg $Ca(NO_3)_2$/1000 mL water; flow rate, 0.7 mL/min; elution temperature, 85°C; detection, refractive index, 4× attenuation; detection temperature, 25°C; integrator, HP 3380A; injection volume, 25 μL.

Quantitate sugars via internal standard method and graphical interpretation of results. Take 1:0, 2:3, 1:1, and 1:3 dilution of sugar standard solution 2, plot area vs concentration, and fit curve with linear regression model for each individual sugar and for internal standard:

$$C = \alpha + \beta \times A \qquad (4)$$

where C is concentration, mg/mL; A is peak area; α is curve intercept, and β is curve slope. Calculate concentration of each individual sugar and internal standard in prepared sample from peak area and equation (*4*). Sugar concentration, C_s, in original sample can be calculated by the formula:

$$C_s\ (mg/mL) = C'_s \times C_{is} \times DF/C'_{is} \qquad (5)$$

where DF is dilution factor, C'_s is concentration of sugar in question in prepared sample, C'_{is} is concentration of internal standard after sample preparation, and C_{is} is concentration of internal standard in sample before sample preparation.

Anthocyanins

Sample preparation.—Adsorb pigments contained in 3–7 mL juice sample (depending on anthocyanin concentration) onto activated Sep-Pak C_{18} cartridge. Wash bed with 5 mL 0.01% HCl and elute pigments with minimum required volume of acidified methanol (4–6 mL) in 10 mL beaker. Evaporate methanol to ca 2 mL under nitrogen stream and dilute pigments to ca 5 mL with 0.01% HCl. Filter isolated pigments through 0.45 μm Millipore filter (type HA) and immediately inject onto LC system. Store sample in the dark in ice bath between injections.

LC determination.—Operate liquid chromatograph (Perkin-Elmer 400) under following conditions: column, Supelcosil LC-18 fitted with ODS-10 Micro-Guard column; mobile phase—solvent A, 15% acetic acid, solvent B, 15% acetic acid in methanol; flow rate, 1.2 mL/min; elution progam, 100% A isocratic for 10 min followed by a 0–5% linear gradient with B for 8 min and holding with 5% B for an additional 7 min (isocratic), equilibrate column to initial conditions for 5 min between injections; detection, visible at 515 nm, 0.2 absorbance unit full scale (AUFS); integrator, Perkin-Elmer LCI-100; injection volume, 25 μL.

Calculate anthocyanin ratios from area percentage of individual anthocyanins.

Anthocyanidins

Sample preparation.—Prepare samples for LC determination of anthocyanidins according to procedure described by Hong and Wrolstad (7).

Table 2. Compilation of general properties of red raspberry juice

Ref.	No. of samples	°Brix			pH		Titrat. acidity, g citric acid/100 mL		
		Min.	Max.	Mean	Min.	Max.	Min.	Max.	Mean
12	12	9.0	15.0	11.3			1.01[a]	1.82[a]	1.23[a]
12	54	7.2	14.4	10.8					
13	16	8.2	10.9	8.9			1.38	2.01	1.88
14	260						1.15[b]	1.70[b]	1.45[b]
—[c]	49				2.9	3.4			

[a] Original data expressed as mL 0.1 NaOH/100 mL.
[b] Titrated to pH 7.0; original data expressed as tartaric acid.
[c] G. W. Varseveld (1969) Oregon State University, Dept of Food Science and Technology, Corvallis, OR, unpublished data.

LC determination.—Operate liquid chromatograph (Perkin-Elmer Series 400) under following conditions: column, Nucleosil C-18 column fitted with ODS-10 Micro-Guard column; mobile phase—solvent A, 10% acetic acid, solvent B, acetonitrile–methanol (2 + 1); elution, isocratic 84% A and 16% B; flow rate, 2.0 mL/min; elution temperature, 25°C; detection, visible at 530 nm, 0.2 AUFS; integrator, Perkin-Elmer LCI-100; injection volume, 25 μL.

Calculate anthocyanidin ratios from area percentage of individual anthocyanidins.

Results and Discussion

General Properties

Table 1 shows yield, degree Brix, pH, titratable acidity, and $\delta^{13}C$ values for the juice samples prepared in our pilot plant. Table 2 shows °Brix, pH, and titratable acidity data compiled from the literature.

There were 2 replications for Meeker (ORNWS) and Willamette (ORNWS) samples, which showed considerable variation in juice yield and °Brix. Factors contributing to this variation would have been the relatively small sample size, and washing of the fruit and/or washing of the equipment between pressing. Normalization of the yield to 10° Brix (Table 1) shows close agreement between replications, which implicates dilution as the cause of variation in yield.

Degree Brix values range from 5.6 to 10.7 with a mean of 7.8. These values are lower than those reported in the literature (Table 2) and well below the 10.5° Brix standard for red raspberries specified by the U.S. Department of Agriculture jelly standard. However, 10.0° Brix is commonly used for single strength red raspberry juice within the fruit juice industry, and all of the analytical data were normalized to that value. Normalization facilitates compositional comparisons between samples and circumvents the problem of yield variation and sample dilution.

Values for pH range from 2.94 to 3.23 and agree with those previously reported (Table 2).

Titratable acidity ranges from 1.40 to 1.86 g citric acid per 100 mL juice, which is similar to values reported in the literature (Table 2). There is a large difference in the titratable acidity between the replications of the Meeker (ORNWS) samples. However, if these values are normalized to 10° Brix they show no difference, again suggesting that dilution was the primary cause of the yield differences between replications.

The $\delta^{13}C$ values show a narrow range and low CV. These data provide a reference value which can be used for estimating the content of Hatch-Slack sweeteners such as cane sugar or corn syrup in a red raspberry product. Krueger et al. (15) reported −26.1 and −24.6‰ as $\delta^{13}C$ values for 2 red raspberry juice samples.

The European samples (Table 1) exhibit similar °Brix, pH, and titratable acidity characteristics.

Nonvolatile Acid Analyses

Red raspberries contain citric acid as the major acid, low levels of malic acid (14, 16–19), and trace amounts of isocitric acid (14); trace amounts of lactic and succinic acids have also been reported (19).

Reverse phase LC quantitation of isocitric acid is difficult because it elutes very close to malic acid, and it is present at very low levels. Adequate resolution of isocitric acid from malic acid can be achieved, however, by using two C-18 columns in series. Figure 1A shows a chromatogram of red raspberry nonvolatile acids, using UV detection at 227 nm and a 2 column system. Sample preparation involved only cleanup with a Sep-Pak C_{18} cartridge. In addition to malic acid (peak 1), isocitric (peak 2), and citric (peak 5), the following acids were tentatively identified: α-ketoglutaric (peak 3), shikimic, and ascorbic coeluting under peak 4, succinic (peak 6), and fumaric (peak 7). Identification of α-ketoglutaric acid was based on its retention time and production of symmetrical peak in spiked samples. Presence of α-ketoglutaric and shikimic acids has not been previously reported. Quantitation of isocitric acid on the basis of this procedure was about 4 times higher than quantitation on the basis of enzymatic analysis. This was probably due to coelution of an unidentified, highly UV-absorbing compound with isocitric acid. Moreover, late-eluting compounds with high response in the UV region made the total time of analysis very long, about 1 h. Modification in the sample preparation using anion exchange resin resulted in no change in the measured concentration of isocitric acid. Thus, the interfering compound is believed to be acidic and not a neutral compound.

A typical chromatogram of the red raspberry acids obtained by using refractive index detection and anion exchange cleanup is shown in Figure 1B. The more uniform response of refractive index detection to analytes allowed for quantitation of isocitric acid and shortened the total time of analysis to less than 25 min. The percent recoveries of individual acids in a standard solution containing 12 g citric, 500 mg malic, and 100 mg isocitric/L, were: citric acid 89.9, SD 2.8; malic acid 83.7, SD 2.7; and isocitric acid 87.3, SD 9.4.

Tables 3 and 4 show the results of the LC and enzymatic analyses, respectively, of the nonvolatile acids for the juice samples prepared in our pilot plant and for the European samples. Agreement between the 2 analytical methods is reasonably good. LC values for citric and malic acids are consistently lower than enzymatic results by 5–8%. LC determinations for isocitric acid show a wider range than do enzymatic determinations. However, statistical analysis using the paired t-test shows that the concentrations of citric and malic acids as well as total acid content by liquid chromatography are significantly different from those by enzymatic analysis at the 0.05 level. Citric and malic acid concentrations expressed as percent of total acids (by summation) do not differ significantly by the 2 different methods.

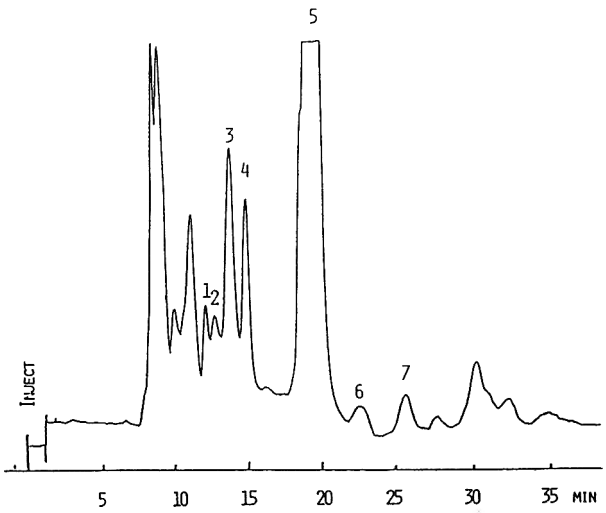

Figure 1A. LC separation of red raspberry nonvolatile acids: UV detection at 227 nm. Peak identification: 1, malic; 2, isocitric; 3, α-ketoglutaric; 4, shikimic and ascorbic; 5, citric; 6, succinic; 7, fumaric.

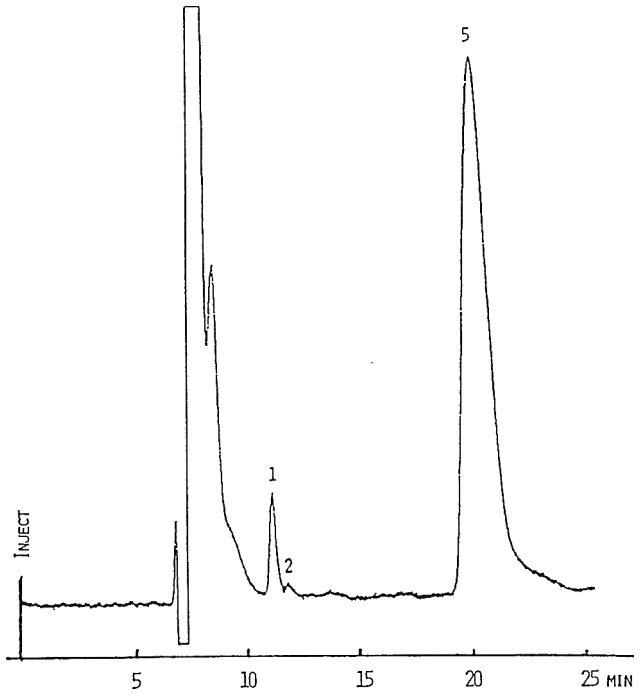

Figure 1B. LC separation of red raspberry nonvolatile acids: detection by refractive index. See Figure 1A for peak identification.

The German Richwerte und Schwankungsbreiten bestimmer Kennzahlen (RSK) values are a major data base available for red raspberries (14). The values reported in 1981 were based on 260 industrially produced samples as well as information from the scientific literature. The median values of citric, malic, and isocitric acids were 15 g/L, 500 mg/L, and 100 mg/L, respectively. The ranges for malic and isocitric contents were 200–1200 mg/L and 60–220 mg/L, respectively. Our data exhibit wider ranges and higher mean values. The RSK citric-to-isocitric ratio had a median of 150 and range between 80 and 200. Our range is narrower but our mean value is similar for this ratio. It is worth noting that one European sample (Romanian, Tables 3 and 4) exhibits atypical malic and isocitric content compared with the RSK values. The malic content, however, expressed in terms of the actual °Brix is in agreement with the RSK values.

The acid profile of red raspberry is simple; citric acid accounts for up to 97% of the acids. Synthetic citric acid is the most likely acidulant to be used in adulteration of red raspberry juice, and its detection would be difficult. The low levels of malic concentrations limit the use of malic acid as a potential adulterant. A very high citric-to-isocitric ratio would suggest adulteration with citric acid. Addition of isocitric acid is unlikely because of its relative high cost. Microbial activity has been reported to preferentially reduce the isocitric content (14) and could also account for a high citric-to-isocitric ratio.

Sugar Analyses

A typical chromatogram of red raspberry sugars is shown in Figure 2. Removal of acids from the juice via an anion exchange minicolumn is required, however, because interactions of acids with the calcium of the resin cause calcium leaching. The resulting protonated resin and the high elution temperature (85°C) catalyze on-column sucrose hydrolysis. This can be prevented by addition of calcium to the mobile phase; however, interactions between calcium and carboxyl groups result in increased retention of the nonvolatile acids and their coelution with sugars. The percent recovery of individual sugars in a standard solution, containing 0.4 g sucrose and 1.2 g each of glucose, fructose, and mannitol (internal standard), was: sucrose 95.9, SD 1.9; glucose 94.8, SD 1.7; fructose 94.2, SD 1.7; and mannitol 94.9, SD 1.8.

The results of the sugar analyses of juice samples prepared in our pilot plant are shown in Table 5. There is considerable variation in the individual and total sugar contents but the glucose-to-fructose ratio and the individual sugar content (with the exception of sucrose) expressed as percent of total show much less variation. All samples contained slightly more fructose than glucose, hardly any sucrose, and no sorbitol. The glucose-to-fructose ratio shows essentially an invert sugar pattern.

The red raspberry sugar profile, as compiled from the literature by Wrolstad and Shallenberger (20), showed much higher sucrose levels and lower glucose and fructose levels. The total sugar content and the glucose-to-fructose ratio are in agreement with our data. The sucrose levels reported in the literature are high for fruit but low for juice. The replicates of Meeker (ORNWS) and Willamette (ORNWS) samples (Table 5) show different sucrose levels. Traces of sucrose were detected in one replication of Meeker (ORNWS) but not in the other; low levels of sucrose (0.08 g/100 mL) were measured only in one replication of Willamette (ORNWS). Analysis of 50° Brix concentrates prepared in our laboratory from Skeena (ACRS) and Willamette (ACRS) juices showed about 7% decrease in the sucrose content. Similar findings have been reported by Fitelson (21) who found no sucrose in authentic red raspberry juice concentrates. Although invertase activity has not been documented for red raspberry fruit, it is very likely that it is responsible for the low sucrose content of processed juice relative to that of the fruit.

The sugar composition of the European samples (Table 5) exhibits the same pattern with a trend for slightly higher total sugar content and glucose-to-fructose ratio.

The sugar profile has limited utility in checking authenticity of red raspberry juices and juice concentrates. It is a simple invert profile, and invert syrups or fruits with invert pattern could be difficult to detect. Presence of sorbitol, however, is a very useful indicator of dilution with a sorbitol-containing juice such as cherry, plum, apple, or pear.

Table 3. Nonvolatile acid composition[a] of red raspberry juice—LC determinations

Sample	Citric, g/L	Malic mg/L	Isocitric, mg/L	Total, g/L	Percent of total			Citric/ malic	Citric/ isocitric	Malic/ isocitric
					Citric	Malic	Isocitric			
North American and New Zealand samples[b]										
Meeker (ORNWS)										
(Repl. 1)	(19.1)	(496)	(186)							
(Repl. 2)	(18.4)	(480)	(172)							
Av. of repl. 1, 2	18.8	488	179	19.4	96.6	2.5	0.9	38.4	104.7	2.7
Meeker (ACRS)	18.7	884	119	19.7	94.9	4.5	0.6	21.1	156.7	7.4
Meeker (WWREC)	13.1	393	75	13.5	96.5	2.9	0.6	33.3	174.3	5.2
Willamette (ORNWS)										
(Repl. 1)	(23.3)	(561)	(181)							
(Repl. 2)	(24.1)	(539)	(169)							
Av. of repl. 1, 2	23.7	550	175	24.4	97.0	2.3	0.7	43.0	135.2	3.1
Willamette (ACRS)	27.1	1903	231	29.2	92.7	6.5	0.8	14.2	117.4	8.2
Skeena (ACRS)	21.1	812	123	22.1	95.8	3.7	0.6	26.0	171.8	6.6
Marcy (DSIRNZ)	24.8	1075	205	26.1	95.1	4.1	0.8	23.1	121.1	5.2
Min.	13.1	393	75	13.5	92.7	2.3	0.6	14.2	104.7	2.7
Max.	27.1	1903	231	29.2	97.0	6.5	0.9	43.0	174.3	8.2
Mean	21.0	872	158	22.1	95.5	3.8	0.7	28.5	140.2	5.5
Std dev.	4.4	476	51	4.8	1.4	1.4	0.1	9.5	25.6	1.9
CV, %	20.7	54.6	32.0	21.6	1.4	35.7	18.2	33.2	18.3	34.8
European samples										
Bavarian	21.9	742	119	22.7	96.2	3.3	0.5	29.5	183.9	6.2
Romanian	14.8	1326	trace[c]	16.1	91.8	8.2	0.0	11.1	—	—
Hungarian	19.1	483	91	19.6	97.1	2.5	0.5	39.5	209.6	5.3

[a] Results normalized to 10° Brix.
[b] Samples prepared at Oregon State University pilot plant.
[c] Less than 50 mg/L.

Anthocyanin Analyses

The anthocyanins of red raspberries have been thoroughly investigated with the use of thin layer chromatography (TLC) or paper chromatography. Four major anthocyanin pigments have been identified (22–27): cyanidin-3-sophoroside (cyd-3-soph), cyanidin-3-glucoside (cyd-3-glu), cyanidin-3-glucorutinoside (cyd-3-glurut), and cyanidin-3-rutinoside (cyd-3-rut). All four do not occur in all varieties and selections. Related pelargonidin (pgd) anthocyanins and cyanidin-3,5-diglucoside (cyd-3,5-diglu) have also been reported as minor pigments (25).

Table 4. Nonvolatile acid composition[a] of red raspberry juice—enzymatic analysis

Sample	Citric, g/L	Malic, mg/L	Isocitric, mg/L	Total, g/L	Percent of total			Citric/ malic	Citric/ Isocitric	Malic/ isocitric
					Citric	Malic	Isocitric			
North American and New Zealand samples[b]										
Meeker (ORNWS)										
(Repl. 1)	(20.4)	(529)	(159)							
(Repl. 2)	(19.8)	(511)	(165)							
Av. of repl. 1, 2	20.1	520	162	20.7	96.7	2.5	0.8	38.6	123.8	3.2
Meeker (ACRS)	20.1	887	128	21.1	95.2	4.2	0.6	22.6	156.7	6.9
Meeker (WWREC)	13.8	411	83	14.3	96.5	2.9	0.6	33.6	166.1	5.0
Willamette (ORNWS)										
(Repl. 1)	(24.7)	(638)	(182)							
(Repl. 2)	(25.5)	(614)	(174)							
Av. of repl. 1, 2	25.1	626	178	25.9	96.9	2.4	0.7	40.1	141.1	3.5
Willamette (ACRS)	29.0	1928	179	31.1	93.2	6.2	0.6	15.0	161.7	10.8
Skeena (ACRS)	22.4	856	119	23.4	95.8	3.7	0.5	26.1	188.1	7.2
Marcy (DSIRNZ)	26.7	1062	184	28.0	95.5	3.8	0.7	25.2	145.2	5.8
Min.	13.8	411	83	14.3	93.2	2.4	0.5	15.0	123.8	3.2
Max.	29.0	1928	184	31.1	96.9	6.2	0.8	40.1	188.1	10.8
Mean	22.4	899	148	23.5	95.7	3.7	0.6	28.7	154.7	6.0
Std dev.	4.7	470	35	5.1	1.2	1.2	0.1	8.4	19.0	2.4
CV, %	20.9	52.3	24.1	21.7	1.2	33.1	13.1	29.3	12.3	39.6
European samples										
Bavarian	22.86	786	111	23.76	96.22	3 31	0.47	29.1	205.9	7.1
Romanian	15.42	1445	45	16.91	91.19	8 55	0.27	10.7	342.7	32.1
Hungarian	19.81	532	103	20.45	96.89	2 60	0.50	37.2	192.3	5.2

[a] Results normalized to 10° Brix.
[b] Samples prepared at Oregon State University pilot plant.

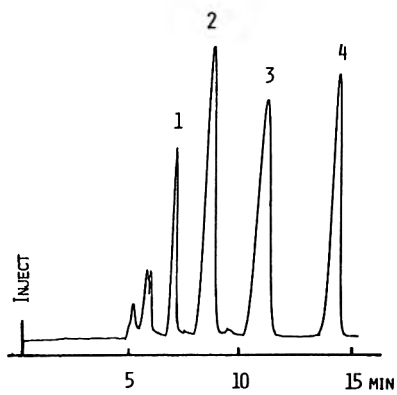

Figure 2. LC separation of red raspberry sugars. Peak identification: 1, sucrose; 2, glucose; 3, fructose; 4, mannitol (internal standard).

The use of reverse phase liquid chromatography for separation of anthocyanins and related substances has been reported for a number of commodities such as elderberries, cranberries, and cowberries (28–30) but not for red raspberries. Most of the reported systems include gradient elution with methanol or a combination of methanol and acetonitrile as organic modifiers. Initial investigations in our laboratory showed that such systems did not have enough selectivity to resolve the anthocyanins of red raspberry. For example, cyd-3-glurut, cyd-3-glu, and pgd-3-soph coeluted as one peak when the following conditions were used: solvent A = 10% acetic acid, solvent B = methanol; elution program, isocratic with 7% B and flow rate 1.5 mL/min. We succeeded in resolving the anthocyanins of red raspberries through use of acetic acid as an organic modifier, increasing its concentration to 15%. The solvent in which the isolated anthocyanin pigments were dissolved was found to strongly influence the separation. If the injected anthocyanins were dissolved in acidified methanol, double peaks for the major anthocyanins and asymmetrical peaks with gradual up slope and sharp down slope for minor anthocyanins resulted. The methanol injected with the pigments moves faster than the mobile phase in the column and creates a local methanolic gradient. This front of methanol causes faster pigment elution than does the mobile phase and may account for the poor peak shape observed. Evaporation of methanol and redilution with 0.01% HCl improved peak shape, giving a single peak for each compound. It is worth noting that despite the acidity of the mobile phase (pH of 15% acetic acid 1.9–2.0), there was no loss of resolution or peak shape deterioration over a long period of column use throughout this investigation.

Peak identification was based on (*1*) the chemistry of separation which includes the overall polarity of the anthocyanin molecule, the nature of the attached sugar, and the substitution of the B ring; (*2*) the relative magnitude of the peak; and (*3*) the retention times of anthocyanins contained in sour cherry, blackberry, black currant, and strawberry fruits. Extracts of these fruits were chromatographed under the same condition as that described for red raspberries. Chromatograms of these extracts are shown in Figure 3. Chromatograms of Meeker, Willamette, and Marcy anthocyanins are shown in Figure 4.

Peak 1 is the major peak in all red raspberry samples analyzed (42.0–85.3%). Several investigators (22–27) have certified cyd-3-soph as the major pigment in red raspberries. Peak 2 was identified as cyd-3-glurut, the major pigment of sour cherries (31). Barritt and Torre (25, 26) reported that cyd-3-glurut is present in Meeker but not in Willamette variety. Peak 2 is the major peak in the sour cherry chromatogram, and its presence distinguishes Meeker and Willamette chromatograms (Figures 3A; 4A and B). Peak 3 was identified as cyd-3-glu, which is common to blackberries as the major

Table 5. Sugar composition of red raspberry juice

Sample	Concentration,[a] g/100 mL				Percent of total sugars			Glucose/fructose
	Sucrose	Glucose	Fructose	Total	Sucrose	Glucose	Fructose	
North American and New Zealand samples[b]								
Meeker (ORNWS)								
(Repl. 1)	(0.00)	(2.74)	(3.01)					
(Repl. 2)	(trace)[c]	(2.78)	(3.09)					
Av. of repl. 1, 2	0.00	2.76	3.05	5.81	0.0	47.5	52.5	0.90
Meeker (ACRS)	0.10	2.99	3.37	6.46	1.5	46.3	52.2	0.89
Meeker (WWREC)	0.00	3.44	3.79	7.23	0.0	47.5	52.4	0.91
Willamette (ORNWS)								
(Repl. 1)	(0.00)	(2.27)	(2.56)					
(Repl. 2)	(0.08)	(2.23)	(2.50)					
Av. of repl. 1, 2	0.04	2.25	2.53	4.82	0.8	46.7	52.5	0.89
Willamette (ACRS)	0.17	2.23	2.62	5.02	3.4	44.4	52.2	0.85
Skeena (ACRS)	0.91	2.69	3.04	6.64	13.7	40.5	45.8	0.88
Marcy (DSIRNZ)	0.00	1.80	2.41	4.21	0.0	42.3	57.2	0.75
Min.	0.00	1.80	2.41	4.21	0.0	40.5	45.8	0.75
Max.	0.91	3.44	3.79	7.23	13.7	47.5	57.2	0.91
Mean	0.17	2.59	2.97	5.74	2.8	45.1	52.1	0.87
Std dev.	0.31	0.51	0.46	1.02	4.6	2.5	3.1	0.05
CV, %	175.6	19.5	15.4	17.7	165.5	5.5	5.9	6.0
European samples								
Bavarian	0.36	2.76	3.21	6.32	5.7	43.6	50.8	0.86
Romanian	0.00	3.51	3.69	7.19	0.0	48.8	51.2	0.95
Hungarian	0.16	3.11	3.69	6.96	2.3	44.7	53.1	0.84

[a] Expressed as single strength juice normalized to 10.0° Brix.
[b] Samples prepared at Oregon State University pilot plant.
[c] Less than 0.02 g/100 mL.

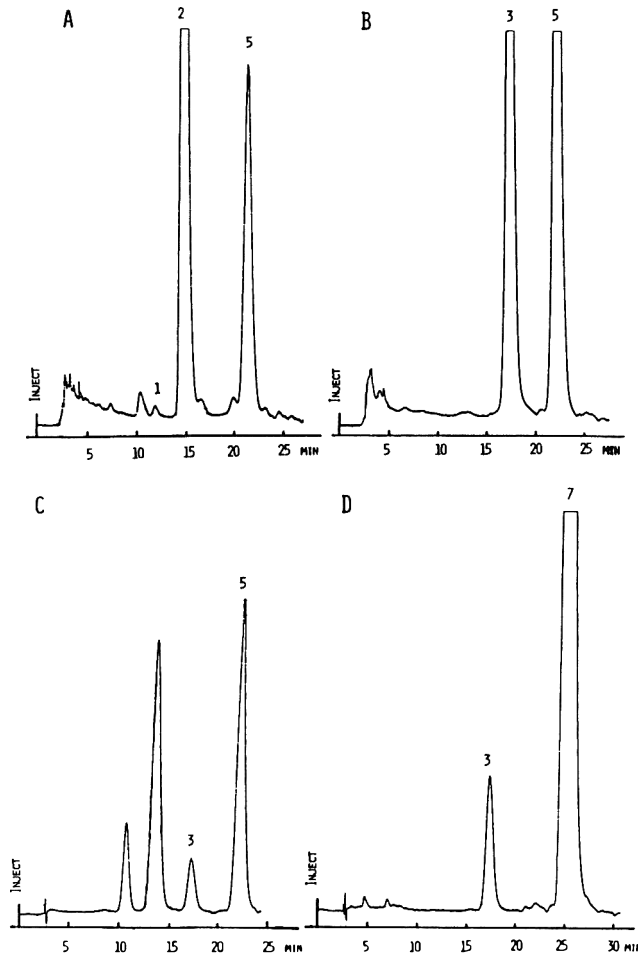

Figure 3. LC separation of anthocyanins from A, sour cherry; B, blackberry; C, black currant; D, strawberry. Peak identification: 1, cyd-3-soph; 2, cyd-3-glurut; 3, cyd-3-glu; 5, cyd-3-rut; 7, pgd-3-glu.

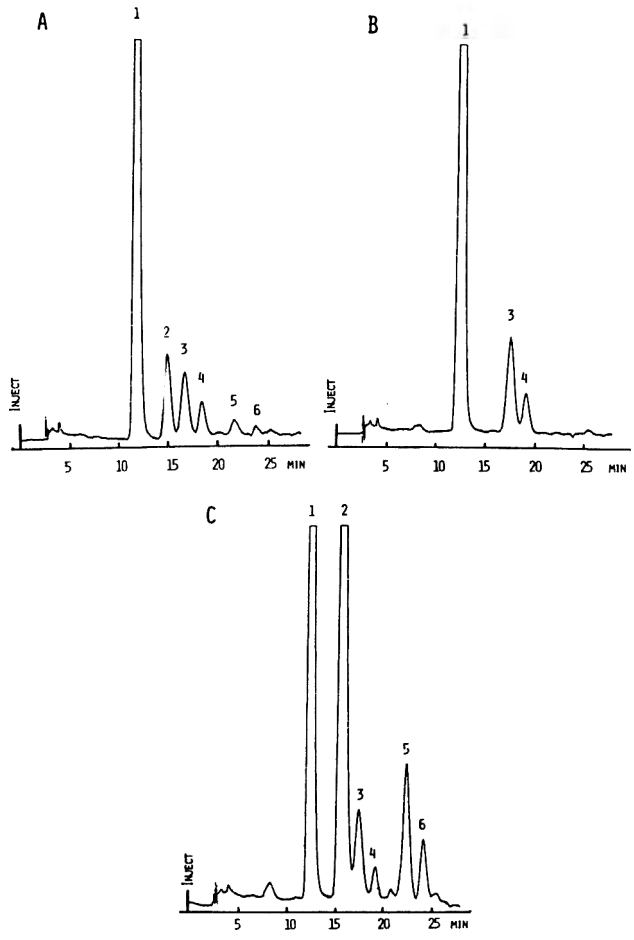

Figure 4. LC separation of red raspberry anthocyanins: A, Meeker (ORNWS); B, Willamette (ORNWS); C, Marcy (DSIRNZ). Peak identification: 1, cyd-3-soph; 2, cyd-3-glurut; 3, cyd-3-glu; 4, pgd-3-soph; 5, cyd-3-rut; 6, pgd-3-glurut; 7, pgd-3-glu.

pigment (23, 26). Cyd-3-glu has also been found in strawberries and black currant as a minor pigment (32) and in raspberries at intermediate levels (23–27). Peak 3 is the only peak common to the chromatograms of these fruits, and it is the major peak in blackberry, minor peak in strawberry and black currant, and intermediate peak in red raspberry (Figures 3B, C, and D; and 4A, B, and C). Peak 4 was not detected in varieties with trace amounts of pelargonidin, and it was tentatively identified as pgd-3-soph. Of the pelargonidin pigments in red raspberries, pgd-3-soph is contained in largest quantities (25). Peak 5 was identified as cyd-3-rut, the major pigment in black currant (32), Marion blackberry (23), and sour cherry (31) and the minor pigment in Meeker red raspberry cultivar; it is not present in Willamette variety (25, 26). Peak 5 fits this matrix. Peak 6 was tentatively identified as pgd-3-glurut (see below). Peak 7 was identified as pgd-3-glu because it is the major peak in the strawberry chromatogram (Figure 3D).

Summarizing, the elution order of the cyanidin pigments was as follows: cyd-3-soph, cyd-3-glurut, cyd-3-glu, and cyd-3-rut. This elution order suggests that the hydrophobic CH_3 group of rhamnose causes increased retention of the rutinose glycosides and reverses the general rule that the elution order is tri-, di-, mono-saccharide of the same aglycone. Considering the effect of the rhamnose CH_3 group on anthocyanin retention, the elution order for the pelargonidin pigments should be the following: pgd-3-soph, pgd-3-glurut, pgd-3-glu, and pgd-3-rut. This tentatively identifies peak 6 as pgd-3-glurut. Combining the effect of sugar moiety, determined by the number of sugar units and the presence of the CH_3 group, with the effect of B ring substitution on the overall polarity of the anthocyanin molecule gives the resulting elution order: cyd-3-soph, cyd-3-glurut, cyd-3-glu, pgd-3-soph, cyd-3-rut, pgd-3-glurut, pgd-3-glu, and pgd-3-rut. The presence of the trace pelargonidin pigments was readily detected by injection of more concentrated anthocyanin preparations. In these cases, cyd-3-soph exceeded the dynamic range of the detector, which would not allow for accurate quantitation of all pigments.

The pigment assignment by LC retention certified previous identifications of red raspberry anthocyanins. No additional pigments were detected. Cyd-3,5-diglu, which was tentatively identified on the basis of TLC retentions by Barritt and Torre (25) as a minor pigment in some varieties, was not found in any of the samples that we analyzed. From our analyses we could not conclusively determine whether this pigment was absent or coelutes, possibly with cyd-3-soph, because we do not have a standard source for determination of retention time. All other anthocyanin pigments that have been previously reported in red raspberry varieties were detected and separated. Similarly, the LC profiles for black currant, sour cherry, blackberry, and strawberry confirmed the published anthocyanin composition of these fruits (23, 31, 32).

The results of the anthocyanin analyses of the samples pressed in our pilot plant are shown in Table 6. Table 7 shows the quantitation of cyanidin pigments by TLC densitometry as determined by Barritt and Torre (25) for 37

Table 6. Anthocyanin and anthocyanidin composition (%) of red raspberry juice

Sample	Anthocyanins						Anthocyanidins	
	Cyanidin				Pelargonidin			
	3-soph	3-glu	3-glurut	3-rut	3-soph	3-glurut	Cyanidin	Pelargonidin
North American and New Zealand samples[a]								
Meeker (ORNWS)								
(Repl. 1)	(79.1)	(10.9)	(3.7)	(1.1)	(5.2)	trace[b]	(96.0)	(4.0)
(Repl. 2)	(78.7)	(11.5)	(3.5)	(1.1)	(5.2)	trace	(96.4)	(3.6)
Av. of repl. 1, 2	78.9	11.2	3.6	1.1	5.2	0.0	96.2	3.8
Meeker (ACRS)	75.7	10.8	8.6	1.9	2.9	0.0	98.1	1.9
Meeker (WWREC)	75.8	8.9	9.3	1.7	4.2	0.0	97.2	2.8
Willamette (ORNWS)								
(Repl. 1)	(85.5)	(10.7)	(0.0)	(0.0)	(3.8)	(0.0)	(97.1)	(2.9)
(Repl. 2)	(85.2)	(10.5)	(0.0)	(0.0)	(4.2)	(0.0)	(96.9)	(3.1)
Av. of repl. 1, 2	85.3	10.6	0.0	0.0	4.0	0.0	97.0	3.0
Willamette (ACRS)	85.2	12.6	0.0	0.0	2.2	0.0	98.5	1.5
Skeena (ACRS)	76.1	23.9	0.0	0.0	0.0	0.0	100.0	trace
Marcy (DSIRNZ)	42.0	7.6	38.3	7.0	2.0	3.2	95.1	4.9
Min.	42.0	7.6	0.0	0.0	0.0	0.0	95.1	0.0
Max.	85.3	23.9	38.3	7.0	5.2	3.2	100.0	4.9
Mean	74.2	12.2	8.6	1.7	2.9	0.5	97.4	2.6
Std dev.	13.7	5.0	12.7	2.3	1.6	1.1	1.5	1.5
CV, %	18.4	40.9	148.5	136.8	54.9	244.9	1.5	58.0
European samples								
Bavarian	85.1	14.9	0.0	0.0	0.0	0.0	100.0	trace
Romanian	53.1	13.0	21.5	9.5	1.5	1.4	98.4	1.6
Hungarian	80.1	14.4	0.0	0.0	5.5	0.0	97.6	2.4

[a] Samples prepared at Oregon State University pilot plant.
[b] Less than 1%.

cultivars and collections. Related pelargonidin pigments were listed as trace (less than 2%). Our data show higher mean values for cyd-3-soph and cyd-3-glurut and lower values for cyd-3-glu and cyd-3-rut. Partial hydrolysis or polymerization of anthocyanins during the more rigorous pigment isolation for TLC analysis as well as quantitation by densitometry could account for these differences.

The results of the anthocyanin analyses of the European samples are also shown in Table 6. These samples had undergone considerable anthocyanin degradation as evidenced by the lower monomeric pigment and higher percentage of polymeric color (Table 8). The anthocyanin profiles, however, are very similar to those for the juices processed in our pilot plant.

The anthocyanin profile of red raspberries is distinctive with 2 different patterns predominating. Varieties such as Willamette and Skeena and the Bavarian and Hungarian samples possess a simpler profile with 2 major cyanidin pigments predominating. Varieties such as Meeker and Marcy and the Romanian sample have a more complex pattern with 4 cyanidin pigments present in substantial quantities (Figure 4). There is considerable variation in the percentages of the cyanidin pigments between samples (Table 6).

Table 7. Red raspberry anthocyanin composition (%) as determined by Barritt and Torre (25)

Statistic	Cyanidin				
	3-soph	3-glu	3-glurut	3-rut	3-diglu
Min.	20	11	0	0	0
Max.	72	45	43	32	6
Mean	47.2	22.4	18.9	10.9	0.6
Std dev.	15.2	8.2	13.4	9.4	1.4
CV, %	32	37	71	86	250

Most anthocyanin-containing fruits have qualitatively and quantitatively different anthocyanin profiles from red raspberry and should be detected by this analysis.

Anthocyanidin Analyses

The results of the anthocyanidin analyses are shown in Table 6 and a typical chromatogram in Figure 5. Cyanidin, which is the only compound detected in some samples, predominates; pelargonidin has a maximum level of 5%. Anthocyanidin analysis would be useful in detecting adulterations with colorants containing malvinidin, delpinidin, peonidin, or petunidin.

Spectral Characteristics and Hunter Parameters

The color characteristics of the samples pressed in our pilot plant are shown in Table 8. Anthocyanin concentrations range between 23.8 and 101.0 mg/100 mL. In their analysis of fruit, Torre and Barritt (26) found a lower range of anthocyanin concentration between 20 and 60 mg/100 g of fruit. Variety, origin, and maturity can all account for differences in anthocyanin levels. Our data show that Willamette is about 50% higher in pigment than Meeker if comparisons are made within the same geographic region. Torre and Barritt (26) similarly found Willamette fruit to be about 50% higher in anthocyanin pigment than Meeker. The number of samples we analyzed, however, is too limited to ascertain the primary source of variation in anthocyanin pigment content. Measurements of color density, percent polymeric color, and browning index indicate that juices contain a low level of polymerized anthocyanin pigments. There was very little difference between replications for the color indices (samples Meeker ORNWS and Willamette ORNWS).

The effect of concentration (under laboratory conditions) on monomeric and polymeric color is shown in Table 8

Table 8. Color analyses of red raspberry juice

Sample	Anthocyanin concn,[a,b] mg/100 mL	Color density[b]	Polymeric color[b]	Percent polymeric color	Browning index[b]
North American and New Zealand samples[c]					
Meeker (ORNWS)					
(Repl. 1)	(68.2)	(22.6)	(1.0)	(4.6)	(0.64)
(Repl. 2)	(68.9)	(24.5)	(1.5)	(5.9)	(0.89)
Av. of repl. 1, 2	68.6	23.6	1.3	5.3	0.77
Meeker (ACRS)	29.0	13.5	1.4	10.1	0.86
Meeker (WWREC)	31.6	13.1	0.8	6.0	0.45
Willamette (ORNWS)					
(Repl. 1)	(98.6)	(32.9)	(1.4)	(4.3)	(0.91)
(Repl. 2)	(103.5)	(34.2)	(1.6)	(4.7)	(1.01)
Av. of repl. 1, 2	101.0	33.6	1.5	4.6	0.96
Willamette (ACRS)	53.0	24.6	1.7	7.1	1.05
Skeena (ACRS)	23.8	11.3	0.7	6.6	0.45
Marcy (DSIRNZ)	57.1	19.9	2.1	10.7	1.26
Min.	23.8	11.3	0.7	4.6	0.5
Max.	101.0	33.6	2.1	10.7	1.3
Mean	52.0	19.9	1.4	7.2	0.8
Std dev.	25.2	7.4	0.5	2.2	0.3
CV, %	48.4	37.2	34.0	30.3	33.6
Authentic samples concentrated to 50° Brix					
Meeker (ORNWS Repl. 1)	63.1	26.8	2.5	9.3	1.56
Willamette (ACRS)	45.9	24.2	3.4	14.0	2.09
Skeena (ACRS)	19.9	12.2	1.4	11.9	0.93
Marcy (DSIRNZ)	53.2	22.2	3.2	14.5	1.98
European samples					
Bavarian	9.2	8.0	2.9	36.3	1.71
Romanian	9.6	4.7	1.0	20.2	0.65
Hungarian	10.0	6.2	2.0	32.2	1.31

[a] Expressed as cyd-3-glu (ϵ = 29 600).
[b] Results normalized to 10° Brix.
[c] Samples prepared at Oregon State University pilot plant.

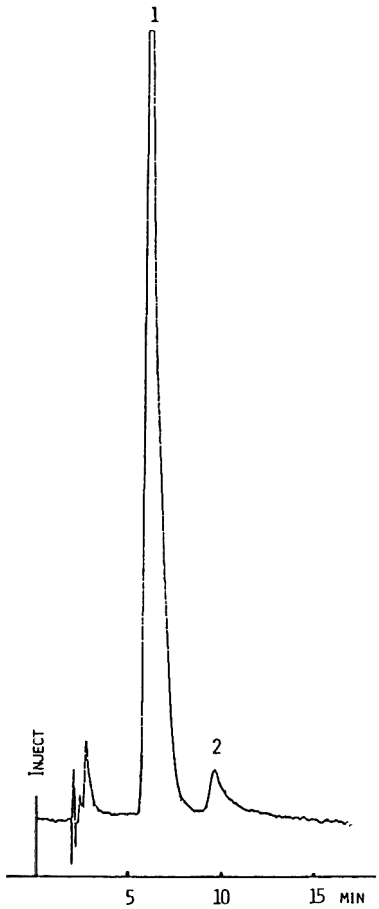

Figure 5. LC separation of red raspberry anthocyanidins. Peak identification: 1, cyanidin; 2, pelargonidin.

(samples concentrated to 50° Brix). Monomeric color decreases by an average of 11% while color density increases by an average of 9%. Polymeric color, percent polymeric color, and browning index increase by even more than 100%.

Anthocyanin concentration and color density of the European samples (Table 8) show that these samples are low in anthocyanin pigment and high in polymeric color. Pigment degradation with processing and/or storage would at least partially account for the low monomeric pigment content, although varietal and geographic influence could also contribute.

The spectral characteristics of red raspberry juice are shown in Table 9; the Hunter parameters are shown in Table 10. The European samples show higher L values due to their lighter color.

Summary

Methodology was developed for sugar, nonvolatile acid, and anthocyanin analysis by liquid chromatography in red raspberry juice. Adequate resolution of malic acid from isocitric by reverse phase C-18 chromatographic media can be achieved through use of 2 reverse phase C-18 columns in series. The more uniform response of analytes to refractive index detection allows for quantitation of isocitric acid. For sugar analysis, removal of acids in the juice via ion-exchange treatment is recommended. Anthocyanin pigments were resolved through use of 15% acetic acid in the mobile phase where it functioned as selective organic modifier.

The nonvolatile acid, sugar, and anthocyanidin profiles are simple, limiting their utility in detecting adulterations. Presence of sorbitol or detection of anthocyanidins other than cyanidin and low levels of pelargonidin, however, would be substantive evidence for adulteration. The anthocyanin profile is qualitatively distinctive, with 2 principal quantitative patterns found. Anthocyanin analysis should prove useful in detecting adulteration of red raspberries with other anthocyanin-containing fruits. Total anthocyanin pigment content exhibited a very wide range with variety, geographic origin, and processing as influencing factors.

It is recommended that in future work the data base be extended to include more authentic samples and that the

Table 9. Spectral characteristics of red raspberry juice

Mode	Wavelength range, nm: Maxima	Minima
Absorbance	513–515	
	273–278	
First derivative	664–665	537–541
	477–479	282–283
	391–392	224–230
	258–260	207–209
		202–204
Second derivative	550–551	503–504
	284–285	264–274
	226–232	210–211
	215–216	
	204–209	

Table 10. Hunter and CIE[a] parameters of red raspberry juice

Sample	Hunter parameters			CIE parameters			Hue	SI[b]	Hue/SI
	L	a	b	Y	X	Z			
North American and New Zealand samples[c]									
Meeker (ORNWS)									
(Repl. 1)	(22.3)	(48.5)	(15.3)	(5.0)	(10.9)	(0.1)	(72.5)	(50.9)	(1.4)
(Repl. 2)	(24.2)	(51.4)	(16.6)	(5.8)	(12.6)	(0.3)	(72.1)	(54.0)	(1.3)
Av. of repl. 1, 2	23.3	50.0	16.0	5.4	11.8	0.2	72.3	52.5	1.4
Meeker (ACRS)	35.2	61.3	24.1	12.4	24.1	0.3	68.6	65.9	1.0
Meeker (WWREC)	30.0	58.3	20.7	9.0	18.5	0.1	70.5	61.9	1.1
Willamette (ORNWS)									
(Repl. 1)	(20.2)	(44.8)	(13.7)	(4.1)	(9.0)	(0.1)	(73.0)	(46.8)	(1.6)
(Repl. 2)	(20.0)	(44.5)	(13.6)	(4.0)	(8.8)	(0.1)	(73.0)	(46.5)	(1.6)
Av. of repl. 1, 2	20.1	44.7	13.7	4.1	8.9	0.1	73.0	46.8	1.6
Willamette (ACRS)	30.3	58.2	20.9	9.2	18.8	0.1	70.3	61.8	1.1
Skeena (ACRS)	37.7	63.0	25.2	14.2	27.1	0.8	68.2	67.9	1.0
Marcy (DSIRNZ)	32.5	59.1	22.2	10.6	21.0	0.3	69.4	63.1	1.1
Min.	20.1	44.7	13.7	4.1	8.9	0.1	68.2	46.8	1.0
Max.	37.7	63.0	25.2	14.2	27.1	0.8	73.0	67.9	1.6
Mean	29.9	56.4	20.4	9.3	18.6	0.3	70.3	60.0	1.2
Std dev.	5.8	6.1	3.9	3.3	6.0	0.2	1.7	7.0	0.2
CV, %	19.4	10.8	18.9	35.9	32.0	85.2	2.4	11.7	15.6
European samples									
Bavarian	36.2	53.5	19.9	13.1	23.6	3.3	69.6	57.1	1.2
Romanian	47.5	56.3	22.4	22.5	36.9	8.7	68.3	60.6	1.1
Hungarian	40.6	54.8	22.1	16.5	28.5	4.3	68.1	59.1	1.2

[a] Commission Internationale de l'Èclairage.
[b] SI = Saturation Index.
[c] Samples prepared at Oregon State University pilot plant.

developed methodology be applied to commercial samples to evaluate its effectiveness.

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MYCOTOXINS

Optimum Conditions for Formation of Aflatoxin M_1-Trifluoroacetic Acid Derivative

ROBERT D. STUBBLEFIELD
U.S. Department of Agriculture, Northern Regional Research Center, Agricultural Research Service, Peoria, IL 61604

Because thin-layer chromatographic (TLC) confirmation of identity and reverse-phase liquid chromatographic (LC) determination with fluorescence detection of aflatoxin M_1 both require the derivative formed in the reaction of M_1 and trifluoroacetic acid (TFA), various reaction conditions were studied to obtain complete derivative formation. Of the various organic solvents tested, the reaction between M_1 and TFA proceeded best in the nonpolar solvents hexane and isooctane. Other parameters investigated were reaction temperature and time, aflatoxin M_1 concentration, and solvent volume. The following procedure is considered optimum: 200 μL each of hexane and trifluoroacetic acid are mixed with M_1 standard in a silylated glass vial or with milk residue in a regular glass vial with a Teflon-lined screw cap and heated 10 min at 40°C. The mixture is evaporated to dryness under N_2, and the derivative is saved for TLC or LC. No unreacted aflatoxin M_1 was detected by reverse-phase LC after this procedure was incorporated for analysis of milk samples.

The hemiacetal derivatives of aflatoxins B_1 and G_1 (B_{2a} and G_{2a}) have been used for thin-layer chromatographic (TLC) confirmation of identity since 1971 (1). The derivatives are highly fluorescent and used extensively for the quantitation of these aflatoxins by reverse-phase liquid chromatography (LC), because B_1 and G_1 are only weakly fluorescent in aqueous mobile phases. Derivatives B_{2a} and G_{2a} are readily prepared by the trifluoroacetic acid (TFA)-catalyzed hydration of aflatoxins B_1 and G_1 at room temperature (2). This is not true with aflatoxin M_1 because the reaction rarely goes to completion. The first reverse-phase LC method for determining aflatoxin M_1 in milk and milk products, which measured the fluorescence of the M_1-TFA derivative (designated M_{2a}), was reported by Beebe and Takahashi in 1980 (3). No formal evidence has been published that definitely identifies this derivative as the hemiacetal; however, for consistency with other authors, it is referred to here as M_{2a}. Beebe and Takahashi were successful in the reproducible derivatization of aflatoxin M_1 to M_{2a}; however, other scientists have encountered incomplete derivatization and have incorporated different reaction conditions (4–11). Consequently, some of these workers have chosen to quantitate aflatoxin M_1 directly, even though M_1 is less fluorescent than M_{2a}.

In this report, the reaction parameters for derivatization of aflatoxin M_1 or M_{2a} are examined, and the procedure that produces complete conversion of M_1 to the M_{2a} derivative is given.

Experimental

Apparatus and Reagents

(a) *Solvents.*—All are reagent grade.

(b) *Trifluoroacetic acid (TFA).*—Purity 99+%.

(c) *Dichlorodimethylsilane (DDS).*—Prepare 5% (v/v) solution in toluene.

(d) *Aflatoxin standard solutions.*—Aflatoxin M_1 standards were 0.5, 7.04, and 8.37 μg/mL in acetonitrile–benzene (1 + 9). Aflatoxin B_1 and G_1 standard solution was 8.0 μg each/mL in acetonitrile–benzene (2 + 98).

(e) *Mobile phase.*—Mix water–isopropyl alcohol–acetonitrile (80 + 12 + 8). Degas with ultrasonic probe.

(f) *Heating block.*—Reacti-therm heating module (Pierce Chemical Co., Rockford, IL 61105) or equivalent.

(g) *Silylated vials.*—Add 5% DDS solution to 1–1.5 dram vials and heat ca 40 min at 45–55°C. Discard solution and rinse vials 3 times with toluene and then 3 times with methanol. Heat vials at 75°C for 20–30 min to evaporate methanol. Cap vials (with Teflon liners) and store.

(h) *LC system.*—Spectra-Physics Model 8700, equipped with injector (Rheodyne Model 7125) with 2.0 mL loop, recorder/integrator (Spectra-Physics SP4270), fluorescence detector (Kratos FS970), set at 365 nm for excitation and 418 nm for emission, and 4.3 mm id × 25 cm Zorbax ODS LC column (DuPont, Wilmington, DE 19898). Mobile phase, water–isopropyl alcohol–acetonitrile (80 + 12 + 8), 1.0 mL/min.

Initial Derivative Formation

The following were mixed in a Teflon-lined screw-cap vial: 200 μL solvent, 50 μL TFA, and 50 μL aflatoxin M_1 standard. The mixture was allowed to react at room temperature for 15 min and then was evaporated under nitrogen. The residue was dissolved in 2000 μL water–acetonitrile (75 + 25) for LC. The injection volume was 50 μL.

Results and Discussion

This study was initiated by duplicating the reaction conditions of Beebe and Takahashi (3). They used *n*-hexane as a base solvent to dissolve the dairy extract residue containing aflatoxin M_1. Although hexane is an excellent solvent for this purpose, it is a poor solvent for the polar aflatoxin M_1 when present in pure form (e.g., aflatoxin M_1 standard). Therefore, other solvents were substituted for hexane to determine if any improvement could be obtained (Table 1). Solvents were chosen that scanned the elutropic series, with values of −0.25 to 0.95 (12). The data indicate that the solvents that produced the most M_{2a} and the least unreacted M_1 were the nonpolar *n*-hexane, isooctane, and 1,1,2-trichlorotrifluoroethane. Generally, as the solvent polarity increased, the M_1 conversion decreased. Unreacted aflatoxin M_1 was detected with all solvents. When water was added to the reaction mixture, poor yields of M_{2a} were obtained (data not shown). Even though aflatoxin M_1 is less soluble in nonpolar solvents, the reaction was more complete with them. In this study, aflatoxin M_1 standard was added to the solvent–TFA mixture in 50 μL aliquots. This technique prevented adsorption of M_1 to the glass vial—a problem encountered with aflatoxin standard solutions (13) and discussed in more detail later.

Next, the optimum reaction temperature was determined for hexane and isooctane as base solvents (Table 2). 1,1,2-Trichlorotrifluoroethane was not tested because it is not a

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Table 1. Comparison of the effects of various solvents on the aflatoxin M_1-TFA reaction

Solvent	E^o (Al_2O_3)[a]	Aflatoxin peak area, counts/μL[b] M_{2a}	M_1
1,1,2,-Trichlorotrifluoroethane	−0.25	1239	1
Hexane	0.00	1289	9
Isooctane	0.00	1299	10
Toluene	0.29	651	214
Chloroform	0.40	935	144
Methylene chloride	0.42	1049	55
Ethyl ether	0.38	292	303
Tetrahydrofuran	0.45	257	292
Acetone	0.56	528	237
Ethyl acetate	0.58	274	348
Acetonitrile	0.65	551	158
2-Propanol	0.82	140	334
Methanol	0.95	239	226

[a] Elutropic value of solvent on alumina (12).
[b] Each value represents average of 5 samples.

common laboratory chemical. The reaction conditions were identical to those given in the text and for the data in Table 1, except the temperature was varied from 10 to 50°C. As the temperature increased, aflatoxin M_{2a} was produced in greater yield until a decrease was detected at 50°C. The optimum reaction temperature was 40°C. No unreacted M_1 could be detected at temperatures above ambient; however, some must have been present at 30 and 50°C because less M_{2a} was present. Aflatoxin M_1 is not as fluorescent as M_{2a} in polar mobile phases, so small, undetected amounts of M_1 could be present.

The effect of increased reaction times is given in Table 3. The reaction temperature was held at 40°C, and hexane was selected as the residue solvent because most aflatoxin laboratories use it routinely in their assays. No unreacted M_1 was found after 15 min, and maximum aflatoxin M_{2a} peak areas occurred at 30–45 min. This was a longer reaction time than desired, so the TFA volume was increased from 50 to 200 μL, and the experiment was repeated. With equal volumes (200 μL) of hexane and TFA, the reaction was complete at 10 min, and only M_{2a} was detected. On the basis of these data, it appears that the best conditions for forming M_{2a} from M_1 are mixing equal 200 μL portions of hexane and TFA with the dairy extract residue and letting the mixture react at 40°C for 10 min.

These reaction parameters were tested with increasing quantities of aflatoxin M_1 to determine the maximum amount of toxin that would react. The results are given in Table 4. Only aflatoxin M_{2a} was visible when quantities of less than

Table 2. Effect of temperature on the aflatoxin M_1-TFA reaction[a]

Solvent	Temp, °C	Aflatoxin peak area, counts/μL[b] M_{2a}	M_1
Hexane	10	1029	79
	33	1171	ND
	40	1241	ND
	50	935	ND
Isooctane	10	978	81
	33	1197	ND
	40	1228	ND
	50	1025	ND

[a] Reaction carried out as specified in text except for temperatures given in column 2.
[b] As determined by reverse-phase LC; ND = not detected; each value = average of 10 samples.

Table 3. Effect of reaction time on the aflatoxin M_1-TFA reaction in hexane[a]

TFA, μL	Time, min	Aflatoxin peak area, counts/μL[b] M_{2a}	M_1
50	5	970	47
50	10	1038	43
50	15	1158	39[c]
50	20	1183	ND
50	25	1109	ND
50	30	1219	ND
50	45	1236	ND
50	60	1161	ND
200	5	1072	trace
200	10	1217	ND
200	15	1209	ND

[a] Reaction carried out as specified in text except as given above and temperature = 40°C.
[b] As determined by reverse-phase LC; ND = not detected; each value = average of 3 samples.
[c] Only 2 samples had unreacted M_1 present; aflatoxin M_1 was not detected in other 8 samples.

or equal to 150 ng or 300 μL M_1 standard were treated with TFA. The data suggest that this volume (300 μL) of standard solution diluted the reaction mixture excessively. To determine if this was true, a concentrated M_1 solution (1675 ng in 200 μL) was treated, and a very small unreacted M_1 peak—63 area counts—was detected (Table 4). Therefore, the quantity of aflatoxin M_1 is not a critical factor in the derivatization, but dilution of the reactants is to be avoided. With most of the current methods, the dairy product extract is evaporated before it is treated with hexane and TFA; therefore, the proposed technique will successfully form the M_{2a} derivative.

If aflatoxin M_1 standards are evaporated in a glass vial, M_1 is irreversibly adsorbed to the glass (13). Glass vials should be silylated to avoid adsorption of M_1 during evaporation. Standard solutions (benzene–acetonitrile) of less than 200 μL can be added to the hexane–TFA mixture directly (Table 4), and the reaction will go to completion. The data in Table 4 show that 1408 ng aflatoxin M_1 reacted completely when the 200 μL concentrated standard solution was evaporated in a silylated vial prior to the reaction. Therefore, it is best to use silylated vials for the derivatization of aflatoxin M_1 standards. This is not necessary with dairy samples because aflatoxin M_1 will completely redissolve when present with

Table 4. Effect of aflatoxin M_1 quantities on the reaction with TFA in hexane[a]

Aflatoxin M_1 added μL	ng	Unreacted M_1 peak area, counts/μL[b]
100	50	ND
150	75	ND
200	100	ND
300	150	160
400	200	340
100	837	ND
200	1675	63
200[c]	1408[c]	ND

[a] Reaction carried out as specified in text except as shown above and temperature = 40°C, time = 10 min, and 200 μL TFA.
[b] As determined by reverse-phase LC; M_{2a} was off scale in all experiments; ND = not detected; each value = average of 2 samples.
[c] Added to silylated vial and evaporated under N_2 before reacting with hexane and TFA.

contaminants and impurities commonly found in dairy extracts (12).

The derivatization procedure was tried also with standard solutions containing aflatoxins B_1 and G_1, and it converted them completely to B_{2a} and G_{2a}. In a recent international collaborative study (14), four collaborators obtained incomplete reaction of aflatoxin M_1 with TFA, probably because the specified procedure did not use heat. Since then, the 4 collaborators have successfully used the procedure given in this paper (private communications).

In summary, the optimum conditions for converting aflatoxin M_1 to aflatoxin M_{2a} are to add equal 200 μL volumes of hexane and TFA to dry dairy extract in a screw-cap vial, mix well, heat the vial at 40°C for 10 min, evaporate the mixture under N_2, and save the residue for either TLC or reverse-phase LC determinations. This procedure also will successfully form aflatoxins B_{2a} and G_{2a} from aflatoxins B_1 and G_1. Standard aflatoxin solutions should be transferred to silylated vials, prior to forming the derivatives, to prevent irreversible adsorption of the aflatoxins to the glass; however, sample extracts do not need silylated vials.

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Optimization of Chick Embryotoxicity Bioassay for Testing Toxicity Potential of Fungal Metabolites

DAN B. PRELUSKY, ROBERT M. G. HAMILTON, BRIAN C. FOSTER, H. LOCKSLEY TRENHOLM, and BRIAN K. THOMPSON[1]

Agriculture Canada, Animal Research Centre, Ottawa, Ontario K1A 0C6, Canada

The optimization of a simple, sensitive procedure using a chick embryotoxicity screening test (CHEST) bioassay for detection of toxic compounds is presented. Dosing protocols of eggs, using several mycotoxins (aflatoxin B_1, deoxynivalenol, T-2 toxin) and appropriate controls, were evaluated for embryonic sensitivity, overall practicality of the procedure, and consistency of results. It was found that both type of carrier solvent and volume injected could significantly affect overall embryonic mortality. The chick embryo was most sensitive to the effects of toxins and solvents after 1 or 2 days of incubation; a rapid decrease in response was observed as the age of the embryo at dosing increased. Following administration of the toxins just below the shell membrane by way of a small hole (<0.5 mm diameter) punched in the shell, a good dose-response (% mortality) could be obtained regardless of the site of injection (except directly into the yolk), although dosing via the air sac position resulted in a slightly better statistical outcome. Although some variations in calculated LD_{50} values were found among repeated assays, statistical analyses showed that the differences were not due to dosing protocol but to the variations in embryo sensitivities among batches of eggs. Thus, if standard reference toxins for comparison are run concurrently, the CHEST assay can prove to be a very satisfactory model, as well as having considerable flexibility to be adapted to the needs and resources of many laboratories.

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[1] Engineering and Statistical Research Centre.

The identification of unfamiliar fungal metabolites and determination of their potential toxicities can be an arduous task. To aid in the isolation of these unknown mycotoxins, a biological assay is usually required to monitor the analytical progress from the initial identification of the toxic source, through purification of the extracts, to the establishment of relative toxicity of the purified compound. A number of approaches have been reported in the literature, such as lethality to brine shrimp (1), army worm larvae (2), mosquito larvae (3), and day-old ducklings (4); rabbit skin sensitivity test (5); in vitro reticulocyte lysate assay (6); and even the use of mammalian tissue cultures (7).

One such bioassay that has become highly regarded because of its simplicity and sensitivity and because it avoids many of the criticisms leveled at the nature and waste of mammalian (small-animal) LD_{50} determinations and the limitations of in vitro testing is the chick embryo toxicity assay commonly referred to as the CHEST (chick embryotoxicity screening test) bioassay (8–11). Several studies have shown a high predictive value with the CHEST assay when compared to classic rodent acute toxicity tests (10–13). However, a review of the literature indicated an absence of standardization in the assay procedure among different laboratories. Although the Association of Official Analytical Chemists (AOAC) has a recommended protocol for use of a CHEST bioassay for determining aflatoxin B_1 levels (14) which appears to have adequate sensitivity and precision (15), we believe the stringent requirements of the method, difficult

Table 1. Incidence of unhatched eggs (%) of controls and following treatment with carrier solvents

For 144 control eggs set (no treatment), unhatched eggs = 14.6%

Treatment		No. of eggs set[a]	% Unhatched[b] and site of injection[c,d]			
			Air sac	45° angle	Equator	Yolk
Puncture (<0.5 mm)		48	16.7	12.5	16.7	—
Puncture + beeswax		96	14.6	16.7	16.7	—
Sham injection (no solvent)		48	12.5	16.7	16.7	29.2*
Test solvent injections, μL[e]						
Normal saline:[f]	5	72	15.3	16.7	16.7	33.3*
	20	48	22.9	22.9	25.0	
30% ETOH:	5	72	18.1	16.7	12.5	41.7*
	20	48	20.8	20.8	16.7	
	100	64[g]	60.9*	64.1*	57.8*	—
95% ETOH:	5	72	22.2	25.0	16.7	50.0*
	20	72	43.1*	37.5*	25.0	—
	100	48	100.0*	95.8*	100.0*	
100% ETAc:	5	48	12.5	16.7		
	20	48	33.3*	29.2*		
100% CH_2Cl_2:	5	48	16.7	12.5		
5% DMSO:	5	64	45.8*	31.3*		
20% DMSO:	5	48	50.0*	37.5*		
90% DMSO:	5	48	54.2*	41.7*		
Distilled water:[h]	10	72	12.5			
	100	72	29.2*			

[a] For each of yolk tests, 48 eggs were set.
[b] Includes ca 4–5% of eggs that were infertile.
[c] See Figure 1; injections made on day 1 of incubation.
[d] Approximate standard error of percentage (P) is given by $[P(100 - P)/10\,000 \times N]^{1/2}$ where N is the number of eggs set.
[e] ETOH = ethanol; ETAc = ethyl acetate; CH_2Cl_2 = dichloromethane; DMSO = dimethyl sulfoxide.
[f] 0.9% NaCl, sterile.
[g] For air sac, 72 eggs set.
[h] Sterile.
* Denotes significantly higher lethality than the controls, based on Dunnett's test by column at the 5% level.

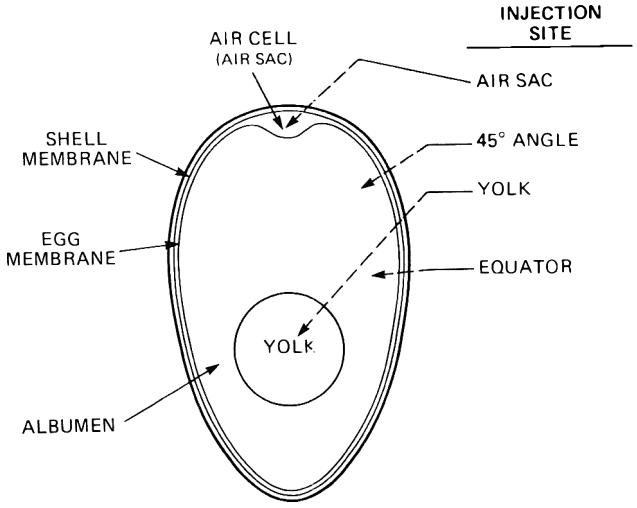

Figure 1. Sites of injection tested for administration of toxins to chick embryos.

injection protocol, and requirement of large numbers of eggs make it impractical as a routine screening procedure. Therefore, this study describes a simplified and adaptable, but completely practical, approach to the CHEST bioassay for routinely predicting the toxic potential of isolated metabolites. Depending on the application, the bioassay either can be used as a 7 day test to establish whether a substance is nontoxic, moderately toxic, or very toxic, or can be taken until the chicks hatch at full term (21 days) to determine more accurately the comparative LD_{50} values of test compounds. Many of the protocol variables that could affect overall embryonic mortality were investigated.

Experimental

Analytically pure aflatoxin B_1 and T-2 toxin were purchased from Sigma Chemical Co. (St. Louis, MO). Deoxynivalenol (DON, vomitoxin) was provided by R. Greenhalgh, Plant Research Centre, Agriculture Canada, Ottawa, Ontario. Solvents used as carrier vehicles were LC grade. Water was double-distilled in glass and filtered through Millipore filters HA-0.45 mM (Millipore Ltd, Mississauga, Ontario); just prior to injection, all distilled water and normal saline solutions were filter-sterilized using Millipore Millex-GS sterilizing filter units. Dimethyl sulfoxide (DMSO), (purity 90%, medical grade) was obtained from Diamond Laboratories (Calgary, Alberta) as Domoso® (D.I.N. 3.29584). Fertile eggs from White Leghorn hens of a random-bred control stock (strain 10) maintained at the Animal Research Centre were collected for up to 8 days prior to incubation. The eggs were stored on fiber trays at 4°C and 100% relative humidity until set in the incubator. At the time of setting, eggs were distributed into groups of 24, so that each group contained a similar number of eggs from each day's collection. After being numbered sequentially, the eggs were set in a Jamesway incubator (Model 252B) where the temperature was maintained at 39°C dry bulb and 28–29°C wet bulb; the eggs were turned automatically through a 90° arc every 4 h.

Aflatoxin B_1, deoxynivalenol, and T-2 toxin were selected as test compounds. The toxins were dissolved in carrier solvent (30% ethanol) and administered 1, 2, 3, 5, or 7 days after the start of incubation. Unless indicated otherwise, 5 μL of the test solution was injected per egg at one of 4 possible injection sites (Figure 1). Injections were made through a small hole (<0.5 mm) punctured in the shell with a common kitchen egg punch used to put holes in eggs prior to hard-boiling to prevent cracking of the shell. Needle (27 gauge) depth was 1–2 mm below the surface of the shell, except when administration directly into the yolk required a 50 mm needle.

On day 7, the eggs were candled. Eggs that appeared clear or to contain a dead embryo were counted, removed, and stored at 4°C. The remaining eggs were returned to the incubator and candled again on days 14 and 18. On day 18, eggs were transferred from the turning racks in the incubator and placed in compartmental baskets for hatching in a second incubator where the dry- and wet-bulb temperatures were 39°C and 30–31°C, respectively. Frequency of hatching was noted, and any eggs that remained unhatched by day 23 were classified as dead and were examined; extra incubation time of several days was included to allow for any "late-hatched" chicks. All eggs with nonviable embryos were opened within 1 to 2 days of removal from the incubators and examined for fertility and/or day of death (developmental stage) (16).

The mortality data of Tables 1 and 2 were analyzed using least-squares analyses of variance with the response for each egg coded as 0 if it hatched and 1 otherwise. This approach was taken rather than using contingency tables, partly because of complexities of design arising from the different batches, but also to facilitate hypothesis testing involving single degree-of-freedom comparisons and multiple comparison tests. Examples of this type of analysis are common in the literature (see, for example, Harvey [17]). Analyses of

Table 2. Effect of time of injection and site of injection on overall mortality of carrier solvents

	% Unhatched[a] and site of injection[b,c]												
	Air sac						45° angle					Equator	Yolk sac
Time of injection, day	5 μL Normal saline	20 μL Normal saline	20 μL 30% ETOH	100 μL 30% ETOH	20 μL 95% ETOH	20 μL 95% DMSO	5 μL Normal saline	20 μL Normal saline	20 μL 30% ETOH	100 μL 30% ETOH	100 μL 95% ETOH	100 μL 30% ETOH	5 μL 30% ETOH
1	15.3 (72)	22.9 (48)	20.8 (48)	59.7* (72)	43.1* (72)	40.0* (40)	16.7 (72)	22.9 (48)	20.8 (48)	64.1* (64)	95.8* (48)	57.8* (64)	41.7* (48)
2	16.7 (48)	25.0 (48)	12.5 (48)	62.5* (64)	50.0* (48)	40.0* (40)	20.8 (48)	18.7 (48)	14.6 (48)	73.4* (64)	87.5* (48)	37.5* (64)	47.9* (48)
3	16.7 (24)	27.1 (48)	12.5 (48)	66.7* (48)	59.7* (72)	66.7* (24)	16.7 (24)	25.0 (48)	12.5 (64)	31.2 (48)	38.9* (72)	25.0 (48)	50.0* (48)
5	12.5 (24)	16.7 (24)	13.9 (72)	50.0* (64)	50.0* (48)	62.5* (40)	16.7 (24)	14.6 (48)	14.1 (64)	31.2 (48)	20.8 (48)	25.0 (48)	25.0 (48)
7	12.5 (24)	12.5 (24)	13.9 (72)	12.5 (72)	14.1 (64)	14.3 (56)	16.7 (24)	14.6 (48)	12.5 (64)	16.7 (48)	16.7 (48)	17.2 (64)	12.5 (48)

[a] Mortality of control (no treatment) eggs = 14.5% and includes 4–5% infertile eggs.
[b] See Figure 1.
[c] No. of eggs set (N) given in parentheses; approximate standard error for percentage (P) given by $[P(100 - P)/10\,000 \times N]^{1/2}$.
* Denotes significantly higher lethality than controls, based on Dunnett's test by row at the 5% level.

variance were applied to various subsets of the data, but generally the treatments with high mortality were excluded to reduce the heterogeneity of variance. In fact, exclusion of these values had little impact on interpretation because they were so obviously higher. Results in the columns of Table 1 and the rows of Table 2 were compared against the controls using Dunnett's test described by Steel and Torrie (18). Some minor changes to the method were necessary to take into account the unequal sample sizes. Estimates of variance for the test were taken from the analyses of variance but differ little from those derived from the formulas given in Tables 1 and 2.

The dose-response curves were analyzed using single-line probit analyses in the manner described by Finney (19). Several transformations of dose level were tried; the log dose was the most satisfactory and was applied throughout. Parallel-line probit analyses were used to compare both the same assays conducted at different times and similar assays conducted at the same time but with different injection dates. No evidence could be found of differences in hatchability of the nonpunctured eggs included with the assays conducted at different times. Hence, the pooled measure of mortality for these eggs was used as the background mortality for all probit analyses.

Statistical tests noted in the text were conducted at the 5% significance level, unless otherwise designated.

Results and Discussion

The aim of this study was to provide a simplified, sensitive, and practical protocol for the chick embryotoxicity bioassay. Optimization of the procedure used for administering the toxic materials was carried out using 3 test mycotoxins—aflatoxin B_1, T-2 toxin (T-2), and deoxynivalenol (DON). The main parameters investigated were site and day (incubation age of embryo) of injection, type of carrier solvent, and volume injected. The need to cover the injection site with beeswax was also examined.

Several sets of assays were repeated during a period of approximately 8 months. In instances where a certain treatment obviously interfered with the bioassay (e.g., caused unacceptably high mortality levels) or was found eventually to have no effect, the treatment was eliminated from subsequent assays. These 2 criteria helped speed up development of the assay protocol, improved overall sensitivity, and saved resources.

In all instances, groups of control eggs which were set as received from the hens were included to determine fertility, hatchability, and effect of physical treatment. When dose-response data were interpreted, these normal background control values, determined as nonhatchability by 23 days' incubation time, were subtracted from results before final mortality was determined. Table 1 summarizes the parameters tested. A mortality of about 15% for control eggs, which included approximately 4–5% infertility, agreed with high hatchability of eggs set that was previously reported for this strain of White Leghorn hen (20). Hatchability did not change throughout the 8-month duration of this study (no batch effect) before the hens were forced molted, nor was there any apparent problem observed, as expected (21), using eggs stored up to 8 days prior to incubation.

Of initial interest was whether any effect was produced by puncturing a small hole (<0.5 mm) through the shell and membranes to allow entry of the needle for injection of the test toxins. As indicated in Table 1, no increase in mortality occurred, regardless of the site on the shell where the puncture was made (Figure 1) and whether beeswax was applied as a sealant. The absence of any effect due to puncturing supports the results of Hamilton and Thompson (22) who investigated the results of puncturing eggs with up to 4 holes of size similar to that used in the current study and found no significant change in the overall hatchability of the eggs from Leghorn hens. Application of beeswax was not required, since minor punctures of this type quickly reseal, presumably because of albumen congealing around the opening (except when the puncture site was above the air sac). The ease and practicality of this puncture method avoids all of the obvious problems associated with earlier reported methods that required precision drilling into the shell for the removal of sections of shell (up to 15 × 10 mm) prior to dosing, as well as having to aseptically reseal these openings (8, 9, 13, 14, 23, 24). Using the procedure presented in this paper, it was possible for 2 people to dose a tray of 180 eggs in about 35–40 min. Rapid dosing was aided by the use of Hamilton repeating syringes (Model PB-600).

It was interesting to note that embryos were very sensitive to solvent type, and particularly to the volume of injection,

Table 3. Effect of time and site of injection on overall mortality[a] of chick embryos for various mycotoxins[b]

Day of injection	LD_{50},[c] μg toxin/egg								
	Aflatoxin B_1			T-2 Toxin			Deoxynivalenol		
	Air sac	45° Angle	Equator	Air sac	45° Angle	Equator	Air sac	45° Angle	Equator
1	0.097 (0.071–0.118)	0.085 (0.062–0.108)	0.099 (0.096–0.102)	0.70 (0.51–0.87)	0.37 (0.25–0.50)	1.28 (1.04–1.49)	5.15 (3.77–6.37)	3.14 (2.24–4.03)	15.30 (14.34–16.39)
2	0.074 (0.058–0.092)	0.026 (0.017–0.036)	0.072 (0.055–0.087)	0.88 (0.68–1.06)	0.34 (0.24–0.43)	0.76 (0.57–0.91)	4.79 (3.43–6.04)	8.18 (6.16–9.53)	7.33 (5.52–8.64)
3	0.129 (0.096–0.161)	0.077 (0.058–0.093)	0.088 (0.066–0.111)	3.59 (3.00–5.36)	3.87 (3.27–8.65)	6.75 (—)[d]	17.78 (15.00–20.88)	19.81 (18.18–21.65)	26.38 (—)[d]
Parallel lines?[e]	yes	no	no	yes	no	yes	yes	no	no
Same intercepts?	no	—[f]	—	no	—	no	no	—	—
Satisfactory model?	yes	yes	yes	yes	yes	yes	yes	yes	yes

[a] "Background" mortality of 14.6% incorporated into assay; toxins administered in 5 μL 30% ethanol.
[b] See Figure 1.
[c] 95% confidence limits for LD_{50} in parentheses, unless otherwise indicated.
[d] Confidence limits not calculated because regression was not significant.
[e] Tests of parallel lines for the probits within a column.
[f] Tests for the same intercept appropriate only when parallelism criterion met.

a fact only a few other research groups (24, 25) have addressed. Of the solvents/volumes tested (Table 1), the least toxic effect was observed with 10 μL distilled water and 5 μL each of normal saline, 30% ethanol, ethyl acetate, or dichloromethane. Generally, analysis of variance showed mortality to be significantly higher ($P < 0.05$) with 20 μL injections than with 5 μL injections, but the differences for 30% ethanol and normal saline were sufficiently small that the 20 μL level could be considered borderline acceptable. All other solvent/volume combinations caused unacceptable increases in mortality ($P < 0.05$). McLaughlin and coworkers (24) also observed that normal saline (0.9% NaCl) and 95% ethanol were slightly more toxic than distilled water, and mortality of the embryos increased significantly as the amount of NaCl or ethanol injected was increased.

Except for directly into the yolk, site of injection (air sac, 45° angle, or equator) did not influence ($P > 0.05$) the mortality caused by solvent injections. A significantly toxic solvent/volume combination remained toxic regardless of the site where it was administered. However, a sham injection into the yolk produced a response that far exceeded acceptable background mortality levels, and administration of any solvent at this site only increased the overall lethality. For this reason, the yolk was eliminated as a practical site for administration. Jelinek (10) arrived at the same conclusion about yolk injections, but for different reasons; there was serious concern about the reproducibility of this method, not knowing if and in what form or concentration an administered compound reaches the embryos (26, 27).

Results shown in Table 2 indicate that overall sensitivity to solvent and/or volume was affected by the age of the chick embryo at time of injection; as age increased, toxicity decreased. By day 7 after injection, no solvent tested was significantly toxic regardless of the volume used (up to 100 μL). In the most severe circumstance—100 μL of 95% ethanol injected at 45° angle—mortality decreased from 96% on day 1 to approximately 17% on day 7. Thus, in instances where a larger volume of injection is necessary because of the solubility characteristics of the toxin, an older embryo may have to be used. However, as shown in Table 3, a considerable loss in sensitivity of the bioassay to certain toxins will probably result with the use of older embryos. Estimation of conventional LD_{50} values indicated that T-2 toxin and DON were most lethal when administered at 1 or 2 days of incubation; by day 3, sensitivity of the embryos to the toxins had decreased sufficiently that LD_{50} values had risen severalfold (Table 3), and by day 4 and in subsequent days, LD_{50} values could not be determined below the maximum dosages administered (4.0 μg/egg and 32.0 μg/egg for T-2 and DON, respectively) (data not shown in table). Because of the greater amounts of toxins required, no attempt was made to establish LD_{50} values in eggs beyond 4 days' incubation time. However, with aflatoxin B_1—the most toxic of the 3 compounds examined—the decline in sensitivity as a function of embryonic age was not as rapid as that observed with the 2 trichothecene toxins. Over the initial 3 days' incubation period, LD_{50} values for B_1 remained relatively consistent (Table 3), and when administered on days 5 and 7, 0.1 μg B_1/egg still produced a 50% and 38% mortality (corrected for background), respectively, regardless of the injection site (data not shown in table).

In contrast to the effect of carrier solvent, which showed no significant variation in toxicity among injection sites, sensitivity of the embryo (based on LD_{50} values) to certain mycotoxins could vary with the location of injection. Typically, when day 1 injections were compared, LD_{50} values for T-2 and DON were always consistently higher following administration at the equator than at the air sac or 45° angle (Tables 3 and 4). The toxicity of B_1 did not appear affected by the injection site.

Similarly, it was of interest to note that the probit model provided a satisfactory fit to the dose-response curve for all injection sites, but the overall response seemed to be inferior for injection at a 45° angle and the equator, because the parallel line test between days of injection was positive for all 3 toxins only with air sac injections (Table 3). Dosing at the equator (DON, T-2) appeared to produce a threshold-type pattern in which a minimal response occurs over a wide dose range until sufficient toxin has been added to cause a very rapid increase in mortality. The explanation for these differences is unclear, but there is an indication that early embryonic development begins at or about the equator of

Table 4. Reproducibility of chick embryotoxicity bioassay[a]

Date of test	LD_{50} values, μg toxin/egg[b]								
	Aflatoxin B_1			T-2 Toxin			Deoxynivalenol		
	Air sac	45° Angle	Equator	Air sac	45° Angle	Equator	Air sac	45° Angle	Equator
Feb. 21		0.053 (0.044–0.065)			0.52 (0.38–0.67)			3.26 (2.30–4.26)	
Mar. 24	0.097 (0.071–0.118)	0.085 (0.062–0.108)	0.099 (0.096–0.102)	0.49 (0.33–0.70)	0.41 (0.28–0.54)	1.59 (1.51–1.68)	5.53 (4.04–6.98)	4.45 (3.12–5.88)	6.13[c] (—)[d]
June 9	0.064 (0.052–0.077)	0.050 (0.036–0.066)	0.074 (0.054–0.093)	0.70 (0.51–0.87)	0.37 (0.25–0.50)	1.28 (1.04–1.49)	5.15 (3.77–6.37)	3.14 (2.24–4.03)	15.30 (14.34–16.59)
Sept. 11	0.074 (0.057–0.091)	0.055 (0.041–0.069)		0.44 (0.30–0.60)	0.40 (0.27–0.54)	2.55 (1.97–3.27)	6.59 (4.64–8.35)		
Parallel lines?[e]	yes	yes	no	yes	yes	yes	yes	yes	no
Same intercepts?	no	no	—[f]	yes	yes	no	no	yes	—
Satisfactory model?	yes	yes	yes	yes	yes	yes	yes	yes	no

[a] Injections carried out on day 1 of incubation; "background" mortality of 14.6% incorporated into assay. Toxins administered in 5 μL 30% ethanol.
[b] 95% confidence limits for LD_{50} in parentheses.
[c] Significant lack of fit ($P < 0.05$) for single probit analysis (χ^2 test for goodness-of-fit).
[d] Confidence limits not calculated because regression was not significant.
[e] Tests of parallel lines for probits within a column.
[f] Tests for the same intercept appropriate only when parallelism criterion met.

the yolk (24, 28); consequently, dosing at the equator would deposit the toxin anywhere from very close to the embryo to the furthest point from the embryo on the opposite side of the yolk. This random administration of the toxin relative to the developing embryo could contribute to the peculiar dose-response profile obtained with injections at the equator. Only the highly toxic aflatoxin displayed somewhat consistent LD_{50} values, regardless of location or day (1, 2, or 3) of injection; a "threshold" response was not observed for B_1 administered at the equator. Furthermore, the solubility or binding characteristics of certain toxins in the egg albumen can also affect the rate and extent of the response. This raises questions as to the fate of certain toxins if they are administered in such a manner as to impede delivery of the substance to the embryo. For example, Bata et al. (29) found that both T-2 toxin and diacetoxyscirpenol (DAS) injected into the yolk underwent extensive metabolism upon incubation of the egg; the proportions of resulting metabolites varied with the length of incubation. Miura (30) observed that the extent of metabolism of aflatoxin B_1 was influenced dramatically by the location of the injection into the egg.

The reproducibility of the bioassay was investigated by applying parallel-line probit analyses to the results from assays repeated several times during a period of 8 months (Table 4). The resulting LD_{50} values (intercepts of dose-response data) did show significant differences among repeat assays ($P < 0.05$), but the slopes (probit scale) remained consistently parallel for injections into the air sac and at a 45° angle (i.e., the shape of dose-response curves remained similar). This indicates that, although the toxicity levels may vary among duplicate assays, the extent and direction of any changes occur equally for each toxin being tested. Thus, it appears that a combination of factors that influence the tolerance of the embryo—such as age of the flock producing the fertile eggs, storage time of eggs prior to incubation (1–8 days in the present study), time of the year when the bioassay was done, or even changes in personnel performing the assays—may all contribute to changes in the sensitivity of the assay. Fortunately, though, the impact of most of these variables can be eliminated if results from an assay are expressed relative to an adequate standard reference compound run concurrently with the toxins being tested. The reliability of this procedure among several laboratories remains to be established.

As part of the present study, the reliability of the dose-response was determined when embryo mortality was examined at 7 or 14 days after administration of toxins (day 1) instead of taking the incubation to our standard 23 days. A shorter time period would expedite the assay and would eliminate the need for investigators to have access to hatching facilities. A comparison to the standard protocol (full incubation, 1–23 days) was made by establishing the time of embryonic death following dosing and then examining dose-response curves based on percent mortality after 1–7 days' incubation and 1–14 days' incubation. It was observed that a reduced incubation period can produce slightly different results (LD_{50} values), which may or may not be acceptable for all experimental protocols.

As shown in Table 5, using mortality data for doses that led to about 50 and 95% mortality (above controls) as examples, the mortality pattern following T-2 or DON administration (air sac, day 1) had a >90% incidence of all deaths occurring during the initial 7 day period. Only a few later deaths occurred, which was similar to the pattern of natural mortality observed with the untreated controls. In contrast, the pattern following B_1 administration showed that a higher proportion of deaths occurred after the initial 7 day period. Even at the high dose level, over 25% of all deaths still resulted during the final week of incubation with the majority occurring between days 8 and 14. Thus, when compared to 23 day LD_{50} data, values based on mortality by day 7 or 14 would translate to only a very minor difference for DON or T-2, but a significant increase (1.8- to 3.3-fold) in the LD_{50} value for B_1 (Table 6). In all practicality though, it should be recognized that in attempting to establish whether a compound is nontoxic, toxic, or very toxic, a 3-fold difference in calculated LD_{50} values may not be all that important. Such differing results could typically be obtained upon repetition, or if the study were to be conducted in different laboratories. Subsequently, depending on the objective of the toxicity test-

Table 5. Time of death of chick embryos, based on dose levels which led to approximately 50% and 95% mortality[a]

Toxin	Mortality level[a] %	No. of eggs set[b]	Total no. of embryo deaths	Number of embryos that died within a certain period (days incubation) 1–7 days	8–14 days	15–23 days
Control		115	12	6	2	4
DON	50	92	49	42	3	4
	95	91	87	83	2	2
T-2	50	89	47	39	2	6
	95	92	88	82	4	2
B_1	50	114	62	6	7	49
	95	92	88	17	46	25

[a] Data chosen from 4 or 5 runs where mortality, after adjustment for background mortality, approximated 50% or 95% lethality.
[b] Viable eggs only, infertile eggs excluded from the counts.

ing, a shortened incubation time could be completely acceptable. Furthermore, by the time doses reach 2–3 times the LD_{98} dose level, all deaths occurred within the initial 7-day period regardless of the toxin involved (not shown). This prevents values determined at 7 days from being more than just a few-fold higher than those calculated at the end of the incubation period (day 23).

Conclusion

The results obtained demonstrate the importance of a standardized bioassay procedure. Although the overall toxicity of the chick embryo to the highly toxic compound aflatoxin B_1 did not appear to change considerably with the varying methodologies tested, the results for the other toxins (T-2, DON) could be affected substantially. Differences associated with the site of injection (Table 1) could be due to the solubility, binding, or diffusion characteristics of the toxin and to how rapidly detoxification/metabolism occurs, if at all. Miura and Aibara (25) noted that overall toxicity could be influenced by the effect of the carrier solvent on the egg environment surrounding the injection site, thus leading to a disturbed diffusion of the toxin. In the current study, administration at the site of the air sac appears to minimize much of the variation. This could be due to delivering the toxin to a constant area (air sac) in relation to the developing embryo, whereas injection at the equator would allow some variation to occur between where the embryo develops and where the toxin is deposited.

Age-dependent changes in toxicity to the embryo also occurred (Table 2). Those changes as a function of age were consistent with other studies that have generally demonstrated that sensitivities decrease as embryo age increases (23, 31), although recommendations for dosing as late as 6 (25), 7 (19), or 10 (4) days into the incubation period have been reported. The current study showed that greatest susceptibility occurred on day 1 or 2 of incubation (Table 2). By day 3, the effect of T-2 and DON had declined considerably; B_1 retained a relatively higher toxicity during the embryo's initial week of development.

In summary, the chick embryotoxicity assay has excellent capabilities for supporting existing chromatographic methods used in the isolation and identification of potentially toxic substances. The CHEST method adapted by our laboratory has demonstrated practical advantages over existing procedures, the foremost being the simplicity of the injection technique utilizing a common household egg punch to permit entry into the egg, thus greatly increasing the speed of the dosing procedure. The assay protocol established for optimum sensitivity, dose-response, and reproducibility involves injection into air sac on day 1 or 2 of incubation, using an absolute minimum amount of carrier solvent (usually <10 μL) which has been pretested for nontoxicity against the chick embryo. A minimum of 20 viable eggs per dose level provides sufficient numbers to examine the dose response, although under certain circumstances, such as pretesting of carrier solvents or preliminary approximations of toxicity (i.e., very toxic, toxic, nontoxic) prior to further investigation, fewer eggs could be used. However, if attempting to determine conventional LD_{50} values, too few eggs per dose will understandably reduce the precision of the bioassay. To obtain a useful picture of the dose-response curve itself, it is important to include a number of dose levels in the area where intermediate response is expected. Finally, to minimize the effect of certain factors pertaining to diffusion or metabolism, eggs should be allowed to incubate for the full period, although under limitations of resources or time constraints associated with practical acute toxicity testing discussed earlier, a shorter incubation period would be acceptable.

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Table 6. Estimated LD_{50} values from parallel-line probit assays, based on embryo mortality as determined over 3 different time periods[a]

Toxin	LD_{50},[b] μg toxin/egg Time period, days 1–7	1–14	1–23	Parallel lines	Same intercepts	Satisfactory model
DON	5.54 (4.18–6.80)	5.47 (4.03–6.76)	5.17 (3.77–6.37)	yes	yes	yes
T-2	0.75 (0.56–6.94)	0.72 (0.53–6.89)	0.70 (0.50–0.87)	yes	yes	yes
B_1	0.21 (—)[c]	0.12 (0.091–0.150)	0.064 (0.052–0.077)	no	[d]	no

[a] Based on June 9 bioassay, injection into air sac on day 1 of incubation; background mortality of 8.3%, 12.5%, and 14.6% incorporated into assay for respective time periods.
[b] 95% confidence limits for LD_{50} in parentheses.
[c] Confidence limits not calculated because regression was not significant.
[d] Test for the same intercept appropriate only when parallelism criterion met.

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PESTICIDE FORMULATIONS

Gas Chromatographic Determination of Alachlor in Microencapsulated Formulations: Mini-Collaborative Study

DAVID F. TOMKINS
Monsanto Co., PO Box 473, Muscatine, IA 52761

Collaborators: F. Brill; T. Riggs; R. Speth; L. Torma; R. Wiley; J. L. Hansen (*Statistical Consultant*)

An isothermal gas chromatographic method for measuring alachlor in Micro-Tech® (microencapsulated) formulations was tested by 5 collaborators. The samples were prepared in acetone, and alachlor was determined using a gas chromatographic column of 10% SP-2250 on 100-120 mesh Supelcoport. Di-*n*-pentylphthalate was used as the internal standard. Collaborators made single determinations on 5 samples distributed as blind duplicates. The mini-collaborative study generated 47 data points. The coefficient of variation (CV_o-pooled) was 1.35%, and CV_x-pooled was 0.73%. The method was simple to use and did not reveal any interferences in samples tested. The method has been adopted official first action as an AOAC-CIPAC method.

Alachlor (2-chloro-2',6'-diethyl-*N*-(methoxymethyl) acetanilide) is the active ingredient in Lasso® Micro-Tech®, a microencapsulated herbicide for weed control in soybeans and other listed crops. It is used 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. (2). A method described in that paper was adopted by AOAC (3). This collaborative study evaluated a GC method developed by B. W. Mueller and D. F. Tomkins for alachlor microencapsulated formulations (4).

Collaborative Study

Five pairs of alachlor microencapsulated samples (blind duplicates marked 1–10 in random order), together with standards, practice samples, and detailed guidelines, were sent to 5 collaborators. 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 (with duplicate injections) on each of the 10 samples. 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.

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This report of the Associate Referee was presented at the 100th AOAC Annual International Meeting, Sept. 15–18, 1986, at Scottsdale, AZ.

The recommendation of the Associate Referee was approved interim official first action by the General Referee, the Committee on Pesticide Formulations and Disinfectants, and the Chairman of the Official Methods Board. The method was adopted official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) **71**, January/February issue.

Alachlor in Microencapsulated Pesticide Formulations

Gas Chromatographic Method

First Action

AOAC-CIPAC Method

Principle

Sample is dissolved in acetone contg di-*n*-pentyl phthalate as internal std, analyzed by gas chromatgy with flame ionization detection, and quantitated by comparison of integrated peak areas.

Safety

LD_{50} of alachlor has been found to be 930 mg/kg in rat acute oral studies (Monsanto Co., 1985, MSDS No. 015972608). Alachlor has been detd to produce tumors in laboratory animals. Wear protective clothing to avoid excessive exposure.

Apparatus

(a) *Gas chromatograph.*—With flame ionization detector and on-column injection ports. Operating conditions: 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.*—Glass, 6 ft × 2 mm id (on-column configuration), packed with 10% SP-2250 on 100–120 mesh Supelcoport (Supelco Inc.), or equiv. SP-2250 is methyl-phenyl silicone (50 + 50).

Reagents

(a) *Acetone.*—Pesticide grade (Fisher or equiv.).

(b) *Di-n-pentyl phthalate internal and std soln.*—Weigh 5.3 g di-*n*-pentyl phthalate (CTC Organics, PO Box 6933, Atlanta, GA 30315) into 1 L vol. flask. Dissolve in acetone and dil. to vol. with acetone.

(c) *Alachlor std soln.*—Recrystallize alachlor (Monsanto Co., PO Box 473, Muscatine, IA 52761) from MeOH. Accurately weigh 0.2 g recrystd alachlor into small flask. Add by pipet 30.0 mL internal std soln and shake mixt. to dissolve.

Instrument Setup and Calibration

Condition chromatgc column ovenight at 250° with He flow at 35 mL/min. Suggested conditions represent best compromise for sepn and quantitation of cmpds of interest. Some minor adjustments may be required in other instruments and columns. Column, when working properly, should generate 4000–5000 plates calcd as follows: $N = 16(x/y)^2$, where N = no. of theoretical plates, x = distance from point of injection to peak max., and y = distance along baseline between intercept points of lines drawn tangent to slope of peak, with x and y measured in same units. Typical retention times for alachlor and internal std are ca 6 and 11.5 min, resp. Impurity in internal std (peak C), which elutes at ca 9.9 min, should be completely resolved from internal std peak at ca 11.5 min (Fig. **6:D1**). Internal std contains another impurity that elutes slightly after internal std causing slight tail on that peak. Careful control of integrator conditions is required to integrate internal std peak.

After instrument equilibration, make ≥3 injections of std soln before calibration.

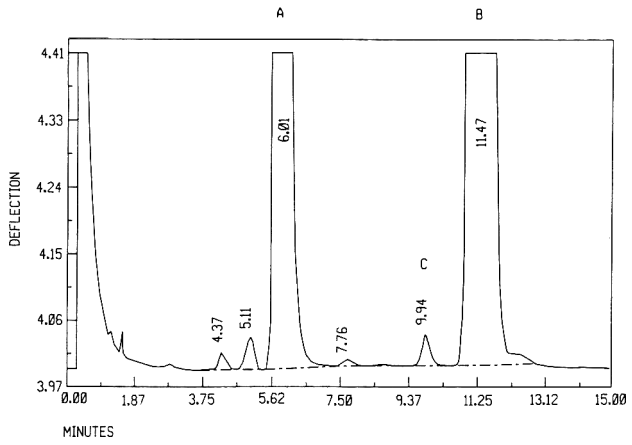

FIG. 6:D1–GC chromatogram of alachlor standard (A), internal standard di-*n*-pentyl phthalate (B), and unknown from internal standard (C).

Determination

Accurately weigh, to nearest 0.1 mg, ca. 0.45 g alachlor microencapsulated formulation into 2 oz sample bottle. Avoid spilling sample on inside wall or neck of bottle; entire sample should be on bottom of bottle.

Pipet 30.0 mL internal std soln **(b)** into sample bottle. To reduce stirring time, use liq. stream from pipet to remove most of alachlor sample from bottom of bottle. Add mag. stirring bar (13 × 15 mm) and cap bottle with polyethylene-lined cap.

Mag. stir mixt. until sample is completely removed from inside wall and bottom of bottle. During stirring, aggregated sample turns fluffy and easily floats in acetone. For most samples, this requires ca 2–3 min moderately fast stirring. Then place bottles on shaker and shake 10 min at high speed.

Let solids settle and pipet off clear acetone soln.

Make replicate 1 μL injections of alachlor std soln and measure response ratio, R (area alachlor peak/area internal std peak) for each injection. Repeat until consecutive response ratios agree within 0.5%.

Make duplicate injections of acetone 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.

CAS-15972-60-8 (alachlor)

Table 1. Results from collaborative study of alachlor (%) in microencapsulated formulations by using an isothermal GC method[a]

		Collaborator				
Sample[b]		1	2	3	4	5
1	A	44.25[c]	42.89	41.77	42.12	42.43
	B	42.10	42.97	42.16	43.00	42.31
2	A	45.57[c]	42.86	41.60	42.10	42.31
	B	42.62	42.68	41.65	43.19	42.57
3	A	44.26[c]	42.79	41.87	42.17	42.36
	B	42.15	42.93	42.34	43.16	42.86
4	A	43.60	42.44	41.76	42.54	41.65
	B	43.97	42.40	41.48	42.39	41.94
5	A	42.35	43.18	42.29	42.98	42.21
	B	42.58	43.01	42.22	43.08	42.38

[a] Each result is the average of 2 injections.
[b] Samples were randomly numbered blind duplicate pairs.
[c] Outlier by Dixon test at 95%; not included in statistical evaluation.

Table 2. Statistical summary of collaborative results for GC determination of alachlor in microencapsulated formulation

	Sample				
Statistic	1	2	3	4	5
Data points	9	9	9	10	10
Mean, %	42.39	42.42	42.48	42.42	42.63
S_o	0.344	0.402	0.429	0.180	0.112
S_L	0.292	0.375	0.070	0.841	0.397
S_x	0.451	0.550	0.434	0.860	0.413
CV_o	0.812	0.947	1.01	0.424	0.263
CV_x	1.07	1.30	1.02	2.03	0.968

Results and Discussion

Results were reviewed and tabulated and were statistically analyzed by the methods committee statistician. Calculations with 47 data points from 5 laboratories were used for evaluation and are presented in Table 1. Using 95% confidence limits, results from collaborator 1 (samples 1A, 2A, 3A) were omitted as outliers. The outliers are all marked in Table 1.

Although a few results were 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 S_x values from 0.413 to 0.860 and S_o values from 0.112 to 0.429. The coefficients of variation (CV_x) were from 0.968 to 2.03%. The CV_x value of 2.03% for sample 4 was nearly twice the value for the other samples. The pooled CV_x was 1.35% for the 5 samples. The pooled statistic is calculated as follows:

$$\text{S-pooled} = \left[\sum_{i=1}^{n} \frac{(f_i \times s_i^2)}{f}\right]^{1/2}$$

where f_i = degrees of freedom at i level;
f = sum of individual degrees of freedom;
s_i = standard deviation at i level.

Notes on instrument setup and calibration are included in the method. Minor adjustments may be necessary to obtain optimum separation and quantitation. An AOAC committee on gas chromatography of pesticide formulations discussed column preparation which included calculation of theoretical plates (5). A properly working column should generate 4000–5000 plates.

Recommendation

It is recommended that the gas chromatographic method for determining alachlor in microencapsulated formulations be adopted official first action as an AOAC-CIPAC method.

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Russ Wiley, Monsanto Co., Muscatine, IA

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Liquid Chromatographic Determination of Cholecalciferol in Rodent Baits

CONNIE C. GEHRIG and RODGER W. STRINGHAM
Purdue University, Department of Biochemistry, West Lafayette, IN 47907

Cholecalciferol (vitamin D_3) is extracted in acetonitrile on a Goldfisch apparatus, diluted to volume, and determined by reverse phase liquid chromatography (LC). The sum of the peak heights of pre-vitamin D_3 and *cis*-vitamin D_3 is used for quantitation. The method was tested for precision, linearity, and recovery. Quadruplicate analyses of 5 formulation samples gave relative standard deviations of 1.56–2.65%. Linearity was excellent with regression and correlation coefficients of 0.9997 and 0.9998, respectively. Recovery was 98.0 ± 2.7%. The method is applicable to 0.075% cholecalciferol rodent baits.

The recent introduction of cholecalciferol (vitamin D_3) as a rodenticide requires the development of a suitable method of analysis. Vitamin D_3 in feeds has been determined by liquid chromatography (LC) (1–6); LC has been shown to be the most specific method for identifying the D_3 isomers (3). The official AOAC method (7) for vitamin D_3 in feeds requires complex extractions and cleanup procedures because of the complex matrixes and the presence of interfering compounds. These extraction and cleanup steps might be circumvented in the analysis of rodent baits because cleanup is not needed, and no other ingredients of interest are present.

In the method presented here, the sample is subjected to a simple extraction and cleanup procedure and analyzed by reverse phase liquid chromatography. The method has been tested for linearity, precision, and recovery.

METHOD

Apparatus and Reagents

(a) *Extractant.*—0.1 mg butylated hydroxytoluene (BHT)/mL acetonitrile (LC grade).

(b) *Extraction apparatus.*—Goldfisch Fat Extractor with 100 mL beakers and 19 × 9 mm cellulose extraction thimbles (Labconco Corp., Kansas City, MO 64132).

(c) *Liquid chromatograph.*—Varian Model 5060 equipped with Varian UV-50 variable-wavelength detector set at 265 nm and 0.2 AUFs, and Valco C6U injection valve, or equivalent (Varian Assoc., Palo Alto, CA 94303).

(d) *LC column.*—250 × 4.6 mm Alltech C18, 10 μm or equivalent (Alltech Assoc., Inc., Deerfield, IL 60015).

(e) *Mobile phase.*—CH_3CN–2% acetic acid–tetrahydrofuran (90 + 5 + 5, v/v/v); flow rate, 1.5 mL/min.

Extraction and Liquid Chromatography

(a) *Sample.*—Method is suitable for 0.075% rodent baits. Grind pelleted samples manually with mortar and pestle. Weigh 4.0 g freshly ground pellets or unground seeds into extraction thimble, tap down sample, plug with cotton, and extract with 40 mL extractant for 4 h on Goldfisch apparatus. Cool sample 5 min, transfer quantitatively to 50 mL volumetric flask, and dilute to volume with extractant. Filter through glass fiber filter pad (Gelman Sciences, Inc., Ann Arbor, MI 48106) and inject 50 μL into chromatograph.

(b) *Standard.*—Prepare same day as analysis. Weigh 15 mg standard (Sigma Chemical Co., St. Louis, MO 63178) into extraction thimble and extract as for sample. Quantitatively transfer to 50 mL volumetric flask and dilute to volume with extractant. Dilute 10 mL to 50 mL with extractant; filter and inject into chromatograph. Bracket every 3 samples with standard injections.

Calculations

Use sum of pre-vitamin D_3 and *cis*-vitamin D_3 peak heights as the cholecalciferol response; bioactivity is due to both isomers (2). For standard response, use average of standards bracketing sample. Calculate as follows:

$$\text{Cholecalciferol, \%} = (R/R') \times (W'/W) \times (V/V') \times \text{\% std purity}$$

where R and R' = response for sample and standard, respectively; W and W' = weight of sample and standard respectively; V and V' = volume of sample and standard, respectively. Here, V = 50 and V' = 250.

Results and Discussion

Figure 1 represents a typical chromatogram of cholecalciferol extracted from a 0.075% rodent bait. Heating during the extraction results in the isomerization of the vitamin D_3 into the pre- and *cis*-forms. It is thus essential to treat the standard in the same manner as the samples. The ratio of peak heights for the standard and sample were constant at 2 different wavelengths, implying the absence of impurities under the sample peaks.

Linearity of the method was tested by chromatographing dilutions of the standard ranging in concentration from 0.03 to 0.07 mg/mL. Least-squares analysis gave regression and correlation coefficients of 0.9997 and 0.9998, respectively. Recovery of diluted standard added to a ground sample (n = 5) was calculated to be 98.0 ± 2.7%.

Table 1 shows the results of quadruplicate analyses of cholecalciferol in 5 rodent baits and a single standard. Sample 86-679 is an unground, mixed, whole-seed bait, whereas the others are pellets. The larger relative standard deviation (RSD) obtained for the seed bait is to be expected due to the heterogeneity of the sample. The precision is quite good for samples with this level of analyte. It cannot be determined if the difference between label claim and analyte found in some samples is due to incomplete extraction or to incomplete formulation.

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Purdue Agricultural Experiment Station No. 11,029.

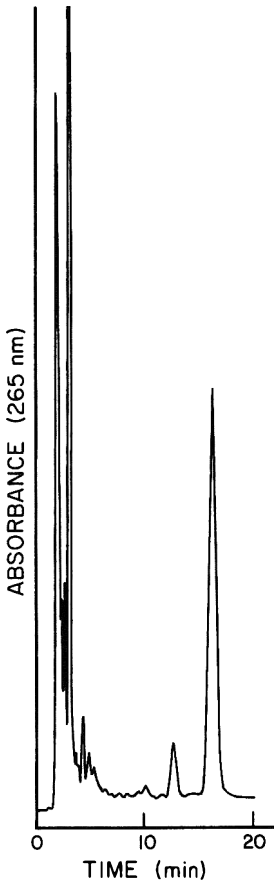

Figure 1. Typical chromatogram of cholecalciferol rodent bait. Peaks used for quantitation were pre-vitamin D_3 (ca 13 min) and *cis*-vitamin D_3 (ca 18 min).

Grinding the samples immediately before analysis increases analyte recovery. Evidently, ground samples that are allowed to sit become oxidized or are degraded in the light. Care was taken to minimize exposure to light throughout the analysis. BHT was included in the extractant to prevent oxidation. The Goldfisch apparatus continuously provides cool extractant, thus minimizing the extraction of interfering substances. Furthermore, the cellulose extraction thimbles serve to absorb wax from the extract. Acetonitrile was chosen as the extractant because it was strong enough to extract the cholecalciferol, but not strong enough to extract unwanted compounds from the sample matrix.

Table 1. Results of quadruplicate analyses of cholecalciferol in rodent bait formulations

Formulation	Label claim, %	Found, %
86-679	0.075	0.0581
		0.0556
		0.0557
		0.0546
Av. ± SD (RSD)		0.0560 ± 0.0015 (2.65%)
86-680	0.075	0.0750
		0.0746
		0.0768
		0.0770
Av. ± SD (RSD)		0.0759 ± 0.0012 (1.61%)
12646	0.075	0.0747
		0.0716
		0.0737
		0.0737
Av. ± SD (RSD)		0.0734 ± 0.0016 (1.78%)
12757	0.075	0.0705
		0.0683
		0.0682
		0.0694
Av. ± SD (RSD)		0.0691 ± 0.0012 (1.56%)
13756	0.075	0.0662
		0.0639
		0.0650
		0.0659
Av. ± SD (RSD)		0.0653 ± 0.0012 (1.59%)

The method presented here is simple and has excellent precision, recovery, and linearity for the analysis of cholecalciferol in rodent baits.

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ALCOHOLIC BEVERAGES

Titrimetric Determination of Carbon Dioxide in Wine: Collaborative Study

ARTHUR CAPUTI, JR, and DURWARD R. WALKER
E. & J. Gallo Winery, PO Box 1130, Modesto, CA 95353

Collaborators: S. Blittman; R. Bunnell; M. Burns; R. Dowrie; S. Fike; B. Gump; R. Jones; B. Masuda; M. Salehi

The AOAC official methods for determination of carbon dioxide in wine are time-consuming, relatively complex, and may not be suitable for newer wine products with higher levels of carbonation. A different procedure was collaboratively studied. In this method, NaOH is added to convert CO_2 in the wine to the carbonate form. The solution is then titrated with a standard H_2SO_4 solution, and the titer is recorded between pH 8.6 and 4.0. A degassed sample of the same wine is analyzed in the same manner to provide a blank, and that titer is subtracted before the CO_2 concentration is calculated. Ten laboratories participated in the collaborative study. Each laboratory received 6 pairs of coded samples covering a range of approximately 200 to 380 mg CO_2/100 mL. The results from 2 laboratories were excluded as outliers. Although the samples used were commercially produced wines whose carbon dioxide content can vary due to normal bottling equipment variations, average standard deviations for reproducibility and repeatability were 10.97 and 9.96, respectively. The method has been adopted official first action.

The determination of carbon dioxide in wine has become more frequently required with the advent of an increasing number of lightly carbonated wine products. The 3 AOAC official methods for this determination (1) are all very time consuming for routine use where many samples must be analyzed. The manometric method, **11.062-11.066**, has never progressed beyond first action status, and, because of its complexity, it is seldom used. The volumetric method, **11.067-11.069**, has been declared surplus and also remains in first action status. Problems that occur with the third method, **11.070-11.071**, an enzymatic method, have been reported previously (2). It was designated surplus at one time. Furthermore, that method was designed to accommodate CO_2 concentrations on the order of 250 mg/100 mL. Levels of almost 400 mg/100 mL are now commonly encountered.

The method tested in this collaborative study, which has been described previously (2, 3), avoids most of the difficulties found in the present AOAC methods. In brief, 50% NaOH is added to adjust the sample pH to 10–11, and the solution is titrated with a standard acid solution in the presence of carbonic anhydrase. The titer between pH 8.6 and 4.0 is recorded, and the equivalent titer of a degassed sample of the same wine is subtracted as a blank. The difference is used to calculate the CO_2 content of the sample. Carbonic anhydrase is added to the blank sample prior to degassing to catalyze the reaction $H_2CO_3 = CO_2 + H_2O$. Data developed in our laboratory indicate that blank values as much as 1% lower are obtained using this approach instead of first degassing the blank sample, then adding the enzyme after pH adjustment with NaOH.

Submitted for publication June 10, 1987.

This report of the Associate Referee, A. Caputi, Jr, was presented at the 100th AOAC Annual International Meeting, Sept. 15–18, 1986, at Scottsdale, AZ.

The recommendation of the Associate Referee was approved interim official first action by the General Referee, the Committee on Foods II, and the Chairman of the Official Methods Board. The method was adopted official first action at the 101st AOAC Annual International Meeting, Sept. 14–17, 1987, at San Francisco, CA. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1988) **71**, January/February issue.

Data in this laboratory had shown a coefficient of variation for this technique which was consistently under 1% when the sample was repeatedly analyzed. However, this collaborative study necessitated the use of a great number of commercially bottled wines. Some difference can exist in the CO_2 concentration from one bottle to another of the same wine type due to mechanical factors in the filling operation. To estimate the degree of error that would be introduced into the study by this inherent problem, a case (12 bottles) of one of the test products was analyzed; each sample was checked twice. The duplicate analyses were all within 2 mg CO_2/100 mL, but the averages of the duplicates of the 12 samples had a mean of 368.9 mg CO_2/100 mL with a range of 17 and a standard deviation of 4.34 (CV 1.2%). This experiment was repeated with another 12 bottles and gave the following results: mean 367.3 mg CO_2/100 mL, range 27, and standard deviation 8.15 (CV 2.2%). This unavoidable lack of homogeneity in the samples must be kept in mind when the results of the study are evaluated.

Collaborative Study

Ten laboratories participated in the collaborative study. Each was supplied with the necessary samples as 6 blind duplicate pairs and corresponding duplicates for the blanks, fresh carbonic anhydrase which was to be refrigerated on receipt, and a sample of sodium carbonate in water that contained the equivalent of 315 mg CO_2/100 mL. Each collaborator was asked to practice the procedure by analyzing the sodium carbonate sample until the correct result was consistently obtained. Only then was the analyst to proceed to analyze the sample. To learn as much as possible about this method when it was used by chemists unfamiliar with it, the report form was more extensive than usual. In addition to reporting the final results for each sample, we asked each analyst to record the pH of each after addition of NaOH, as well as the titers for the samples of the corresponding blanks.

Carbon Dioxide in Wines

Titrimetric Method

First Action

(Applicable to wines contg ≤400 mg CO_2/100 mL)

Principle

Sample is made alk. with NaOH and carbonate formed is titrd with stdzd H_2SO_4. Degassed sample is titrd as blank.

Apparatus and Reagents

(a) *pH meter.*—Stdzd at pH 4 and 10 with appropriate buffers.

(b) *Buret.*—25 mL with automatic zero.

(c) *Magnetic stirrer.*

(d) *Sulfuric acid.*—0.0455N H_2SO_4. At this normality, 1 mL = 20 mg CO_2/100 mL.

(e) *Carbonic anhydrase soln.*—0.1 mg/mL H_2O. Keep refrigerated; soln is stable for 1 week. (Use carbonic anhydrase from bovine erythrocytes. Available from Sigma Chemical Co., Cat. No. C-7500.)

(f) *Sodium hydroxide soln.*—50% soln of reageant grade NaOH.

Table 1. Individual collaborative results for study of CO_2 in blind duplicate wine samples (mg CO_2/100 mL sample)

	Dry white table wine		Rosé table wine		Semisweet table wine		Wine cooler		Special natural wine		Special natural wine 2		Stan-
Coll.	A	L	B	H	C	G	D	I	E	J	F	K	dard
1	221	218	220	223	201	201	370	371	299	296	159	160	316
2	215	205	215	215	201	199	364	392	301	305	160	117[a]	319
3	231	217	200	214	188	199	371	373	301	303	161	156	316
4	216	220	220	235	182	196	363	365	296	300	157	157	320
5	204	206	208	218	188	191	362	362	285	288	152	152	316
6	208	243	210	212	178	194	355	371	287	300	143	135	338
7	223	231	202	224	207	202	367	367	298	308	153	173	314
8[b]	212	237	204	247	154	227	380	406	282	292	150	171	318
9	237	222	244	223	215	177	352	347	307	290	176	164	317
10[c]	198	200	190	188	176	178	355	355	263	265	114	116	318
$\bar{x}$	219.8		217.7		194.9		365.8		297.8		154.7 (157)		319.2
SD	11.4		11.2		10.4		10.1		7.0		14.1 (10.2)		7.7
CV(r), %	4.88		4.39		5.80		2.24		2.08		8.05 (4.28)		
CV(R), %	5.21		5.18		5.80		2.81		2.38		9.16 (6.48)		

[a] Sum of this value and its duplicate did not meet Dixon's criteria as an outlying pair; the value was included in the statistical evaluation. Values in parentheses show the effect of deleting this pair.

[b] Results from this laboratory eliminated from statistical evaluation on basis of Steiner's test for variation between replicates (4).

[c] Results from this laboratory eliminated from statistical evaluation on basis of Youden's ranking test (4).

Determination

Cool sample to ca 5°, open bottle, and add equiv. of 5 mL NaOH soln per 375 mL sample. Immediately recap bottle, mix contents, pipet 10 mL portion of sample into 40 mL H_2O, add 3 drops carbonic anhydrase soln, and titr. to pH 8.6 with 0.0455N H_2SO_4. Refill buret and continue titrn to pH 4.0. Record titer between pH 8.6 and 4.0.

Obtain blank as follows: Degas 25 mL duplicate of sample with agitation ca 1 min under ≥28 in. vac. in 500 mL filter flask contg 3 drops carbonic anhydrase soln. Add 0.33 mL NaOH soln. Pipet 10 mL degassed sample into 150 mL beaker contg 40 mL H_2O. Stir and titr. as for sample detn.

Calc. as follows:

$$\text{mg } CO_2/100 \text{ mL} = (\text{mL sample} - \text{mL blank}) \times 20 \times 1.013$$

CAS-124-38-9 (carbon dioxide)

Results and Recommendation

The raw data from the study are given in Table 1, along with the sample codes. The ranked results, shown in Table 2, indicated that data from laboratory 10 should be eliminated for the subsequent statistical computations. The results from this laboratory are extremely interesting. The variation between replicates for their results is the best of any of the collaborators. Their results are generally about 10% low, and frequent additional conversations with the analyst have failed to locate the source of the error, although the raw data show consistently high values for their blank titrations. Incomplete degassing of the blank sample is a possible explanation for the discrepancy, because this laboratory was using a water aspirator which may not have provided sufficient vacuum. In addition, Steiner's (4) test for variation between replicates disqualified data from laboratory 8. If it had been possible to obtain samples for this study which contained more uniform concentrations of carbon dioxide, this laboratory's results might have been acceptable. Nevertheless, the results from these 2 laboratories were excluded from further calculations. The means, standard deviations, and repeatability and reproducibility coefficients of variation are shown in Table 1 with results for these 2 laboratories excluded. The unusually high CVs for special natural wine 2 can be attributed to a second value of laboratory 2 for this sample. Although it is obviously an anomalous result the sum with its corresponding duplicate did not meet Dixon's criteria as an outlying pair. The values in parentheses (Table 1) demonstrate the statistical effect of deleting this duplicate pair from these calculations.

The results from the analysis of variance are shown in Table 3. Neither the between-laboratories variance ratio nor the laboratory-sample interaction is significant at the 95% confidence level (5). It seems likely that if the samples used in the study could have been more homogeneous in respect to CO_2 content, these variances would have been even lower. Nevertheless, the results of this study warrant the recommendation that this method be adopted as official first action.

Table 2. Ranked results for collaborative study of CO_2 in wine[a]

	Sample						
Lab.	Dry white table wine	Rosé table wine	Semi-sweet table wine	Wine cooler	Special natural wine	Special natural wine 2	Total
1	5	7	9	7	5	7	40
2	3	6	8	9	9.5	2	37.5
3	6	2	6	8	8	6	36
4	4	9	3	5	6	5	32
5	2	4.5	4	3	2	4	19.5
6	8	3	2	4	4	3	24
7	9	4.5	10	6	9.5	9	48
8	7	8	5	10	3	8	41
9	10	10	7	1	7	10	45
10	1	1	1	2	1	1	7

[a] Results ranked by Youden's ranking test (4).

Table 3. Analysis of variance for collaborative study of CO_2 in wine

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between labs	1867.8	7	266.8 (MS_L)	2.92 (MS_L/MS_{LS})
Between (adjusted) samples	1001	5	—	—
Lab.–sample interaction	3190.7	35	91.2 (MS_{LS})	0.83 (MS_{LS}/MS_O)
Between replicates	5293	48	110.2 (MS_O)	—
Total	11 352.5	95		
Est. of repeatability	SD 9.96			
Est. of reproducibility	SD 10.97			

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Rick Jones, San Martin Winery, San Martin, CA

Jeffery Kasavan and Bernard Masuda, Seagram Wine Co., Gonzales, CA

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FOOD ADDITIVES

Comparison of Methods for Determination of Lactose (Nonfat Dry Milk) in Meat Products

P. CHRISTOPHER ELLIS
Rhode Island Department of Health Laboratories, Food Chemistry Laboratory, 50 Orms St, Providence, RI 02904
ARTHUR G. RAND, JR
University of Rhode Island, Food Science, Nutrition, and Dietetics, Kingston, RI 02881

Four enzymatic methods were compared with the official AOAC yeast fermentation procedure for determining nonfat dry milk (NFDM) as lactose in meat products. The four enzymatic procedures included a commercially available UV test kit, 2 colorimetric assays using kits prepared in the laboratory, and a commercially available instrument with an immobilized enzyme electrode to detect lactose electrochemically. The enzyme procedures were compared with the AOAC method in terms of analysis time, accuracy, precision, sensitivity, specificity, and ease of performance. The UV kit and immobilized enzyme electrode appeared to be the most promising alternatives to the AOAC method on the basis of statistical data and analysis time. Particular needs of a laboratory would dictate the method of choice, but either one could be used as a screening procedure for regulatory purposes. Samples containing levels of NFDM in excess of 3.5% by weight could then be verified by the AOAC yeast method.

Nonfat dry milk (NFDM), consisting of approximately 50% lactose (1), is a nutritive ingredient added to processed meat products as a filler or extender to reduce costs and as a binder for textural improvement. NFDM does not adversely affect any flavor characteristics of the meat at the levels permitted by the U.S. Food and Drug Administration (FDA). NFDM is allowed by itself or with fillers such as soy or cereal up to 3.5% by sample weight (2, 3). Some manufacturers tend to use NFDM in excess of 3.5% because it is less expensive than meat. To ensure that the 3.5% level is not violated, regulatory laboratories test for lactose in processed cold cuts and sausage-type products to determine NFDM content.

The official AOAC procedure (4) uses a yeast suspension to ferment all sugars in the meat products except lactose. Lactose is then proportionately measured by a stoichiometric titration based on the amount of copper reduced in a boiling Benedict solution. However, the AOAC method is nonspecific, time-consuming, and tedious, and requires numerous reagents. The method lacks specificity because the yeast cannot ferment maltose from corn syrup or corn syrup solids, which are common additives in processed meats (5). The AOAC method includes a lengthy maltose-yeast acclimation procedure to help the yeast ferment maltose but some researchers have had difficulty accomplishing this (6). The presence of other nonfermentable reducing substances in corn syrup solids, besides maltose, can further complicate the assay (7). The desired assay for testing NFDM either on a routine or a nonroutine basis in the laboratory should be rapid, accurate, precise, yet economical. The AOAC yeast method does not satisfy all these criteria.

In addition to the standard AOAC yeast procedure, lactose has been conventionally determined using chemical methods such as Fehling titrations (another copper reagent), optical methods including polarimetry and refractometry, and chromatographic techniques such as gas, thin-layer, paper, and liquid (8). The use of enzymatic methods to determine lactose in meat products might offer significant advantages over the AOAC yeast method in terms of simplicity, shorter analysis times, higher sensitivity, and greater specificity (9–11). Other methods that have proven successful for lactose determination include liquid chromatography (12) and amperometric analysis (13–15). Liquid chromatography is sensitive but requires more complex instrumentation. One of the methods tested in the study uses a commercial sugar analyzer that takes advantage of both amperometric and enzyme analysis for specific sugar quantitation.

The research presented is the result of a comparative study involving 4 enzymatic methods and the official AOAC method for determining lactose in meat products. Meat samples, lactose standards, and meat samples spiked with lactose were analyzed by all the methods and the data were statistically treated to determine any significant differences in the procedures in terms of accuracy and precision. A study of analysis time was also done on all the methods.

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METHOD

Principles

The AOAC yeast procedure and the 4 enzymatic reactions are outlined below and in Scheme 1. The AOAC method breaks down lactose chemically, while 2 of the enzymatic methods (UV and B-Gal/GAX) involve hydrolysis of lactose to glucose and galactose with the enzyme β-galactosidase. Both hexoses could be used to assay lactose content; galactose is the sugar of choice because of high glucose blanks in many foods (16, 17). The UV method (18; Boehringer-Mannheim

Scheme 1. Reactions for 5 lactose methods

(1) Yeast Method:

(a) Lactose + fermentable sugars (maltose-acclimated yeast) → lactose – fermented sugars
(b) Lactose + Benedict solution (heat) → oxidized lactose + red cuprous oxide precipitate
(c) Red cuprous oxide precipitate + excess I_2/KI solution (dilute H_3PO_4) → liberated I_2
(d) Liberated I_2 + starch indicator solution (blue) ($Na_2S_2O_3$) → starch indicator solution (light green)

(2) UV Method:

(a) Lactose + H_2O (beta-galactosidase) → glucose + beta-galactose
(b) Beta-galactose + NAD^+ (beta-galactose dehydrogenase) → galactonolactone + NADH + H^+

(3) B-Gal/GAX Method:

(a) Lactose + H_2O (beta-galactosidase) → glucose + beta-galactose
(b) Beta-galactose + O_2 (galactose oxidase) → D-galactohexodialdose + H_2O_2
(c) H_2O_2 + reduced chromagen (ABTS) (peroxidase) → oxidized chromagen (ABTS)

(4) GAX Method:

(a) Lactose + O_2 (galactose oxidase) → H_2O_2 + oxidation product
(b) H_2O + reduced chromagen (ABTS) (peroxidase) → oxidized chromager (ABTS)

(5) YSI Method:

(a) Lactose + O_2 (galactose oxidase) → H_2O_2 + oxidation product
(b) H_2O_2 (platinum anode) → $2H^+ + O_2 + 2e^-$
(c) $4H^+ + O_2 + 4e^-$ (silver cathode) → $2H_2O - 4e^-$

"Product Highlights," July 1983) uses the enzyme β-galactose dehydrogenase plus the coenzyme nicotinamide adenine dinucleotide (NAD^+) to oxidize β-galactose to galactonolactone and NADH. The amount of NADH formed is proportional to the lactose content and can be measured at 340 nm. The B-Gal/GAX method (19) uses galactose oxidase to oxidize β-galactose from lactose hydrolysis to hydrogen peroxide (H_2O_2) and D-galactohexodialdose. The H_2O_2 formed is reduced by peroxidase in the presence of the chromagen 2,2'-azino-di-(3-ethylbenzthiazoline)-6-sulfonate (ABTS) which is oxidized to its colored form; the increase in absorbance is read at 425 nm. In the GAX (20) and YSI methods (21; J. Huntington, Yellow Springs Instrument Co., 1978), the enzyme galactose oxidase is used to oxidize lactose to H_2O_2 and a galactose dialdehyde derivative. The GAX method colorimetrically determines lactose as described in the B-Gal/GAX method. The YSI method uses an immobilized galactose oxidase enzyme system incorporated into the YSI Model 27 Industrial Analyzer, which electrochemically detects the H_2O_2 formed as a proportional measurement of the lactose present. This is displayed on an LED as mg/dL, previously calibrated with a known amount of lactose.

Reagents and Apparatus

(1) Yeast Method:

Reagents.—See **24.072** (4). Modify (**a**). *Acclimated yeast suspension (for use in presence or absence of maltose)* as follows: 2 packages of Fleishmann's All Natural Dry Yeast (¼ oz or 7 g package) per 100 mL yeast solution (prepared in lieu of 2 cakes of bakers yeast).

(2) UV Method:

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

(**b**) *Lactose standard.*—0.5 g/L. Weigh 0.527 g lactose monohydrate (Allied Chemical Corp., Morristown, NJ 07960) and dilute to 1 L with water. Use 0.1 mL of this solution for assay.

(**c**) *Spectrophotometer.*—Bausch and Lomb Spectronic 2000, double beam.

(3) B-Gal/GAX Method:

(**a**) *Beta-galactosidase.*—Liquid Maxilact LX 2000 (2000 NLU/g or 8000 ONPG U/g), GB Fermentation Industries, Inc., Des Plaines, IL. Dilute 0.1 g Maxilact with 10.0 mL water for test. Prepare daily.

(**b**) *Potassium phosphate buffer.*—0.1M, pH 6.8. Dissolve 6.8 g KH_2PO_4 and 8.7 g K_2HPO_4 in water and dilute to 1 L with water in volumetric flask. Check and adjust to pH 6.8 if necessary. Store in refrigerator and prepare weekly.

(**c**) *Peroxidase.*—Type 1, horseradish, 50 000 U, 500 mg solid, 100 U/mg solid (Sigma Chemical Co., St. Louis, MO 63178, Cat. No. P-8125). Dissolve 25 mg peroxidase in 10 mL buffer (**b**). Freeze separately 1.0 mL aliquots in plastic test tubes with cap (12 × 75 mm clear with cap, Falcon, Oxnard, CA). When ready to use, dilute solution to 5.0 mL with 4.0 mL buffer (**b**). Solution contains peroxidase with 50 U/mL activity.

(**d**) *Galactose oxidase.*—Type 5, 450 U, 90 U/mg solid (Sigma Chemical Co., Cat. No. G-3385). Dissolve contents of vial in 4.5 mL buffer (**b**) and dispense 0.4 mL aliquots in plastic test tubes for freezing. When ready to use, thaw and reconstitute tube contents with 1.6 mL buffer. Solution contains galactose oxidase with 20 U/mL activity.

(**e**) *2,2'-Azino-di-(3-ethylbenzthiazoline)-6-sulfonate (ABTS).*—Sigma Chemical Co., Cat. No. A-1888. Dissolve 50 mg ABTS in 10.0 mL water. Store in amber bottle and refrigerate. Prepare monthly. Solution contains 5.0 mg/mL of ABTS.

(**f**) *Galactose standards.*—Weigh 0.1 g D(+)galactose anhydrous (Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH 45212) and dilute to 100 mL with water (1.0 mg/mL). Pipet 1, 2, 3, 4, 5, and 6 mL of this solution into separate test tubes and dilute to 10 mL with water (100, 200, 300, 400, 500, and 600 μg/mL of galactose, respectively). Use 0.1 mL of each solution for test. Store stock solution in refrigerator and prepare weekly. Prepare working solutions daily.

(**g**) *Spectrophotometer.*—See UV method.

(4) GAX Method:

(**a**) *Lactose standards.*—Weigh 0.527 g lactose monohydrate and dilute to 100 mL with water (5 mg/mL). Pipet 1, 2, 3, 4, 6, and 8 mL of this solution (5 mg/mL) into separate test tubes and dilute to 10 mL with water (0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 mg/mL of lactose, respectively). Use 0.1 mL of each solution for test. Store stock solution in refrigerator and prepare weekly. Prepare working solution daily.

(**b–e**) *Reagents.*—See B-Gal-GAX method.

(**f**) *Spectrophotometer.*—See UV method.

(5) YSI Method:

Apparatus.—YSI Model 27 Industrial Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH 45387) with accompanying membranes, reagents, and standards.

Determination

(1) Yeast method.—Follow sections **24.072-24.073** (4) to determine lactose, using acclimated yeast suspension.

(2) UV method.—Follow instructions with lactose/galactose kit No. 176303 for enzymatic UV determination of lactose.

(3) B-Gal/GAX and (4) GAX methods.—Follow section **24.073** (4), up to "Pipet 40 mL filtrate into 50 mL vol. flask. . . ." Instead, pipet 25 mL filtrate into 50 mL beaker and adjust to pH 7.0, using pH meter and 2–3 mL 5N NaOH followed by dropwise addition of 1N NaOH. If pH is >7.0, use 1N HCl to return to neutral. Add 5.0 mL buffer to stabilize pH and quantitatively transfer with water to 50 mL volumetric flask.

Reagent sequence for B-Gal/GAX method: To test tube add 0.2 mL buffer, 0.1 mL sample solution or lactose standard solution, and 0.05 mL β-galactosidase; let stand 10 min at room temperature. Add 2.35 mL buffer, 0.1 mL ABTS solution, and 0.1 mL peroxidase solution; equilibrate 5 min in 37°C water bath. Add 0.1 mL galactose oxidase solution; gently mix, and incubate 10 min at 37°C. After 10 min, stop color development with 0.1 mL 4N HCl, vortex-mix, and read *A* (green) at 425 nm against a reagent blank of water instead of sample.

Reagent sequence for GAX method: To test tube add 2.6 mL buffer, 0.1 mL of either sample solution, galactose standard solution to determine galactose standard curve, or lactose standard solution to determine lactose standard curve, 0.1 mL ABTS solution; and 0.1 mL peroxidase solution; equilibrate 5 min at 37°C in a water bath. Add 0.1 mL galactose oxidase solution, gently mix, and incubate 60 min at 37°C in a water bath to determine lactose in sample or lactose standard solution. Incubate galactose standard solution 10 min and use galactose standard curve to determine galactose from lactose as determined by the B-Gal/GAX method. Stop colorimetric reaction with 0.1 mL 4N HCl, vortex-mix, and read *A* at 425 nm against reagent blank of water instead of sample. To determine percent recovery for B-Gal/GAX

Table 1. Ranges and standard deviations for multiple analyses of galactose and lactose standards by GAX method[a]

Galactose					Lactose				
		A_{425}					A_{425}		
Std, μg	Trials	Range	Mean	SD	Std, μg	Trials	Range	Mean	SD
10	16	0.070–0.125	0.101	0.014	50	6	0.080–0.204	0.135	0.045
20	4	0.145–0.200	0.181	0.026	100	11	0.181–0.380	0.263	0.067
30	17	0.255–0.380	0.311	0.028	150	2	0.340–0.345	0.343	—
40	4	0.365–0.410	0.389	0.025	200	8	0.410–0.650	0.523	0.087
50	13	0.475–0.585	0.531	0.038	250	1	0.565	—	—
60	2	0.565–0.575	0.570	—	300	6	0.680–0.885	0.773	0.073
					400	5	0.810–1.096	0.929	0.107

[a] Regression equations:

X(μg) = 1.471 + 92.859 Y (A_{425})
Correlation coefficient, r = 0.985
Total trials = 56 pairs of standard X, Y values

X(μg) = 0.773 + 397.278 Y (A_{425})
r = 0.964
Total trials = 39 pairs of standard X, Y values

method, prepare 60 μg total lactose solution by pipeting 6.0 mL of 1.0 mg/mL lactose standard into test tube and diluting to 10 mL with water. A 0.1 mL aliquot of this solution represents 60 μg and is used to determine percent recovery.

(5) YSI method.—Place 5 g homogenized sample in 100 mL volumetric Kohlrausch sugar flask, add 70 mL warm (60°C) water, agitate to break up sample, and heat 30 min on steam bath with frequent shaking. Let cool to room temperature, dilute to mark with water, and mix. Place in refrigerator 30 min to precipitate fat and then filter through Whatman No. 1 paper. Use clear or slightly turbid solution for assay as prescribed by YSI Model 27 Industrial Analyzer instructions. Dilute if necessary.

Calculations

(1) AOAC method, from USDA laboratory guidebook (5):

$$\% \text{ Lactose} = (\text{mL } I_2 - [A \times \text{mL } Na_2S_2O_3]) \times B \times 100/C$$

in which $A = I_2/Na_2S_2O_3$ ratio, where I_2 = volume of I_2 added to flask and $Na_2S_2O_3$ = volume of $Na_2S_2O_3$ solution required to titrate 20 mL I_2; B = lactose/I_2 ratio, where lactose represents mg lactose in 10 mL aliquot of standard lactose solution and I_2 = volume of $Na_2S_2O_3$ required to back-titrate I_2; C = mg sample in aliquot = (10 g/100 mL × 10 mL × (40/50) × 1000 mg/g) = 800 mg.

$$\% \text{ NFDM} = (\% \text{ lactose} \times 2) - \text{correction}$$

where correction = 0.4% in absence of corn syrup solids or corn syrup, or 0.8% in presence of corn syrup solids or corn syrup.

(2) UV method, from instructions in lactose/galactose kit:

Sample concentration = 5 g/100 mL or 50 g/L

$$\% \text{ Lactose} = (\text{g lactose/L})(\text{g sample/L}) \times 100$$

(3) B-Gal/GAX method:

$$\% \text{ Lactose} = (\mu\text{g galactose from galactose std curve} \times 1.9/\mu\text{g sample}) \times 100$$

where 1.9 = factor to convert galactose formed from lactose to original lactose present = (MW lactose/MW galactose) = 342/180.

$$\mu\text{g sample in final solution} = (10 \text{ g}/100)(25/50)(1/10)(10^6 \mu\text{g/g}) = 5000 \mu\text{g}$$

(4) GAX method:

$$\% \text{ Lactose} = (\mu\text{g lactose from lactose std curve}/\mu\text{g sample}) \times 100 / \mu\text{g sample in final solution} = 5000 \mu\text{g}$$

(5) YSI method:

$$\% \text{ Lactose} = (\text{mg lactose/dL})/(\text{mg sample/dL}) \times 100$$

mg sample/dL = 5 g/100 mL = 5000 mg/dL

Results and Discussion

The calibration curve data used to determine galactose and lactose concentrations by the GAX method are presented in Table 1. The range and standard deviation of values at each concentration assayed are included along with the line of best fit as determined by the least squares method (22). The predicted absorbances (Y values) for the curves can be determined by substituting the appropriate galactose and lactose concentrations in the regression equation. The wide ranges and standard deviations obtained from the numerous analyses indicate that standard curves should be run on each test occasion to account for enzyme and test variability. Time studies on the reagents for the galactose oxidase methods showed them both to be stable for at least 60 days when refrigerated at 0–5°C. The diluted Maxilact β-galactosidase was prepared daily. A β-galactosidase suspension from Boehringer-Mannheim could also be obtained with a shelf life of at least 1 year at 4°C. On the basis of sensitivity and linearity testing performed in the laboratory, ABTS was chosen over *o*-dianisidine HCl and *o*-tolidine as the chromagen in the B-Gal/GAX and GAX methods. ABTS is also noncarcino-

Table 2. Recovery comparison for AOAC and enzymatic lactose analysis methods

Statistic	AOAC	UV	B-Gal/GAX	GAX	YSI
Lactose spike,[a]	263 mg	527 mg/L	623 mg/L	1054 mg/L	211 mg/dL
Mean % lactose recd	88.15	93.47	100.54	105.62	99.83
Std dev.	0.94	3.33	4.68	26.80	2.25
95% Confidence limits, %	1.17	1.77	2.70	18.88	1.12
Range, %	1.65	11.78	12.68	71.51	8.50
Coeff. of var., %	1.07	3.56	4.65	25.37	2.25
No. of trials	5	16	14	11	18

[a] Lactose monohydrate.

Table 3. Precision and accuracy of AOAC and enzymatic methods for bologna spiked with lactose at 1.0 and 2.5% levels

	Spiking level, %									
	AOAC		UV		B-Gal/GAX		GAX		YSI	
Statistic	1.0	2.5	1.0	2.5	1.0	2.5	1.0	2.5	1.0	2.5
Sample weight, g	10	10	5	5	5	5	5	5	5	5
Lactose spike,[a] mg	105	263	53	132	53	132	53	132	53	132
No. of trials	5	5	5	5	5	5	5	5	5	5
Mean % lactose recd	99.06	90.54	85.00	92.00	73.57	81.13	62.20	78.32	100.80	103.52
Std dev.	6.37	2.50	3.51	2.24	2.64	2.49	3.03	6.13	8.90	5.79
95% Confidence limits	7.91	3.10	4.39	2.78	2.44	2.61	3.76	7.61	11.04	7.19
Range, %	16.00	6.60	9.00	6.00	9.00	6.00	8.00	14.40	20.00	15.20
Coeff. of var., %	6.43	2.76	4.12	2.43	4.87	3.07	3.59	7.82	8.82	5.59

[a] Lactose monohydrate, MW 360, was used as spike (1.053 g lactose monohydrate is equivalent to 1.000 g anhydrous lactose).

genic, making it less of a health risk compared to the carcinogenic *o*-dianisidine HCl and *o*-tolidine.

Recovery of lactose monohydrate was determined for the 5 lactose analysis methods, and the results are presented in Table 2. The GAX method gave a recovery of 105.62% followed by the B-Gal/GAX method at 100.54%, YSI method at 99.83%, UV method at 93.47%, and AOAC method at 88.15%. The YSI and B-Gal/GAX methods gave the best recovery, while the AOAC and YSI methods were the most precise on the basis of standard deviation, 95% confidence limits, range, and coefficient of variation. The 2 colorimetric methods, B-Gal/GAX and GAX, were the least reproducible while the UV method was in the middle, both for accuracy and precision.

The precision and accuracy of the 5 lactose analysis methods, using bologna spiked with lactose monohydrate at 1.0 and 2.5% levels by weight, are presented in Table 3. The 1.0% lactose spike represented a level of NFDM (2.0%) commonly found in meat products, while the 2.5% lactose spike (5.0%) would be considered high for extenders (3.5% NFDM is the limit) by Rhode Island meat standards. The bologna contained a low level of NFDM, so the sample background was first determined then subtracted from the final results to obtain percent recovery.

The lactose recovery results for a sample matrix substantially differed from the data presented in Table 2. The GAX and B-Gal/GAX methods gave significantly lower recoveries, 62.20 and 73.57% with a 1.0% spike, 78.32 and 81.13% with a 2.5% spike, compared to the 105.62 and 100.54% recoveries in the absence of a sample matrix. The UV procedure, which uses the enzyme β-galactose dehydrogenase instead of galactose oxidase, did not show the dramatic decline in recovery (85.00% with a 1.0% spike, 92.00% with a 2.5% spike, 93.47% for nonmatrix recovery). Some component in the sample matrix may have interfered with the enzymatic galactose oxidase/peroxidase reaction involving H_2O_2 formation in the B-Gal/GAX and GAX procedures. Ascorbic acid, often added to cured meats (including the bologna used in the sample spikes) as a preservative, might account for the recovery discrepancies; it can act as a reducing substance and interfere in the reaction involving galactose oxidase (Yellow Springs Instrument Co., 1983). The specificity of galactose oxidase, which would be better for galactose than for lactose, might have contributed along with matrix interferences to the lower GAX recoveries. The AOAC method gave similar recovery results at the same spiking levels with and without sample matrix (90.54% with a 2.5% spike, and 88.15% using 263 mg lactose monohydrate from Table 2 as the nonmatrix equivalent to a 2.5% spike); at the 1.0% spike level a higher 99.06% recovery was obtained. Except for the AOAC test, the lactose recoveries of the enzymatic methods were greater with a 2.5% spike than with a 1.0% spike. Excluding the GAX procedure, the methods were more precise with a 2.5% spike. On the basis of these results, variability is less and recovery is greater with a larger lactose sample size. The YSI, AOAC, and UV methods were the most accurate, with the YSI clearly superior at both the 1.0% and 2.5% spiking levels (100.80%, 103.52%).

The YSI was also the most variable of the 3 according to the precision statistics. The inherent error in the YSI analyzer

Table 4. Comparison of AOAC and lactose enzyme methods for determination of lactose and NFDM concentrations in a variety of formulated meat products[a]

	AOAC		UV		B-Gal/GAX		GAX		YSI	
Sample	% Lac.	% NFDM[b]	% Lac.	% NFDM[b]	% Lac.	% NFDM[b]	% Lac.	% NFDM[b]	% Lac.	% NFDM[b]
Frankfurter 1	1.47	2.54	1.33	2.66	1.78	3.56	1.52	3.04	2.15	4.30
Frankfurter 2[c]	0.50	0.20	0.44	0.88	0.65	1.30	0.24	0.48	1.09	2.18
Knockwurst[c]	0.51	0.22	0.41	0.82	0.62	1.24	0.19	0.38	1.03	2.06
Liverwurst[c]	1.94	3.08	1.45	2.90	1.22	2.44	1.04	2.08	2.27	4.54
Bockwurst	5.95	11.50	6.28	12.56	6.63	13.26	7.44	14.88	6.98	13.96
Linguica	1.04	1.68	1.13	2.26	1.32	2.64	0.89	1.78	3.24	6.48
Chourico	1.61	2.82	1.12	2.24	1.42	2.84	1.10	2.20	1.79	3.58
Olive loaf[c]	2.52	4.24	1.90	3.80	1.89	3.78	2.78	5.56	4.76	9.52
Bologna[c]	0.58	0.36	0.42	0.84	0.13	0.26	0.61	1.22	0.84	1.68
Luncheon loaf[c]	3.67	6.54	2.84	5.68	3.02	6.04	0.92	1.84	4.20	8.40
Imitation chicken loaf[c]	2.06	3.26	1.68	3.36	1.85	3.70	2.19	4.38	2.44	4.88

[a] Each value is the average of 5 analyses.

[b] Approximate NFDM was determined by multiplying % lactose by 2. For AOAC method, % NFDM = (% lactose × 2) − 0.4% if no corn syrup was present, or % NFDM = (% lactose × 2) − 0.8% if corn syrup was present.

[c] Corn syrup declared on label.

Table 5. Analysis times for AOAC and enzymatic lactose methods tested

Step	AOAC	UV	B-Gal/GAX	GAX	YSI
Sample prepn, h	1	1	1	1	1
Reagent prepn,[a] h	2	0[b]	0.83	0.83	0.83
Yeast prepn, h	5	—	—	—	—
Yeast incubation, h	48	—	—	—	—
Sample incubation, h	3	—	—	—	—
Direct analysis, h[c]	0.83	0.50	0.75	1.42	0.05
Total analysis, h	59.83	1.50	2.58	3.25	1.88
Total analysis, h[d]	4.83	1.50	1.75	2.42	1.05

[a] Preparation time required to make fresh reagents.
[b] Commercial kit was used, so reagent preparation was minimal.
[c] Includes sample plus any standards required to run complete analysis.
[d] Excludes preparation of reagents.

(±2% from 0 to 200 mg/dL, ±5% from 200 to 500 mg/dL) could account for this. Therefore, higher concentrations can yield greater errors. To avoid this, further sample dilutions are sometimes necessary.

A variety of meat products containing NFDM were analyzed for their lactose content by using the 5 lactose analysis methods (Table 4). The NFDM value was obtained by multiplying the lactose content by 2, assuming that NFDM was approximately 50% lactose. The AOAC calculation employed a subtraction correction factor of 0.4% when corn syrup solids were not present, and 0.8% when they were contained in the product. This correction factor accounted for interference associated with corn syrup/corn syrup solids which could cause erroneously high readings. The majority of the meat products tested contained corn syrup/corn syrup solids according to the label (Table 4).

Viewing the results in Table 4 from a regulatory standpoint, there were some obvious violations of the 3.5% NFDM cutoff level (no other fillers were declared on the ingredient labels of the meat products tested). All 5 methods found NFDM in excessive amounts of the regulatory level in the bockwurst and olive loaf while all but the GAX method detected violations in the luncheon loaf. Although the AOAC procedure showed no other samples exceeding the 3.5% regulatory limit, the YSI method detected 5 additional violations (frankfurter 1, liverwurst, linguica, chourico, and imitation chicken loaf), the B-Gal/GAX method detected 2 (frankfurter 1 and imitation chicken loaf), and the GAX method detected one (imitation chicken loaf). The inconsistencies of the B-Gal/GAX and GAX methods with spiked lactose recoveries placed some doubt on the validity of their respective estimates for NFDM in the meat products. However, the YSI data are very interesting. It would appear the YSI method is a more sensitive method of measuring NFDM content in meat products than the AOAC method, on the basis of the small sample population tested.

Aside from statistical parameters, analysis time would dictate the best method for determining NFDM as lactose in meat products. The times required for each method, incorporating sample and reagent preparation time, direct analysis, and total analysis time, are illustrated in Table 5. Analysis times were determined over multiple test occasions as conducted by an analyst familiar with the methods involved. The main disadvantage of the AOAC procedure was the length of time required for a complete analysis. Final results could not be obtained for 3 days if fresh reagents needed preparation, while reagent preparation times for the enzymatic methods were considerably shorter. Even without reagent preparation, the AOAC test took approximately 5 h to complete. The UV method required the shortest analysis time but was also the most expensive because of the cost of the enzymes involved, in particular, β-galactosidase. The β-galactosidase also makes the B-Gal/GAX assay more expensive than the AOAC method. The GAX method was inexpensive because fewer enzymes were needed, but it was also the least desirable analytical choice on the basis of the data obtained. The YSI method provided a rapid (second to the UV method in total analysis and reagent preparation) and inexpensive mode of analysis once the initial cost of the equipment was accounted for; the enzyme membranes can be used repeatedly.

The YSI and UV methods appear to be promising alternatives to the AOAC yeast procedure on the basis of statistical analyses and time and cost factors. The enzymatic UV kit provides a simple, accurate, and precise means of detecting lactose with minimal instrumentation. The kit is stable for at least 3 months at 4°C. The UV kit would be a good spot check assay for infrequent testing of meat products for NFDM. The YSI method showed good agreement with the AOAC method, offering simplicity of operation, fast analysis times, accuracy, and precision. As evidenced by the high percent lactose recoveries and percent lactose determined on the meat products tested, the immobilized enzyme system offers many advantages over the colorimetric and UV reactions because of reduced interferences from color, turbidity, refractive index, specific gravity, temperature, and presence of other nutrients. The immobilized enzyme membrane is stable for about 1 week once installed in the analyzer; this makes it suitable for handling large sample loads over a short period of time. The needs of the laboratory dictate the method of choice, but in either case, the UV and YSI procedures can be used to screen possible NFDM violations for retesting by the official AOAC yeast method. The data obtained from the YSI method concerning NFDM violations that were not detected by the AOAC procedure suggest there is a more accurate method available for determining NFDM from lactose.

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CHEMICAL CONTAMINANTS MONITORING

Sample Accountability Quality Assurance for the "Integrated Air Cancer Project" Research Program of the U.S. Environmental Protection Agency

RANDALL R. WATTS and LARRY T. CUPITT[1]
U.S. Environmental Protection Agency, Health Effects Research Laboratory, Research Triangle Park, NC 27711

A sample accountability quality assurance (QA) program is described for a field and laboratory research effort which resulted in collection of approximately 2000 samples for analysis by several EPA and contractor laboratories. A QA program was specifically developed for this research program to include sample transfer from collection site to storage maintenance, record development, transfer to researchers, and sample tracking at all stages. A sample identification system and sample custody records are described for field and laboratory application. The functions of a sample coordinator are also described as relating to sample custody, coordination of sample analysis with researchers, and development of computer record files to facilitate research and sample tracking.

The Integrated Air Cancer Project (IACP) of the U.S. Environmental Protection Agency (EPA) was established in 1984 as a multiyear research program in response to the Agency's mandate for regulating the emission of carcinogens into the air. Past research on hazardous air pollutants was generally conducted on individual primary pollutants at sites selected for specific reasons. The IACP has adopted a different approach of monitoring ambient residential neighborhood air for all chemical species likely to be mutagenic and/or carcinogenic. The goals of the project are to (*1*) identify the principal airborne carcinogens, (*2*) determine which emission sources are the major contributors of carcinogens to ambient air, and (*3*) improve the estimate of comparative human cancer risk from specific pollution emission sources. Meeting these goals required the IACP planners to bring together the multidisciplinary talents of chemists, engineers, and health scientists to conduct a series of coordinated field studies of emission sources and of atmospheric transport and transformation and toxicological studies to address the complex issues involved. This mix of scientific capability was accomplished by combining expertise from the 4 EPA Office of Research and Development laboratories at Research Triangle Park, NC.

Phase 1 (1985) of the sampling program of the IACP studied simplified residential air sheds in 2 neighborhoods heavily impacted by woodsmoke. The objectives of this first phase were to (*1*) identify and quantitate carcinogens in ambient air resulting from residential wood combustion and motor vehicles, (*2*) quantitate the relative contributions of specific emission sources to the mutagenic activity, organic, and fine particulate mass of ambient airborne pollutants, and (*3*) develop and test exposure assessment methodology for selected carcinogens. The diversified goals of this phase 1 study resulted in 25 research projects and collection of about 2000 air samples.

The complexity of coordinating this large sampling effort with the numerous researchers and research tasks involved was recognized during study planning. Sample tracking and coordination was a particular concern because 2 or more laboratories were often expected to perform different functions on the same sample, i.e., extraction, determination of total extractable organic matter, analytical fractionation, characterization, and bioassay. The study planners therefore directed the development of a sample accountability quality assurance (QA) program. Detailed procedures were to be established for storage and transfer of samples and related documentation so that strict integrity of the study would be maintained.

A committee was appointed to establish procedures, develop forms, and designate key personnel to institute a QA program for sample transfer from collection site to storage maintenance, record development, transfer to researchers, and sample tracking at all stages. Quality assurance plans were also adopted for management of the field sampling program but will not be discussed here.

Data management was also of primary concern. Research findings would be forthcoming from the various laboratories with data that would often apply to the same samples or sample sets. A central data management office was therefore established to receive copies of all sample records coming from the field sampling site as well as research results from individual laboratories and researchers.

The purpose of this paper is to relate the QA procedures developed and information gained from this first phase of the IACP and to make recommendations relevant to QA plans for similar field sampling and research programs. The procedures described proved adequate and are recommended for research programs involving a number of research tasks and many field and laboratory personnel. Not all aspects of this QA plan, however, would be appropriate for regulatory investigations. An excellent review of other forms and applications of QA programs may be found in an AOAC publication on quality assurance (Garfield, F. M. [1984] *Quality Assurance Principles for Analytical Laboratories,* AOAC, Arlington, VA).

Sample Identification

The starting point of this sample accountability QA program was the development of a sample description/identification form that would be used at the field sampling sites. An example of the subsequently developed form is shown in Figure 1. The form provides 2 types of sample information. The top portion relates all pertinent sample collection information, and the lower portion is devoted to assignment of an alphanumeric sample identification number.

The sample numbering system was given careful consideration because several hundred samples would be generated in the field, and the same sample might be subject to analytical operations in 2 or more laboratories. The system needed to be simple for field use and flexible enough to reflect subsequent analytical manipulations as later discussed. A sample numbering system was developed which would (*1*) identify the sample program, year, and collection site, (*2*)

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[1] Atmospheric Sciences Research Laboratory, EPA, Research Triangle Park, NC 27711.

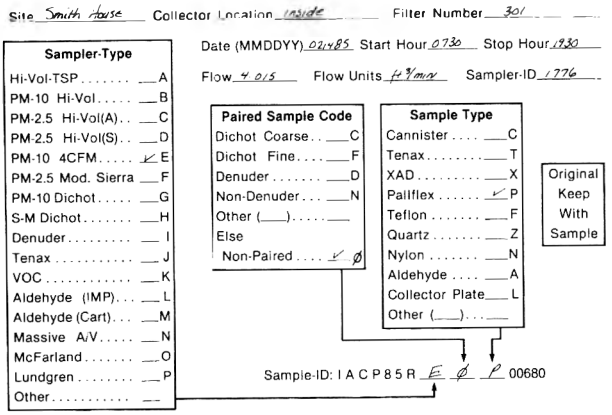

Figure 1. Sample identification form.

relate descriptive information about the sample, and (*3*) contain a distinguishing 5 digit number. The sample form as supplied to the field contained a preassigned sample identification number with the exception of the 3 descriptor spaces as later described, e.g., IACP85R_____00680 (see Figure 1). This simple precaution of supplying forms with serialized identification numbers precluded the possibility of the sampling team issuing duplicate numbers. The IACP first portion of the preprinted identification number represented the Integrated Air Cancer Project and 85R designated the 1985 sampling program in Raleigh, NC. The next 3 letter characters related the sample descriptive information and were to be assigned by sampling team personnel. The first space, selected from the field indicated on the form in Figure 1, identified the sampler type. The second letter entry, selected from the "paired sample code" field, distinguished between 2 samples collected simultaneously by the same sampler, e.g., C and F entries for a dichot sampler coarse and fine filters. The third space entry identified the sample medium used.

Assignment in the Field

Instructions accompanying the forms to the field gave directions for special case situations as follows: (*1*) sample blanks be indicated by writing "blank" on the "other" line in the "sampler-type" field and a "Z" letter code entered which would also become the first letter of the 3 space identification code number; (*2*) samplers not listed in the sampler-type field would be written in on the "other" line and a "Y" letter code also entered which would become the first letter of the 3 space identification code number; and (*3*) sample types not listed would be described on the "other" line of the third field and a "Y" letter code entered in the third space.

A quantity of the preserialized identification forms were prepared as 2 part duplicates and supplied to the sampling site along with detailed instructions for their use. After the form was completed, it was separated at the perforation and one portion was transmitted to the central data management office; the duplicate portion was kept with the sample for subsequent transport and receipt by the laboratory sample custodian.

Laboratory Modifications/Expansions

Instructions for the sample identification system also included special case situations for the analytical laboratory as follows: (*1*) blank samples generated from the laboratory would be accompanied by a new sample identification form with the first of the 3 descriptor letters again designated with a "Z"; and (*2*) samples or extracts that were *composited* in the laboratory would also have a new identification form prepared with a "Z" designation in the middle, or "paired sample code," field. The new identification form would also contain a listing of parent samples used to make up the composite.

Two other analytical operations were considered which affected the sample identification number, namely, the use and transfer of aliquots and the creation of additional subsamples through some analytical fractionation scheme. Since these operations did not change the accuracy of the information associated with the original sample, new identification numbers were not required. Instead, these operations were indicated by suffixes added to the original sample identification number. The suffix rules adopted directed alternate aliquot field, fractionation field, aliquot field, fractionation field, etc. Specific format was as follows.

Aliquot field.—This was denoted by 3 characters, Ann, where "A" is a mnemonic for "Aliquot" and "nn" was a 2 digit number. The bulk sample was assumed to be A00. Even if no aliquots were taken and the whole sample were used for fractionation, the Sample ID suffix would be written ID#-A00-FXnn.

Fractionation field.—This field consisted of 4 characters, FXnn, where "F" represented "Fractionation," "X" was a letter code for the type of fractionation, and "nn" was a 2 digit number. The letter codes for the type of fractionation were: N—normal phase fraction, R—reverse phase fraction, and G—silica gel fraction. The 2 digit number represented the various "cuts" from the fractionation. "Cut" points were to be documented in the laboratory's internal records. The use of the number 00 had a special meaning. If no fractionation was actually performed, but an aliquot was taken from a previous aliquot, then the suffix "FX00" was used as a "place keeper" to separate the aliquot fields. For example, a sample identification number with multiple aliquots and/or fractionations would take the form:

IACP85RXXXnnnnn-Ann-FXnn-Ann-FXnn.

where "X" represents an alphanumeric character and "n" represents an appropriate digit. Note that the aliquot fields and the fractionation fields must alternate.

Sample Accountability

The IACP Steering Committee developed overall goals for the sample accountability program from sampling site storage and maintenance, transport and transfer to the laboratory sample custodian, transfer to individual researchers, and sample tracking/record keeping. Although this first phase of the IACP was not directly related to a regulatory activity, the committee determined that sample custody procedures would be used and that one person should be designated as a sample coordinator. This person was responsible for sample custody and tracking from the field sampling site through all analytical operations. Collected samples were properly labeled and immediately stored at the sampling site under cryogenic conditions (−80°C). Sample transfers from the collection sites were made with samples contained in a cooler packed with dry ice. Sample custody forms and identification forms for all transferred samples were included with samples received by the laboratory sample custodian. Samples were immediately stored in a cryogenic freezer subsequent to distribution to individual researchers.

Sample Custody and Transfer

All sample transfers were recorded on a custody form as shown in Figure 2. A "pedigree" file consisting of at least a

Integrated Air Cancer Project
Sample Custody Form

Sample ID No.					
Description:					
Released by	Received by	Date	Time	Reason for Transfer	Storage and/or Transfer Conditions
Comments: (Parent ID No., etc.)					

Figure 2. Sample custody form.

IACP Sample Operations Record Page_____

Sample ID No.	Sample Description	Amount of Original Sample	Date Received	Received by

Date	Analyst and Organization	Sample Analysis Procedure	Amount of Sample Consumed	Amount of Sample Remaining	Result or Data	Storage Location or Disposition

Figure 3. Sample operations record.

sample identification form and a custody record accompanied each sample during transfer. One copy of the signed custody form was forwarded to the sample custodian, a second copy was retained by the person releasing the sample, and the original form was transferred with the sample to the laboratory analyst.

Sample Operations Record

The "pedigree" file transferred with the sample could also contain a sample operations record which detailed preceding laboratory manipulations or measurements. A copy of a suggested operations record is shown in Figure 3. This particular format was not mandatory because laboratories usually had their own specialized forms which better reflected their particular operations.

Sample Coordination

The sample coordinator was responsible for 3 primary functions: sample custody, tracking all sample operations from collection site through the research tasks, and working with researchers to develop and assemble specific sets of samples required for special research tasks. A sample tracking computer file that used a Lotus software program was developed by the sample custodian. An excerpted portion of the file is shown in Figure 4. This file represents the total inventory of samples available and also relates pertinent sample collection parameters, i.e., sampler flow rate, total collection time, and fine particle mass concentrations for that time period. This added information gave invaluable assistance to analysis planning since samples could be ordered by concentration or amount collected. The file also contained sample custody information with fields for transfer date, received by, and reason for transfer. This portion of the file was periodically updated as custody sheets were received for processing. Fields were chosen to allow maximum flexibility in sorting or ordering samples by various parameters such as the 5 digit portion of the identification number, the 3 letter sample descriptor numerics from the identification number, collection date, individuals releasing or receiving samples, transfer dates, laboratory operations, or sample site. A description or notes field was also included to give added flexibility to sample records.

Additional computer files were also prepared for specific research tasks that required compilation of special sets of samples. For example, a residential sampling portion of the program required development of a file to show matched samples collected inside homes with samples from like collectors immediately outside the home and at the primary collection site located some blocks away. The sample coordinator assembled these samples as laboratory investigational units and coordinated their analyses with the various laboratories involved.

Conclusion and Recommendations

This first phase of the IACP provided valuable experience regarding sample accountability QA for a large field and laboratory research program. The QA procedures and formats described here proved adequate; however, they were not completely instituted until after the field sampling had begun. This initial delay caused some confusion for both field and laboratory personnel during the early part of the program. This phase 1 effort, however, was intended to be a learning experience in combining expertise and coordinating the work of 4 separate laboratories within EPA.

From the experience gained in the first phase of the IACP, some general thoughts and specific recommendations might now be made for future studies. QA considerations for sample handling, coordination, and tracking should be of primary importance to program planners. A sample coordinator should be appointed during preliminary planning. A committee consisting of both field sampling and laboratory personnel should also be established in early phases of planning to ensure development and finalizing of sample accountability procedures far in advance of actual field work. These QA considerations should include sample labeling and storage at field site, development and use of sample identification forms and custody forms for sample transfer, sample transfer conditions, receipt and storage of samples by the laboratory sample custodian, sample coordination and tracking, and data management.

Specific recommendations resulting from this study relate to sample coordination and use of the sample identification form which also contains the vital sampling parameter information. The 2 part duplicate form as used in this study led to a few copying errors. This form should consist of an

Integrated Air Cancer Project—Raleigh, NC, 1985 (IACP85R...)
Sample Inventory and Disposition

Date Coll'd	IACP Code	IACP#	Filter#	AM PM	Site	Flow m^3/min	Total Min	Fine $\mu g/m^3$	T'fer Date	Rec'd by	Reason	Notes
850119	COP	00567	2	PM	PRI	1.088	789.5	78	860400	R. Williams	Extraction	Alaska Biostudy 3
850119	COP	00563	5	PM	PRI	1.357	789.6	78				
850119	COP	00568	6	PM	PRI	1.217	789.5	78	851224	R. Williams	Task 20	Task 20, Follow-up Study
850119	COP	00566	3	PM	PRI	1.322	789.6	78				

Figure 4. Lotus file: Sample inventory and disposition.

original and 2 carbon copies to be completed by field personnel. The original should always be kept with the sample and one copy should be retained by the sample coordinator. The remaining copy would be forwarded to the data management staff. The sample coordinator should develop and continually update a sample inventory/custody record computer file. It is necessary for the sample coordinator to have an active part in planning the sample accountability portion of the QA plan and also be an active participant in specific research tasks requiring development of special sample sets and interlaboratory coordination for the various aspects of sample analysis.

The sample identification number expansion system for identification of analytical fractions was found to be inflexible since only forms of column chromatography were considered. An improved version would account for other fractionation techniques, e.g., liquid/liquid partitioning. An "O" letter for "other" could be used to indicate a fractionation technique other than those specifically mentioned.

Expanding and Tracking the Capabilities of Pesticide Multiresidue Methodology Used in the Food and Drug Administration's Pesticide Monitoring Programs

BERNADETTE M. McMAHON and JERRY A. BURKE
Food and Drug Administration, Division of Contaminants Chemistry, Washington, DC 20204

Foods analyzed for pesticide residues in the monitoring programs of the U.S. Food and Drug Administration (FDA) are most often examined by using one or more of the multiresidue methods developed for this purpose over the years. Because no single method can be used for all potential residues, each commodity is examined by a method or methods which will identify and/or determine the chemicals most likely to have been used. FDA conducts research to develop new multiresidue methods, which are included in monitoring programs as needed to cover additional chemicals. FDA's multiresidue methods have undergone continuous study over a 20 year period to ascertain which compounds can and cannot be recovered by them. FDA continues to perform tests to discover a compound's analytical characteristics. Protocols have been published to direct the testing of additional compounds so that new information can be added to the existing compilations. Methods capable of determining residues of single pesticides are used to analyze selected commodities for residues of high priority that cannot be determined by existing multiresidue methods. Pestrak, a computerized listing of pesticide analytical information, has been developed by FDA to keep track of the capabilities of multiresidue methods and the coverage of residues by the single residue methods used in FDA monitoring.

In the United States, the Environmental Protection Agency (EPA) has responsibility for registering (approving) pesticides for use, under the requirements of the Federal Insecticide, Fungicide and Rodenticide Act. Where uses may lead to residues in food or feed, EPA has the responsibility under the Federal Food, Drug and Cosmetic Act to set tolerances which limit the amount of pesticide residue that may legally be present in food or feed moving in interstate commerce. Enforcement of these tolerances is the responsibility of the U.S. Food and Drug Administration (FDA), except for meat and poultry, for which the U.S. Department of Agriculture is responsible.

Tolerances or exemptions from the requirement for a tolerance are required for the residues of all pesticides which are registered for use on foods or animal feedstuff; tolerances are specific for individual commodities or groups of commodities. Tolerances for residues on foods are currently established for over 300 pesticides in the United States (1). Many uses result in residues which include alteration products that are included in the tolerance description when they are considered to be toxicologically significant.

Because each food or feed item for which a pesticide use is approved has a separate tolerance, the number of legal residue/commodity combinations is in the thousands. (For the remainder of this article the term "food" will include human food as well as feed for food-producing animals.)

Foods containing residues of pesticides at levels higher than the established tolerances are in violation of the law and are subject to regulatory action. The presence of a pesticide residue on a commodity for which no tolerance exists is also in violation. Therefore, FDA must be able to identify and measure residues of pesticides that are not registered in the United States as well as residues on commodities for which no tolerances exist.

In addition to prescribed tolerances, action levels (administratively established regulatory limits) have been established to protect the consumer from unavoidable residues of certain persistent pesticides that may be present in some foods (e.g., fish) as a consequence of their presence in the environment (2). Industrial chemical contamination of food items is also sometimes detected by analytical methods used for the determination of pesticide residues.

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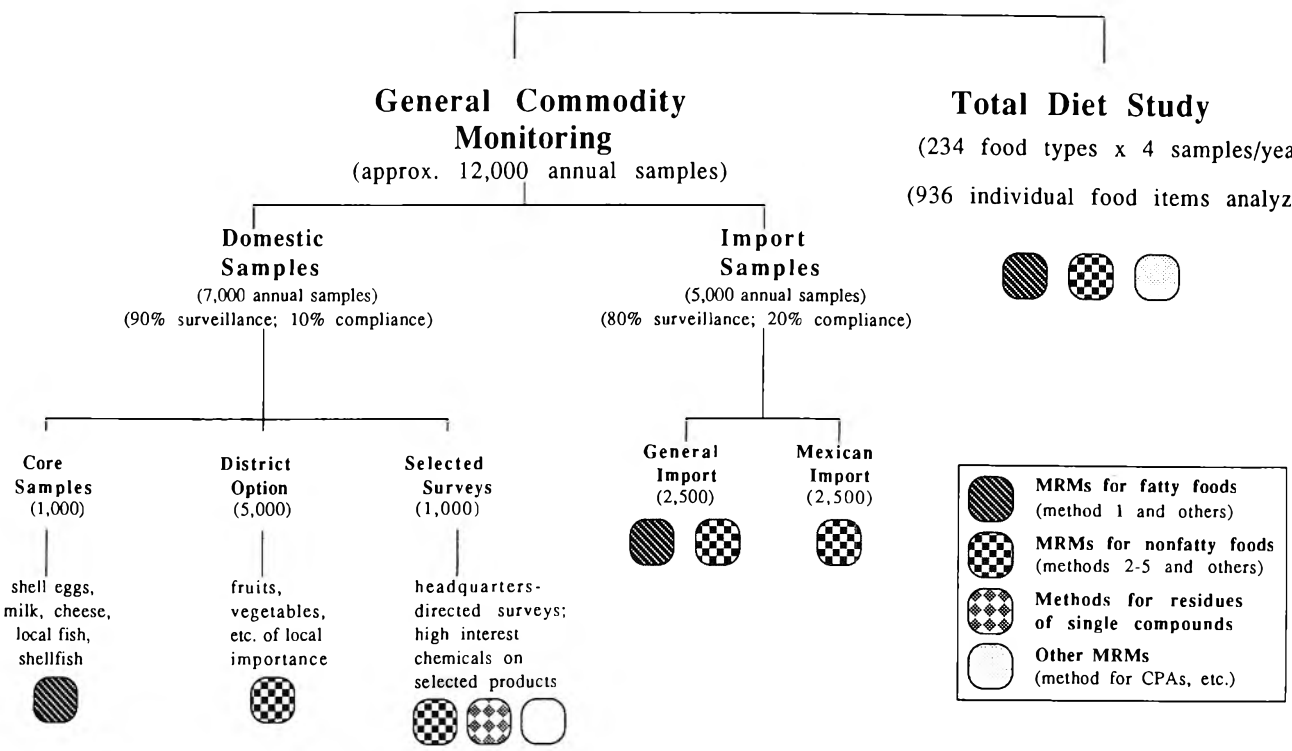

Figure 1. Various types of analytical methods used in FDA monitoring programs.

Note that all numbers of samples are approximate and refer to an average year. Codes for analytical methods refer to those most often used for analysis of the samples in any category. Abbreviations: MRM = multiresidue method; CPA = chlorophenoxy acid. Definitions: compliance samples = samples collected from shipments for which there was prior evidence or suspicion of illegal pesticide residues (i.e., subjective samples); surveillance samples = samples collected from shipments for which there was no prior evidence or suspicion of illegal pesticide residues (i.e., objective samples).

FDA enforces tolerances and action levels for pesticide residues by a program which includes analysis of selected domestic and imported foods. The design and strategy of FDA's pesticide program are described in detail by Reed et al. (3). The monitoring is done with 2 goals in mind: first, to remove from the market those foods containing illegal pesticide residues, according to the definitions provided in the law; and second, to gather information on the incidence and levels of pesticide residues in the food supply.

Two main programs support these goals: (*1*) general commodity monitoring, i.e., regular sampling and analysis of raw agricultural commodities, processed foods, and animal feeds in interstate commerce; and (*2*) the Total Diet Study, which determines the dietary intake of residues in foods as consumed, i.e., washed, cooked, or otherwise prepared for eating.

Most samples analyzed in the FDA monitoring programs are of unknown treatment history, i.e., they are selected randomly, generally without specific knowledge of which pesticides may have been used during their growth or preparation. It is therefore essential to analyze these samples by using one or more multiresidue methods capable of determining residues of many pesticides in a single test portion. These multiresidue methods are described in the FDA *Pesticide Analytical Manual,* Volume I (PAM I), which is compiled and issued by FDA (4, 5).

Other commodities analyzed for pesticide residues are chosen because of some background information on their treatment history. As the situation dictates, these foods may be analyzed by methods capable of measuring the residues of the specific compound known to have been used, if these residues cannot be determined using multiresidue methods.

To ensure that a method is available for the residues of each pesticide for which food tolerances have been established, FDA and EPA cooperate in producing the FDA *Pesticide Analytical Manual,* Volume II (PAM II) (4), which contains such methods. Most PAM II methods were developed by pesticide manufacturers to determine the residues of a single pesticide. They are considered to be the methods of choice for the determination of residues not amenable to quantitation by multiresidue methodology.

The paper by Reed et al. (3) explains how FDA decides to expend the time and resources available to its pesticide programs. That paper discusses the considerations which cause FDA to use multiresidue methods for analysis of most foods, while also directing the use of the generally less efficient single compound methods when the importance of the residue or commodity requires.

Figure 1 displays an overview of the commodity types chosen and the methods used in the FDA monitoring programs in an average year. The categories of products are more fully described by Reed et al. (3).

The present paper is intended to complement that by Reed et al. by describing the application of FDA's multiresidue methods in its pesticide programs. In particular, it describes FDA's continuing effort to increase its analytical coverage of potential pesticide residues in foods. Since 1959, FDA's ability to identify and measure pesticide residues with multiresidue methods has increased from determination of the 13 organochlorine residues by the multiresidue method of Mills (6) to determination of the current 321 total pesticides or pesticide-related compounds by at least one of the 5 multiresidue methods shown in Table 1.

This improvement was brought about by continual testing of the behavior of pesticidal compounds through the methods in use to increase the known scope of these methods and by adding new methods to the program to provide monitoring

Table 1. Information on commonly used FDA pesticide multiresidue methods

Common title	Original ref.	PAM I (ref. 4) sec(s)	AOAC (ref. 7) secs	No. of compounds with AOAC official status	No. of compounds with recovery tested[a]	No. of compounds recovered through method[a,b]
Mills fatty food method	6	211.1, 252	29.001–29.002, 29.005, 29.008–29.010, 29.012, 29.014–29.018, 29.044–29.049	11	200	149
Mills, Onley, & Gaither nonfatty food method	8	212.1, 252	29.001–29.002, 29.005, 29.008–29.011, 29.015, 29.018, 29.044–29.049	20	274	194
Storherr method	9	232.3	29.054–29.058	5	95	94
Luke method	10, 11	232.4, 212.2	29.A01–29.A04 (ref. 12)	8	192	191
Krause method	13	242.2	29.A05–29.A13 (ref. 12)	9	33	33

[a] Data as of December 2, 1986. "Compounds" refers to parent pesticides plus their components, metabolites, impurities, and photoproducts.
[b] Includes recovery through all versions of the methods; includes recoveries of any percentage.

for additional chemical types. (It should be noted that an increase in the number of pesticides that FDA is obliged to monitor has occurred concommitantly.)

This paper also discusses other aspects of the application of multiresidue methods in FDA programs and describes an internal FDA system for tracking the overall status of pesticide program capabilities.

Increasing Knowledge of Current Method Capabilities

At the present time, FDA's 5 most commonly used multiresidue methods are each described in detail in PAM I (4). Each method was published in the scientific literature before inclusion in PAM I, and each has undergone collaborative testing under the auspices of AOAC.

Table 1 lists the 5 methods. References to the original literature, PAM I, and the *Official Methods of Analysis* (7) are included for each. Table 1 also lists the number of compounds for which each method is considered official by AOAC.

Because the most efficient and effective way to analyze samples is to use multiresidue methods, considerable research effort is invested in multiresidue methods beyond their initial development. If a method is found to be useful, additional work is done to apply it to other commodities and other residues. In some cases, alternative steps in the method are needed to extend its applicability (e.g., 11, 14–18).

A large effort is also expended on the testing of many compounds through the steps of the multiresidue methods, so that a database of each method's capabilities can be compiled. Such a compilation is valuable for several reasons. Most important, the information permits evaluation of the agency's monitoring programs in regard to the residues that are covered by the analyses conducted and those that can therefore be inferred to be absent from a particular sample or set of samples.

The analyst examining a sample is also better able to identify residues if information on method capabilities is available. FDA's Division of Contaminants Chemistry in the Center for Food Safety and Applied Nutrition has for many years led the effort to collect, compile, and publish such data so that analytical chemists will be provided with as much assistance as possible in use of the methods.

Table 1 also includes the number of compounds for which method performance data are available for each of the 5 multiresidue methods. The last column in Table 1 lists the number of compounds which are at least partially recovered, since some of the compounds tested have been found *not* to be recovered by the methods. Note that these numbers represent many more compounds than just the parent pesticides. Metabolites, impurities, and photoproducts of pesticides are included, as are individual components of parent pesticides that are made up of several distinct components.

These data have been accumulated by pesticide residue chemists in many FDA laboratories. Chemists in headquarters and in FDA's 16 field laboratories that conduct pesticide residue analyses have contributed to the data. Some of the work was done as a routine adjunct to the monitoring program in each laboratory, whereas other studies were conducted as a part of special research projects. Many of the results are published in FDA's internal Laboratory Information Bulletin series. Others are reported directly to the Division of Contaminants Chemistry, where the data compilations are maintained. Results of some of these studies are published in the scientific literature.

A particularly interesting source of new data is from the identification of residues originally labeled as "unidentified analytical responses" (UARs) by the laboratories which detected them. The increasing use of mass spectrometry has facilitated the identification of residues whose presence was originally detected as peaks in gas or liquid chromatograms (19).

Factors that are used in deciding which of the routinely encountered UARs will be further examined include a particularly large detector response, especially if the detector is one with a high degree of selectivity; repeated occurrence of the peak in chromatograms from analyses of different samples; and the importance of the commodity in which the residue occurred. Once the UAR is chosen for further work, samples in which it occurred are prepared for mass spectral examination.

A number of residues have been identified in this way; results of this type of research are usually published in the scientific literature (e.g., 20–23). If a reference standard is available for the newly identified UAR, further work is done to establish the behavior of the compound through multiresidue methods. If no standard is available, notation is made of the fact that the compound is recovered through the method, but with no known percentage recovery.

FDA field and headquarters laboratories will continue a program of testing additional compounds through the currently used multiresidue methods. The eventual goal is to know the analytical behavior of all pesticidal and industrial chemicals with potential for contaminating food. Compounds whose chemistry suggests the likelihood of recovery through these methods will be tested first. Highest priority is given to chemicals classified as being of particular importance by FDA's Surveillance Index (SI) program (3, 24).

It is hoped that in the future other users of PAM I methods will also contribute method behavior data to the compilation. Protocols have been developed by FDA to describe an efficient means of determining the behavior of compounds tested through the steps of the multiresidue methods. These protocols have recently been published in PAM I (4) as Appendix II. Laboratories using these methods are invited to contribute to FDA data collected according to these protocols, so that the database available to all PAM I users will be increased.

EPA has recently issued a requirement (25) that organizations submitting food tolerance petitions or reregistration data must include with their data the analytical behavior of their compound through FDA multiresidue methods. The protocols in PAM I, Appendix II, are to be used to develop these data. In this way, FDA will eventually be able to describe analytical behavior through these methods for all chemicals with newly established food tolerances.

Compiling the Data

Development of analytical behavior data for many potential residues is of little value unless that information is readily available. Both the FDA staff who evaluate the overall findings of the monitoring program and the field analysts need to be able to consult tables in which the data are compiled.

Most of the data on behavior of compounds through the multiresidue methods, collected over the last 20 years, are published in PAM I in several tabular formats. Tables 201-A, 201-H, 201-I, and 201-J list all the data available on the recovery of compounds through PAM I methods 211.1 and 212.1, 232.3, 232.4, and 242.2, respectively. These data include values for the percent recovered of those compounds that are determined as well as information on compounds known *not* to be recovered through the method. Additional details, such as the eluate in which the compound elutes from a cleanup column, are included.

A computerized listing of analytical method data is also included in PAM I as Appendix I. This table combines most of the data from other PAM I tables. The entry for each chemical includes, where available, relative retention times (RRTs) on each of the 4 gas chromatography (GC) columns; detector responses to the compound for the commonly used GC detectors; and recoveries through 3 of the 5 methods listed in Table 1 of this paper.

The recent revision (Oct. 1987) of these tables included data for a fourth method, the Luke method, PAM I, Sec. 232.4. Since the Krause method, PAM I, Sec. 242.2, relies on liquid chromatography [LC] for determination of most residues, there are no current plans to include data for it in Appendix I.

The individual entries in Appendix I are printed in different orders in 6 tables: alphabetically by name, numerically by standard number (referring to repositories of reference standards maintained by EPA or FDA), and 4 tables in order by the RRTs on each of the 4 GC columns included.

The latter lists are especially useful for the analyst who has used a method, has injected the test solution into a particular GC column, and is making a tentative identification of the peaks in the chromatogram. A typical page from the table showing pesticides ordered by OV-101 RRTs is included as Figure 2.

Using this table, the analyst compares the RRTs of the chromatogram peak(s) to those listed in the table. Certain possible identities (compounds with similar RRTs) can be eliminated or supported, based on the information in the table on known recovery of these compound(s) through the method that was used. Once tentative identification is made, the detector response data in the table provide some indication of the approximate amount of the residue.

The molecular formula for each compound assists in choosing other element-selective detectors for confirmation of the original tentative identification. The RRTs on other GC columns can also be useful for confirmatory analysis. Usually, one of the other columns provides separation of chemicals which elute close to one another on the first column used.

Separate tables of GC data (RRTs and detector responses) were previously published in PAM I, in tables numbered 331-A, etc. These tables are no longer being updated for publication, because all the information in them is presented in a more efficient format in Appendix I. Some of these tables contain RRTs on columns not included in Appendix I. These should be retained for reference in case data for these GC columns are needed.

The tables in the 201 series are updated and the revisions are published in PAM I on an irregular schedule dictated by the amount of new data collected. Appendix I has been revised approximately every 2 years (most recently October 1987). Updated versions of Appendix I are often distributed to FDA laboratories in the interim between their publications in PAM I.

Special effort to share data is made within FDA whenever a UAR is identified. Information on the analytical characteristics of the residue is sent to all field laboratories by electronic mail so that any other FDA laboratory encountering the same residue will be able to recognize it without repeating the laborious identification process. The data are also supplied to the Division of Contaminants Chemistry for inclusion in the next published compilation of data.

Tracking the Methods' Expansion

The efforts to expand the capabilities of FDA multiresidue methods have continued in FDA since the 1960s. During those years when data were increasing, the U.S. Congress, public interest groups, and other organizations evaluating public health made frequent requests for information on FDA's enforcement of pesticide tolerances, in terms of the number of chemicals covered by multiresidue methods in use. Response to each of those requests required a manual count of the number of chemicals in the PAM I tables.

During 1983–1984, FDA created a computerized system to help track the ability of the monitoring programs to determine residues in the food supply. The system, named Pestrak, contains separate databases for pesticides and their metabolites, divided according to the tolerance status of the pesticides.

Thus, Pestrak includes 5 types of lists for pesticides, their metabolites, and other related compounds: (*1*) with food tolerances listed in 40 CFR 180; (*2*) with interim tolerances (40 CFR 180.319); (*3*) with pending tolerances; (*4*) with temporary tolerances and action levels; and (*5*) with no tolerances. The last list contains certain selected pesticides considered to be of current or potential FDA interest because of rescinded tolerances, known usage in foreign countries, or other reasons.

Complete lists of the Pestrak databases can be printed for use as reference. A typical page from the first list of Pestrak is displayed as Figure 3.

Included for each entry in the Pestrak lists are codes and other information used by FDA in planning monitoring programs and in reporting results. Information on the ability of the 5 multiresidue methods to determine each compound is included, as is reference to special surveys which have in-

PESTICIDES BY OV-101 RRT (CHLORPYRIFOS) 23:29 MONDAY, MARCH 18, 1985

RRT/C OV-101	RRT/C OV-225	RRT/C OV-17	RRT/C DEGS	NI63-ECD SENS	H3-ECD SENS	TID SENS	PREFERRED NAME	MOLECULAR FORMULA	FAT METHOD	NONFAT METHOD	MIXED ETHER	CH2CL2	CARBON METHOD	STD NO
1.14	1.03	1.14	.97	8	150.	4.	PIRIMIPHOS-ETHYL	C13H24N3O3PS	C	-	15	-	C	E5642
1.15	4.7	-	-	6.	6.	-	CYPROMID	C10H9CL2NO	-	-	NR	2	-	X 948
1.17	-	1.01	-	8	1.	-	ISODRIN	C12H8CL6	C	C	6	1	-	E4045
1.17	1.58	-	-	-	3.5	4.	CHLORFENVINPHOS, ALPHA	C12H14CL3O4P	NR	-	-	-	-	F 458
1.17	-	-	-	-	200.	-	METHYL 4-CHLOROINDOLYL-3-ACETATE (PEA GRWTH HORMN)	C11H10CLNO2	-	P	50	NR	-	X 942
1.19	1.15	1.20	-	8	12.	-	TDE, O, P'- OLEFIN	C14H9CL3	-	-	-	-	-	F 207
1.20	-	1.85	-	2.	1.5	-	CAPTAN	C9H8CL3NO2S	-	P	50	3	-	E1020
1.21	-	-	-	-	-	150.	THIABENDAZOLE	C10H7N3S	-	NR	-	-	-	E6660
1.21	2.73	-	6.5	-	-	2.	DES N-ISOPROPYL ISOFENPHOS	C12H18NO4PS	-	P	50	-	-	X 972
1.23	-	-	-	-	3.	-	FOLPET (EV)	C9H4CL3O2NS	P	C	1550	23	C	E3660
1.24	-	1.47	-	7.	7.	-	ANILAZINE (RV)	C9H5CL3N4	P	C	15	2	-	E2920
1.25	-	1.41	-	3.3	-	70.	TOLYLFLUANID	C10H13CLFNOS	-	-	-	-	-	E6700
1.25	-	1.67	2.92	8	5.	3.	PHENTHOATE	C12H17O4PS2	-	C	1550	-	C	F 530
1.26	3.5	-	-	-	4.	-	SULPHENONE	C12H9CL2OS	-	-	2025	3	-	F 134
1.26	1.93	-	-	-	2.5	4.	CHLORFENVINPHOS, BETA	C12H14CL3O4P	-	-	-	-	-	F 459
1.29	1.93	1.50	-	-	-	6.	CHLORFENVINPHOS*	C12H14CL3O4P	-	NR	NR	NR	C	E1300
1.29	1.22	1.15	-	1.	1.	-	HEPTACHLOR EPOXIDE	C10H5CL7O	C	C	6	2	-	E3880
1.33	.94	1.05	-	1.	1.	-	OCTACHLOR EPOXIDE	C10H4CL8O	C	C	6	1	-	E5200
1.36	1.22	-	-	8.	-	-	ALLETHRIN	C19H26O3	C	-	50	-	-	E 100
1.36	1.73	-	2.12	20.	-	2.	ISOFENPHOS	C15H24NO4PS	-	C	1550	-	-	X 971
1.37	2.85	1.74	5.6	-	-	11.	CROTOXYPHOS	C14H19O6P	-	NR	-	-	C	E1500
1.37	-	-	-	12.	-	-	PROCYMIDONE	C13H11Cl2NO2	P	C	15	-	-	E5741
1.39	1.62	1.54	-	8	2.	-	CHLORBENSIDE (ERV)	C13H10CL2S	P	P	6	1	-	F 19
1.43	3.33	2.11	-	10.	-	7.	METHIDATHION	C6H11N2O4PS3	P	P	50	3	C	E6340
1.43	-	-	-	-	2.	-	PHOTODIELDRIN B	C13H9CL5O	-	-	-	-	-	X 914
1.44	-	-	-	-	60.	-	NITROFEN, AMINO	C12H9CL2NO	-	-	-	-	-	X 921
1.45	1.36	-	-	-	7.	-	TDE, P, P'-, OLEFIN	C14H9CL3	C	C	6	1	-	E1800
1.49	1.46	1.34	-	1.	1.	-	CHLORDANE, TRANS	C10H6CL8	C	C	6	1	-	E1240
1.50	3.00	-	-	-	2.	-	GENITE 923	C12H8CL2O3S	C	C	15	-	-	F 186
1.50	6.7	2.39	-	15.	-	10.	DISULFOTON SULFONE	C8H19O4PS3	-	NR	-	-	C	F 616
1.51	1.42	-	1.33	3.	-	4.	BROMOPHOS-ETHYL	C10H12BRCL2O3PS	P	C	6	-	-	E 860
1.53	.95	-	-	9.	40.	-	PERTHANE OLEFIN	C18H19CL	C	C	6	1	-	F 493
1.54	3.6	-	-	-	20.	-	2,4-D PROPYLENE GLYCOL * BUTYL ETHER ESTER	C15H20CL2O4	-	-	-	-	-	X 908
1.55	1.28	1.51	-	1.5	2.	-	DDE, O, P'-	C14H8CL4	C	C	6	1	-	E1840
1.55	3.8	2.37	-	-	10.	21.	METHYL TRITHION OXYGEN ANALOG	C9H12CLO3PS2	-	-	-	-	-	F 448
1.57	-	-	-	-	4000.	-	TEPP* (RV)	C8H20O7P2	-	-	-	-	P	F 168
1.58	2.72	1.97	4.4	5.	3.	8.	GARDONA	C10H9CL4O4P	-	NR	-	NR	C	E3740
1.58	-	-	-	-	-	400.	PROMECARB	C12H17NO2	-	-	-	-	-	E5752
1.60	2.04	1.84	-	8	180.	-	DDA, P, P'-, METHYL ESTER	C15H12CL2O2	-	-	-	-	-	F 294
1.61	-	-	-	-	1.5	-	ENDOSULFAN*	C9H6CL6O3S	-	-	-	-	-	E3180
1.61	3.41	2.20	-	-	3.	-	OVEX	C12H8CL2O3S	C	C	15	2	-	X 906
1.63	1.38	1.47	-	1.5	1.5	-	ENDOSULFAN I	C9H6CL6O3S	C	C	15	2	-	E3200
1.66	1.54	1.48	-	1.	1.	-	CHLORDANE, CIS	C10H6CL8	C	C	6	1	-	E1220
1.66	3.7	2.41	-	-	-	7.	FENAMIPHOS (OV-17 GCV)	C13H22NO3PS	-	-	NR	NR	C	E3470
1.66	1.18	-	-	-	60.	-	2,4-D BEP ESTER*	C17H24CL2O4	-	-	-	-	-	F 225
1.75	.95	1.42	-	1.5	2.	-	NONACHLOR, TRANS	C10H5CL9	C	C	6	1	-	E5080

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Figure 2. Sample page from PAM I Appendix I table showing pesticides in order by relative retention time (RRT) on an OV-101 column.

volved analysis for particular residues by other methods. Inclusion of the latter allows users of Pestrak to measure more easily the overall scope of the monitoring program.

Computer programs have been written for searching the Pestrak lists according to many different criteria. Such searches provide FDA personnel with the following capabilities:

(*1*) Information is readily available to evaluate the findings of the monitoring programs. The Pestrak tables include general information on whether or not a particular pesticide or related compound is being analyzed for in the current programs. Use of these tables provides a reasonably complete search for pesticides likely to be used in the United States on foods, since the tables contain all pesticides with tolerances, at all stages of the tolerance-setting process.

(*2*) Information is readily available to provide answers to questions concerning the capabilities of the current monitoring programs. Examples of the responses to such searches are shown in Figures 4 and 5. The system allows the user at a terminal to determine the number of chemicals which meet particular specified criteria; alternatively, printouts containing any or all of the information for each chemical can be obtained (Figures 4 and 5).

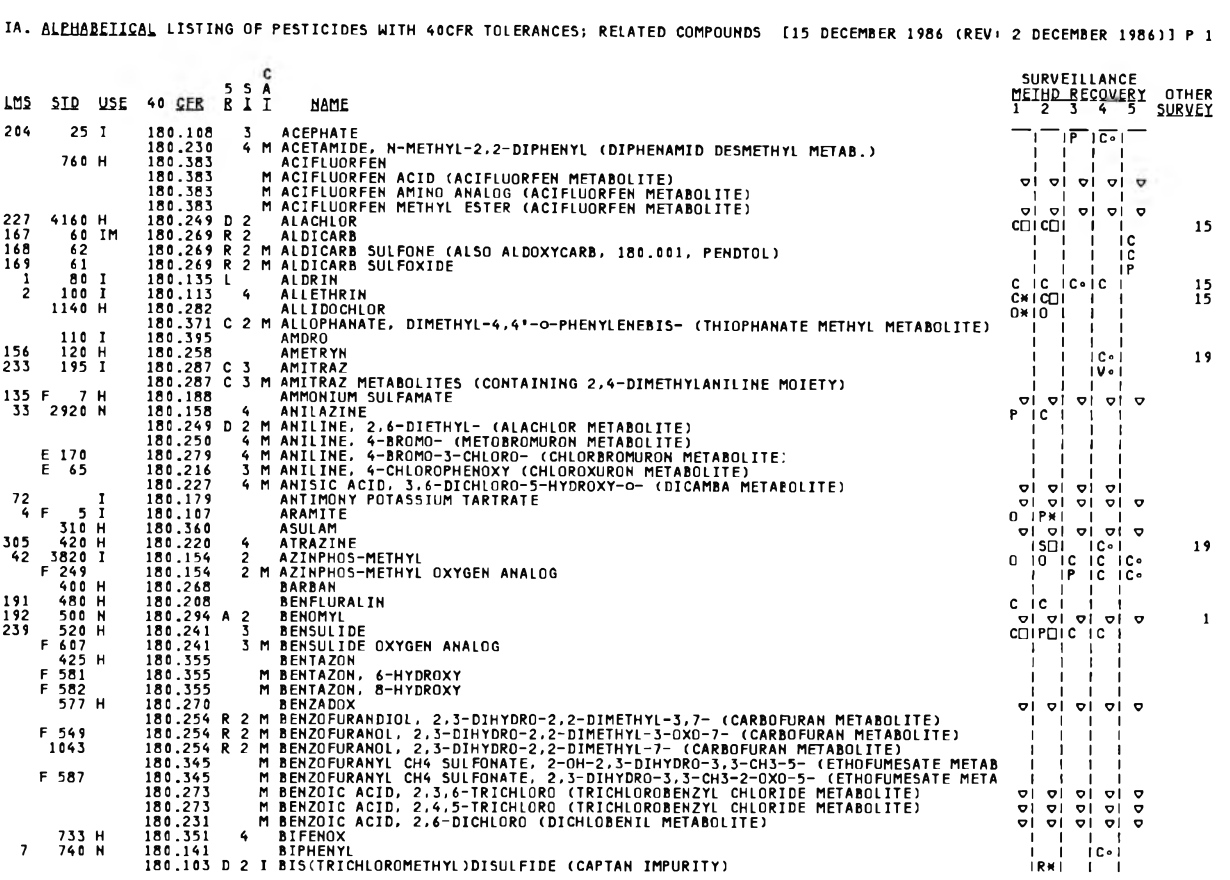
(*3*) Pestrak can be used to help plan future work on expanding the method behavior data for FDA's multiresidue methods. Gaps in the data are readily identified in these listings, and the classifications assigned to chemicals that have undergone FDA's SI process are included among the data in Pestrak. The SI classification indicates the relative importance, based on potential hazard, given to the need for including the particular chemical in the monitoring programs. The Pestrak search programs permit the data to be sorted by SI class and therefore can produce lists which include a system of assessing priority. Figure 6 is an example of the results of such a search and sort.

(*4*) Pestrak is used internally in FDA to provide the agency with a means of tracking work in progress on the continuing expansion of the method behavior data. Notations are made of which laboratory has agreed to test a compound for its analytical behavior. Compounds recommended for testing through one or more of the 5 usual multiresidue methods, through other multiresidue methods, or through a single compound method are also noted.

Adding Other Multiresidue Methods

Increasing the data for the recovery of chemicals through existing methods is only one aspect of the effort to extend coverage to as many chemicals as possible in FDA programs. At least as important is the development of other multiresidue methods capable of determining residues not amenable to determination by existing methods. The 5 methods currently used most often by FDA were themselves developed and added to the programs as the need for them arose.

It is expected that the need to develop additional multiresidue methods will continue as pesticide manufacturers develop products of new chemical types, and as older pesticides are found to require closer surveillance. The chlorophenoxy acid (CPA) herbicides and ethylene bisdithiocarbamate fungicides are good examples of the latter.



IA. ALPHABETICAL LISTING OF PESTICIDES WITH 40CFR TOLERANCES; RELATED COMPOUNDS [15 DECEMBER 1986 (REV: 2 DECEMBER 1986)] P 1

LMS	SID	USE	40 CFR	S R	S I	C A I	NAME	SURVEILLANCE METHD 1	RECOVERY 2	3	4	5	OTHER SURVEY
204	25	I	180.108			3	ACEPHATE			P	C∘		
			180.230			4 M	ACETAMIDE, N-METHYL-2,2-DIPHENYL (DIPHENAMID DESMETHYL METAB.)						
	760	H	180.383				ACIFLUORFEN						
			180.383			M	ACIFLUORFEN ACID (ACIFLUORFEN METABOLITE)	▽	▽	▽	▽	▽	
			180.383			M	ACIFLUORFEN AMINO ANALOG (ACIFLUORFEN METABOLITE)						
			180.383			M	ACIFLUORFEN METHYL ESTER (ACIFLUORFEN METABOLITE)	▽	▽	▽	▽	▽	
227	4160	H	180.249	D	2		ALACHLOR	C□	C□				15
167	60	IM	180.269	R	2		ALDICARB					C	
168	62		180.269	R	2	M	ALDICARB SULFONE (ALSO ALDOXYCARB, 180.001, PENDTOL)					C	
169	61		180.269	R	2	M	ALDICARB SULFOXIDE					P	
1	80	I	180.135	L			ALDRIN	C	C	C∘	C		15
2	100	I	180.113		4		ALLETHRIN	C✱	C□				15
	1140	H	180.282				ALLIDOCHLOR	O✱	O				
			180.371	C	2	M	ALLOPHANATE, DIMETHYL-4,4'-O-PHENYLENEBIS- (THIOPHANATE METHYL METABOLITE)						
	110	I	180.395				AMDRO						
156	120	H	180.258				AMETRYN				C∘		19
233	195	I	180.287	C	3		AMITRAZ				V∘		
			180.287	C	3	M	AMITRAZ METABOLITES (CONTAINING 2,4-DIMETHYLANILINE MOIETY)						
135	7	H	180.188				AMMONIUM SULFAMATE	▽	▽	▽	▽	▽	
33	2920	N	180.158		4		ANILAZINE	P	C				
			180.249	D	2	M	ANILINE, 2,6-DIETHYL- (ALACHLOR METABOLITE)						
			180.250		4	M	ANILINE, 4-BROMO- (METOBROMURON METABOLITE)						
	E 170		180.279		4	M	ANILINE, 4-BROMO-3-CHLORO- (CHLORBROMURON METABOLITE)						
	E 65		180.216		3	M	ANILINE, 4-CHLOROPHENOXY (CHLOROXURON METABOLITE)						
			180.227		4	M	ANISIC ACID, 3,6-DICHLORO-5-HYDROXY-O- (DICAMBA METABOLITE)	▽	▽	▽	▽		
72		I	180.179				ANTIMONY POTASSIUM TARTRATE	▽	▽	▽	▽	▽	
4	F 5	I	180.107				ARAMITE	O	P✱				
	310	H	180.360				ASULAM	▽	▽	▽	▽	▽	
305	420	H	180.220		4		ATRAZINE		S□		C∘		19
42	3820	I	180.154		2		AZINPHOS-METHYL	O	O	C	C	C∘	
	F 249		180.154		2	M	AZINPHOS-METHYL OXYGEN ANALOG			P	C	C∘	
	400	H	180.268				BARBAN						
191	480	H	180.208				BENFLURALIN	C	C				
192	500	N	180.294	A	2		BENOMYL	▽	▽	▽	▽	▽	1
239	520	H	180.241		3		BENSULIDE	C□	P□	C	C		
	F 607		180.241		3	M	BENSULIDE OXYGEN ANALOG						
	425	H	180.355				BENTAZON						
	F 581		180.355			M	BENTAZON, 6-HYDROXY						
	F 582		180.355			M	BENTAZON, 8-HYDROXY						
	577	H	180.270				BENZADOX	▽	▽	▽	▽	▽	
			180.254	R	2	M	BENZOFURANDIOL, 2,3-DIHYDRO-2,2-DIMETHYL-3,7- (CARBOFURAN METABOLITE)						
	F 549		180.254	R	2	M	BENZOFURANOL, 2,3-DIHYDRO-2,2-DIMETHYL-3-OXO-7- (CARBOFURAN METABOLITE)						
	1043		180.254	R	2	M	BENZOFURANOL, 2,3-DIHYDRO-2,2-DIMETHYL-7- (CARBOFURAN METABOLITE)						
			180.345			M	BENZOFURANYL CH4 SULFONATE, 2-OH-2,3-DIHYDRO-3,3-CH3-5- (ETHOFUMESATE METAB						
	F 587		180.345			M	BENZOFURANYL CH4 SULFONATE, 2,3-DIHYDRO-3,3-CH3-2-OXO-5- (ETHOFUMESATE META						
			180.273			M	BENZOIC ACID, 2,3,6-TRICHLORO (TRICHLOROBENZYL CHLORIDE METABOLITE)	▽	▽	▽	▽	▽	
			180.273			M	BENZOIC ACID, 2,4,5-TRICHLORO (TRICHLOROBENZYL CHLORIDE METABOLITE)	▽	▽	▽	▽	▽	
			180.231			M	BENZOIC ACID, 2,6-DICHLORO (DICHLOBENIL METABOLITE)	▽	▽	▽	▽	▽	
	733	H	180.351		4		BIFENOX						
7	740	N	180.141				BIPHENYL				C∘		
			180.103	D	2	I	BIS(TRICHLOROMETHYL)DISULFIDE (CAPTAN IMPURITY)	R✱					

Figure 3. Sample page from Pestrak alphabetical list of chemicals with tolerances on foods.

Current research efforts involve the consideration of compounds, grouped according to their chemistry, to determine those which might be measured by a single multiresidue method. Modern instrumental techniques for compound separation and measurement are being explored for potential use in the field of residue analysis. Of particular interest are various versatile LC techniques, including those using photoconductivity and electrochemical detectors.

Groups of pesticides for which new or improved multiresidue methods are in use or under development include the CPAs and certain pesticides and metabolites containing the urea moiety. Methodology for CPA residues has recently been included in PAM I (4) as Sec. 221.2. The method includes extraction from an acidified sample, cleanup by gel permeation chromatography, methylation with diazomethane, and determination by electron-capture (EC) GC. The method is capable of determining 6 CPAs and pentachlorophenol. Other acids are being tested for recovery. Improvements in this method have recently been developed. Ion-pair alkylation can now replace the potentially hazardous diazomethane methylation step in this procedure (26).

A method for residues containing the phenylurea moiety is also under development (27, 28). When available, this method will permit analysis for residues of at least 6 parent compounds with current U.S. food tolerances, as well as other similar compounds used elsewhere. The potential also exists for expansion of this method to other compounds whose structure will permit quantitation by the determinative step devised for the phenylureas (27).

Other groups of chemicals which seem amenable to multiresidue methodology are the organotin compounds and the synthetic pyrethroids. These compounds are also of particular interest to FDA in its goal of regular monitoring for important pesticide residues. Long-term activities will certainly include the testing of those pesticides likely to be determined through these methods once basic methods are established.

Multiresidue Methods in FDA Monitoring Programs

Usage

FDA's multiresidue methods form the backbone of the agency's monitoring programs. Detailed instructions for their application are published in PAM I. FDA laboratories may choose from among these methods, or from other variations if needed, to analyze the samples collected as part of the monitoring program (3). Certain modifications to these methods have been developed and applied in field laboratories over the years. For example, certain residues, such as that of methamidophos, are known to be only partially recovered through the Luke procedure, PAM I, Sec. 232.4. A triple extraction must be used to achieve quantitative recovery of the compounds from the matrix (29). Such a modification is used when there is evidence or suspicion that the test sample contains such a residue.

There are also other cases in which a multiresidue method can be used to identify a residue but not to measure it quantitatively. Once the original analysis indicates the presence of such a residue, however, an additional analysis of the sample can be conducted with a method that is known to produce a quantitative result for that residue in that commodity. The example of methamidophos given above is one approach to this follow-up analysis.

The monitoring program directions, issued annually to field laboratories, include a number of specific cases for which such additional analyses are required. For example, terbufos,

PESTRAK INFORMATION ON CHEMICALS REQUESTED 15 DECEMBER 1986 PAGE 1
(PESTRAK LAST UPDATED 2 DECEMBER 1986)
FOLLOWING LIST(S) SEARCHED:TOLERANCES (624 TOTAL CHEMICALS)

LISTED CHEMICALS MEET ALL THE FOLLOWING CRITERIA (NUMBER OF CHEMICALS MEETING THIS AND EACH PRECEDING CRITERIA):
CATEGORY OF CHEMICAL: PARENT COMPOUND (312 CHEMICALS)
RECOVERY THROUGH PAM I 242.2; HPLC+DERIV.+FLUORES. DETN: C, P (20 CHEMICALS)

THIS LIST INCLUDES 20 ENTRIES

THE CFR NUMBERS USED IN THIS LIST HAVE THE FOLLOWING MEANINGS:

180.102-180.XXX(EXCEPT 180.319): SECTION OF 40CFR IN WHICH THE TOLERANCES ARE LISTED.
180.319: INTERIM TOLERANCES LISTED IN A SPECIAL SECTION SET ASIDE FOR THAT PURPOSE.
180.001: PENDING TOLERANCES; ARBITRARY NUMBER CHOSEN.
180.002: TEMPORARY TOLERANCES; ARBITRARY NUMBER CHOSEN.
180.000: NO TOLERANCES; ARBITRARY NUMBER CHOSEN.

SID	USE	40 CFR	S R	S I	C A I	NAME	SURVEILLANCE METHD RECOVERY 1	2	3	4	5	OTHER SURVEY
60	IM	180.269	R		2	ALDICARB					C	
3820	I	180.154			2	AZINPHOS-METHYL	O	O	C	C	C•	
960	I	180.255			4	BUFENCARB					C	
1060	I	180.169	C		3	CARBARYL			C•	C•	C	
1040	I	180.254	R		2	CARBOFURAN				C•	C	
2080	I	180.153	R			DIAZINON	C	C	C	C	C•	15
2420	I	180.204	C		3	DIMETHOATE		O	C	C•	C•	
4260	I	180.111				MALATHION	C	C	C	C	C•	
4500	I	180.320			3	METHIOCARB				C•	C	15
4520	I	180.253			2	METHOMYL				C•	C	
4640	I	180.157			3	MEVINPHOS		O✱	C	C•	C•	
2010	H	180.328				NAPROPAMIDE				C•	C•	
5186	I	180.303			3	OXAMYL					C	
5245	I	180.121				PARATHION	C	C	C	C	C•	15
4580	I	180.121				PARATHION-METHYL	C	C	C	C	C•	15
5520	I	180.263				PHOSALONE	C	C	C	C	C•	
5620	I	180.127	C		4	PIPERONYL BUTOXIDE					C•	
4880	R	180.309				NAPHTHALENEACETAMIDE					IP•	
5642	I	180.308			4	PIRIMIPHOS-ETHYL	C✱	I	C	C	IP•	
F 681	I	180.407				THIODICARB					IP	

Figure 4. Example of printout resulting from search of Pestrak database.

Only the list of chemicals with 40 CFR 180 tolerances was searched; selected chemicals are parent compounds which are completely (C) or partially (P) recovered through the Krause method (method 5).

an organophosphorus pesticide, produces metabolites which are included in the tolerance and are expected to constitute major portions of the total residue, but are not amenable to determination by multiresidue methodology. Field analysts are directed to reanalyze samples found to contain the parent terbufos if the level exceeds 30% of the established tolerance. Reanalysis in this case is to done by the PAM II method, which converts the total residue to one compound for identification, quantitation, and comparison to the tolerance value.

Reporting Results

As a part of FDA's pesticide programs, residue results are reported by each laboratory into a computerized system,

PESTRAK INFORMATION ON CHEMICALS REQUESTED 15 DECEMBER 1986 PAGE 1
(PESTRAK LAST UPDATED 2 DECEMBER 1986)
SEARCH PERFORMED ON ALL LISTS OF CHEMICALS (840 TOTAL CHEMICALS)

LISTED CHEMICALS MEET ALL THE FOLLOWING CRITERIA (NUMBER OF CHEMICALS MEETING THIS AND EACH PRECEDING CRITERIA):
CATEGORY OF CHEMICAL: METABOLITE (273 CHEMICALS)
RECOVERY THROUGH AT LEAST ONE SURVEILLANCE METHOD (68 CHEMICALS)
SI CLASS: 1, 2, 3 (37 CHEMICALS)

THIS LIST INCLUDES 37 ENTRIES

S I	NAME	SURVEILLANCE METHD RECOVERY 1	2	3	4	5	OTHER SURVEY
2	ALDICARB SULFONE (ALSO ALDOXYCARB, 180.001, PENDTOL)					C	
2	ALDICARB SULFOXIDE					P	
2	AZINPHOS-METHYL OXYGEN ANALOG			P	C	C•	
2	DISULFOTON OXYGEN ANALOG (ALSO DEMETON-S, 180.105)		O✱	C	C		7
2	DISULFOTON SULFONE		O✱	C	C		7
2	ENDRIN ALCOHOL	C□	P□				
2	ENDRIN ALDEHYDE	C✱	P✱				
2	ENDRIN KETONE	C	C				
2	ETHYLENE THIOUREA (METABOLITE OF ZINEB AND OTHER ETHYLENEBIS DITHIOCARBAMATES)			V•			10
2	PENTACHLOROANILINE (QUINTOZENE METABOLITE)	C	C				
2	PENTACHLOROBENZENE (QUINTOZENE METABOLITE)	C✱	C✱				
2	PENTACHLOROPHENYL METHYL ETHER (QUINTOZENE METABOLITE)	C	C				
2	PENTACHLOROPHENYL METHYL SULFIDE (QUINTOZENE METABOLITE)	C	C				
2	PHORATE OXYGEN ANALOG			C		C•	6
2	PHORATE SULFONE			C	C•		6
2	PHORATE SULFOXIDE				C•		6
2	3-HYDROXYCARBOFURAN (CARBOFURAN METABOLITE)					C	
2	4-CYCLOHEXENE-1,2-DICARBOXIMIDE, CIS- (CAPTAN HYDROLYSIS PRODUCT)	O✱	O✱		C		
3	CARBOPHENOTHION OXYGEN ANALOG		O	C			
3	CARBOPHENOTHION SULFONE			C	C		
3	CHLORPYRIFOS OXYGEN ANALOG		O✱	C			
3	DEMETON-S SULFONE			C	C•		
3	DES N-ISOPROPYL ISOFENPHOS (ISOFENPHOS METABOLITE)		S✱		C		
3	DIMETHOATE OXYGEN ANALOG (ALSO SEE TABLE V, NO TOLERANCES, OMETHOATE)	O✱	O	C	C•		
3	FENSULFOTHION OXYGEN ANALOG		O✱	C			
3	FENSULFOTHION SULFONE		O✱	C			
3	FENTHION OXYGEN ANALOG	O	O		C		
3	FENTHION OXYGEN ANALOG SULFOXIDE	O	O		C		
3	FENTHION SULFONE	O	O		C		
3	IPRODIONE ISOMERIC METAB. [3-(1-ME,ET)-N-(3,5-CL2PHENYL)-2,4-DIOXO-1-IMIDAZOLIDINECARBOXIMIDE]			C			15
3	METHAMIDOPHOS (ACEPHATE METABOLITE)			P	C•		
3	METHIOCARB SULFOXIDE			S•	C		15
3	OXYDEMETON-METHYL SULFONE			C•			
3	2,3,5,6-TETRACHLOROANISIDINE (TECNAZENE METABOLITE)	R					
3	2,3,5,6-TETRACHLORONITROANISOLE (TECNAZENE METABOLITE)	R					
3	3-(3,4-DICHLOROPHENYL)-1-METHOXYUREA (DIURON AND LINURON METABOLITE)	O✱	O✱	R			
3	3,4-DICHLOROANILINE (DIURON AND LINURON METABOLITE)		O✱	V			

Figure 5. Example of printout resulting from search of Pestrak database.

All 5 lists in Pestrak were searched; selected chemicals are the metabolites recovered through at least 1 of the 5 common multiresidue methods and classified as 1, 2, or 3 in the Surveillance Index project (24).

PESTRAK INFORMATION ON CHEMICALS REQUESTED 15 DECEMBER 1986 PAGE 1
(PESTRAK LAST UPDATED 2 DECEMBER 1986)
FOLLOWING LIST(S) SEARCHED:TOLERANCES (624 TOTAL CHEMICALS)

LISTED CHEMICALS MEET ALL THE FOLLOWING CRITERIA (NUMBER OF CHEMICALS MEETING THIS AND EACH PRECEDING CRITERIA):
CATEGORY OF CHEMICAL: PARENT COMPOUND (312 CHEMICALS)
ASSIGNMENT TO: **1 (34 CHEMICALS)
SI CLASS: 1, 2, 3, 4, 5, (34 CHEMICALS)

THIS LIST INCLUDES 34 ENTRIES

S I	SID	NAME	SURVEILLANCE METHD RECOVERY 1	2	3	4	5	ASN
3	F 715	FLUCYTHRINATE				C		**1
3	4050	ISOFENPHOS		C*		C		**1
4	2920	ANILAZINE	P	C				**1
4	940	BUTYLATE						**1
4	1100	CARBOXIN	O*	O*				**1
4	1491	CHLORTHIOPHOS	C□	C□	C	C		**1
4	1552	CYANAZINE		O*		P∘		**1
4	1591	CYCLOATE				C∘		**1
4	1600	CYCLOHEXIMIDE						**1
4	2006	DESMEDIPHAM						**1
4	2560	DINOCAP	P*	P*		C∘		**1
4	3620	FLUOMETURON						**1
4	3940	HEXACHLOROPHENE	O*	O*				**1
4	7001	HEXAZINONE						**1
4	4620	METOLACHLOR		P*				**1
4	5148	ORYZALIN						**1
4	5300	PEBULATE						**1
4	5742	PROFENOFOS	P□	P□	C	C		**1
4	5940	PYRETHRINS	C*	C*				**1
4	F 440	TCMTB						**1
5	6630	TETRASUL	C	C				**1
	2902	CHLORPYRIFOS-METHYL			C			**1
	F 740	CHLORSULFURON						**1
	1615	CYPRAZINE						**1
	F 669	DIMETHIPIN						**1
	2653	DIPROPETRYN						**1
	F 754	FLUVALINATE						**1
	4446	MEFLUIDIDE						**1
	5410	PHENMEDIPHAM						**1
	5925	PYRAZON						**1
	6068	SETHOXYDIM				C		**1
	6408	TEBUTHIURON						**1
	3980	TERBUTRYN						**1
	6661	THIDIAZURON						**1

Figure 6. Example of printout resulting from search of Pestrak database.

Only the list of chemicals with tolerances was searched; selected chemicals are parent compounds which have been designated as worth testing further through multiresidue methods ("Assignment to **1"); these have been grouped by Surveillance Index classification, but no compounds classified as 1 or 2 occur in this list.

along with codes which refer to the method(s) used. Separate codes are used for the extraction-cleanup portion of the analytical method and for the determinative step.

The complete set of data from all laboratories for the monitoring samples is then examined and reported. Separate reports are prepared to describe the general commodity monitoring program (e.g., ref. 30, which describes results from several years of monitoring) and the Total Diet Study (31, 32). Descriptions of the numbers and amounts of pesticide residues found in the U.S. food supply are derived from these data and included in the reports.

A complete listing of residues which were *not* found, but which could have been found had they been present, is much more difficult to prepare. This kind of report is usually not included in descriptions of the results of the overall programs, but is done when there is particular interest in the residues of a specific pesticide.

Tables of method capabilities, such as PAM I Tables 201 and Appendix I, can be used to determine which compounds were not present in any given sample. Examining residue data in this way is very time consuming, however. Each combination of extraction-cleanup code and determinative step code is capable of determining different residues. The nature of the commodity analyzed can also affect the method's ability to determine particular residues. The Pestrak system does not contain sufficiently detailed method capability data to be useful in this type of data examination.

Total Diet Study Application of Methods

Methods used to analyze the samples collected and prepared for the Total Diet Study are somewhat different from those used in the general commodity monitoring program. The Total Diet Study currently uses all 5 methods in Table 1, but some of these are adjusted to permit the identification and measurement of residues at a lower limit of quantitation than that used in commodity monitoring. In some cases, additional cleanup of the sample is required to achieve these lower limits, which are generally about one-fifth of the quantitation limits in the commodity monitoring. The PAM I, Sec. 221.1, method for CPAs is also included in the Total Diet analyses.

(Total Diet samples are examined for a number of toxicologically and nutritionally important elements and radionuclides, in addition to residues of pesticides and industrial chemicals.)

Quality Assurance

Both intra- and interlaboratory quality assurance (QA) programs are maintained by FDA. Results from the early years of these studies (33) support the adequacy of the methods used as well as the performance of the laboratories. These quality assurance measures have continued in the years since that report was published. If a QA program uncovers problems with a laboratory's application of a method, corrective action is taken.

Method Limits of Quantitation

Methods used in FDA's commodity monitoring program have been develcped and are applied to provide quantitation at residue levels sufficiently below tolerance limits to generate reliable data on the incidence and levels of residues in the food supply. Residues below tolerance levels are quantitated, and the data are compiled. Because there are so many compounds to consider and because the analytical response varies for the different compounds determined by a multiresidue method, it is not possible to achieve the same quantitation limit for all pesticides and their alteration products. As a broad generalization, the quantitation limits for commodity

monitoring are in the range 0.01–0.1 ppm. As mentioned above, quantitation limits in the Total Diet Study are lower, generally ranging from 0.002 to 0.02 ppm, so that lower residue levels can be measured for the calculation of actual dietary intakes.

FDA strives to maintain uniform limits of quantitation for a given pesticide among its field laboratories conducting monitoring analysis. To achieve this, laboratories are guided on key method operating parameters affecting quantitation limits. For the GC and LC multiresidue methods, the target quantitation limit is described in terms of detector response of a specified magnitude to a particular compound; minimum magnitude of detector response that will be considered for quantitation; and weight equivalent of test portion that will be injected into the gas or liquid chromatograph for analysis. The limit of quantitation for other compounds in the multiresidue analysis is then based on the response of the same detector to these compounds at the specified conditions.

For example, Sec. 143 of PAM I defines limit of quantitation by specifying that the GC electron-capture detector be set to produce half-scale recorder deflection when responding to 1 ng heptachlor epoxide. One-tenth scale recorder deflection is specified as the lowest quantitatable response. Preparation and cleanup are designed so that 20–30 mg equivalent of the test portion can be introduced to the chromatographic detection system, resulting in a quantitation limit of approximately 0.01 ppm heptachlor epoxide.

Although the tritium source electron-capture detector referred to in PAM I, Sec. 143, is now obsolete, this mechanistic approach remains applicable to defining how a method will be used to achieve a consistent quantitation limit in different laboratories. Chlorpyrifos, a pesticide containing chlorine, phosphorus, nitrogen, and sulfur in the molecule, is now used as the compound to which the response is standardized for halogen-, phosphorus-, nitrogen-, and sulfur-selective GC detectors, as well as for the less selective nickel source electron-capture detector.

This approach to defining limit of quantitation was adopted so that the limits of quantitation achieved in each FDA laboratory would be consistent, sufficiently below tolerance limits to provide data on incidence and levels of residues in the food supply, and realistic in terms of effort required for each analysis. This last provision is intended to avoid unnecessary expenditure of resources in pursuit of ever-decreasing and toxicologically insignificant residue levels.

With the availability of more analytical approaches and determinative techniques, it is now more difficult to maintain a generally uniform and acceptable quantitation limit among a large group of laboratories. For example, some detection techniques used today must be operated in their most sensitive mode to achieve adequate limits of quantitation for the residues of interest. The parameters of operation may vary from one instrument to another for a variety of reasons, such as purity of available reagents or operator experience. As a result, some laboratories may be capable of achieving the desired limit of quantitation while others may not. Such variations affect only the degree to which the quantitation limit is below the tolerance or action level; applications of methodology which do not provide quantitation limits that are somewhat below the regulatory levels of pesticides of interest are not used.

FDA maintains a centralized program of analytical methods development. Among its several objectives is a continuing effort to provide field laboratories with the guidance necessary to achieve the necessary quantitation limits, to maintain uniform quantitation limits among the laboratories, and to do so without expending resources merely to measure lower levels of residues.

Future Applications of Multiresidue Methodology

FDA has made a concerted effort in recent years to extend the coverage of its pesticide programs to chemicals which are not amenable to analysis by multiresidue methods. Each year, field districts are required to survey some locally important commodity for selected residues which require single compound methodology.

There is no question, however, that most products will continue to be examined by the various multiresidue methods available to the agency. In fact, it is expected that development and use of multiresidue methods will increase as the agency attempts to maintain an adequate level of monitoring in the face of budgetary constraints.

The evaluation of additional chemicals for recoverability by the current multiresidue methods will continue indefinitely. The increase in knowledge regarding which chemicals can be recovered strengthens the efficacy of the FDA pesticide programs.

As mentioned previously, research will continue to develop multiresidue methods for compounds that are not determinable by current methods because of their chemical structure. Newly developed instrumental techniques will be examined for potential application to the determination of residues that are not quantitatively recovered through current methods. New instrumentation may also lead to the development of completely new analytical methods.

The trend toward miniaturization of existing and future methods is expected to continue because of the cost savings inherent in the use of smaller amounts of solvents and in the shorter analysis times usually involved. A study currently under way in FDA will provide statistical information to guide the choice of the smaller test portion size used in these methods (L. R. Kamps, S. J. V. Young, & J. Link, FDA, Division of Contaminants Chemistry, 1987).

The application of automation to multiresidue methodology is being explored and may eventually provide a means of increasing the number of samples that FDA can analyze (34–36). Certain methods already include automated steps, and that trend is expected to continue (37).

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Residues of Insecticides, Fungicides, and Herbicides on Ontario-Grown Vegetables, 1980-1985

RICHARD FRANK, HEINZ E. BRAUN, and BRIAN D. RIPLEY
Ontario Ministry of Agriculture and Food, Provincial Pesticide Residue Testing Laboratory, Agricultural Laboratory Services Branch, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Between 1980 and 1985, 354 composite vegetable samples representing 9 vegetable commodities were collected from farm deliveries to the market place in Ontario, Canada. All samples were analyzed for insecticides, 275 for fungicides, and 135 for herbicides. The analyses included organochlorine, organophosphorus, synthetic pyrethroid, and *N*-methylcarbamate insecticides; dithiocarbamate, acylalanine, phthalimide, dicarboximide, and other fungicides; and, chlorophenoxy acid, chlorobenzoic acid, bipyridilium, phenylurea, carbamate, and other herbicides. The commodities tested included asparagus, beans, carrots, cauliflower, cucumbers, onions, potatoes, sweet corn, and tomatoes.

In most samples, pesticide residues were below the detection limits (i.e., 0.005 to 0.1 mg/kg), and most of the positive findings were a fraction (i.e., <1 to 20%) of the maximum residue limit (MRL) permitted for each commodity under the Canadian Food and Drugs Act and Regulations. A small number of samples had residues that exceeded the MRL, and these involved aldicarb and linuron on potatoes and chlorobromuron on carrots.

A wide range of vegetables is grown within the Province of Ontario. The following 9 are among the most important: asparagus, wax and green beans, carrots, cauliflower, cucumbers, onions, potatoes, sweet corn, and tomatoes. According to 1983 statistics, these 9 vegetables were grown on 55 500 ha and had a combined market value of $200 million (1). The number of pesticides that were registered and recommended for use in the production of each of these 9 commodities in 1980 and in 1985 (2) appears in Table 1. In this study, we monitored 34 of 36 registered insecticides; 13 of 15 fungicides; 28 of 46 herbicides and growth regulators; and none of the 3 registered nematocides. In 1983, 39 herbicides, 12 fungicides, and 29 insecticides were used in vegetable production in Ontario, and the quantities of materials used were 101, 155, and 101 tonnes, respectively (3).

The analytical program reported here was undertaken to monitor for (*1*) insecticides and fungicides, because these treatments were mainly foliar and treatments were made close to harvest, (*2*) chlorophenoxy and chlorobenzoic acid herbicides because of possible spray drift to edible crops, (*3*) phenylurea herbicides because of damage to carrots and potatoes from previous field use in other crops, and (*4*) herbicides used in vegetable production (2) because few data exist on their residues in edible food stuffs. Overall, the data indicate if pesticide residues were present in Ontario-produced vegetables, if these residues were in violation of maximum residue limits (MRL) permitted under the Canadian Food and Drugs Act and Regulations (4), and if the current vegetable production recommendations (2) resulted in excessive residues.

METHOD

Field Collections

Composite samples of vegetables were collected either at the farm gate or on delivery to farmers' wholesale markets at Leamington and Toronto, Ontario. Asparagus, cauliflower, cucumbers, sweet corn, and tomatoes were sampled at the farmers' markets. Beans and potatoes were collected when farm deliveries were made to each of 2 processors. Onions and carrots were collected at the farm gate on the Holland-Bradford Marsh at time of shipment to a cooperative wholesale outlet. Each composite sample consisted of 5 kg potatoes, 2 kg carrots, onions, or tomatoes, 0.5 kg beans or asparagus, and 5–10 cauliflower heads, cucumbers, and sweet corn cobs. Samples had already been prepared for market by the pro-

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Table 1. Summary of pesticides registered and recommended for use on each of 9 crops in Ontario in 1980 and 1985

Commodity	Pesticides registered and recommended (Ontario)	
	1980	1985
Asparagus	15	20
Beans	15	17
Carrots	17	19
Cauliflower	25	31
Cucumbers	23	20
Onions	24	27
Potatoes	39	42
Sweet corn	21	28
Tomatoes	29	29

ducer, and other than for sweet corn, analyses were performed on the samples as received, that is, as shipped to the market place. In the case of sweet corn, husks were removed and kernels stripped from cobs prior to analysis. All samples were shipped to the laboratory within 2 days of collection, and analyses were commenced within 2 days of receipt. Samples were refrigerated until delivery and analysis.

Analytical Procedure

Vegetables were sliced and composited into small pieces using a Hobart food chopper; subsamples were taken for analyses using the following 12 analytical procedures. Compounds analyzed and detection limits are shown in Table 2. The number of samples and the analyses are shown in Table 3. Residue results are uncorrected for recoveries.

(a) *Organochlorine, organophosphorus, and synthetic pyrethroid insecticides, and phthalimide fungicides.*—50 g macerated sample was blended with 200 mL acetonitrile–water (2 + 1) and was filtered. A one-half aliquot was removed (5), was diluted with 500 mL water and 25 mL saturated NaCl solution, and was extracted with two 50 mL portions of dichloromethane. Dichloromethane was dried by percolation through anhydrous Na_2SO_4, isooctane was added, and the extract was concentrated just to dryness using a rotary vacuum evaporator. The residue was redissolved in 5.0 mL isooctane.

A portion of concentrated sample (4.5 mL) was cleaned up and fractionated for organochlorine and pyrethroid insecticides and phthalimide fungicides according to the procedure of Mills et al. (6) as described by Braun and Stanek (7). The remaining portion was used without cleanup for determination of organophosphorus insecticides by packed column gas chromatography (GC) with a flame photometric detector (FPD). Recoveries ranged from 85 to 97%.

(b) *Acephate, methamidophos, and oxydemeton-methyl.*—Acephate and methamidophos were extracted according to the procedure described for methamidophos by Braun et al. (8). Oxydemeton-methyl was extracted as in **(a)** along with the other organophosphorus insecticides; a portion of the extract was oxidized to convert oxydemeton-methyl to its sulfone (9) prior to GC/FPD analysis. Recoveries were 81–93%.

(c) *N-methylcarbamate insecticides, acylalanine and dicarboximide fungicides, and allidochlor.*—50 g macerated vegetable tissue was extracted as described in **(a)**. All compounds were determined without cleanup by capillary column GC with a nitrogen–phosphorus detector (NPD) as described by Ripley and Braun (10). Some potatoes were analyzed for metalaxyl by using the method of Bruin et al. (11). Recoveries ranged from 85 to 110%.

Table 2. Compounds used and monitored between 1980 and 1985 and their detection limits

Group	Name	Detection limit, mg/kg
	Insecticides (I)	
Organochlorine[a] (OCl)	(dieldrin), lindane (DDT, DDE, TDE), endosulfan[b]	0.001
	chlordane[c], dicofol	0.002
	methoxychlor	0.01
Organophosphorus[d] (OPI)	diazinon, disulfoton, fonofos, mevinphos, phorate, terbufos, chlorpyrifos, demeton, dimethoate, ethion, isofenphos. malathion	0.005
	parathion	0.01
	chlorfenvinphos, methidathion, phosalone	0.02
	fensulfothion	0.05
	naled	0.1
	phosmet	0.2
	azinphos-methyl	0.5
Special OPI	oxydemeton-methyl[e]	0.1
	methamidophos	0.02
N-methylcarbamate (MCI)	carbaryl, carbofuran, pirimicarb	0.01
Special MCI	aldicarb[e]	0.1
Synthetic pyrethroids	permethrin, fenvalerate, cypermethrin, deltamethrin	0.005
	Herbicides (H) or fungicides (F)	
Bipyridilium (BPH)	diquat, paraquat	0.02
Chloroacetamide and triazine (ATH)	atrazine, alachlor, metribuzin, metolachlor, prometryn, simazine, cyanazine	0.01
Chlorophenoxy (CPH) and chlorobenzoic acids (CBH)	chloramben, 2,4-D, 2,4-DB, dichlorprop, MCPA, MCPB, dicamba, diclofop-methyl	0.01
Phenylurea (PUH)	chlorbromuron, diuron, linuron, monolinuron, metobromuron	0.01
Carbamate (TCH)	butylate, chlorpropham, EPTC, pebulate	0.01
Dalapon		0.01
Allidochlor		0.01
Acylalanine (AAF)	metalaxyl	0.01
Dicarboximide	iprodione	0.01
Organochlorine (OCF)	captan, captafol, folpet, dichloran	0.002
Special OCF (CTF)	chlorothalonil	0.01
Dithiocarbamates (DCF)	maneb, mancozeb, metiram, zineb, ziram, thiram	0.1

[a] Dieldrin not used since 1969; DDT not used since 1970.
[b] Total endosulfan—endosulfan I, II, and sulfate.
[c] Total chlordane—*cis* and *trans* chlordane, heptachlor epoxide, and nonachlor; not used since 1978.
[d] Includes some oxon, sulfoxide, and sulfone metabolites.
[e] Total—parent, sulfoxide, and sulfone; determined as sulfone.

(d) *Aldicarb.*—100 g macerated potato was extracted and oxidized using the procedure of Maitlen et al. (12) prior to GC/FPD (S-mode) analysis. Recoveries from fortified potatoes were 65–85%.

(e) *Chlorothalonil.*—Chlorothalonil was determined using procedure (c) or as described by Northover and Ripley (13). The latter analysis included the parent compound and the 4-hydroxy metabolite with recoveries of 85–88%.

(f) *Dithiocarbamate fungicides.*—These fungicides were determined as their zineb equivalent by using the carbon disulfide evolution technique based on the method of Pease

(14) with modifications described by Ripley (15). Recoveries were 90–98%.

(g) *Bipyridilium herbicides.*—Potatoes were analyzed using the reduction with sodium borohydride method described by King (16). Determinations were made by packed column GC with N-P detection. Recoveries from fortified potato were 50–75%.

(h) *Chloroacetamide and triazine herbicides.*—25 g macerated vegetable tissue was extracted, and residues were quantitated using the procedure of Ramsteiner et al. (17) as described by Sirons et al. (18). Recoveries were 70–80%.

(i) *Chlorophenoxy and chlorobenzoic acid herbicides.*—50 g samples of vegetables were extracted, and residues were quantitated by gas chromatography of their methyl esters using a procedure described by Yip (19) and modified by Sirons et al. (20). Mean recovery for residues above 0.01 mg/kg was 80%.

(j) *Phenylurea herbicides.*—25 g macerated vegetables was extracted with hexane–dichloromethane and cleaned up on a Florisil column. Herbicides were quantitated on a liquid chromatograph with a UV detector (254 nm). The analytical procedure was described by Frank et al. (21) and was adapted from Lawrence (22) and Farrington et al. (23). Recoveries varied from 85 to 90% using fortified vegetable samples.

(k) *Carbamate herbicides.*—50 g macerated vegetables was acidified and then extracted with isooctane, and thiocarbamate residues were determined by GC/FPD (S-mode) as described by Frank et al. (24); chlorpropham was determined by GC with an electron capture detector. In the latter 3 years of this survey, method (c) was used. Recoveries were 85–110%.

(l) *Dalapon.*—50 g macerated vegetable tissue was used to determine dalapon residues by GC according to a method described by Getzendaner (25, 26). Recoveries were 85–95%.

The methods used represent routine standard procedures within the laboratory, and recoveries were periodically confirmed as part of an ongoing quality assurance/quality control program. Duplicate field samples were also analyzed at random, and internal laboratory checks were performed. Fortification levels ranged from the detection limit of the procedure to greater than the maximum residue limit (MRL). Detection limits (Table 2) represent the lowest limit to which the individual procedure was reproducible, and these varied somewhat depending on the particular instrumentation used and the substrate being analyzed. Confirmation techniques were applied when residues were high enough to allow alternative procedures to be used. These included the use of (*1*) element-specific GC detectors, e.g., electrolytic conductivity (or Hall) detection in the Cl- or N-specific modes or flame photometric detection in the P- or S-specific mode, (*2*) alternate-column gas chromatography, e.g., using column packings of different polarity so that characteristic retention times were significantly changed, or (*3*) a capillary gas chromatograph and mass selective detector using either full scan or single- or multiple-ion monitoring.

Results

Table 1 shows the number of pesticides available for the production of vegetables during the study period; however, individual producers would use only a few of these to protect their crops. Because the treatment history of the produce was unknown, surveillance was designed to check for as many of these pesticides as possible (Table 2).

Whenever possible, multiresidue procedures were applied to determine several pesticides in the vegetable samples. Every effort was made to examine Ontario-produced vegetables for the recommended pesticides (2) that could have been applied. Individual procedures were used for those vegetables that may have been treated with chemicals not determined with the screening methodologies. The multiresidue procedures could also detect numerous other pesticides that were not recommended but may have been present through nonregistered use, residue carry-over from past use or drift from other treatments in nearby fields. Not all commodities were examined for all pesticides described. Of the 100 pesticides that may have been used (Table 1), analytical procedures could detect 76, including most of the major chemicals used during 1980–1985 (Table 2). Notable exceptions were the insecticide methomyl, fixed copper fungicides, and the herbicide trifluralin.

The results of the analysis of the 354 vegetable samples appear in Table 3 and are compared to the MRLs as they appear in the Canadian Food and Drugs Act and Regulations (4).

Insecticides

The 354 vegetable samples were analyzed for the following insecticide classes: 251 for organochlorines, 258 for organophosphorus, 23 for synthetic pyrethroids, and 151 for *N*-methylcarbamates (Table 4). Five organochlorine insecticide residues were identified to a detection limit of 0.01 to 0.005 mg/kg (Table 3). These included dieldrin, DDT, and chlordane, which were not applied to any of the commodities between 1980 and 1985 but were present as residues in soils from use in past years. Registrations of dieldrin, DDT, and chlordane were cancelled in 1969, 1970, and 1978, respectively. For all 3 compounds, residues averaged between 0.01 and 0.02 mg/kg. Dicofol, endosulfan, lindane, and methoxychlor are the only remaining organochlorine insecticides still being recommended (2) and used; only residues of dicofol and endosulfan were detected. Dicofol was present in a single sample of cucumber at 0.02 mg/kg. Endosulfan was identified on cauliflower, cucumbers, and tomatoes; however, the concentrations were well below the MRL.

Four organophosphorus insecticides were identified (2 on carrots and 2 on tomatoes). Diazinon and parathion were found on some carrots but at a concentration well below the MRL (Table 3). Chlorpyrifos and malathion were each identified in one tomato sample but, again, below the MRL.

Three carbamate insecticides were identified. Carbaryl was observed at low levels in some samples of asparagus, cauliflower, and tomatoes (Table 3). Carbofuran was identified on one sample of tomatoes at a concentration well below the MRL. Aldicarb residues were detected in 79 of 96 potato samples, with 71 below the 0.5 mg/kg MRL and 8 above. The incidence and level of aldicarb residues appear in Table 5.

Analysis for synthetic pyrethroid insecticides commenced in 1984, and no residues were identified.

Fungicides

Of 275 composite samples analyzed for fungicides, 224 were analyzed for phthalimides, 70 for chlorothalonil, 9 for acylalanines, and 53 for dithiocarbamates (Table 4).

Captan was identified on tomatoes at residue concentrations that were a fraction of the 5.0 mg/kg MRL; chlorothalonil was identified on cauliflower, cucumbers, and tomatoes also at only a fraction of the MRL (Table 3). No acylalanine fungicides were identified. Dithiocarbamate fungicides were detected in cauliflower, cucumbers, and tomatoes; however, residues were low in relation to the MRL. No other fungicides were detected.

Table 3. Summary of vegetable samples analyzed for pesticides, number of sample composites with no detectable residues, and type of pesticides detected, Ontario, 1980–1985

Commodity (no. of samples)	Pesticide group[a]	Composites: No. analyzed	Composites: No. below detection limit	Pesticides detected: Identity	Pesticides detected: No. of samples	Pesticides detected: Mean ± SD, mg/kg	MRL, mg/kg
Asparagus	OCI, OPI	22	22	—	—	—	—
(24)	MCI	5	2	carbaryl	3	0.41 ± 0.83	10
	CPH, CBH	6	5	MCPA	1	0.06	0.1
	OCF	22	22	—	—	—	—
	DCF, CTF	5	5	—	—	—	—
Green & wax beans	OCI, OPI	40	40	—	—	—	—
(40)	OCF	40	40	—	—	—	—
Carrots	OCI	15	15	—	—	—	—
(26)	OPI	15	8	diazinon	4	0.04 ± 0.02	0.75
				parathion	4	0.06 ± 0.04	0.70
	SPI	3	3	—	—	—	—
	PUH	26	8	chlorbromuron	9	0.03 ± 0.03	0.1
				chlorbromuron	3	0.14 ± 0.04	0.1
				monolinuron	1	0.03	0.1
				linuron	5	0.05 ± 0.03	0.1
	OCF	15	15	—	—	—	—
Cauliflower	OCI	21	20	endosulfan	1	0.01	1.0
(27)	OPI	21	21	—	—	—	—
	MCI	2	1	carbaryl	1	0.70	5.0
	CPH, CBH	14	13	2,4-D	1	0.02	0.1
	OCF	21	21	—	—	—	—
	DCF	2	0	zineb eq.	2	1.0 ± 0.8	7.0
	CTF	4	0	chlorothalonil	4	0.42 ± 0.52	5.0
Cucumber	OCI	49	22	dicofol	1	0.02	3.0
(52)				dieldrin	9	0.02 ± 0.02	0.1
				endosulfan	26	0.04 ± 0.04	1.0
	OPI	49	49	—	—	—	—
	MCI	34	34	—	—	—	—
	CPH, CBH	12	12	—	—	—	—
	OCF	22	22	—	—	—	—
	AAF	3	3	—	—	—	—
	DCF	4	1	zineb eq.	3	0.1	4.0
	CTF	12	10	chlorothalonil	2	0.01 ± 0.01	5.0
Onions	OCI, OPI	10	10	—	—	—	—
(10)	CPH, CBH	2	2	—	—	—	—
	allidochlor	14	14	—	—	—	—
	OCF	10	10	—	—	—	—
Potatoes[c]	OCI	17	12	DDT	4	0.01 ± 0.01	0.5
(96)				chlordane	1	0.01	0.1
	OPI	24	24	—	—	—	—
	OPI-special	14	14	—	—	—	—
	MCI	31	31	—	—	—	—
	MCI-special	96	17	aldicarb	71	0.23 ± 0.13	0.5
				aldicarb	8	0.67 ± 0.15	0.5
	SPI	20	20	—	—	—	—
	BPH	40	38	diquat	2	0.02	0.1
	CPH, CBH	5	5	—	—	—	—
	dalapon	18	18	—	—	—	—
	PUH	33	30	linuron	3	1.61 ± 1.87	0.1
	ATH	25	25	—	—	—	—
	TCH	33	31	chlorpropham	2	0.18	15
	OCF	17	17	—	—	—	—
	AAF	6	6	—	—	—	—
	CTF, DCF	31	31	—	—	—	—
Sweet corn	OCI, OPI	23	23	—	—	—	—
(28)	MCI	5	5	—	—	—	—
	CPH, CBH	7	6	dicamba	1	0.02	0.1
	OCF	23	23	—	—	—	—
	CTF, DCF	5	5	—	—	—	—
Tomatoes	OCI	54	29	endosulfan	25	0.06 ± 0.13	1.0
(63)	OPI	54	52	chlorpyrifos	1	0.02	0.1
				malathion	1	0.01	3.0
	MCI	9	7	carbaryl	1	0.06	5.0
				carbofuran	1	0.01	0.1
	CPH, CBH	14	12	dicamba	1	0.03	0.1
				2,4-D	1	0.02	0.1
	OCF	54	50	captan	4	0.03 ± 0.04	5.0
	DCF	6	4	zineb eq.	2	0.1 ± 0.1	4.0
	CTF	13	8	chlorothalonil	5	0.07 ± 0.13	4.0

[a] OCI, organochlorine insecticides; OPI, organophosphorus insecticides; MCI, carbamate insecticides; SPI, synthetic pyrethroid insecticides; CPH, chlorophen-

Table 4. Numbers of composite vegetable samples containing pesticide residues below and above MRL, Ontario, Canada, 1980–1985

Pesticide class (No. of samples)	Pesticide group[a]	Composites: No. analyzed	Composites: No. below detection limit	Pesticides detected: No. below MRL	Pesticides detected: No. above MRL
Insecticide (354)	OCI	251	164	87	
	OPI	258	248	10	
	Special OPI	14	14	—	
	SPI	23	23	—	
	MCI	86	80	6	
	Special MCI	96	17	71	8
Herbicide (135)	CPH, CBH	60	55	5	
	BPH	40	38	2	
	allidochlor	14	14	—	
	dalapon	18	18	—	
	PUH	59	38	15	6
	ATH	25	25	—	
	TCH	33	31	2	
Fungicide (275)	OCF	224	220	4	
	CTF	70	59	11	
	AAF	9	9	—	
	DCF	53	46	7	

[a] See Table 3 footnote [a].

Herbicides

Of 135 composite vegetable samples analyzed for herbicides, 60 were analyzed for chlorophenoxy and chlorobenzoic acids, 40 for bipyridilium, 59 for phenylureas, 25 for chloroacetamides and triazines, 33 for carbamates, 14 for allidochlor, and 18 for dalapon (Table 4).

Two chlorophenoxy and one chlorobenzoic herbicides were identified—MCPA on asparagus, 2,4-D on cauliflower and tomato, and dicamba on sweet corn and tomatoes. Residue levels were well below the negligible limit (0.1 mg/kg) permitted under the Canadian Food and Drugs Act (4). The residues on cauliflower and tomatoes were most likely derived from spray drift when other crops were treated. No hormone injury symptoms were observed on the product at the time of sampling.

Diquat was identified on 2 potato samples but at trace levels. Three phenylurea herbicides (chlorbromuron, monolinuron, and linuron) were identified on carrots. Three samples containing chlorbromuron were slightly above the MRL, whereas all the others were below the limit. Three of 33 potato samples had linuron residues, and all were above the MRL. No chloroacetamide, triazine, dalapon, or allidochlor residues were found on commodities analyzed for these herbicides.

Discussion

Other than aldicarb on potatoes, most of the insecticides used in the production of vegetables were below the detection levels at harvest time. Those samples that contained detectable residues had levels that ranged from <1 to 20% of the maximum residue limit (MRL). Aldicarb appeared in most potatoes sampled, and 7% were above the 0.5 mg/kg MRL. These residues appeared lower in 1984 than in 1983 and 1985.

No fungicide residues were found on asparagus, beans, onions, or sweet corn. Fungicide residues found on other vegetables were at a fraction of the permitted MRL, ranging from <1 to 14% of the limits.

Residues of phenylurea herbicides exceeded the MRL in a few carrot and potato samples. One of these herbicides, chlorbromuron, was removed from the market by the manufacturer in 1983.

In general, the use of pesticides on vegetables in Ontario resulted in edible commodities that contained no detectable or very low levels of residues. In the few cases where excessive residues were found, changes in recommendations on rate of application, e.g., aldicarb from 3.3 to 2.2 kg/ha, or preharvest interval, e.g., diazinon from 7 to 14 days, have been sufficient to reduce residues to permitted levels. Occasionally, a use has been discontinued because of the persistence of high residues, e.g., endosulfan on greenhouse lettuce.

Many of the pesticide recommendations for preharvest intervals were determined from field dissipation studies under local conditions. Since residue levels in samples from the farm gate are acceptable it appears that current recommendations are satisfactory to achieve low or nondetectable terminal residues on foods and that growers are not using more chemical than is recommended on the label.

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Table 5. Residues of aldicarb in potato tubers, 1983–1985[a]

Year	Potato samples	Aldicarb found, mg/kg (mean ± SD)	Number of samples: Not detected, <0.02 mg/kg	Below MRL	Mean ± SD, mg/kg	Above MRL	Mean ± SD, mg/kg
1983	36	0.27 ± 0.21	7	25	0.28 ± 0.13	4	0.66 ± 0.14
1984	28	0.13 ± 0.15	9	18	0.17 ± 0.12	1	0.57
1985	32	0.27 ± 0.28	1	28	0.23 ± 0.24	3	0.71 ± 0.16

[a] Maximum residue limit = 0.5 mg/kg.

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oxy acid herbicides; CBH, chlorobenzoic acid herbicides; PUH, phenylurea herbicides; BPH, bypyridilium herbicides; ATH, chloroacetamide and triazine herbicides; TCH, carbamate herbicides; OCF, organochlorine fungicides; DCF, dithiocarbamate fungicides; CTF, chlorothalonil fungicide; AAF, acylalanine fungicides.

[b] Potatoes analyzed in 1983–1985.

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TECHNICAL COMMUNICATIONS

Determination of Malic Acid, Lactic Acid, Citric Acid, Sodium, Potassium, Magnesium, Calcium, and Chloride in Wine: Summary of Collaborative Study of the International Office of Wine (OIV)

CHARLOTTE JUNGE
Office International de la Vigne et du Vin, 11, rue Roquépine, Paris (8e), France

Thirty-six laboratories analyzed 4 wines—1 white, 2 reds, and 1 dessert wine—in the following order: The first determination on each commodity, then the second determination on each commodity, etc., until 5 tests had been performed on each commodity.

Malic, lactic, and citric acids were determined by enzymatic procedures (OIV) and, in comparison, by the following chemical methods:

Citric acid was determined by precipitation by barium ion, decolorization, oxidation by lead(IV) acetate, and colorimetric determination, using diazotized sulfanilic acid (OIV No. A29 common method with modifications/EEC No. 1108/82).

Malic acid and lactic acid were separated by an ion-exchange column and determined colorimetrically (OIV No. A33 for malic acid by determination with chromotropic acid; OIV No. A27 for lactic acid by oxidation with ceric sulfate to acetaldehyde and determination with sodium nitroprusside and piperidine).

Sodium and potassium were determined by atomic absorption spectrometry, and, in comparison, by the following flame photometric methods: sodium, OIV No. A25; potassium, OIV No. A8.

Calcium and magnesium were determined by atomic absorption spectrometry (AAS) and by the complexometric method, OIV No. A26.

Chloride was determined by the potentiometric micromethod, OIV No. A15.

Collaborators were instructed to report malic and lactic acids as g/L to 2 decimals; citric acid as mg/L; and sodium, calcium, magnesium, and potassium as mg/L.

Statistical analysis was done by ISO 5725-1981, calculating the following data: mean (in appropriate units as indicated with each method); within-laboratory (s_r) and among-laboratory (s_R) standard deviations obtained by 1-way analysis of variance, material by material; ISO repeatability critical value ($r = 2 \cdot 2^{1/2} s_r$) and reproducibility critical value ($R = 2 \cdot 2^{1/2} s_R$). The numbers in parentheses in the tables are the associated degrees of freedom. The standard deviations are converted to relative standard deviations (coefficients of variation) by multiplying the corresponding standard deviations by (100/mean).

The following outlier test was used: Check the 5 replicates by the Grubbs test (2-sided) at the 95% confidence level. If the critical value is exceeded, make 3 additional determinations. Check the 8 values at the 99% confidence level. If the critical value is exceeded, omit the extreme value from the calculation, but report it.

Performance parameters are given in Table 1. For sodium by flame photometry, a product dependence must be assumed for repeatability: r = 1.4 (red and white) and 2.0 (dessert). There is a linear correlation for reproducibility with concentration, C_i: $R = 4.7 + 0.08C_i$ (mg/L). For sodium by AAS, there is a linear correlation for repeatability and reproducibility with concentration, C_i: $r = 1.0 + 0.024C_i$; $R = 2.5 + 0.054C_i$. We conclude that both methods work well but that flame photometry gives better results within laboratories and unsatisfactory results between laboratories. Therefore, flame photometry cannot be recommended for determination at the limit value of 60 mg/L.

Since the standard deviations for magnesium by AAS are compatible, the following values can be assigned: r = 3.1 and R = 7.8 mg/L. Because of considerable differences between laboratories of means and standard deviations for the complexometric determination of magnesium, a large number of laboratories were eliminated, so the evaluation of this method is not satisfactory.

In the determination of calcium by AAS, the repeatability depends on the concentration: for <60 mg/L, r = 2.7; for >60 mg/L, r = 4.0. There is a linear correlation for reproducibility with concentration, C_i: $R = 0.114C_i - 0.5$. For the complexometric determination, again, the considerable differences between laboratories eliminated too many laboratories for a satisfactory evaluation of the method.

For potassium by flame photometry, the following values are calculated: r = 17; R = 66 mg/L. For potassium by AAS, the following values are calculated: r = 35; R = 66 mg/L.

The chemical method OIV A27 gives high values and high reproducibility critical values. Therefore, this method is not recommended for lactic acid. For lactic acid by the enzymatic method, there is a linear correlation for repeatability and reproducibility with concentration, C_i: $r = 0.02 + 0.07C_i$; $R = 0.05 + 0.125C_i$.

The purpose of the malic acid study was to determine whether the addition of DL-malic acid could be detected using a combination of chemical and enzymatic methods. This does not appear to be possible because the chemical method gives high results. Therefore, only the enzymatic method is recommended. For the latter method, there is a linear correlation for repeatability and reproducibility with concentration, C_i: $r = 0.03 + 0.034C_i$; $R = 0.05 + 0.071C_i$.

The reproducibility of the first collaborative study of the colorimetric method OIV A29 for citric acid was poor. The ranges of means of the 4 wines were: white, 458–640; red 1, 80–210; red 2, 788–985; and dessert, 725–882 mg/L. Method OIV A29 modified to include a separation on an ion-exchange column also did not work well. OIV is abandoning both methods.

For the citric acid enzymatic method, standard deviations for repeatability and reproducibility are compatible, so the following values are calculated: for <400 mg/L, r = 14, R = 39; for >400 mg/L, r = 28, R = 65. OIV recommends this method as the official method for European Economic Commission regulation (EEC No. 1108/82).

Standard deviations for repeatability and reproducibility for potentiometric micromethod for chlorides are compatible, so the following values are calculated: r = 1.2 mg/L; R = 4.1 mg/L.

Table 1. Design and performance parameters for various analytes in wine

Product	Mean	Original no. of labs	Outlying labs removed		Repeatability		Reproducibility		Ratio
			No.	%	r	RSD_r, %	R	RSD_R, %	R/r
Analysis: sodium (mg/L) by flame photometry									
White wine	89	15	2	13	1.6 (55)	0.62	12. (67)	4.8	7.6
Red wine	18	15	1	7	1.2 (55)	2.4	6.3 (68)	12.3	5.2
Dessert wine	69	15	2	13	2.0 (55)	1.0	9.8 (67)	5.0	4.9
Analysis: sodium (mg/L) by atomic absorption spectrometry									
White wine	90	11	2	18	3.1 (36)	1.2	7.5 (44)	3.0	2.4
Red wine	18	11	3	27	1.4 (32)	2.7	3.5 (39)	6.9	2.5
Dessert wine	69	11	3	27	2.8 (32)	1.4	5.9 (39)	3.0	2.1
Analysis: magnesium (mg/L) by atomic absorption spectrometry									
White wine	63	20	5	25	3.0 (60)	1.7	7.1 (74)	4.0	2.3
Red wine	104	20	7	35	3.0 (52)	1.0	8.1 (64)	2.7	2.7
Dessert wine	88	20	8	40	3.3 (51)	1.3	8.4 (62)	3.4	2.5
Analysis: calcium (mg/L) by atomic absorption spectrometry									
White wine	86	20	8	40	4.1 (50)	1.7	8.6 (61)	3.5	2.1
Red wine	155	20	8	40	3.9 (54)	0.89	17.5 (65)	4.0	4.5
Dessert wine	57	20	8	40	2.7 (51)	1.6	6.6 (62)	4.1	2.4
Analysis: potassium (mg/L) by flame photometry									
White wine	928	15	4	27	15 (47)	0.56	62 (57)	2.3	4.1
Red wine	1035	15	5	33	10 (40)	0.34	62 (49)	2.1	6.2
Dessert wine	825	15	5	33	20 (43)	0.84	70 (52)	3.0	3.5
Analysis: potassium (mg/L) by atomic absorption spectrometry									
White wine	926	11	1	9	41 (43)	1.58	67 (52)	2.5	1.6
Red wine	1031	11	2	18	34 (36)	1.15	61 (44)	2.1	1.8
Dessert wine	825	11	2	18	28 (42)	1.18	73 (50)	3.1	2.6
Analysis: lactic acid (g/L) by chemical method OIV A27									
White wine	2.08	16	0	0	0.22 (65)	3.8	0.56 (80)	9.5	2.5
Red wine	4.29	15	2	13	0.35 (54)	2.9	1.29 (66)	10.6	3.7
Dessert wine	0.46	15	4	26	0.07 (62)	5.7	0.17 (76)	12.8	2.4
Analysis: L-lactic acid (g/L) by enzymatic method									
White wine	1.56	15	3	20	0.12 (48)	2.7	0.23 (59)	5.1	1.9
Red wine	3.60	15	5	30	0.28 (43)	2.7	0.5 (52)	5.0	1.8
Dessert wine	0.29	15	2	13	0.05 (55)	6.1	0.10 (67)	12.1	2.0
Analysis: DL-malic acid (g/L) by chemical method OIV A33 (common method)									
White wine	3.40	15	4	27	0.24 (50)	2.5	0.49 (60)	5.1	2.0
Red wine 1	0.94	14	3	21	0.17 (46)	6.2	0.49 (56)	18.2	2.9
Red wine 2	2.36	12	3	25	0.28 (36)	4.2	0.44 (44)	6.6	1.6
Dessert wine	1.96	14	4	29	0.20 (40)	3.6	0.34 (49)	6.2	1.7
Analysis: L-malic acid (g/L) by enzymatic method									
White wine	3.07	16	2	13	0.134 (61)	1.5	0.238 (74)	3.1	2.0
Red wine 1	0.27	16	2	13	0.036 (59)	4.8	0.071 (72)	9.2	2.0
Red wine 2	1.03	15	2	13	0.072 (52)	2.5	0.117 (64)	4.0	1.6
Dessert wine	1.09	16	4	25	0.068 (48)	2.2	0.124 (59)	4.0	1.8
Analysis: citric acid (mg/L) by colorimetric method OIV A29									
White wine	548	12	4	33	34 (36)	2.2	94 (39)	6.0	2.6
Red wine 1	137	11	4	36	31 (41)	8.0	69 (46)	17.8	1.9
Red wine 2	924	12	4	33	38 (36)	1.5	174 (39)	6.6	4.6
Dessert wine	773	12	3	25	56 (40)	2.5	114 (44)	5.2	2.0
Analysis: citric acid (mg/L) by colorimetric method modified with ion-exchange									
White wine	493						143	10.3	
Red wine 1	179						66	13.0	
Red wine 2	964						214	7.8	
Dessert wine	985						169	6.1	
Analysis: citric acid (mg/L) by enzymatic method (first collaborative study)									
White wine	567	12	4	33	30 (32)	1.8	40 (39)	2.5	1.3
Red wine 1	961	12	4	33	28 (38)	1.0	79 (45)	2.9	2.8
Red wine 2	173	12	4	33	12 (34)	2.4	34 (41)	6.8	2.8
Dessert wine	832	12	4	33	24 (32)	1.0	62 (39)	2.6	2.6

Table 1. Continued

Product	Mean	Original no. of labs	Outlying labs removed		Repeatability			Reproducibility			Ratio R/r
			No.	%	r		RSD_r, %	R		RSD_R, %	
Analysis: citric acid (mg/L) by enzymatic method (second collaborative study)											
White wine	493	12	1	8	24	(44)	4.9	33	(54)	2.3	1.3
Red wine 1	167	12	1	8	16	(47)	3.4	42	(57)	8.8	2.6
Red wine 2	976	12	3	25	32	(38)	1.2	58	(46)	2.1	1.8
Dessert wine	974	12	5	42	16	(28)	0.59	58	(34)	2.1	3.6
Analysis: chlorides (mg/L) by potentiometric micromethod (OIV A15)											
White wine	23.0	17	3	18	1.4	(55)	2.4	4.3	(68)	7.1	3.1
Red wine	34.3	17	4	24	1.1	(51)	1.1	3.9	(63)	4.0	3.5
Dessert wine	66.1	17	4	24	1.2	(54)	0.6	4.0	(66)	2.3	3.3

Determination of Density, Alcohol Content, and Extract in Alcoholic Beverages: Summary of Collaborative Study

CHARLOTTE JUNGE

Max von Pettenkofer-Institut des Bundesgesundheitsamtes, Abteilung: Chemie der Lebensmittel und Bedarfsgegenstände, Postfach 33 00 13, D-1000 Berlin 33, Federal Republic of Germany

Collaborators: Bundesmonopolverwaltung für Branntwein, Offenbach; Bundesmonopolverwaltung für Branntwein, Berlin; Zolltechnische Prüfungs-und Lehranstalt, Berlin; Institut für Gärungsgewerbe und Biotechnologie, Berlin; Bundesgesundheitsamt, Berlin; and research laboratories in Berlin, Münster, Würzburg, Mainz, Wiesbaden, Stuttgart, Braunschweig, Karlsruhe, Lübeck, Firma Eckes, Nieder-Olm

Thirteen laboratories analyzed 5 alcoholic beverages—fruit juice liqueur (25% alcohol by volume), light spirit (Klarer; 32%), brandy (38%), rum (53%), and raw spirit (Rohbrand; 63%) for the following parameters: alcohol content (as % by volume), density (mass per unit volume), and extract, all by the pycnometric method described in "Chemisch-Technische Bestimmungen der Bundesmonopolverwaltung für Branntwein, M 3."

The collaborators were instructed as follows:

Statement of results.—Report density (mass per unit volume) to 5 decimal places; report alcohol content (as % by volume) to 2 decimal places calculated from the mass per unit volume of the distillate determined by the pycnometer method calculated to 5 decimal places; report extract (g/100 mL) to 2 decimal places, calculated from the density of the residue from distillation calculated to 5 decimal places.

Table 1. Design and performance parameters for pycnometric determination of alcohol content, density, and extract of alcoholic beverages

Product	Mean	Original no. of labs	Outlying labs removed		Repeatability			Reproducibility			Ratio R/r
			No.	%	r		RSD_r, %	R		RSD_R, %	
Analysis: mass per unit volume at 20°C by pycnometer											
Fruit juice liqueur	1.08315	13	3	23	0.00015	(43)	0.0047	0.00046	(52)	0.0150	3.2
Light spirit (Klarer)	0.95943	13	2	15	0.00012	(58)	0.0044	0.00025	(68)	0.0093	2.1
Rum	0.92234	13	2	15	0.00012	(48)	0.0046	0.00020	(58)	0.0078	1.7
Brandy	0.95644	13	2	15	0.00013	(51)	0.0049	0.00023	(61)	0.0085	1.8
Raw spirit	0.89992	13	4	31	0.00012	(43)	0.0047	0.00027	(51)	0.0105	2.2
Average or (total)		(65)	(13)	20	0.00013		0.0047	0.00028		0.0102	2.2
Omit liqueur		(52)	(10)	19	0.00012		0.0047	0.00024		0.0090	1.9
Analysis: alcohol content (% by volume)											
Fruit juice liqueur	25.11	13	2	15	0.14	(47)	0.20	0.23	(57)	0.33	1.6
Light spirit	32.33	13	1	8	0.19	(55)	0.21	0.30	(66)	0.33	1.6
Rum	53.99	13	2	15	0.224	(45)	0.15	0.397	(55)	0.260	1.8
Brandy	38.17	13	3	23	0.207	(43)	0.19	0.343	(52)	0.318	1.7
Raw spirit	63.90	13	1	8	0.274	(60)	0.15	0.475	(71)	0.263	1.7
Average or (total)		(65)	(9)	14	0.07		0.18	0.349		0.298	1.7
Omit raw spirit		(52)	(8)	15	0.19			0.32			1.7
Analysis: extract (g/100 mL)											
Fruit juice liqueur	30.78	13	1	8	0.186	(50)	0.21	0.268	(60)	0.307	1.4
Light spirit	0.082	13	2	15	0.0430	(50)	18.5	0.0531	(60)	22.9	1.2
Rum	0.102	13	1	8	0.0356	(51)	12.4	0.0436	(61)	15.1	1.2
Brandy	1.52	13	2	15	0.0591	(48)	1.38	0.0982	(58)	2.28	1.7
Raw spirit	0.0										

Testing of outliers.—Make 5 tests. Check the 5 replicate values by the 2-sided Grubbs test at the 95% confidence level. If the critical value is exceeded, make 3 additional determinations. Check the 8 values at the 99% confidence level. If the critical value is exceeded, omit the extreme value from the calculation, but report it.

Calculation of the Grubbs statistic.—Grubbs statistic = |Extreme value − average of all values|/standard deviation of all values.

Order of determinations.—Make the first determination from each of the 5 commodities, then the second determination from each of the 5 commodities, etc., until all 25 determinations have been performed.

After outlier testing was performed by each laboratory as directed above, the within-laboratory variances of all the laboratories were checked for homogeneity by the Bartlett test. The mean values from all the laboratories were checked for systematic deviations by 1-way analysis of variance. Flagged outliers are not included in the final calculations of the parameters, but the percentage of laboratories removed is indicated in Table 1.

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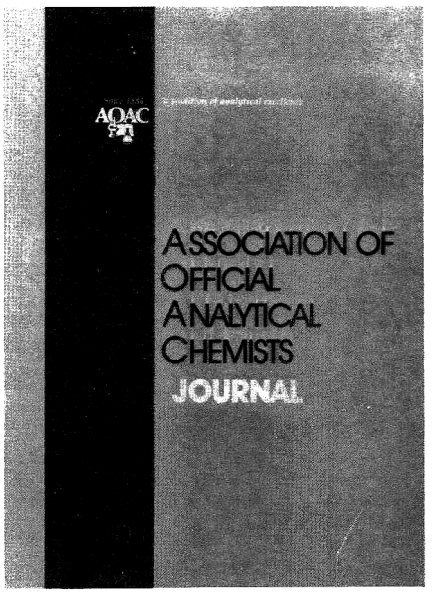

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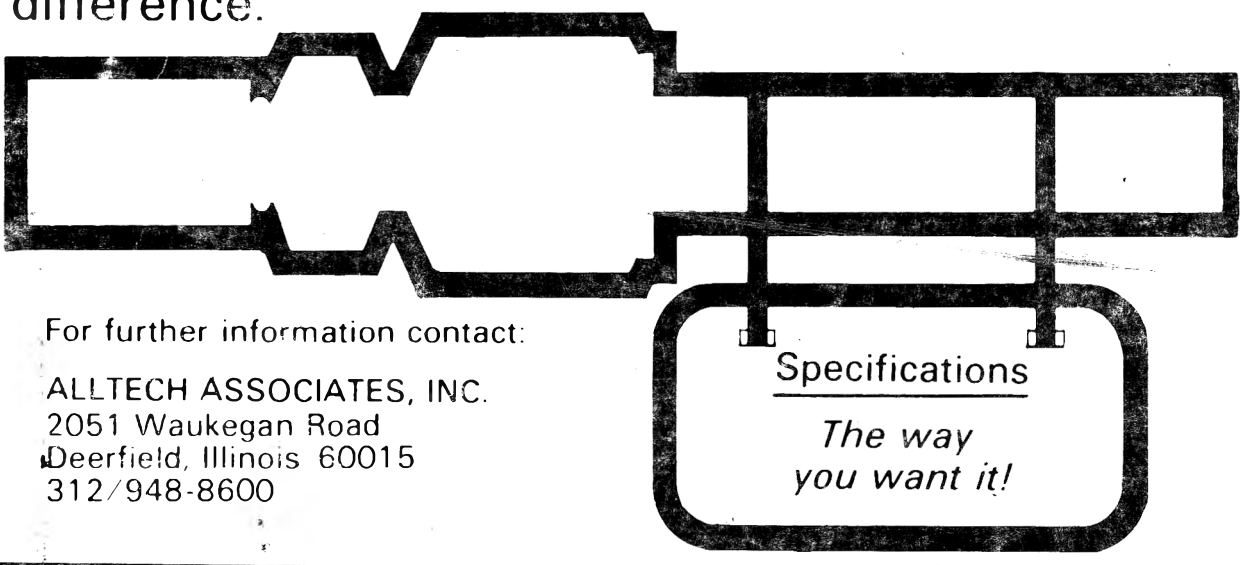

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